Inhibition of N-(4-hydroxyphenyl)retinamide-induced autophagy at a lower dose enhances cell death in malignant glioma cells

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The question whether chemotherapy-induced autophagy is causative to the demise of the cells or a part of the survival mechanism activated during cellular distress is unclear. Others and we have previously demonstrated apoptosis-inducing capacity of N-(4-hydroxyphenyl)retinamide (4-HPR) in malignant glioma cells. We provide evidences of 4-HPR-induced autophagy at a lower concentration (5 μM). Suboptimal dose of 4-HPR treatment of malignant glioma cell lines increased G2/M arrest, whereas cell accumulated in S phase at a higher concentration. 4-HPR-induced autophagy was associated with acidic vacuole [acidic vesicular organelle (AVO)] formation and recruitment of microtubule-associated protein light chain 3 (LC3). At a higher concentration of 10 μM of 4-HPR, glioma cells undergoing apoptosis manifested autophagic features indicated by autophagosome formation, AVO development and LC3 localization. Autophagy inhibition at an early stage by 3-methyl adenine inhibited the AVO formation and LC3 localization with an enhancement in cell death. Bafilomycin A1, a specific inhibitor of vacuolar type H⁺-ATPase also prevented AVO formation without effecting LC-3 localization pattern and also enhanced the extent of 4-HPR-induced cell death. 4-HPR activated c-Jun and P38MAPK at both 5 and 10 μM concentrations, whereas increased activation of extracellular signal-regulated kinase 1/2 and NF-kappaB was seen only at lower dose. Inhibiting phosphoinositide 3-kinase and mitogen-activated protein kinases pathways modulated 4-HPR-induced cell death. This is the first report that provides evidences that besides apoptosis induction 4-HPR can also induce autophagy. These results indicate that 4-HPR-induced autophagy in glioma cell may provide survival advantage and inhibition of autophagy may enhance the cytotoxicity to 4-HPR.

Material and methods

Reagents

4-HPR and 5,5′,6,6′-tetrachlo-1-1′,3′,3′-tetrathylbenzimidazolycarbocyanine iodide (JC-1) were purchased from Calbiochem (San Diego, CA). Dimethyl sulfoxide (DMSO), 2′,7′-dichlorofluorescein diacetate (DCF-DA), propidium iodide (PI), Hoechst-33258, acridine orange (AO), 3-methyl adenine (3-MA) and bafilomycin A1 (Baf-A1) were purchased from Sigma Chemical Co. (St Louis, MO). P38MAPK inhibitor SB203580, 1 μM PD98059, 10 nM of Baf-A1 were added to glioma cells U87MG (p53 wild-type) were procured from National Center for Cell Science, Pune (India). Cells were cultured in minimum essential media (Invitrogen Corporation, Carsbad, CA) supplemented with non-essential amino acids, sodium pyruvate 10 mM, 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Invitrogen, Scotland). Cells were treated with drugs, 24 h of plating in fresh minimum essential media. To detect cell death in combination with 3-MA/Baf-A1/mitogen-activated protein kinases (MAPKs) inhibitors, U373MG cells were cultured in a medium containing 4-HPR for 24 h, and then 1 mM of 3-MA/10 mM of Baf-A1 was added in culture medium or preincubated with or without 1 μM PD98059, 1 μM SP600125 or 10 μM SB203580 for 1 h, followed by 4-HPR treatment. For morphological analysis, the cells were directly viewed under phase-contrast inverted microscope.

Cell viability assay

The cytotoxic effect of 4-HPR alone or in combination of inhibitors on glioma cell lines was determined by counting the viable cells by excluding the dead cancer cells by triggering a non-apoptotic cell death program. Furthermore, in glioma cells arsenic trioxide has been shown to induce autophagic cell death at lower concentration (6) and apoptosis at a higher concentration (7). In some cases, apoptotic and autophagic cell death coincide in vivo in certain tissues, as shown during deprivation of neural growth factor-induced simultaneous autophagic and apoptotic cell death in primary sympathetic neurons (8), and in rare cases, both morphologies may coincide within the same cells (9). However, the mutual relationship between apoptotic and autophagic death is debated (10,11) and some autophagic cell death events might have been attributed to apoptosis. N-(4-hydroxyphenyl)retinamide (4-HPR), a synthetic retinoid has emerged as a beneficial chemotherapeutic agent that exerts its growth inhibitory effect by activating multiple pathways of apoptosis in a cell type-dependent manner (12). Earlier, we have shown that 4-HPR (≥10 μM) induces apoptosis in glioma cell lines via mitochondria-mediated pathway and endoplasmic reticulum stress generation (13). However, achievement of inhibitory concentration50 at a higher concentration as compared with other cancer cell lines and inability of 4-HPR to initiate apoptosis at a lower cytotoxic concentration led us to investigate the reason behind it. Here, we report for the first time that at non-apoptotic growth inhibitory concentrations 4-HPR induces autophagy. 4-HPR induces autophagy and apoptosis in a concentration-dependent manner in glioma cells U373MG. Moreover, the cells undergoing apoptosis at a higher concentration of 4-HPR also show features of autophagy. Specific inhibition of autophagy enhances cell death and sensitizes glioma cells to a lower concentration of the drug. Thus, the present study offers additional mechanistic information, which could help to optimize the dosage of 4-HPR as a single agent or combinations thereof.

Introduction

Growing amount of evidence suggest that cancer cells face the onslaught of anticancer therapy by undergoing autophagic recycling of molecules for biosynthetic or metabolic reactions (1). Macropathology (referred as autophagy hereafter) has emerged as one of the defense mechanisms offered by cancer cells in response to radiation (2,3) or chemotherapeutic agent like temozolomide (4). In several cases exemplified by ceramide and arsenic trioxide in glioma (5,6), autophagy is irreversible and plays a central role in the elimination of doubling time. We provide evidences of 4-HPR-induced autophagy at a lower dose.

Abbreviations:

AO, acridine orange; AVO, acidic vesicular organelle; Baf-A1, bafilomycin A1; CMFDA, 5-chloromethylfluorescein diacetate; DCF-DA, 2′,7′-dichlorofluorescein diacetate; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; 4-HPR, N-(4-hydroxyphenyl)retinamide; JC-1, 5,5′,6,6′-tetrachlo-1-1′,3′,3′-tetrathylbenzimidazolylcarbocyanine iodide; JNK, c-Jun N-terminal kinase; LC3, light chain 3; LTR, Lysotracker red; 3-MA, /C21 nitride 3-kinase; ROS, reactive oxygen species.
cell by trypsin blue staining. Briefly, cells (2 × 10⁶ cells per well) were seeded in 24-well plate. After 24 h of plating, the cells were exposed to various concentrations of 4-HPR alone or in combination for 72 h, both attached and non-attached cells were collected and resuspended in phosphate-buffered saline (PBS). Viable cells were counted in a Coulter counter after staining with trypan blue dye (dead cells take up trypsin blue stain and are excluded from counting).

Flow cytometry
After defined treatment, the 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 µg/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 the treated cells. Data were analyzed as single parameter frequency histogram using CellQuest Alias software.

CMFDA staining to detect vacuoles
Cells cultured on coverslips were stained with CMFDA (1 µM) for 15 min at 37°C in dark, with PBS and followed by fluorescence microscopic assessment (×60 magnification) (Nikon Eclipse 80i; Nikon Instech Co. Ltd, Kawasaki, Kanagawa, Japan).

LTR to detect acidic lysosomes
To label lysosomes, LTR (500 nM) was added to the cultures for 30 min, washed, followed by fluorescence microscopic assessment at ×60 with Nikon Eclipse 80i.

Detection and quantification of acidic vesicular organelles with AO staining
4-HPR-treated cells were stained with AO to detect and quantify acidic vesicular organelles (AVOs) (2,4). In brief, cultured cells were incubated with AO (1 µg/ml) for 15 min. In AO-stained cells, the acidic compartments fluoresce bright red and the intensity of the fluorescence is proportional to the degree of acidity. Therefore, the volume of the cellular acidic compartment can be quantified. Photographs (×60 magnification) were obtained with a confocal microscope (Radiance 200100, Bio-Rad Laboratories, Hercules, CA). To quantify the formation of AVOs, AO-stained cells were detached and immediately analyzed. Red (650 nm, FL-3) fluorescence emission with blue (488 nm) excitation light was measured for 10,000 cells with a FACScalibur (Becton Dickenson) using CellQuest Alias software.

Immunocytochemistry
For immunofluorescence staining, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100, washed three times in PBS containing 0.01% Triton X-100 and 10% bovine serum albumin and followed by incubation with different primary antibodies such as anti-rabbit light chain 3 (LC3) (1:1000) (kind gift from Dr Tamotsu Yoshimori), anti mouse NF-kappaB (NF-kB) (Santa Cruz Biotechnology), anti mouse NF-kappaB (NF-κB) (kind gift from Dr Dumi Ernst), phosphorylated anti-rabbit antibodies against p-jun (#9164 Cell signaling Technology, Boston, MA), p38MAPK (sc-7975-R Santa Cruz Biotechnology), ERK1/2 (sc-16982 Santa Cruz Biotechnology). Fluorescein isothiocyanate-labeled rabbit/tetramethyl rhodamine isothiocyanate-labeled mouse secondary antibodies (Santa Cruz Biotechnology) were used for the detection and images were captured at ×60 with Nikon Eclipse 80i microscope.

Measurement of reactive oxygen species and mitochondrial membrane potential (∆Ψm)
Intracellular reactive oxygen species (ROS) generation and ∆Ψm were measured using DCF-DA and JC-1, respectively, as described earlier (13). Briefly, cells were plated in a six-well plate. One hour before the cells were harvested, DCF-DA (10 µM) or 15 min prior to 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 emits at 530 nm (FL-1) channel, whereas JC-1 emits at two wavelengths, JC-1 monomer at 527 nm (FL-1 channel) and J-aggregate emits at 590 nm (FL-2 channel). For microscopic evaluation of ∆Ψm, cells were grown on coverslips and stained with JC-1, counterstained with Hoechst-33258 and live cells were viewed under confocal microscope (Radiance 200100, Bio-Rad).

Transmission electron microscopy
The cells were harvested by trypsinization, washed twice with PBS, and 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 postfixed 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 procedures. Specimens were photographed in a FET Tecnai-12 Twin electron microscope at 80 kV.

Western blot analysis
The western blotting was performed as described previously (13). Briefly, the cells were harvested after 24 and 48 h of treatment. After washing twice with PBS, whole-cell lysate was prepared by lysing cells by freeze–thaw in 50 mM phosphate buffer with protease inhibitor cocktail. Proteins (50 µg) were resolved on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane in electro-transblot apparatus (Amersham Biosciences, Buckinghamshire, UK). Membranes were incubated with primary antibody. Rabbit polyclonal anti-beclin-1 antibody was a kind gift (Dr Tamotsu Yoshimori) and α-tubulin as well as mouse monoclonal anti-Bcl-2 antibodies were from Santa Cruz Biotechnology. The bands were detected by using horseradish peroxidase-conjugated secondary antibodies to primary immunoglobulin using enhanced chemiluminescence system (Amersham Biosciences). Density analysis was performed using ImageJ software.

Statistical analysis
All the experiments were repeated at least three times. The data were expressed as means ± SE. Statistical analysis was performed by using Student’s t-test (two-tailed). The criterion for statistical significance was taken as P < 0.05.

Results
4-HPR-induced cell death in glioma cells
The preliminary experiments were performed in glioma cells U373MG and U87MG to study the correspondence of 4-HPR-induced extent of cell death with apoptosis in a concentration-dependent manner. Data indicate non-correspondence between cell death and apoptosis at low dose of 4-HPR in both the glioma cell lines, whereas almost a complete correspondence was seen at high dose (Figure 1A). A significant number of cells (>30%) were undergoing death even at a concentration of 5 µM in U373MG; however, cell death could not be attributed to apoptosis and significant apoptosis was induced at a concentration >10 µM of 4-HPR (Figure 1A). In U87MG that appeared to be more sensitive to 4-HPR-induced cell death, a significant apoptosis (~16%) was taking place even at 5 µM; however, the extent of cell death (~32%) could not be attributed completely to apoptosis (Figure 1A). These results suggested the existence of an alternative cell death mode at low dose.

Effect of 4-HPR on cell cycle arrest
To investigate whether 4-HPR induces cell cycle arrest in glioma cells, we performed the flow cytometric analysis of PI-stained cells (Figure 1B). 4-HPR treatment (5 µM, 72 h) increases the population of U373MG and U87MG cells in the G2/M phase with a decrease in the population of G1 phase. At a higher concentration, i.e. 10 µM of 4-HPR, cells accumulated in S phase of the cell cycle with a reduction in G1 phase (Figure 1B). These results indicate that 4-HPR induces cell cycle arrest in a concentration-dependent manner in malignant glioma cell lines.

Reversibility effect of 4-HPR on U373MG and U87MG
To determine the effective concentration of 4-HPR that can lead to irreversible growth arrest, the growth of U373MG and U87MG cells was studied using lower (5 µm) and higher (10 µm) cytotoxic concentration of 4-HPR (Table I). Glioma cells that were pretreated with 5 µM 4-HPR recovered and began to proliferate again; however, the cells treated with a higher concentration (10 µM) for >12 h could not recover and continued to die (Table I). Thus, suggesting that lower dosage of 4-HPR induces reversible cell death, whereas higher concentration induces irreversible mode of cell death.

4-HPR induced mode of cell death in U373MG glioma cells at lower concentration
To study the mode of cell death at a lower concentration, U373MG cells were chosen since they were less sensitive to 4-HPR-induced apoptosis as compared with U87MG cells. Morphological analysis revealed that at
A concentration of 5 μM of 4-HPR, U373MG cells shrink, show large vacuoles and remained attached to the substratum (data not shown). In addition, cells manifested cytoplasmic vacuolization detectable as ‘holes’ not staining with CMFDA (Figure 1Ca–c) and correlated with an increase in the volume of acidic vesicles exhibiting a positive fluorescence for the lysosomal marker LTR (Figure 1Da–c).

**Induction of autophagy in 4-HPR-treated U373MG cells**

The reduction in cell size following 4-HPR treatment at non-apoptotic concentration along with the development of vacuoles detected by CMFDA staining and LTR positivity (Figure 1C and D) suggested the possibility that the cells are undergoing macroautophagy as an alternative mode of cell death. After exposure to 4-HPR (5 or 10 μM)
for 48 h, U373MG cells were collected and ultrastructure of the cells was analyzed by electron microscopy. Control U373MG cells exhibited characteristic features of proliferating cancer cell (Figure 2a–c). After 48 h of 4-HPR treatment, various features of autophagy were observed in 4-HPR-treated cells (Figure 2d–i). The presence of numerous autophagic vacuole, empty vacuoles and secondary lysosomes was observed in cells treated with 5 µM concentration of 4-HPR. Most of the autophagosomes contained lamellar structures or residual digested materials. The persistent formation of autophagosomes is correlated with a progressive reduction of intracellular organelles in response to drug treatment. Very few ribosomes and the Golgi/endoplasmic reticulum network were observed in any of the sections examined. The electron-dense membranous autophagic vacuoles, myelin whorls, multivesicular bodies, condensed mitochondria as well as engulfment of entire organelles are also seen (Figure 2d–f). The nucleus displayed a reduced number of nuclear pores, reduced nucleolar size and dense heterochromatin. However, chromatin condensation or fragmentation characteristic of apoptosis was not observed (Figure 2d). At a higher concentration (10 µM), ultrastructure of a number of cells reveal coexistence of autophagy and apoptosis features in treated cells (Figure 2g–i). In a same cell, typical morphological features of apoptosis, e.g. cell shrinkage, margination and condensation of chromatin as well as autophagy, e.g. autophagosomes and autophagic vacuoles are seen (Figure 2g). On the other hand, untreated tumor cells showed

<table>
<thead>
<tr>
<th>Time in days</th>
<th>4-HPR 5 µM</th>
<th>4-HPR 10 µM</th>
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<tr>
<td></td>
<td>0 day</td>
<td>1st day</td>
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<tr>
<td>U373MG cells (n x 10⁶)</td>
<td></td>
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<tr>
<td>6 h</td>
<td>10.2 ± 0.78</td>
<td>30.2 ± 2.91</td>
</tr>
<tr>
<td>12 h</td>
<td>12.4 ± 0.67</td>
<td>28.2 ± 2.13</td>
</tr>
<tr>
<td>24 h</td>
<td>11.4 ± 0.92</td>
<td>26.3 ± 1.74</td>
</tr>
<tr>
<td>48 h</td>
<td>12 ± 0.86</td>
<td>20.1 ± 1.43</td>
</tr>
<tr>
<td>U87MG cells (n x 10⁶)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 h</td>
<td>12.2 ± 0.69</td>
<td>28.2 ± 2.96</td>
</tr>
<tr>
<td>12 h</td>
<td>13.5 ± 0.89</td>
<td>26.5 ± 2.02</td>
</tr>
<tr>
<td>24 h</td>
<td>10.3 ± 0.43</td>
<td>29.6 ± 1.84</td>
</tr>
<tr>
<td>48 h</td>
<td>11.5 ± 0.92</td>
<td>16.4 ± 0.94</td>
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Cells were washed free of 4-HPR and cell number was monitored for further 3 days. The cells were trypsinized and the number of viable cells was counted using trypan blue dye. Data represent the means ± SE of three independent experiments.

Fig. 2. Electron microscopy images showing ultrastructural features of a control cell (a–c), morphological features of autophagy in U373MG cells exposed to 4-HPR (5 µM) (d–f) and coexistence of morphological features of apoptosis and autophagy in U373MG cells exposed to 4-HPR (10 µM) (g–i) for 48 h. a, d and g = 1 µm; b, e and h = 10 nm; c, f and i = 5 nm. N indicating nucleus, open white arrow for endoplasmic reticulum, filled thick arrow for autophagic vacuole, diamond arrow for mitochondria, asterisk for myelin whorls and black circle showing numerous vacuoles.
few autophagic features (Figure 2b and c). These results suggest that cancer cells showed a varied response to drug treatment by eliciting both autophagy and apoptosis. The extent of each response seems to be dose dependent.

4-HPR-induced AVO formation and LC-3 localization

Further to quantify autophagy, we performed the following assays. First to quantify AVOs that include autophagic vacuoles and lysosomes (14), we used AO staining. Confocal microscopy and flow cytometric analysis using AO was performed after 24 h of 4-HPR treatment of U373MG cells. In control cells, less accumulation of AO was indicated by weak AO fluorescence that increases on treatment with 4-HPR at 5 μM (~4-fold) as well as 10 μM (~3.18-fold) (Figure 3A and B) signify development of AVOs. It is interesting to note that AVO develops even at apoptotic concentration that further suggests the existence of features of autophagy in the apoptotic cells. Moreover, the addition of both early- and late-stage autophagy inhibitors; 3-MA (15) and Baf-A1 (15,16) suppressed 4-HPR-induced AVO generation (Figure 3B).

Secondly, we determined the induction of autophagy by localizing an autophagosome-specific protein LC-3 that is recruited to the autophagosome membrane during autophagy (17). Immunocytochemistry using LC-3-specific antibody was performed in U373MG cells (Figure 3C). Determined by fluorescence microscopy, LC-3-stained cells showed diffuse distribution of green fluorescence in the absence of 4-HPR (Figure 3Ca). In contrast, treatment with 4-HPR at lower concentration (5 μM) increased a punctuate pattern in number and fluorescence intensity, representing autophagic vacuoles (Figure 3Cb), indicating that LC3 is recruited in 4-HPR-induced autophagy. At apoptotic concentration (10 μM) also, 4-HPR induced punctuate pattern of LC3 in majority of the cells (Figure 3Cc). As seen in Figure 4d–f, the appearance of punctuate pattern of LC3 was suppressed by 3-MA at both the concentrations of 4-HPR. On the other hand, Baf-A1 (10 nM) did not suppress localization of LC3 induced by 4-HPR (Figure 3Ch–i) at any of the concentration. The number of cells expressing a punctuate pattern of LC3 was significantly increased (P < 0.05) in tumor cells treated with 4-HPR alone or its combination with Baf-A1 compared with the control or 3-MA alone (Figure 3C) (P < 0.05). These

Fig. 3. (A and B) Development of AVO in 4-HPR-treated U373MG cells. (A) Micrographs showing AVOs formation after 4-HPR treatment for 24 h using AO in U373MG cells; (a) untreated, (b) 5 μM 4-HPR and (c) 10 μM 4-HPR. (B) Detection of red fluorescence (630 nm, FL-3) in AO-stained cells using fluorescence activated cell sorting analysis. U373MG cells were treated with 4-HPR (5 and 10 μM) for 24 h and then the cells were further treated with 3-MA (1.0 mM) or Baf-A1 (10 nM) for another 48 h. Data are represented as fold increase in FL-3 fluorescence and are representative of three independent experiments; bars, ±SE. *P < 0.01, compared with cells treated with 4-HPR alone. (C) Involvement of LC3 in 4-HPR-induced autophagy in malignant glioma cells. U373MG cells were treated with 4-HPR (5 and 10 μM) for 24 h and further treated with 3-MA (1.0 mM) and Baf-A1 (10 nM) for 48 h. (a) Control, (b) 5 μM 4-HPR, (c) 10 μM 4-HPR, (d) 3-MA, (e) 5 μM 4-HPR + 3-MA, (f) 10 μM 4-HPR + 3-MA, (g) Baf-A1, (h) 5 μM 4-HPR + Baf-A1 and (i) 10 μM 4-HPR + Baf-A1. LC-3 appears diffused in control cells, whereas a punctuate pattern can be observed when it translocates to autophagosomal membrane. Representative images of cells obtained after 48 h are presented. (D) Effect of 3-MA or Baf-A1 on 4-HPR-induced cytotoxicity in U373MG cells. At 24 h after exposure to 4-HPR (5 and 10 μM), 3-MA (1.0 mM) or Baf-A1 (10 nM) was added and cultured for additional 48 h. For cell viability assay, the cells were trypsinized and the number of viable cells was counted using trypan blue exclusion assay. The viability of the untreated cells was taken as 100%. Data representative of means ± SE of three independent experiments. *P < 0.01, compared with cells treated with 4-HPR alone.
findings indicate that Baf-A1 inhibits autophagy after autophagosome membrane association of LC3.

**Inhibition of 4-HPR-induced autophagy enhances cell death in malignant glioma cells**

We next tested whether the continued degradation of metabolic substrate within the autophagosome/lysosome system was required to maintain cell viability after 4-HPR treatment (Figure 3D). Thus, we used inhibitors of early and late stage of macroautophagy to determine the effect on 4-HPR-induced cell death. Treatment with 3-MA (1 mM)/Baf-A1 (10 nM) alone had no significant effect on survival of U373MG cells growth. The presence of 3-MA and Baf-A1 enhanced the cell death induced by 4-HPR treatment both at autophagic and apoptotic concentration, as determined by trypan blue staining.

These results indicate that cell death is enhanced in 4-HPR-treated U373MG cells when autophagy is inhibited.

**Involvement of change in ΔΨₘ and hydroperoxide generation in 4-HPR-induced autophagy**

In response to the appropriate stimulation, depolarized/injured mitochondria are known to move into autophagic vacuoles (18). We have previously shown that ΔΨₘ changes and hydroperoxide plays an important role in 4-HPR-induced apoptosis in glioma cells (13). Thus, to study the involvement of change in ΔΨₘ and hydroperoxide in 4-HPR-induced autophagy, the change in ΔΨₘ was quantified using JC-1 and hydroperoxide was quantified using DCF-DA by flow cytometry. A time-dependent decrease in ΔΨₘ was seen at both lower (5 μM) and higher concentrations (10 μM) of the drug. At lower concentration, the loss of ΔΨₘ was slower as compared with that observed at higher concentration (Figure 4A).

Further a dose-dependent increase in hydroperoxide generation was observed after 4-HPR (1–15 μM) treatment with a significant increase at a concentration >7.5 μM (Figure 4B).

To check whether inhibition of autophagy resulting in increased cell death is mediated through mitochondrial permeability alterations, experiments were performed. Effect of 3-MA or Baf-A1 was determined on ΔΨₘ and ROS generation. At 12 h post treatment 4-HPR at 5 μM, 3-MA or Baf-A1 alone did not affect the ΔΨₘ significantly; however, combined treatment of 4-HPR (5 μM) with 3-MA or Baf-A1 decreased ΔΨₘ significantly (P ≤ 0.05) and comparably with 10 μM 4-HPR treatment at 12 h. Similarly, 3-MA or Baf-A1 alone did not enhance hydroperoxide levels. At lower concentration, 4-HPR caused no significant increase in hydroperoxide generation that was significantly (P ≤ 0.05) enhanced upon 4-HPR (5 μM) plus 3-MA or Baf-A1 combined treatment (Table II). These results indicate that combination of 4-HPR with 3-MA or Baf-A1 induces mitochondrial changes conducive for cell death via enhancing the loss of ΔΨₘ and hydroperoxide generation that may be a critical step of 4-HPR-induced cell death.

**Involvement of MAPKs and PI3K in 4-HPR-induced cell death**

The involvement of MAPK pathway in regulating cell death/survival in cancer cells is well documented (19). To investigate the role of MAPKs in 4-HPR-induced cytotoxicity, the activation of c-jun, P38MAPK and ERK1/2 was studied using the phosphorylated antibodies that determine the active form of these enzymes. As shown in the overlay images (Figure 5A), 24 h after 4-HPR treatment (5 and 10μM), both phosphorylated c-jun (Figure 5Aa–c) and phosphorylated P38MAPK (Figure 5Ad–f) protein expressions were augmented significantly as compared with the control cells, which were mainly restricted to nuclei (appear in cyan color due to overlay of green with blue). It is interesting to note that the phosphorylated ERK1/2 expression selectively augmented at autophagic but not apoptotic concentration of 4-HPR. Further, to confirm the involvement of MAPKs, different inhibitors were used and the extent of cell death was studied by trypan blue staining. The cells were preincubated with or without 1 μM PD98059, 1 μM SP600125 or 10 μM SB203580 for 1 h, followed by 4-HPR treatment for 72 h. Both SP600125 and SB203580 downregulated cell death at 5 and 10 μM, while PD98059 enhanced 4-HPR-induced cell death at 5 μM, whereas no significant change was

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**Table II. Characterization of 4-HPR-induced cell death**

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<thead>
<tr>
<th></th>
<th>Control</th>
<th>4-HPR (5 μM)</th>
<th>4-HPR (10 μM)</th>
<th>Baf-A1</th>
<th>4-HPR (5 μM) + Baf-A1</th>
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<tr>
<td>Percentage of cells with JC-1 aggregates (12 h)</td>
<td>97.4 ± 7.6</td>
<td>92.4 ± 8.9</td>
<td>63.5 ± 5.6</td>
<td>95.4 ± 9.1</td>
<td>61.2 ± 7.2</td>
</tr>
<tr>
<td>Fold increase in DCF intensity (6 h)</td>
<td>1</td>
<td>1.32 ± 0.15</td>
<td>2.7 ± 0.19</td>
<td>1.05 ± 0.08</td>
<td>2.9 ± 0.18</td>
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Disruption of mitochondrial membrane potential and hydroperoxide generation in U373MG cells by 4-HPR/3-MA/Baf-A1. Mitochondrial membrane potential was measured by JC-1 and hydroperoxide production was measured by DCF-DA, fluorescence was measured using fluorescence activated cell sorting. Data represent means ± SE of three independent experiments.

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**Fig. 4. 4-HPR-induced ΔΨₘ and hydroperoxide generation in U373MG cells.**
(A) U373MG cells were treated with or without 4-HPR (5 and 10 μM) on a time-dependent manner and stained with JC-1 to quantify the changes in ΔΨₘ by fluorescence activated cell sorting analysis. Percentage of JC-1 aggregates indicate cells with high ΔΨₘ. (B) Concentration-dependent 4-HPR-induced hydroperoxide production after 6 h was measured by DCF-DA and fluorescence was measured using fluorescence activated cell sorting. Data represent the means ± SE of three independent experiments. *P < 0.01, control versus treated cells.
after observed at 10 μM in 4-HPR-treated cells, suggesting that JNK and P38 MAPK contributed to 4-HPR-induced cell death at both the concentrations of 4-HPR, but ERK1/2 antagonized cell death at a lower concentration (Figure 5B).

Further, to confirm the involvement of PI3K pathway in 4-HPR-induced cell death besides 3-MA, we also used LY294002, inhibitor of PI3K, and performed cell viability assay. Similar to 3-MA, 10 μM LY294002 also significantly enhanced the extent of 4-HPR-induced cell death at both the concentrations of 4-HPR (data not shown).

PI3K/Akt signaling also involves the activation of NF-κB pathway, a survival pathway that enables cancer cell types to resist cytotoxic insults (20,21). Composed of p65 (RelA) and p50 proteins, NF-κB is normally present in the cytoplasm in an inactive state in a complex with members of the IκB inhibitor protein family, when activated (when Inhibitory KappaB is phosphorylated and degraded) it translocates to nuclei and acts as a transcription factor. To study whether NF-κB is activated during 4-HPR-induced cell death, immunocytochemistry of NF-κB (p65/RelA antibody) using tetramethyl rhodamine isothiocyanate-labeled secondary antibody was performed in U373MG cells after 48 h of treatment. Nuclei were counterstained with Hoechst-33258 (Figure 5C).

Fig. 5. Involvement of MAPKs and PI3K in 4-HPR-induced cell death in U373MG cells. (A) Overlay of immunocytochemistry of phosphorylated antibodies of c-jun, p38MAPK and ERK1/2 detected with fluorescein isothiocyanate-labeled secondary antibody (green) with Hoechst-33258 (blue) in 4-HPR-treated U373MG glioma cells. The translocation to nuclei appears as cyan color (indicated as red arrow). (B) Influences of MAPKs inhibitors on 4-HPR-induced cell death after 72 h of treatment determined by trypan blue staining. The viability of the untreated cells was taken as 100%. Data representative of means ± SE of three independent experiments. *P < 0.01, compared with cells treated with 4-HPR alone. (C) Overlay of immunofluorescence staining of NF-κB and nuclei. Red, immunostaining of NF-κB with anti-NF-κB (p65 antibodies) detected with TRITC-labeled secondary antibody; blue, Hoechst-33258 stained nuclei and traslocated NF-κB appears as magenta. (D) Western blotting for beclin-1 and Bcl-2 of the whole-cell lysate prepared from 5 μM of 4-HPR-treated U373MG cells after 24 and 48 h of treatment.
Induction of autophagy by 4-HPR in glioma cells

translocated to the nuclei (appears a magenta color) in majority of the cells (~80%) at autophagic concentration, which was inhabitable with 3-MA, a PI3K inhibitor (Figure 5C). Interestingly, at apoptotic concentration there was no significant translocation of NF-κB to the nuclei, suggesting a dose-specific activation of NF-κB (Figure 5C).

The Beclin-1 is part of a class III PI3K complex that is involved in autophagy and shown to negatively interact with Bcl-2 (22,23). Of particular interest was the role of Bcl-2 family proteins, specifically beclin-1 and Bcl-2, and the onset of autophagic cell death. Thus, we performed western blot analysis to detect any change in the protein expression of beclin-1 and Bcl-2 at a concentration of 5 μM of 4-HPR treatment in U373MG cells (Figure 5Da and b). Results demonstrate a significant increase in beclin-1 after 24 and 48 h, whereas a significant decrease in Bcl-2 levels was observed after 48 h of 4-HPR treatment.

Taken together, these results suggest that 4-HPR induces both autophagy and apoptosis in a dose-related manner in U373MG cells.

Discussion
In spite of efforts to develop and optimize the new therapies, the success rate in preventing glioblastoma recurrence has seen little success. Malignant glioma cells are resistant to apoptosis and associated proapoptotic effects of drugs. Emerging role of autophagy has been demonstrated in response to the various chemotherapeutic agents and radiation in glioma; however, its role in cell death or survival is debatable (24).

4-HPR predominately induces anticancer effect by inducing apoptosis (12). We have previously shown that 4-HPR induces apoptosis at a higher concentration (10–15 μM) in glioma cells through mitochondrial-mediated pathway and endoplasmic reticulum stress (13). However, the phase II clinical trial conducted at lower concentration of the drug (600 mg/m2 b.i.d.) was associated with lack of activity, whereas when used at a higher doses (900 mg/m2 bid) patients showed a durable radiologic response and remained progression free, with no substantial toxicity after 13 cycles of therapy (25). This finding has raised question regarding the glioma cells acquiring resistance to its proapoptotic effect.

The present study was performed on malignant glioma cell line U373MG, which was selected as it carries mutation in PTEN and p53 genes. Malignant gliomas frequently carry mutations in the PTEN and p53 tumor suppressor genes are associated with higher tumor grade, lower levels of apoptosis and an adverse clinical outcome in the case of human gliomas (26).

Here, we provide evidences that the stress induced by 4-HPR triggers two different pathways of programed cell death: autophagy and apoptosis in glioma cells, autophagy being dominant at lower dose and apoptosis at a higher dose. Our finding supports the previous studies, where a concentration-dependent induction of autophagy or apoptosis has been shown in glioma cells and leukemia cells by arsentic trioxide (6,27,28).

Additionally, both apoptotic and autophagic mode of cell death has been reported with the use of temozolomide on glioma cells (4,29). In malignant glioma cells, up to 20 Gy irradiation induced only autophagy, not apoptosis (30), and on the other hand, 10 Gy irradiation induced massive autophagy in retinoblastoma cells, which are radiation sensitive (31). It looks that choice between the cell death program depends on the cell types and stimuli.

In the present study, malignant glioma cells treated with autophagic concentration of 4-HPR began to proliferate after removal of the drug, whereas at apoptosis-inducing concentration cell death program induced was irreversible. Further, using pharmacological inhibitors of autophagy 3-MA and Baf-A1, we found that inhibition of 4-HPR-induced autophagy enhanced cell death in glioma cells. In the present system, it seems that autophagy may rather antagonize or delay apoptosis, and inhibition of autophagy may increase the sensitivity of the cells to cell death signals. Similarly, enhancement of cell death has been shown in the case of sulindac sulfide drug-induced apoptosis where autophagy inhibitors increased their sensitivities to apoptosis (33). Literature further suggests that inhibition of radiation/arsenic trioxide/temozolamide-induced autophagy decreases the survival of glioma cells (2,4,6,30), which corresponds to our result. The present study supports the growing amount of evidences suggesting that once cancer cells are exposed to radiation or chemotherapeutic agents, besides apoptosis that leads cells to death, autophagy that may lead to death or survival will be induced. The cancer cells trigger reversible autophagic process that sequesters and degrades unnecessary molecules for their survival and contribute to resistance (3,34). It appears that 4-HPR-induced autophagy provides a mechanism of self-defense and a possible contributing factor for drug resistance.

Further, we found that autophagy inhibitors prevented 4-HPR-induced AVO formation, suggesting that the AVO formation may share similar pathways that regulate autophagy in glioma cells. It has been suggested that moderate AVO formation may have a role in providing survival advantage (2) and inhibition of AVO formation may serve as a tool to enhance cell death. Present study also demonstrates that the autophagy inhibitors not only inhibited the AVO formation but also enhanced cell death, further strengthening the role of AVO in promoting survival.

In the present study, we also found that 4-HPR-induced autophagy is associated with slow loss of ΔΨm, whereas apoptosis is associated with rapid loss of ΔΨm. Further, when autophagy is inhibited, enhanced cell death is coupled with increase in mitochondrial depolarization and ROS generation. To explain this, in mitochondria, certain types of stress can lead to mitochondrial permeability transition that promotes oxidative stress, thereby increasing the mitochondrial damage and resulting in the release of cell death-inducing molecules (35). It has been suggested that such damaged mitochondria are degraded through macroautophagy (18). Thus, autophagy is activated as a novel strategy for reducing mitochondrial damage and ROS and provides a protection from damage to cell (36). If the strength of the stimulus is low, the depolarized mitochondria are sufficiently removed by the cells and the release of apoptotic molecules is prevented. However, if the strength of the stimulus is high, the cell may not remove sufficient number of mitochondria and a certain level of apoptotic molecules may be released by depolarized mitochondria, activating programmed cell death (36). Thus, it appears that inhibiting autophagy prevents the removal of damaged mitochondria promoting loss of ΔΨm and subsequent ROS generation, thereby accelerating cell death.

Our results also indicate that even at a higher concentration, at which apoptosis is induced (10 μM), the features of autophagy exist in apoptotic cells. The cells manifest typical morphological features of apoptosis (cell shrinkage, margination and condensation of chromatin) and autophagy (autophagosomes and autophagic vacuoles) in the same cell. Besides, morphological features, AVO formation, and a possible contributing factor for drug resistance. The available data as reviewed indicate that the dying cell may present features of both autophagy and apoptosis or both the programs may run in parallel (37). The coexistence of autophagy and apoptosis has also been shown in MCF-7 cells on treatment with camptothecin (38), suggesting a considerable overlap or interdependence of both programs of cell demise.
We also found that 4-HPR leads to activation of NF-

Acknowledgements

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