Mechanism of 4-HPR-induced apoptosis in glioma cells: evidences suggesting role of mitochondrial-mediated pathway and endoplasmic reticulum stress

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4-HPR treatment increased the free cytosolic Ca\(^{2+}\) as well as mitochondrial Ca\(^{2+}\). Chelation of extracellular Ca\(^{2+}\) by EGTA did not prevent Ca\(^{2+}\) elevation, thus suggesting involvement of intracellular calcium stores in the release. Buffering of intracellular calcium by BAPTA-AM did not prevent 4-HPR-induced apoptosis; however, blocking the release of Ca\(^{2+}\) from ER by heparin inhibited apoptosis, indicating the role of depletion of Ca\(^{2+}\) from ER stores in apoptosis. 4-HPR treatment also resulted in an increase in Bax levels along with its translocation to mitochondria that promote mitochondrial membrane permeabilization. 4-HPR-induced apoptosis was further associated with the release of cytochrome c and apoptosis-inducing factor (AIF) from mitochondria to cytosol and nucleus, respectively, along with caspase-3 and caspase-7 activation. However, AIF nuclear translocation, peripheral chromatin condensation and apoptosis were not completely prevented by general caspase inhibitors, thus suggesting involvement of a caspase-dependent and caspase-independent pathway in 4-HPR-induced apoptosis. Taken together, these results suggest the role of mitochondrial-mediated pathway and ER stress as a key event in 4-HPR-induced apoptosis in glioma cells.

Introduction

Retinoids are a class of natural and synthetic compounds with pleiotropic effects including antitumor activity. Retinoids have demonstrated their ability to inhibit growth and induce differentiation in a variety of malignancies; however, many retinoids like all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9-cisRA), have limited therapeutic utility because of side effects (1,2). N-(4-Hydroxyphenyl)retinamide (4-HPR) is a synthetic retinoid that was developed as retinoid acid receptor (RAR) β and RAR-γ selective agonist and has emerged as a potential chemotherapeutic agent owing to its effectiveness relative to toxicity in a variety of malignancies (3–5). Its anticancer activity is attributed to its ability to inhibit tumor cell growth and apoptosis induction. 4-HPR initiates cell-type-specific responses, and the apoptotic pathways involved vary overlapping pathways (5).

4-HPR may act through RAR-dependent (3), RAR-independent (6), or both the pathways (7,8). 4-HPR leads to activation of caspases in a variety of cancer cells through intrinsic (9–11) as well as extrinsic pathway (12); recent report also suggests involvement of caspase-independent pathway (13). Modulations of members of Bcl-2 family proteins (11), p53-independent (14) and ceramide-mediated pathways (8) have been implicated in 4-HPR-induced apoptosis. Various reports have suggested that 4-HPR-induced apoptosis is predominantly associated with generation of reactive oxygen species (ROS) and loss of mitochondrial membrane potential (ΔΨ\(_{m}\)) (5,15–19). ROS levels and ΔΨ\(_{m}\) are known to regulate intracellular Ca\(^{2+}\) homeostasis. Disruption of cellular Ca\(^{2+}\) homeostasis has been proposed to be a critical event in both apoptosis and necrosis (20,21). However, the role of Ca\(^{2+}\) in 4-HPR-induced apoptosis is not determined. Mitochondria play an important role during apoptosis by releasing various apoptotic proteins that leads to apoptosis induction. In 4-HPR-induced apoptosis, mitochondria play a central role by releasing cytochrome c from intermembrane space, which thereby initiates apoptosis by activating executioner caspases (9–11). Apoptosis-inducing factor (AIF), another such protein that resides in mitochondria and under various conditions, translocates to nucleus and results in apoptosis induction in a caspase-independent manner (22). AIF-mediated apoptosis has been reported in the case of synthetic retinoid CD437 in human bronchial epithelial cells (23); its role in 4-HPR-induced apoptosis needs to be evaluated. Further, growing evidence suggests that besides mitochondria endoplasmic reticulum (ER) are also a major point of integration for damage

Abbreviations: ΔΨ\(_{m}\) mitochondrial membrane potential; 4-HPR, N-(4-hydroxyphenyl)retinamide; 9-cis RA, 9-cis retinoic acid; AA, ascorbic acid; AIF, apoptosis-inducing factor; ATRA, all-trans retinoic acid; BAPTA-AM, 1,2-bis(O-aminophenoxylethene)-N,N,N,N-tetraacetic acid acetomethyl ester; CsA, cyclosporin A; DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycol-bis(β-aminoethyl)-N,N',N'-tetraacetic acid; EM, electron microscopy; ER, endoplasmic reticulum; GSH, reduced glutathione; KRB, Krebs–Ringer buffer; L-NAMe, N\(^{5}\)-nitro-l-arginine methyl ester; MMP, mitochondrial membrane permeabilization; MN, mannitol; MPT, membrane permeability transition; PBS, phosphate-buffered saline; PI, propidium iodide; RAR, retinoic acid receptor; RPA, rat primary astrocytes; ROS, reactive oxygen species; RXR, retinoic X receptor.
sensing, pro-apoptotic signaling and caspase activation (24). Thus, the underlying mechanism(s) in 4-HPR-induced apoptosis seems to remain unclear. Therefore, the present study was conducted to get a better understanding of some of the possible mechanisms involved in 4-HPR-induced apoptosis required for its full clinical evaluation.

Malignant gliomas are aggressively growing brain tumors that are resistant to treatments including chemotherapy and other adjuvant therapies. Till date, chemotherapeutic agent for malignant gliomas still needs to be identified. Better efficacy of 4-HPR than natural retinoids has been shown in apoptosis induction in glioma cell lines that involves down-regulation of Bcl-2 and Bcl-XL and PARP cleavage (25–27). However, the mechanisms involved in glioma cells are not well understood.

We investigated the molecular mechanism of 4-HPR-induced apoptosis in glioma cell lines, U87MG and U373MG. 4-HPR-induced apoptosis was associated with ROS generation, loss of ΔΨm and disruption of Ca2+ homeostasis along with mitochondrial swelling and ER dilation. 4-HPR increased Bax expression and its translocation to mitochondria, leading to release of cytochrome c and AIF. Downstream events further involved caspase-3 and caspase-7 activation; however, apoptosis also takes place in the presence of caspase inhibitors via AIF nuclear translocation. Taken together, our results suggest that 4-HPR-induced apoptosis involves two parallel pathways emanating from mitochondria and also leads to ER stress.

Materials and methods

Cell lines and reagents

The human glioma cell lines U87MG (p53-wild-type) and U373MG (p53-mutant) were procured from National Center for Cell Science, Pune (India). 4-HPR, 5,5′,6,6′-tetrahydro-1′,3′,3′,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and caspase inhibitors were purchased from Calbiochem (San Diego, CA). ATRA, 9-cisRA, reduced glutathione (GSH), mannitol (MN), ascorbic acid (AA), N′-nitro-arginine methyl ester (L-NAM), cyclosporin A (CSA), dimethyl sulfoxide (DMSO), 2′,7′-dichlorofluorescein diacetate (DCF-DA), ethylene glycol-bis(β-aminopropyl)tetra-acetic acid (EGTA), 1,2- bis (O-aminophenoxy)ethane-N,N,N,N′-tetraacetic acid acetyl ester (BAPTA-AM), heparin (low molecular weight sodium salt) and propidium iodide (PI) were purchased from Sigma Chemicals (St Louis, MO). Fluoro-3AM, Hoechst 33588 and Mitotracker Red CM-H2Xros were from Molecular Probes (Eugene, OR). ApoAlert caspase-3 detection kit was from Clontech (Palo Alto, CA). Retinoids were dissolved in DMSO at a concentration of 10−2 M and were stored in aliquots at −80°C. Other chemicals, drugs, probes and stains used in this work were of molecular biological grade.

Cell culture and drug treatment

Rat primary astrocytes (RPA) were developed from neonatal rat cerebellum with institutional animal ethics committee permission. The single cells were obtained by mechanical disruption and cultured in Dulbecco’s modified Eagle’s medium with fetal calf serum (FCS) (10%). Glial origin of cells was confirmed by glial fibrillary acidic protein staining (Sigma).

U87MG and U373MG cells were cultured in Minimal Essential Medium (MEM) with non-essential amino acids, containing glutamine (4 mM), and penicillin–streptomycin (50 IU/ml) with FCS (10%), at 37°C in a humidified atmosphere of 5% CO2. For experimental purposes, exponentially growing cells were seeded at the densities that allowed untreated cells to reach a nearly confluent state (~70–80%) at the end of the experiment. Cells were treated with drugs, 24 h of plating in fresh MEM. Retinoids were replenished after 48 h. Untreated cells received the same amount of DMSO (0.1%) as the treated cells and were used as control.

Cell viability assay

The cytotoxic effect of retinoids on glioma cell lines was determined by counting the viable cells with trypan blue staining. Briefly, cells (2 × 105 cells/well) were seeded in 24-well plate. After 24 h of plating the cells were exposed to various concentrations of retinoids for 72 h, and both attached and non-attached cells were collected and resuspended in phosphate-buffered saline (PBS). Viable cells were counted using a Coulter counter after staining with trypan blue dye. The IC50 concentrations were determined from concentration response curve. For further experiments, unless specified otherwise, IC50 concentrations were used.

Flow cytometry for quantification of apoptosis

Cells were harvested and fixed in 70% ethanol at −20°C. Pelleted cells were stained for 1 h in 0.5 ml of staining solution (40 μg/ml PI, 0.5% Triton X-100 and 0.1 mg/ml RNAse-A in PBS). PI fluorescence was detected in FL-2 mode in a FACScan (Becton Dickinson, San Jose, CA). Approximately 10,000 events (cells) were evaluated from each sample. The sub-G1 fraction (apoptotic) was estimated by gating hypodiploid cells in the DNA histogram using non-apoptotic population (cells treated with DMSO) as a reference to compare with treated cells. Data were analyzed as single parameter frequency histogram using cell Quest Alias software.

DNA fragmentation

Cells (~106) were pelleted and re-suspended in hypotonic buffer (25 mM Tris-HCl (pH 7.4), 25 mM EDTA and 0.5% Triton X-100) for 30 min on ice. After centrifugation at 10,000 g, 0.1 mg/ml RNAse-A was added to supernatant for 2 h followed by 0.1 mg/ml of proteinase-K for 4 h at 37°C. DNA was extracted using phenol-chloroform and then precipitated with ethanol and sodium acetate. Pelleted DNA was dissolved in Tris-EDTA (pH 8.0) and electrophoresed on a 1.8% agarose gel in the presence of ethidium bromide (0.5 μg/ml).

Measurement of ROS and ΔΨm

Intracellular ROS generation and ΔΨm were measured using DCF-DA and JC-1 (28), respectively. Briefly, cells were plated in a six-well plate. One hour before the cells were harvested, DCF-DA (10 μM), or 15 min before, JC-1 (5 μM), was added directly to the culture medium at 37°C in dark. Then, the cells were harvested in PBS and analyzed immediately by FACScan equipped with a 488 nm laser. Forward and side scatter were used to gate the viable population of cells. DCF-DA emit at 530 nm (FL-1 channel), whereas JC-1 emit at two wavelengths: JC-1 monomer at 527 nm (FL-1 channel), indicating cells with low ΔΨm, and J-aggregate emit at 590 nm (FL-2 channel), indicating cells with high ΔΨm.

Assay for measuring free cytosolic calcium ([Ca2+]c)

Free cytosolic calcium ([Ca2+]c) was measured using cell permeant Ca2+-sensitive fluorescent dye Fluo-3AM (29). Wherever indicated, EGTA (1 mM), BAPTA-AM (10 μM) and heparin (5 mg/ml) were added to the culture medium for 24, 4 and 12 h, respectively, before the loading with Fluo-3AM. The medium was removed from the tissue culture plates and replaced with 4 μM Fluo-3AM diluted in Krebs-Ringer buffer (KRB) [110 mM NaCl, 120 mM NaHCO3, 4.5 mM KCl, 1.7 mM CaCl2 and 0.5 mM MgCl2 (pH 7.4 at 37°C)] for 20 min. The dishes were washed once with 5 ml KRB to remove the residual dye. The cultures were then treated for the indicated times with 4-HPR or the vehicle diluted in KRB including 2 mM CaCl2. The cells were then harvested by trypsinization, washed with 5 ml of Ca2+-free PBS at 37°C and analyzed immediately for Fluo-3AM fluorescence intensity at 530 nm (FL-1) by flow cytometry. Forward and side scatter were used to gate the viable population of cells.

To localize mitochondria Ca2+, Fluo-3AM-loaded live cells were incubated with Mitotracker Red (200 mM) for 45 min and visualized under UV-fluorescence microscopy.

Loading of heparin inside the cells to inhibit IP3-mediated Ca2+ release was performed by cytoplasmic hypo-osmotic pinocytic lysis procedure (30). Briefly, cells were incubated in hypertonic solution polyethylene glycol 1000 (10%, v/v), 0.5 M sucrose and heparin (5 mg/ml) for 5 min in MEM to a final volume of 1 ml to form heparin-containing pinocytic vesicles. Cells were exposed to hypo-osmotic media (six parts MEM to four parts water) for 2 min to promote pinocytic lysis and the distribution of heparin throughout the cytoplasm. Cells were further rinsed and cultured in normal MEM. This procedure resulted in the loading of ~90% cells as determined by cytoplasmic localization of fluorescent fluorescein isothiocyanate-dextran, and did not contribute to cell toxicity (data not shown).

Immunofluorescence

Cells were grown on coverslips, were fixed in 4% paraformaldehyde for 30 min at 4°C and permeabilized with pre-chilled 0.2% Triton X-100/PBS. Primary antibodies: mouse monoclonal against N-terminal of Bax YTH-6A7 (Trevesen, Gaithersburg, MD) and goat polyclonal anti-AIF E-1 (Santa Cruz Biotechnology, Santa Cruz, CA). Before labeling with anti-Bax, cells were
permeabilized for 5 min with 0.0125% CHAPS/PBS to prevent artificial activation of Bax. Secondary antibodies: FITC-conjugated immunoglobulins (DAKO, Denmark). Nuclei were counterstained with Hoechst-33258 (10 μg/ml) or PI (25 μg/ml), after incubation in 0.5% mg/ml RNase-A (Sigma). Cells were photographed under conventional UV-fluorescence microscope.

Transmission electron microscopy
Cells were fixed in a mixture of 4% glutaraldehyde/2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 4 h at 4°C. Cells were washed and the samples were post-fixed in 2% osmium tetroxide for 2 h. Cells were dehydrated, embedded in epon/plastic mixture, sectioned using ultramicrotome (Leica ULTRACUT UCT) and stained using standard electron microscopy (EM) procedures. Specimens were photographed in an FET Tecnai-12 Twin electron microscope at 80 kV.

Colorimetric assay for caspase-3 activation
Caspase-3-like activity was quantified by colorimetric assay according to the manufacturer’s protocol (Clontech, Palo Alto, CA). Briefly, 1 ×10^6 cells were lysed in 50 μl of lysis buffer, and supernatant was collected after centrifugation at 10 000 g for 10 min. Fifty microliter of the 2x reaction buffer/DTT mix was added to 50 μl of the cell lysate. Then, 5 μl of 1 mM of caspase-3 substrate (DEVD-pNA) was added to each sample and incubated at 37°C for 1 h. A parallel control reaction was set up that did not contain conjugated substrate. The samples were read at 405 nm in a microplate reader.

Western blot analysis
The cells were harvested after 24 and 48 h of treatment. We did not perform western blotting of 72 h treated cells, as after 72 h majority of the cells were detached and entered secondary necrosis and the cellular contents were lost from the cells. After washing twice with PBS, whole cell lysate was prepared by lysis cells by freeze-thaw in 50 mM phosphate buffer with protease inhibitor cocktail. For cytotoxic fractions, cells were homogenized in buffer (20 mM HEPES–KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium-EGTA and 1 mM dithiothretol, 250 mM sucrose and protease inhibitor cocktail) (20 mM HEPES–KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium-EGTA and 1 mM dithiothretol, 250 mM sucrose and protease inhibitor cocktail) (20 mM HEPES–KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium-EGTA and 1 mM dithiothretol, 250 mM sucrose and protease inhibitor cocktail). For cytosolic fractions, cells were homogenized in buffer (20 mM HEPES–KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium-EGTA and 1 mM dithiothretol, 250 mM sucrose and protease inhibitor cocktail) (20 mM HEPES–KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium-EGTA and 1 mM dithiothretol, 250 mM sucrose and protease inhibitor cocktail). The samples were run on 8% pre-cast gels (Amersham Biosciences, Buckinghamshire, UK). Membranes were incubated with primary antibody, Rabbit polyclonal anti-caspase-3, Bax, Bcl-xl, β-actin and mouse monoclonal anti-Bcl-2 antibodies were from Santa Cruz Biotechnology; and rabbit polyclonal anti-capase-7 and mouse monoclonal anti-cytochrome c (7H8.2C12) antibodies were kind gifts from Dr Xiao Miu Sung (University of Leicester, UK) and Dr R Jemmerson (University of Minnesota, USA), respectively. Antibodies for RAR-α (No. 443) and retinoid X receptor (RXR) α (No. 444) were kindly gifted by Dr P Chambon (IGBMC, De Strasbourg, France). The bands were detected by using HRP-conjugated secondary antibodies to primary immunoglobulin using enhanced chemiluminescence system (Amersham Biosciences).

Statistical analysis
Differences in mean were calculated using Student’s t-tests. P-value < 0.05 was considered as statistically significant.

Results
Enhanced cytotoxic effect of 4-HPR over ATRA and 9-cisRA in glioma cell lines
The comparative cytotoxic effects of retinoids were studied on high-grade glioma cell lines U87MG and U373MG in comparison with normal astrocytes; RPA developed from neonatal rat cerebellum.

We found a better cytotoxic potential of 4-HPR than ATRA and 9-cisRA in glioma cell lines as determined by cell viability assay (Figure 1A). The IC₅₀ of 4-HPR in U87MG and U373MG cells was 10 and 15 μM, respectively. In contrast, ATRA and 9-cisRA showed a weak cytostatic effect and even at higher concentration (50 μM) failed to induce a growth inhibitory effect on U87MG and U373MG cells similar to 4-HPR. On RPA all the retinoids exerted weak cytostatic effect (Figure 1A) with no cell death observed even at higher concentration of 50 μM (data not shown), suggesting that glioma cells were more sensitive to retinoid-induced growth inhibitory effects in comparison with normal astrocytes.

At higher concentrations, that is, IC₅₀ and above, 4-HPR induced cell death by apoptosis in both the glioma cell lines but not in normal astrocytes as determined by microscopic observation of Hoechst-33258 nuclear staining. Typical features of apoptosis like condensed and fragmented nuclei were observed (data not shown). Further, to quantify the extent of apoptosis, hypodiploid peak of PI-stained cells was measured and DNA laddering pattern was also analyzed (Figure 1B and C). 4-HPR induced apoptosis at IC₅₀ concentration in >50% of the glioma cells after 72 h of treatment along with a 180 bp laddering pattern. A similar extent of apoptosis was not achieved by ATRA and 9-cisRA even at higher concentration (50 μM).

4-HPR-induced apoptosis is associated with ROS generation and loss of ΔΨₘ in glioma cell lines
Several characteristic early apoptotic events involve enhanced ROS production and loss of ΔΨₘ compared to normal astrocytes; RPA developed from neonatal astrocytes (Figure 2A). The comparative cytotoxic effects of retinoids were studied on high-grade glioma cell lines U87MG and U373MG in comparison with normal astrocytes; RPA developed from neonatal rat cerebellum.

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effectively prevented ROS generation (Figure 2C) and subsequent apoptosis (Figure 2D), suggesting that 4-HPR-induced hydroperoxide generation and loss of \( \Delta \Psi_{m} \) are related events and associated with apoptosis induction.

**4-HPR promotes distinct cellular and subcellular alterations in glioma cells**

Since loss of \( \Delta \Psi_{m} \) was associated with 4-HPR-induced apoptosis, we were interested in the ultrastructural changes in mitochondria and also other subcellular organelles like ER that would possibly provide additional clues to the underlying stress mechanism. Thus, we performed EM of U87MG cells exposed to 4-HPR for 48 h, the time when the cells are in execution phase. Cells with representative characteristics are presented in Figure 4. The control U87MG cells displayed a well-defined plasma membrane and nuclear envelope containing nucleoli (Figure 4A). The mitochondria of these cells were healthy with well-defined cristae (Figure 4C). Rough ER could be seen through the cytoplasm of the cell. A 48 h exposure to 4-HPR led to cytoplasmic and nuclear shrinkage (Figure 4B). The nucleoli were absent and chromatin condensation was evident along with clusters of vacuolization in the cytoplasm. A higher magnification confirmed that the larger vacuoles were the remnants of the mitochondria, which appeared abnormally enlarged or swollen, along with accumulation of electron-dense material (Figure 4D) compared with those observed in the control cells. The cristae were absent, and the disruption of outer membrane integrity was evident. These mitochondrial changes appeared to be consistent with MPT induction. The ER was dilated and could also be identified in close proximity to the mitochondria. The smaller vacuoles surrounding the mitochondria presumably represented what remained of the ER. Furthermore, in addition to chromatin condensation, the dilation of the ER appeared to be a common subcellular feature in 4-HPR exposed...
cells, implying that ER stress was associated with apoptosis induction.

**Role of intracellular Ca2+ in 4-HPR-induced apoptosis**

EM studies suggested the involvement of mitochondrial swelling and ER stress in 4-HPR-induced apoptosis. In cells, the ER and mitochondria are the two major sites that are directly involved in the storage and regulation of intracellular Ca2+ homeostasis (33). Although enhanced ROS generation and MPT are well associated with 4-HPR-induced apoptosis, an alteration in Ca2+ homeostasis, which is a related event, has not been studied yet. Thus, we investigated whether 4-HPR could increase [Ca2+]c as a surrogate indicator of ER stress using the Ca2+-sensitive dye Fluo-3AM by flow cytometry. 4-HPR increased the Fluo-3-AM intensity in U87MG and U373MG cells (Figure 5A). Time-course analysis in U87MG cells showed a significantly increased [Ca2+]c within initial hours of 4-HPR treatment, which further increased with time (Figure 5B), and was sustained with the DNA fragmentation (data not shown). We also determined the effect of antioxidants and MPT inhibitor on the alterations of [Ca2+]c (Figure 5C). Both antioxidants and CsA could prevent the rise in [Ca2+]c, suggesting the role of ROS and MPT in regulating intracellular Ca2+ levels (Figure 5C).

We further examined the Fluo-3AM-loaded cells that were counter-stained with Mitotracker Red under fluorescence microscope, to investigate whether 4-HPR also increases the mitochondrial Ca2+ levels. Results indicate that 4-HPR treatment not only increases [Ca2+]c, but also increases mitochondrial calcium uptake observed as yellowing of the mitochondria due to overlay of green and red (Figure 5D).

**Effect of calcium chelators in 4-HPR-induced cell death**

In order to determine the source of increased Ca2+, we pre-treated the cells with EGTA at a subtoxic concentration of 1 mM to chelate the extracellular Ca2+ that brings extracellular Ca2+ concentration in micromolar range (34). Chelation of extracellular Ca2+ did not prevent the increase in [Ca2+]c and subsequent apoptosis after 4-HPR treatment (Figure 5E and F), suggesting involvement of intracellular Ca2+ stores for [Ca2+]c elevation. To test whether increase in [Ca2+]c is involved in progression of apoptosis, we used a cell permeant

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**Fig. 2.** HPR-induced ROS generation and effect of various antioxidants on ROS generation and apoptosis. Glioma cells were treated with 4-HPR at the indicated concentration, retinoids or DMSO for indicated times. Then, 10,000 events were evaluated for ROS production by flow cytometry using DCF-DA. (A) U87MG and U373MG cells were treated with 4-HPR (10 and 15 μM, respectively) or DMSO for 4 h. DCF-fluorescence histograms showing overlay of control and 4-HPR treated cells. (B) Time-course analysis of U87MG cells treated with 4-HPR (10 μM), ATRA (50 μM), 9-cisRA (50 μM) or DMSO for the indicated time intervals (up to 360 min). DCF fluorescence expressed as fold increase as compared with control. (C and D) U87MG cells were treated with 4-HPR (10 μM) alone or pretreated with GSH (100 μM), MN (100 μM), AA (500 μM), L-NAME (100 μM) or DMSO for 12 h or co-treated with 5 μM CsA. (C) Treatment was continued for 6 h; cells were incubated with DCF-DA 1 h before harvestation. ROS generation was measured by flow cytometry. Data are expressed as fold increase in DCF levels obtained from three individual experiments ± SE (error bars). (D) Apoptosis was detected as percentage of hypodiploid cells by flow cytometry after 72 h of treatment. Data represent the mean of triplicate experiments ± SE (error bars). **P < 0.05, ***P < 0.01 compared with cells treated with 4-HPR alone.
cells were incubated with JC-1 and percentage of cells with low fluorescence histograms plotted against cell number showing overlay of monomers. (B) U87MG cells were treated with 4-HPR (10 μM) for the indicated time intervals (up to 360 min), and ΔΨm was analyzed by flow cytometry using JC-1, by measuring increase in FL-1 fluorescence (JC-1 monomers). (C) U87MG and U373MG cells were treated with retinoids at the indicated concentration or DMSO. ΔΨm was analyzed by flow cytometry using JC-1, by measuring increase in FL-1 fluorescence (JC-1 monomers). Data represent the mean of triplicate experiments ± SE (error bars). *P < 0.05, **P < 0.01 compared with cells treated with 4-HPR alone.

Fig. 3. 4-HPR-induced loss of ΔΨm and effect of various inhibitors on ΔΨm. U87MG and U373MG cells were treated with retinoids at the indicated concentration or DMSO. ΔΨm was analyzed by flow cytometry using JC-1, by measuring increase in FL-1 fluorescence (JC-1 monomers). (A) U87MG and U373MG cells were treated with 4-HPR (10 and 15 μM, respectively) or DMSO for 4 h. JC-1 monomers fluorescence histograms plotted against cell number showing overlay of control and 4-HPR treated cells. (B) U87MG cells were treated with 10 μM 4-HPR for the indicated time intervals (up to 360 min), and ΔΨm was expressed as percentage of cells with low ΔΨm. (C) U87MG cells were treated with 4-HPR (10 μM) alone or pretreated with GSH (100 μM), MN (100 μM), AA (500 μM), L-NAME (100 μM) or DMSO for 12 h or co-treated with 5 μM CsA. Treatment was continued for 12 h, cells were incubated with JC-1 and percentage of cells with low ΔΨm was detected by flow-cytometric analysis. Data are plotted by measuring the percentage of cells with low ΔΨm (JC1 monomers). Data represent the mean of triplicate experiments ± SE (error bars). *P < 0.05, **P < 0.01 compared with cells treated with 4-HPR alone.

Ca2+ chelator, BAPTA-AM (10 μM) (29,34,35). It prevented the rise in intracellular Ca2+ but further increased 4-HPR-induced apoptosis (Figure 5E and F). The results are difficult to interpret given the reports of its anomalous effects on drug-induced Ca2+ homeostatic perturbations and apoptosis in various cancer cells. One possible explanation may be the permeability of BAPTA-AM and its Ca2+ chelating ability both in cytosolic and ER milieu. Its ability to deplete ER Ca2+ by its chelation has a potential to cause apoptosis (35). To resolve the issue, we made the 4-HPR-treated cells permeable to heparin, a blocker of IP3R on ER membrane, to prevent the release of Ca2+ from ER to cytosol. We found that heparin effectively inhibited the rise in Ca2+ as well as apoptosis induction by 4-HPR (Figure 5E and F). Thus, our findings suggest that ER Ca2+ depletion can contribute to apoptosis process. Furthermore, we found that heparin also prevented ROS generation and MPT (data not shown) again, suggesting that ROS generation, MPT and Ca2+ are associated events.

Modulation of Bcl-2, Bcl-XL and Bax expression by 4-HPR

Bcl-2 family proteins like Bcl-2, Bcl-XL and Bax regulate apoptosis by maintaining the mitochondrial membrane integrity (36). Looking at the loss of ΔΨm in 4-HPR-induced apoptosis, we analyzed the expression levels of Bcl-2, Bcl-XL and Bax proteins by immunoblotting followed by densitometry analysis of U87MG and U373MG after 4-HPR treatment. We did not find a significant change in the levels of Bcl-2 and Bcl-XL (data not shown), which is in contrast with the observations shown recently (27). However, 4-HPR significantly increased (P < 0.01) the levels of pro-apoptotic member Bax in a time-dependent manner (Figure 6A). Since the translocation of Bax from cytosol to mitochondria leads to mitochondrial membrane permeabilization (MMP) and further promotes loss of ΔΨm, immunocytochemistry was performed. An antibody recognizing the Bax N-terminus, which is exposed by the activation of Bax and its insertion into the mitochondrial membrane (a, b in Figure 6B), was used, and nuclei were counterstained with Hoechst-33258 (c, d in Figure 6B). In control U87MG cells with non-apoptotic nuclei, Bax staining was undetectable. After 4-HPR treatment Bax labeling was bright and punctuate in nature in the cells with fragmented nuclei, thus suggesting the role of Bax in 4-HPR-induced apoptosis regulating the MMP.

Cytochrome c and AIF release and activation of caspases by 4-HPR treatment

Loss of ΔΨm and MMP promotes the release of cytochrome c and AIF to cytosol and nucleus, respectively (37). Therefore, western blotting of cytosolic fraction was performed to detect the release of cytochrome c to cytosol (Figure 6C), and immunocytochemistry was performed to see the nuclear translocation of AIF in Figure 6D). A time-dependent increase in the release of cytochrome c was observed after 4-HPR treatment in U87MG and U373MG. After 4-HPR treatment, AIF labeling was observed in the nucleus of cells with morphological features of apoptosis. We further pretreated the cells with antioxidant (AA) and MPT inhibitor (CSA) to study the involvement of ROS generation and MPT in AIF nuclear translocation (e–h in Figure 6D). Both antioxidants and CSA inhibited the nuclear translocation of AIF and apoptosis.

Release of cytochrome c from mitochondria leads to the formation of apoposome, which results in activation of...
executioner caspases, namely caspase-3 and caspase-7 (38). Therefore, activation of caspase-3 and caspase-7 was assessed by colorimetric assay for caspase-3-like activity and western blotting in U87MG and U373MG cells. 4-HPR treatment induced significant increase in caspase-3-like activity in a time-dependent manner (Figure 7A). A decrease in the pro-caspase-3 levels were also detected (a in Figure 7B), which indicates an increase in its activated form and corroborates with the increasing caspase-3-like activity. Further, an increase in the active caspase-7 subunit was detected after 4-HPR treatment (b in Figure 7B). The general and caspase-3-specific inhibitor, but not caspase-8 inhibitor, prevented the cells from 4-HPR-induced intranucleosomal fragmentation; however, peripheral chromatin condensation still took place in ~40% of the cells, as assessed by nuclear morphology of PI-stained cells (Figure 7C).

Presently, we also found that 4-HPR treatment leads to nuclear translocation of AIF (c, d in Figure 6D), and AIF is known to induce apoptosis independent of caspases activity (22). Thus, we performed immunocytochemistry of AIF in the presence of general caspase inhibitor. Results indicate that AIF translocation was not inhibited after treatment with general caspase inhibitors (i, j in Figure 6D); however, the nuclear apoptotic pattern differed. In the presence of general caspase inhibitor, which prevented the generation of active caspase-3 subunit (data not shown), peripheral chromatin condensation in the absence of fragmentation was observed, thus indicating the involvement of both caspase-dependent and caspase-independent pathway 4-HPR-induced apoptosis in glioma cell.

4-HPR increased RAR-γ and RXR-α expression
4-HPR shows a selective retinoid receptor activation profile that induces transcriptional activation preferentially via RAR-γ and to some extent by RAR-β, but not RAR-α, and induces the receptor expression (3). To determine if 4-HPR-mediated signals in glioma cultures resulted in increased expression of RAR-β and RAR-γ and its receptor partner, RXR-α, as a downstream effect, we assessed the expression of the receptor by western blotting (Figure 7D). Both the cell lines showed a basal level expression of all these receptors that significantly increased for RAR-γ as well as for RXR-α in a time-dependent manner at the same drug concentration at which apoptosis was induced. The levels of RAR-β remained unchanged after 4-HPR treatment.

Discussion
4-HPR has shown its chemotherapeutic potential in clinical trials in a variety of cancers (5). Although much interest and effort have been focused on the apoptotic potential of
4-HPR as a means of eliminating cancer cells, the mechanisms underlying 4-HPR-induced cell death are complex and still remain elusive.

Gliomas are predominantly aggressively growing malignant brain tumors and are associated with poor prognosis. Majority of chemotherapeutic agents have shown negative outcomes in malignant glioma (39). To improve the glioma treatment, a better understanding of the molecular mechanism involved in the drug action is required (40). The sensitivity or resistance of cancer cells to various drugs depends on the activation of various routes to apoptosis involving different signaling pathways. The present study was performed to get a better insight into the molecular pathway involved in 4HPR-induced apoptosis using glioma cell lines U87MG and U373MG.

![Fig. 5. HPR induced changes in calcium homeostasis in glioma cells.](https://academic.oup.com/carcin/article-abstract/27/10/2047/2391867)

4-HPR was treated as indicated, and levels of $[\text{Ca}^{2+}]_c$ were determined by flow cytometry using Fluo-3AM. (A) U87MG and U373MG cells were treated with 4-HPR (10 and 15 μM, respectively) or DMSO for 4 h. Fluo-3AM fluorescence histograms showing overlay of control and 4-HPR-treated cells. (B) U87MG cells were treated with 4-HPR (10 μM), ATRA (50 μM), 9-cisRA (50 μM) or DMSO (control) for the indicated times, and then levels of $[\text{Ca}^{2+}]_c$ were determined by flow cytometry. (C) U87MG cells were treated with 4-HPR (10 μM) alone or pretreated with GSH (100 μM), MN (100 μM), AA (500 μM), L-NAME (100 μM) or DMSO for 12 h or co-treated with 5 μM CsA. Levels of $[\text{Ca}^{2+}]_c$ were determined by flow cytometry. Data are expressed as fold increase in fluo-3AM intensity. (D) Fluorescent microscopy images of U87MG cells treated with 4-HPR for 24 h were loaded with Fluo-3 (green fluorescence) and Mitotracker Red (red fluorescence) and were excited at 488 nm. Images show the overlay of the two individual images. Green fluorescence shows increased $[\text{Ca}^{2+}]_c$, red localizes mitochondria, and colocalization of $\text{Ca}^{2+}$ and mitochondria is represented in shades of yellow owing to overlay of green fluorescence with red. (E and F) U87MG cells were pretreated with DMSO (control), 1 mM EGTA for 24 h or 10 μM BAPTA-AM for 4 h or 5 mg/ml heparin for 12 h and then treated with 10 μM 4-HPR for indicated times in the presence or absence of 1 mM EGTA or 10 μM BAPTA-AM, 5 mg/ml heparin. (E) Free $[\text{Ca}^{2+}]_c$, was measured after 6 h of treatment by flow cytometry and expressed as fold increase. (F) Apoptosis (hypodiploid peak) using PI staining after 72 h, by flow cytometry. Data are expressed as mean of triplicate experiments ± SD (error bars). *$P \leq 0.05$ compared with cells treated with 4-HPR alone.
Here, we demonstrate that 4-HPR induced apoptosis in glioma cells, but not in normal astrocytes, suggesting its cancer cell-specific action as shown previously (10). Results further demonstrate that 4-HPR induced apoptosis through a similar mechanism in both the glioma cell lines U87MG (p53-wild-type) and U373MG (p53-mutant), which differed in their p53 status, thus supporting earlier findings (14) showing involvement of a p53-independent pathway.

4-HPR, which is a synthetic derivative of ATRA, induces apoptosis in a wide variety of cancer cells as a major effector mechanism; however, ATRA and 9-cisRA are known to induce differentiation and mediate distinct biological outcomes. 4-HPR seems to surmount the problems associated with natural retinoid-induced differentiation and increased resistance to chemotherapy, owing to its apoptosis-inducing capacity (5). In glioma cells, in comparison with 4-HPR, apoptosis was not induced with ATRA and 9-cisRA, even at higher concentration at which they showed growth inhibitory action. Our results further suggested that 4-HPR acts by a distinct mechanism from the one ascribed to ATRA and 9-cisRA by involving ROS generation, loss of ΔΨm and elevation of Ca2+.

4-HPR-induced apoptosis via a pro-oxidant mechanism, even better than newer synthetic retinoid, like SHetA2 (41). In glioma cells also the pro-oxidant capacity of 4-HPR was sustained and hydroperoxide production was associated with apoptosis induction as suggested previously in a variety of cell types (9,10,17–19). 4-HPR also led to a loss of ΔΨm as a subsequent event to ROS generation, and both were associated events that lead to apoptosis in glioma cells as shown earlier in metastatic squamous cell carcinoma cells (15) and in human cervical carcinoma C33A cells (9).

During various conditions, ROS and MPT are often associated with Ca2+ homeostasis, and all three are cohort events. Any alteration therein plays an important role in apoptosis induction (20,21). Chemotherapeutic drugs like triterpenoid-CDDO have been shown to act via a mechanism involving modulation of Ca2+ homeostasis leading to caspase activation and apoptosis (29). In the present study, we also determined the role of alteration in Ca2+ homeostasis in 4-HPR-induced apoptosis. Here, we provide an illustration of a novel mechanism for the possible anticancer activity of 4-HPR. This is the first report indicating that 4-HPR induced rise in [Ca2+] in glioma cells, which came from intracellular Ca2+ stores (Figure 5). We also found an association between Ca2+ increase, ROS generation and MPT. In the eukaryotic cells, the ER and mitochondria are the primary sites that are utilized for Ca2+ storage and the regulation of intracellular Ca2+ homeostasis.


Ca\textsuperscript{2+} homeostasis (21). The sustained Ca\textsuperscript{2+} release from the ER can trigger Ca\textsuperscript{2+} uptake by the mitochondria, which possess an exceptional capacity to sequester Ca\textsuperscript{2+} and buffer local variations in [Ca\textsuperscript{2+}]\textsubscript{c} levels (21,37). Indeed, 4-HPR treatment resulted in an increase in the mitochondria Ca\textsuperscript{2+} levels in glioma cells (Figure 5D). Furthermore, the ultrastructural changes also indicated the close proximity of the mitochondria to the ER (Figure 4D). It has been suggested that close contacts exist between mitochondria and the sites of ER Ca\textsuperscript{2+} release, such that ER Ca\textsuperscript{2+} release leads to rapid Ca\textsuperscript{2+} accumulation in mitochondria, which may promote the loss of mitochondrial permeability and further play an important role in apoptosis induction (37). We further found that 4-HPR-induced apoptosis in glioma cells was inhibited by heparin, which

**Fig. 7.** Activation of caspases and RARs by 4-HPR. (A) Caspase-3-like activity in whole cell lysates, measured by using ApoAlert caspase-3 detection kit (clontech). Data are expressed as mean of triplicate experiments ± SE (error bars). (B) Pro-caspase-3 or caspase-7 (pro and active form) by western blotting. (C) U87MG cells and U373MG cells were pretreated with Z-VAD (50 μM), a general caspase inhibitor; Z-DEVD-fmk (50 μM), inhibitor for caspase-3; or Z-IETD-fmk (50 μM), caspase-8 inhibitor, for 4 h followed by treatment with 4-HPR (IC\textsubscript{50}) or were left untreated for 72 h. Percentage apoptosis was determined by counting the apoptotic nuclei of PI-stained cells under microscope. Data are expressed as mean of triplicate experiments ± SD (error bars). *P ≤ 0.05, **P ≤ 0.01 compared with cells treated with 4-HPR alone. (D) Western blotting for RAR-β, RAR-γ, RXR-α and β-actin showing loading control. Experiment was repeated three times independently. Level of significance was drawn from densitometric analysis of the blots.
blocks the release of Ca\(^{2+}\) from ER, but not BAPTA-AM, which prevents the rise of [Ca\(^{2+}\)]\(_{\text{cyt}}\). Thus, our results suggest an important role of ER Ca\(^{2+}\) pool depletion and its trafficking to cytosol and to mitochondria in 4-HPR-induced apoptosis. Furthermore, 4-HPR treatment resulted in ER vacuolization (Figure 4B). It is suggested that the ER Ca\(^{2+}\) pool depletion may compromise its structural and functional integrity that may result in its vacuolization. As apoptosis is accompanied by blebbing, it is likely that loss of ER integrity may direct the bleb formation through cytoskeleton rearrangement (42). Furthermore, the disruption of intracellular calcium homeostasis and/or ER stress can also promote the activation of caspase-12, which has been implicated in activation of caspases 8, 9 and 3 (43). However, in the present study we did not study the role of caspase-12 activation that needs to be investigated.

Loss of \(\Delta \Psi_m\) and MMP are early events in apoptosis (32). 4-HPR-induced loss of \(\Delta \Psi_m\), which is associated with apoptosis in glioma cells, consistent with earlier studies showing that 4-HPR acts directly on mitochondria to induce apoptosis (9,11,15,16). The opening of the mitochondrial ‘permeability transition pore’ is regulated by endogenous effectors, including members of the Bcl-2 family proteins such as Bax (36). The pro-apoptotic Bax is normally present in the cytosol, which promotes programmed cell death by its insertion into mitochondrial membrane (44). In glioma cells 4-HPR upregulated expression as well as mitochondrial translocation of Bax. In mitochondria, Bax has pore-forming properties (44) that can promote the loss of \(\Delta \Psi_m\) and release cytochrome \(c\) and AIF from mitochondria (32,45). Indeed, 4-HPR leads to release of cytochrome \(c\) and AIF in glioma cells. The release of cytochrome \(c\) from the mitochondria and its interaction with cytosolic factors including Apaf-1 and dATP can activate caspase-9 and then caspases 3 and 7 (46). Caspases are involved in 4-HPR-induced apoptosis, and the activation of caspase-3 subsequent to cytochrome \(c\) release has been previously assessed (5,9–11). We also found 4-HPR-induced activation of caspase-3 and caspase-7 in glioma cells (25). However, we found that in the presence of pan-caspase inhibitor oligonucleosomal fragmentation was prevented but peripheral chromatin condition still took place, suggesting that 4-HPR may also trigger the caspase-independent pathway. Recent reports have also suggested involvement of caspase-independent apoptosis by 4-HPR in human B lymphoma cells (13). Another mechanism for apoptosis induction, independent of caspase activity, is through AIF, a mitochondrial protein that is translocated into the nucleus and leads to apoptosis induction (22). It is also suggested that the nuclear translocation of AIF can be a caspase-dependent event (47). Within the nucleus, AIF, in cooperation with a mitochondrial protein Endo G, induces peripheral chromatin condensation and large-scale DNA fragmentation (22). We found that 4-HPR-induced AIF nuclear translocation in the presence of caspase inhibitors, which leads to peripheral chromatin condensation in the absence of nuclear fragmentation. AIF nuclear translocation was inhibited by antioxidants and CsA, suggesting role of ROS generation and MPT in AIF-mediated apoptosis. Thus, the results suggest involvement of parallel pathways of chromatin processing in which nuclear fragmentation is governed by caspases and peripheral chromatin condensation by AIF as suggested earlier, where role of AIF has been shown in the first stage of apoptosis and caspases in the second stage (48). There are growing amount of evidences that suggest involvement of caspase-independent pathways in synthetic retinoid-induced apoptosis (13,23), which may provide additional advantage to overcome the resistance due to caspase inactivation in cancers.

In the present study, use of antioxidants, MPT as well as caspases inhibitors did not completely prevent 4-HPR-induced apoptosis, suggesting role of additional mechanisms involving ceramide (8) or RAR-mediated pathway (3). Presently, we did not study the role of ceramide pathway, but the levels of RAR-\(\beta\), RAR-\(\gamma\) and RXR-\(\alpha\) in 4-HPR-induced apoptosis were determined. 4-HPR led to upregulation of RAR-\(\gamma\) and RXR-\(\alpha\) levels in glioma cells. It has been suggested that RAR-\(\gamma\) and its receptor partner RXR-\(\alpha\) activation can lead to apoptosis induction. The increased RAR-\(\gamma\) expression may further enhance apoptosis (3). Thus, the receptor-dependent effect of 4-HPR seems to be relevant in glioma cells as shown previously in other cell types (3,5–7). The ability of 4-HPR to induce apoptosis in retinoid-resistant tumor cells (6,49) suggested that 4-HPR can mediate its effects in a receptor-independent manner. However, in the present study, it is not clear whether the upregulation of receptor is associated with apoptosis, as we could not use the RAR-antagonists that are needed for further evaluation.

In conclusion, the present study brings insight into some of the unrevealed pathways of 4-HPR-induced apoptosis. Looking at its ability to target multiple sites of apoptotic pathways, further studies are warranted using 4-HPR as a single or combinational agent to eliminate cancer cells.

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