Fenretinide via NOXA Induction, Enhanced Activity of the BCL-2 Inhibitor Venetoclax in High BCL-2-Expressing Neuroblastoma Preclinical Models

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Abstract

Recurrent high-risk neuroblastoma is a childhood cancer that often fails to respond to therapy. Fenretinide (4-HPR) is a cytotoxic retinoid with clinical activity in recurrent neuroblastoma and venetoclax (ABT-199) is a selective inhibitor of the antiapoptotic protein B-cell lymphoma-2 (BCL-2). We evaluated activity of 4-HPR + ABT-199 in preclinical models of neuroblastoma. Patient-derived cell lines and xenografts from progressive neuroblastoma were tested. Cytotoxicity was evaluated by DIMSCAN, apoptosis by flow cytometry, and gene expression by RNA sequencing, quantitative RT-PCR, and immunoblotting. 4-HPR + ABT-199 was highly synergistic against high BCL-2–expressing neuroblastoma cell lines and significantly improved event-free survival of mice carrying high BCL-2–expressing patient-derived xenografts (PDX). In 10 matched-pair cell lines [established at diagnosis (DX) and progressive disease (PD) from the same patients], BCL-2 expression in the DX and PD lines was comparable, suggesting that BCL-2 expression at diagnosis may provide a biomarker for neuroblastomas likely to respond to 4-HPR + ABT-199. In a pair of DX (COG-N-603x) and PD (COG-N-623x) PDXs established from the same patient, COG-N-623x less responsive to cyclophosphamide + topotecan than COG-N-603x, but both DX and PD PDXs were responsive to 4-HPR + ABT-199. Synergy of 4-HPR + ABT-199 was mediated by induction of NOXA via 4-HPR stimulation of reactive oxygen species that induced expression of ATF4 and ATF3, transcription factors for NOXA. Thus, fenretinide + venetoclax is a synergistic combination that warrants clinical testing in high BCL-2–expressing neuroblastoma.

Introduction

Neuroblastoma is an extracranial malignant tumor, arising along the sympathetic chain most commonly in the adrenal gland and is the most common extracranial solid tumor in childhood. Age, MYCN amplification, stage, DNA ploidy, and histology are used to stratify patients with neuroblastoma into low, intermediate, and high risk. Patients with high-risk tumors are those with genomic amplification of the MYCN oncogene, patient age > 18 months at diagnosis, higher stage, diploid, and unfavorable histology (1). Despite intensive treatment with surgery, chemotherapy, myeloablative chemotherapy, and radiotherapy, followed by maintenance therapy with 13-cis-retinoic acid and anti-GD2 antibody + cytokines, the 5-year overall survival rate for patients with high-risk neuroblastoma is still approximately 50% (1, 2). Therefore, investigating novel drug combinations is crucial for improving the therapy of high-risk neuroblastoma.

B-cell lymphoma-2 (BCL-2) family of proteins consists of 25 proapoptotic and antiapoptotic members (3). Proapoptotic BCL-2 proteins play vital roles in inducing apoptosis in response to external stress signals, while antiapoptotic Bcl-2 family proteins promote cancer cell survival by antagonizing apoptosis and thus provide therapeutic targets (4). BCL-2 overexpression is frequent in various cancers, including neuroblastoma, and BCL-2 modulation can increase sensitivity to chemotherapeutic agents (5–7). ABT-737 (a preclinical version of navitoclax = ABT-263) is active against preclinical models of hematologic malignancies (8–10), neuroblastoma (5, 11), and small cell lung cancer (12). Clinical development of navitoclax (an orally bioavailable version of ABT-373) was curtailed because of dose-dependent thrombocytopenia resulting from the inhibition of BCL-XL by ABT-263 (13). This led to the development of venetoclax (ABT-199), a selective BCL-2 protein inhibitor that does not inhibit BCL-XL, thus avoiding thrombocytopenia (13). ABT-199, which is well-tolerated by patients achieving 2 μmol/L plasma concentrations, was granted an FDA-registered indication for treating chronic lymphocytic leukemia (CLL) with a 17p deletion (14, 15) and is being investigated in clinical trials of multiple hematologic
malignancies (16), as well as preclinically for solid tumors (17) including neuroblastoma (6, 18, 19). Fenretinide (4-HPR), a synthetic retinoid, can safely achieve 10–40 μmol/L plasma levels in children and has shown multiple complete responses among heavily pretreated neuroblastoma patients (20). 4-HPR can activate apoptosis independent of retinoid receptors and p53 (21), an essential consideration as loss of p53 function has been shown to be a mechanism of multidrug resistance in neuroblastoma (22). 4-HPR increases accumulation of toxic dihydroceramides in neuroblastoma by activating de novo dihydroceramidase synthetic enzymes, while simultaneously inhibiting dihydroceramide desaturase (23). The increased dihydroceramides in response to 4-HPR occur selectively in malignant cells and minimally in nonmalignant cells (24). Another primary mechanism of 4-HPR inducing cell death is via increase in reactive oxygen species (ROS) generation (21). We have previously demonstrated that ABT-737 significantly increased the activity of fenretinide in vitro and in vivo models of neuroblastoma (11) and acute lymphoblastic leukemia (9).

BCL-2 is essential for survival of some cancer cells, including a subset of BCL-2-dependent neuroblastomas, and high BCL-2 expression is a potential biomarker of sensitivity to BCL-2 inhibitors (5, 25, 26). However, it is often not feasible to obtain the tumor biopsies from patients with recurrent neuroblastoma to determine the BCL-2 expression level due to ethical and clinical reasons. We determined whether ABT-199 can synergize with 4-HPR against neuroblastoma. We also assessed BCL-2 expression as a potential biomarker of sensitivity to ABT-199 + 4-HPR and we determined the mechanism of synergistic antineuroblastoma activity between 4-HPR and ABT-199.

**Materials and Methods**

**Chemicals**

Fenretinide (4-HPR) for in vitro experiments and fenretinide oral powder formulation in Lym-X-Sorb were from the NCI. Venetoclax (ABT-199) was from AbbVie. Ketoconazole (keto) was from TEVA Pharmaceuticals. Ascorbic acid was from Sigma Aldrich. H2DCFDA was obtained from Thermo Fisher Scientific. Cyclophosphamide (Cyclo) was obtained from Baxter Healthcare. Topotecan (Topo) was from Sagent Pharmaceuticals. ABT-199 was dissolved in 60% plasol 50 (Thermo Fisher Scientific). 30% peg 400 (Sigma Aldrich), and 10% ethanol (Sigma-Aldrich) for dosing of mice. All-trans retinoic acid (ATRA) was from Sigma Aldrich.

**Cell culture**

All cell lines used in this study are described in Supplementary Table S1 and were obtained from the COG/ALSF Childhood Cancer Repository (www.CCcells.org). Neuroblastoma cell lines used in the study were cultured in complete medium of Isove’s Modified Dulbecco Medium (Thermo Fisher Scientific) supplemented with 20% FBS (Gibco Life Technologies), 4 mmol/L l-glutamine (Corning Cellgro), insulin and transferrin (20 mg/mL each), and selenous acid (20 ng/mL; ITS Culture Supplement; BD Biosciences). Cell line and patient-derived xenograft (PDX) identities were verified via short tandem repeat profiling by GenePrint 10 System from Promega validated against a searchable online database at www.CCcells.org, before starting and at the conclusion of the experiments, and were confirmed to be free of Mycoplasma with MycoAlert Mycoplasma Detection Kit from Lonza. Cells were cultured and treated in a 37°C humidified incubator gassed with 5% CO2 and 90% N2, to achieve bone marrow level hypoxia of 5% O2 (27). Cell lines with the ‘h’ suffix were established in and only cultured in 5% O2.

**Cytotoxicity assay**

We plated neuroblastoma cells in 96-well plates for 24 hours before treating with either 4-HPR (0, 1.25, 2.5, 5, and 10 μmol/L), ABT-199 (0, 0.625, 1.25, 2.5, and 5 μmol/L), or the combination of 4-HPR + ABT-199 at 1:2 drug concentration ratio. There were six replicates per drug concentration, and the control was treated with drug vehicle. After 96 hours of drug incubation, cell viability was measured using the DIMSCAN assay as described previously (28–30).

**Protein expression analysis**

Cells were collected and washed twice with PBS before lysing with lysis buffer. RIPA (Thermo Fisher Scientific) + Protease Inhibitor (Sigma-Aldrich) + phosphatase inhibitors (NAF and Na3VO4). The protein concentration was quantified by BCA Assay (Thermo Fisher Scientific), then diluted with NuPAGE LDS Sample Buffer (Thermo Fisher Scientific) and Dithiothreitol (Thermo Fisher Scientific), and subjected to SDS-PAGE. Protein was transferred from the gel onto the Amersham Protran 0.45 NC nitrocellulose Western blotting membrane using the GE Healthcare Amersham TE 70 Semi-Dry Transfer Unit. The nitrocellulose membrane was blocked by 1% BSA solution for 1 hour, and then incubated with primary antibody overnight. The next day, the membrane was washed three times (10 minutes each) in 1× TBST solution before incubating with secondary antibody for 1 hour. Then the membrane was washed three times (10 minutes each) with TBST before developing for chemiluminescent signal using Amersham ECL Western Blotting Detection Reagent (GE Healthcare) and HyBlot CL Autoradiography Film (Denville Scientific) or Amersham Hyperfilm ECL (GE Healthcare).

Primary antibodies, anti-BIM (2933), anti-BAD (9292), anti-NOXA (14766), anti-PUMA (12450), anti-BAX (6947), anti-BAX (2772), anti-BIK (4592), anti-BETA ACTIN (8457), anti-ATF4 (11815), anti-BCL-2 (2872), anti-BCL-W (2724), and anti-BCL-XL (2764), were from Cell Signaling Technology. Anti-ATF-3 (SC81189) was from Santa Cruz Biotechnology, and anti-MCL-1 (559207) was from BD Biosciences. Anti-FLAG (F3165) antibody was from Sigma-Aldrich. Horseradish peroxidase–conjugated secondary antibodies (anti-rabbit-7074 and anti-mouse-7076) were from Cell Signaling Technology. For communoprecipitation, NOXA (Myc-DDK tagged) was immunoprecipitated by anti-FLAG beads (Sigma-Aldrich). 3X FLAG Peptide (Sigma-Aldrich) was used to elute NOXA (Myc-DDK–tagged protein) from the anti-FLAG beads.

**Real-time RT-PCR**

RNA was as extracted by RNaseasy Mini Kit (Qiagen) as per the manufacturer’s instructions. The primers used in the study are: ATF-4 (Hs00231069_m1, Thermo Fisher Scientific), ATF-3 (Hs00231069_m1, Thermo Fisher Scientific), NOXA (Hs00560402_m1, Thermo Fisher Scientific), and GAPDH (Hs PT.39a.22214836, Integrated DNA Technologies). Real-time RT-PCR reactions were performed in triplicate in a 96-well plate with TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems) using QuantStudio 12K Flex. The cycling conditions were 30 minutes at 48°C, 10 minutes at 95°C, then 45 cycles of 15 seconds at 95°C, and 1 minute at 60°C.
RNA sequencing
RNA-sequencing (RNA-seq) libraries were prepared from polyA-selected RNA and sequenced on an Illumina HiSeq 2500 as described in (31). The FASTQ-formatted sequence reads were aligned to GRCh37.69 using the STAR RNA Seq aligner (v2.3.1; ref. 32) and transcripts were assembled and quantified using Cufflinks v2.1.1 (33) and reported as fragments per kilobase of gene length per million reads.

IHC
Tissue sections (4 μm) were deparaffinized in xylene and hydrated by ethanol. For antigen retrieval, sections were steamed at 95°C with citrate buffer (pH = 6.0) for 30 minutes. After cooling for 5 minutes, endogenous peroxidase was blocked with dual endogenous enzyme blocking agent (Dako, S2003) for 10 minutes. Sections were incubated with anti-BCL-2 (M0887, Dako, 1:50) primary antibody for 1 hour in a humidified chamber at room temperature, followed by incubation with secondary antibody (8125S, Cell Signaling Technology) for 30 minutes, and detected with signal stain DAB Substrate Kit (Cell Signaling Technology) after 10 minutes. All wash steps were performed with 1 × TBS containing 0.1% Tween-20 for 5 minutes. Sections were then counterstained with hematoxylin, rehydrated, and mounted. Mounted slides were imaged using Olympus BX51.

Apoptosis (DNA fragmentation) assay
Cells were plated in triplicate before treating with 4-HPR (10 μmol/L), ABT-199 (2 μmol/L), or 4-HPR (10 μmol/L) + ABT-199 (2 μmol/L). The amount of DNA fragmentation is quantified by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay (APO-DIRECT KIT from BD Biosciences). We followed the company established protocol, except the incubation time with DNA-Labeling Solution which was adjusted to 3 hours to maximize the staining of neuroblastoma cell lines. Then, the cells were analyzed by BD LSR II Flow Cytometer with BD FACSDiva Software (version 6.1.3) by gating for singlets and detecting percent of FITC-positive population with emission at 520 nm.

Xenograft studies
Patient-derived xenografts (PDXs) used in this study are described in Supplementary Table S1 and were obtained from the COG/ALSF Childhood Cancer Repository (www.CCcells.org). Six- to 8-weeks old female nu/nu mice (Envigo) were subcutaneously injected with 10–20 × 10^6 cells from a progressively growing PDX. Then, tumor-bearing mice were randomized into control and treatment groups at 100–300 mm³ tumor volume, which was measured by 1/2 length × width × height as described previously (34). Mice were sacrificed once the tumor volume exceeded 1,500 mm³ or if moribund from toxicity or illness.

ROS detection assay
ROS generation was detected using 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) and flow cytometry (9), with cells washed after staining with 1 × PBS (to eliminate interference from phenol red dye in the medium) and reconstituted in PBS before analysis; 100 μmol/L of H_2O_2 was used as a positive control. Vitamin C at 150 μmol/L was used to antagonize the ROS generated by 4-HPR treatment.

Statistical analysis
Antip apoptotic protein expression and combination index value (CIN) was compared using unpaired two-tailed t-test with Welch correction. The difference in dose–response curves of ABT-199 versus ABT-199 plus doxycycline treatment for PCW-empty vector and PMAIP1-PCW stably infected cell lines was compared by the ratio-paired two-tailed t test. The difference in percent of apoptotic cells between 4-HPR, ABT-199, and 4-HPR + ABT-199 was compared by one-way ANOVA with Tukey multiple comparisons test. The differences in fold change in DCFDA fluorescence between the control, 4-HPR, and 4-HPR + vitamin C were compared by one-way ANOVA with Tukey multiple comparisons test. The differences in mRNA expression of NOXA, ATF3, and topotecan by intraperitoneal injection at 0.6 mg/kg. Cyclophosphamide and topotecan (Cyclo + Topo) were given for 5 days (day 1–5) every 21 days cycle (total three cycles). Mouse survival was analyzed by log-rank test of event-free survival (EFS), in which an event was tumor volume exceeding 1,500 mm³, mouse being sacrificed for humane reasons (illness or toxicity), or death from any cause. The protocol was approved by the TTIHSC Laboratory Animal Resources Center and the Institutional Animal Care and Use Committee.

Stable knockdown or overexpression of genes
Lentiviruses containing either short hairpin RNA (shRNA) plasmid (plko.1) or EGFP-KD (plko.1-non-targeting plasmid) were packaged using Lentivpak Packaging Kit from OriGene, following the manufacturer’s instructions with minor modifications. Cells were plated in 6-well plates; then the virus-containing medium was added and incubated for 72 hours. The transduced cells were selected with 1.5 μg/ml of puromycin dihydrochloride (Sigma) until stable clones were established. PMAIP1 (TRCN0000151311) TRC lentiviral shRNA plasmid was from Dharmacon. PMAIP1 (TRC202071) Human cDNA ORF Clone plasmid was ordered from OriGene then subcloned into a doxycycline-inducible plasmid, pCW57-MCS1-2A-MCS2. pCW57-MCS1-2A-MCS2 was a gift from Adam Karpf (Eppley Institute, University of Nebraska Medical Center; Addgene plasmid # 71782).

Transient knockdown of ATF3 and ATF4 by siRNA
Cells were plated in complete growth medium 24 hours before the transfection. Growth medium was replaced with Opti-MEM (Thermo Fisher Scientific) before transfection. Lipofectamine RNAiMAX was used as the transfection agent along with 150 nmol/L of ON-TARGETplus Non-targeting Pool (Dharmacon), 150 nmol/L of SMARTpool: ON-TARGETplus ATF3 siRNA (Dharmacon), or 150 nmol/L of SMARTpool: ON-TARGETplus ATF4 siRNA (Dharmacon). Cells for incubated with the siRNA for 12 hours before the transfection medium was replaced by complete growth medium and treated with 5 μmol/L of 4-HPR for 6 hours prior to use in experiments.
ATF4 were compared by paired two-tailed t test. The difference in survival curves was compared by log-rank (Mantel–Cox) test. The difference in the dose–response curves of 4-HPR, ABT-199, and 4-HPR + ABT-199 with or without the presence of vitamin C was compared by the ratio-paired two-tailed t test. The differences in BCL-2 protein expression, ABT-199 fraction affected, and the 4-HPR + ABT-199 CIₐ for the high BCL-2 and low BCL-2 groups, as well as MYCN-amplified and MYCN non-amplified groups were compared by non-paired two-tailed t test. All statistical analysis was computed using GraphPad Prism 7.01. The CIₐ value (37) and the concentration cytotoxic or inhibitory for 50% of cells (ICₐ) were used in the combination response curves of 4-HPR, ABT-199, and ABT-263 (ref. 26), the association shown to be a biomarker for the sensitivity to BCL-2 family inhibitors (ABT-737 and ABT-263; ref. 26), the association between the expression of antiapoptotic BCL-2 family of proteins to ABT-199 was examined. In each of the seven neuroblastoma cell lines most sensitive and resistant to ABT-199 and sensitivity to ABT-199 was examined. In each of the seven neuroblastoma cell lines, we assessed expression of the antiapoptotic BCL-2 family of proteins and sensitivity to ABT-199 was examined. In each of the seven neuroblastoma cell lines most sensitive and resistant to ABT-199 (Fig. 1C), we assessed expression of the antiapoptotic BCL-2 family of proteins (Fig. 1D). BCL-2 and BCL-W protein levels in neuroblastoma cell lines most sensitive and resistant to ABT-199 (Fig. 1A). Because BCL-2 expression has been shown to be a biomarker for the sensitivity to BCL-2 family inhibitors (ABT-737 and ABT-263; ref. 26), the association between the expression of antiapoptotic BCL-2 family of proteins and sensitivity to ABT-199 was examined. In each of the seven neuroblastoma cell lines most sensitive and resistant to ABT-199 (Fig. 1C), we assessed expression of the antiapoptotic BCL-2 family of proteins (Fig. 1D). BCL-2 and BCL-W protein levels in the 199 synergy in neuroblastoma cell lines

BCL-2 is a biomarker of ABT-199 sensitivity and 4-HPR + ABT-199 synergy in neuroblastoma cell lines

4-HPR and ABT-199 showed synergistic activity in multiple neuroblastoma cell lines (Fig. 1A and B; Supplementary Figs. S1 and S2), within the clinically achievable concentrations for both agents. We classified cell lines with ABT-199 ICₐ >5 μmol/L (2.5 times clinically achievable concentration) as relatively resistant to ABT-199. Cell lines relatively sensitive to single-agent ABT-199 (defined as ABT-199 ICₐ <5 μmol/L) showed greater synergy between 4-HPR + ABT-199 compared with cell lines relatively resistant to ABT-199 (Fig. 1A). Because BCL-2 expression has been shown to be a biomarker for the sensitivity to BCL-2 family inhibitors (ABT-737 and ABT-263; ref. 26), the association between the expression of antiapoptotic BCL-2 family of proteins and sensitivity to ABT-199 was examined. In each of the seven neuroblastoma cell lines most sensitive and resistant to ABT-199 (Fig. 1C), we assessed expression of the antiapoptotic BCL-2 family of proteins (Fig. 1D). BCL-2 and BCL-W protein levels in the 199 synergy in neuroblastoma cell lines

BCL-2 is a biomarker for 4-HPR + ABT-199 activity in neuroblastoma PDXs

We tested activity of 4-HPR + ABT-199 using two PDXs, both established at postmortem from progressive disease after therapy: Felix-PDX (low BCL-2 expression) and COG-N-452x (high BCL-2 expression; Fig. 2A; Supplementary Fig. S3A). In COG-N-452x, ABT-199 + keto significantly extended EFS relative to vehicle control (P < 0.001) and EFS of mice treated with 4-HPR + ABT-199 + keto was greater than 4-HPR + keto (93 vs. 43 days; P < 0.0001) or ABT-199 + keto (93 vs. 46 days; P < 0.0001; Fig. 2B and C). Felix-PDX (low BCL-2 expression) did not show significant improvement in EFS for ABT-199 + keto versus control or for 4-HPR + ABT-199 + keto relative to the 4-HPR + keto or ABT-199 + keto (Fig. 2B and C).

BCL-2 expression is consistent from diagnosis to progressive disease in the same patient

Because obtaining tumor biopsies at time of progressive disease to analyze BCL-2 protein expression as a biomarker can be challenging, we compared BCL-2 protein expression in a panel of neuroblastoma-matched pairs (cell lines established at diagnosis and progressive disease from the same patient) from 10 patients. BCL-2 protein expression was consistent between diagnosis and relapse cell lines established from the same patient (Fig. 3A). We also compared BCL-2 protein expression in a matched pair of PDXs established at diagnosis and progressive disease from the same patient, COG-N-603x (diagnosis) and COG-N-623x (progressive disease). The matching cell lines for these PDXs were COG-N-603x and COG-N-623x. We observed that COG-N-603x and COG-N-623x, both expressed high BCL-2 protein levels (Fig. 3B; Supplementary Fig. S3B), which is consistent with the expression of BCL-2 in the matching cell lines (Fig. 3A). 4-HPR + ABT-199 + keto was more active against both diagnosis (DX) and relapse (PD) PDXs relative to 4-HPR + keto (P < 0.0001) or to ABT-199 + keto (P < 0.001; Fig. 3C; Supplementary Fig. S4A). These data are consistent with our observations in cell lines showing that BCL-2 is a biomarker for 4-HPR + ABT-199 activity, and that BCL-2 protein expression is consistent between diagnosis and progressive disease. In the matched pair PDXs we assessed the activity of CYCLO + TOPO (one of the treatment regimens for neuroblastoma; ref. 38). COG-N-623x (PD, median mouse survival = 38 days) was significantly more resistant (P < 0.05) to CYCLO + TOPO treatment relative to COG-N-603x (DX, median mouse survival = 71 days; Fig. 3D; Supplementary Fig. S4B), demonstrating that 4-HPR + ABT-199 is noncross-resistant with standard cytotoxic chemotherapy for neuroblastoma.

4-HPR enhanced ABT-199 apoptosis by inducing NOXA expression

We observed that 4-HPR + ABT-199 induced greater caspase 3 cleavage (Fig. 4A and B) and higher DNA fragmentation measured by TUNEL assay (P < 0.0001; Fig. 4C and D) compared with both single agents. These data suggest that apoptosis plays a major role in the combination cytotoxicity of 4-HPR + ABT-199. To study the specific mechanism of apoptosis, changes in expression of the proapoptotic BCL-2 family of proteins, myeloid differentiation factor 88 (MCL-1) protein (Supplementary Fig. S5). These data suggest that an increase in NOXA expression by 4-HPR plays an important role in the enhanced apoptosis observed with the 4-HPR + ABT-199 combination.

Knockdown of NOXA abolished 4-HPR + ABT-199 synergy, while overexpressing NOXA enhanced ABT-199 cytotoxicity

To determine whether NOXA can enhance the apoptosis induced by ABT-199, we transduced two neuroblastoma cell lines, COG-N-415x and COG-N-623x, with either empty vector or a PMAIP1 (encoding NOXA) doxycycline-inducible vector (PMAIP1-myc-DDK). Doxycycline (0.5 μg/mL) induced NOXA protein expression without significantly affecting the cell viability in both cell lines (Fig. 5A and B). ABT-199 (in the presence of doxycycline) induced much higher cytotoxicity in cells transduced with PMAIP1-myc-DDK relative to the cells transduced with empty vector in both tested cell lines (Fig. 5C and D).

To further confirm the role of NOXA in the combination cytotoxicity of 4-HPR + ABT-199, we stably knocked down NOXA and conducted in vitro cytotoxicity experiments in COG-N-415x

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Figure 1.
BCL-2 protein expression as a marker of ABT-199 sensitivity and 4-HPR + ABT-199 synergy in neuroblastoma cell lines. A, CIN values of all tested neuroblastoma cell lines for the combination cytotoxicity of ABT-199 and 4-HPR showing data for 15 ABT-199 relatively sensitive cell lines (ABT-199 IC_{50} < 5 \text{ μmol/L}), black symbols and 17 ABT-199 relatively resistant cell lines (ABT-199 IC_{50} > 5 \text{ μmol/L}), red symbols. CIN < 1 (synergy), CIN = 1 (additive effect), and CIN > 1 (antagonism; ref. 37). Fixed-ratio dose–response curves used to generate data for A are shown in Supplementary Figs. S1 and S2. B, Example of DIMSCAN assay dose–response curves for ABT-199 relatively sensitive (COG-N-452h) and ABT-199 relatively resistant (Felix-h) cell lines. Cells were treated with varying (fixed-ratio) concentrations of 4-HPR, ABT-199, and 4-HPR + ABT-199 (six replicates per drug concentration) for 96 hours then analyzed by the DIMSCAN assay. C, Dose–response curves of seven ABT-199 relatively sensitive and seven ABT-199 relatively resistant cell lines selected for determining the difference in basal expression of antiapoptotic proteins. D, Expression of antiapoptotic proteins in seven ABT-199 relatively sensitive cell lines and seven ABT-199 relatively resistant cell lines. E, Dot plots quantitating immunoblotting data shown in C. The BCL-2 and BCL-W protein levels were significantly higher in the ABT-199 relatively sensitive group versus the ABT-199 relatively resistant group. n.s., not significant. F, CIN values for the seven selected ABT-199 relatively sensitive cell lines compared with seven selected ABT-199 relatively resistant cell lines. 4-HPR + ABT-199 CIN values of the ABT-199 relatively sensitive lines was significantly lower than the CIN values of the ABT-199 relatively resistant lines.
and COG-N-623h. We found that NOXA knockdown reduced the cytotoxicity of single-agent ABT-199 and decreased the synergy of 4-HPR + ABT-199 in both cell lines (Fig. 5E and F). NOXA knockdown also diminished the enhanced apoptosis observed by 4-HPR + ABT-199 relative to single agents (Fig. 5G). Together, these data demonstrate that NOXA induction by 4-HPR is a key mechanism of 4-HPR + ABT-199 synergy.

NOXA binds to MCL-1 while having little affinity to other antiapoptotic proteins (39, 40). To verify NOXA affinity for MCL-1 in our system, NOXA immunoprecipitation was conducted in COG-N-623h transduced with PMAIP1-Myc-DDK or the empty vector. Only MCL-1, not BCL-2, BCL-W, or BCL-XL, was detected in NOXA pull-down samples (Fig. 5H). NOXA is also known as a sensitizer proapoptotic protein, which does not directly bind to activated BAX/BAK oligomerization but instead enhances apoptosis by inhibiting MCL-1 (4). We also found that NOXA does not directly bind to BAX or BAK (Fig. 5H). These data suggest that 4-HPR enhanced ABT-199 activity by inducing NOXA, which specifically binds to and inhibits the MCL-1 protein.

4-HPR induced NOXA through the ATF3 and ATF4 pathway

To determine the mechanism of NOXA induction by 4-HPR, we analyzed control and 4-HPR–treated (5 μmol/L for 15 hours) CHLA-119 cells by RNA-seq. The transcription factors binding to the PMAIP1 (NOXA) promoter were identified using transcription factor analysis available with the PROMO database (41, 42). Expression of ATF3 (a transcription factor binding to CTACGTCA sequence of the PMAIP1 promoter) and ATF4 (a transcription factor forming a complex with ATF3 to activate PMAIP1) were elevated in 4-HPR–treated cells relative to control cells (Fig. 6A). Even though the PROMO database did not identify ATF4 as a potential transcription factor of the PMAIP1 promoter, we found that ATF4 has also been reported to bind to the CTACGTCA sequence of the PMAIP1 promoter (43) and that mutation of this sequence reduced the PMAIP1 promoter activation by ATF4 (43). Moreover, ATF4 has been reported to activate the ATF3 promoter (44). In multiple neuroblastoma cell lines treated with 4-HPR, ATF-3, ATF-4, and NOXA mRNA were increased (Fig. 6B; Supplementary Fig. S6A and S6B), and 4-HPR also increased ATF-3, ATF-4, and NOXA protein expression (Fig. 6C). ATF3 and ATF4 knockdown by siRNA reduced NOXA protein induced by 4-HPR, while ATF4 knockdown had greater effect in reducing 4-HPR–induced NOXA expression compared with ATF3 knockdown (Fig. 6D). ATF4 knockdown also reduced the level of ATF3 protein induced by 4-HPR, while ATF4 knockdown had greater effect in reducing 4-HPR–induced NOXA expression compared with ATF3 knockdown (Fig. 6D). ATF4 knockdown also reduced the level of ATF3 protein induced by 4-HPR (Fig. 6D), confirming that ATF4 is upstream of ATF3 and NOXA. In mice bearing the COG-N-623x PDX treated with either vehicle or 4-HPR + keto for 4 days ATF3, AT and NOXA proteins were induced in the PDX tumors by 4-HPR (Fig. 6E).

ROS have been shown to be one of the mechanisms of 4-HPR cytotoxicity, and vitamin C, an antioxidant capable of penetrating into mitochondria, can neutralize the ROS generated by 4-HPR (34, 45). ATF4 is reported as an ROS responsive gene (46), which suggests that ROS generated by 4-HPR is upstream of the
Figure 3.
BCL-2 protein expression at DX and PD are consistent and may provide a biomarker for neuroblastoma tumors likely to respond to the combination of 4-HPR + ABT-199. A, BCL-2, NOXA, and MCL-1 protein expression for matched pair cell lines (established at DX and PD from 10 patients). High BCL-2–expressing cell lines established at DX and later at PD from the same patient show comparable BCL-2 protein expression. B, High BCL-2 protein expression was observed for both PDXs of the matched pair PDX models, COG-N-623x (DX) and COG-N-603x (PD). C, Tumor growth curves and EFS of the control, 4-HPR + keto, ABT-199 + keto, and 4-HPR + ABT-199 + keto groups for COG-N-603x and COG-N-623x. Both models showed significant improvement in survival for 4-HPR + ABT-199 + keto versus the 4-HPR + keto and ABT-199 + keto groups. D, EFS of the control and CYCLO + TOPO groups for COG-N-603x (DX) and COG-N-623x (PD) showing the increased resistance of COG-N-623x to CYCLO + TOPO compared with COG-N-603x.
ATF4, ATF3, and NOXA pathway. COG-N-623h and COG-N-452h were pretreated with vitamin C for 1 hour before incubating with 4-HPR for 12 hours prior to immunoblotting. Vitamin C attenuated the expression of ATF-3, ATF-4, and NOXA induced by 4-HPR (Fig. 6F) and significantly decreased the cytotoxicity of 4-HPR + ABT-199 (Supplementary Fig. S7). ATF-4, ATF-3, and NOXA were not induced by ATRA (Supplementary Fig. S6E). Together, our data suggest that 4-HPR induced NOXA through generating ROS, which activates the ATF4 and ATF3 pathway.

4-HPR–induced NOXA is independent of MYCN

MYCN has been shown to activate the NOXA promoter [6]; however, treatment of 4-HPR decreased MYCN protein in MYCN-amplified cell lines (Supplementary Fig. S8A). We also observe that vitamin C can reverse the effect of 4-HPR reducing MYCN protein expression. Our data suggested that NOXA induced by 4-HPR is independent of MYCN protein expression and that 4-HPR can reduce the MYCN protein expression in neuroblastoma models.

BCL-2 is a more robust predictor than MYCN amplification status for ABT-199 single agent sensitivity and 4-HPR + ABT-199 combination synergy

To confirm BCL-2 as the marker of 4-HPR + ABT-199 combination synergy, we separated all neuroblastoma cell lines (n = 28) with available BCL-2 protein expression into either high BCL-2 protein expression or low BCL-2 protein expression group (Supplementary Fig. S9A). We used ABT-199...
Figure 5.
NOXA as a crucial mediator of 4-HPR + ABT-199 synergy. A and B, COG-N-415h and COG-N-623h, transduced with either empty vector or PMAIP1-Myc-DDK, were treated with different concentrations of doxycycline for 48 hours before being subjected to immunoblotting for NOXA (Myc-DDK tagged) protein. Cells were also treated with different concentrations of doxycycline (six replicates per drug concentration) for 96 hours then analyzed for cytotoxicity by DIMSCAN. Doxycycline (0.5 μg/mL) was selected to induce NOXA-Myc-DDK expression for both COG-N-415h and COG-N-623h. (Continued on the following page.)
fraction affected (Fa) at 1.25 μM/L as the marker of ABT-199 sensitivity because not all cell lines were able to achieve IC50 at the maximum tested concentration of ABT-199. We found that the high BCL-2 protein expression group showed significantly higher sensitivity to single-agent ABT-199 (Supplementary Fig. S9B) and significantly higher synergy to the combination of 4-HPR + ABT-199 (Supplementary Fig. S9C). Using the same group of cell lines, we separated them into either MYCN-amplified or MYCN-nonamplified groups. We found that there were no significant differences in ABT-199 single-agent sensitivity (Supplementary Fig. S9D) or 4-HPR + ABT-199 combination synergy (Supplementary Fig. S9E) between the MYCN-amplified or nonamplified groups of lines.

**Discussion**

Neuroblastoma, especially high-risk neuroblastoma remains a therapeutic challenge (1, 47). Identifying well-tolerated drug combinations that are not cross-resistant with currently employed therapy would enable testing in patients with progressive disease and eventual incorporation into upfront treatment regimens. We demonstrated that combining 4-HPR + ABT-199 is both highly synergistic in vitro at concentrations that are achievable in the plasma for each single agent in patients and was more active than either of the single agents against multiple high BCL-2–expressing PDXs established from patients who developed progressive disease on currently available therapy. ABT-199 + 4-HPR was also active against neuroblastoma PDXs established from patients at time of progressive disease (using dosing comparable with that used for single agents in patients). ABT-199 + 4-HPR was equally active against a neuroblastoma PDX that developed resistance to CYCLO + TOPO and a PDX from the same patient established at diagnosis that was sensitive to CYCLO + TOPO. 4-HPR has shown tolerable toxicity and achieved multiple complete responses in a phase 1 clinical trial for high-risk neuroblastoma (20). ABT-199 received FDA approval for treating CLL with 17p deletion, and it showed manageable systemic toxicities (14, 15). Thus, combining these two agents in patients with neuroblastoma with high BCL-2 expression may provide a regimen with minimal systemic toxicities that is active against progressive neuroblastoma.

High BCL-2 expression is known as a marker of sensitivity to BCL-2 inhibitors in cancer cells, including neuroblastoma (5, 25, 26). We demonstrated that BCL-2 protein levels are consistent between tumors cells obtained at diagnosis and at progressive disease (the latter all from blood or bone marrow) using paired cell lines and a PDX pair from 10 patients. This opens the possibility of assessing BCL-2 protein expression in tumor biopsies of neuroblastoma obtained at diagnosis to identify patients with progressive disease that are more likely to respond to 4-HPR + ABT-199.

The survival of neuroblastoma cells depend on either MCL-1 or BCL-2 protein (5). Targeting only BCL-2 protein has been shown to cause neuroblastoma cells to depend on MCL-1 for survival (5). Consistent with observations by others (6, 18, 19), we found that in some neuroblastoma cell lines and PDXs single-agent ABT-199 induced cytotoxicity but ABT-199 single-agent activity is often modest. Thus, clinical development of ABT-199 has focused on combinations with traditional cytotoxic agents for which tolerability in patients has yet to be demonstrated and may be limited by enhanced marrow toxicity (48). Thus, development of combinations of agents with ABT-199 that have minimal marrow toxicity, such as 4-HPR, are warranted.

The synergism between 4-HPR and ABT-199 is similar to synergism we observed between 4-HPR and ABT-737 (11), with 4-HPR benefitting from inhibition of BCL-2 while at the same time inhibiting MCL-1. Here we have demonstrated that the mechanism of MCL-1 inhibition is via induction of NOXA, which specifically binds to MCL-1 and prevents MCL-1 from interfering with the proapoptotic function of ABT-199. Moreover, knocking down and also overexpressing NOXA confirmed that induction of NOXA is key to enhancing ABT-199 activity against neuroblastoma.

Inducing NOXA through MYCN has been shown to increase ABT-199 activity in MYCN-amplified neuroblastoma cell lines (6). However, we have demonstrated that 4-HPR induction of NOXA protein is greater than NOXA expression seen in MYCN-amplified cell lines. Moreover, we also observed that 4-HPR can reduce MYCN protein in MYCN-amplified cell lines indicating that the mechanism of 4-HPR inducing NOXA production is independent of MYCN. The increase in NOXA protein expression, either by 4-HPR treatment or through the NOXA plasmid transduction, significantly increased the activity of ABT-199 in MYCN-amplified cell lines. Moreover, we also observed that BCL-2 expression is a stronger predictor compared with MYCN genomic amplification for BCL-2 single-agent sensitivity or for 4-HPR + ABT-199 combination synergy. Taken together our data indicate that the enhanced cytotoxicity of ABT-199 when combined with 4-HPR is independent of the MYCN amplification status of neuroblastoma cells.

In investigating how 4-HPR induced NOXA protein expression, we discovered that 4-HPR induced NOXA through generating ROS which can activate the ATF4 and ATF3 transcription factors. ATF3 and ATF4 have been shown to transcriptionally activate PMAIP1, the gene encoding NOXA, the ATF4 and ATF3 induction are generally associated with endoplasmic reticulum stress which can be induced by proteasome inhibitors (49). Our study is the first to demonstrate that 4-HPR induces NOXA protein expression via ATF4 and ATF3. It has been suggested that 4-HPR generates ROS by inhibiting the mitochondrial electron complexes (50) and previous studies have shown that vitamin C, a mitochondrial-penetrating antioxidant, can effectively attenuate 4-HPR toxicity.
Induction of ATF-3 and ATF-4 transcription factors by 4-HPR–induced ROS mediate induction of NOXA expression in neuroblastoma. A, RNA-seq was carried out with control and 4-HPR–treated (5 μmol/L for 15 hours) CHLA-119 cells; expression of selected genes are shown in the heatmap. We used the PROMO database and PMAIP1 promoter sequence (−500 to 0 of the ATG transcription start site) to further narrow down RNA-seq data to transcription factors binding to the PMAIP1 promoter. ATF3, a transcription factor known to activate PMAIP1 promoter (43), showed the highest increase in response to 4-HPR. ATF4, a transcription factor known to activate ATF3 and PMAIP1 promoter (44, 45), is also upregulated. Heatmap scale was adjusted to emphasize differences in transcription factor expression. Relative differences in expression are better visualized in B. B, 4-HPR treatment significantly upregulated NOXA, ATF3, and ATF4 mRNA expression compared with controls. CHLA-119 was treated with 4-HPR (5 μmol/L) or vehicle control for 6 hours before being harvested for measuring changes in mRNA expression by real-time RT-PCR. C, 4-HPR treatment at 5 and 10 μmol/L for 12 hours induced both ATF3 and ATF4 protein expression along with NOXA protein expression in multiple tested neuroblastoma cell lines. D, Knockdown of ATF3 and ATF4 using siRNA reduced NOXA induction by 4-HPR treatment in multiple neuroblastoma cell lines. Cell lines were transfected with either nontargeted control (NTC) siRNA, ATF3 siRNA, or ATF-4 siRNA for 12 hours before being treated with 4-HPR (5 μmol/L) for 6 hours, harvested, and blotted for ATF-3, ATF-4, and NOXA. E, Mice bearing COG-N-623x (PDX) were treated with either vehicle or 4-HPR + keto for 4 days before the tumors being harvested and subjected to immunoblotting for NOXA, ATF3, and ATF4 protein. Figure shows representative data from tumors in 2 mice, control and 4-HPR treated. F, Vitamin C (Vit. C) reduced ATF3, ATF4, and NOXA induced by 4-HPR treatment. Cells were pretreated with vitamin C (150 μmol/L) for 1 hour before being treated with 4-HPR for 12 hours. G, Mechanism of 4-HPR + ABT-199 mechanism of synergy.
cytotoxicity (34, 46). We showed that vitamin C significantly reduced induction by 4-HPR of ATF3, ATF4, and NOXA, and also reduced 4-HPR + ABT-199 cytotoxicity, thus implicating 4-HPR–generated ROS in the induction of NOXA and the synergy of 4-HPR + ABT-199.

In summary, 4-HPR + ABT-199 demonstrated synergistic activity in preclinical models of neuroblastoma with high BCL-2 protein expression via induction of NOXA by 4-HPR. In our preclinical models, BCL-2 protein expression identified neuroblastomas with high sensitivity to 4-HPR + ABT-199. BCL-2 protein expression was consistent from diagnosis to progressive disease in pairs of cell lines and PDXs established from the same patients. These data support carrying out early phase clinical trials of 4-HPR + ABT-199 in neuroblastomas with high BCL-2 expression in tumor biopsies obtained at any time during the course of therapy.

Disclosure of Potential Conflicts of Interest
C.P. Reynolds is a chief scientific officer (paid consultant) at, and has ownership interest (including patents) in, CerRx, Inc. Dr. Kang is a consultant (unpaid) to CerRx, Inc. No potential conflicts of interest were disclosed by the other authors.

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Fenretinide via NOXA Induction, Enhanced Activity of the BCL-2 Inhibitor Venetoclax in High BCL-2–Expressing Neuroblastoma Preclinical Models

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