Reactive oxygen species mediates the synergistic activity of fenretinide combined with the microtubule inhibitor ABT-751 against multi-drug-resistant recurrent neuroblastoma xenografts

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Authors' Disclosure of Potential Conflicts of Interest

The Children's Hospital Los Angeles (CHLA) holds patents and/or patent applications on Fenretinide/LYM-X-SORB™ (LXS) oral powder (the study drug). CHLA and co-inventors of Fenretinide/LXS oral powder, Drs. Barry J. Maurer and C. Patrick Reynolds, Texas Tech University Health Sciences Center, Lubbock, TX, may potentially benefit financially from the development and future use of the study drug.
Abstract

ABT-751 is a colchicine-binding site microtubule inhibitor. Fenretinide (4-HPR) is a synthetic retinoid; both agents have shown activity against neuroblastoma in laboratory models and clinical trials. We investigated the antitumor activity of 4-HPR + the microtubule-targeting agents ABT-751, vincristine, paclitaxel, vinorelbine, or colchicine in laboratory models of recurrent neuroblastoma. Drug cytotoxicity was assessed \textit{in vitro} by a fluorescence-based assay (DIMSCAN) and in subcutaneous xenografts in nu/nu mice. Reactive oxygen species levels (ROS), apoptosis, and mitochondrial depolarization were measured by flow cytometry; cytochrome c release and proapoptotic proteins by immunoblotting. 4-HPR + ABT-751 showed modest additive or synergistic cytotoxicity, mitochondrial membrane depolarization, cytochrome c release, and caspase activation compared to single agents \textit{in vitro}; synergism was inhibited by antioxidants (ascorbic acid, \(\alpha\)-tocopherol). 4-HPR + ABT-751 was highly active against four xenograft models, achieving multiple maintained complete responses. The median event-free survival (days) for xenografts from 4 patients combined were control = 28, 4-HPR = 49, ABT-751 = 77, and 4-HPR + ABT-751 > 150 \((P < 0.001)\). Apoptosis (TUNEL) was significantly higher in 4-HPR + ABT-751-treated tumors than with single agents \((P < 0.01)\) and was inhibited by ascorbic acid and \(\alpha\)-tocopherol \((P < 0.01)\), indicating that ROS from 4-HPR enhanced the activity of ABT-751. 4-HPR also enhanced the activity against neuroblastoma xenografts of vincristine or paclitaxel but the latter combinations
were less active than 4-HPR + ABT-751. Our data support clinical evaluation of 4-HPR combined with ABT-751 in recurrent and refractory neuroblastoma.
Introduction

Retinoids are active against neuroblastoma both as differentiation inducers and as cytotoxic agents. The differentiation inducer 13-cis-retinoic acid (13-cis-RA) achieved complete responses in a high-risk neuroblastoma phase I study of high-dose (2 weeks on, 2 weeks off) after myeloablative therapy (1), and a randomized phase III study demonstrated that maintenance therapy of high-risk neuroblastoma with 13-cis-RA after completion of cytotoxic consolidation therapy significantly enhanced event-free survival (2). Outcome in a subsequent non-randomized study employing 13-cis-RA as maintenance therapy was consistent with a benefit from 13-cis-RA maintenance therapy (3). A phase III trial demonstrated that maintenance therapy that combined 13-cis-RA with ch14.18 antibody + cytokines further improved outcome (4).

The synthetic retinoid N-(4-hydroxyphenyl)retinamide (fenretinide; 4-HPR) is cytotoxic for neuroblastoma in vitro by p53-independent and caspase-dependent and -independent mechanisms that involve increases of reactive oxygen species (ROS) and dihydroceramides (5, 6). Clinical activity in recurrent neuroblastoma of 4-HPR when given as sub-optimally bioavailable capsules was modest in early-phase studies (7-9). Incorporation of 4-HPR into a lipid matrix (LYM-X-SORB™) and formulation as an oral powder (4-HPR/LXS) increased bioavailability and anti-neuroblastoma activity in mouse xenografts (10). A pediatric phase I trial of 4-HPR/LXS demonstrated significantly higher 4-HPR exposures than previously achieved with the capsule formulation and documented four complete responses in recurrent neuroblastoma (11).
ABT-751 is a sulfonamide microtubule inhibitor that binds to the colchicine-binding site on beta-tubulin, inhibiting microtubule polymerization (12, 13). In cell lines, exposure to ABT-751 leads to a cell cycle block at G2/M and induces apoptosis (13). As ABT-751 is not a substrate for P-glycoprotein, it is active against tumor models resistant to other microtubule inhibitors, such as vincristine and paclitaxel, and ABT-751 has been shown to be active against neuroblastoma xenografts (14). Phase I clinical trials with ABT-751 achieved stable disease but few objective responses in recurrent neuroblastoma (15-17). However, a Children's Oncology Group phase II study of ABT-751 failed to demonstrate an increase in progression-free survival when compared to historical controls (18).

Although there has been a consistent improvement in outcome for neuroblastoma patients over the past 2 decades, many patients with high-risk neuroblastoma develop disease progression that is refractory to further therapy (19). New drugs and drug combinations active against recurrent, multidrug-resistant neuroblastoma are needed to improve survival. Moreover, well-tolerated agents that demonstrate clinical activity against recurrent neuroblastoma may be used to further improve outcome by employing them in post-consolidation maintenance therapy (2, 4, 20). Both 4-HPR/LXS and ABT-751 are well-tolerated, orally available drugs with differing mechanisms of action and non-overlapping systemic toxicities that have shown single-agent activity in recurrent neuroblastoma preclinical models (5, 10, 14, 17). Thus, we investigated whether combining 4-HPR with ABT-751 could enhance anti-
neuroblastoma activity against neuroblastoma cell lines and multidrug-resistant neuroblastoma xenografts established from patients with progressive disease.

Materials and Methods

Chemicals

4-HPR and 4-HPR/LXS (the LYM-X-SORB™ powder formulation of 4-HPR) (10) were provided by the Developmental Therapeutics Program of the National Cancer Institute (NCI; Bethesda, MD). ABT-751 was supplied by Abbot Laboratories (Abbot Park, IL). Vincristine sulfate salt (VCR), vinorelbine ditartrate (VRL), paclitaxel (PTX), colchicine (CLC), ascorbic acid (vitamin C; Vit C), α-tocopherol (vitamin E; Vit E), n-acetylcysteine (NAC), sodium thiosulfate (STS), fluorescein diacetate (FDA), eosin Y, dimethyl sulfoxide (DMSO), and ethanol were purchased from Sigma-Aldrich. For in vivo administration, vincristine was manufactured by Mayne Pharma Inc., colchicine was manufactured by West-Ward Pharmaceutical Corp., and paclitaxel (Taxol) was manufactured by Bristol Myers Squibb Co. Paraformaldehyde was from USB Corporation (Cleveland, OH). MitoProbe JC-1 and 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) were from Invitrogen (Carlsbad, CA). 4-OH-fenretinide was from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). References to chemical structures are 4-HPR (21), ABT-751 (22), vincristine, vinorelbine, and colchicine (23).
Cell Culture

Human neuroblastoma cell lines, SMS-KCNR, SMS-SAN, CHLA-15, CHLA-20, CHLA-90, CHLA-119, CHLA-136, and CHLA-140 have been described previously (24-28). SMS-SAN and CHLA-15 were established at diagnosis prior to therapy, SMS-KCNR at progressive disease after dual-agent induction chemotherapy. CHLA-20, CHLA-119, and CHLA-140, are multidrug-resistant cell lines established at time of progressive disease; CHLA-119 was established from a rapidly fatal marrow relapse after a single course of 13-cis-RA after complete response to CCG-3891 consolidation chemotherapy (2). FU-NB-2006 was established from post-mortem blood at time of progressive disease after intensive multi-agent chemotherapy and single-agent oral fenretinide. CHLA-90 and CHLA-136 are multidrug-resistant cell lines established at relapse after myeloablative chemotherapy and autologous bone marrow transplant. CHLA-90 and CHLA-119 bear TP53 loss-of-function mutations (26); CHLA-90, CHLA-119, CHLA-136, and CHLA-140 overexpress the MDR1 gene relative to neuroblastoma cell lines established at diagnosis (Reynolds, unpublished).

SMS-SAN and SMS-KCNR were cultured in RPMI-1640 (Mediatech Inc., Herdon, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS). CHLA-15, CHLA-20, CHLA-90, CHLA-119, CHLA-136, CHLA-140, and FU-NB-2006 were cultured in Iscove’s modified Dulbecco’s medium (Cambrex, Walkersville, MD) supplemented with 3 mM L-glutamine, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenous acid, and 20% heat-inactivated FBS. Cell lines used in this study were tested to be Mycoplasma-free and maintained at
37°C in a humidified incubator containing 95% room air and 5% CO₂ atmosphere. Cell line identities were validated at time of experimentation by short tandem repeat (STR) profiling (29) as compared to the Children’s Oncology Group cell line and xenograft STR database (www.COGcell.org).

**Cytotoxicity Assay**

Cytotoxicity dose-response curves (4 days post start of drug exposure) were determined using the DIMSCAN digital imaging microscopy assay system as previously described ((30, 31). Concentration ranges tested *in vitro* (modeled on clinically obtainable plasma levels of active agents and taking into account protein-binding considerations) were:: 4-HPR, 0-10 µM; ABT-751, 0-500 ng/mL CLC, 0-10 µM; VCR, 0-10 µM; VRL, 0-10 µM; and PTX; vitamin C, 0-250 µM; vitamin E, 0-250 µM; n-acetylcysteine 0-500 µM; sodium thiosulfate 0-500 µM. Cells (4000 in 100 µL/well) were seeded in 96-well plates 16 to 24 hours before 100 µL of drugs (stock solutions: 10 mM 4-HPR in 99.5% ethanol; 1 mg/mL ABT-751 in DMSO) were added to each well (n = 12 replicates). For antioxidant studies, cells were pretreated for 3 hours with antioxidants before addition of 4-HPR and ABT-751.

**Determination of Reactive Oxygen Species Production**

CHLA-119 cells (1 × 10⁶) were treated with 0.62 µM 4-HPR, 62.5 ng/mL ABT-751, and 0.62 µM 4-HPR + 62.5 ng/mL ABT-751, alone or in combination with antioxidants (250 µM vitamin C, 250 µM vitamin E, 500 µM n-acetylcysteine
(NAC), or 500 µM sodium thiosulfate (STS)) for 3 hours. For some experiments concentrations of NAC to 10 mM and of STS to 5 mM were employed. Cells were incubated in 1 mL of medium containing 50 µM of the ROS-sensitive probe carboxy-H$_2$DCFDA ((5) for 25 minutes at 37°C. For a positive control, H$_2$O$_2$ was added to the cells at a final concentration of 100 µM for 15 minutes. Cells were centrifuged, re-suspended in 500 µl of medium, and analyzed by a BD LSR II flow cytometer.

**Measuring Apoptosis by TUNEL**

TUNEL (terminal deoxynucleotidyltransferase dUTP nick end labeling; APO-DIRECT kit, BD Biosciences) was used to assess apoptosis. CHLA-119 cells (1 × 10$^6$) exposed for 20-hours to 0.62 µM 4-HPR, 62 ng/mL ABT-751, or 0.62 µM 4-HPR + 62 ng/mL ABT-751 (minimal drug concentrations demonstrating strong synergy) with or without a 3 hour pretreatment with (and continued exposure to) antioxidants (250 µM vitamin C, 250 µM vitamin E, 500 µM n-acetylcysteine, or 500 µM sodium thiosulfate). Cells were fixed with 1% (w/v) paraformaldehyde in PBS, stored in 70% ethanol at -20°C, washed and incubated in 50 µl of the TUNEL staining solution (2 hours, 37°C), washed twice, and 500 µl of propidium iodide/RNase added before flow cytometry using bandpass filters: 525 ± 25 nm (FITC), 610 ± 10 nm (propidium iodide).
Analysis of Mitochondrial Membrane Depolarization

Cells were incubated in 1 mL of medium containing 1 µM JC-1 mitochondrial probe (Invitrogen) for 30 minutes at 37°C, washed once with PBS and analyzed by flow cytometry. Mitochondrial membrane depolarization was indicated by a decrease in the red (590 ± 10 nm) to green (525 ± 25 nm) fluorescence intensity ratio.

Cytochrome c Release and Pro-Apoptotic Protein Expression

CHLA-119 cells (10 × 10^6 cells) were treated with 0.62 µM 4-HPR, 62.5 ng/mL ABT-751, and 0.62 µM 4-HPR + 62.5 ng/mL ABT-751 +/- 250 µM vitamin E for 24 hours and subjected to subcellular fractionation (BioVision Mitochondria/Cytosol Fractionation Kit, Mountain View, CA) to generate cytosolic (supernatant) and mitochondrial (pellet) fractions. For cytochrome c detection, 35 µg of the cytosolic fraction was separated by a 4% - 12% Bis-Tris precast gel (Invitrogen), transferred to a nitrocellulose membrane (Protran, Keene, NH), and incubated with 1:500 dilution of mouse monoclonal anti-cytochrome c (BD Biosciences) followed by 1:2000 dilution of horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Antibody binding was visualized with chemiluminescent substrate (Pierce, Rockford, IL) and autoradiography film (Denville Scientific, Inc, Metuchen, NJ), imaged with the VersaDoc 5000 MP Imaging System (Bio-Rad Laboratories, Hercules, CA) linked with Quantity One (version 4.6.6, Bio-Rad Laboratories) software. Cytochrome c release relative to vehicle control was normalized to β-actin expression. For pro-apoptotic protein expression cells were cells were
lysed in radioimmunoprecipitation (RIPA) buffer (Upstate, Lake Placid, NY) containing 1 mM phenylmethanesulphonylfluoride (PMSF, Sigma), and 1 µg/mL of protease inhibitor cocktail (Sigma) consisting of aprotinin, bestatin, leupeptin, and pepstatin. Lysates were incubated on ice for 30 minutes and sonicated briefly before centrifugation at 12,000 g for 30 minutes. Protein quantification of the supernatants employed the BCA protein assay kit (Pierce). Equal amounts of protein were resolved on 4% - 12% Bis-Tris precast gels (Invitrogen), transferred to a nitrocellulose membrane, and incubated with primary antibodies: rabbit polyclonal anti-cleaved caspase-9 at 1:1000 dilution (Cell Signaling Technology, Danvers, MA), rabbit polyclonal anti-cleaved caspase-3 at 1:1000 dilution (Cell Signaling Technology), rabbit polyclonal anti-poly (ADP-ribose) polymerase (PARP) at 1:1000 dilution (Santa Cruz Biotechnology), and mouse monoclonal anti-β-actin at 1:2000 dilution (Santa Cruz Biotechnology), followed by 1:2000 dilution of HRP-conjugated anti-mouse and anti-rabbit IgGs (Santa Cruz Biotechnology). Antibody binding was visualized with chemiluminescent substrate and autoradiography. Densitometry was used to evaluate changes in protein expression.

**Tumor Xenograft Testing**

Six- to eight-week-old athymic (nu/nu) mice (The Jackson Laboratory, Bar Harbor, MA) were injected subcutaneously between the shoulder blades with 12-15 million human neuroblastoma cells (SMS-KCNR, CHLA-90, CHLA-136, and CHLA-140 (25-28) mixed in Matrigel Matrix HC (BD Biosciences). Xenografted
mice were randomized into four treatment groups (5-6 mice per group): vehicle, 4-HPR/LXS, ABT-751, and 4-HPR/LXS + ABT-751. Drug treatment was begun when progressively growing tumors measured 100 to 200 mm$^3$. 4-HPR/LXS (240 mg 4-HPR/kg/day; slurried in water) and ABT-751 (75 mg/kg/day; dissolved in 95% D5W, 0.6% HCL, and 3.9% ethanol) were administered by gavage, 4-HPR/LXS twice daily divided doses and ABT-751 as a single daily dose, five days per week. Control animals received powdered LYM-X-SORB matrix slurried in 96% D5W, 0.6% HCL, and 3.4% ethanol. Dosing schedules for other microtubule inhibitors were as follows:; CLC, 0.05 mg/kg/day in divided daily doses 5 days a week by oral gavage; VCR, 0.75 mg/kg/day once a week by intraperitoneal injection (i.p.); and PTX, 10 mg/kg/day once a week by i.p.injection. Tumor growth and mouse weight was assessed twice weekly by caliper measurement and tumor volumes were calculated as $0.5 \times$ height $\times$ width $\times$ length (32). Mice were sacrificed by CO$_2$ narcosis when tumor volumes exceeded 1500 mm$^3$ or serious morbidity was observed. Event-free survival was time from initial xenografting until death from any cause. Animals were housed and treated according to protocols approved by the Institutional Animal Care and Use Committee.

**Assessment of Apoptosis In Vivo**

When xenograft tumors measured 300 to 500 mm$^3$, 96-hour treatment of the mice was begun as described for xenograft testing. Mice were sacrificed 4 hours after the last treatment, tumors excised and fixed in formalin. Paraffin
sections (5 μm) were stained for apoptosis via terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Ten high power fields for each treatment group were scored for apoptotic cells.

To examine the effect of antioxidants on the in vivo apoptosis of 4-HPR/LXS + ABT-751, nu/nu mice (3 per group) bearing 300 to 500 mm$^3$ SMS-KCNR xenografts were randomized into four treatment groups: vehicle, vitamins C+E, 4-HPR/LXS + ABT-751, and 4-HPR/LXS + ABT-751 + vitamins C+E. Mice were pretreated for 2 days with 3 mg/day of both vitamins C and E that continued during 48-hours of 180 mg/kg/day 4-HPR/LXS + 75 mg/kg/day ABT-751. Mice were sacrificed 4 hours after the last treatment and tumors assessed for apoptosis by TUNEL.

**Statistical Analyses**

Combination index (CIN) determined by CalcuSyn software (version 2.1, Biosoft, Cambridge, United Kingdom), was used to assess drug synergism (33), based on the CIN values (calculated for each concentration level): CIN > 1.10, antagonism; CIN of 0.9 to 1.10, additive; CIN < 0.9, synergism. Statistical significance of differences in means was determined by the one-way analysis of variance (ANOVA) test using SigmaPlot software (version 11.0, Systat Software Inc., San Jose, CA). Xenograft event-free survival employed Kaplan-Meier log-rank analysis. All $P$ values were two-sided and statistical significance was defined as $P < 0.05$. 

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Results

Cytotoxicity of 4-HPR + ABT-751 in neuroblastoma cell Lines

We determined the cytotoxicity of 4-HPR, ABT-751, and fixed-ratio concentration combinations of both drugs, in nine human neuroblastoma cell lines using the DIMSCAN cytotoxicity assay. Dose-response curves to 4-HPR, ABT-751, and 4-HPR + ABT-751 in the neuroblastoma cell lines are shown in Fig. 1. ABT-751 was more active than 4-HPR in some lines (CHLA-15, CHLA-20, and FU-NB-2006), while 4-HPR was more active than ABT-751 in others (SMS-SAN, SMS-KCNR, CHLA140, and CHLA-119), but the combination was active (achieving at least 2 logs of cell kill) in all the lines, though often activity of the combination was not significantly higher than of 4-HPR as a single agent. Cell lines established at time of progressive disease after chemotherapy were as sensitive as CHLA-15 and SMS-SAN (established prior to therapy), and the activity of ABT-751 +/- 4-HPR was more active in CHLA-20 (post-chemotherapy) than in CHLA-15 (pre-therapy line from the same patient as CHLA-20). Combining 4-HPR and ABT-751 showed a modest additive effect in most of the cell lines with drug synergy apparent in only one cell line, CHLA-119. 4-HPR + ABT-751 exhibited synergistic cytotoxicity in CHLA-119 at two fixed-ratio concentrations (4-HPR 0.62 µM, ABT-751 62.5 ng/mL; and 4-HPR 1.25 µM, ABT-751 125 ng/mL) with CIN values of 0.73 and 0.76, respectively, achieving 2 to 4 logs of tumor cell kill. Combination indices for the nine cell lines tested with 4-HPR + ABT-751 are shown in Supplementary Table S1.
Antitumor Activity of 4-HPR/LXS + ABT-751 in neuroblastoma xenografts

The in vivo activity of 4-HPR/LXS oral powder + ABT-751 was assessed in four subcutaneous human neuroblastoma xenograft models (CHLA-90, CHLA-136, CHLA-140, and SMS-KCNR), all established at time of progressive disease after chemotherapy or myeloablative chemoradiotherapy. Mice received 4-HPR/LXS and ABT-751, alone or in combination, by oral gavage 5 days/week. In contrast to the modest combination effect observed for most cell lines in vitro, activity of 4-HPR/LXS + ABT-751 was substantially greater than for either single agent in all four xenograft models (Fig. 2; individual mouse curves in Supplementary Fig. S1). In CHLA-90, SMS-KCNR, and CHLA-140 there were occasional mice treated with ABT-751 that maintained complete responses (MCR) > 60 days. However, multiple MCR were observed in SMS-KCNR, CHLA-136, and CHLA-140 with 4-HPR/LXS + ABT-751. Mouse event-free survival (EFS) for 4-HPR/LXS + ABT-751 was significantly greater than for either single agent for CHLA-90, SMS-KCNR, and CHLA-136 (P = 0.01), but not for CHLA-140 (Fig. 3). Median survival for all 4 xenograft models combined (n = 21 per cohort) was 28 days for the control cohort, 49 days for the 4-HPR-treated cohort, 77 days for the ABT-751-treated cohort, and > 150 days for the 4-HPR/LXS + ABT-751-treated cohort (Fig. 3E), with the EFS of the combination significantly greater than either single agent (P < 0.001).

The enhanced cytotoxicity observed in vitro from combining 4-HPR + ABT-751 was less than the enhanced anti-tumor effect of the combination in vivo. A
possible explanation for this could be that a metabolite of 4-HPR generated only
*in vivo* was more effective at promoting cytotoxicity of ABT-751 than 4-HPR, and
a 4-OH-fenretinide metabolite has been reported to have anti-microtubule activity
(34). However, we tested this hypothesis by comparing the ability of 4-HPR and
4-oxo-fenretinide to synergize with ABT-751 *in vitro* (fixed ratio dose-response
curves in both 20% and 2% oxygen using DIMSCAN) in the CHLA-119 and FU-
NB-2006 cell lines and found no significant difference between 4-HPR and 4-OH-
fenretinide in cytotoxic synergy with ABT-751 *in vitro* (data not shown).

**Specific antioxidants reduced cytotoxicity of 4-HPR + ABT-751 *in vitro***

As 4-HPR single agent cytotoxicity is partially mediated through increased
ROS levels (5, 35, 36), we examined the effects of antioxidants on the
cytotoxicity of 4-HPR + ABT-751 in the CHLA-119 cell line, which showed the
highest synergy *in vitro* between 4-HPR + ABT-751. Cells were pretreated for 3
hours with vitamin C, vitamin E, n-acetylcysteine (NAC), or sodium thiosulfate
(STS) before addition of 4-HPR and ABT-751 (Fig. 4A). Vitamin C and vitamin E,
but not NAC or STS, significantly diminished the cytotoxicity of 4-HPR and 4-
HPR + ABT-751 (*P* < 0.001); antioxidants did not alter cytotoxicity of ABT-751.
Dose response curves of 4-HPR + ABT-751 +/- antioxidants are shown in Fig.
4B. We repeated these this experiment using higher concentrations of NAC (.5
to 10 mM) and STS (.5 to 5 mM), which (unlike vitamins C and E) failed to
decrease the cytotoxicity of 4-HPR + ABT-751 (Supplementary Fig. S2).
Antioxidants reduced apoptosis induced by 4-HPR and 4-HPR + ABT-751

CHLA-119 cells were treated with 4-HPR, ABT-751, or 4-HPR + ABT-751 for 20 hours (+/- antioxidants added 3 hours prior to 4-HPR) and then analyzed for apoptosis by TUNEL (flow cytometry) (Fig. 4C). Apoptosis was seen in 11.5% of control cells (data not shown), 68.5% with 4-HPR, 37.7% with ABT-751, and 76.5% with 4-HPR + ABT-751. 4-HPR + ABT-751 somewhat increased apoptosis relative to single agent-treated cells, suggesting the enhanced cytotoxicity observed with the drug combination in vivo could be due to enhanced apoptosis. Vitamins C and E, but not NAC or STS, significantly decreased the percentage of apoptotic cells in 4-HPR- and combination-treated cells (Fig. 4C; P < 0.01). Of note, in cells treated with 4-HPR + ABT-751, vitamins C and E reduced apoptosis to the level of cells treated with ABT-751 alone. Antioxidants did not statistically alter the percentage of apoptotic cells in ABT-751-treated cells.

4-HPR + ABT-751 increased reactive oxygen species

We determined if combining 4-HPR with ABT-751 increased reactive oxygen species relative to single agents. CHLA-119 cells were treated with 4-HPR +/- ABT-751 for 3 hours and ROS levels were measured via flow cytometry using DCFDA. Mean DCFDA intensity values were: vehicle = 1821, 4-HPR = 3847, ABT-751 = 1847, 4-HPR + ABT-751 = 4432 for (Fig. 4D, left panel). DCFDA intensity in cells treated with 4-HPR + ABT-751 was ~15% greater than
4-HPR alone ($P = 0.04$) while mean DCFDA intensity of ABT-751-treated cells was not statistically different from controls. Vitamins C, E, and NAC significantly reduced the mean DCFDA intensity of 4-HPR + ABT-751-treated cells (Fig. 4D, right panel, $P < 0.01$). STS did not statistically alter the ROS level of 4-HPR + ABT-751-treated cells ($P = 0.10$). These results are roughly concordant with the observed effects of antioxidants on apoptosis induced in vitro (Fig. 4C).

**Effects of 4-HPR + ABT-751 on mitochondrial membrane depolarization, cytochrome c release, and caspase and PARP cleavage**

We examined the effects of 4-HPR + ABT-751 (20 hour exposure) (+/- antioxidants) on mitochondrial membrane depolarization (JC-1 staining) and cytochrome c release in CHLA-119 cells. Mitochondrial depolarization was ~22 % in vehicle control cells, ~46 % with 4-HPR, ~39 % with ABT-751, and ~57 % with 4-HPR + ABT-751 (Supplementary Fig. S3A). Vitamins C and E significantly decreased depolarization in 4-HPR-treated cells to ~25 % and ~26 % respectively, and in 4-HPR + ABT-751-treated cells to ~35 % and ~31 % respectively ($P < 0.01$). NAC or STS did not statistically alter mitochondrial membrane depolarization.

Cytochrome c release from mitochondria into the cytosol (immunoblotting) was assessed in CHLA-119 cells treated with 4-HPR, ABT-751, and 4-HPR + ABT-751 for 24 hours +/- vitamin E (Supplementary Fig. S3B). Densitometry values (normalized to β-actin, vehicle control set to 1.00) for cells treated with vitamin E = 0.9, 4-HPR = 2.0, ABT-751 = 3.5, 4-HPR + ABT-751 = 7.9, and 4-
HPR + ABT-751 + vitamin E = 3.7. Cytochrome c release was increased in 4-HPR- and ABT-751-treated cells, and to a greater degree in cells treated with both drugs. The addition of vitamin E to 4-HPR + ABT-751-treated cells decreased cytochrome c release into the cytosol.

Caspases-9 and -3 are major executors of the mitochondrial apoptotic cascade (37) (Supplementary Fig. S3B). Densitometry values (normalized to β-actin, vehicle control set to 1.00) for cleaved-caspase-9 for cells treated with vitamin E = 0.7, 4-HPR = 1.8, ABT-751 = 2.9, 4-HPR + ABT-751 = 4.8, 4-HPR + ABT-751 + vitamin E = 1.4. For cleaved-caspase-3: vitamin E = 1.2, 4-HPR = 4.8, ABT-751 = 14.0, 4-HPR + ABT-751 = 23.3, 4-HPR + ABT-751 + vitamin E = 6.5. For cleaved-PARP: vitamin E = 0.8, 4-HPR = 2, ABT-751 = 4.2, 4-HPR + ABT-751 = 5.7, 4-HPR + ABT-751 + vitamin E = 2.7. Expression of cleaved-caspase-9, cleaved-caspase-3, and cleaved-PARP (at 85 kDa) was increased in 4-HPR- and ABT-751-treated cells and to a greater extent in cells treated with 4-HPR + ABT-751. Similar to the cytochrome c release data, co-treatment of the drug combination with vitamin E reduced expression of cleaved-caspases and cleaved-PARP.

Antioxidants reduced apoptosis induced by 4-HPR + ABT-751 in neuroblastoma xenografts

We assessed apoptosis in SMS-KCNR xenografts from mice treated for 96 hours with control vehicle, 4-HPR/LXS, ABT-751, or 4-HPR/LXS + ABT-751. The mice were sacrificed and tumors stained for apoptosis via TUNEL. The
average numbers of apoptotic cells in 10 high power fields of tumors were: control = ~8, 4-HPR = ~27, ABT-751 = ~30 and 4-HPR + ABT-751 = ~74 (Fig. 5A). The enhanced apoptosis from combining 4-HPR + ABT-751 was observed in neuroblastoma tumor cells and not in tumor stroma. Apoptosis induced by 4-HPR/LXS + ABT-751 was significantly higher than in control and single agent-treated tumors ($P < 0.01$). To assess the involvement of reactive oxygen species (ROS) in the synergy of 4-HPR/LXS + ABT-751, mice bearing SMS-KCNR xenografts were pretreated for 2 days with vitamins C + E and throughout 48-hour treatment with 4-HPR/LXS + ABT-751. The average number of apoptotic cells in 10 high power fields of tumors were: vehicle = ~8, vehicle + vitamins C + E = ~12, 4-HPR + ABT-751 = ~64, and 4-HPR + ABT-751 + vitamins C + E = ~27 (Fig. 5B). Vitamin C + E treatment significantly reduced apoptosis from 4-HPR/LXS + ABT-751 ($P < 0.01$).

**Activity of various microtubule-targeting agents when combined with 4-HPR.**

We compared drug synergy between 4-HPR and five microtubule inhibitors (ABT-751, CLC, VCR, VRL, and PTX) in SMS-KCNR, CHLA-119, and CHLA-90 (Supplementary Fig. S4). Drug synergy was also observed in SMS-KCNR when 4-HPR was combined with CLC (CI = 0.69) or VCR (CI = 0.71) and in CHLA-140 when 4-HPR was combined with CLC (CI = 0.76), VCR (CI = 0.57), or PTX (CI = 0.75). Both vitamin E and vitamin C decreased the synergistic toxicity observed in vitro between 4-HPR and all 5 tested microtubule inhibitors (Supplementary
Fig. S5). The IC$_{90}$ values and combination indices for the 5 tested microtubule inhibitors combined with 4-HPR in 7 neuroblastoma cell lines are shown in Supplementary Table S2.

The *in vivo* efficacy of 4-HPR/LYM-X-SORB oral powder combined with four microtubule inhibitors (ABT-751, VCR, PTX, and CLC) was assessed in SMS-KCNR xenograft models (Fig. 6). The xenografts received the indicated doses of 4-HPR/LXS and microtubule inhibitors, alone or in combination, until treatment endpoint or morbidity. 4-HPR/LXS + ABT-751 was highly active relative to vehicle or single-agent treatments (Fig. 6A). Combining 4-HPR/LXS with three other microtubule inhibitors, vincristine (VCR), paclitaxel (PTX), or colchicine (CLC), was less active than what we observed with ABT-751. Treatment with VCR alone was only slightly better than vehicle and 4-HPR/LXS treatment (Fig. 6B); single-agent PTX (Fig. 6C) or CLC (Fig. 6D) displayed no activity over vehicle-only treatment. Combining 4-HPR/LXS with VCR and PTX was more effective in suppressing tumor growth than the single-agent treatments; however, the combinations did not achieve the level of activity observed with the 4-HPR/LXS + ABT-751. CLC + 4-HPR/LXS was not more active than 4-HPR/LXS alone.
Discussion

Through a series of early-phase and then randomized trials, intensive induction chemotherapy, local control with surgery and radiation, consolidation with myeloablative therapy, and post-consolidation maintenance therapy with 13-cis-retinoic acid + ch14.18 antibody + cytokines has been defined as the current optimal therapy for patients with high-risk neuroblastoma (2, 4, 19). However, even with optimal therapy about one half of patients will develop progressive disease that is almost always less responsive to therapy than disease prior to progression and progressive disease on or after therapy is often fatal for high-risk neuroblastoma. Thus, there is a critical need for new drugs and novel drug combinations that are active against recurrent and refractory neuroblastoma.

In preclinical and clinical studies, the orally available colchicine binding site microtubule inhibitor, ABT-751, was well-tolerated and showed signals of activity against recurrent neuroblastoma (14-17), but in a neuroblastoma phase II clinical trial, ABT-751 achieved few objective responses and did not increase time-to-progression over historical controls (18). Fenretinide also showed activity against recurrent neuroblastoma in preclinical studies (5, 6, 10), signals of activity in early phase trials of a suboptimal capsule formulation (7-9), and multiple complete responses in a phase I trial of a novel lipid matrix oral powder formulation (4-HPR/LXS) that increased 4-HPR exposures (11). Based on known mechanisms of action, we postulated that 4-HPR and ABT-751 would demonstrate additive antitumor activity in vivo, but it was not anticipated that 4-HPR + ABT-751 would be synergistic, per se. Indeed, our in vitro testing
performed after pilot xenograft experiments evidenced a high activity of the 4-HPR + ABT-751 combination indicated only modest additive to synergistic activity in most lines and high synergy in only a single cell line. However, testing in multiple neuroblastoma xenograft models confirmed that the two drugs together achieved a striking activity (with multiple maintained complete responses) not observed with either single agent. Combining 4-HPR/LXS + ABT-751 significantly prolonged mouse survival relative to control and single agent treatments with the majority of mice treated with the combination surviving progression-free $\geq 100$ days. The precise mechanism(s) of the discrepancy in results between these in vitro and in vivo models awaits future elucidation, but our data excluded metabolism to 4-OH-fenretinide (34) and cytotoxicity for tumor stroma as potential mechanisms.

It has been well documented that mechanisms of 4-HPR cytotoxicity in cell lines from multiple cancer types in vitro can involve the induction of reactive oxygen species (ROS) (5, 36, 38). Thus, we examined the effect of antioxidants on the synergistic cytotoxicity between 4-HPR and ABT-751, and we found that the addition of vitamins C or E abrogated the enhanced cytotoxicity seen with 4-HPR, 4-HPR + ABT-751, but not with ABT-751 alone. Interestingly, thiol antioxidants (sodium thiosulfate and n-acetylcysteine) did not antagonize the cytotoxicity of 4-HPR + ABT-751, even though n-acetylcysteine significantly decreased ROS in cells treated with 4-HPR + ABT-751. We speculate that the differential effects of these antioxidants may be due to their differing cellular compartmentalization. Vitamins C and E, two naturally-occurring antioxidants,
are capable of entering the mitochondria (39-41), where the mitochondrial respiratory chain is a major source of intracellular ROS generation, as well as, an important target of ROS damage (42). However, N-acetylcysteine and sodium thiosulfate, two thiol antioxidants that aid in the replenishment of glutathione (43, 44), have not been shown to enter mitochondria.

As single agents, both 4-HPR and ABT-751 induced apoptosis, and we observed enhanced apoptosis in vitro and in vivo when combining the two agents. In cell lines in vitro, 4-HPR + ABT-751 resulted in increased ROS, activation of the mitochondrial apoptotic pathway, and greater mitochondrial membrane depolarization, cytochrome c release, caspase activation, and apoptosis than either agent alone. These enhanced effects of 4-HPR + ABT-751 were blocked by mitochondrial-penetrant antioxidants in vitro and in vivo. Thus, our data suggest that 4-HPR-induced an increase of ROS, and the activity of 4-HPR +/- ABT-751 that promotes apoptosis, may be occurring in mitochondria.

As 4-HPR/LXS + ABT-751 was very well tolerated in mice where complete responses were achieved in tumor xenografts, the effect of the combination on enhancing cell death mechanisms is largely restricted to malignant cells. Our data indicate a novel mechanism of action for fenretinide, enhancing activity of anti-microtubule via the generation of ROS in selective cellular compartments of malignant cells.

We compared activity of 4-HPR in combination with other microtubule disruptors (vinristine, vinorelbine, and colchicine) and the microtubule stabilizing taxane paclitaxel. Like ABT-751, the other 4 microtubule inhibitors showed
additive or synergistic cytotoxicity in vitro that was mediated by 4-HPR-generated ROS. However, in xenograft testing activity of vincristine or paclitaxel was much less than observed with ABT-751, consistent with previously reported data for the single agent activity of vincristine or taxanes against neuroblastoma xenografts (45, 46). Colchicine + 4-HPR/LXS showed no increase in activity against xenografts compared to 4-HPR alone, likely due to the limited doses required in mice for colchicine due to systemic toxicity.

In summary, these data demonstrate that: 1) combining 4-HPR with ABT-751 increased activation of the mitochondrial apoptotic cascade in vitro; 2) the increases in cytotoxicity and apoptosis of the 4-HPR + ABT-751 combination were mediated by increased ROS, likely of mitochondrial origin, as activity was suppressed by vitamin C and vitamin E and not thiol antioxidants; and 3) the combination of 4-HPR/LXS + ABT-751 was highly active against several multidrug-resistant, recurrent neuroblastoma xenograft models in immunodeficient mice. Early-phase trials of 4-HPR/LXS and ABT-751 as single agents have demonstrated drug exposures as high or higher than those obtained in mice (8, 9, 11, 47), that both 4-HPR/LXS and ABT-751 are well-tolerated as single agents (8, 9, 11, 15, 16, 18), and that multiple complete responses in recurrent high-risk neuroblastoma have been observed with 4-HPR/LXS (11). Thus, together, the preclinical and clinical data support that the combination of 4-HPR/LXS and ABT-751 warrants clinical investigation in recurrent and refractory high-risk neuroblastoma.
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References


Figure 1. Cytotoxicity of N-(4-hydroxyphenyl)retinamide (4-HPR), ABT-751, and 4-HPR + ABT-751 in neuroblastoma cell lines. Fixed-ratio dose-response curves for 4-HPR (o, empty circles), ABT-751 (Δ, empty triangles), and 4-HPR + ABT-751 (■, filled squares) using the fluorescence-based DIMSCAN cytotoxicity assay in eight neuroblastoma cell lines. Cytotoxicity was evaluated after treatment of cells with vehicle, 4-HPR, ABT-751, or 4-HPR + ABT-751, for four days. Survival fraction was determined by dividing the mean fluorescence of treated cells by the mean fluorescence of control cells. Symbols represent the mean survival fraction of twelve replicates and error bars represent 95% confidence intervals. Error bars smaller than the size of the symbol are not shown.

Figure 2. Activity of 4-HPR/LXS and ABT-751 in recurrent neuroblastoma xenografts. Four human neuroblastoma cell lines CHLA-90, CHLA-136, SMS-KCNR, and CHLA-140, were established as subcutaneous xenografts in nu/nu mice. Xenografts were randomized into 4 treatment groups: vehicle (Ctrl, ●, filled circles), 4-HPR/LXS (H, Δ, empty triangles), ABT-751 (A, ▲, filled triangles), and 4-HPR/LXS + ABT-751 (H + A, o, empty circles). Symbols represent mean tumor volume and error bars correspond to 95% confidence intervals. Error bars smaller than the size of the symbol are not shown. Mice
received by oral gavage 240 mg 4-HPR/kg/day as 4-HPR/LXS in 2 divided daily doses and/or 75 mg/kg/day ABT-751 once daily, for 5 days a week, for stated duration of therapy. **A)** CHLA-90 mice (n = 6) treated for 11 weeks. **B)** CHLA-136 mice (n = 5) treated for 11 weeks. **C)** SMS-KCNR mice (n = 5) treated for 9 weeks. **D)** CHLA-140 mice (n = 5) treated for 28 weeks. Drug treatment was begun when tumors measured 100 to 200 mm$^3$ and tumor volumes were calculated as $0.5 \times \text{height} \times \text{width} \times \text{length}$. Animals were sacrificed when tumors exceeded 1500 mm$^3$ or serious morbidity occurred.

**Figure 3.** Kaplan-Meier event-free survival (EFS) analysis of xenografts treated with 4-HPR/LXS and ABT-751. Four human neuroblastoma cell lines CHLA-90, CHLA-136, SMS-KCNR, and CHLA-140, were established as subcutaneous xenografts in nu/nu mice. Xenografted mice were randomized into four treatment groups: vehicle (Ctrl, ——, thin line), 4-HPR/LXS (H, ·····, dotted line), ABT-751 (A, −−, dashed line), and 4-HPR/LXS + ABT-751 (H + A, ---, bold line). Mice were treated as described Fig. 2. **A)** CHLA-90 mice (n = 6), **B)** CHLA-136 mice (n = 5), **C)** SMS-KCNR mice (n = 5), **D)** CHLA-140 mice (n = 5), **E)** Combined EFS plot for CHLA-90, CHLA-136, SMS-KCNR, and CHLA-140 subcutaneous xenografts (n = 21); EFS for mice treated with 4-HPR + ABT-751 was significantly greater than control mice or mice treated with either single agent (P < 0.001).
Figure 4. Effect of antioxidants on induction of cytotoxicity, apoptosis, and ROS by 4-HPR + ABT-751. A) Cytotoxicity of 4-HPR (H), ABT-751 (A), and 4-HPR + ABT-751 (H + A) in CHLA-119 cells treated with vehicle, vitamin C (Vit C), vitamin E (Vit E), n-acetylcysteine (NAC), or sodium thiosulfate (STS). Cells were pretreated with 250 µM Vit C, 250 µM Vit E, 500 µM NAC or 500 µM STS for 3 hours before treatment with 2.5 µM 4-HPR, 250 ng/mL ABT-751, or 2.5 µM 4-HPR + 250 ng/mL ABT-751 for 4 days. Survival fraction was determined via DIMSCAN by dividing the mean fluorescence of treated cells by the mean fluorescence of control cells. Each condition was tested in 12 replicates with bars representing mean survival fraction and error bars corresponding to 95% confidence intervals. B) Dose response curves of 4-HPR + ABT-751 in combination with the four antioxidants as described in Fig. 4A. C) Induction of apoptosis by 4-HPR (H), ABT-751 (A), and 4-HPR + ABT-751 (H + A) in combination with vehicle, Vit C, Vit E, NAC, or STS in CHLA-119 cells. Cells were pretreated with 250 µM Vit C, 250 µM Vit E, 500 µM NAC or 500 µM STS for 3 hours before treatment with 0.62 µM 4-HPR, 62.5 ng/mL ABT-751, and 0.62 µM 4-HPR + 62.5 ng/mL ABT-751 for 20 hours. After fixation and staining, the cells were analyzed for apoptosis by TUNEL/FITC and PI staining using flow cytometry. Bars represent the percentage of apoptotic cells from three separate experiments, and error bars correspond to 95% confidence intervals. D) Reactive oxygen species (ROS) induced by vehicle, 0.62 µM 4-HPR, 62.5 ng/mL ABT-751, and 0.62 µM 4-HPR + 62.5 ng/mL ABT-751 (H+A) in CHLA-119 cells (left panel); and by 4-HPR + ABT-751 in combination with 250 µM Vit C, 250 µM
Vit E, 500 µM NAC or 500 µM STS (right panel). CHLA-119 cells were pretreated with antioxidants for 3 hours before 3-hour incubation of 4-HPR, ABT-751, or 4-HPR + ABT-751. The cells were then incubated with 50 µM carboxy-H_2DCFDA and ROS, as measured by mean DCFDA fluorescence intensity, analyzed by flow cytometry. Bars represent mean DCFDA intensity of triplicate samples and are representative of results obtained from three separate experiments; error bars represent 95% confidence intervals.

PARP. β-actin was used as a control for equal protein loading. Data shown are representative of results obtained from three separate experiments.

**Figure 5. Evaluation of apoptosis in neuroblastoma xenografts treated with 4-HPR/LXS and ABT-751.**

**A)** SMS-KCNR (n = 3) animals were administered vehicle, 180 mg 4-HPR/kg/day as 4-HPR/LXS, 75 mg/kg/day ABT-751, or 180 mg 4-HPR/kg/day as 4-HPR/LXS + 75 mg/kg/day ABT-751 for 96 hours. **B)** SMS-KCNR (n = 3) animals were administered vehicle, 3 mg/day vitamin C + 3 mg/day vitamin E (Vit C + Vit E), 180 mg 4-HPR/kg/day as 4-HPR/LXS + 75 mg/kg/day ABT-751, or 3 mg/day vitamin C + 3 mg/day vitamin E combined with 180 mg 4-HPR/kg/day as 4-HPR/LXS + 75 mg/kg/day ABT-751. Tumor samples were excised and stained for apoptosis via TUNEL immunohistochemistry (dark brown stain). Bars represent average apoptotic cell number of 10 high power field images; error bars represent 95% confidence intervals. Representative images for each group are presented to the right of each graph.
Figure 6. Activity of 4-HPR/LYM-X-SORB combined with microtubule disruptors (ABT-751, vincristine (VCR), or colchicine (CLC)) or with the microtubule stabilizing taxane paclitaxel (PTX)) in recurrent neuroblastoma murine xenografts. SMS-KCNR cells were established as subcutaneous xenografts in nu/nu mice. Xenografts were randomized into 4 treatment groups in cohorts of 5: vehicle (filled circles), 4-HPR/LYM-X-SORB (empty triangles), microtubule inhibitor (filled triangles), and 4-HPR/LYM-X-SORB plus microtubule inhibitor (empty circles). Symbols represent mean tumor volume and error bars correspond to standard deviation. Error bars smaller than the size of the symbol are not shown. Dosing schedules were as follows: A) 240 mg/kg/day 4-HPR/LYM-X-SORB in divided daily doses and 75 mg/kg/day ABT-751 once daily 5 days a week by oral gavage for 9 weeks. B) 240 mg/kg/day 4-HPR/LYM-X-SORB in divided daily doses 5 days a week by oral gavage and 0.75 mg/kg/day vincristine (VCR) once a week by intraperitoneal injection (i.p.) for 8 weeks. C) 240 mg/kg/day 4-HPR/LYM-X-SORB in divided daily doses 5 days a week by oral gavage and 10 mg/kg/day paclitaxel (PTX) once a week by i.p. for 7 weeks. D) 240 mg/kg/day 4-HPR/LYM-X-SORB and 0.05 mg/kg/day colchicine (CLC) in divided daily doses 5 days a week by oral gavage for 5 weeks. Drug treatment was begun when tumors measured 100 to 200 mm$^3$ and tumor volumes were calculated as 0.5 $\times$ height $\times$ width $\times$ length. Animals were sacrificed when tumors exceeded 1500 mm$^3$ or serious morbidity was present.
Figure 1

The figure shows survival fraction plots for different cell lines under varying concentrations of 4-HPR and ABT-751. Each plot compares the effect of 4-HPR (in μM) and ABT-751 (in ng/mL) on the survival fraction of SMS-SAN, CHLA-15, CHLA-20, SMS-KCNN, CHLA-140, CHLA-90, FU-NB-2006, CHLA-119, and CHLA-136 cell lines. The survival fraction is plotted on a logarithmic scale against the concentration of 4-HPR and ABT-751.
Figure 2

A. CHLA-90

B. CHLA-136

C. SMS-KCNR

D. CHLA-140

Mean Tumor Volume (mm$^3$)

Days

0 20 40 60 80 100

Mean Tumor Volume (mm$^3$)

0 500 1000 1500 2000

Ctrl

H

A

H + A

CHLA-90

CHLA-136

SMS-KCNR

CHLA-140

Days

0 20 40 60 80 100

Mean Tumor Volume (mm$^3$)

0 500 1000 1500 2000

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Figure 4

A. Survival Fraction

B. CHLA-119

C. % Apoptosis

D. Mean DCFDA Intensity (X 1,000)

* P < 0.001

* P < 0.01

* P = 0.043

* P < 0.01
Figure 5

A

![Bar graph showing average apoptotic cell number.](#)

*P < 0.01

- Control
- 4-HPR
- ABT-751
- 4-HPR + ABT-751

![Images showing control and treated samples.](#)

B

![Bar graph showing average apoptotic cell number.](#)

*P < 0.01

- Control
- Vit C + Vit E
- 4-HPR + ABT-751
- Vit C + Vit E + HPR + ABT

![Images showing control and treated samples.](#)
Figure 6

A

![Graph A: Mean Tumor Volume (mm³) vs Days]

- Ctrl
- HPR
- ABT-751
- HPR + ABT-751

B

![Graph B: Mean Tumor Volume (mm³) vs Days]

- Ctrl
- HPR
- VCR
- HPR + VCR

C

![Graph C: Mean Tumor Volume (mm³) vs Days]

- Ctrl
- HPR
- PTX
- HPR + PTX

D

![Graph D: Mean Tumor Volume (mm³) vs Days]

- Ctrl
- HPR
- CLC
- HPR + CLC
Molecular Cancer Therapeutics

Reactive oxygen species mediates the synergistic activity of fenretinide combined with the microtubule Inhibitor ABT-751 against multi-drug resistant recurrent neuroblastoma xenografts.

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