Mechanism of Synergy of N-(4-Hydroxyphenyl) Retinamide and ABT-737 in Acute Lymphoblastic Leukemia Cell Lines: Mcl-1 Inactivation

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Background
ABT-737 is a pan-Bcl-2 inhibitor that has a wide range of single-agent activity against acute lymphoblastic leukemia (ALL) cell lines and xenografts. A relationship between expression of myeloid cell leukemia 1 (Mcl-1), an antiapoptotic member of the Bcl-2 family of proteins, and resistance to ABT-737 has been reported for various cancers. The synthetic cytotoxic retinoid N-(4-hydroxyphenyl)retinamide (4-HPR) is known to generate reactive oxygen species (ROS), and ROS have been shown to activate c-Jun kinase (JNK), which in turn phosphorylates and inhibits Mcl-1. Thus, we investigated whether 4-HPR-mediated inactivation of Mcl-1 could act synergistically with ABT-737 to promote leukemia cell death.

Methods
Cytotoxicity was determined using the fluorescence-based DIMSCAN assay. Synergy was defined as a combination index (CIN) less than 1. The expression of Bcl-2 family messenger RNAs was measured by real-time reverse transcription–polymerase chain reaction, and caspase activity was measured enzymatically. Changes in Bcl-2 family proteins and release of mitochondrial cytochrome c were detected by immunoblotting. ROS, apoptosis, mitochondrial membrane depolarization, and phospho-JNK were measured by flow cytometry. Gene silencing was by small interfering RNA (siRNA). All statistical tests were two-sided.

Results
ABT-737 decreased Mcl-1 protein expression in ABT-737-sensitive ALL cell lines but not in ABT-737-resistant lines. Using the antioxidant ascorbic acid and siRNA-mediated knockdown of JNK, we showed that 4-HPR decreased Mcl-1 via ROS generation (that phosphorylates JNK) in ABT-737-resistant cell lines. Combining ABT-737 with 4-HPR enhanced the mitochondrial apoptotic cascade (percentage of cells with depolarized mitochondrial membrane at 6 hours, ABT-737 vs ABT-737 plus 4-HPR: 24.5% vs 45.5%, difference = 20.1%, 95% CI = 18.9% to 13.9%; \( P < .001 \)) and caused caspase-dependent, synergistic multilog cytotoxicity in all seven ALL cell lines examined (mean CIN = 0.57, 95% CI = 0.37 to 0.87), with minimal cytotoxicity for normal lymphocytes.

Conclusions
An increase of Mcl-1 protein in response to ABT-737 is one mechanism of ABT-737 resistance that can be overcome by 4-HPR, resulting in synergistic cytotoxicity of ABT-737 combined with 4-HPR in ALL cell lines.
Conversely, high Bcl-2 expression has been associated with the lack of responsiveness to induction chemotherapy in ALL (14). Thus, the antiapoptotic members of the Bcl-2 family of proteins provide an attractive therapeutic target for ALL.

ABT-737 is a small-molecule chemical that mimics the direct binding of Bad to Bcl-2, Bcl-X<sub>L</sub>, and Bcl-w (another antiapoptotic Bcl-2 family member). It has been reported that heterodimerization of antiapoptotic Bcl-2 proteins with proapoptotic Bcl-2 family proteins (eg, Bid, Bim, Bax, or Bak) prevents them from initiating apoptosis via mitochondrial membrane depolarization (11,15). Upon binding of ABT-737, proapoptotic Bcl-2 proteins, such as Bid and Bim (direct activators of Bax or Bak), are prevented from forming heterodimers with antiapoptotic Bcl-2 family proteins, thereby promoting Bax and Bak activation (via oligomerization) (15). ABT-737 markedly increases the cytosolic response of cancer cells to radiation by reducing the median effective concentration (EC<sub>50</sub>) value for cytotoxicity (15) and has shown preclinical activity as a single agent or in combination with other chemotherapeutic agents against acute myeloid leukemia (AML) (16,17), multiple myeloma (18), lymphoma (19), chronic lymphocytic leukemia (20), small cell lung cancer (15,21,22), and ALL (23). Myeloid cell leukemia 1 (Mcl-1) and branched flossetless 1 (Bfl-1, also called A1, BFL-1/A1, and Bcl2A1) are Bcl-2 family members to which ABT-737 has relatively low binding affinity (15). A high basal level of Mcl-1 expression in small cell lung cancer cells (21,24) and in other types of cancer cells (20,25,26) has been associated with resistance to ABT-737.

Because Mcl-1 overexpression is associated with resistance to ABT-737 (via unidentified mechanisms) (20,21,24–26), inhibition of Mcl-1 expression may potentiate the cytotoxicity of ABT-737. Inactivation of Mcl-1 via phosphorylation by c-Jun kinase (JNK) has been reported in leukemia cells in response to oxidative stress (27). Because one of the cytotoxicity mechanisms for the synthetic retinoid N-(4-hydroxyphenyl)retinamide (4-HPR; also known as fenretinide) is via generation of reactive oxygen species (ROS) (28), we investigated the effect of 4-HPR on Mcl-1 and subsequently demonstrated the mechanism of Mcl-1 inhibition by 4-HPR. 4-HPR is cytotoxic to cancer cells but not nonmalignant cells (29,30). 4-HPR induces Bak expression (31) and the release of cytochrome c from mitochondria into the cytosol through a mitochondrial pathway (32,33). As a single agent, 4-HPR is cytotoxic in vitro to a variety of cancer cell types and has modest systemic toxicity in vivo (30,31,34–41). However, in clinical trials, tumor responses to 4-HPR have been less than expected based on the in vitro data, likely because the oral (capsule) formulation has limited bioavailability, resulting in suboptimal systemic 4-HPR exposure (34,41–44). Therefore, we investigated whether 4-HPR-mediated inactivation of Mcl-1 could act synergistically with ABT-737 to promote leukemia cell death.

**Materials and Methods**

**Chemicals**

ABT-737 was supplied by Abbott Laboratories (Abbott Park, IL), and 4-HPR was provided by the Developmental Therapeutics Program of the National Cancer Institute (NCI; Bethesda, MD). ABT-737 was supplied by Abbott Laboratories (Abbott Park, IL), and 4-HPR is cytotoxic in vitro to a variety of cancer cell types and has modest systemic toxicity in vivo (30,31,34–41). However, in clinical trials, tumor responses to 4-HPR have been less than expected based on the in vitro data, likely because the oral (capsule) formulation has limited bioavailability, resulting in suboptimal systemic 4-HPR exposure (34,41–44). Therefore, we investigated whether 4-HPR-mediated inactivation of Mcl-1 could act synergistically with ABT-737 to promote leukemia cell death.

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**Study design**

Molecular studies in seven human ALL cell lines to study the mechanisms of resistance to ABT-737 and the synergistic effects of 4-HPR on the cytotoxicity of ABT-737.

**Contribution**

In ABT-737–resistant ALL cell lines, Mcl-1 protein levels increased with ABT-737 treatment and decreased with 4-HPR treatment. 4-HPR–induced inhibition of Mcl-1 expression occurred via c-Jun kinase phosphorylation downstream of ROS generation. Treatment with ABT-737 plus 4-HPR enhanced the mitochondrial apoptotic cascade and caused caspase-dependent, synergistic multilog cytotoxicity in all seven ALL cell lines examined but had minimal cytotoxicity for normal lymphocytes.

**Implications**

Clinical trials of ABT-737 in combination with 4-HPR are warranted.

**Limitations**

The small sample size (n = 7 cell lines) was not sufficient to determine a statistically significant correlation between the expression of Bcl-2 family members and ABT-737 sensitivity. There was no direct demonstration of Mcl-1 phosphorylation by 4-HPR. The mechanisms underlying changes in Mcl-1 expression in response to ABT-737 remain to be elucidated. 

Ascorbic acid, p-nitroaniline (pNA), Ficoll solution, dimethyl sulfide (DMSO), and ethanol were purchased from Sigma (St Louis, MO). IETD-fmk, a caspase-8 inhibitor, was from BD Biosciences (San Jose, CA). Boc-d-fmk, a pan-caspase inhibitor, was purchased from IMGenex (San Diego, CA).

**Cell Culture**

Human ALL cell lines COG-LL-317 (human T-cell leukemia established from a child at time of relapse) and COG-LL-319 (human pre-B leukemia established at diagnosis from a child before therapy) were established in our laboratory as previously described (45) from clinical samples that were provided by the Children’s Oncology Group (COG) under written informed consent. Cell lines were maintained in Iscove’s modified Dulbecco’s medium (Cambrex, Walkersville, MD) supplemented with 3 mM l-glutamine, 5 µg/mL insulin, and 20% heat-inactivated fetal bovine serum (FBS). The pre-B ALL cell line NALM-6 (obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen [German Collection of Microorganisms and Cell Cultures], Braunschweig, Germany); RS4;11 (pre-B ALL); and the CCRF-CEM, MOLT-3, and MOLT-4 (all T-cell ALL) cell lines (all from the American Type Culture Collection, Manassas, VA) were

**CONTEXT AND CAVEATS**

**Prior knowledge**

ABT-737 is a pan-Bcl-2 inhibitor that is cytotoxic for acute lymphoblastic leukemia (ALL) cell lines and xenografts. Resistance to ABT-737 is associated with expression of myeloid cell leukemia 1 (Mcl-1), an antiapoptotic Bcl-2 family member, in some cancers. The synthetic retinoid N-(4-hydroxyphenyl)retinamide (4-HPR) acts via generation of reactive oxygen species (ROS) and may inhibit Mcl-1 expression.

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cultured in RPMI-1640 medium (Mediatech Inc., Herdon, VA) supplemented with 10% heat-inactivated FBS at 37°C in a humidified incubator containing 5% O₂ and 5% CO₂.

To prepare peripheral blood mononuclear cells (PBMCs), we obtained excess cells (obtained with written informed consent) from leukapheresate that was being used for autologous stem cell transplant, subjected them to carbonyl ion depletion of monocytes (30), diluted them 1:1 with phosphate-buffered saline (PBS), and centrifuged the mixture over Ficoll at 375g for 30 minutes at room temperature. T-lymphocytes were isolated from the normal lymphocytes that remained after monocyte depletion of PBMCs with the use of a Pan T-Cell Isolation kit II (Miltenyi Biotec, Auburn, CA). We assessed the purity of the T-lymphocytes by staining them with two mouse monoclonal antibodies—fluorescein isothiocyanate (FITC)—conjugated anti-CD3 and phycoerythrin (PE)—conjugated anti-CD2 (both from BD Biosciences)—and subjecting them to flow cytometry as described below. To stimulate T-lymphocyte proliferation, T cells were resuspended at a concentration of 1–2 × 10⁶ cells per mL in RPMI-1640 medium containing 5% FBS and 50 U/mL of IL-2 (BD Biosciences) and added to six-well tissue culture plates (2 mL per well) that had been coated with mouse monoclonal antibodies against CD3 and phycoerythrin (PE)–conjugated anti-CD2 (both from BD Biosciences) and centrifuged the mixture over Ficoll at 375g for 30 minutes at room temperature. T-lymphocytes were isolated from the normal lymphocytes that remained after monocyte depletion of PBMCs with the use of a Pan T-Cell Isolation kit II (Miltenyi Biotec, Auburn, CA). We assessed the purity of the T-lymphocytes by staining them with two mouse monoclonal antibodies—fluorescein isothiocyanate (FITC)—conjugated anti-CD3 and phycoerythrin (PE)—conjugated anti-CD2 (both from BD Biosciences) and CD28 (clone 9.3, 2 µg/mL, BD Biosciences) and CD28 (clone 9.3, 2 µg/mL, BD Biosciences) in PBS overnight at 4°C and washed three times with PBS. The cells were incubated with drugs in a 37°C incubator with 5% O₂ (bone marrow–level lines used were mycoplasma free and were cultured and treated for 2 weeks, depending on their proliferation rate. The actively proliferating T-lymphocytes (ie, T-lymphoblasts) were subcultured for cytotoxicity assays. Mycoplasma testing was performed at the Bioreagents and Cell Culture Core, University of Southern California (Los Angeles, CA). All of the cells and cell lines used were mycoplasma free and were cultured and treated with drugs in a 37°C incubator with 5% O₂ and 5% CO₂) to stimulate proliferation. After the stimulation, the lymphocytes were transferred to new plates that had not been coated with any antibodies and incubated in medium containing IL-2 (50 U/mL, ebioscience, San Diego, CA) for approximately 2 weeks, depending on their proliferation rate. The actively proliferating T-lymphocytes (ie, T-lymphoblasts) were subcultured for cytotoxicity assays. Mycoplasma testing was performed at the Bioreagents and Cell Culture Core, University of Southern California (Los Angeles, CA). All of the cells and cell lines used were mycoplasma free and were cultured and treated with drugs in a 37°C incubator with 5% O₂ and 5% CO₂, 5% CO₂, and 90% N₂. Cell line identities were confirmed by short tandem repeat profiling as previously described (48); short tandem repeats were unique for all cell lines. Studies using human specimens were approved by the Children’s Hospital Los Angeles committee for protection of human subjects.

**Cytotoxicity Assay**

We determined the cytotoxic effect of ABT-737 and 4-HPR, alone and in combination, in all seven ALL cell lines and in T-lymphocytes and T-lymphoblasts with the use of DIMSCAN (49,50), a semiautomated fluorescence-based digital image microscopy system that quantifies viable cells in tissue culture multwell plates on the basis of their selective accumulation of fluorescein diacetate (FDA). Each drug was assayed alone and in combination (at a 1:1 molar ratio) at concentrations that ranged from 0 to 10 µM. Cells were seeded into 96-well plates in 100 µL of complete medium (15,000 cells per well) and incubated for 16–24 hours. ABT-737 (stock solution: 10 mM ABT-737 in DMSO) and/or 4-HPR (stock solution: 10 mM in 100% ethanol) was added in 100 µL of culture medium per well at appropriate concentrations, and the cells were incubated for 48 hours at 37°C in 12 replicates. Each assay was repeated twice. We then added 50 µL of FDA in 0.5% eosin Y to each well (final concentrations: FDA = 10 µg/mL, eosin Y = 0.1% [w/v]) and incubated the cells for 15 minutes at 37°C. Total fluorescence in each well was measured with the use of DIMSCAN, and the results were expressed as surviving fractions of treated cells compared with control cells that were exposed to vehicle (<0.1% DMSO and/or ethanol in medium).

**Real-Time Reverse Transcription–Polymerase Chain Reaction**

We used real-time reverse transcription–polymerase chain reaction (RT-PCR) and an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) to quantify basal gene expression of Bcl-2 family members in each of the seven ALL cell lines used. Primers and probes (Supplementary Table 1, available online) were designed and synthesized with the use of Primer Express software (version 1.5; Perkin-Elmer Applied Biosystems, Foster City, CA) using sequences obtained from the GenBank database of Integrated DNA Technologies, Inc (Corvalle, IA). The probes were labeled on the 5′ nucleotide with the fluorescent reporter dye 6-carboxy-fluorescein and on the 3′ nucleotide with the fluorescent quenching dye 6-carboxy-tetramethyl-rhodamine. The primers were designed so that the amplicon spanned at least two exons to avoid amplification of genomic DNA.

All RT-PCR assays were performed in triplicate in 96-well plates using Master Mix Reagents (TaqMan one-step RT-PCR Master Mix Reagents kit; Applied Biosystems), 200 nM forward primer, 200 nM reverse primer, 100 nM of probe, and 50 ng of total RNA in a total volume of 25 µL. Total RNA was extracted from each of the seven ALL cell lines with the use of TRIzol reagent (Invitrogen, Carlsbad, CA). The cycling conditions were 30 minutes at 48°C for reverse transcription, 10 minutes at 95°C for initial activation, and 40 cycles of 15 seconds at 95°C and 60°C for 1 minute. TaqMan real-time RT-PCR data were analyzed with the use of Sequence Detector V1.7 software (Perkin-Elmer Applied Biosystems). The RNA level for each sample was normalized to the glyceraldehyde-3-phosphate dehydrogenase messenger RNA (mRNA) level, which was measured on the same plate but not in the same wells as the gene of interest.

**c-Jun Kinase Gene Silencing by Small Interfering RNA**

We used a previously described electroporation method (51) with minimal modification to transfect CCRF-CEM cells with a small interfering RNA (siRNA) targeted against the JNK gene (Validated Stealth RNAi duplex, Invitrogen). The sense and antisense sequences of the JNK siRNA were 5′-AUCUGAAGACUUCUGCAAAAGAUUG-3′ and 5′-C AAAUCUUUGCC AAGUGAUCAAG-3′, respectively. As a control siRNA, we used the Stealth RNAi Negative Control Lo GC siRNA (Invitrogen Corporation, proprietary sequence, catalog No. 45-2002), which is not homologous to any sequence in the vertebrate transcriptome and does not induce a stress response. Electroporation was carried out using 0.5 mL of cells (at a concentration of 1 × 10⁶ cells per mL in serum-free culture medium) mixed with either 0.5 nmol of JNK siRNA or control siRNA in...
a 0.4-cm cuvette with the use of a GenePulser Xcell device (Bio-Rad Laboratories, Hercules, CA) at 260 V (capacitance = 1050 µF) for 10 ms. Immediately after electroporation, the cells were plated in complete medium (RPMI-1640 containing 10% FBS), incubated for 16 hours at 37°C, and then treated for 6 hours with ABT-737, 4-HPR, or the combination, as described above. The cells were then analyzed by immunoblotting for the levels of JNK and Mcl-1.

**Immunoblot Analysis of Protein Expression**

Cells were lysed in radioimmunoprecipitation (RIPA) lysis buffer consisting of 10% (vol/vol) 20 x RIPA lysis buffer (Upstate, Lake Placid, NY), 1 mM phenylmethanesulphonylfluoride (PMSF), 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 µg/mL pepstatin. The lysates were incubated on ice for 15 minutes, sonicated briefly, and centrifuged at 12 000 g for 15 minutes. The amount of protein in the supernatants was determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL), and equal amounts of protein were resolved by electrophoresis on a 4%–20% Tris–Glycine precast gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane (Protran, Keene, NH), and the membrane was incubated with primary antibodies (anti-Bax, anti-Bad, and anti-Bid [all from BD Biosciences]; anti–cytochrome c [Santa Cruz Biotechnology, Santa Cruz, CA]; or anti-Mcl-1 [Cell Signaling Technology, Danvers, MA], each at 1:1000 dilution, and anti-Noxa [Cell Signaling Technology] at 1:500 dilution), followed by horseradish peroxidase (HRP)–conjugated secondary antibodies (anti-mouse IgG [Sigma], anti-rabbit [BD Biosciences], or anti-goat IgG [BD Biosciences]) at 1:2000 dilution. Antibody binding was then detected with the use of a chemiluminescent substrate (Pierce Biotechnology) and visualized on autoradiography film (Denville Scientific, Inc, Metuchen, NJ). For the analysis of Mcl-1 expression after treating with ABT-737 in cells resistant to the agent, we used a densitometry technique to estimate the changes in expression. The immunoblots were scanned using Epson Expression 1680 (Long Beach, CA) linked with Quantity One (version 4.4.1, Bio-Rad Laboratories) software, and the expression of Mcl-1 in response to ABT-737 treatment relative to vehicle control (0.025% DMSO) treatment was estimated after normalizing the data with β-actin.

To examine the effect of retinoids on Mcl-1 expression, CCRF-CEM cells (7.5 x 10⁶ cells in 15 mL medium) were treated with vehicle (0.1% ethanol), 2.5 µM ABT-737, 10 µM 13-cis-retinoic acid (13-cis-RA), 10 µM all-trans-retinoic acid (ATRA), 10 µM 4-HPR, or the combination of ABT-737 and retinoids for 12 hours. Cells were lysed in RIPA lysis buffer, incubated on ice for 15 minutes, sonicated briefly, and centrifuged at 12 000 g for 15 minutes. The supernatant was collected, and protein levels were quantified using BCA protein assay kit. Equal amounts of protein (30 µg) were separated on a 4%–20% Tris–Glycine precast gel and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with anti-Mcl-1 antibody at 1:1000 dilution, followed by HRP-conjugated anti-rabbit IgG antibody at 1:2000 dilution. Antibody binding was visualized with a chemiluminescent substrate and autoradiography.

**Phosphatase Treatment of Cell Lysates**

CCRF-CEM, RS4;11, and COG-LL-319 cells (15 mL at 0.5 x 10⁶ cells per mL) were treated with vehicle or ABT-737 (2.5 µM), 4-HPR (10 µM), or the combination for 6–12 hours, collected in 15-mL conical tubes, washed twice with 1 mL of ice-cold PBS, and pelleted by centrifugation at 300g for 5 minutes. The cells were then lysed in phosphate buffer (150 mM NaCl, 10 mM Tris–HCl [pH 7.5], 1 mM EDTA, 1% Nonidet P-40, 1 mM dithiothreitol [DTT], 1 mM PMSF, and 1.5% aprotinin) for 10 minutes on ice and then centrifuged at 12 000 g for 10 minutes to remove cellular debris. The resulting supernatants were incubated with or without α-protein phosphatase (2 U/µL; New England Biolabs, Ipswich, MA) according to the manufacturer’s instructions. The reaction was terminated by adding sodium dodecyl sulfate (SDS) sample buffer (containing 50 mM Tris–HCl [pH 6.8], 10% glycerol, 2% SDS, 1% β-mercaptoethanol, 12.5 mM EDTA, and 0.02% bromophenol blue) and boiling for 3 minutes. The levels of Mcl-1 protein relative to β-actin were determined by immunoblot analysis, as described above. The primary antibody (rabbit polyclonal anti-Mcl-1 antibody) was used at 1:1000 dilution and the secondary antibody (HRP-conjugated anti-rabbit IgG) at 1:2000 dilution.

**Apoptosis Assays**

**Analysis of Mitochondrial Membrane Potential.** COG-LL-317 cells (2 mL at 0.5 x 10⁶ cells per mL) were treated with ABT-737 (2.5 or 5 µM) and/or 4-HPR (2.5 or 5 µM) for 6 hours. The cells were collected in 5-mL polystyrene tubes, centrifuged at 300g for 5 minutes, resuspended in 1 mL of medium containing 10 µg/mL of JC-1, a fluorescent dye whose accumulation in mitochondria is a marker for mitochondrial membrane depolarization, and incubated at 37°C for 10 minutes. The cells were then analyzed by flow cytometry. A fluorescence emission shift from green (band-pass filter: 525 ± 10 nm) to red (61 ± 10 nm) indicates mitochondrial membrane depolarization (52). For flow cytometry, we used a BD LSR II system (BD Biosciences) that was driven by DIVA software (version 4.1.2; BD Biosciences). FlowJo software (Tree Star Inc., Ashland, OR) was used for data analyses.

**Detection of Cytochrome c Release From Mitochondria.** COG-LL-317 cells were incubated with ABT-737 and/or 4-HPR for 4 hours, as described above. As a positive control for the release of cytochrome c from mitochondria (ie, to validate the apoptosis assay), COG-LL-317 cells were exposed to UV-B irradiation for 7 minutes on a Bio-Rad UV transilluminator 2000 (Bio-Rad). After drug or UV-B treatment, the cells were subjected to a digitonin-based subcellular fractionation technique (53) to generate supernatant (cytosolic) and pellet (mitochondrial) fractions. The supernatants were subjected to protein quantitation, and the level of cytochrome c in the supernatant was analyzed by immunoblotting, as described above.

**Caspase Activation.** We used a colorimetric assay kit (ApoAlert Caspase-3 and Caspase-8 colorimetric assay kits, BD Biosciences; Caspase-9 colorimetric assay kit, Chemicon International Inc., Temecula, CA) according to the manufacturer’s instructions to detect activation of caspase-9, -3, and -8 in COG-LL-317 cells. The cells (15 mL at 0.5 x 10⁶ cells per mL) were incubated with...
vehicle or ABT-737, 4-HPR, or the combination for 1, 2, 3, 4, 6, or 12 hours, then chilled cell lysis buffer (supplied with the kits) was added to the cells (60 µL per 2.4 × 10⁶ cells), and the mixture was incubated on ice for 10 minutes. The cells were centrifuged at 12000g for 10 minutes at 4°C to remove debris and nuclei. The supernatant was transferred to a 1.5-mL microfuge tube. Protein concentration in the supernatant was quantified using a bicinchoninic acid protein assay kit (Pierce Biotechnology). The supernatants (50 µg of protein) were transferred to a 96-well microplate, and lysis buffer was added to each well to make a total volume of 50 µL. Fifty microliters of 2x reaction buffer containing 1,4-DTT (final DTT concentration: 10 mM) and caspase substrates (DEVD-pNA for caspase-3, IETD-pNA for caspase-8, and LEHD-pNA for caspase-9), with or without the preadded specific caspase inhibitors (DEVD-fmk, a caspase-3 inhibitor; IETD-fmk, a caspase-8 inhibitor; Ac-LEHD-CHO, a caspase-9 inhibitor), were added to each well of the microplate as instructed by the manufacturer. The microplate was incubated at 37°C for 2 hours, and the cleavage of the chromophore pNA from the caspase substrates was measured by reading the absorbance in each well at 405 nm in a microtiter plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA). Negative controls (buffer blank containing reaction buffer with DTT only), samples with inhibitor (reaction buffer containing DTT, caspase substrate, caspase inhibitor, and sample to validate assay), and samples without a substrate (substrate blank containing reaction buffer containing DTT and caspase substrate) were analyzed to assure the quality of the assays. Each sample was tested in triplicate, and the assays were repeated twice.

Phosphatidylserine Detection. Apoptosis was evaluated by analyzing the subdiploid DNA content and annexin V binding, which detects phosphatidylserine inversion in cells that are undergoing apoptosis, using flow cytometry. COG-LL-317 cells were treated with ABT-737 (2.5 µM) and/or 4-HPR (5 µM) for 6 hours, washed twice with PBS, and resuspended in 100 µL of binding buffer (10 mM HEPES–NaOH [pH 7.4], 140 mM NaCl, 25 mM CaCl₂) at a final concentration of 0.5 × 10⁶ cells per 50 µL. Annexin V conjugated with fluorescein (annexin V–FITC: 10 µL; BD Biosciences) was added to the cell suspension, and the mixture was incubated for 10 minutes at room temperature. The cells were washed and resuspended in 390 µL of binding buffer. Just before flow cytometry analysis, 5 µL of a 20-µg/mL propidium iodide (PI) stock solution was added for counterstaining to distinguish cells in the early stage of apoptosis (annexin V–positive cells) from cells that have a subdiploid DNA content (PI-positive) and that are therefore considered to be in the late stage of apoptosis. The cells were analyzed by flow cytometry with band-pass filters of 525 ± 25 nm for FITC and 610 ± 20 nm for PI.

Analysis of Phosphorylated c-Jun Kinase Level CCRF-CEM cells (2 mL at a concentration of 0.5 × 10⁶ cells per mL) were pretreated with ascorbic acid (400 µM) or vehicle control (1% normal saline) for 2 hours, followed by 10 µM 4-HPR or vehicle control (0.1% ethanol) for 12 hours. The cells were then collected in 5-mL polystyrene tubes, and an equal volume of prewarmed (37°C) Cytofix buffer (BD Biosciences) was added immediately to the cell suspension to fix the cells. The cells were incubated at 37°C for 10 minutes and pelleted by centrifugation at 300g for 5 minutes, and the supernatant was removed. The cells were then permeabilized by the addition of 1 mL of PhosFlow Perm Buffer III (BD Biosciences) followed by a 30-minute incubation on ice. The cells were then washed twice with staining buffer (BD Biosciences), centrifuged at 300g for 5 minutes, and resuspended in 50 µL of staining buffer. Then, 10 µL of a purified rabbit polyclonal antibody directed against JNK that is phosphorylated at residues T183 and Y185 (anti-p-JNK; BD Biosciences) was added to 50 µL of cells (0.5 × 10⁶ cells), and the cells were incubated for 30 minutes and washed with staining buffer. PE-conjugated anti-rabbit IgG (10 µL) was then added to the cells, and the mixture was incubated for 30 minutes in the dark at room temperature. The cells were then washed, resuspended in 500 µL of staining buffer, and then analyzed by flow cytometry using a band-pass filter of 575 ± 26 nm for PE.

Analysis of Reactive Oxygen Species Production CCRF-CEM cells (1 × 10⁶ cells in 2 mL medium per well) were plated in 6-well plates. The cells were pretreated with vehicle (0.1% normal saline) or 400 µM ascorbic acid for 2 hours followed by treatment with 10 µM 4-HPR for 12 hours. The cells were centrifuged at 300g for 5 minutes and resuspended in warm (37°C) medium containing 10 µM 2′,7′-dichlorofluorescein diacetate (DCFDA, diluted from a 50-mM stock solution in DMSO; Invitrogen) and incubated for 20 minutes at 37°C. The cells were pelleted by centrifugation at 300g for 5 minutes and resuspended in 0.5 mL of culture medium. In the presence of ROS, which were generated by treating cells with 4-HPR, DCFDA is oxidized to emit green fluorescence. As a positive control, hydrogen peroxide (H₂O₂) was added to cells at a final concentration of 100 µM 15 minutes before flow cytometry analysis. Cells positive for green fluorescence were detected by flow cytometry using a band-pass filter of 525 ± 25 nm.

Statistical Analyses Analysis of DIMSCAN cellular cytotoxicity experiments and computation of the combination index (CIN) were based on the methods of Chou and Talalay (54); drug-induced cytotoxic synergy was analyzed with the use of CalcuSyn software (Biosoft, Cambridge, UK). CIN computation is a method for quantifying combination cytotoxicity effect of two or more drugs. The creators of CalcuSyn software have proposed that CIN values be interpreted as follows: antagonistic effect when CIN > 1.1, additive effect when CIN = 0.9–1.1, slight synergism when CIN = 0.7–0.9, synergism when CIN = 0.3–0.7, strong synergism when CIN = 0.1–0.3, and very strong synergism when CIN < 0.1. CIN values can be calculated at different “effect levels” or “fraction affected” levels (eg, at LC₅₀, LC₉₀, or LC₉₀ [ie, concentration lethal to 50%, 90%, or 99% of the cells]) and may vary depending on the fractional effect level at which it is calculated.

Associations between mRNA expression of Bcl-2 family members and LC₉₀ values for 4-HPR and ABT-737 were determined using Spearman correlation analysis; P values were computed from the null permutation distribution of the correlation coefficient.

Differences in mean pNA levels were assessed by unpaired two-sided Student t tests. The effect of ascorbic acid on the cellular
Figure 1. Effect of ABT-737 on Mcl-1 expression and basal expression of Bcl-2 family proteins in seven acute lymphoblastic leukemia (ALL) cell lines. A) Dose–response curves for selected ALL cell lines treated with ABT-737 as a single agent. The concentrations of ABT-737 tested were 1 × 10⁻⁸ to 1 × 10⁻⁴ M in three-fold changes (1 × 10⁻⁸, 3 × 10⁻⁸, 1 × 10⁻⁷, 3 × 10⁻⁷, 1 × 10⁻⁶, 3 × 10⁻⁶, 1 × 10⁻⁵, 3 × 10⁻⁵, 1 × 10⁻⁴, 3 × 10⁻⁴, 1 × 10⁻³ M). The cells were incubated with vehicle control (dimethyl sulfoxide [DMSO]) or ABT-737 for 48 hours. Dose–response curves were determined by DIMSCAN. Each condition was tested in 12 replicates, and symbols represent the mean fractional survival; error bars correspond to 95% confidence intervals. Fractional survival was determined by mean fluorescence of the treated cells/mean fluorescence of control cells. B) Immunoblot analysis of Mcl-1 expression. The two most ABT-737–resistant cell lines (CCRF-CEM and NALM-6) and the two most ABT-737–sensitive cell lines (COG-LL-319 and RS4;11) were treated with vehicle (C, 0.025% DMSO), 2.5 µM ABT-737 for 6, 9, and 12 hours and then subjected to immunoblot analysis for Mcl-1. β-actin was used as a control for equal protein loading. C) Immunoblot analysis of basal expression of Bcl-2 family proteins in all seven ALL cell lines. Cells were collected in log-scale growth and lysed, and the same amount of protein was loaded for immunoblotting of Bcl-2 family proteins.

Results

Association Between Changes in Myeloid Cell Leukemia 1 Expression and ABT-737 Resistance in Acute Lymphoblastic Leukemia Cells

We first examined the cytotoxicity of ABT-737 as a single agent against seven human ALL cell lines and explored the relationship between ABT-737 cytotoxicity and Mcl-1 expression. Two cell lines (CCRF-CEM and NALM-6) were highly resistant to ABT-737 (the LC₉₀ were 8.5 µM [95% CI = 7.5 to 9.6 µM] and 5.8 µM [95% CI = 5.0 to 6.7 µM], respectively), and two (RS4;11 and COG-LL-319) were highly sensitive (the LC₉₀ were 0.1 µM [95% CI = 0.07 to 0.11 µM] and 0.1 µM [95% CI = 0.06 to 0.2 µM], respectively) (Figure 1, A and Table 1). In the ABT-737–resistant cell lines, Mcl-1 protein levels increased with increasing duration of exposure to ABT-737 (fold increase over 12 hours in Mcl-1 protein levels by ABT-737 compared with vehicle control for CCRF-CEM and NALM-6 were 1.2-fold [95% CI = 1.0- to 1.6-fold] and 4.3-fold [95% CI = 4.2- to 4.5-fold], respectively) (Figure 1, B). By contrast, in the two ABT-737–sensitive cell lines, Mcl-1 levels decreased with increasing duration of exposure to ABT-737 as a single agent.

Previous reports have suggested that high basal Bcl-2 and Bcl-X₇ expression is associated with sensitivity to ABT-737 in small cell lung cancer (21) and that basal Bcl-2 expression is associated with sensitivity to ABT-737 in acute myeloid leukemia (17). We therefore measured basal RNA and protein expression for Bcl-2 family members in the ALL cell lines (Table 2 and Figure 1, C). The basal mRNA and protein levels for Bcl-2 were both highest in the two cell lines that were the most sensitive to ABT-737 (ie, COG-LL-319 and RS4;11; Table 2, Figure 1, C). However, the correlation coefficient between Bcl-2 mRNA expression and the LC₉₀ for ABT-737 was not statistically significant across all seven cell lines (P = .09), and there were no statistically significant correlations between the LC₉₀ for either ABT-737 or 4-HPR and the mRNA expression levels of any of the Bcl-2 family members (P ≥ .09). There was also no apparent relationship between basal protein expression of any of the Bcl-2 family members and ABT-737 sensitivity (Figure 1, C). These data suggest that although Mcl-1 protein level is increased by ABT-737 in ALL cell lines that are relatively resistant to the drug and decreased by ABT-737 in ALL cell lines that are sensitive to the drug, sensitivity to ABT-737 or 4-HPR is not correlated with the expression of Bcl-2 family members.

Effect of N-(4-Hydroxyphenyl)Retinamide on Myeloid Cell Leukemia 1 Expression in Acute Lymphoblastic Leukemia Cell Lines

Treatment of the ABT-737–resistant ALL cell lines NALM-6 and CCRF-CEM with 4-HPR resulted in a concentration-dependent...
Because RS4;11 and COG-LL-319 cell lines had >90% cell kill by ABT-737 at 1 µM (the lowest concentration test for other ALL cell lines), the LC 90 values were

† Interpretation of CIN values: <0.1 = very strong synergism, 0.1 – 0.3 = strong synergism, 0.3 – 0.7 = synergism, 0.7 – 0.9 = slight synergism, 0.9 – 1.1 = additive
effect, and >1.1 = antagonism.

‡ Because RS4;11 and COG-LL-319 cell lines had >90% cell kill by ABT-737 at 1 µM (the lowest concentration test for other ALL cell lines), the LC 90 values were
defined in RS4;11 and COG-LL-319 using lower concentrations of ABT-737 (20–200 nM).

decrease in Mcl-1 protein expression (Figure 2, A). We therefore examined the effect of 4-HPR in combination with ABT-737 on Mcl-1 expression in the ALL cell lines. Combination treatment with 4-HPR plus ABT-737 resulted in decreased Mcl-1 protein expression in both ABT-737–resistant (ie, CCRF-CEM) and ABT-737–sensitive (ie, RS4;11 and COG-LL-319) ALL cell lines (Figure 2, B). Phosphatase treatment of lysates prepared from the cells that were treated with ABT-737 plus 4-HPR restored the Mcl-1 band that had disappeared in cells treated with ABT-737 plus 4-HPR, suggesting that phosphorylation of Mcl-1 was responsible for the decreased expression of Mcl-1 protein in all three cell lines (Figure 2, B).

We hypothesized that the putative phosphorylation of Mcl-1 in response to 4-HPR treatment of ALL cells occurred via JNK phosphorylation on the basis of a previous report that showed that Mcl-1 was phosphorylated and inactivated by oxidative stress in human embryonic kidney cells (55). To investigate this hypothesis, we examined Mcl-1 protein levels in CCFR-CEM cells that had been transfected with an siRNA designed to knock down JNK phosphorylation. To test whether this knockdown of JNK expression or a control siRNA and then treated with ABT-737, either alone or in combination with 4-HPR. Transfection of cells with JNK siRNA prevented the decrease in Mcl-1 protein level in CCFR-CEM cells that had been transfected with the control siRNA did not (Figure 2, C), suggesting that inhibition of Mcl-1 expression occurs downstream of JNK.

Next examined whether the putative phosphorylation of Mcl-1 is specific to 4-HPR treatment or reflects a general property of retinoids by incubating CCFR-CEM cells with ATRA or 13-cis-RA with or without ABT-737; 4-HPR (at 10 µM) was used as a positive control to confirm the assay conditions used to decrease Mcl-1. Mcl-1 in cells treated with 13-cis-RA, ATRA, or either drug in combination with ABT-737 was not decreased at 12 hours, whereas it was decreased at this time by 4-HPR, both alone and in combination with ABT-737 (Figure 2, D). Combining 13-cis-RA or ATRA with ABT-737 did not increase the cytotoxicity of ABT-737 in CCFR-CEM cells (data not shown). Thus, the inhibition of Mcl-1 expression that is induced by 4-HPR occurs via JNK and is a unique property of 4-HPR that is not observed with other retinoids.

Because 4-HPR is reported to activate JNK via ROS generation (56) and because we demonstrated that JNK plays a role in inhibiting Mcl-1 expression, we next investigated whether 4-HPR–stimulated ROS generation is involved in JNK phosphorylation in CCFR-CEM cells. Pretreatment of CCFR-CEM cells with the antioxidant ascorbic acid abrogated the generation of ROS induced by 4-HPR. The mean channel fluorescence values for ROS generation (FITC) by vehicle, 4-HPR (10 µM), and ascorbic acid plus 4-HPR were 87.3 (95% CI = 76 to 98), 250 (95% CI = 224 to 276), and 49.3 (95% CI = 40.5 to 58.1), respectively (Figure 3, A). Ascorbic acid pretreatment of CCFR-CEM cells also abrogated phosphorylation of JNK (Figure 3, B) and the decrease in Mcl-1 (Figure 3, C) induced by 4-HPR plus ABT-737, suggesting that the ROS generated by 4-HPR act to decrease

### Table 1. Combination cytotoxicity of ABT-737 and N-(4-hydroxyphenyl)retinamide in acute lymphoblastic leukemia cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ABT-737 µM (95% CI)</th>
<th>4-HPR µM (95% CI)</th>
<th>CIN (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCRF-CEM</td>
<td>8.5 (7.5 to 9.6)</td>
<td>1.5 (1.1 to 2.1)</td>
<td>0.22 (0.16 to 0.32)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>NALM-6</td>
<td>5.8 (5.0 to 6.8)</td>
<td>6.7 (5.2 to 8.8)</td>
<td>0.75 (0.70 to 0.80)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>COG-LL-317</td>
<td>4.6 (4.0 to 4.7)</td>
<td>2.9 (2.3 to 3.6)</td>
<td>0.74 (0.70 to 0.78)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>1.4 (0.9 to 2.0)</td>
<td>6.0 (3.7 to 9.6)</td>
<td>0.74 (0.71 to 0.78)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>MOLT-3</td>
<td>1.2 (0.7 to 2.4)</td>
<td>4.6 (3.3 to 5.7)</td>
<td>0.92 (0.83 to 1.03)</td>
<td>.13</td>
</tr>
<tr>
<td>RS4;11‡</td>
<td>0.1 (0.07 to 0.11)</td>
<td>0.4 (0.1 to 1.2)</td>
<td>0.29 (0.14 to 0.58)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>COG-LL-319‡</td>
<td>0.1 (0.06 to 0.2)</td>
<td>7.4 (4.5 to 12.1)</td>
<td>0.81 (0.66 to 0.99)</td>
<td>.038</td>
</tr>
</tbody>
</table>

* LC 90 = drug concentration that was lethal for 90% of the cells; CI = confidence interval; 4-HPR = N-(4-hydroxyphenyl)retinamide; CIN = combination index.

† Interpretation of CIN values: <0.1 = very strong synergism, 0.1 – 0.3 = strong synergism, 0.3 – 0.7 = synergism, 0.7 – 0.9 = slight synergism, 0.9 – 1.1 = additive effect, and >1.1 = antagonism.

### Table 2. Real-time reverse transcription–polymerase chain reaction assessment of mean basal mRNA levels (with 95% confidence intervals) for Bcl-2 family members in acute lymphoblastic leukemia cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Bcl-2</th>
<th>Bcl-X L</th>
<th>Bcl-w</th>
<th>Mcl-1</th>
<th>Bfl-1/A1</th>
<th>Bax</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCRF-CEM</td>
<td>0.10 (0.08 to 0.13)</td>
<td>0.98 (0.78 to 1.18)</td>
<td>0.14 (0.06 to 0.22)</td>
<td>0.43 (0.37 to 0.49)</td>
<td>10.51 (2.95 to 18.07)</td>
<td>0.82 (0.67 to 0.98)</td>
</tr>
<tr>
<td>NALM-6</td>
<td>0.23 (0.19 to 0.27)</td>
<td>0.73 (0.72 to 0.75)</td>
<td>0.28 (0.17 to 0.39)</td>
<td>0.83 (0.81 to 0.84)</td>
<td>0.15 (0.02 to 0.28)</td>
<td>0.52 (0.48 to 0.56)</td>
</tr>
<tr>
<td>COG-LL-317</td>
<td>0.13 (0.09 to 0.16)</td>
<td>1.32 (1.08 to 1.56)</td>
<td>–</td>
<td>0.32 (0.28 to 0.36)</td>
<td>0.76 (0.30 to 1.22)</td>
<td>0.98 (0.77 to 1.19)</td>
</tr>
<tr>
<td>MOLT-3</td>
<td>0.23 (0.12 to 0.34)</td>
<td>1.21 (1.01 to 1.41)</td>
<td>–</td>
<td>0.28 (0.24 to 0.32)</td>
<td>0.75 (0.64 to 0.85)</td>
<td>0.64 (0.54 to 0.73)</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>0.08 (0.05 to 0.11)</td>
<td>0.66 (0.53 to 0.79)</td>
<td>–</td>
<td>0.29 (0.26 to 0.32)</td>
<td>0.08 (0.03 to 0.13)</td>
<td>0.32 (0.28 to 0.36)</td>
</tr>
<tr>
<td>RS4;11</td>
<td>2.37 (2.16 to 2.58)</td>
<td>0.63 (0.43 to 0.84)</td>
<td>0.40 (0.13 to 0.67)</td>
<td>0.76 (0.74 to 0.79)</td>
<td>0.84 (0.72 to 0.96)</td>
<td>1.31 (1.11 to 1.50)</td>
</tr>
<tr>
<td>COG-LL-319</td>
<td>0.92 (0.74 to 1.10)</td>
<td>0.81 (0.40 to 1.22)</td>
<td>0.45 (0.40 to 0.51)</td>
<td>0.84 (0.66 to 1.02)</td>
<td>0.51 (0.45 to 0.56)</td>
<td>1.29 (1.17 to 1.42)</td>
</tr>
</tbody>
</table>

* Mean values (in arbitrary units) are for triplicate samples from three independent experiments. – = the gene was not amplified in these cell lines.
Mcl-1 via phosphorylation of JNK. In addition, cytotoxicity of ABT-737 plus 4-HPR was statistically significantly attenuated by ascorbic acid. The fractional survival of cells treated with ABT-737 plus 4-HPR with ascorbic acid pretreatment was higher than that of cells treated with ABT-737 plus 4-HPR without ascorbic acid pretreatment (74% vs 46%; difference = 28%, 95% CI = 21% to 34%, P < .001) (Figure 3, D).

Thus, 4-HPR appears to act via the generation of ROS to inactivate Mcl-1 via JNK phosphorylation and enhance the cytotoxicity of ABT-737 in ALL cell lines. We also examined whether the differential effects of 13-cis-RA, ATRA, and 4-HPR on Mcl-1 protein expression were due to differences in the abilities of these retinoids to stimulate ROS generation and found that neither ATRA nor 13-cis-RA induced ROS generation in CCRF-CEM cells (data not shown). Thus, we conclude that the generation of ROS is upstream of JNK phosphorylation and inhibition of Mcl-1 expression by 4-HPR and that ATRA and 13-cis-RA do not generate ROS, which may be the reason that these retinoids do not affect Mcl-1.

**Combination Cytotoxicity of ABT-737 and N-(4-Hydroxyphenyl)retinamide in Acute Lymphoblastic Leukemia Cell Lines and in Normal Lymphocytes**

Our observations—that Mcl-1 protein expression in ABT-737–treated ALL cells correlated with resistance to ABT-737 and that 4-HPR acts via JNK phosphorylation to inhibit the expression of Mcl-1 and enhance the cytotoxicity of ABT-737—suggested that the combination of 4-HPR plus ABT-737 might have a synergistic effect on cytotoxicity in ALL. We therefore examined the cytotoxicity profiles of ABT-737, 4-HPR, and ABT-737 plus 4-HPR in the seven ALL cell lines, proliferating (nonmalignant) T-lymphoblasts, and normal nonproliferating lymphocytes (Figure 4, A–C). In the ALL cell lines, the ABT-737 single-agent LC₉₀ values ranged from 100 nM for COG-LL-319 and RS4;11 cells to low micromolar concentrations for the other five ALL cell lines (Table 1). Because RS4;11 and COG-LL-319 cells are 50- to 80-fold more sensitive to ABT-737 than CCRF-CEM or NALM-6 cells, 1 µM ABT-737 (which is the lowest concentration test for all other ALL cell lines) killed more than 90% of the cells. Consequently, the LC₉₀ determined using the concentration range (1–10 µM) may not be accurate in the two cell lines. Thus, the LC₉₀ values and combination cytotoxicity were redefined for RS4;11 and COG-LL-319 cells using lower concentrations of ABT-737 (20–200 nM). 4-HPR synergistically enhanced the cytotoxicity of ABT-737 in all seven ALL cell lines: CIN values at LC₉₀ ranged from 0.22 (for CCRF-CEM cells) to 0.92 (for MOLT-3 cells) (mean CIN = 0.57, 95% CI = 0.37 to 0.87).

We also assessed the cytotoxic activity of 4-HPR plus ABT-737 in actively proliferating nonmalignant T-lymphoblasts and in normal resting lymphocytes (Figure 4, C). 4-HPR alone and 4-HPR plus ABT-737 showed substantial cytotoxicity against actively proliferating T-lymphoblasts, but ABT-737 alone did not (Figure 4, C, left). However, neither ABT-737 nor 4-HPR was cytotoxic as a single agent against nonproliferating lymphocytes at concentrations up to 10 µM, and the combination was only minimally cytotoxic (Figure 4, C, right). Consistent with the latter observation, whereas exogenous H₂O₂ treatment of normal resting lymphocytes increased ROS generation, 4-HPR treatment did not induce generation of ROS (Figure 4, D) or phosphorylation of JNK (Figure 4, E). We also tested the cytotoxicity of 4-HPR plus ABR-737 in other human hematopoietic cell lines. In two human acute myelogenous leukemia cell lines (CHLA-221 and Kasumi-1), ABT-737 plus 4-HPR showed greater cytotoxicity than either drug alone (data not shown). Two lymphoid cell lines (HuT-78 and GA-10) were relatively insensitive to ABT-737, and 4-HPR enhanced ABT-737 cytotoxicity but only at relatively high concentrations (10 µM for both ABT-737 and 4-HPR) (data not shown).
Effect of ABT-737 Plus N-(4-Hydroxyphenyl)Retinamide on Mitochondrial Membrane Depolarization, Cytochrome c Release, and Apoptosis

We next examined the effect of 4-HPR plus ABT-737 on mitochondrial membrane potential and the release of cytochrome c from mitochondria to the cytosol in COG-LL-317 cells. Cells treated with ABT-737 plus 4-HPR displayed greater mitochondrial membrane depolarization (Figure 5, A, upper panels) and increased apoptosis, as demonstrated by annexin V binding (Figure 5, A, lower panels), than cells treated with either drug alone. Mitochondrial membrane depolarization was substantially and statistically significantly greater in cells treated with ABT-737 plus 4-HPR than in cells treated with ABT-737 as a single agent (% depolarized at 6 hours, ABT-737 vs ABT-737 plus 4-HPR: 24.5%...
Figure 4. Effect of ABT-737, N-(4-hydroxyphenyl)retinamide (4-HPR), and ABT-737 plus 4-HPR on cytotoxicity in acute lymphoblastic leukemia (ALL) cell lines and human hematopoietic cells. A) Dose–response curves for ABT-737 (empty circles), 4-HPR (empty triangles), and ABT-737 plus 4-HPR (filled triangles) in leukemia cell lines. Cytotoxicity was evaluated after treating cells with vehicle, ABT-737, 4-HPR, or ABT-737 plus 4-HPR for 48 hours. The fractional survival was determined by mean fluorescence of the treated cells/mean fluorescence of control cells. Each point represents the mean value for 12 replicates, error bars correspond to 95% confidence intervals.

B and C) Dose–response curves for ABT-737 (empty circles), 4-HPR (empty triangles), and ABT-737 plus 4-HPR (filled triangles) in ALL cell lines with the highest sensitivity to ABT-737 and in T-lymphocytes and normal resting lymphocytes. Because RS4;11 and COG-LL-319 cell lines were highly sensitive to ABT-737 as a single agent relative to other ALL cell lines, a lower range of concentrations of ABT-737 (20–200 nM) was employed for these two cell lines. Normal T-lymphoblasts and normal lymphocytes were tested using the same concentrations of 4-HPR and ABT-737 that were used for the ALL cell lines in panel A. In cytotoxicity experiments, each point represents the mean value for 12 replicates, and error bars represent 95% confidence intervals. Error bars that are smaller than the size of the symbol are not shown. D and E) Reactive oxygen species (ROS) generation and c-Jun kinase (JNK) phosphorylation by 4-HPR in normal lymphocytes. Normal resting lymphocytes were treated with 10 µM 4-HPR (D, right, solid line) or vehicle (D, dotted line) for 6 hours, and ROS were detected by incubating cells with 2,7′-dichlorofluorescein diacetate for 15 minutes, and the green fluorescence emitted from cells was measured by flow cytometry. The left panel of (D) shows the effect of hydrogen peroxide (H₂O₂, positive control, solid line) in ROS measurement relative to vehicle effect (dotted line). Phosphorylated JNK levels in cells treated with 4-HPR (E, right, solid line) or vehicle (E, dotted line) were detected using rabbit polyclonal antibody that recognizes JNK (primary antibody) and PE–conjugated anti-rabbit IgG (secondary antibody) that binds to the primary antibody. After incubating cells with the primary and the secondary antibodies in sequence, cells were washed and resuspended in 500 µL of staining buffer and then analyzed by flow cytometry using a band-pass filter of 575 ± 26 nm for PE. The left panel shows the isotype control used for detecting nonspecific binding of the antibody (for our experiment, it is p-JNK). Histogram of isotype control is not shifted to the right compared with control, indicating a lack of nonspecific binding. The histograms shown are representative of results obtained in three separate experiments.
Figure 5. Effect of N-(4-hydroxyphenyl)retinamide (4-HPR) plus ABT-737 on mitochondrial membrane depolarization, annexin V binding, cytochrome c release, and expression of proapoptotic Bcl-2 family proteins of acute lymphoblastic leukemia (ALL) cells. A) Mitochondrial membrane depolarization and apoptosis. COG-LL-317 cells were incubated with vehicle (Control, 0.025% dimethyl sulfoxide [DMSO] and 0.1% ethanol), ABT-737 (2.5 µM), 4-HPR (5 µM), or the combination for 6 hours in 5% oxygen. Cells were stained with JC-1 (upper panels) to assess the loss of mitochondrial membrane potential or with propidium iodide (PI) and annexin V–fluorescein isothiocyanate (FITC) (lower panels) to assess phosphatidylserine exposure, an early marker of apoptosis, and subjected to flow cytometry. Diagonal lines in the upper panels separate cell populations with green fluorescence (indicator of mitochondrial membrane depolarization) from those with red fluorescence. The transition from red to green fluorescence indicates a loss of mitochondrial membrane potential (percentages indicate the proportion of the cells with depolarized [green] mitochondrial membrane). In the lower panels, cells in the lower right quadrants are annexin V–FITC–positive and PI-negative (defined as early apoptosis) and those in the upper right quadrants are positive for both annexin V–FITC and PI (defined as late apoptosis). B) Mitochondrial membrane depolarization. Loss of mitochondrial membrane potential in response to treatment of COG-LL-317 cells with vehicle (Control, 0.05% DMSO and 0.05% ethanol), 5 µM ABT-737, 5 µM 4-HPR, or the combination at 2, 4, and 6 hours was detected using the JC-1 probe and flow cytometry. The intensity of JC-1 green fluorescence represents the depolarized mitochondrial membrane; the analysis was limited to the live cell fraction, which was selected using narrow angle forward light scatter (forward scatter) and side angle scatter (side scatter). Mean values and 95% confidence intervals (error bars) are shown for duplicate samples assayed in three separate experiments. Statistically significant differences from controls were observed beginning at 2 hours of drug exposure with ABT-737 and with ABT-737 plus 4-HPR (P < .001 for both conditions compared with control; two-sided Student t test). C) Cytochrome c release. COG-LL-317 cells were incubated with vehicle (Control, 0.05% DMSO and 0.05% ethanol), ABT-737 (2.5 or 5 µM), 4-HPR (2.5 or 5 µM), or the combination (2.5 or 5 µM for both) for 6 hours and then used to prepare cytosolic extracts, which were subjected to immunoblot analysis with a monoclonal antibody to cytochrome c. Immunoblotting for β-actin was used as a control for equal protein loading. Data shown are representative of those obtained in three separate experiments. D and E) Proapoptotic Bcl-2 family protein expression in COG-LL-317 cells (D) and RS4;11 cells (E). Cells were treated with ABT-737 (2.5 µM), 4-HPR (5 µM), or the combination for 6, 9, and 12 hours (COG-LL-317) or 3, 6, and 9 hours (RS4;11). Whole cell lysates from the two cell lines were prepared and subjected to immunoblot analysis with antibodies to Bax, Bad, and Bid. Bax-L = the long form of Bax, Bax-S = the short form of Bax (an active form of Bax), tBid = truncated Bid (active form of Bid). Data are representative of three experiments. Arrow indicates dephosphorylated Bad (24 kDa).
Effect of 4-HPR on Activation and Expression of Proapoptotic Bcl-2 Family Proteins

We next evaluated the effect of ABT-737 and 4-HPR, alone and in combination, on the expression of proapoptotic Bcl-2 family proteins in COG-LL-317 (Figure 5, D) and RS4;11 (Figure 5, E) cells. In both cell lines, the short form of Bax (Bax-S), a key protein in cytochrome c release (57), accumulated steadily with increasing times of exposure to ABT-737 and, to a greater extent, to ABT-737 plus 4-HPR. In COG-LL-317 cells, ABT-737 activated Bid (to its truncated form, tBid) and 4-HPR did so to a lesser extent, whereas 4-HPR plus ABT-737 produced the most tBid. In RS4;11 cells, the level of tBid increased to a similar extent in response to increasing time of exposure to ABT-737 and ABT-737 plus 4-HPR but not in response to 4-HPR alone.

In RS4;11 cells treated with ABT-737 or ABT-737 plus 4-HPR, the level of phosphorylated Bad (25 kDa) was lower than that in control-treated cells, and the levels of dephosphorylated Bad (24 kDa), the active form of this protein (8,58), increased to readily detectable levels. In COG-LL-317 cells, the levels of phosphorylated Bad was also decreased by ABT-737 and was even further decreased by ABT-737 plus 4-HPR compared with control (Figure 5, D). These results show that ABT-737 and ABT-737 plus 4-HPR increase levels of proapoptotic Bcl-2 family proteins, suggesting that the intrinsic apoptosis pathway is the major cytotoxic mechanism for ABT-737, both alone and in combination with 4-HPR.

Effect of ABT-737 Plus N-(4-Hydroxyphenyl)Retinamide on Activation of Caspase-9, -3, and -8

Caspases are the key players in the execution of apoptotic cascade, and they are activated during apoptosis (57). The two major pathways of apoptosis (intrinsic and extrinsic) converge on activation of caspases, resulting in the morphologic and biochemical changes associated with apoptosis (59). Caspase-9, -3, and -8 were activated almost simultaneously in COG-LL-317 cells treated with ABT-737 or with ABT-737 plus 4-HPR, as measured by the release of pNA from the caspases substrates. The concentrations of pNA released were statistically significantly higher in cells treated with ABT-737 plus 4-HPR than in cells treated with ABT-737 alone at 6 hours for all three caspases (pNA released from caspase-3 substrate, ABT-737 vs ABT-737 plus 4-HPR: 6.3 nM vs 9.0 nM, difference = 2.7 nM, 95% CI = 2.0 to 3.4 nM, P = .02; pNA released from caspase-8 substrate ABT-737 vs ABT-737 plus 4-HPR: 7.9 nM vs 3.2 nM, difference = 4.7 nM, 95% CI = 3.3 to 6.2 nM, P < .001; pNA released from caspase-9 substrate ABT-737 vs ABT-737 plus 4-HPR: 5.2 nM vs 9.2 nM, difference = 4.0 nM, 95% CI = 2.2 to 5.8 nM, P < .001) (Figure 6, A).

To investigate whether caspase activation plays a role in cytotoxicity of ABT-737 or 4-HPR as single agents or in combination, we pretreated the cells with the pan-caspase inhibitor Boc-d-fmk before the cells were treated with ABT-737, 4-HPR, or the combination. Fractional survival was then measured using the DIMSCAN system. Only 41.2% of cells treated for 9 hours with ABT-737 alone survived, compared with 87.7% of cells pretreated with Boc-d-fmk before ABT-737 treatment (difference = 46.5%, 95% CI = 41.2% to 50.8%; P < .001) (Figure 6, B). By contrast, 4-HPR-induced cytotoxicity was not reversed by pretreatment with Boc-d-fmk, suggesting that the main cytotoxic effect of 4-HPR in COG-LL-317 cells was caspase independent. Only 11.4% of cells treated for 9 hours with ABT-737 plus 4-HPR survived, compared with 73.3% of cells pretreated with Boc-d-fmk before treatment with ABT-737 plus 4-HPR (difference = 62.9%, 95% CI = 59.7% to 67.3%; P < .001) (Figure 6, B). Thus, the cytotoxicity of ABT-737 alone or in combination with 4-HPR was, in large part, caspase dependent, and the fraction of combination cytotoxicity that was not reversed by a pan-caspase inhibitor (ie, caspase independent) was likely due to 4-HPR.

Discussion

ABT-737 is a small-molecule inhibitor of the Bcl-2 family of proteins (60,61). Inhibition of the antiapoptotic members of the Bcl-2 family of proteins is a novel and promising approach to cancer chemotherapy. Because ABT-263, an orally bioavailable form of ABT-737, is now being tested in clinical trials (ie, trial number NCI-07-C-0006, available at http://www.clinicaltrials.gov), it is important to define the molecular mechanisms that determine the sensitivity to the drug(s). We have previously reported that susceptibility to ABT-737 varied widely in a panel of ALL cell lines and xenografts (23). In this study, we found that 4-HPR inhibited Mcl-1 first via ROS generation and second via JNK phosphorylation. We also demonstrated that combining ABT-737 with 4-HPR resulted in greater cytotoxicity, cytochrome c release, caspase activation, and apoptosis (measured by annexin V binding) than either single agent.

We found that the levels of antiapoptotic Bcl-2 family members in ALL cell lines were not associated with sensitivity to ABT-737. The basal expression level of Mcl-1, to which ABT-737 has a low affinity (15), has been associated with resistance to ABT-737 in many cancer types (17,19–21,25,26). We found that in ALL cell lines, resistance to ABT-737 was associated with an increase in Mcl-1 protein levels in response to ABT-737 rather than with the basal level of Mcl-1 expression. In addition, we observed that the increase of Mcl-1 by ABT-737 in ABT-737–resistant ALL cell lines was inhibited by the addition of 4-HPR. Our finding that the decrease in Mcl-1 by 4-HPR was not a general property of retinoids is consistent with a previous report that ATRA increases Mcl-1 levels in NB4 and R4 leukemia cells (62). The inhibition of Mcl-1
expression by 4-HPR is a novel observation, and the inhibition of Mcl-1 expression is likely to underlie, at least in part, the synergistic cytotoxicity between ABT-737 and 4-HPR in ALL cell lines.

We demonstrated that the inhibition of Mcl-1 expression by 4-HPR is via ROS generation and JNK phosphorylation. Activation of mitogen-activated protein kinases by 4-HPR–induced ROS has been reported (27,63), and oxidative stress induced by exogenous hydrogen peroxide can phosphorylate and inactivate Mcl-1 via activation of JNK (55). We showed that the decrease in Mcl-1 by 4-HPR occurs via generation of ROS by abrogating 4-HPR–induced ROS generation using ascorbic acid. Abrogation of ROS also resulted in inhibition of 4-HPR–induced Mcl-1 phosphorylation. Thus, ROS generation is upstream of JNK activation and subsequent Mcl-1 inhibition by 4-HPR. The siRNA experiments showed that JNK phosphorylation is in turn upstream of Mcl-1 inhibition. Thus, ROS generation appears to be the main upstream mechanism by which 4-HPR caused JNK activation and the inhibition of Mcl-1 expression. When ROS generation was abrogated, the cytotoxicity of 4-HPR was attenuated in ALL cells. In normal lymphocytes, however, 4-HPR did not generate ROS or phosphorylate JNK. This finding is consistent with the observation that 4-HPR minimally affected the survival of normal lymphocytes and with previous observations that 4-HPR cytotoxicity is malignancy specific (29). Together, these results suggest that ROS generation plays a critical role in the selected nature of 4-HPR cytotoxicity for cancer cells relative to normal cells.

The two drugs we studied—4-HPR and ABT-737—have been reported to have relatively tumor-specific cytotoxicity in clinical trials (4-HPR) (41) and minimal systemic toxicity for normal tissues in animal studies (ABT-737) (15). The concentrations of 4-HPR (up to 10 μM) used in this study were within the concentration range that we have achieved in children in clinical trials (41,64); the ABT-737 concentrations that we tested were in the range that was found to be tolerable and active in mouse xenograft models (15). Although neither drug alone nor the combination was highly cytotoxic for normal resting lymphocytes, 4-HPR and 4-HPR plus ABT-737 (but not ABT-737 alone) were cytotoxic for normal T-lymphocytes that were stimulated to proliferate as lymphoblasts. The minimal toxicity of 4-HPR in normal lymphocytes is consistent with the minimal systemic (including hematopoietic) toxicity that has been observed in patients who were treated with 4-HPR (41,64) and suggests that the combination of ABT-737 plus 4-HPR may have minimal hematopoietic toxicity in vivo.

The cytotoxicity of ABT-737 as a single agent and the combination cytotoxicity of ABT-737 plus 4-HPR in ALL cell lines occurred largely via caspase-dependent apoptosis. In addition, the

Figure 6. Effect of N-(4-hydroxyphenyl)retinamide (4-HPR) and ABT-737 on caspase activation. A) Caspase-9, -3, and -8 activation. COG-LL-317 cells were incubated with vehicle (Control, 0.1% dimethyl sulfoxide + 0.05% ethanol), ABT-737 (2.5 μM), 4-HPR (5 μM), and the combination for 1, 2, 3, 4, 6, and 12 hours. Caspase activity was measured by using caspase substrates labeled with p-nitroaniline (pNA) using colorimetric assay that detects pNA released by activated caspase in lysates of the cells treated with drugs. The symbols represent vehicle control (filled circles), ABT-737 (empty circles), 4-HPR (empty triangles), and ABT-737 plus 4-HPR (filled triangles).

The data represent the mean values for triplicate measurements from three experiments; error bars correspond to 95% confidence intervals. B) Reversal of cytotoxicity by the pan-caspase inhibitor Boc-d-fmk. COG-LL-317 cells were seeded in 96-well microplates, pretreated with Boc-d-fmk (80 μM) for 1 hour, and then incubated with ABT-737 (5 μM), 4-HPR (5 μM), or ABT-737 plus 4-HPR (both at 5 μM) for 9 hours. Cytotoxicity was measured using the DIMSCAN system. The fractional survival was determined by mean fluorescence of the treated cells/mean fluorescence of control cells treated with vehicle. Each point represents the mean value for 12 replicates (three wells that were unequivocal statistical outliers were excluded); error bars represent 95% confidence intervals. *P < .001 (two-sided Student t test).
loss of mitochondrial membrane potential and annexin V binding (apoptosis) were statistically significantly greater with ABT-737 plus 4-HPR than with either drug alone. Levels of several proapoptotic Bcl-2 family proteins (ie, Bax-s, tBid, dephosphorylated Bad) increased in response to ABT-737 in both ABT-737–sensitive and ABT-737–resistant ALL cell lines and, to a greater extent, in response to ABT-737 plus 4-HPR in an ABT-737–resistant ALL cell line. These results indicate that 4-HPR enhanced the activity of ABT-737 to activate Bax and promote truncation of Bid in cells resistant to ABT-737.

We have previously shown that ABT-737 alone and in combination with 1-asparaginase, vincristine, and dexamethasone (a backbone regimen in the treatment of leukemia) caused synergistic cytotoxicity in ALL cell lines and statistically significantly increased event-free survival in nonobese diabetic/severe combined immunodeficient (nod/SCID) mouse xenograft models of ALL [23]. However, we were unable to demonstrate a statistically significant increase in event-free survival of mice carrying human ALL xenografts treated with ABT-737 in combination with 4-HPR [given as Lymxsorb/4-HPR oral powder] and that 4-HPR plasma concentrations achievable in nod/SCID mice were less than 5 µM (V. Khankalidyan, BS, N. Harutyunyan, BS, B. J. Maurer, MD,PhD, C. P. Reynolds, MD,PhD, M. H. Kang, PharmD, unpublished observations). Thus, the 4-HPR levels required to achieve Mcl-1 inhibition in ALL cells in vitro (approximately 10 µM, see Figure 2, A) could not be achieved in nod/SCID mice. We have previously shown that the optimal way to achieve 4-HPR levels that inhibit the growth neuroblastoma xenograft tumors in nu/nu mice is with the use of Lymxsorb/4-HPR oral powder [4-HPR formulated in a novel organized lipid matrix (26)], which achieves 4-HPR plasma levels of approximately 9 µM but much lower levels in most tissues (39). By contrast, we have recently demonstrated in adult human clinical trials that it is possible to achieve steady-state plasma levels of 4-HPR greater than 50 µM and that 4-HPR at such levels is well tolerated and active against refractory lymphoid malignancies (65). Thus, our inability to replicate the reversal of ABT-737 resistance by 4-HPR (which was observed in all ALL cell lines treated with ABT-737 plus 4-HPR) in ALL xenograft models is likely due to the limited 4-HPR exposures achievable in mice, which are approximately 10-fold lower than the 4-HPR levels obtained with a novel intravenous formulation in humans. Because of these limitations in using mouse models of ALL to test 4-HPR, the ability of 4-HPR to reverse resistance to BH3-mimetic drugs in vivo will have to be assessed in clinical trials.

This study has several limitations. First, the sample size (n = 7 cell lines) was not sufficient to determine a statistically significant correlation between the expression of Bcl-2 family members and sensitivity to ABT-737; although Bcl-2 mRNA levels were higher in cell lines that were sensitive to ABT-737 than in cell lines that were resistant to ABT-737, they were not statistically significantly higher. Second, we could not directly demonstrate Mcl-1 phosphorylation by 4-HPR because we did not have suitable reagents. Instead, we could only show indirectly that the decrease in Mcl-1 protein was reversed when cell lysates were treated with λ-phosphatase. Future development of a stable antibody against phospho-Mcl-1 should enable direct detection of phosphor-Mcl-1. Third, the mechanisms underlying different responses of Mcl-1 (increase or decrease) to ABT-737 observed in the tested ALL cell lines remain to be elucidated. We are currently examining whether mutation of the phosphorylation sites in Mcl-1 affects its degradation in response to ABT-737 in sensitive cell lines and whether the mechanism for the increased expression of Mcl-1 in response to ABT-737 in resistant cell lines is transcriptional, posttranscriptional, or posttranslational. In addition, future experiments need to identify which of the 10 isoforms of JNK identified to date (66–68) are responsible for regulating Mcl-1 expression.

In summary, we have demonstrated that 1) in ABT-737–resistant ALL cell lines, Mcl-1 protein levels increased when cells were treated with ABT-737 and decreased when cells were treated with 4-HPR; 2) the inhibition of Mcl-1 expression by 4-HPR occurred via JNK phosphorylation downstream of ROS generation; 3) ABT-737 plus 4-HPR was synergistically cytotoxic against ALL cell lines that were cultured in oxygen conditions that replicate the hypoxic conditions found in bone marrow (5% oxygen), and the combination cytotoxicity was mainly via caspase-dependent apoptosis; and 4) 4-HPR plus ABT-737 was minimally cytotoxic for normal lymphocytes. Thus, combining ABT-737 with 4-HPR achieved a striking synergistic cytotoxicity against ALL cell lines via a novel mechanism, namely, the inactivation of the majority of antiapoptotic Bcl-2 family proteins. Our data suggest that further preclinical studies as well as clinical trials of BH3-mimetic drugs in combination with 4-HPR—including testing this novel combination together with other antineoplastic agents—are warranted.

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