Increase of Ceramide and Induction of Mixed Apoptosis/Necrosis by N-(4-Hydroxyphenyl)-retinamide in Neuroblastoma Cell Lines

Barry J. Maurer, Leonid S. Metelitsa, Robert C. Seeger, Myles C. Cabot, C. Patrick Reynolds

Background: The synthetic retinoid N-(4-hydroxyphenyl)retinamide (4-HPR or fenretinide) is toxic to myeloid leukemia and cervical carcinoma cell lines, probably in part due to its ability to increase levels of reactive oxygen species (ROS). We have studied the effects of 4-HPR on neuroblastoma cell lines. Since neuroblastomas commonly relapse in bone marrow, a hypoxic tissue compartment, and many chemotherapeutic agents are antagonized by hypoxia, our purpose was to study in these cell lines several factors influencing 4-HPR-induced cytotoxicity, including induced levels of ROS, effects of physiologic hypoxia and antioxidants, levels of ceramide, and the mechanism of cell death. Methods: ROS generation was measured by carboxy-dichlorofluorescein diacetate fluorescence. Ceramide was quantified by radiolabeling and thin-layer chromatography. Immunoblotting was used to assess p53 protein levels. Apoptosis (programmed cell death) and necrosis were analyzed by nuclear morphology and internucleosomal DNA fragmentation patterns. Cytotoxicity was measured by a fluorescence-based assay employing digital imaging microscopy in the presence or absence of the pan-caspase enzyme inhibitor BOC-d-fmk. Statistical tests were two-sided. Results/Conclusions: In addition to increasing ROS, 4-HPR (2.5–10 µM) statistically significantly increased the level of intracellular ceramide (up to approximately 10-fold; P<.001) in a dose-dependent manner in two neuroblastoma cell lines, one of which is highly resistant to alkylating agents and to etoposide. Cell death induced by 4-HPR was reduced but not abrogated by hypoxia in the presence or absence of an antioxidant, N-acetyl-L-cysteine. Expression of p53 protein was not affected by 4-HPR. Furthermore, the pan-caspase enzyme inhibitor BOC-d-fmk prevented apoptosis, but not necrosis, and only partially decreased cytotoxicity induced by 4-HPR, indicating that 4-HPR induced both apoptosis and necrosis in neuroblastoma cells. Implications: 4-HPR may form the basis for a novel, p53-independent chemotherapy that operates through increased intracellular levels of ceramide and that retains cytotoxicity under reduced oxygen conditions. [J Natl Cancer Inst 1999;91:1138–46]

Retinoids are natural or synthetic derivatives of vitamin A that have been shown to be important modulators of cellular growth and differentiation. Since 13-cis-retinoic acid has recently been demonstrated in a phase III clinical trial to significantly increase the event-free survival of children with advanced stage neuroblastoma (1), intensive study of retinoids with anti-neuroblastoma activity is warranted. The retinoid N-(4-hydroxyphenyl)retinamide (4-HPR; fenretinide) has been shown to inhibit carcinogenesis in a variety of animal cancer models, including breast cancer (2), bladder cancer (3), lymphoma (4), prostate cancer (5–7), and lung cancer (8,9). Clinically, low-dose 4-HPR (1–3 µM serum levels) has been studied as a chemopreventive agent in breast (10,11), bladder (12), and oral cavity (13–15) cancers and has shown minimal toxicity (16,17). Preclinical studies have shown that 4-HPR is cytotoxic to a variety of cancer cell lines, including neuroblastoma (18–20), colorectal cancer (21), head and neck cancer (22), breast cancer (23,24), prostate cancer (25–28), small-cell lung cancer (29), ovarian cancer (30–32), cervical cancer (33,34), and leukemia or lymphoma (35,36). Because effective cytotoxicity against tumor cells in vitro requires levels of 4-HPR greater than 3 µM, phase I clinical trials are currently under way in both adult and pediatric populations to determine the maximally tolerated dose of 4-HPR given orally twice daily in 1-week courses (37).

It has been reported that reactive oxygen species (ROS) contribute to the induction of apoptosis by 4-HPR in cervical carcinoma (34) and myeloid leukemia (38) cell lines. Therefore, we determined if 4-HPR induced ROS in neuroblastoma cells and whether ROS was the sole mechanism of 4-HPR cytotoxicity.

Ceramide is a sphingosine-based lipid second messenger involved in the regulation of diverse cellular responses, including the generation of hydrogen peroxide in mitochondria (39,40) and apoptosis (41,42). Ceramide can be generated de novo by ceramide synthase or from sphingomyelin breakdown through the activation of neutral or acidic sphingomyelinase by drugs, such as daunorubicin and doxorubicin, or the drug resistance modulator SDZ PSC 833; through activation of receptors such as tumor necrosis factor-α or CD95/Fas/APO-1; or by ionizing radiation, ultraviolet-C, heat shock, or oxidative stress (41,43,44). Ceramide has been reported to initiate apoptosis under hypoxic conditions in a p53-independent manner via caspase-3 activation (45), causing the activation of the pro-death JNK/SAPK cascade, which is opposed by spongiosine-1-phosphate and ERK1/2 activation (46–48). To date, no chemotherapeutic agent has been reported whose cytotoxicity is ascribed principally to the generation of intracellular ceramide.

Because neuroblastomas commonly relapse in bone marrow (49), a tissue with a low oxygen tension (50), and since many common chemotherapeutic agents are antagonized by hypoxia (51,52), we are seeking to identify agents that retain cytotoxicity in reduced-oxygen environments for use in the treatment of neuro-

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b lastoma. To this end, we have examined the cytotoxic properties of 4-HPR in human neuroblastoma cell lines. We have determined 1) if 4-HPR increased ROS, 2) the effects of physiologic hypoxia (PO2, approximately 15 mm Hg) and the antioxidant N-acetyl-L-cysteine (NAC) on 4-HPR cytotoxicity, 3) the extent to which ceramide is induced by 4-HPR, and 4) whether 4-HPR induced cell death by an apoptotic or necrotic mechanism(s).

MATERIALS AND METHODS

Materials

4-HPR was provided by R. W. Johnson Pharmaceuticals (Spring House, PA). Fluorescein diacetate (FDA) was from the Eastman Kodak Company, Rochester, NY. Eosin Y. N-acetyl-d-glucosamine (C2, ceramide), NAC, and thin-layer chromatography (TLC)-grade organic solvents were from Sigma Chemical Co., St. Louis, MO. 5-(and-6)-Carboxy-2',7'-dichlorofluorescein diacetate (carboxy-DCFDA) was from Molecular Probes, Inc., Eugene, OR. Ecmo scintillation cocktail was from ICN Biomedicals, Inc., Costa Mesa, CA. [9,10-3H(N)]Palmitic acid (50 Ci/mmol) was from Dupont.

Cell Culture

Human neuroblastoma cell lines SMS-LHN (53) and SMS-KCNR (54) were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS (whole medium). The human neuroblastoma cell line CHLA-90 (55) was established from a tumor relapse in bone marrow after myeloablative chemoradiation therapy supported by autologous bone marrow transplant and was maintained in IMDM supplemented with 0.7 mM l-glutamine, insulin, and transferrin (5 μg/mL each), selenium (5 ng/mL), and 20% heat-inactivated FBS (whole medium). Cell lines were studied during passages 15 through 40 and cultured at 37 °C in a humidified incubator containing 95% room air plus 5% CO2 atmosphere. Cells were cultured without antibiotics to facilitate the detection of Mycoplasma, for which all cell lines tested negative. Cells were detached without trypsin from culture plates with the use of a modified Puck’s Solution A plus EDTA (Puck’s EDTA), which contains 140 mM NaCl, 5 mM KCl, 5.5 mM glucose, 4 mM NaHCO3, 0.8 mM ethylene-diamine tetraacetic acid (EDTA), 13 μM Phenol Red, and 9 mM HEPES buffer (pH 7.3) (54).

For cytotoxicity assays (described below) under reduced oxygen conditions, cells were seeded into 96-well plates to a final concentration of 1.5 × 104 cells per well, in a final volume of 200 μL. H202 (20 μM) was added, and the plates were incubated for 24 hours. For the experiments described above, tubes used for making drug dilutions were frequently flushed with 2% oxygen during the preparation of the cytotoxicity, FDA (stock solution of 1 mg/mL in DMSO) was added, in 50 μL of whole medium, to a final concentration of 10 μM in 20-μL volumes of whole medium to various final drug concentrations as described above. Plates were assayed at 4-5 days (CHLA-90 and SMS-KCNR) and 7 days (SMS-LHN cells) after initiation of drug exposure, depending on the growth properties of each cell line, to allow for maximum cell death and outgrowth of surviving cells. CHLA-90 cells were visibly rounded and detaching by 24 hours after drug addition, whereas SMS-LHN cells were not observed to begin to round up and detach before 48-72 hours after drug addition. For the measurement of the cytotoxicity, FDA (stock solution of 1 mg/mL in DMSO) was added, in 50 μL of whole medium, to a final concentration of 10 μM in 20-μL volumes of whole medium. The plates were incubated for an additional 15–30 minutes at 37 °C, and then 30 μL of eosin Y (0.5% in normal saline) was added per well. Total fluorescence of each well was then measured with the use of digital imaging microscopy as previously described (58). Results were analyzed and expressed as the surviving fractions of treated cells compared with those of control cells with the use of Excel software (Microsoft, Seattle, WA) and graphed with the use of SIGMAPlot 4.0 (Jandel Scientific, San Rafael, CA). The stability of 4-HPR in our tissue culture system for 7 days is not known.

Aptoptosis and Necrosis Detection

The Apoptosis DNA-Laddering Kit (Boehringer Mannheim GmbH, Mannheim, Germany) was used to distinguish between apoptotic and necrotic cell death by detecting alterations in the DNA-fragmentation pattern visualized by gel electrophoresis following the manufacturer’s instructions. U937 human myeloid leukemia cells, which express CD95/Fas/APO-1, were treated for 16 hours at 37 °C with 250 ng/mL of the anti-Fas IgM antibody CH-11 to serve as a positive control for apoptotic, internucleosomal DNA fragmentation. The pan-caspase inhibitor BOC-d-fmk was used to block the apoptotic component of cell death in both U937 and CHLA-90 cells. Cells were preincubated with either 20 μM or 40 μM BOC-d-fmk, respectively, for 1 hour before the addition of CH-11 in serum-free medium (U937 cells) or 10 μM 4-HPR in whole medium (CHLA-90 cells). DNA-fragmentation patterns were then examined after 24 hours. As an additional control, to demonstrate that 4-HPR did not inhibit BOC-d-fmk, we also preincubated U937 cells with 20 μM BOC-d-fmk and then treated them with 250 ng/mL of CH-11 plus 5 μM 4-HPR. In addition, CHLA-90 cells were pretreated with or without 40 μM BOC-d-fmk for 1 hour prior to the addition of 3–10 μM 4-HPR (six wells per drug concentration) for 24 hours and assayed by DIGISCAN to assess the effect of caspase inhibition on viability. Control cells were treated

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with vehicle solvents of 0.1% ethanol (4-HPR) and/or 0.2% DMSO (BOC-d-fmk). For further examination of cells treated with 4-HPR in the presence or absence of BOC-d-fmk, CHLA-90 cells were plated into Lab Tek chamber slides (Nunc, Naperville, IL), allowed to attach for 24 hours, and then treated with drugs as described above. Morphologic features of apoptosis (DNA condensation and/or apoptotic bodies) were visualized with the use of the blue nuclear fluorescence induced by the supravital DNA stain Hoechst 33342 (10 μg/mL for 30 minutes at 37 °C), whereas necrotic cells and advanced apoptotic cells were recognized by red fluorescent staining with propidium iodide (0.5 μg/mL) (59). Cells were observed by sequential use of filters appropriate for each dye on an Olympus Vanox epifluorescence microscope. Assessment of apoptosis by flow cytometry used propidium iodide in a hypotonic lysis buffer (60) to identify cells with a sub-G_{0}/G_{1} DNA content (61). The stained nuclei were analyzed on a Coulter Epics ELITE flow cytometer with a 488-nm Argon laser and a 20-nm band-pass filter centered on 610 nm.

**Determination of ROS**

Production of ROS was detected by use of carboxy-DCFDA (34,38,62). A stock solution of carboxy-DCFDA (15 mM) was prepared in DMSO and stored over nitrogen vapor. CHLA-90 cells (1 × 10^{5} in 5 mL of whole medium per T25 flask) were cultured in 20% oxygen and exposed to 5 μM 4-HPR for either 4 or 6 hours at 37 °C. Medium was discarded and, under low light conditions, replaced for 30 seconds and centrifuged at 150 g for 5 minutes at room temperature, and the buffer was aspirated. To each sample vial were added 1.2 mL of methanol–2% acetic acid, 1.0 mL of distilled water, and 1.2 mL of chloroform. The vial was vigorously vortexed for 30 seconds and centrifuged at 150g for 5 minutes at room temperature, and the lower (organic) phase was transferred to a new vial. Viable organic solvents were evaporated under a nitrogen stream, and samples were stored at −20 °C. For analysis, 100 μL of chloroform–methanol (2:1) was added to each vial, and the vial was vortexed. A 10-μL aliquot was assayed for tritium; from this, the tritium in the total lipid sample was calculated. Commercial lipid standards (5 μg per lane) were co-spotted onto the TLC plates with cellular "H-lipids (10-μL aliquots). Ceramide was resolved in a solvent system containing chloroform–acetic acid (90:10, vol/vol). The lipid standards were visualized by iodine vapor, and the co-migrating tritiated lipid sample was assayed by scraping the TLC plate in the area of interest, adding 0.5 mL of water and 4.5 mL of Ecolume scintillation cocktail, vortexing, and measuring cpm tritium by liquid scintillation counting. This value was then corrected for the amount of the original sample previously removed for other assays. Ceramide increases are expressed as the mean fold increase in three drug-treated samples as compared with that of three matched controls.

**Immunoblotting**

SMS-LHN cells cultured as above were exposed to 4-HPR (10 μM) or cisplatin (10 μg/mL) in whole medium for the indicated time, and whole-cell protein lysates were prepared by standard procedures. Protein lysates were quantified with the use of the BCA Protein Assay™ (Pierce Chemical Co.), and 12-μg aliquots were separated in precast 15% Tris–HCl polyacrylamide gels (Bio-Rad Laboratories), transferred to Protran® nitrocellulose membranes, and hybridized with anti-human p53 mouse monoclonal antibody by standard procedures. Immunoblotting results were detected by chemiluminescence using ECL™ detection reagents (Amersham Life Science, Inc.) with x-ray film and stored digitally by use of the ScanJet IICXT scanner (Hewlett-Packard, Palo Alto, CA).

**Statistical Analysis**

All data on cytotoxicity and ceramide are presented as means ± 95% confidence interval. The 95% confidence interval was calculated as 1.96σ/√n, where σ = standard deviation of ungrouped data and n = number of trials. The linearity of ceramide increases with dose and time was determined by the Spearman rank correlation test. The statistical significance of the differences in mean cytotoxicity, apoptosis, and necrosis induced by 4-HPR, NAC, and BOC-d-fmk treatments was evaluated by the unpaired, two-sided Student’s t test with the use of Microsoft® Excel 97 software. All P values are two-sided. All experiments were performed at least twice.

**Results**

**Effect of 4-HPR on ROS Levels**

4-HPR has been reported to increase ROS levels in a time- and dose-dependent manner in HL-60 myeloid leukemia (38) and C33A cervical carcinoma cells (34). To determine if 4-HPR increased ROS levels in neuroblastoma cells, we exposed CHLA-90 cells to 5 μM 4-HPR for 4 or 6 hours and measured ROS by using carboxy-DCFDA. The results at both time points were similar. Fig. 1 shows that 4-HPR exposure increased the mean fluorescence signal approximately 2.5-fold compared with that of the controls in CHLA-90 neuroblastoma cells. As a further control, 4-HPR (5 μM) was co-incubated with carboxy-DCFDA in whole, cell-free medium for 90 minutes, without activation of carboxy-DCFDA fluorescence (data not shown).

![Fig. 1. N-(4-Hydroxyphenyl)retinamide (4-HPR) increased reactive oxygen species (ROS) in the CHLA-90 neuroblastoma cell line. ROS was assayed with the use of 5-(and-6)-carboxy-2',7'-dichlorofluorescin diacetate (carboxy-DCFDA) fluorescence in CHLA-90 cells: negative control = untreated, ethanol vector only; 4-HPR (5 μM) for 4 hours; positive control = hydrogen peroxide (100 μM) for 15 minutes. Cells were washed free of 4-HPR-containing medium prior to the addition of carboxy-DCFDA (see “Materials and Methods” section). However, as a further control, 4-HPR (5 μM) was co-incubated with carboxy-DCFDA in whole, cell-free medium for 90 minutes, without activation of carboxy-DCFDA fluorescence (data not shown).](image-url)
Effects of Hypoxic and Antioxidant Conditions on Cytotoxicity of 4-HPR

4-HPR has been previously reported to be cytotoxic to neuroblastoma cell lines (18–20) and to be antagonized by the antioxidants NAC and l-ascorbic acid in HL-60 myeloid leukemia cells (38,65) and by pyrrolidine dithiocarbamate in C33A cervical carcinoma cells (34). To ascertain whether 4-HPR cytotoxicity for neuroblastoma was antagonized by antioxidants and/or hypoxia equivalent to that found in bone marrow (50), we determined cytotoxicity with the use of the DIMSCAN assay in 20% oxygen (normoxic) and 2% oxygen (hypoxia, approximately 15 mm Hg of oxygen) and in the presence of the antioxidant NAC (1 mM). NAC at 1 mM was chosen because it was shown previously to be sufficient to abrogate the ROS-mediated cytotoxicity induced in neuroblastoma cells by buthionine sulfoximine treatment (66).

As shown in Fig. 2, 4-HPR-mediated cytotoxicity was statistically significantly reduced under hypoxic conditions compared with normoxic conditions for CHLA-90 cells (at 1 μM, 2 μM, and 5 μM 4-HPR, P < .001; at 10 μM 4-HPR, P = .007), SMS-KCNR cells (at 5 μM 4-HPR, P < .001; at 10 μM 4-HPR, P = .03; at 15 μM 4-HPR, P = .003), and in SMS-LHN cells (at 1 μM 4-HPR, P = .05; at 2 μM 4-HPR, P = .02; at 5 μM and 10 μM 4-HPR, P < .001). Although hypoxia reduced the activity of 4-HPR, dose escalation still achieved a substantial cell kill in all cell lines. As shown in Fig. 2, A, the antioxidant NAC (1 mM) statistically significantly reduced the cytotoxicity of some, but not all, 4-HPR concentrations tested under both normoxic and hypoxic conditions in CHLA-90 cells (in 20% oxygen, at 2 μM 4-HPR, P < .001; at 5 μM 4-HPR, P = .02; and in 2% oxygen, at 2 μM 4-HPR, P = .13; at 5 μM 4-HPR, P = .07; at 10 μM 4-HPR, P = .008), but 4-HPR still achieved a statistically significant cell kill despite the combined antagonism of hypoxia and NAC (P < .001). Similarly, in Fig. 2, B, NAC (1 mM) statistically significantly reduced the cytotoxicity of high 4-HPR concentrations, most but not all, under both normoxic and hypoxic conditions for SMS-LHN cells (in 20% oxygen, at 1 μM 4-HPR, P = .50; at 2 μM 4-HPR, P = .03; at 10 μM 4-HPR, P = .05; and in 2% oxygen, at 1 μM 4-HPR, P = .68; at 2 μM 4-HPR, P = .02; at 10 μM 4-HPR, P < .001), yet significant cytotoxicity was retained in the presence of NAC and hypoxia at 10 μM 4-HPR (P < .001). NAC (1 mM) had no appreciable effect on 4-HPR cytotoxicity in SMS-KCNR cells under either normoxic or hypoxic conditions (Fig. 2, C).

Thus, both hypoxia and the antioxidant NAC can decrease cytotoxicity of 4-HPR, especially at lower 4-HPR concentrations. However, even in hypoxia and/or NAC, all three cell lines still showed a statistically significant cytotoxicity in response to higher concentrations of 4-HPR (≥5 μM), suggesting that ROS was not the sole mechanism of 4-HPR-mediated cytotoxicity.

Effect of 4-HPR Dose on Ceramide Levels

Because hypoxia and NAC, conditions expected to substantially reduce ROS, did not completely abolish 4-HPR cytotoxicity in neuroblastoma cells, a second mechanism of cytotoxicity was sought. Intracellular ceramide levels were directly assayed in SMS-LHN and CHLA-90 cells after a 6-hour exposure to increasing concentrations of 4-HPR. As shown in Fig. 3, A, 4-HPR induced a statistically significant increase in ceramide in a dose-dependent manner at 6 hours in both neuroblastoma cell lines (P < .001). 4-HPR did not influence the cellular uptake of [3H]palmitic acid (data not shown).

Effect of 4-HPR Exposure Time on Ceramide Levels

Ceramide levels were assayed in SMS-LHN and CHLA-90 cells after varying times of exposure to 4-HPR (10 μM). As shown in Fig. 3, B, the level of ceramide was found to increase in a time-dependent manner up to at least 48 hours (to approximately 13-fold in CHLA-90 cells, ρ = .90, R = .037 [Spearman test]; to approximately sevenfold in SMS-LHN cells, p = .70, R = .19 [Spearman test]; however, P < .001 for SMS-LHN cells compared with controls at 48 hours [Student’s t test]). 4-HPR did not influence the cellular uptake of [3H]palmitic acid (data not shown). Both cell lines exhibited a biphasic increase, with an early ceramide peak occurring at +6 hours. CHLA-90 cells were rounded and detaching at +36 hours after 4-HPR exposure. At +48 hours, the cytotoxic effects of 4-HPR were morphologically apparent in only a minority of the SMS-LHN cells, but cells became predominantly rounded and detached by +96 hours (data not shown).

Effect of 4-HPR on p53 Protein Expression

4-HPR (10 μM) did not appreciably increase p53 protein levels in SMS-LHN cells after up to 72 hours of exposure in 20% oxygen (Fig. 3, C), a time at which SMS-LHN cells were visibly dying. This result suggests that 4-HPR cytotoxicity is not primarily dependent on p53 induction.
4-HPR in the presence of [3 H]palmitic acid. 20% oxygen to the indicated concentration of in a dose-dependent manner. SMS-LHN and dependent manner but did not increase p53 HPR) increased ceramide in a dose- and time-dependent manner. Immunoblotting of SMS-LHN and CHLA-90 cells under conditions of 20% oxygen for the time (hours) indicated. Total cellular protein was prepared, and 12-μg aliquots were separated by polyacrylamide gel electrophoresis. Immunoblotting with anti-p53 monoclonal antibody was followed by the detection of bound antibody by a chemoluminescent indicator system. Both drugs induced rounding and detachment of cells at 72 hours. Bars represent 95% confidence intervals.

in neuroblastoma cells. In contrast, cisplatin (10 μg/mL) readily increased p53 protein levels in SMS-LHN cells, confirming that this cell line can increase p53 protein in response to another chemotherapeutic agent (Fig. 3, C).

4-HPR and Induction of Death by Mixed Apoptosis and Necrosis

To determine whether 4-HPR caused death in neuroblastoma cells by apoptosis or necrosis, we assessed morphologic evidence of apoptosis and necrosis as well as internucleosomal DNA-fragmentation patterns for CHLA-90 cells in the presence or absence of the neural cell-penetrant, pan-caspase enzyme inhibitor BOC-d-fmk. As shown in Fig. 4, A, the cytotoxicity of 4-HPR was significantly reduced by 40 μM BOC-d-fmk across all 4-HPR concentrations (<0.001), but 4-HPR still induced significant cytotoxicity in the presence of 40 μM BOC-d-fmk (at 3 μM 4-HPR, P = .002; at >3 μM 4-HPR, P <0.001). The addition of BOC-d-fmk (40 μM) significantly reduced (P = .001) the morphologic nuclear changes indicative of apoptosis (Fig. 4, B and C), typified by condensed nuclear chromatin and fragmentation of the nuclei into apoptotic bodies in cells that had not lost membrane integrity. 4-HPR alone induced significant apoptosis (P = .006), but apoptosis in cells treated with 4-HPR plus BOC-d-fmk was not significantly different from that of controls (P = .48). However, the statistically significant morphologic evidence of necrosis (P = .002) induced by 4-HPR (loss of membrane integrity demonstrated by propidium iodide staining, cell rounding, and cell detachment) was minimally changed by BOC-d-fmk and was still statistically significant (P = .016) relative to that of controls (Fig. 4, B and C). BOC-d-fmk (40 μM) also abrogated the minimal DNA fragmentation induced by 4-HPR in CHLA-90 cells as detected by flow cytometry (Fig. 4, D) and gel electrophoresis (Fig. 4, E) but only partially decreased the cytotoxicity of 4-HPR (Fig. 4, A). This finding suggests that 4-HPR-induced cell death in neuroblastoma cell lines proceeds predominantly by necrosis at higher drug concentrations. A similar result was observed in neuroblastoma cells with the use of exogenous C2 ceramide (Metelitsa LS, Keshelava N, Reynolds CP, Durden D, Seeger RC; manuscript in preparation).

**DISCUSSION**

Advanced neuroblastoma is a tumor of children associated with substantial mortality. Tumors are typically hypoxic (56,67–69), and neuroblastomas commonly relapse in bone marrow (49), a tissue with a low oxygen tension (50). Since many common chemotherapeutic agents are antagonized by hypoxia (51,52), identifying agents that are active in reduced-oxygen environments may be particularly important for the treatment of neuroblastoma.

The retinoid 4-HPR as a chemopreventative agent is minimally toxic in human adults in chronic daily dosing of approximately 12 mg/kg orally (serum levels, 1–3 μM) and minimally toxic in animal models up to chronic daily dosing of approximately 800 mg/kg (8). We speculate that, since 4-HPR has not been reported to induce its own metabolism (8), in vivo levels similar to or higher than those of 13-cis-retinoic acid (5–10 μM) (70) will be achievable in patients, especially by use of a pulse-dosing schedule. High levels of 4-HPR (3–10 μM) have been reported to have in vitro cytotoxic activity against neuroblastoma cell lines (18–20), including those resistant to 13-cis- and trans-retinoic acid (71). For these reasons, we are investigating its mechanism(s) of cytotoxicity and its potential activity under hypoxic conditions less than the oxygen tension found in bone marrow, a common site of neuroblastoma metastases.

4-HPR has been reported to increase ROS in myeloid leukemia and cervical carcinoma cell lines (38,34), and we now confirm ROS generation in neuroblastoma cells. However, our dose–response studies in hypoxia and in the presence of the antioxidant NAC suggested additional mechanisms of 4-HPR cytotoxicity.

Our observation that 4-HPR induced a sustained increase in intracellular ceramide in neuroblastoma cell lines of up to approximately 10-fold in a time- and dose-dependent manner is novel. Recently, 4-HPR (3 μM) was reported to cause only a transient increase in ceramide in HL-60 leukemia cells and a modest 1.5-fold increase in the MCF7
breast cancer cell line (72). Other chemotherapeutic agents, at clinically achievable levels, such as the cyclosporine analogue SDZ PSC 833 (44), daunorubicin (73,74), doxorubicin (43), and irinotecan (CPT-11) (75), have been shown to increase intracellular ceramide, but to levels much lower than those that we observed in response to 4-HPR. Furthermore, our observation that p53 protein was not increased by 4-HPR in SMS-LHN neuroblastoma cells (which possess a p53 protein that is readily increased by cisplatin) suggests that 4-HPR cytotoxicity is generally p53 independent, which is in agree-

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ment with observations of 4-HPR cytotoxicity in HL-60 myeloid leukemia (65), small-cell lung cancer (76), and non-small-cell lung cancer (77) cell lines.

The mixed apoptosis–necrosis induced by 4-HPR in neuroblastoma cells is also noteworthy. There have been reports in numerous cell types of apoptosis induced by low-dose 4-HPR (approximately 3 μM) (20,22,27,29,30,34,36,38,65). These conclusions have been based mainly on evidence of internucleosomal fragmentation of DNA. However, the effect of caspase inhibitors (which block apoptotic cell death) on 4-HPR cytotoxicity has only been minimally investigated (72). Herein we report that, while 4-HPR-mediated apoptosis was abrogated and cytotoxicity was significantly reduced by the pan-caspase enzyme inhibitor BOC-d-fmk, significant dose-dependent cytotoxicity was retained compared with findings in controls, even in the presence of 40 μM BOC-d-fmk (Fig. 4). These observations suggest that 4-HPR induced a nonapoptotic cytotoxicity (necrosis) in neuroblastoma cell lines, especially at higher drug concentrations. Recently, 4-HPR (3 μM) has been shown to induce apoptosis in lymphoblastoid cell lines, but higher concentrations of 4-HPR (10–20 μM) induced necrosis (78), and 10 μM 4-HPR has been shown to induce both apoptosis and necrosis in an embryonal carcinoma cell line (59). The prominence of necrotic death observed with high-dose 4-HPR suggests that it might be able to induce cell death not only in p53-defective tumors but also in those with defective caspase cascades. In addition, CHLA-90 is a cell line derived after myeloablative therapy that shows considerable resistance to alkylating agents and etoposide (55,79). The observation that 4-HPR demonstrated cytotoxicity in such a cell line suggests an ability to circumvent resistance to commonly used chemotherapeutic agents in at least one tumor type.

The exact mechanism by which 4-HPR increased intracellular ceramide is not yet clear. Our preliminary experiments showed that pretreatment with Fumonisin B1 (100 μM) inhibited the 4-HPR-mediated increase in ceramide seen in CHLA-90 cells (data not shown), suggesting that 4-HPR (10 μM) may target the de novo ceramide synthesis pathway (72,80). Experiments are in progress that directly assay the effect of 4-HPR on ceramide synthase and neutral and acidic sphingomyelinase.

It is also not clear if 4-HPR initially increased ROS or ceramide. It is possible, depending on their intracellular compartmentalization, that these second messengers reciprocally stimulate their production. Our results with hypoxia and the antioxidant NAC, which should minimize ROS-induced cytotoxicity, suggest that 4-HPR might initially induce an increase in ceramide.

The discovery that high-dose 4-HPR induced a sustained increase in ceramide in neuroblastoma cell lines suggests that a similar increase might be induced in tumors in vivo. We have found 4-HPR to be highly active against neuroblastoma cell lines resistant to trans- and 13-cis-retinoic acid in vitro (71). This observation suggests that 4-HPR might be employed sequentially after 13-cis-retinoic acid in neuroblastoma treatment (1) to further eliminate minimal residual disease induced by high-dose chemotherapy by targeting tumor cells resistant to 13-cis-retinoic acid. Since high-dose 4-HPR has in vitro activity against other tumors that relapse from states of minimal residual disease (24,26,36,76), clinical trials in these tumors, similar to the 13-cis-retinoic acid trial in advanced neuroblastoma (1), would seem to be warranted.

Therefore, should it be clinically tolerated, high-dose 4-HPR may form the basis for a new, ceramide-based chemotherapeutic agent, which would have the advantages of being p53 and caspase independent and functional under conditions of reduced oxygen.

**References**


(20) Mariotti A, Marcara E, Banone G, Costa A,


NOTES
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