Novel cell death pathways induced by N-(4-hydroxyphenyl)retinamide: therapeutic implications

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Abstract

We previously reported that N-(4-hydroxyphenyl)retinamide (4HPR) inhibits retinoblastoma tumor growth in a murine model in vivo and kills Y79 retinoblastoma cells in vitro. In this work, we assayed different cell death–related parameters, including mitochondrial damage and caspase activation, in Y79 cells exposed to 4HPR. 4HPR induced cytochrome c release from mitochondria, caspase-3 activation, and oligonucleosomal DNA fragmentation. However, pharmacologic inactivation of caspases by the pan-caspase inhibitor BOC-D-fmk, or specific caspase-3 inhibition by Z-DEVD-fmk, was not sufficient to prevent cell death, as assessed by loss of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction, lactate dehydrogenase release, disruption of mitochondrial transmembrane potential (∆ψm), and ATP depletion. We found that 4HPR causes lysosomal membrane permeabilization and cytosolic relocation of cathepsin D. Pepstatin A partially rescued cell viability and reduced DNA fragmentation and cytosolic cytochrome c. The antioxidant N-acetylcysteine attenuated cathepsin D relocation into the cytosol, suggesting that lysosomal destabilization is dependent on elevation of reactive oxygen species and precedes mitochondrial dysfunction. Activation of AKT, which regulates energy level in the cell, by the retinal survival factor insulin-like growth factor I was impaired and insulin-like growth factor I was ineffective against ATP and ∆ψm loss in the presence of 4HPR. Lysosomal destabilization, associated with mitochondrial dysfunction, was induced by 4HPR also in other cancer cell lines, including PC3 prostate adenocarcinoma and the vascular tumor Kaposi sarcoma KS-Imm cells. The novel finding of a lysosome-mediated cell death pathway activated by 4HPR could have implications at clinical level for the development of combination chemoprevention and therapy of cancer. [Mol Cancer Ther 2007;6(1):286–98]

Introduction

The cancer chemopreventive and therapeutic retinoid N-(4-hydroxyphenyl)retinamide (4HPR; ref. 1) has extensively been studied in preclinical models and clinical trials of cancer prevention due to its favorable toxicity profile in comparison with natural retinoids (2). 4HPR induces cell death in a number of tumor cell types, and different molecular mediators have been identified, suggesting the activation of multiple mechanisms depending on dose and cell type. In several cellular systems, exposure to 4HPR results in reactive oxygen species (ROS)–mediated cell death (3, 4). However, ROS-independent mechanisms have been also reported (5). 4HPR can induce mitochondrial dysfunction and cytochrome c release, leading to caspase-dependent apoptosis (6, 7). Other mechanisms include the implication of ceramide/sphingomyelin pathways (8–11), 12-LOX (8), Bax and/or Bak (7), c-jun NH2-terminal kinase (12), c-fos (13), GADD153 (14), and caspase-8 (15).

The identity of the retinoblastoma cell for origin and the definite molecular events leading to retinal cell transformation in humans are still debated (16). Defective cell death–regulating pathways in multipotential stem cells of the developing retina lacking the tumor suppressor pRB-1 have been indicated as a cause of retinoblastoma insurgence in humans (17). Current treatment of intraocular retinoblastoma includes a three-drug regimen of high-dose chemotherapy, in addition to radiotherapy and intensive local therapy. Because of concerns with the systemic toxicity of conventional chemotherapy agents and the related risk of...
developing extraocular tumors, the identification of novel molecular targets for the development of more effective and less toxic therapeutic interventions is of great importance to control retinoblastoma progression. Recently, 4HPR has been shown to induce cell death in brain tumor cells, including glioblastoma and primary meningeal cells (18), and has been tested as a chemotherapy drug in gliomas (19).

We previously reported that inhibition of retinoblastoma tumor growth by 4HPR in a murine model in vivo is associated with an increase in the intracellular levels of ROS leading to a necrosis-like form of death (20).

In this work, we show that 4HPR activates caspase-dependent and caspase-independent cell death pathways through lysosomal membrane permeabilization. We observed induction of lysosomal damage also in other cancer cell lines, including PC3 prostate adenocarcinoma and Kaposi sarcoma KS-Imm cells. Recently, the activation of mechanisms of cell death alternative to classic apoptosis by cancer therapeutics has been highlighted as a promising tool to overcome drug resistance following conventional chemotherapy (21). Because 4HPR as a single agent gave encouraging results in children affected by neuroblastoma, a pediatric tumor of neuroectodermal origin (22), the data shown here could represent an experimental background to plan novel drug combination strategies for the treatment of pediatric neurogenic tumors.

Materials and Methods

Chemicals

4HPR was kindly provided by Dr. James A. Crowell (Division of Cancer Prevention, National Cancer Institute, Bethesda) and Dr. Gregg Bullard (McKesson Bio, Rockville, MD). The following reagents were used: the pan-caspase inhibitor Boc-Asp(Ome)-CH2F (BOC-D-fmk), the caspase-3 inhibitor Z-Asp(Ome)-Glu(OMe)-Val-Asp(Ome)-CH2F (Z-DEVD-fmk), and the caspase-9 inhibitor Z-Leu-Glu(OMe)-His-Asp(Ome)-CH2F (Z-LEHD-fmk) were from Calbiochem (San Diego, CA); bafilomycin A1, the cathepsin D inhibitor pepstatin A, dl-buthionine-[S,R]-sulfoximine, N-acetyl-L-cysteine, and desferrioxamine mesylate were from Sigma-Aldrich (Milan, Italy).

Cell Cultures and Treatments

Human retinoblastoma Y79 cells (ATCC HTB-18) were propagated in suspension in RPMI 1640 supplemented with 15% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, and 100 mmol/L penicillin/streptomycin (complete medium). For adherent cultures (23), cells were seeded on plastic dishes coated with poly-D-lysine (Sigma) at 5 μg/cm² for 24 h, then the medium was replaced by a chemically defined neuronal medium containing the N1 supplement (transferrin 62.5 mmol/L, selenium 30 mmol/L, insulin 5 μg/mL; Life Technologies, San Giuliano Milanese, Italy; ref. 24). Prostate adenocarcinoma PC3 and DU145 cells and Kaposi sarcoma KS-Imm cells were grown in complete RPMI 1640 supplemented with 10% fetal bovine serum. Cells were treated with 4HPR dissolved in ethanol in a 10 mmol/L stock solution and used at the final concentrations indicated in figure legends. The following reagents were used at the indicated final concentration: BOC-D-fmk at 10 to 40 μmol/L (stock solution 4 mmol/L in DMSO); Z-DEVD-fmk and Z-LEHD-fmk at 1 to 50 μmol/L (stock solution 5 mmol/L in DMSO); bafilomycin A1 at 0.25 to 0.5 μmol/L (stock solution 10 mmol/L in DMSO); pepstatin A at 200 μmol/L (stock solution 25 mmol/L in DMSO); DL-buthionine-[S,R]-sulfoximine at 150 μmol/L (stock solution 100 mmol/L in water); N-acetylcysteine at 10 mmol/L (stock solution 1 mol/L in culture medium, pH 7.4); and desferrioxamine mesylate at 200 μmol/L (stock solution 50 mmol/L in water).

Cell Viability and Cytotoxicity Assays

Y79 cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (20). Cytotoxicity was measured as cell membrane lysis and release of lactate dehydrogenase (LDH) into the culture medium. Y79 cells in suspension were seeded at 1 × 10⁶ to 1.5 × 10⁶ per well in poly-d-lysine-coated 96-well microtiter dishes in 100 μL of complete medium. The medium was replaced after 24 h with 100 μL of defined N1 medium per well. 4HPR dissolved in ethanol (0.1% final ethanol concentration) was added to the cells and incubated for 4 or 24 h at 37°C. The LDH assay was carried out with the colorimetric CytoTox 96 assay kit (Promega, Milan, Italy) following the instructions of the manufacturers. The data obtained were expressed as percent LDH release relative to total LDH in culture. Absorbance at 570 nm (MTT) or 490 nm (LDH) was determined with an automatic microtiter plate reader (Molecular Devices Corp., Sunnyvale, CA).

Detection of Apoptosis and Necrosis by Flow Cytometry, Histone-Associated DNA Fragments, and Cytochrome c Release

Fluorescence-activated cell sorting (FACS) analysis was done using an Annexin V-FITC/propidium iodide apoptosis detection kit (Oncogene Research, Boston, MA) based on the method described by Vermes et al. (25), with 5 × 10⁵ cells from adherent cultures of Y79 cells, as previously described (20). Analysis was done on 10,000 gated cells using a Coulter Epics XL FACS with excitation set at 488 nm and emission at 518 nm (FITC detector) or 620 nm (phycoerythrin fluorescence detector).

Apoptotic cell death was determined by an enzyme-linked immunosassay kit (Cell Death Detection ELISAPLUS, Roche, Mannheim, Germany) to detect fragmented DNA and histones (mononucleosomes and oligonucleosomes). Cell lysates, prepared from 5 × 10⁵ cells seeded on poly-D-lysine–coated 24-well plates and treated with 4HPR in combination with different inhibitors for 24 h, were processed following the instructions of the manufacturers. Cytochrome c release in the cytosolic fractions isolated from 5 × 10⁵ cells was measured with a commercial ELISA kit (Oncogene Research) following the instructions of the manufacturers and expressed as nanograms per milliliter.

Western Blot Analysis of Proteins

Total cell lysates were prepared by syringe homogenization of the cells in lysis buffer containing Tris-HCl...
20 mmol/L (pH 7.5), NaCl 150 mmol/L, EDTA 1 mmol/L, EGTA 1 mmol/L, Triton X-100 1%, sodium pyrophosphate 2.5 mmol/L, glyceral 2-phosphate 1 mmol/L, sodium orthovanadate 1 mmol/L, leupeptin 1 μg/mL, phenylmethylsulfonyl fluoride 1 mmol/L. A mitochondrial/lysosomal−enriched fraction was isolated from 5 × 10⁶ cells with a cell fractionation kit (MBL, Watertown, MA) following the instructions of the manufacturers. The cytosolic fraction isolated after a 10,000 × g centrifugation was further centrifuged at 30,000 × g and the supernatant was used for cytosolic cytochrome c and cathepsin D immunodetection. Protein quantitation in cell lysates and cytosolic and membrane fractions was done with the DC Protein Assay Kit (Bio-Rad, Richmond, CA). Forty micrograms of total cellular proteins, diluted in reducing SDS sample buffer, were resolved on 12.5% SDS polyacrylamide gels and transferred on nitrocellulose membranes by Western blotting. The membranes were then incubated with antibodies at the appropriate dilutions in 5% powdered skim milk dissolved in 25 mmol/L TBS-Tween containing 0.15 mol/L NaCl, 0.1%Tween 20 (BLOTTO), except if stated otherwise. The following antihuman antibodies were used at the indicated dilution: mouse monoclonal anti–cathepsin D (Ab-1; Calbiochem), 1:200 diluted in 10% horse serum, TBS-Tween 0.1%; rabbit polyclonal anti–actin (Sigma), 1:200 in BLOTTO; monoclonal anti–caspase-3 (3G2), polyclonal anti–cleaved poly(ADP-ribose)polymerase (Sigma), 1:200 in BLOTTO; monoclonal anti–caspase-3 (Ser2448), AKT, mammalian target of rapamycin (mTOR), and cathepsin D monoclonal antibodies (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), 1:1,000 in BLOTTO.

A mouse monoclonal anti–cytochrome c antibody (1:1,000) was provided with the cell fractionation kit for the isolation of mitochondria (MBL) and used at 1:200 dilution in BLOTTO. All the antibodies were reacted with the nitrocellulose membranes overnight at 4°C. Secondary horseradish peroxidase−labeled antirabbit or antimouse antibodies (Amersham Pharmacia Biotech, Milan, Italy) were used at 1:10,000 dilution in BLOTTO. The immunoreaction was revealed by the enhanced chemiluminescence−plus detection system (Amersham Pharmacia Biotech).

**Caspase-3 Activity Assay**

Protease activity for caspase-3/caspase-7 was measured with the Caspase-Glo luminescent Assay (Promega) on 4 × 10⁶ cells seeded in 96-well white-walled clear-bottom luminometer microplates. Briefly, Y79 cells were seeded in complete medium and treated with 4HPR in defined medium for 4, 8, 16, and 24 h, with or without a 2-h preincubation with inhibitors. Ten micrograms of whole-cell lysates were incubated at room temperature in the dark for 1 h with the luminogenic substrate Z-DEVD-aminoluciferin. Following incubation, luminescence was measured using a Wallac 1420 MicroBeta Trilux luminometer (Perkin-Elmer Life and Analytical Sciences, Milan, Italy). The amount of luminescence detected was expressed as relative light units. Because the luminescent assay for caspase-3 also measures caspase-7 activity, we analyzed caspase-7 by Western blotting, with no detectable caspase-7 processing in the presence of 4HPR (data not shown). We then considered the results obtained with the luminescent assay, or with Z-DEVD-fmk, as indicative of caspase-3 activity. All samples were assayed in triplicate in at least three independent experiments.

**Fluorescence and Confocal Microscopy**

Cells seeded on poly-d-lysine−coated multiwell chamber slides (LabTek, Nunc, Naperville, IL) were cultured for 24 h at 37°C, pretreated for 2 h with different inhibitors, and incubated with 4HPR for 1 or 3 h. At the end of the incubation, lysosomes were stained for 30 min at 37°C with the acidotropic fluorescent probe LysoTracker Red and mitochondria were stained with MitoTracker Green (Molecular Probes) diluted in culture medium at 200 nmol/L. Cells were then counterstained with 1 μg/mL 4',6-diamidino-2-phenylindole (Sigma-Aldrich) in PBS and analyzed on an IX81 microscope with the confocal equipment FV500 (Olympus BioSystem). The objectives used and magnifications were 100× oil 1.35 numerical aperture, 60× oil 1.40 numerical aperture, and 40× oil 1.00 numerical aperture. For the immunofluorescence detection of lysosomal cathepsin D, cells previously fixed and permeabilized with cold methanol for 4 min at −20°C and blocked in PBS-10% horse serum for 10 min were incubated for 1 h with anti–cathepsin D monoclonal antibodies (clone Ab-1; Calbiochem) used at 1:100 dilution in PBS-1% horse serum. Cells were washed thrice with PBS, incubated with fluoresceinated secondary antimonouse antibodies (Amersham Pharmacia Biotech) at 1:200 dilution in PBS-1% horse serum for 30 min, and counterstained with 4',6-diamidino-2-phenylindole (1 μg/mL) for 5 min. The slides were mounted with ProLong antifade reagent (Molecular Probes) and viewed under a Leica DM L epifluorescence microscope at ×40 or ×100 magnification.

**Electron Microscopy**

To analyze the morphology of mitochondria and of the intracellular acidic compartment, 10⁶ cells were harvested and the cell pellet was washed with c cacodylate buffer 0.1 mol/L (pH 7.5) for 5 min, centrifuged at 5,000 rpm for 5 min, prefixed in Karnovsky’s solution (1% paraformaldehyde, 2% glutaraldehyde, 2 mmol/L calcium chloride, 0.1 mol/L cacodylate buffer, pH 7.5) for 2 h at 4°C, and finally washed with cacodylate buffer. Postfixing was carried out in 1% osmium tetroxide in cacodylate buffer for 1 h. After dehydration with 70% to 100% alcohol, the cells were embedded in Poly/Bed 812 resin (Polysciences, Inc., Warrington, PA) and polymerized. The ultrathin sections were stained with 5% uranyl acetate in 50% ethanol followed by 0.4% aqueous lead citrate and examined in a Zeiss LEO 900 electron microscope operating at 80 kV.

**Measurement of Volume of Acidic Compartment**

Triplet samples of cells (10⁶) were incubated for 1 and 3 h with 2.5 μmol/L 4HPR or vehicle and loaded with...
LysoTracker Red at 200 nmol/L for 20 min. Cells were then washed and suspended in phenol red–free RPMI 1640 supplemented with L-glutamine. Healthy cells were gated based on size and the fluorescence was measured by flow cytometry (FACSORT, Becton Dickinson, Buccinasco, Milan, Italy) with CellQuest acquisition and analysis software. Mean fluorescence intensity was used as the relative value of volume of the acidic compartment as described (26).

**ROS and Mitochondrial Transmembrane Potential Detection**

Y79 cells (5 × 10^5) seeded on poly-D-lysine–coated 24-well plastic dishes were treated with 2.5 μmol/L 4HPR for the times indicated in figure legends. Pretreatment of the cells with various inhibitors was carried out for 2 h before 4HPR addition. Twenty minutes before the end of the treatment, the cells were incubated with 50 μmol/L dichlorofluorescein diacetate (H2DCFDA; Molecular Probes) for detection of ROS, or with 200 nmol/L MitoTracker Red chloromethyl-X-rosamine (CMX Ros) or 200 nmol/L 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1; Molecular Probes) to assess the mitochondrial transmembrane potential (ΔΨ_m). The cells were then washed twice with phenol-free HBSS, resuspended in the same medium, and analyzed with a flow cytometer (FACScan or FACSORT, Becton Dickinson) or a microplate fluorometer (Gemini XPS, Molecular Devices) with excitation set at 488 nm and emission at 530 nm for H2DCFDA, or with excitation at 579 nm and emission at 599 nm for MitoTracker Red CMX Ros. The FACS analysis was done on 10,000 gated cells.

**ATP Determination**

ATP was determined luminometrically by the CellTiter-Glo Luminescent Cell Viability assay (Promega) on 4 × 10^4 cells as described for the caspase activity assay. ATP concentration in control cells was calculated from a calibration curve and expressed as nanomoles per milligram of protein.

**Statistical Analysis**

Data are expressed as mean ± SD. The statistical significance between two experimental groups was determined by two-tailed, unpaired Student’s t test using the PRISM software. One-way ANOVA followed by Tukey’s test was used in the analysis of three or more groups. P values of <0.05 and <0.005 were considered significant.

**Results**

**Cell Death Induced by 4HPR Is Partially Caspase Independent**

We previously reported that a mixed form of apoptotic and necrotic cell death, characterized by phosphatidylserine exposure and loss of cell membrane integrity, occurs within 4 h on 4HPR administration in Y79 cells (20). To investigate the contribution of caspases to 4HPR cytotoxicity, we compared the effect of the irreversible pan-caspase inhibitor BOC-D-fmk on cell viability parameters. We measured MTT reduction and cell membrane damage as measured by LDH release. BOC-D-fmk at 10 μmol/L was unable to block the release of LDH (Fig. 1A) or to rescue the loss of cell viability (Fig. 1B) caused by 4HPR at the higher doses (2.5–5 μmol/L).

We then investigated whether 4HPR can induce oligonucleosomal DNA fragmentation that, in the paradigmatic model of apoptotic cell death, is mediated by effector caspase-3. General caspase inhibition by BOC-D-fmk significantly, but not completely, inhibited 4HPR-induced oligonucleosome release at 1 to 10 μmol/L (Fig. 1C). Similarly, specific inhibition of caspase-3 by Z-DEVD-fmk (1–10 μmol/L) only partially prevented DNA fragmentation (Fig. 1D). BOC-D-fmk at 40 μmol/L and Z-DEVD-fmk at 50 μmol/L were more effective (Fig. 1C and D); however, at those higher concentrations, methyl ketone peptide inhibitors could nonspecifically inhibit other proteases, including cysteine proteases of the cathepsin family and calpain (27).

![Figure 1](https://mct.aacrjournals.org/content/6/1/289/F1.large.jpg)
These data indicated that DNA fragmentation induced by 4HPR is partially caspase independent. We tried to directly inactivate caspase-3 with small interfering RNA in Y79 cells using different standard transfection methods (electroporation, lipofection); however, none of the methods used proved effective in our system, giving very low transfection efficiency (<5%). We then used different experimental approaches in parallel to assess the role of caspase-3 in response to 4HPR. We verified caspase-3 activation in Y79 cells treated with 2.5 μmol/L 4HPR over time (Fig. 2). Maximal activity was detectable at 24 h (Fig. 2A) and, as expected, it was totally abolished by 10 μmol/L Z-DEVD-fmk (Fig. 2B). Immunodetection by Western blotting of the 89-kDa fragment of poly(ADP-ribose) polymerase (PARP) at the corresponding times is shown. Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) immunostaining was done as a protein loading control. D, caspase-3 inhibition by Z-DEVD-fmk does not attenuate cell membrane damage induced by 2.5 μmol/L 4HPR after 24 h of treatment. E, MTT reduction in cells treated as in (D). Representative of three independent experiments run in sextuplicate. Columns, mean; bars, SD. F, cellular morphology of control (CTRL) Y79 cells incubated with vehicle alone or treated with 2.5 μmol/L 4HPR for 24 h (4HPR), and examined by phase-contrast light microscopy (magnification, ×40). Y79 cells grown in monolayer acquire a characteristic rod-like morphology and form "rosette" colonies. 4HPR-treated cells show apoptotic-like cell shrinkage and detachment from the surface, which is not prevented by pretreatment with broad-spectrum or caspase-3 inhibitors.

We observed a remarkable difference in the modulation of the cell viability parameters by using low (1 μmol/L) or higher (2.5–5 μmol/L) doses of 4HPR, which correspond to average peak plasma levels in patients in chemopreventive (2) or therapeutic (22) clinical trials, respectively. The data obtained with BOC-D-fmk, in particular, suggested the induction of a form of cell death progressively independent of caspase activation with higher doses of 4HPR (Fig. 1A and B).

Because the induction of nonapoptotic cell death by 4HPR in retinoblastoma cells could potentially overcome resistance to common chemotherapeutic agents, we subsequently focused on the minimum dose capable of inducing apparently caspase-independent cell killing (2.5 μmol/L).

**Mechanisms of 4HPR-Induced Cell Death**

Elevation of ROS has been shown to play a critical role in 4HPR-induced necrosis-like cell death in different tumor cell types (9), including Y79 cells (20). Because ROS-induced lysosomal rupture (28, 29) determines loss of plasma membrane integrity (26), which was a sign of cell death induced by 4HPR (Fig. 1), we considered the involvement of lysosomes in 4HPR cytotoxicity. The cathepsin D inhibitor pepstatin A was as effective as BOC-D-fmk in rescuing cell viability, as measured by LDH release (Fig. 3A) and MTT reduction (Fig. 3B), and caused a significant decrease of 4HPR-induced DNA fragmentation (Fig. 3C). On the contrary,
inhibition of cysteine proteases by E64 (10 μmol/L) or calpain by Z-LLL-fmk (10 μmol/L) was ineffective (data not shown). To obtain a more general protection of lysosomal structural integrity, we tried to overexpress heat shock protein 70, which prevents lysosomal membrane permeabilization (30), in Y79 cells. Again, the low efficiency of transfection and failure of selection due to the high mortality of transfected Y79 cells did not allow us to use genetic methods to prevent lysosomal damage. We then took advantage of the iron chelator desferrioxamine, which, on internalization by endocytosis and selective accumulation into lysosomes, protects cells from cell death mediated by oxidative stress (31, 32). Interestingly, desferrioxamine mesylate protected against 4HPR-induced cytolyis, loss of MTT reduction, and DNA fragmentation more effectively than pepstatin A (Fig. 3A–C).

Cytosolic relocation of cathepsin D was detected into the cytosol of 4HPR-treated cells by Western blot analysis at 3 h on 4HPR administration (Fig. 3D and E), confirming the involvement of lysosomal destabilization in this paradigm of cell death. Cytosolic cytochrome c, indicative of mitochondrial membrane permeabilization, was also detectable. Cytosolic cytochrome c and cathepsin D declined with time (Fig. 3D), probably as a consequence of cell membrane rupture and leakage into the extracellular medium (33).

To define whether caspase activity affects lysosomal permeabilization, we probed cathepsin D released into the cytosolic fractions of cells treated with caspase inhibitors. Specific caspase-9 and caspase-3 inhibition by Z-LEHD-fmk or Z-DEVD-fmk (10 μmol/L) did not significantly affect the amount of cytosolic cathepsin D (Fig. 3E), indicating that caspase-9 and caspase-3 do not influence lysosomal destabilization, and this event probably precedes the activation of the mitochondrial apoptotic pathway.

4HPR Induces Lysosomal Destabilization in Retinoblastoma Cells

To confirm the involvement of lysosomal damage in 4HPR-induced cell death, we analyzed cytosolic cathepsin D release in Y79 cells by immunofluorescence detection. Cathepsin D immunostaining revealed a granular pattern in control Y79 cells, consistent with a lysosomal distribution (Fig. 4). Cathepsin D immunostaining after 1 or 3 h of treatment with 4HPR showed a more diffuse distribution indicative of a cytosolic relocation of the enzyme. A more diffuse staining of cathepsin D was also observed with bafilomycin A1, a specific inhibitor of vacuolar Na+/H+ ATPase, which can induce lysosomal destabilization, used as a control (Fig. 4).

Figure 3. Inhibition of cathepsin D and attenuation of lysosomal damage protect Y79 cells against 4HPR cytotoxicity. A, LDH release induced by 2.5 μmol/L 4HPR at 24 h was measured in cells pretreated with pepstatin A (pepA; 100 μmol/L) or desferrioxamine mesylate (DFX; 200 μmol/L) for 2 h before 4HPR addition. BOC-D-fmk (10 μmol/L) was included to compare the effect of caspase inhibition. B, MTT reduction was rescued close to control levels by desferrioxamine mesylate. C, DNA fragmentation was attenuated by pepstatin A and by desferrioxamine mesylate. Columns, means of three independent experiments run in sextuplicate; bars, SD. *, P < 0.05; **, P < 0.005. D, Western blot analysis of cytochrome c (cyt c) and cathepsin D (CathD) in the cytosolic fractions isolated from Y79 cells treated with 4HPR for 3 h. Immunostaining of the integral mitochondrial and lysosomal proteins cytochrome c oxidase IV (COXIV) and Lamp-1, respectively, was used to exclude membrane contamination in the cytosolic fractions. E, inhibition of caspase-9 by Z-LEHD-fmk (LEHD; 10 μmol/L) or caspase-3 by Z-DEVD-fmk (10 μmol/L) does not significantly affect cytosolic relocation of cathepsin D induced by 4HPR. Quantification by image analysis was carried out with the NIH Image 1.4 software; columns, average of three experiments; bars, SD.
To probe lysosomal and mitochondrial integrity, we stained Y79 cells with the fluorescent dyes LysoTracker Red, a lysosomotropic and acidotropic reagent, and MitoTrackerGreen. Confocal microscopy analysis showed enlargement of the acidic compartment, as indicated by a more intense staining with LysoTracker Red, and localization of the fluorescent dye into large, clustered vesicles detectable as early as 1 h after 4HPR administration (Fig. 5A). This was paralleled by decreased MitoTracker Green staining and mitochondrial stretching, suggestive of impaired mitochondrial function. A similar perturbation of lysosomes and of the mitochondrial network by 4HPR was detectable in other cancer cell lines including prostate adenocarcinoma PC3 and Kaposi sarcoma KS-Imm cells (see Supplementary Fig. S1A and B, respectively) and in DU145 cells (data not shown), suggesting a more general effect of 4HPR on lysosomal stability. Lysosomal enlargement correlated with an increased volume of the acidic compartment, which reflects the size of lysosomes (26), as determined by LysoTracker Red uptake and quantitative evaluation by flow cytometry (Fig. 5B). Interestingly, increased volume of the acidic compartment has been indicated as a determinant of plasma membrane disruption in necrotic cell death (26).

Transmission electron micrographs showed intense cytosolic vacuolization with the appearance of clusters of large electron lucent organelles into the cytosol of cells as early as 1 h after 4HPR addition, together with decreased nuclear volume, dilated cisternae of the nuclear envelope, and swollen mitochondria (Fig. 6). Taken together, these data suggest that lysosomal destabilization is an early event and accompanies mitochondrial damage in 4HPR-induced cell death.

**N-Acetylcysteine Reduces Cathepsin D and Cytochrome c Release into the Cytosol of Y79 Cells**

Because 4HPR is a pro-oxidant drug, the antioxidant N-acetylcysteine can reduce ROS generation and cytotoxicity induced by 4HPR (9, 20). Here we monitored ROS increase in 4HPR-treated cells with time, in the presence or absence of N-acetylcysteine (Fig. 7A).

Elevation of ROS, as detected by the increase of H$_2$DCFDA fluorescence, was an early event, doubling within 15 min of exposure to the drug (average of 2.1 ± 0.7-fold increase over the control). The ROS content reached a maximum at ~6 h of treatment (5.2 ± 0.5-fold over the control) and declined at 16 h.

We then verified whether 4HPR-induced lysosomal permeabilization was ROS dependent.

Western blot analysis showed that cytosolic cathepsin D released after 3 h of exposure to 4HPR is significantly reduced in cells pretreated with N-acetylcysteine for 2 h (Fig. 7B). N-Acetylcysteine also decreased cytochrome c release from mitochondria (Fig. 7B and E) and oligonucleosomal DNA fragmentation (not shown).

The attenuating effect of antioxidants on lysosomal perturbation was confirmed by immunofluorescence detection of cathepsin D. N-Acetylcysteine pretreatment partially preserved the punctated lysosomal distribution of cathepsin D, which was lost in 4HPR-treated cells (Fig. 7C).

We then investigated whether lysosomal injury in response to 4HPR could affect mitochondrial function and integrity. Protection of lysosomal integrity by desferroxamine mesylate significantly reduced ROS increase in cells at 15 min (not shown) and 2 h on 4HPR administration (average inhibition at 2 h, 52.8 ± 3.9%; Fig. 7D), indicating that lysosomal labilization caused by 4HPR can in turn affect mitochondrial ROS production. Pepstatin A, on the contrary, did not influence the amount of intracellular ROS (Fig. 7D).

A cytochrome c ELISA assay done on the cytosolic fractions isolated from Y79 cells pretreated with N-acetylcysteine (10 mmol/L) or pepstatin A (100 μmol/L) and then with 4HPR (2.5 μmol/L) for 3 h showed that both agents can decrease the release of cytochrome c in 4HPR-treated cells (Fig. 7E).

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6Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
Collectively, these data suggest that attenuation of the pro-oxidant effects of 4HPR and ROS generation can reduce mitochondrial and lysosomal damage induced by 4HPR, and that the release of lysosomal proteases can influence the integrity of mitochondria in Y79 cells.

**4HPR Induces ATP Depletion and Irreversible Loss of Δψₘ**

Energy level, as indicated by ATP concentration, can switch cell death from apoptosis to necrosis (34). Because 4HPR induced early signs of apoptosis, rapidly followed by plasma membrane disruption, we measured intracellular ATP levels with time in cells treated with 4HPR. ATP levels were maintained for the first 4 h, declined starting at 8 h (data not shown), and reached ~50% (41.3 ± 8.3%) of control at 24 h (Fig. 8A; average ATP concentration in control cells was 1.2 ± 0.3 nmol/mg of protein). Desferrioxamine mesylate per se caused a decline in ATP level in control cells. Interestingly, iron chelators, which are neuroprotective (35, 36), have been shown to influence energy metabolism by decreasing ATP formation via oxidative phosphorylation while increasing glucose consumption (37). However, N-acetylcysteine, pepstatin A, and desferrioxamine mesylate were not able to significantly rescue ATP loss caused by 4HPR (Fig. 8A).

To correlate ATP depletion with bioenergetic parameters indicative of mitochondrial dysfunction, we measured the Δψₘ. We stained the cells with the fluorescent dye MitoTracker Red CMX Ros (200 nmol/L) and evaluated total fluorescence intensity by cytofluorimetric analysis. 4HPR at 2.5 μmol/L caused transient mitochondrial hyperpolarization within 15 min of treatment, as indicated by an increased fluorescence associated with CMX Ros retention, temporally related with initial ROS elevation, which has been reported to be an early and reversible step in the activation of cell death (38); following this, Δψₘ was reduced to ~50% (50.2 ± 10.7%) of controls after 6 h and remained stable on these levels through 24 h (Fig. 8B). A similar late decrease of Δψₘ resulted by measuring voltage-dependent JC-1 fluorescent at 16 h after 4HPR administration (Fig. 8C). N-Acetylcysteine, which was able to prevent mitochondrial permeabilization, cytochrome c release, and ROS elevation occurring within 3 h of exposure to 4HPR (Fig. 7A, B, and E), was ineffective against the subsequent loss of Δψₘ (Fig. 8D). Pretreatment of the cells with caspase inhibitors, desferrioxamine mesylate, or pepstatin A gave results similar to those observed for ATP depletion, as none of the drugs affected loss of Δψₘ induced by 4HPR (data not shown).

**4HPR Compromises Insulin-like Growth Factor I–Stimulated AKT Survival Pathway**

Growth factor stimulation of the serine/threonine kinase AKT protects cells against cell death by maintaining ATP levels (39) and preventing mitochondrial damage, including mitochondrial permeability transition (40). We then assessed whether activation of AKT by the insulin-like growth factor I (IGF-I), which is a major survival factor in the retina (41), was able to rescue ATP loss and Δψₘ disruption induced by 4HPR.

We incubated the cells with IGF-I (100 ng/mL) for 2 h and then with 2.5 μmol/L 4HPR for 24 h. ATP levels in cells treated with 4HPR, as expected, were remarkably lower in cells treated with 4HPR, as expected, were remarkably lower.

**Figure 5.** 4HPR induces enlargement of lysosomes and increase of volume of the acidic compartment at early times of treatment. A, confocal microscopy analysis of lysosomal and mitochondrial morphology was done in Y79 cells treated for 1 or 3 h and stained with MitoTracker Green (MTG) and LysoTracker Red (LTR; 200 nmol/L). Intense staining with LysoTracker Red in cells treated with 4HPR was localized in large vesicles. B, volume of the acidic compartment (VAC) was measured by LysoTracker Red uptake followed by flow cytometric analysis. Columns, mean fluorescence intensity of the cells calculated from two independent experiments run in triplicate; bars, SD.
than in controls (40.5 ± 5.3%; Fig. 9A). ATP content in control cells treated with IGF-I alone was increased by ~2-fold (2.3 ± 1.1). However, IGF-I was unable to prevent ATP depletion (Fig. 9A) or Δψm loss (Fig. 9B) in cells treated with 4HPR.

We then verified whether activation of AKT by IGF-I was impaired in Y79 cells treated with 4HPR. IGF-I induced rapid phosphorylation of AKT at Ser473 and of mTOR at Ser2448, which was sustained over time (Fig. 9C). Phosphorylation of AKT was almost completely suppressed by 4HPR at 24 h. mTOR phosphorylation was decreased but was less affected. These data suggest that, in the presence of 4HPR, the AKT-mediated survival pathway is compromised, and this could account for the lack of protection by IGF-I against 4HPR cytotoxicity.

Discussion

Due to the increasing clinical application of 4HPR as a cancer chemopreventive and therapeutic agent, it is important to understand in detail how this retinoid promotes cell death in tumor cells. The induction of a mixed form of apoptotic and necrotic cell death, as a consequence of oxidative stress, has previously been described in tumor cells derived by neurogenic tumors (9, 20).

The molecular mechanisms identified in this work suggest that oxidative stress triggered by 4HPR operates in parallel on lysosomal perturbation and mitochondrial dysfunction, both leading to a form of necrotic cell death. In this model, early events occurring within 4 h on 4HPR administration include ROS production, phosphatidylserine extrusion (20), mitochondrial outer membrane permeabilization, release of cytochrome c into the cytosol, and cell membrane damage. Other events, including ATP and disruption of Δψm, indicative of irreversible mitochondrial dysfunction, occur later in the process (16–24 h on 4HPR addition). Several lines of evidence indicate that a caspase-independent pathway is activated in response to 4HPR in Y79 cells. First, we observed that general caspase inactivation by BOC-D-fmk and specific caspase-3 inactivation by low doses of Z-DEVD-fmk had limited activity in oligonucleosomal DNA fragmentation. Most importantly, caspase inhibitors were ineffective in preventing loss of cell viability caused by 4HPR.

Based on early studies showing lysosomal labilization in response to oxidative stress (42), increasing evidence establishes lysosomal proteases, in particular cathepsin D and B, as central regulators of programmed cell death in response to oxidative stress (28, 29). Indeed, we detected biochemical and morphologic signs of lysosomal destabilization, including cathepsin D translocation into...
the cytosol, enlargement of lysosomes, and increased volume of the acidic compartment, as early as 1 h on 4HPR administration. In addition, the cathepsin D inhibitor pepstatin A reduced the amount of cytosolic cytochrome c and, accordingly, was as effective as BOC-D-fmk in protecting against 4HPR-induced DNA fragmentation.

Because pepstatin A offered only partial protection against loss of cell viability, indicating the involvement of lysosomal proteases other than cathepsin D (43), we took advantage of the iron chelator desferrioxamine mesylate to attain structural protection of lysosomes. Desferrioxamine mesylate, in fact, has been shown to specifically accumulate in lysosomes by fluid phase endocytosis and to prevent lysosome-mediated cell death induced by oxidative challenge (31, 32). Notably, desferrioxamine mesylate was the most effective protective agent against 4HPR cytotoxicity. Desferrioxamine mesylate chelates lysosomal iron, which represents the major intracellular iron pool, thus inhibiting iron-dependent Fenton-type reactions in hydrogen peroxide-mediated events that render lysosomes particularly vulnerable to oxidative stress (42). Further, iron chelators have been shown to exert antioxidant and neuroprotective activity (36) through induction of the transcription factor hypoxia-inducible transcription factor 1α, which increases glucose uptake (36).

Therefore, more than one mechanism might contribute to desferrioxamine mesylate–induced preservation of cell viability in 4HPR-treated Y79 cells. We found that ROS elevation affects lysosomal stability and that, conversely, preservation of lysosomal integrity reduces 4HPR-induced oxidative stress. ROS elevation, in fact, was the earliest measurable event after 4HPR administration (doubling within 15 min); however, sustained ROS production continued over time (maximal increase after 6 h) after cytochrome c release (detectable at 3 h). N-Acetylcysteine not only decreased the amount of cytosolic cytochrome c but also reduced cytosolic cathepsin D, suggesting that preservation of a reducing environment and inhibition of oxidative stress attenuate both mitochondrial

Figure 7. ROS generation by 4HPR detected by flow cytometric analysis of H₂DCFDA fluorescence. A, pretreatment with N-acetylcysteine (NAC; 10 mmol/L) for 2 h decreases ROS production at early times. Cells treated for 6 h with L-buthionine-(S,R)-sulfoximine (BSO; 100 μmol/L) and hydrogen peroxide (H₂O₂; 100 μmol/L) were used as positive controls. Columns, mean fluorescence intensity of the cells calculated from two independent experiments run in triplicate; bars, SD. B, N-acetylcysteine reduces cytosolic cathepsin D and cytochrome c in 4HPR-treated cells. A membrane fraction enriched in mitochondria and lysosomes (m/l) and a cytosolic fraction (cyt) were isolated from Y79 cells treated with 2.5 μmol/L 4HPR. Cytochrome c oxidase IV immunostaining was used as a loading control for the membrane fractions and to exclude membrane contamination in the cytosolic fractions. C, N-acetylcysteine partially preserves the punctate cathepsin D staining pattern in Y79 cells treated with 4HPR for 3 h. D, N-acetylcysteine and desferrioxamine mesylate (200 μmol/L), but not pepstatin A, attenuate ROS increase induced by 4HPR. Y79 cells were treated with 2.5 μmol/L 4HPR for 2 h and processed as described in (A). Fluorescence intensity was detected with a fluorescence microtiter plate reader with excitation set at 488 nm and emission set at 518 nm. E, pepstatin A and N-acetylcysteine reduce the release of cytochrome c into the cytosolic fractions of Y79 cells treated with 2.5 μmol/L 4HPR for 3 h, as determined by ELISA assay. Columns, mean of the data collected from two independent experiments run in triplicate; bars, SD.
and lysosomal membrane permeabilization in 4HPR-treated cells. Further, the results of the present study indicate that lysosomal destabilization precedes evident mitochondrial dysfunction, in agreement with other models of lysosome-dependent cell death (44, 45). In fact, we observed cytosolic relocation of cathepsin D and morphologic signs of lysosomal destabilization in Y79 cells after 1 h of 4HPR administration, whereas loss of Δψm and ATP depletion occurred at later times (6–16 h).

In an attempt to order the sequence of events activated by 4HPR, we speculate that, because lysosomes are acidic organelles that are very sensitive to oxidative stress (46), initial elevation of ROS of mitochondrial origin affects lysosomal integrity before sustained mitochondrial damage occurs. Thus, ROS produced at mitochondrial level might induce limited lysosomal damage, possibly by reacting with lysosomal iron, and leakage of proteases, which generates a positive feedback loop enhancing subsequent mitochondrial dysfunction, ROS production, and further lysosomal damage. This model is supported by our data showing a reduction by 50% of ROS content in 4HPR-treated cells where oxidative stress was attenuated by desferrioxamine mesylate.

Our data show that early mitochondrial outer membrane permeabilization and release of cytochrome c occur several hours before irreversible mitochondrial dysfunction, indicated by dissipation of Δψm and ATP loss, which is in agreement with previous studies showing that mitochondrial functions can be maintained after cytochrome c release (47). However, blocking several early events, including ROS production, cytochrome c and cathepsin D release by N-acetylcysteine, or even preservation of cell membrane integrity by desferrioxamine mesylate, could not delay or prevent the later disruption of Δψm and ATP depletion induced by 4HPR.

Interestingly, our data show that stimulation of the AKT survival pathway by IGF-I is compromised in Y79 cells exposed to 4HPR at a time coincident with Δψm and ATP loss. Because AKT and its substrate mTOR are prominent regulators of energetic metabolism by modulating ATP levels in the cell (39), we speculate that 4HPR interferes with the molecular machinery in the control of protection from cell death through energy regulation in the cell.

In summary, the biochemical features of cell death induced by 4HPR in our system depict a complex pathway distinct from classic caspase-dependent apoptosis.

The involvement of intracellular organelles other than mitochondria in retinoid-mediated cell death, at least in some cell types, has previously been reported. For example, a lysosomal pathway has been implicated in cell death induced by the synthetic retinoid CD437 (48). In this work, we documented that the induction of lysosomal destabilization by 4HPR occurs in several cancer cell lines, in addition to Y79 cells. These data emphasize the opportunity to explore alternative pathways of cell death in future studies on retinoids to optimize the therapeutic use of these compounds.
Recently, the discovery of novel caspase-independent forms of cell death as potential targets to overcome impairment of apoptosis in cancer cells opened new perspectives for the improvement of cancer treatment (21). 4HPR gave significant results in experimental and clinical trials of cancer chemoprevention (2). Despite the limited efficacy displayed in experimental and clinical contexts of late-stage disease, 4HPR as a single agent has progressed into clinical evaluation as a cancer therapy drug in advanced tumors. The knowledge that different cell death paradigms can be triggered in a dose- and cell type–specific manner could be of importance to reduce the risk of inefficacy or resistance due to the administration of suboptimal doses of the drug. The present study shows that 4HPR can activate caspase-independent pathways at concentrations (2.5–5 μmol/L) that are more elevated than chemopreventive doses (1 μmol/L). These concentrations are, however, clinically achievable, as shown by the good tolerability of therapeutic doses of 4HPR administered to children affected by neuroblastoma in a phase I clinical trial (average peak plasma concentration of 12.9 μmol/L, comparable to cytotoxic doses in neuroblastoma cell lines; ref. 22).

The data shown can also contribute to the clinical improvement of 4HPR in combination with anticancer agents acting through nonoverlapping mechanisms (e.g., tyrosine kinase receptor inhibitors specific for tissue-relevant growth factors that can protect cancer cells from caspase-dependent apoptosis). The experimental evidence of multiple programs integrating and converging to final cell demise (21) provides, in fact, the rationale for combination regimens at the clinical level to control cancer development and progression (49).

Figure 9. Energy depletion is not prevented by IGF-I in cells treated with 4HPR. A, ATP levels were determined in cells treated with IGF-I (100 ng/mL) for 2 h and with 4HPR (2.5 μmol/L) for 24 h. B, ΔΨm disruption is also unaffected by IGF-I. C, IGF-I induces rapid phosphorylation of AKT at Ser473 and of mTOR at Ser2448, which is sustained over time. The 24-h time point from two independent samples was loaded. Phosphorylation of AKT is almost completely suppressed in the presence of 4HPR at 24 h. Poly(ADP-ribose) polymerase cleavage was followed to detect signs of cell death. Extracellular signal–regulated kinase 1/2 (ERK1/2) and glyceraldehyde-3-phosphate dehydrogenase immunostaining was done as a protein loading control.

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