

Synergistic Activity of Fenretinide and the Bcl-2 Family Protein Inhibitor ABT-737 against Human Neuroblastoma

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Abstract

Purpose: Fenretinide (4-HPR) is a cytotoxic retinoid with minimal systemic toxicity that has shown clinical activity against recurrent high-risk neuroblastoma. To identify possible synergistic drug combinations for future clinical trials, we determined whether ABT-737, a small-molecule BH3-mimetic that inhibits most proteins of the antiapoptotic Bcl-2 family, could enhance 4-HPR activity in neuroblastoma.

Experimental Design: Eleven neuroblastoma cell lines were tested for the cytotoxic activity of 4-HPR and ABT-737 as single agents and in combination using the DIMSCAN fluorescence digital imaging cytotoxicity assay. The effect of these agents alone and in combination on mitochondrial membrane depolarization and apoptosis (by flow cytometry), cytochrome *c* release, caspases, Bax- α , t-Bid, and Bak activation, and subcutaneous xenografts in *nu/nu* mice was also determined.

Results: Multilog synergistic cytotoxicity was observed for the drug combination in all of the 11 neuroblastoma cell lines tested, including MDR lines and those insensitive to either drug as single agents. 4-HPR + ABT-737 induced greater mitochondrial membrane depolarization and mitochondrial cytochrome *c* release, greater activation of caspases, Bax- α , t-Bid, and Bak, and a higher level of apoptosis than either drug alone. *In vivo*, 4-HPR + ABT-737 increased the event-free survival of the MDR human neuroblastoma line CHLA-119 implanted subcutaneously in *nu/nu* mice (194.5 days for the combination vs. 68 days for ABT-737 and 99 days for 4-HPR).

Conclusion: Thus, the combination of 4-HPR with a BH3-mimetic drug warrants clinical trials in recurrent neuroblastoma. *Clin Cancer Res*; 17(22); 7093–104. ©2011 AACR.

Introduction

Neuroblastoma is an aggressive childhood tumor of the sympathetic nervous system accounting for 8% to 10% of all childhood cancers and approximately 15% of cancer deaths in children (1). Treatment of high-risk neuroblastoma (stage 4 patients above 1 year at diagnosis and stage 3 disease with *MYCN* amplification or unfavorable histopathology) with multiagent chemotherapy, radiotherapy, and myeloablative chemotherapy supported stem cell trans-

plant followed by treatment of minimal residual disease by 13-*cis*-retinoic acid (13-*cis*-RA; refs. 2, 3), and more recently, anti-GD2 antibody, cytokines, and 13-*cis*-RA have significantly improved the outcome for these patients (4, 5). However, as many high-risk patients still ultimately die from tumor that is refractory to initial therapy or recurrent, resistant disease, novel therapies effective against MDR neuroblastoma are needed.

4-HPR is a synthetic derivative of retinoic acid that has a broad spectrum of cytotoxic activity against primary tumor cells, cell lines, and/or xenografts of various cancers (including neuroblastoma; refs. 6–9) and has been tested in early phase clinical trials in recurrent neuroblastoma (10–12). In contrast to retinoids, such as all-*trans*-retinoic acid (ATRA) and 13-*cis*-RA, that cause arrest of cell growth and morphologic differentiation of human neuroblastoma cell lines, 4-HPR induces cell death in neuroblastoma cells via both apoptotic and nonapoptotic mechanisms (6–8). The observation that 4-HPR remains cytotoxic in neuroblastoma cell lines that are resistant to ATRA, 13-*cis*-RA, alkylating agents, and etoposide suggests that it may be active in high-risk neuroblastoma patients who are resistant to standard therapy (7, 9, 13). Indeed, a recent phase I trial in patients with refractory/recurrent neuroblastoma of a novel oral powder 4-HPR

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Translational Relevance

Fenretinide (4-HPR) is a synthetic retinoid that has broad-spectrum *in vitro* cytotoxicity against various cancers and clinical activity against recurrent high-risk neuroblastoma. Cell lines established from patients with recurrent neuroblastoma exhibit increased levels of Bcl-2 expression which may confer drug resistance. We determined whether ABT-737, a BH3-mimetic small-molecule inhibitor of Bcl-2, Bcl-XL, and Bcl-w, could enhance activity of 4-HPR against laboratory models of neuroblastoma. We show a positive synergistic interaction between 4-HPR and ABT-737 in neuroblastoma cell lines through their inhibitory activity against caspase-dependent apoptosis involving both intrinsic and extrinsic pathways. Consistent with their synergistic *in vitro* activity, we show a similar antineuroblastoma activity in a mouse xenograft model of recurrent, MDR neuroblastoma. These data support clinical trials combining 4-HPR with BH3-mimetic drugs in children with recurrent neuroblastoma.

formulation (4-HPR LXS oral powder) that improves 4-HPR exposures documented 4 complete responses (11).

Overexpression of antiapoptotic Bcl-2 family proteins is a common mechanism by which cells become resistant to conventional chemotherapy, providing attractive therapeutic targets (14, 15). Neuroblastoma cell lines obtained from patients with recurrent disease after treatment exhibit increased levels of Bcl-2 expression, which may be responsible for their drug resistance (16). Strong Bcl-2 immunoreactivity was also detected in islets of residual neuroblastoma cells in *MYCN* nonamplified primary tumors of treated patients (17). These observations suggest that an increased expression of antiapoptotic Bcl-2 family of proteins may be one mechanism for the resistance of neuroblastoma cells to cytotoxic agents including 4-HPR.

ABT-737 is a small molecule that mimics the direct binding of the BH3-only protein Bad to antiapoptotic Bcl-2 family proteins such as Bcl-2, Bcl-X_L, and Bcl-w and therefore displaces BH3-only death-activating proteins (Bim, Bid; ref. 14). By binding to proteins of the Bcl-2 family, ABT-737 prevents proteins, such as Bid and Bim (direct activators of Bax and Bak), from forming heterodimers with antiapoptotic Bcl-2 family proteins, thereby promoting Bax and Bak activation via oligomerization (18). ABT-737 has been reported to be cytotoxic as a single agent and to sensitize a wide variety of cancer cells to several chemotherapeutic agents *in vitro* and *in vivo* (14, 19–23). We have previously shown that ABT-737 synergistically enhances 4-HPR cytotoxicity in acute lymphoblastic leukemia cells but that 4-HPR + ABT-737 was not toxic for normal nonproliferating lymphocytes (24).

We therefore hypothesized that the combination of an inhibitor of proteins of the Bcl-2 family (such as ABT-737)

with 4-HPR would be synergistic against neuroblastoma with minimal systemic toxicities. To test this hypothesis, we evaluated the activity of ABT-737 and 4-HPR (alone and in combination) on a panel of human neuroblastoma cell lines and in a xenograft model of recurrent MDR human neuroblastoma.

Materials and Methods

Cell culture

We used a panel of 11 human neuroblastoma cell lines obtained from patients at various stages of disease: 2 cell lines established at diagnosis prior to any therapy (CHLA-15 and SMS-KAN), 7 cell lines obtained at the time of progressive disease during induction therapy [SK-N-BE (2), SK-N-RA, CHLA-119, LA-N-6, CHLA-20, SMS-KCNR, and CHLA-140], and 2 cell lines established at relapse after myeloablative therapy and bone marrow transplantation (CHLA-79 and CHLA-136). All cell lines were established in the senior author's laboratory, except SK-N-RA and SK-N-BE (2) which were a gift of Dr. L. Helson; characterization of these neuroblastoma cell lines has been previously reported (25, 26). Cell line identity was confirmed at time of the experiments using a 15-loci short tandem repeat (STR) assay + amelion for sex determination (27), with the genetic signature compared with the Children's Oncology Group STR database (www.COGcell.org). We also tested the human normal fibroblast cell line CRL-2076 obtained from the American Type Culture Collection.

SMS-KAN, SK-N-BE(2), SK-N-RA, LA-N-6, SMS-KCNR, and CRL-2076 fibroblasts were cultured in RPMI-1640 medium (Irvine Scientific) supplemented with 10% heat-inactivated FBS (Gemini Bio-Products, Inc.). CHLA-15, CHLA-119, CHLA-20, CHLA-140, CHLA-79, and CHLA-136 were cultured in Iscove's Modified Dulbecco's Medium (BioWhittaker) containing 20% heat-inactivated FBS and supplemented with 3 mmol/L L-glutamine (Gemini Bio-Products, Inc.), insulin, and transferrin (5 µg/mL each) and selenium (5 ng/mL; ITS Culture Supplement, Collaborative Biomedical Products). All cell lines were continuously cultured at 37°C in a humidified incubator containing 95% air + 5% CO₂ without antibiotics. Experiments were carried out using neuroblastoma cell lines at passage 15 to 35. Cells were detached from culture plates or flasks with the use of a modified Puck's Solution A plus EDTA (Puck's EDTA), containing 140 mmol/L NaCl, 5 mmol/L KCl, 5.5 mmol/L glucose, 4 mmol/L NaHCO₃, 0.8 mmol/L EDTA, 13 µmol/L phenol red, and 9 mmol/L HEPES buffer (pH = 7.3; ref. 28).

Drugs and reagents

ABT-737 was kindly provided by Abbott Laboratories. 4-HPR was obtained from the Developmental Therapeutics Program of the National Cancer Institute (Bethesda, MD). Fenretinide was formulated as LYM-X-SORB oral powder (3% 4-HPR by weight, 4-HPR LXS) by Avanti Polar Lipids, Inc. (29) and was kindly provided to the investigators by Barry J. Maurer, MD, PhD. Eosin Y was purchased from Sigma Chemical Co., and fluorescein diacetate (FDA)

was obtained from Eastman Kodak Co. Mitochondrial membrane potential probe JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide) was obtained from Molecular Probes; the Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Kit and the caspase-8 enzyme inhibitor Z-IETD-FMK were obtained from BD Biosciences (APO-DIRECT). The pan-caspase enzyme inhibitor Boc-d-fmk was purchased from MP Biomedicals, LLC. Stock solutions of ABT-737 (5 mmol/L), 4-HPR (10 mmol/L), FDA (1 mg/mL), JC-1 (2 mg/mL), Z-IETD-FMK (10 mmol/L), and Boc-d-fmk (20 mmol/L) were dissolved in dimethyl sulfoxide, except 4-HPR which was dissolved in 95% ethanol. All reagents were stored at -20°C .

Cytotoxicity assay

The cytotoxicity of ABT-737, 4-HPR, and their combination (at a 1:1 molar ratio) was determined in 96-well plates using the semiautomatic fluorescence-based Digital Imaging Microscopy System (DIMSCAN; refs. 30, 31). DIMSCAN uses digital imaging microscopy to quantify viable cells, which selectively accumulate FDA. DIMSCAN is capable of measuring cytotoxicity over a 4 log dynamic range by quantifying total fluorescence per well (which is proportional to the number of viable cells) after elimination of the background fluorescence by digital thresholding and eosin Y quenching. Cells were seeded into 96-well plates in 100 μL of complete medium at 5,000 to 10,000 cells per well, depending on cell line growth rate. After overnight incubation, ABT-737, 4-HPR, or their combination was added to each well at various concentrations in 50 μL of culture medium. We used the following drug concentrations: 0, 1.25, 2.5, 5, 10 $\mu\text{mol/L}$ or 0, 2.5, 5, 7.5, 10 $\mu\text{mol/L}$ in replicates of 12 wells for each experimental condition. After incubation with the drugs for 96 hours at 37°C , FDA (final concentration: 10 $\mu\text{g/mL}$) and eosin Y [final concentration: 0.1% (w/v)] were added to each well and the cells were incubated for an additional 20 minutes at 37°C . Total fluorescence per well was then measured using DIMSCAN, and the results were expressed as the ratio of the fluorescence in treated wells to the fluorescence in untreated wells (survival fraction).

Assessment of apoptosis by flow cytometry

Apoptosis in cells was examined by flow cytometry using a commercial TUNEL kit (APO-DIRECT; BD Biosciences) according to the manufacturer's instructions. Cells treated with ABT-737, 4-HPR, or their combination in the presence or absence of the pan-caspase inhibitor Boc-d-fmk (40 $\mu\text{mol/L}$ for 1 hour) were collected, washed, centrifuged, and resuspended in 1% (w/v) paraformaldehyde in PBS (pH 7.4). They were then kept on ice for 60 minutes, washed in PBS, centrifuged, and fixed in 70% (v/v) ice-cold ethanol at -20°C for 12 to 18 hours, before being stained with terminal deoxynucleotidyltransferase (TdT) and fluorescein isothiocyanate (FITC)-labeled dUTP (FITC-dUTP) for 2 hours at 37°C . After washing with PBS, cells were resus-

pending in 0.5 mL of propidium iodide (PI) and RNase containing buffer (5 $\mu\text{g/mL}$ PI, 200 $\mu\text{g/mL}$ RNase). Cells were then incubated in the dark for 30 minutes at room temperature prior to analysis by flow cytometry. The percentage of TdT-mediated fluorescent cells was measured by flow cytometry using band pass filters of 525 ± 25 nm for FITC and 610 ± 25 nm for PI (7, 32) in a BD LSR II system (BD Biosciences) equipped with the DiVA software (version 4.1.2; BD Biosciences).

Determination of mitochondrial membrane potential ($\Delta\psi_m$) transition

Cells were treated with ABT-737, 4-HPR, or the combination, collected in 5 mL polystyrene tubes, centrifuged at $300 \times g$ for 5 minutes, and resuspended in 1 mL of medium containing 10 $\mu\text{g/mL}$ of JC-1, incubated at 37°C for 10 minutes, and analyzed by flow cytometry. The detection of a fluorescence emission shift from red (610 ± 10 nm) to green (525 ± 10 nm) was an indication of mitochondrial membrane depolarization (33).

Western blot analysis

Cells were lysed in radioimmunoprecipitation (RIPA) lysis buffer (Upstate Biotechnology), containing 15 $\mu\text{L/mL}$ of phenylmethylsulfonyl fluoride and 40 $\mu\text{L/mL}$ of Protease Inhibitor Cocktail (Sigma). The lysates were left on ice for 15 minutes, briefly sonicated, and centrifuged at $12,000 \times g$ for 15 minutes. Protein concentration in the supernatants was determined using the BCA Protein Assay Kit (Pierce Biotechnology), and 20 μg of protein in each sample was resolved by electrophoresis in a 10% to 20% gradient acrylamide gel containing 0.1% SDS (Invitrogen). After electrophoresis, the gels were transferred to a protein nitrocellulose transfer membrane (Whatman GmbH). The membrane was hybridized with primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies and immunocomplexes detected by chemiluminescence (Pierce Biotechnology) and visualized on autoradiography film (Denville Scientific, Inc.). Quantification was obtained by scanning the immunoblots using an Epson Expression 1680 system (Epson). Antibodies used were as follows: anti-Bax rabbit polyclonal antibody (#554104) from BD Biosciences; anti- β -actin goat polyclonal antibody, anti-GAPDH mouse monoclonal antibody, and HRP-conjugated secondary anti-mouse, anti-goat, and anti-rabbit antibodies from Santa Cruz Biotechnology; anti-caspase-9 rabbit polyclonal antibody (#9502), anti-caspase-3 rabbit polyclonal antibody (#9662), anti-Bid rabbit polyclonal antibody (#2002), anti-Bcl-2 rabbit polyclonal antibody (#2870), anti-Bcl-XL rabbit polyclonal antibody (#2764), anti-Bcl-w rabbit polyclonal antibody (#2724), anti-Mcl-1 rabbit polyclonal antibody (#5453), and anti-Bak rabbit polyclonal antibody (#3814) from Cell Signaling Technology; anti-Bak mouse monoclonal antibody (Ab-1) which recognizes only conformationally active Bak was from Calbiochem; and anti-cytochrome *c* rabbit polyclonal antibody and anti-OxPhos Complex IV (COX IV) mouse monoclonal antibody were from Clontech. Densitometric

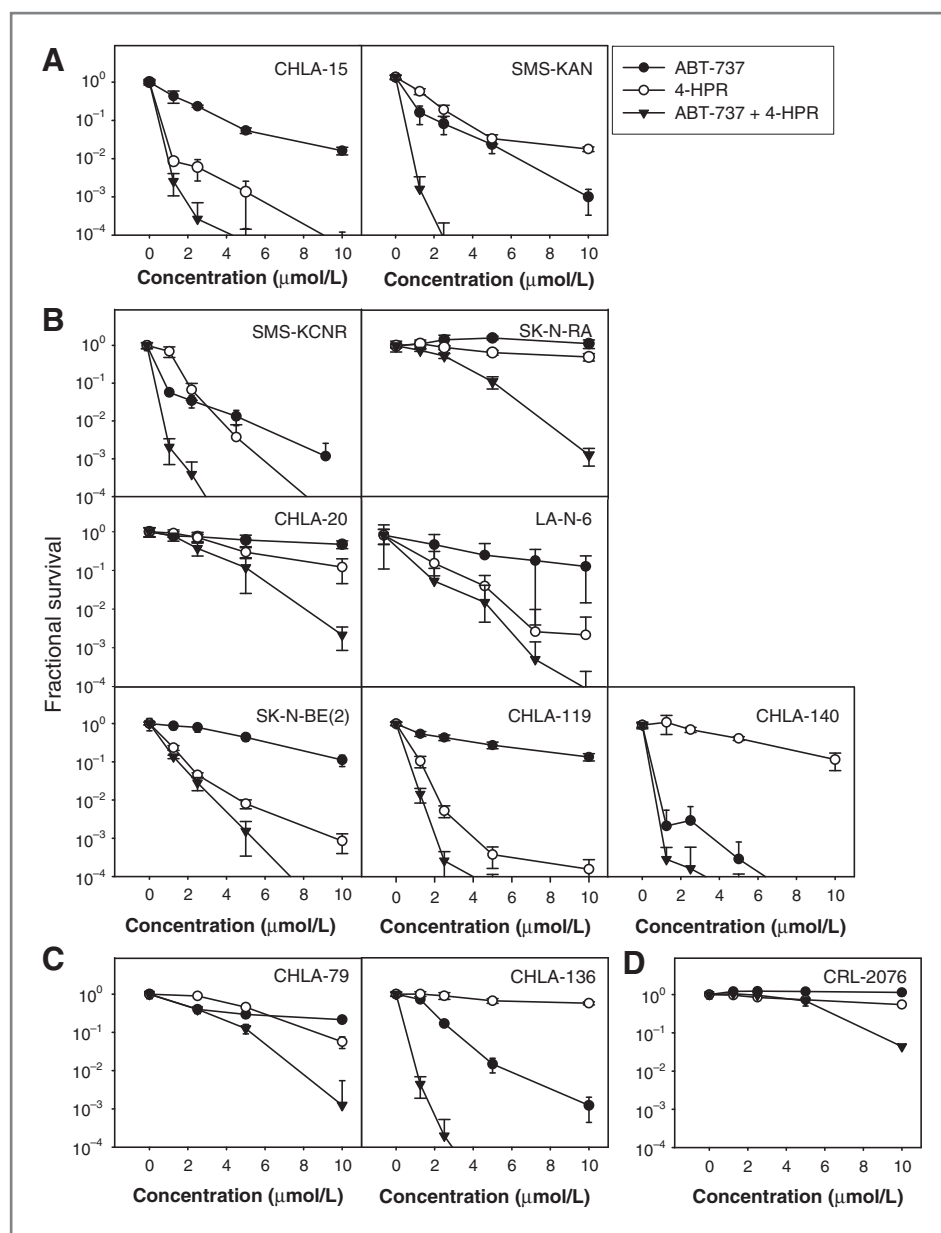


Figure 1. The combination ABT-737 plus 4-HPR is synergistically cytotoxic against neuroblastoma cell lines but not normal fibroblasts cell line *in vitro*. Dose-response curves of neuroblastoma cell lines treated with ABT-737 (●), 4-HPR (○), and the combination (▼) as measured by DIMSCAN. The concentrations applied for the cell lines were 1.25 to 10 $\mu\text{mol/L}$ for ABT-737 and/or 4-HPR (except for LA-N-6 and CHLA-79, from 2.5 to 10 $\mu\text{mol/L}$). Each condition had 12 replicates, and error bars represent SDs. A–C, eleven tested neuroblastoma cell lines were divided into 3 different groups according to therapy received by the patients prior to the line being established. A, cell lines established prior any therapy (CHLA-15 and SMS-KAN); B, cell lines obtained at the time of disease progression during induction therapy (SMS-KCNR, SK-N-RA, CHLA-20, LA-N-6, SK-N-BE(2), CHLA-119, and CHLA-140); C, cell lines established at relapse after myeloablative therapy (CHLA-79 and CHLA-136). D, the human normal fibroblast cell line CRL-2076.

analysis was conducted using ImageJ digital imaging software (NIH, Bethesda, MD).

Detection of cytochrome *c* release from mitochondria

After treatment with ABT-737, 4-HPR, or the combination for 6 or 24 hours, cells were subjected to a digitonin-based subcellular fractionation (34) to separate the cytosol (supernatant) from intact mitochondria (pellet). The pellets were then lysed in RIPA lysis buffer and Western blot analysis conducted as described above.

Caspase-8 activation

A caspase-8/FLICE colorimetric assay (Invitrogen) was used according to the manufacturer's instructions to detect the activation of caspase-8.

RNA interference

Validated siRNAs specific for Bax (5'-GCUCUGAGCA-GAUGAUGAATT-3') and Bak (5'-GCGAAGUCUUUGC-CUUCUCTT-3') were purchased from Qiagen. A nonspecific nonsilencing siRNA (AllStars Negative Control siRNA; QIAGEN) was used as negative control. Neuroblastoma cells were transfected with 100 nmol/L of Bax or Bak siRNA or control siRNA using Lipofectamine iMax transfection reagent (Invitrogen) according to the manufacturer's instructions.

Human neuroblastoma xenograft model

The CHLA-119 cell line, established from a patient with neuroblastoma at time of disease progression during chemotherapy (26), was injected at 17×10^6 cells subcutaneously between the shoulder blades of 4- to 6-week-old

Table 1. IC₉₀ values of 4-HPR and ABT-737, and CI values of ABT-737 at concentrations in combination with 4-HPR

Group	Cell line	Phase of therapy	IC ₉₀ , μmol/L		CI at each concentration, μmol/L			
			4-HPR	ABT-737	1.25	2.5	5	10
A. Prior to any therapy	CHLA-15	DX	3.7	0.5	0.486	0.349	0.416	0.001
	SMS-KAN	DX	2.4	1.4	0.152	0.086	0.073	0.063
B. At progressive disease during or after therapy	SMS-KCNR	PD	2.3	1	0.342	0.414	0.321	0.382
	SK-N-RA	PD	12.3	>20	0.255	0.402	0.474	0.324
	CHLA-20	PD	10.3	>20	1.458	0.648	0.56	0.161
	LA-N-6	PD	3.2	13.7	—	0.794	1.016	0.425
	SK-N-BE(2)	PD	2.2	12.3	0.827	0.543	0.16	0.058
	CHLA-119	PD*	1.1	15.8	0.619	0.353	0.496	0.109
C. At relapse after myeloablative therapy and bone marrow transplantation	CHLA-140	PD*	2.2	0.4	0.521	0.922	0.785	0.86
	CHLA-79	PD-BMT	8.6	>20	—	1.481	0.817	0.332
	CHLA-136	PD-BMT	11.3	2.9	0.237	0.218	0.209	0.238

NOTE: Lines in the panel included those established at diagnosis before therapy (DX), at time of progressive disease during or after non-myeloablative therapy (PD), after therapy with 13-*cis*-RA (PD*), and after myeloablative therapy and bone marrow transplantation (PD-BMT).

A CI less than 0.9 indicates synergism; 0.1, very strong synergism; 0.1 to 0.3, strong synergism; 0.3 to 0.7, good synergism; 0.7 to 0.85, moderate synergism; 0.85 to 0.9, slight synergism; 0.9 to 1.1, additive; and more than 1.1, antagonism. — indicates not applicable.

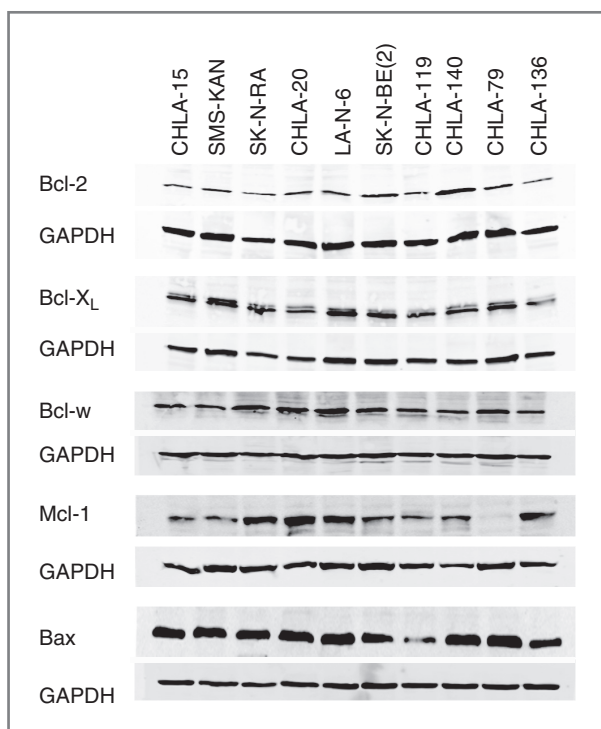


Figure 2. The basal expression of Bcl-2 family members in neuroblastoma cell lines. A, Western blot analysis of basal expression of Bcl-2 family proteins in 10 neuroblastoma cell lines. The data shown are representative of 2 independent experiments showing similar results. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

female athymic (*nu/nu*) mice. Once palpable and progressing tumors of 100 to 200 mm³ had developed, mice were treated with a maximum tolerated dose of 4-HPR LXS oral powder (240 mg/kg/d) and ABT-737 (50 mg/kg/d) for 5 days per week, alone or in combination or with the vehicle as control. When given alone, the dose of ABT-737 was increased to 100 mg/kg/d. 4-HPR LXS was prepared as slurry in sterile water and given orally by gavage, whereas ABT-737 was administered by intraperitoneal injection. Tumor volume was determined from measurements taken twice weekly using the formula 0.5 × height × width × length (35). When tumor volumes reached 1,500 mm³, the mice were sacrificed.

Statistical analysis

For *in vitro* experiments, synergistic drug interactions were determined by fixed ratio dose–response assays of the drugs alone and in combination (4-HPR:ABT-737 = 1:1). The IC₉₀ (the drug concentration that is cytotoxic or growth inhibitory for 90% of a cell population) and the combination index (CI) values were obtained by DIMSCAN analysis and calculated using CalcuSyn software (Biosoft). Calculation of a CI is a method to numerically quantify drug synergism based on the multiple drug–effect equation of Chou–Talalay derived from enzyme kinetic models (36, 37). With this method, a CI lower than 0.9 indicates synergism; a CI of 0.9 to 1.10 indicates additive activity; and a CI greater than 1.10 indicates antagonism.

In vivo data were analyzed using the software GraphPad Prism (GraphPad Software, Inc., version 4.03). Event-free

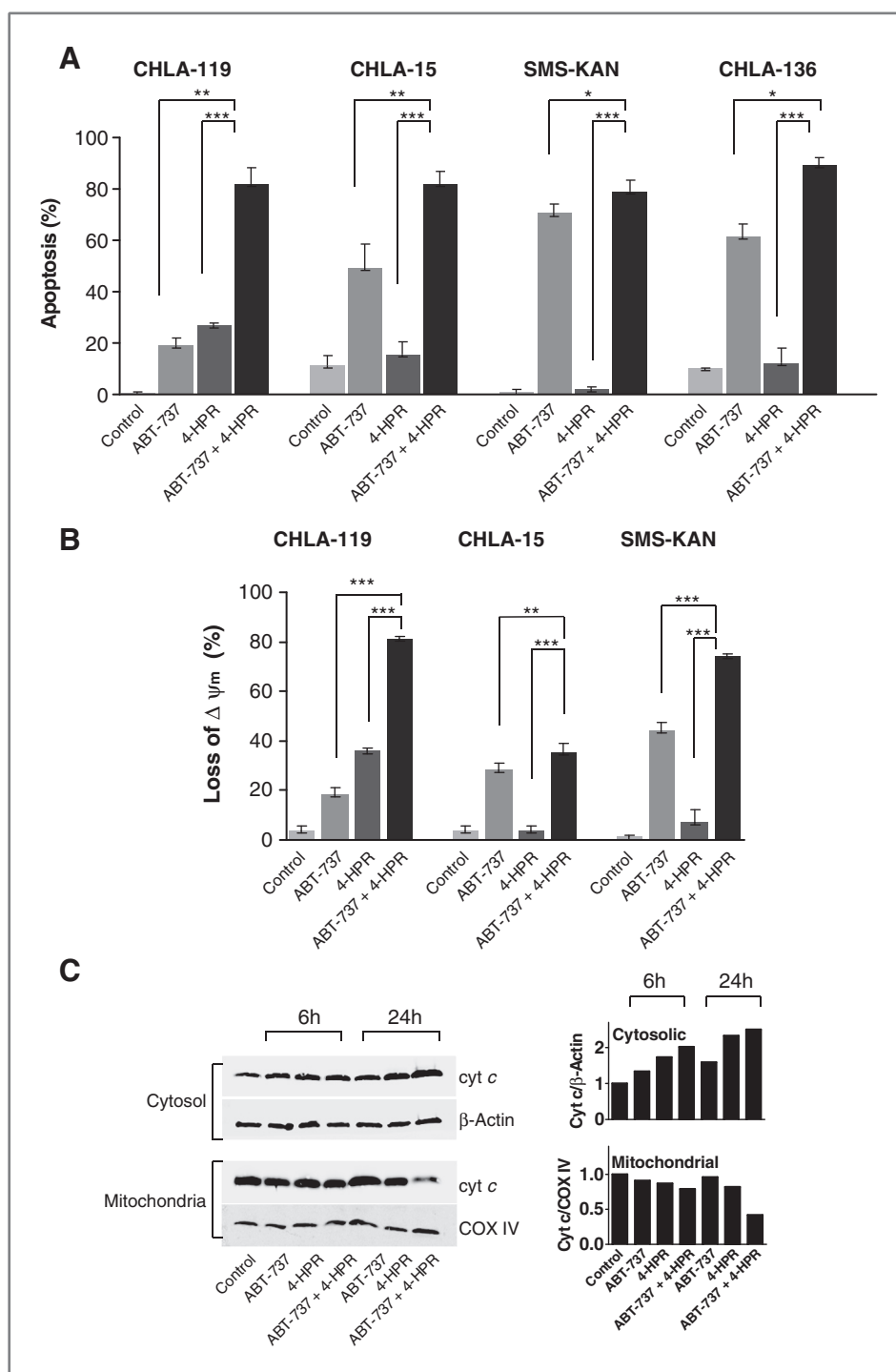


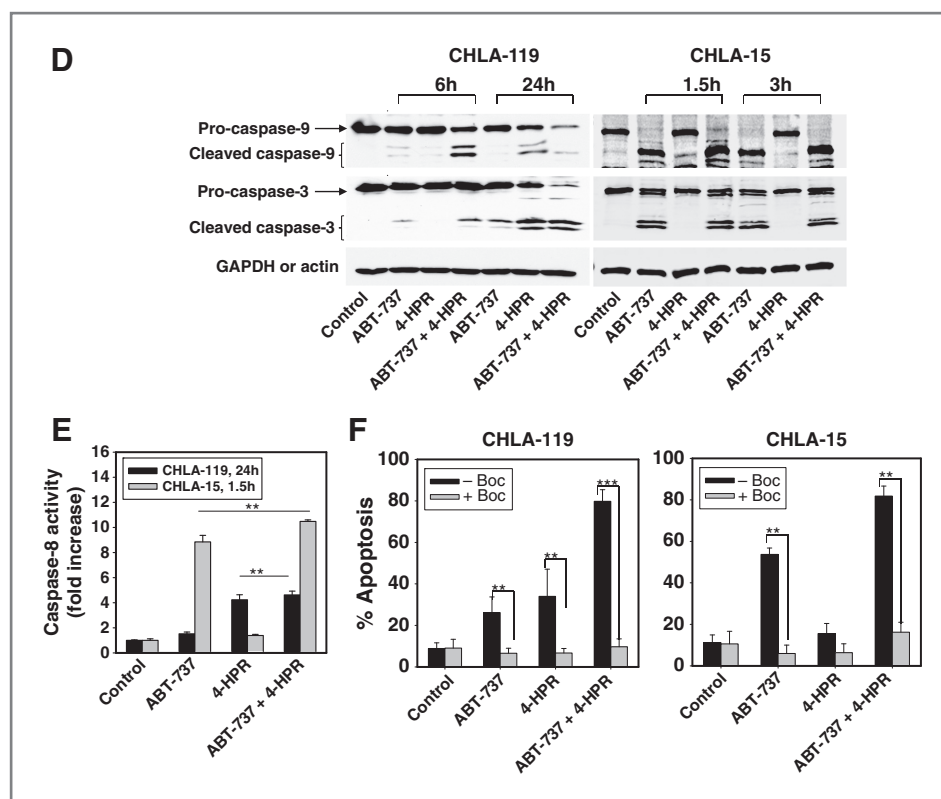
Figure 3. The combination of ABT-737 and 4-HPR induced caspase-dependent apoptosis through mitochondrial membrane depolarization and cytochrome c release. A and B, CHLA-119, CHLA-15, SMS-KAN, or CHLA-136 neuroblastoma cells were treated with ABT-737, 4-HPR, or the combination for 24 hours (for CHLA-15, 3 hours). Drug concentrations used were 2.5 $\mu\text{mol/L}$ for CHLA-119 and CHLA-15 and 5 $\mu\text{mol/L}$ for SMS-KAN and CHLA-136. A, cells were then analyzed for apoptosis (TUNEL assay) by flow cytometry. Bars show the percentages of TUNEL-positive cells defined as apoptotic. B, CHLA-119, CHLA-15, and SMS-KAN cells were incubated with the JC-1 mitochondrial probe and analyzed by flow cytometry. Bars show the percentages of mitochondrial membrane-depolarized cells. C, cytosolic and mitochondrial extracts from CHLA-119 cells incubated for 6 or 24 hours with ABT-737, 4-HPR, or the combination were prepared and immunoblotted with an anti-cytochrome c (cyt c) antibody. β -Actin and COX IV were used as the loading control for cytosolic and mitochondrial fractions, respectively. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$.

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survival (EFS) curves were compared by Kaplan–Meier log-rank test and quantified as the time taken from the initiation of treatment until an event, with events defined as tumor volume reaching 1,500 mm^3 or when mice died or were sacrificed because of treatment-related toxicity. An EFS T/C value was defined as the ratio of the median time to event of the treatment group and the median time to event of the

respective control group (38). For the EFS T/C measure, agents are considered highly active if they met 3 criteria: (a) an EFS T/C greater than 2; (b) a significant difference ($P \leq 0.05$) in EFS distribution between treated and control (or single agent); and (c) a net reduction in median tumor volume for animals in the treated group at the end of treatment as compared with treatment initiation. Agents

Figure 3. (Continued) D–F, CHLA-119 and CHLA-15 cells were incubated with ABT-737, 4-HPR, or the combination for 1.5 and 3 hours (CHLA-15) or 6 and 24 hours (CHLA-119). D, cell lysates were then examined by Western blot analysis for caspase-9 and caspase-3. E, cell lysates from CHLA-119 (24 hours) and CHLA-15 (1.5 hours) were incubated with caspase-8 substrate using a colorimetric assay. Bars show the mean fold increase of caspase-8 activity. F, CHLA-119 and CHLA-15 cells were pretreated with Boc-d-fmk (40 $\mu\text{mol/L}$) for 1 hour before being exposed to 2.5 $\mu\text{mol/L}$ ABT-737, 4-HPR, or the combination for 3 or 24 hours. After treatment, apoptotic cells were measured by flow cytometric TUNEL assay. Bars show the percentage of TUNEL-positive cells defined as apoptotic. Data shown are representative of 2 independent experiments. A, B, E, and F, data represent mean \pm SD of triplicate samples. **, $P < 0.005$; ***, $P < 0.001$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.



meeting the first 2 criteria, but not having a net reduction in median tumor volume for treated animals at the end of the study, are considered to have intermediate activity. Agents with an EFS T/C less than 2 are considered to have low levels of activity.

Relative tumor volumes (RTV) for control (C) and treatment (T) mice (tumor volume T/C values) were calculated on day 21 (38). The mean RTVs for control and treatment mice for each study were then calculated, and the T/C value was the mean RTV for the treatment group divided by the mean RTV for the control group. For the tumor volume T/C response measure, agents producing a T/C of $\leq 15\%$ are considered highly active, those with a mean tumor volume T/C of $\leq 45\%$ but $>15\%$ are considered to have intermediate activity, and those with mean T/C values $>45\%$ are considered to have low levels of activity.

Results with a value of $P < 0.05$ were regarded as significant (log-rank for EFS by GraphPad Prism or unpaired 2-sided Student t test by Microsoft Excel 2000 for other data).

Results

The combination ABT-737 plus 4-HPR was synergistically cytotoxic against neuroblastoma cell lines *in vitro*

We first determined the cytotoxicity of ABT-737, 4-HPR, and ABT-737 plus 4-HPR (0–10 $\mu\text{mol/L}$ for each drug) at 1:1 molar ratio in 11 neuroblastoma cell lines using the DIMSCAN cytotoxicity assay. Representative drug cytotox-

icity dose–response curves are shown in Fig. 1 and CI values calculated at the fixed ratio drug concentrations tested for the cytotoxicity assays and IC_{50} values of both agents are shown in Table 1. 4-HPR + ABT-737 showed synergistic activity in all 11 cell lines ($\text{CI} < 0.9$). In 8 of the 11 cell lines, including those resistant to 4-HPR alone (CHLA-136), ABT-737 alone [SK-N-BE(2) and CHLA-119], or both (SK-N-RA and CHLA-20), 4-HPR + ABT-737 showed very strong to good synergistic activity. Only in 3 cell lines (LA-N-6, CHLA-79, and CHLA-140) did 4-HPR + ABT-737 show only moderate or slight synergistic activity. There was no apparent association between sensitivity to single agents and synergy of the drugs in combination. ABT-737 and 4-HPR as single agents or in combination were minimally toxic for CRL-2076 fibroblasts at concentrations up to 5 $\mu\text{mol/L}$ and the combination showed only modest toxicity at 10 $\mu\text{mol/L}$ for each drug (Fig. 1D).

The basal levels of proapoptotic and antiapoptotic proteins in 10 of these cell lines was examined by Western blot analysis (Fig. 2). There was no apparent association between protein basal levels of Bcl-2, Bcl-XL, Bcl-w, or Mcl-1 and response to 4-HPR, ABT-737, or the 2 drugs in combination (Fig. 1).

The combination of ABT-737 and 4-HPR induced caspase-dependent apoptosis through mitochondrial membrane depolarization and cytochrome *c* release

Because apoptosis is a major mechanism of action for both 4-HPR and ABT-737, we examined the effect of single agents and their combination on apoptosis. In all 4 cell lines

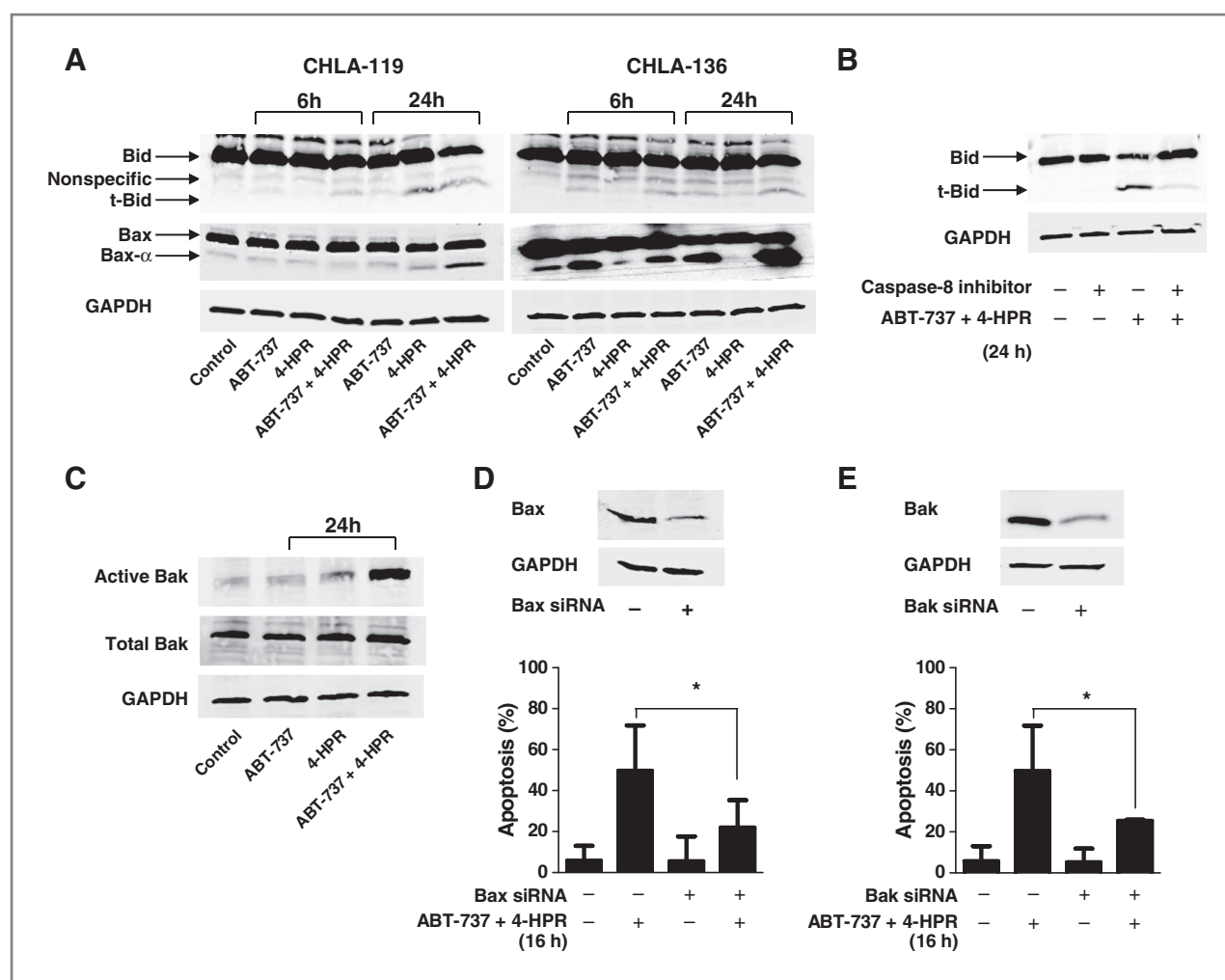


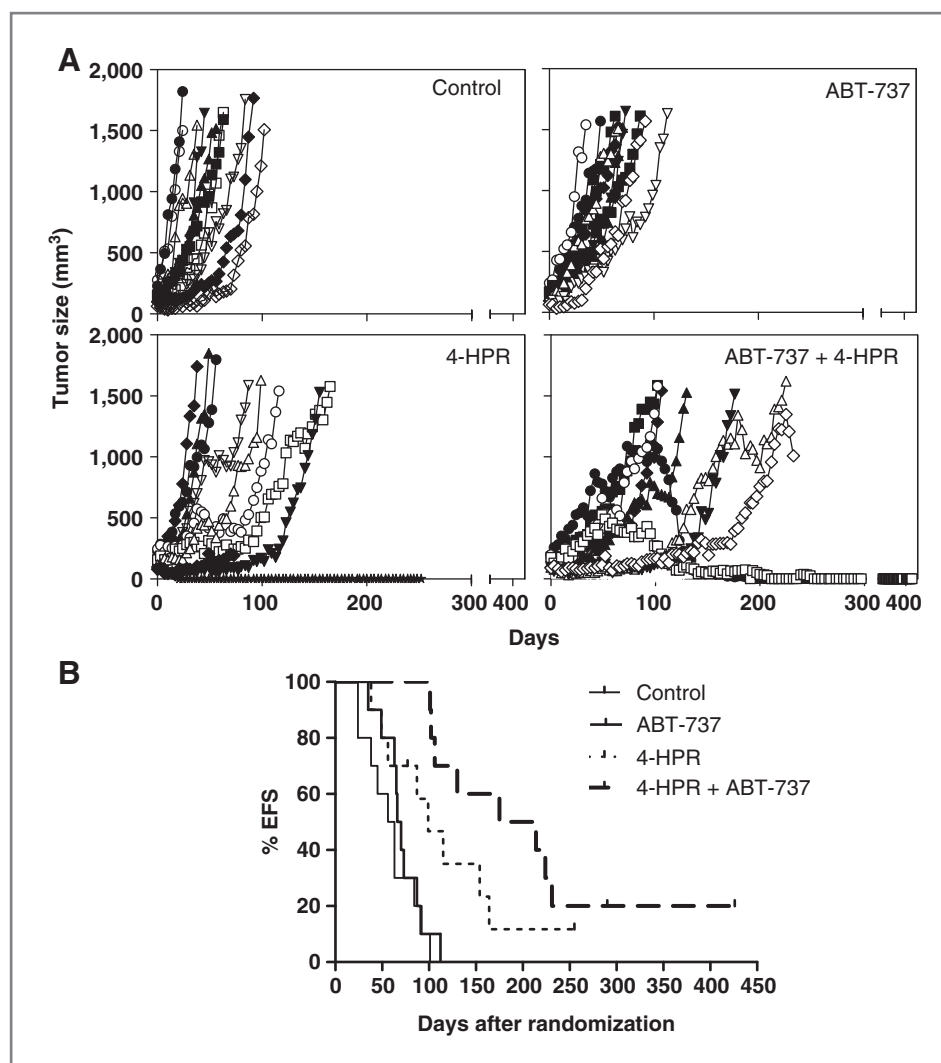
Figure 4. Effects of ABT-737 plus 4-HPR on the activation of proapoptotic proteins. CHLA-119 or CHLA-136 cells were incubated with ABT-737 or 4-HPR (2.5 or 5 $\mu\text{mol/L}$, respectively) or the combination for 6 or 24 hours. A, cell lysates were then examined for the presence of proapoptotic Bid and Bax. B, CHLA-119 cells were pretreated with Z-VAD-FMK (20 $\mu\text{mol/L}$) for 1 hour before being exposed to ABT-737 + 4-HPR (2.5 $\mu\text{mol/L}$ each) for 24 hours. Cell lysates were then examined for Bid cleavage into t-Bid by Western blot analysis. C, CHLA-119 cells were incubated with ABT-737 or 4-HPR (2.5 $\mu\text{mol/L}$ each) or the combination for 24 hours, and the presence of conformationally active Bak and total Bak was detected by Western blot. D and E, CHLA-119 cells were transfected with siRNA against Bax or Bak or control siRNA as indicated in Materials and Methods. Knockdown of Bax (D) and Bak (E) protein expression was assessed by Western blot (top). Forty-eight hours after transfection, cells were treated with ABT-737 + 4-HPR (2.5 $\mu\text{mol/L}$ each) for another 16 hours and apoptosis was determined by flow cytometric TUNEL assay (bottom). The bar graph shows the mean percentage of apoptotic cells (\pm SD) of triplicate samples. All the data shown are representative of 2 independent experiments showing similar results. *, $P < 0.05$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

tested (CHLA-119, CHLA-15, SMS-KAN, and CHLA-136), we observed a higher level of apoptosis when the 2 drugs were combined in comparison with each drug alone (Fig. 3A). For example, in CHLA-119 cells, the percentage of apoptotic cells at 24 hours was $0.6\% \pm 0.4\%$ (control), $19.2\% \pm 2.9\%$ in the presence of 2.5 $\mu\text{mol/L}$ ABT-737, $26.9\% \pm 0.9\%$ in the presence of 2.5 $\mu\text{mol/L}$ 4-HPR, and $82.1\% \pm 6.0\%$ in the presence of both drugs ($P < 0.005$ and $P < 0.001$ relative to ABT-737 and 4-HPR as single agents, respectively). In contrast, 4-HPR did not cause a significant increase of apoptosis in the other cell lines (CHLA-15, SMS-KAN, and CHLA-136), but the combination of 4-HPR and ABT-737 significantly increased apoptosis compared with

ABT-737 alone. ABT-737 and 4-HPR (either alone or in combination) did not induce apoptosis in CRL-2076 normal fibroblasts ($P > 0.05$; Supplementary Fig. S1).

We next investigated the effect of 4-HPR and ABT-737 on the loss of mitochondrial membrane potential ($\Delta\psi_m$) in CHLA-119, CHLA-15, and SMS-KAN cells. Treatment with ABT-737 and 4-HPR resulted in a greater than additive loss of $\Delta\psi_m$ when compared with the sum of the losses observed with each single agent (Fig. 3B). ABT-737 or 4-HPR alone, and especially the combination, caused release of cytochrome *c* from the mitochondria into the cytosol, and a decrease of cytochrome *c* in the mitochondrial fraction in CHLA-119 cells (Fig. 3C).

Figure 5. *In vivo* antitumor activity of ABT-737 combined with 4-HPR against human neuroblastoma cells. Athymic (*nu/nu*) mice carrying CHLA-119 neuroblastoma subcutaneous xenografts were treated with vehicle control (thin lines), ABT-737 (bold lines), 4-HPR (thin dotted lines), or 4-HPR + ABT-737 (bold dotted lines). 4-HPR/LYM-X-SORB oral powder in water (240 mg/kg) was given by oral gavage 5 d/wk. ABT-737 (100 mg/kg) was administered by intraperitoneal injection 5 d/wk. When used in combination, ABT-737 was administered at a dose of 50 mg/kg/d of ABT-737 (half of the dose used in single agent treatment). A, tumor volumes were measured twice per week and tumor volumes calculated as described in Materials and Methods. Each line represents tumor growth in a mouse. Mice were sacrificed when tumors reached 1,500 mm³. B, the EFS of mice was calculated from the time of tumor injection to the time when the tumor volume reached 1,500 m³ or the mice had to be sacrificed because of treatment-related toxicity. Each line represents the proportion of mice remaining event free over time.



Caspase-9 is a key intermediate in the mitochondrial or intrinsic apoptotic pathway, and caspase-3 is the main final "effector caspase" inducing cell death. We therefore examined the effects of ABT-737, 4-HPR, and their combination on the expression and activation of these 2 caspases (Fig. 3D). The data indicated that both caspase-9 and caspase-3 were cleaved and activated upon treatment with ABT-737 alone or in combination with 4-HPR. To determine whether the effect of 4-HPR and ABT-737 solely involved activation of the intrinsic pathway, we also examine their effect on the activation of caspase-8, the major caspase involved in the extrinsic apoptosis (Fig. 3E). Caspase-8 activity was significantly higher in CHLA-119 and CHLA-15 cells treated with the 4-HPR + ABT-737 than in untreated cells or in cells treated with either single agent. These data suggest that ABT-737 plus 4-HPR induced both extrinsic and intrinsic apoptotic pathways.

To confirm that apoptosis induced by 4-HPR, ABT-737, or the combination was caspase dependent, we pretreated the cells with a pan-caspase inhibitor Boc-d-fmk (40 μ mol/L) 1

hour before drug exposure and examined cells for apoptosis by TUNEL assay (Fig. 3F). In all cases (single drug and combination), Boc-d-fmk returned the levels of apoptosis to levels observed in the absence of any drug (control), indicating that the apoptotic effects of ABT-737, 4-HPR, and the combination were caspase dependent.

The combination of ABT-737 and 4-HPR increased expression of members of the proapoptotic Bcl-2 family of proteins

To further explore the mechanisms of apoptosis induced by ABT-737 and 4-HPR, we determined the effects of ABT-737, 4-HPR, and the combination on the expression of proapoptotic proteins Bid and Bax by Western blot analysis (Fig. 4A). The activation of Bid was shown by the presence of the 15 kDa truncated form of Bid (t-Bid), and the activation of Bax by the presence of Bax- α (21 kDa), the most active form of Bax that plays a key role in cytochrome *c* release (14). In CHLA-136 cells (sensitive to ABT-737 and resistant to 4-HPR), ABT-737 activated Bid as indicated by the

presence of t-Bid and Bax- α , and a greater effect was seen with the combination whereas 4-HPR alone had little effect. In CHLA-119 cells, which are relatively sensitive to 4-HPR but resistant to ABT-737, treatment with 4-HPR induced the truncation of Bid in a time-dependent manner, and a maximum activation was observed in the presence of 4-HPR + ABT-737. Bax activation occurred earlier in CHLA-136 than CHLA-119, perhaps due to differences in their relative sensitivity to ABT-737 as a single agent (Fig. 1).

The cleavage of Bid into t-Bid further supported the contribution of the extrinsic apoptotic pathway to the apoptosis induced by 4-HPR and ABT-737 because Bid is a substrate for caspase-8 (39). To confirm this possibility, we tested whether the activation of Bid into t-Bid in cells treated with ABT-737 and 4-HPR could be prevented by the caspase-8 inhibitor Z-VAD-FMK (20 μ mol/L, 1 hour pre-exposure). As shown in Fig. 4B, Z-VAD-FMK markedly decreased Bid cleavage into t-Bid in CHLA-119 cells treated with 4-HPR and ABT-737.

In addition to Bax and Bid activation upon treatment with 4-HPR and ABT-737 (Fig. 4A), activation of another proapoptotic Bcl-2 family protein, Bak, was detected by Western blot using an antibody which specifically recognizes activated Bak (Fig. 4C). To determine whether the activation of Bax and Bak is involved in the apoptosis induced by ABT-737 + 4-HPR, we knocked down Bax or Bak by siRNA and examined apoptosis by TUNEL assay induced by ABT-737 + 4-HPR compared with control siRNA. Knockdown of Bax (Fig. 4D) or Bak (Fig. 4E) significantly decreased apoptosis induced in CHLA-119 cells by ABT-737 + 4-HPR.

Increased *in vivo* activity of ABT-737 and 4-HPR combination to recurrent neuroblastoma xenografts of CHLA-119

To determine whether ABT-737 and 4-HPR would have a synergistic activity *in vivo*, we selected CHLA-119, a cell line derived from a patient whose disease progressed during intensive multiagent chemotherapy (26) and that is sensitive to 4-HPR but resistant to ABT-737 and for which strong synergism was observed *in vitro* (Table 1).

The tumor growth rate over time was recorded (Fig. 5A), and tumor volume T/C on day 21 was calculated to evaluate the capability of inhibiting tumor growth (Supplementary Table S1). We observed that ABT-737 only showed a low activity (tumor volume T/C = 66.8%), but both 4-HPR as a single agent and 4-HPR + ABT-737 showed intermediate activity with tumor volume T/C = 45.9% and 30.9% respectively, which was consistent with CHLA-119 cells being resistant to ABT-737 but sensitive to 4-HPR alone and the combination *in vitro*. Median EFS and EFS T/C values were also calculated to evaluate prolonged survival by the log-rank analysis (Fig. 5B and Supplementary Table S1), and the results were consistent with tumor growth rate analysis. Only 4-HPR (EFS T/C = 1.7) and 4-HPR + ABT-737 (EFS T/C = 3.3) statistically increased EFS ($P = 0.0002$) relative to controls, whereas ABT-737 had no significant effect (EFS

T/C = 1.1, $P > 0.05$). ABT-737 + 4-HPR also increased EFS relative to either single drug alone ($P < 0.001$).

Discussion

The Bcl-2 family of proteins provides one mechanism by which malignant cells can survive various cytotoxic drugs and inhibition of Bcl-2 family antiapoptotic proteins by drugs, such as ABT-737, is a promising approach for the treatment of cancer. ABT-737 is a small-molecule inhibitor of Bcl-2, Bcl-X_L, and Bcl-w that has been reported to be active against lymphoid cancers (19–23) and to have minimal systemic toxicity in animal preclinical models (18). A related orally bioavailable compound, ABT-263, is currently undergoing early phase adult clinical trials (40, 41). We have recently shown that ABT-737 synergizes 4-HPR in lymphoid malignancies *in vitro* (24). 4-HPR is a well-tolerated drug that has shown activity against recurrent neuroblastoma in the laboratory (6, 7) and in patients (10, 11). Here, we showed that ABT-737 is active against most neuroblastoma cell lines *in vitro* and has a synergistic antitumor effect when combined with 4-HPR at a concentration range that is active and tolerable in mouse xenograft models (18). Concentrations of 4-HPR used in this study were within the range achieved in children in clinical trials (10) with little hematopoietic toxicity observed.

In most of the neuroblastoma cell lines and drug concentrations tested in this study, we found that 4-HPR alone had a modest effect on apoptosis, whereas ABT-737 alone readily induced apoptosis in sensitive cell lines. In some cell lines, such as CHLA-119, the cells were found relatively resistant to ABT-737. However, in spite of this resistance, in all cell lines, the combination of the 2 drugs was synergistic *in vitro*. We also found that ABT-737 enhanced the activity of 4-HPR for CHLA-119 *in vivo*. Interestingly, we observed that the combination of these 2 agents had minimal toxic effect on normal human fibroblasts, which is consistent with similar observations made in normal resting lymphocytes (24), and suggests that the combination should have minimal toxicity for nonneoplastic cells *in vivo*.

The combination of 4-HPR and ABT-737 induced the loss of $\Delta\psi_m$ and the release of cytochrome *c* to the cytoplasm (Fig. 3B and C), indicating that this drug combination acts via the mitochondrial death pathway. Activation of caspase-9 and caspase-3 by ABT-737 in the presence or absence of 4-HPR further confirmed that ABT-737 acts via the mitochondria-dependent apoptotic pathway (Fig. 3D). In addition, the observation that caspase-8 was activated by these 2 agents (Fig. 3E), and that ABT-737 + 4-HPR-induced cleavage of Bid into t-Bid was inhibited by a caspase-8 inhibitor, indicate the involvement of the extrinsic apoptotic pathway as well (Fig. 4B). Furthermore, the majority of apoptosis induced by ABT-737 alone and by the combination of ABT-737 + 4-HPR occurred largely via caspase-dependent pathways as indicated by the fact that the pan-caspase inhibitor Boc-d-fmk inhibited the effect of ABT-737 and 4-HPR on apoptosis (Fig. 3B).

The mitochondrial apoptotic pathway is controlled by a balance between the proapoptotic protein members

(i.e., the multidomain proapoptotic Bax, Bak, and BH3-only proapoptotic Bid, Bim, Bad, Bik, Noxa, Puma, Bmf, Hrk) and antiapoptotic protein members (i.e., the multidomain antiapoptotic Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, Bfl/A1) of the Bcl-2 family. Those multidomain Bcl-2 proteins are functionally regulated by the BH3-only proteins (42, 43). Bid is localized in the cytosolic fraction of cells as an inactive precursor (44) and truncated Bid (t-Bid), the active form of Bid, is generated upon proteolytic cleavage by caspase-8 (45, 46).

We showed that ABT-737 together with 4-HPR increased the release of sequestered t-Bid from Bcl-2 and Bcl-X_L and the induction of Bax and Bak oligomerization (Fig. 4). The observation that Bid was cleaved into t-Bid in cells treated with ABT-737 alone or in combination with 4-HPR is similar to the previously reported activity of ABT-737 on leukemic and human pancreatic cancer cells (20, 21). The fact that Bax or Bak knockdown significantly impaired the apoptosis induced by ABT-737 + 4-HPR further confirms the importance of Bax and/or Bak activation in the synergistic effect observed with ABT-737 and 4-HPR. Another antiapoptotic Bcl-1 family protein Mcl-1 has been shown to be overexpressed in cells resistant to ABT-737 (22, 47–50), and a recent study in ALL showed that the combination of ABT-737 and 4-HPR was associated with Mcl-1 inactivation by 4-HPR (24). However, ABT-737 + 4-HPR did not alter Mcl-1 levels in neuroblastoma cells (Supplementary Fig. 2).

We extended our *in vitro* cytotoxicity studies by showing that 4-HPR and ABT-737 enhanced EFS when compared with either drug alone in CHLA-119 tumor-bearing mice, a mouse xenograft model of recurrent, TP53-mutated, p53-nonfunctional, MDR neuroblastoma (Fig. 5). Thus, we have shown a positive synergistic interaction between 4-HPR and the BH3-mimetic agent ABT-737 in neuroblastoma cell

lines, and consistent with the *in vitro* activity, we showed antineuroblastoma activity of this novel combination in a neuroblastoma mouse xenograft model. These data support clinical trials combining 4-HPR with BH3-mimetic drugs in children with recurrent neuroblastoma.

Disclosure of Potential Conflicts of Interest

C.P. Reynolds is a coinventor on a patent covering LXS fenretinide owned by Childrens Hospital Los Angeles. No potential conflicts of interest were disclosed by other authors.

Authors' Contributions

H. Fang, T.M. Harned, Y.A. DeClerck, and C.P. Reynolds designed the research and analyzed the data; H. Fang, T.M. Harned, O. Kalous, and V. Maldonado carried out the research and analyzed the data; H. Fang wrote the manuscript; and Y.A. DeClerck and C.P. Reynolds edited the manuscript.

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