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N-(4-Hydroxyphenyl) retinamide potentiated paclitaxel for cell cycle arrest and apoptosis in glioblastoma C6 and RG2 cells

Rajiv Janardhanan¹, Jonathan T. Butler², Naren L. Banik², and Swapan K. Ray^{1,*}

¹Department of Pathology, Microbiology and Immunology, University of South Carolina School of Medicine, 6439 Garners Ferry Road, Columbia, SC 29209, USA

²Department of Neurosciences, Medical University of South Carolina, 96 Jonathan Lucas Street, P.O. Box 250606, Charleston, SC 29425, USA

Abstract

Glioblastoma grows aggressively due to its ability to maintain abnormally high potentials for cell proliferation. The present study examines the synergistic actions of N-(4-hydroxyphenyl) retinamide (4-HPR) and paclitaxel (PTX) to control the growth of rat glioblastoma C6 and RG2 cell lines. 4-HPR induced astrocytic differentiation was accompanied by increased expression of the tight junction protein e-cadherin and sustained down regulation of Id2 (member of inhibitor of differentiation family), catalytic subunit of rat telomerase reverse transcriptase (rTERT), and proliferating cell nuclear antigen (PCNA). Flow cytometric analysis showed that the microtubule stabilizer PTX caused cell cycle deregulation due to G2/M arrest. This in turn could alter the fate of kinetochore-spindletube dynamics thereby halting cell cycle progression. An interesting observation was induction of G1/S arrest by combination of 4-HPR and PTX, altering the G2/M arrest induced by PTX alone. This was further ratified by the upregulation of tumor suppressor protein retinoblastoma, which repressed the expression of the key signaling moieties to induce G1/S arrest. Collectively, combination of 4-HPR and PTX diminished the survival factors (e.g., rTERT, PCNA, and Bcl-2) to make glioblastoma cells highly prone to apoptosis with activation of cysteine proteases (e.g., calpain, cathepsins, caspase-8, caspase-3) in two glioblastoma cell lines. Hence, combination 4-HPR and PTX can be considered as an effective therapeutic strategy for controlling the growth of heterogeneous glioblastoma cell populations.

Keywords

Apoptosis; Astrocytic differentiation; Cell cycle arrest; Combination therapy; Glioblastoma; N-(4-Hydroxyphenyl) retinamide; Paclitaxel

1. Introduction

After cessation of the development process, only the glial cells retain their inherent ability to proliferate. Hence, it is not surprising that most of the adult neurological tumors are of glial origin (Zhu and Parada, 2002). Glioblastoma is characterized by uncontrolled cell proliferation, diffuse infiltration, lack of differentiation, robust angiogenesis, and intense resistance to

*Corresponding author. Tel.: +1-803-733-1593; Fax: +1-803-733-3192.; Email: E-mail: Swapan.Ray@uscmed.sc.edu (S. K. Ray).

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apoptosis along with increased genomic instability (Furnari et al., 2001). These features along with increased heterogeneity at cytopathological, transcriptional, and genomic levels make it one of the most dreaded cancers with poor prognosis and high rates of morbidity and mortality (Kleihues et al., 2002). The current therapeutic measures for the treatment of glioblastoma have been ineffective necessitating the need to develop innovative therapeutic strategies for successful management of glioblastoma (Parney and Chang, 2003). The inherent ability of glioblastoma cells to avoid terminal differentiation and apoptotic death necessitated the need to develop novel strategies involving combinatorial approach for induction of both differentiation and apoptosis. One approach to control glioblastoma growth might be the synergistic use of a retinoid to induce differentiation and prime the glioblastoma cells to a chemotherapeutic agent for increasing apoptosis.

Retinoid treatment induces morphological changes and prolongs doubling time and this suppression of tumor cell growth by retinoids may not always induce apoptosis and instead may induce an arrest in the G1 phase of the cell cycle (Wu et al., 1997). N-(4-Hydroxyphenyl) retinamide (4-HPR), a synthetic retinoid derived from all-*trans* retinoic acid, effectively suppresses the growth of several tumor cells (Sabichi et al., 1998) by regulating cell proliferation rates. 4-HPR also controls the cell cycle regulating genes like cyclin dependant kinase (Cdk) and its substrate retinoblastoma (Rb) protein. Dephosphorylation of Rb results in reduced expression of the transcriptional factor E2F1, which is known to control the expression of S phase genes in cell cycle (Panigone et al., 2000). Thus, 4-HPR treatment would not only induce astrocytic differentiation in glioblastoma cells but also prime the cells to paclitaxel (PTX), a powerful chemotherapeutic agent, for increasing apoptosis.

PTX belongs to the taxane family of anti-cancer agents and exerts its anti-tumor activity through a unique mechanism by stoichiometrically binding to the microtubule and hyper-stabilizing its structure (Xiao et al., 2006). The resulting complex of microtubule and PTX prevents the progression of mitotic cell cycle from metaphase to anaphase stage leading to non-homologous separation of the chromosomes (Kumar, 1981) due to G2/M arrest and thereby inducing cell cycle exit (Chen et al., 2003). PTX induces apoptosis due to reduced function of the anti-apoptotic Bcl-2 protein (Jordan and Wilson, 2004). The unique ability of PTX to initiate the intrinsic and extrinsic apoptotic cascades along with down regulation of the Bcl-2 protein makes it an excellent candidate for using in combination with 4-HPR for treatment of glioblastoma.

Most of the conventional therapeutic regimens for treating brain tumors involve cytostatic and cytotoxic agents, which usually target interactions among Bcl-2 and inhibitor-of-apoptosis (IAP) families. Our previous report indicated that activation of proteases such as calpain and caspase-3 could also contribute to apoptotic death in glioblastoma (Ray et al., 2002). The ability of combination of 4-HPR and PTX to modulate Bcl-2 and IAP dynamics can activate multiple proteases such as calpain and caspases leading to apoptosis in glioblastoma cells.

In this study, we demonstrated that therapeutic regimen involving 4-HPR and PTX at pharmacologically achievable low concentrations not only reduced cell proliferation but also induced differentiation and increased apoptosis in two glioblastoma cell lines.

2. Results

2.1. Morphological and biochemical features of astrocytic differentiation in glioblastoma cells

Treatment with 500 nM 4-HPR for 72 h induced astrocytic differentiation in rat glioblastoma C6 and RG2 cells (Fig. 1). The morphological features of differentiation were assessed following *in situ* methylene blue staining (Fig. 1A). Induction of astrocytic differentiation was

characterized in cells with small and retracted cell bodies having thin elongated and branched cytoplasmic processes, while the untreated (control) glioblastoma cells maintained wide cell body with short cytoplasmic processes. Induction of astrocytic differentiation in glioblastoma cells was evidenced by not only alterations in morphology but also by increased expression of the tight junction protein e-cadherin, suggesting that astrocytic differentiation was associated with a transition to epithelial phenotype (Fig. 1B). Other biochemical features of astrocytic differentiation were increase in expression of glial fibrillary acidic protein (GFAP) with concomitant decreases in the levels of Id2 (member of inhibitor of differentiation family), rat catalytic subunit of telomerase (rTERT), and proliferation cell nuclear antigen (PCNA). Treatment of cells with 100 nM PTX alone for 24 h did not increase in expression of GFAP. But all biochemical features of differentiation were highly prominent in cells after treatment with combination of 4-HPR (total 72 h) and PTX (last 24 h). Notably, 4-HPR alone and also in combination with PTX increased expression of e-cadherin (marker for epithelial phenotype) and GFAP (marker for astrocytic differentiation). An increase in expression of e-cadherin was found to be correlated with a decrease in expression of Id2. Combination of 4-HPR and PTX greatly decreased expression of rTERT and PCNA leading to inhibition of cell proliferation and promotion of cell death.

2.2. Combination of 4-HPR and PTX reduced the viability of glioblastoma cells

We determined the changes in viability of glioblastoma C6 and RG2 cells after treatment with 4-HPR (500 nM for 72 h), PTX (50 or 100 nM for 24 h), and 4-HPR (total 72 h) + PTX (last 24 h) using the MTT assay (Fig. 2). To state more clearly, we treated the cells first with 500 nM 4-HPR for 48 h and then added 50 or 100 nM PTX to the culture to continue the co-treatment for next 24 h. The MTT assay revealed that although 500 nM 4-HPR alone did not induce a significant decrease in cell viability, treatment with 100 nM PTX alone and combination of 4-HPR and PTX significantly reduced the viability in both C6 and RG2 cells (Fig. 2A). We also used the MTT assay to assess whether treatment with combination of 4HPR and PTX affected the viability of normal astrocytes (Fig. 2B). We found that viability of normal astrocytes was not significantly reduced after treatment with combination of 4-HPR and PTX. Thus, combination therapy mostly affected the viability and growth of glioblastoma cells. In all subsequent experiments, we used 500 nM 4-HPR + 100 nM PTX as an effective combination for inducing apoptosis in glioblastoma cells and examining the alterations in molecular markers that led to this process.

2.3. Combination of 4-HPR and PTX inhibited markers of cell cycle progression

Combination of 4-HPR and PTX caused cell cycle arrest at G1/S phase with alterations in the levels of key signaling molecules governing the G1/S phase mitotic check point (Fig. 3). Flow cytometric analysis of propidium iodide (PI) stained cells following the treatments revealed substantial heterogeneity in the distribution of cells in different phases of cell cycle (Fig. 3A). Treatment of C6 and RG2 cells with 4-HPR alone did not dramatically alter populations in the G0/G1 phase. But treatment of cells with PTX alone substantially decreased populations in G0/G1 phase and highly increased populations in G2/M phase. Interestingly, combination of 4-HPR and PTX induced massive accumulation of cells in the G0/G1 phase (Fig. 3A). These results suggested that combination of 4-HPR and PTX worked synergistically to impose cell cycle arrest at G1/S phase leading to cell cycle exit and promotion of apoptosis.

Strong implementation of cell cycle arrest at G1/S phase by combination of 4-HPR and PTX was correlated with changes in levels of key signaling molecules governing the G1/S mitotic check point (Fig. 3B). Mitogenic signaling to G1 phase cyclins can induce Cdks for phosphorylation of Rb (pRb) for decreasing the function of this tumor suppressor protein. We found that combination 4-HPR and PTX induced a dramatic decrease in the level of Cdk2, an increase in the level of Rb, and concomitant hypo-phosphorylation (Ser780) of Rb in both C6

and RG2 cells (Fig. 3B). The decrease in the level of pRb caused a decrease in the level of the transcriptional factor E2F1 (Fig. 3B). An increased activity of E2F1 is required to promote the synthesis of the S phase genes and their products (Cam and Dynlacht, 2003). Treatment of cells with combination of 4-HPR and PTX triggered the events to decrease the S phase products and thereby induce a systemic inhibition of cell proliferation, contributing to cell cycle arrest at G1/S phase.

2.4. Inhibition of cell proliferation induced mitotic abnormalities

Appearance of mitotic abnormalities is a key event to contribute to cell cycle arrest. Combination of 4-HPR and PTX caused a systemic down regulation of E2F1 followed by sustained reduction in the level of mitotic arrest deficient 2 (MAD2) in both C6 and RG2 cells (Fig. 3B). Also, decreased level of Cdc20 could highly contribute to destruction of the anaphase promoting complex (APC) thereby halting the progression of mitotic cycle. Systemic down regulation of the keystone mitotic molecules (E2F1, MAD2, and Cdc20) in response to treatment with combination of 4-HPR and PTX not only contributed to cell cycle arrest but also initiated the pathways for apoptosis.

2.5. Induction of morphological and biochemical features of apoptotic cell death

The onset of the cell cycle arrest could be accompanied by appearance of the morphological and biochemical features of apoptosis, as we examined by *in situ* Wright staining and flow cytometric analysis (Fig. 4). *In situ* Wright staining enabled us to distinguish the morphological changes between normal cells and apoptotic cells (Fig. 4A). Some of the characteristic morphological features of apoptotic cells were shrinkage of cell volume, chromatin condensation, and membrane-bound apoptotic bodies that appeared prominently following treatment of cells with combination of 4-HPR and PTX (Fig. 4A). *In situ* Wright staining was used for determination of amounts of apoptosis (Fig. 4B). Treatment of cells with 4-HPR alone showed a marginal increase in the percentage of apoptotic cells. In contrast, treatment of cells with combination of 4-HPR and PTX showed a significant increase in the percentage of apoptosis in both C6 and RG2 cells (Fig. 4B). These results were further substantiated by the flow cytometric analysis of Annexin V positive cells. An increase in population of Annexin V positive cells in A4 area indicated occurrence of apoptosis, as shown in C6 cells following treatments (Fig. 4C). Based on flow cytometric analysis of Annexin V positive cells, we determined the percentages of apoptosis in C6 and RG2 cells after the treatments (Fig. 4D). Compared with control C6 or RG2 cells, treatment with 4-HPR alone did not increase the number of Annexin V positive cells but treatment with PTX alone significantly increased the population of Annexin V positive cells (Fig. 4D). Treatment with combination of 4-HPR and PTX very significantly increased the percentages of the Annexin V positive cells in both C6 and RG2 cells (Fig. 4D). The increase in Annexin V positive cells after the treatment was a prominent biochemical feature of apoptosis in glioblastoma cells.

2.6. Activation of calpain and cathepsins to promote apoptotic death

We performed Western blotting to examine the activation of calpain and cathepsins for mediation of apoptotic death in C6 and RG2 cells after the treatments (Fig. 5). Uniform expression of β -actin indicated equal amount of protein loading in each lane. Treatment with combination of 4-HPR and PTX caused the highest increase in calpain expression and activation in both C6 and RG2 cells. Activation of calpain was accompanied by concomitant decrease in level of calpastatin, the endogenous inhibitor of calpain. Also, activation of calpain induced the break down of p35 to its truncated form p25, which was required for increasing the activation of Cdk5. Activation of Cdk5 has been shown to be involved in the induction of differentiation (He et al., 2008) as well as of apoptosis through stable activation of p53 (Lee et al., 2007) in neuronal cells. Our results revealed that combination of 4-HPR and PTX

increased expression and activation of calpain, which then mediated breakdown of p35 to induce activation of Cdk5. Besides, lysosomal proteases such as cathepsin B, cathepsin C, and cathepsin L were also substantially activated to contribute to apoptotic process in both C6 and RG2 cells after treatment with the combination of 4-HPR and PTX (Fig. 5).

2.7. Activation of extrinsic and intrinsic apoptotic pathways

We examined activation of some of the components of the extrinsic and intrinsic apoptotic pathways in C6 and RG2 cells after the treatments (Fig. 6). As an indication of activation of extrinsic apoptotic pathway, our results showed an increase in activation of caspase-8 in both C6 and RG2 cells after the treatments with PTX alone and also combination of 4-HPR and PTX. Active caspase-8 caused the proteolytic cleavage of its substrate 22 kD Bid to generate 15 kD truncated Bid (tBid) (Fig. 6), which could translocate to mitochondria to induce intrinsic apoptotic pathway by releasing cytochrome *c* from mitochondrial inter membrane space (Luo et al., 1998).

Importantly, treatment with combination of 4-HPR and PTX induced the expression of Bax (pro-apoptotic protein) and reduced the expression of Bcl-2 (anti-apoptotic protein) in both C6 and RG2 cells (Fig. 6). The monoclonal antibody that we used in this investigation could recognize both 21 kD Bax and 24 kD Bax. Total expression of Bax was greatly increased whereas expression of Bcl-2 was almost diminished after treatment with combination of 4-HPR and PTX. The stoichiometric increase in the pro-apoptotic Bax can induce the intrinsic apoptotic pathway leading to activation of caspase-3. Our Western blotting showed activation of caspase-3 to a great extent after treatment of cells with combination of 4-HPR and PTX (Fig. 6). Upon activation, caspase-3 cleaved the 45 kD inhibitor of caspase-activated DNase (ICAD) to 35 kD ICAD so as to release and translocate caspase-activated DNase (CAD) to the nucleus for apoptotic DNA fragmentation.

2.8. Induction of astrocytic differentiation and apoptotic death

Based upon the results obtained from our present study, we outlined a schematic diagram indicating the signaling moieties that were activated or altered to induce astrocytic differentiation and apoptotic death in glioblastoma cells after treatment with combination of 4-HPR and PTX (Fig. 7). 4-HPR induced astrocytic differentiation by increasing the levels of GFAP and e-cadherin and by reducing the level of Id2. Astrocytic differentiation, in turn, repressed the cell proliferation markers such as PCNA and rTERT and increased expression of the tumor suppressor Rb. Decrease in phosphorylation of Rb reduced the expression of E2F1 to down regulate the kinetochore sensing protein MAD2, which in turn signaled to inhibit APC activity thereby contributing to cell cycle arrest at G1/S phase. The prolongation of cell cycle arrest at G1/S phase resulted in potentiation of PTX action for activation of caspase-8 in extrinsic apoptotic pathway and also increased expression of Bax to trigger intrinsic apoptotic pathway, ultimately leading to activation of the executioner caspase-3.

3. Discussion

The results obtained from this investigation suggest that combination of the atypical retinoid 4-HPR and the microtubule stabilizer PTX induced astrocytic differentiation as well as apoptosis in two different glioblastoma cells. Astrocytic differentiation not only decreased the rates of cell proliferation but also simultaneously increased the expression of GFAP, e-cadherin, and Rb. Increased expression of Rb resulted in the repression of transcriptional factor E2F1. The repression of E2F1 not only reduced cellular proliferation but also altered the fate of kinetochore-spindle tube dynamics. The reduced activation of Cdc20 ensured that mitotic cycle arrested at metaphase stage. Combination of 4-HPR and PTX acted synergistically to cause cell cycle arrest at G1/S phase leading to appearance of morphological and biochemical

features of apoptosis. Prolongation of cell cycle arrest led to the activation of both extrinsic and intrinsic apoptotic cascades in glioblastoma C6 and RG2 cells.

In various studies, 4-HPR has been used to induce cellular differentiation in carcinomas (Naik et al., 1995) due to its favorable toxicity profile and anti-proliferative activities (Veronesi et al., 2006). We used 4-HPR at low concentration (500 nM) to induce astrocytic differentiation that was accompanied by upregulation of the tight junction protein e-cadherin (Fig. 1). An increase in the level of e-cadherin following treatment with 4-HPR alone or combination of 4-HPR and PTX suggested induction of not only astrocytic differentiation but also glioblastoma cell transformation to epithelial phenotype. Although we observed that PTX alone did not alter the e-cadherin level in glioblastoma cells, previous reports indicated that PTX resistant cells were more prone to epithelial to mesenchymal transition (Wang et al., 2004). Astrocytic differentiation was also accompanied by reduced expression of Id2. This observation is significant since Id proteins repress the expression of differentiation genes by interfering with DNA binding abilities of E proteins (Rothschild et al., 2006). The sustained suppression of Id2 decreased the proliferative potential of glioblastoma (Vandeputte et al., 2002) and we also found that induction of astrocytic differentiation was correlated with the reduced levels of cell proliferation markers such as PCNA and rTERT. To our knowledge, this is the first report correlating a key role of Id2 down regulation in 4-HPR mediated astrocytic differentiation and epithelial phenotype transformation. Treatment with PTX (100 nM) very significantly reduced the viability of differentiated glioblastoma C6 and RG2 cells (Fig. 2).

Flow cytometric analysis revealed that 4-HPR and PTX alone and in combination induced differential distribution of cells in different phases of cell cycle (Fig. 3A). Majority of the 4-HPR treated cells were localized in the G0/G1 phase. In contrast, majority of the PTX treated cells were localized in the G2/M phase. Yet, the treatment with the combination of 4-HPR and PTX caused maximal localization of cells in the G0/G1 phase (Fig. 3A). This observation is indicative of cell cycle arrest at G1/S phase overriding the cell cycle arrest at G2/M phase, which is known to be caused by PTX alone (McDaid and Horwitz, 2001). In this study, we observed for the first time that combination of 4-HPR and PTX mediated cell cycle arrest at G1/S phase in glioblastoma cells. But a previous report showed that retinoid caused reversal of PTX mediated cell cycle arrest at G2/M phase in prostate cancer cells (Verheul et al., 2007). Although 4-HPR and PTX synergistically induced G1/S arrest in both glioblastoma C6 and RG2 cells, yet there was a substantial difference in the distribution of cells (50.80% of C6 and 72.15% of RG2) at G0/G1 phase in response to treatment with combination of 4-HPR and PTX (Fig. 3A), suggesting the existence of differential levels of cell cycle regulating molecules in these glioblastoma cell lines. Astrocytic differentiation by 4-HPR repressed cell proliferation leading to increased expression and stability of the tumor suppressor protein Rb (Fig. 3B). Overexpression of Rb has been reported to cause astrocytic differentiation leading to decreased cell proliferation (Galderisi et al., 2001) through inhibition of E2F family of genes (Ross et al., 2001), whose increased activity is required for expression of the S phase genes (Verschuren and Jackson, 2007). Combination of 4-HPR and PTX caused cell cycle arrest due to upregulation of Rb, less production of pRb, and repression of Cdk2 in both C6 and RG2 cells (Fig. 3).

E2F1 is known to cause repression of inner centromere protein survivin to contribute to improper sister-chromatid segregation and chromosomal instability (Wang et al., 2005). The systemic decrease in expression of kinetochore associated protein MAD2 may induce diffuse signals to anaphase promoting complex (APC), resulting in loss of its activity (Cleveland et al., 2003), thereby contributing to cell cycle arrest at G1/S phase. The APC along with Cdc20 induces proteasomal degradation of spindle check point, which is hindered due to destruction of p27Kip (Binne et al., 2007). Our results showed that combination of 4-HPR and PTX caused down regulation of APC/Cdc20 (Fig. 3B). Decreased stability of Rb could be due to increased

activity of the ubiquitin ligase MDM2 (Miwa et al., 2006). In our study, combination of 4-HPR and PTX enhanced the expression of Rb, which in turn decreased the levels of expression of key signaling proteins (E2F1, MAD2, and Cdc20) to different extents to exert different levels of cell cycle arrest at G1/S phase in C6 and RG2 cells (Fig. 3). This was further evidenced from the fact that combination of 4-HPR and PTX induced differential levels of apoptosis (Annexin V positive) in C6 and RG2 cells (Fig. 4D).

Calpain activity has been extensively correlated with induction of apoptotic death in glioblastoma (Karmakar et al., 2007). Although calpain mediated induction of Cdk5 has been predominantly correlated to apoptosis, recent reports suggest that induction of Cdk5 may also have a role in differentiation (Lee et al., 2000; He et al., 2008). Besides, retinoids have been shown to increase Cdk5 expression (Lee and Kim, 2004). In fact, differentiation is considered to be a curtailed form of apoptosis (Fernando and Megeney, 2007) and a forerunner of apoptosis that occurs with activation of caspase-3, which has been shown to play a role in differentiation as well (Weber and Menko, 2005).

Activation of calpain and cathepsins in glioblastoma cells following treatment with combination of 4-HPR and PTX (Fig. 5) contributed to apoptosis. Treatment with combination of 4-HPR and PTX decreased expression of calpastatin, the endogenous inhibitor of calpain, to maintain a sustained calpain activity for apoptosis in glioblastoma cells (Fig. 5). Recent studies have shown that retinoids activate lysosomal protease cathepsin B (Zang et al., 2001), which may either act as an effector protease (Ospina-Gomez et al., 2006) or promote apoptosis by triggering mitochondrial dysfunction and release of pro-apoptotic proteins (Boya et al., 2003) and activation of caspases (Broker et al., 2005). This process may occur with involvement of Bid (Cirman et al., 2004), which is cleaved to tBid for its translocation to the mitochondria after lysosomal disruption (Ishizaki et al., 1998). This in turn activates intrinsic apoptotic pathway with migration of Bax from cytosol to mitochondria where it cooperates with tBid to release cytochrome c (Murphy et al., 1999). Cytosolic Apaf1 and cytochrome c participate in activation of caspase-9 that subsequently causes activation of caspase-3 (Saleh et al., 1999). Following combination therapy, increase in activation of caspase-3 was associated with cleavage of ICAD (Fig. 6) so as to release CAD, which could be translocated to the nucleus to cause degradation of genomic DNA.

In conclusion, 4-HPR induced astrocytic differentiation and potentiated PTX for activation of multiple pathways that acted in concert for apoptosis in glioblastoma cells, as shown schematically (Fig. 7). We observed that induction of astrocytic differentiation resulted in sustained down regulation of the cell proliferation markers leading to cell cycle arrest. The prolongation of cell cycle arrest primed the glioblastoma cells to PTX mediated activation of extrinsic and intrinsic caspase pathways for apoptosis.

4. Experimental procedures

4.1. Cell culture and treatments

We obtained rat glioblastoma C6 and RG2 cell lines from the American Type Culture Collection (ATCC, Manassas, VA, USA) and procured normal astrocytes from ScienCell Research Laboratories (Carlsbad, CA, USA). We maintained C6 cells in $1 \times$ RPMI and RG2 cells in $1 \times$ DMEM medium, both supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (GIBCO/BRL, Grand Island, NY, USA). Normal astrocytes were maintained in an astrocyte medium (ScienCell Research Laboratories, Carlsbad, CA, USA), supplemented with 10% FBS and 1% penicillin and streptomycin (GIBCO/BRL, Grand Island, NY, USA). All cells were grown in a humidified atmosphere containing 5% CO₂ at 37°C. Other media and serum were purchased from Mediatech (Herndon, VA, USA). 4-HPR (Sigma Chemical, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) to make a stock

solution and aliquots of stock solution were stored in the dark at -70°C . To avoid light sensitivity of 4-HPR, all treatments involving it were performed under subdued lighting for 72 h. All experiments included control cultures, which contained the same volume of DMSO that was used in the 4-HPR treatment. The concentration of DMSO in each experiment was always less than or equal to 0.01%, which did not induce differentiation and cell death. Treatment of glioblastoma cells with PTX was carried out for 24 h. Following the treatments, cells were used for determination of morphological features of astrocytic differentiation or apoptosis, residual cell viability, and expression of specific proteins.

4.2. Detection of morphological features of astrocytic differentiation

Dose-response studies were carried out to optimize the concentration of 4-HPR for inducing astrocytic differentiation in both glioblastoma cell lines. Cells were cultured in monolayer in 9-cm diameter plates in the absence and presence of 500 nM 4-HPR for 72 h. At the end of the treatment, cells were washed twice with ice-cold phosphate-buffered saline (PBS), pH 7.4, before fixing the cells in ice-cold 95% ethanol. Cells were stained with 0.2% (v/v) methylene blue solution (prepared in 50% ethanol) for 30 sec and washed twice with ice-cold distilled water. The plates were dried in air before being examined under the light microscope at 400x magnification.

4.3. Determination of cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

All cells were grown in 6-well plates. The C6 and RG2 cells were treated with 4-HPR (500 nM for 72 h), PTX (50 or 100 nM for 24 h), and 4-HPR (total 72 h) + PTX (last 24 h); while the normal astrocytes were treated with 4-HPR (500 nM for 72 h), PTX (100 nM for 24 h), and 4-HPR (total 72 h) + PTX (last 24 h). The growth medium was supplemented with 5% FBS for the first 48 h and then replaced with fresh medium supplemented with 0.5% FBS. In case of combination, 4-HPR was still present in growth medium during the PTX treatment. After treatments, medium was discarded and replaced with a fresh medium containing MTT (0.2 mg/ml) and cells were incubated for 3 h. Then, DMSO (200 μl) was added to each well to dissolve the formazan crystals and absorbance was measured at 570 nm with background subtraction at 630 nm. Cell viability was presented as percentage of viable cells in total population.

4.4. In situ Wright staining for detection of morphological features of apoptosis

The cells were grown on coverslips and at the end of treatments, both adherent and non-adherent cells were centrifuged at a low rpm to sediment them. The cells were then washed twice with PBS, pH 7.4 before being fixed in 95% ethanol for 20 min. The cells adhering to the coverslips were allowed to dry and subjected to Wright staining. The coverslips were then allowed to dry and mounted on a slide to examine the morphological features of the apoptotic cells under the light microscope, as we described recently (Janardhanan et al., 2008). The morphological features of apoptotic cells included at least one of such characteristics as cell shrinkage, chromatin condensation, and membrane-bound apoptotic bodies.

4.5. Flow cytometry for determination of cell cycle and apoptosis

After the treatments, cells were harvested and washed in PBS (pH 7.4) twice before being fixed with 70% ethyl alcohol for 15 min on ice. Subsequently, alcohol was aspirated and propidium iodide (10 $\mu\text{g/ml}$) was added to the cells before being analyzed on Coulter EPICS-XL-MCL Flow Cytometer for determination of cell cycle. For Annexin V staining, the cells were harvested and washed in PBS (pH 7.4) and processed as per the manufacturer's instructions (Apoptosis Assay Kit, Molecular Probes, Eugene, OR, USA) before being analyzed on Coulter EPICS-XL-MCL Flow Cytometer for determination of apoptosis.

4.6. Antibodies and Western blotting

Monoclonal β -actin antibody (clone AC-15) was purchased (Sigma Chemical, St. Louis, MO, USA) and used to standardize the loading of cytosolic proteins on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, as we described previously (Ray et al., 2006). Both glioblastoma cell lines were treated with 4-HPR (72 h) and PTX (24 h) alone and in combination prior to extraction of protein samples. Protein samples obtained from glioblastoma C6 and RG2 cells were separated by SDS-PAGE and analyzed by Western blotting using the primary IgG antibodies against Bax, Bcl-2, Bid, calpastatin, caspase-8, caspase-3, cathepsin B, cathepsin C, cathepsin L, Cdk2, Cdk5, Cdc20, e-cadherin, GFAP, ICAD, Id2, m-calpain, MAD2, p35/p25, PCNA, retinoblastoma (Rb), phospho (Ser780) Rb (pRb), and rTERT. All the primary antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA) with the exception of GFAP, which was obtained from Chemicon International (Temecula, CA, USA). The horseradish peroxidase conjugated goat anti-mouse or anti-rabbit IgG (ICN Biomedicals, Aurora, OH, USA) was used as secondary antibody. Western blots were incubated with ECL detection reagents (Amersham Pharmacia, Buckinghamshire, UK) and exposed to X-OMAT AR films (Eastman Kodak, Rochester, NY, USA).

4.7. Statistical analysis

Results were analyzed using StatView software (Abacus Concepts, Berkeley, CA, USA) and compared using one-way analysis of variance (ANOVA) with Fisher's post hoc test. Data were presented as mean $\pm$ standard deviation (SD) of separate experiments ($n \geq 3$). Significant difference from control value was indicated by * $P < 0.05$, ** $P < 0.005$, and # $P < 0.001$.

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Fig. 1.

Treatment with 4-HPR decreased cell proliferation and induced morphological and biochemical features of astrocytic differentiation. (A) Decrease in cell proliferation and increase in morphological features of astrocytic differentiation. Cells were treated with 500 nM 4-HPR for 72 h. (B) Western blotting showed changes in biochemical markers of astrocytic differentiation and cell proliferation. Treatments: control, 500 nM 4-HPR for 72 h, 100 nM PTX for 24 h, and 500 nM 4-HPR for 48 h (pretreatment) + 100 nM PTX for 24 h. Expression of β -actin was used as a loading control.

Fig. 2.
Changes in viability of cells following the treatments. Cells were treated with 4-HPR for 72 h, PTX for 24 h, and also pretreated with 4-HPR for 48 h and then treated with PTX for 24 h. After the treatments, changes in cell viability were determined by MTT assay.

Fig. 3.

Cell cycle analysis and changes in levels of cell cycle molecules. Treatments: control, 500 nM 4-HPR for 72 h, 100 nM PTX for 24 h, and 500 nM 4-HPR for 48 h (pretreatment) + 100 nM PTX for 24 h. (A) Flow cytometric analysis of cell cycle after staining of cells with propidium iodide. (B) Western blotting to examine changes in cytosolic levels of molecular markers relating to cell cycle arrest at G1/S phase.

Fig. 4.

Determination of apoptosis in cells after the treatments. Treatments: control, 500 nM 4-HPR for 72 h, 100 nM PTX for 24 h, and 500 nM 4-HPR for 48 h (pretreatment) + 100 nM PTX for 24 h. (A) *In situ* Wright staining to examine morphological features of apoptosis. (B) Determination of percentages of apoptosis based on morphological features revealed by Wrights Staining. (C) Annexin V staining and flow cytometric analysis of apoptotic populations after the treatments. Representative flow cytometric data in C6 cells showing accumulation of apoptotic populations in M4 area. (D) Determination of percentages of apoptosis based on accumulation of Annexin V positive cells in M4 area.

Fig. 5.

Western blotting to examine activation of calpain and cathepsins in C6 and RG2 cells after the treatments. Treatments: control, 500 nM 4-HPR for 72 h, 100 nM PTX for 24 h, and 500 nM 4-HPR for 48 h (pretreatment) + 100 nM PTX for 24 h.

Fig. 6.

Western blotting to examine the molecular components of the extrinsic and intrinsic pathways of apoptosis. Treatments: control, 500 nM 4-HPR for 72 h, 100 nM PTX for 24 h, and 500 nM 4-HPR for 48 h (pretreatment) + 100 nM PTX for 24 h.

Fig. 7.

Schematic presentation of the molecular components and pathways to show induction of differentiation and apoptosis in glioblastoma cells. Treatment with 4-HPR induced differentiation with upregulation of GFAP and e-cadherin, down regulation of Id2, inhibition of phosphorylation (inactivation) of Rb, and also suppression of rTERT and PCNA. Pretreatment with 4-HPR potentiated PTX for cell cycle arrest and induction of both extrinsic and intrinsic pathways of apoptosis in glioblastoma cells.