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N-(4-Hydroxyphenyl) retinamide induced both differentiation and apoptosis in human glioblastoma T98G and U87MG cells

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Abstract

N-(4-Hydroxyphenyl) retinamide (4-HPR) is a synthetic retinoid that has shown biological activity against several malignant tumors and minimal side effects in humans. To explore the mechanisms underlying the chemotherapeutic effects of 4-HPR in glioblastoma, we used two human glioblastoma T98G and U87MG cell lines. *In situ* methylene blue staining showed the morphological features of astrocytic differentiation in glioblastoma cells following exposure to 1 μ M and 2 μ M 4-HPR for a short duration (24 h). Astrocytic differentiation was associated with an increase in expression of glial fibrillary acidic protein (GFAP) and down regulation of telomerase. Wright staining and ApopTag assay indicated appearance of apoptotic features in glioblastoma cells following exposure to 1 μ M and 2 μ M 4-HPR for a long duration (72 h). We found that 4-HPR caused apoptosis with activation of caspase-8 and cleavage of Bid to truncated Bid (tBid). Besides, apoptosis was associated with alterations in expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins resulting in an increase in Bax:Bcl-2 ratio, mitochondrial release of cytochrome *c* and Smac, down regulation of selective baculoviral inhibitor-of-apoptosis repeat containing (BIRC) molecules, an increase in intracellular free [Ca²⁺], and activation of calpain and caspase-3. Taken together, these results strongly suggested that 4-HPR could be used at low doses for induction of both differentiation and apoptosis in human glioblastoma cells.

Keywords

Apoptosis; Differentiation; Calpain; Caspases; Glioblastoma; 4-HPR; Mitochondria

1. Introduction

Chemotherapeutic approaches to glioblastoma, which is the most malignant brain tumor, are presently not successful because of significant toxicity, problems with drug delivery, and the high degree of drug-resistance. There are many clinical trials evaluating emerging therapeutic agents for the treatment of newly diagnosed glioblastoma patients. New agents that target cell characteristics such as differentiation, angiogenesis, invasion, DNA repair, and apoptosis and

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that show acceptable side-effect profiles are presently being investigated for their efficacy against this malignancy (Yung 1994).

N-(4-Hydroxyphenyl) retinamide (4-HPR), also known as fenretinide, is a synthetic derivative of all-*trans* retinoic acid (ATRA) and it induces apoptosis in cancer cell lines, shows minimal side effects in humans and also does not accumulate in the liver (Costa et al., 1995). It is also one of the most promising retinoids in both cell culture and animal models of cancers. Over 25 years ago, 4-HPR proved to be a potent inhibitor of mammary carcinogenesis in the rats (Moon et al., 1979). Since then, 4-HPR has been studied extensively and found to be less toxic and less genotoxic than other retinoids. The Chemoprevention Branch of the National Cancer Institute has active 4-HPR trials for cancers in several organ sites including the prostate, lung, oral cavity, breast, bladder, and cervix (Zou et al., 2003). It has also been found to be highly growth inhibitory in cervical cancer, ovarian cancer, endometrial cancer, lung cancer, non-small cell lung cancer, head and neck squamous cell carcinoma, esophageal carcinoma, prostate cancer, breast carcinoma, colon carcinoma, kidney carcinoma, bladder carcinoma, neuroblastoma, leukemia, non-M3 acute myeloid leukemia, NB4 acute promyelocytic cells, transformed cells such as NIH 3T3 mouse fibroblasts, and F9 embryonal carcinoma cells (Zou et al., 2003).

Moreover, 4-HPR is a valuable tool for defining apoptotic signaling pathways and understanding the mechanisms of synergy with other chemotherapeutic drugs. It has been reported earlier that 4-HPR reduces the expression of human telomerase reverse transcriptase (hTERT) catalytic subunit (Soria et al., 2001), suggesting that hTERT may represent a specific molecular marker for the detection of pre-invasive disease in early carcinogenesis and a potential intermediate biomarker to evaluate the efficacy of chemopreventive agents.

The mechanisms by which 4-HPR exerts its apoptotic effects are not yet clear. Unlike ATRA, 4-HPR induces its apoptotic effects mainly via retinoid receptor-independent mechanisms (Lippman et al., 2000). 4-HPR inhibits cell growth by inducing apoptosis in numerous tumor cell types including ATRA-resistant tumor cells (Ulukaya et al., 2003). However, the signaling mechanisms by which 4-HPR mediates its anti-proliferative effects remain unclear. On the basis of previous reports that 4-HPR induces both differentiation and apoptosis, we have hypothesized that 4-HPR may demonstrate those activities against human glioblastoma cells. Therefore, we examined therapeutic efficacy of 4-HPR against human glioblastoma T98G and U87MG cells.

We examined induction of both differentiation and apoptosis in glioblastoma T98G and U87MG cells following exposure to two low doses (1 μ M and 2 μ M) of 4-HPR for a short time-point (24 h) and a long time-point (72 h). Both doses of 4-HPR for a short exposure (24 h) induced only differentiation but for a long exposure (72 h) caused apoptosis in both glioblastoma cell lines. Induction of apoptosis involved initial caspase-8 activation followed by late mitochondrial release of cytochrome *c* and minor caspase-9 activation, suggesting that caspase-8 activation was the major trigger for apoptosis. Production of pro-apoptotic tBid and overexpression of pro-apoptotic Bax occurred in course of apoptosis. In contrast, levels of anti-apoptotic BIRC-2 to BIRC-6 expression were decreased to favor apoptosis. Taken together, these results indicated that 4-HPR at low doses could be used as a potential chemotherapeutic drug for induction of both differentiation and apoptosis in glioblastoma cells depending on duration of treatment time.

2. Results

2.1. 4-HPR induced differentiation with overexpression of GFAP and down regulation of telomerase

Low doses (1 μ M and 2 μ M) of 4-HPR for a short time (24 h) suppressed cell proliferation and induced morphological and biochemical features astrocytic differentiation in both T98G and U87MG cells (Fig. 1). *In situ* methylene blue staining showed growth restriction and morphology of astrocytic differentiation such as appearance of thin cells with long processes (Fig. 1A). As a biochemical marker of astrocytic differentiation, the GFAP expression was remarkably increased (2 folds) at both mRNA (Fig. 1B) and protein (Fig. 1C) levels in differentiated cells compared with parental T98G and U87MG cells. However, the precise molecular mechanism responsible for the increase in GFAP expression during astrocytic differentiation is uncertain. Since GFAP is a determining factor for astrocytic cell shape, the morphological alterations may be mediated through the induction of GFAP expression (Kumanishi et al., 1992). Also, expression of hTERT, the catalytic subunit of telomerase was examined at the mRNA (Fig. 1D) and protein (Fig. 1E) levels. Our results showed that inhibition of telomerase was associated with induction of differentiation. These findings suggested that 4-HPR induced astrocytic differentiation with overexpression of GFAP and down regulation of telomerase in T98G and U87MG cells.

2.2. Evaluation of viability and also morphological and biochemical features of apoptosis

Residual cell viability and apoptotic features were evaluated after the treatments for 72 h (Fig. 2). Exclusion of trypan blue dye by viable T98G and U87MG cells was evaluated under a light microscope using a hemocytometer. Treatment of T98G and U87MG cells with low doses (1 μ M and 2 μ M) of 4-HPR for a long time (72 h) significantly reduced cell viability (Fig. 2A). Wright staining revealed that 4-HPR induced the characteristic morphological features of apoptosis such as cell-shrinkage with condensation of nucleus and cytoplasm, membrane blebbing, and formation of apoptotic bodies (Fig. 2B). Results obtained from Wright staining were further confirmed by the ApopTag assay (Fig. 2C). Both control cell lines showed little or no brown color, indicating almost absence of ApopTag-positive cells or apoptosis. On the other hand, cells treated with 1 μ M 4-HPR or 2 μ M 4-HPR for 72 h produced apoptotic cells. We counted the apoptotic cells under the light microscope to determine the amounts of apoptosis (Fig. 2D). Compared with control cells, treatment with 4-HPR significantly increased apoptotic cells in both cell lines.

2.3. 4-HPR treatments induced caspase-8 activation and proteolytic cleavage of Bid

Recent studies indicate that caspase-8 activation acts as a key determinant for the extrinsic pathway of apoptosis caused by death-inducing ligands or cytotoxic agents (Stupack et al., 2001). It is known that active caspase-8 uses Bid as a substrate to produce tBid that may be translocated to the mitochondria to induce cell death. We performed Western blotting and colorimetric assay to examine caspase-8 activation and activity in the cells after 4-HPR treatments (Fig. 3). Our results showed that treatment of cells with 4-HPR induced formation of active caspase-8 fragment to cause proteolytic cleavage of Bid to tBid, which was capable of translocating from cytosol to mitochondria (Fig. 3A). Notably, we examined the mitochondria fraction for analysis of tBid. We monitored uniform expression of β -actin as a loading control of cytosolic protein while expression of COX4 as a loading control of mitochondrial protein in each lane (Fig. 3A). In mitochondria, tBid can stimulate more efficient oligomerization of Bax to activate the intrinsic pathway of apoptosis (Cao et al., 2003). Significant increase in total caspase-8 activity following 4-HPR treatment was also further confirmed by colorimetrically assay (Fig. 3B).

2.4. Apoptosis via mitochondria dependent pathway

The intrinsic pathway is regulated at mitochondria, which release cytochrome *c* and other pro-apoptotic factors during different forms of cellular stress (Werdehausen et al., 2007). The release of cytochrome *c* is controlled by proteins of the B-cell lymphoma-2 (Bcl-2) protein family. Upon apoptosis induction, BH3-only proteins activate Bax and Bak, which subsequently undergo a conformational change, leading to their assembly into pore-forming multimers at the outer mitochondrial membrane for cytochrome *c* release. In the cytosol, cytochrome *c* together with caspase 9 induces the formation of the apoptosome, thereby triggering the mitochondria dependent caspase cascade for apoptosis. We examined the involvement of mitochondrial events in course of apoptosis (Fig. 4). Our results showed that treatment of cells with 4-HPR increased Bax expression at mRNA (Fig. 4A) and protein (Fig. 4B) levels. Based on Western blotting, we measured the Bax:Bcl-2 ratio, which was significantly increased in all treatment groups (Fig. 4C). Our results also showed that treatment of cells with 4-HPR promoted disappearance 15 kD cytochrome *c* from the mitochondria fraction (Fig. 4D), indicating that 4-HPR induced mitochondrial release of cytochrome *c*. Because of release from mitochondria, 15 kD cytochrome *c* appeared in the cytosolic fractions (Fig. 4D). We used COX4 as a loading control of mitochondrial protein. Thus, the processes of cell death involved the release of cytochrome *c* from mitochondria, which subsequently could cause activation of caspases. We observed an increase in active caspase-9 fragment in cells following 4-HPR treatments (Fig. 4D). β -Actin expression was used to ensure that equal amount of cytosolic protein was loaded in each lane. Thereafter, significant increase in total caspase-9 activity in apoptotic cells was confirmed by a colorimetric assay (Fig. 4E). These results suggested that caspase-9 activation might be a consequence of cytochrome *c* release from mitochondria. Combined together, we propose that increase in Bax:Bcl-2 ratio, release of cytochrome *c* from mitochondria, and subsequent activation of caspase-9 played key roles for mediation of apoptosis.

2.5. 4-HPR induced mitochondrial release of Smac that could suppress BIRC expression

Like cytochrome *c*, Smac is located in mitochondria and released into the cytosol when cells undergo apoptosis. In response to apoptotic stimuli, Smac is released into the cytosol to bind to BIRC proteins to block their function and thus promote caspase activation (Das et al., 2008). These observations suggested that Smac could be an important regulator of apoptosis. We examined mitochondrial release of Smac into the cytosol and levels of BIRC expression (Fig. 5). Our Western blotting with both mitochondrial and cytosolic fractions showed the mitochondrial release of Smac into the cytosol following 4-HPR treatments (Fig. 5A). Moreover, we examined levels of expression of BIRC-2 to BIRC-8 by RT-PCR (Fig. 5B). Treatment of cells with 4-HPR substantially decreased the levels of BIRC-2 to BIRC-5 but levels of BIRC-6 to BIRC-8 remained relatively unchanged (Fig. 5B). These results indicated that 4-HPR induced mitochondrial release of Smac into the cytosol and decreased expression of BIRC-2 to BIRC-5 for apoptosis in glioblastoma cells.

2.6. 4-HPR increased intracellular free $[Ca^{2+}]$, activated calpain and caspase-3, and down regulated calpastatin

We examined the increase in intracellular free $[Ca^{2+}]$, activation and activity of calpain as well as of caspase-3, and translocation of CAD to the nucleus in T98G and U87MG cells following 4-HPR treatments (Fig. 6). Fura-2 assay showed that 4-HPR treatments caused significant increases in intracellular free $[Ca^{2+}]$ in both T98G and U87MG cells (Fig. 6A), suggesting activation of the Ca^{2+} -dependent protease calpain. Western blotting showed increase in active 76 kD calpain fragment, decrease in 110 kD calpastatin (endogenous calpain inhibitor), and increase in active 20 kD caspase-3 fragment in both cell lines after 4-HPR treatments (Fig. 6B). Caspase-mediated fragmentation of calpastatin has been reported previously (Wang et al.,

1998). The proteolysis of calpastatin suggests a cross-talk between the caspase and calpain systems in course of apoptosis in T98G and U87MG cells. The degradation of 270 kD α -spectrin to 145 kD spectrin breakdown product (SBDP) and 120 kD SBDP has been attributed to increased activities of calpain and caspase-3, respectively (Das et al., 2005,2006,2007, 2008). So, we examined increases in calpain and caspase-3 activities in the formation of 145 kD SBDP and 120 kD SBDP, respectively, on the Western blots (Fig. 6B). During apoptosis, caspase-3 is activated to cleave ICAD to release CAD from the CAD/ICAD (Sakahira et al., 1998). Free CAD is then translocated to the nucleus to degrade chromosomal DNA. Our results showed decrease in 45 kD ICAD in the cytosolic fractions and appearance of 40 kD CAD in the nuclear fractions in T98G and U87MG cells after 4-HPR treatments (Fig. 6B). For analysis of CAD in nuclear fractions, we ran two sets of SDS-PAGE gels at the same time. One set of gels was used for analyzing levels of CAD by Western blotting (Fig. 6B) and other set of gels with resolved proteins was stained with Coomassie blue to confirm equal amounts of nuclear protein loading in all lanes of SDS-PAGE (Fig. 6B). Further, a colorimetric assay confirmed increase in caspase-3 activity (Fig. 6C).

3. Discussion

Among the retinoids, 4-HPR is particularly promising as an anti-tumor agent because it has fewer negative effects than naturally occurring retinoids such as ATRA. Interestingly, 4-HPR can induce apoptosis even in ATRA-resistant cell lines (Ulukaya et al., 2003). The mechanism of action of 4-HPR in human glioblastoma cells appears to differ from that of many other retinoids. Although many retinoids induce differentiation, they do not efficiently induce apoptosis. The ability of 4-HPR to induce both differentiation and apoptosis makes it a fantastic choice for controlling the growth of tumor cells that typically lack differentiation and avoid apoptosis.

To gain insight into the molecular events that allow 4-HPR to function as an inducer of both differentiation and apoptosis, we examined the effects of different concentrations of 4-HPR at different time points in human glioblastoma T98G and U87MG cells. Treatment of cells with 1 μ M and 2 μ M 4-HPR induced differentiation at 24 h (Fig. 1) and apoptosis at 72 h (Fig. 2). Because the growth of many tumors is associated with upregulation of telomerase (Kim et al., 1994), down regulation of telomerase is an important goal in the treatment of tumors. Our results showed that 4-HPR induced differentiation (Fig. 1) as an early process (24 h) with suppression of proliferation, increase in expression of GFAP (a prominent marker of astrocytic differentiation), and down regulation of hTERT (the catalytic subunit of human telomerase) in glioblastoma cells (Fig. 1). Also, 4-HPR decreased cell viability and induced apoptosis (Fig. 2) as a terminal process (72 h) with activation of death receptor-dependent caspase-8 (Fig. 3), calpain, and mitochondria-dependent caspase cascade (Fig. 4–Fig. 6). Therefore, 4-HPR is capable of inducing differentiation with down regulation of telomerase and also apoptosis with activation of multiple proteolytic mechanisms in human glioblastoma cells.

Caspase-8 mediated cleavage of Bid to tBid leads to mitochondrial release of cytochrome *c*, which is an essential component in activation of caspase cascade for apoptosis (Luo et al., 1998). Our data showed that 4-HPR activated caspase-8 and caused proteolytic cleavage of Bid to tBid in T98G and U87MG cells after 4-HPR treatments (Fig. 3). Caspase-8-dependent cleavage of the Bid provides a linkage between the death receptor and mitochondrial pathways of apoptosis. A number of pro-apoptotic and anti-apoptotic members of the Bcl-2 protein family regulate the release of cytochrome *c* and Smac from the mitochondrial intermembrane space into the cytosol (Boise et al., 1993). The anti-apoptotic Bcl-2 forms a heterodimer with the pro-apoptotic Bax to neutralize pro-apoptotic effects of Bax (Yin et al., 1995). Pro-apoptotic Bax is thought to work upstream of the cysteine proteases in the mitochondria-mediated apoptotic pathway (Boise et al., 1993). Chemotherapeutic drugs can increase Bax level to trigger

mitochondrial release of cytochrome *c* into the cytosol, where formation of 'apoptosome' with cytochrome *c*, Apaf-1, and pro-caspase 9 causes ATP-dependent activation of caspase-9 that in turn activates other downstream caspases to orchestrate the execution of cells (Green and Reed, 1998). Our study showed that 4-HPR treatment altered the levels of Bax and Bcl-2, resulting in an increase in Bax:Bcl-2 ratio to promote mitochondrial release of cytochrome *c* and activation of caspase-9 (Fig. 4).

Our results also showed that treatment of T98G and U87MG cells with 4-HPR caused mitochondrial release of Smac that could play an important role in down regulation of selective BIRC molecules (Fig. 5). Our findings supported a direct relationship between an increase in intracellular free $[Ca^{2+}]$ and induction of cell death with activation of calpain in glioblastoma cells following 4-HPR treatments (Fig. 6). Degradation of calpastatin (endogenous calpain inhibitor), increased activities of calpain and caspase-3, and cleavage of ICAD to release and translocate CAD to the nucleus clearly indicated the culmination of the apoptotic process (Fig. 6). Nuclear CAD is capable of causing degradation of the chromosomal DNA. Taken together, our results showed that 4-HPR induced apoptosis in human glioblastoma cells with activation of complex biochemical pathways involving increases in calpain and caspase-3 proteolytic activities. A future challenge will be to unravel this complexity with identification of other components that may contribute to death machinery in glioblastoma cells after 4-HPR treatment.

In conclusion, our investigation indicated that 4-HPR effectively suppressed cell proliferation and induced differentiation as an early process and also apoptosis as a terminal process for controlling the growth of human glioblastoma T98G (containing mutant-type p53) and U87MG (containing wild-type 53) cells. Among the retinoids, 4-HPR with negligible or no organ toxicity profile at low doses should be a promising therapeutic agent for induction of both differentiation and apoptosis in human glioblastoma.

4. Materials and methods

4.1. Cell culture and treatments

Both T98G and U87MG cells were grown in monolayer to sub-confluency in 75-cm² flasks containing 10 ml of RPMI 1640 medium and 10% fetal bovine serum in a fully-humidified incubator containing 5% CO₂ at 37°C. For making stock solution, 4-HPR (Sigma Chemical, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and then stored at -20°C. The stock solution of 4-HPR was diluted into the growth medium immediately before addition to cell cultures. Control cultures received the same amount of DMSO as the treated cultures. Dose-response studies were conducted to determine the appropriate doses of 4-HPR for induction of differentiation and apoptosis. It was decided that cells should be treated with 1 μ M and 2 μ M 4-HPR for 24 h for induction of differentiation and also for 72 h for induction of morphological and biochemical features of apoptosis.

4.2. Methylene blue staining for detection of morphological features of differentiation

Cells are cultured in monolayer in 9-cm diameter plates in absence and presence of 1 μ M and 2 μ M 4-HPR for 24 h. Culture medium was aspirated and washed with ice-cold phosphate-buffered saline (PBS, pH 7.4) two times. Then the plate was placed on ice and 5 ml of ice-cold 50 % (v/v) ethanol was added to fix the cells. Ethanol was aspirated followed by the addition of 5 ml of ice-cold 0.2 % (w/v) methylene blue solution (made up in 50 % ethanol) staining *in situ*. Cells were stained for 30 sec, washed twice with ice-cold water, and the plates were dried in the air. Light microscope was used at 400x magnification to examine the appearance of morphological features of astrocytic differentiation.

4.3. Trypan blue dye exclusion test for determination of residual cell viability

Following the treatments for 72 h, the residual cell viability in attached and detached cell populations was evaluated by trypan blue dye exclusion test, as reported recently (Das et al., 2007; 2008). Viable cells maintained membrane integrity and did not take up trypan blue. Cells with compromised cell membranes took up trypan blue and were counted as dead. At least 800 cells were counted in four different fields and the number of viable cells was calculated as percentage of the total cell population.

4.4. Wright staining and ApopTag assay for detection of apoptotic cells

Cells from each treatment were sedimented onto the microscopic slide and fixed in methanol before examination of apoptosis by Wright staining and ApopTag assay (Das et al., 2007; 2008). Wright staining was used to detect characteristic apoptotic features such as chromatin condensation, cell-volume shrinkage, and membrane-bound apoptotic bodies. ApopTag assay kit (Intergen, Purchase, NY) was used for biochemical detection of DNA fragmentation in apoptotic cells. The nuclei containing DNA fragments were stained dark brown with ApopTag assay and were not counterstained with methyl green that, however, stained normal nuclei pale to medium green. After ApopTag assay, cells were counted to determine the percentage of apoptosis.

4.5. Fura-2 assay for determination of intracellular free $[Ca^{2+}]$

Level of intracellular free $[Ca^{2+}]$ was measured using the fluorescence Ca^{2+} indicator fura-2/AM (Molecular Probes, Eugene, OR), as we described previously (Das et al., 2008), for determination of intracellular free $[Ca^{2+}]$ in T98G and U87MG cells. The value of K_d , a cell-specific constant, was determined experimentally to be $0.387 \mu M$ for the T98G cells and $0.476 \mu M$ for the U87MG cells, using standards of the Calcium Calibration Buffer Kit with Magnesium (Molecular Probes, Eugene, OR).

4.6. Antibodies

Monoclonal IgG antibody against β -actin (Sigma Chemical) was used to standardize cytosolic protein loading on the SDS-PAGE. Cytochrome *c* oxidase subunit IV (COX4) IgG antibody (Molecular Probes, Eugene, OR) was used to standardize the mitochondrial protein levels. COX4 is a membrane protein in the inner mitochondrial membrane and it remains in the mitochondria regardless of activation of apoptosis. Also, IgG antibody against α -spectrin (Affiniti, Exeter, UK) was used to detect the calpain and caspase-3 activities. All other primary IgG antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). We used horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (ICN Biomedicals, aurora, OH) for detecting all primary antibodies except for calpain and α -spectrin antibodies where we used HRP-conjugated goat anti-rabbit IgG secondary antibody (ICN Biomedicals).

4.7. Western blotting

Western blotting was performed as we described previously (Das et al., 2008). The autoradiograms were scanned using Photoshop software (Adobe Systems, Seattle, WA) and optical density (OD) of each band was determined using Quantity One software (Bio-Rad, Hercules, CA).

4.8. Preparations and analyses of cytosolic, mitochondrial, and nuclear fractions

Preparations of cytosolic, mitochondrial, and nuclear fractions were performed by standard procedures (Das et al., 2008). Cytochrome *c* in the supernatants and pellets and also CAD in nuclear fractions were examined by Western blotting.

4.9. Colorimetric assays for caspase-3, caspase -8, and caspase-9 activities

Measurements of caspase-3, caspase-8, and caspase-9 activities in the cell lysates were performed using the commercially available colorimetric assay kits (Sigma). The colorimetric assay is based on the hydrolysis of a specific peptide substrate by a specific caspase activity, resulting in the release of the *p*-nitroaniline (*p*NA) moiety. The *p*-NA has a high absorbance at 405 nm ($\epsilon_{\text{mM}} = 10.5$). The concentration of *p*NA released from the substrate was calculated from the absorbance values at 405 nm. Experiments were performed in triplicate.

4.10. Extraction of total RNA and reverse transcriptase-polymerase chain reaction (RT-PCR)

Extraction of total RNA and RT-PCR were performed according to standard procedure (Das et al., 2008). All human primers (Table 1) for RT-PCR experiments were designed using Oligo software (National Biosciences, Plymouth, MN) and custom synthesized (Operon Technologies, Alameda, CA.). The level of β -actin gene expression served as an internal control.

4.11. Statistical analysis

All results obtained from different treatments of T98G and U87MG cells were analyzed using StatView software (Abacus Concepts). Data were expressed as mean $\pm$ SD of separate experiments ($n \geq 3$) and compared by one-way analysis of variance (ANOVA) followed by Fisher's post hoc test. Difference between two treatments was considered significant at $p \leq 0.05$.

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Fig. 1. Detection of morphological and biochemical features of astrocytic differentiation in T98G and U87MG cells. Treatments (24 h): control (CTL), 1 μ M 4-HPR, and 2 μ M 4-HPR. (A) Methylene blue staining to detect astrocytic morphology. Determination of GFAP expression by (B) RT-PCR and (C) Western blotting. Determination of hTERT expression by (D) RT-PCR and (E) Western blotting.

Fig. 2. Detection of morphological and biochemical features of apoptosis in T98G and U87MG cells. Treatments (72 h): control (CTL), 1 μ M 4-HPR, and 2 μ M 4-HPR. (A) Trypan blue dye exclusion test to assess residual cell viability. (B) Wright staining for examination of morphological features of apoptosis. (C) ApopTag assay to detect apoptotic DNA fragmentation. (D) Bar diagram to show percent apoptosis based on ApopTag assay.

Fig. 3. Determination of caspase-8 activation and activity in T98G and U87MG cells. Treatments (72 h): control (CTL), 1 μ M 4-HPR, and 2 μ M 4-HPR. (A) Western blots to show levels of caspase-8, β -actin, tBid, and COX4. (B) Colorimetric determination of caspase-8 activity.

Fig. 4.

Examination of mitochondrial involvement in apoptosis in T98G and U87MG cells.

Treatments (72 h): control (CTL), 1 μ M 4-HPR, and 2 μ M 4-HPR. Gel pictures with RT-PCR products to show the mRNA (A) and Western blots to show protein (B) levels of Bax, Bcl-2, and β -actin. (C) Densitometric analysis to show the Bax:Bcl-2 ratio. (D) Western blots to show levels of cytochrome *c*, COX4, caspase-9, and β -actin. (E) Colorimetric determination of caspase-9 activity.

Fig. 5. Mitochondrial release of Smac and down regulation of BIRC in T98G and U87MG cells. Treatments (72 h): control (CTL), 1 μ M 4-HPR, and 2 μ M 4-HPR. (A) Representative Western blots to show protein levels of Smac, COX4, and β -actin. (B) Representative gel pictures with RT-PCR products to show BIRC-2 to BIRC-8 and β -actin at mRNA levels.

Fig. 6.

Determination of intracellular free [Ca²⁺] and activities of calpain and caspase-3 in T98G and U87MG cells. Treatments (72 h): control (CTL), 1 μM 4-HPR, and 2 μM 4-HPR. (A) Determination of percent increase in intracellular free [Ca²⁺]. (B) Representative Western blots to show the levels of active calpain fragment, calpastatin, caspase-3, SBDP, ICAD, β-actin, and CAD. (C) Representative SDS-PAGE to show loading of equal amounts of nuclear fraction proteins in all lanes. (D) Determination of caspase-3 activity using a colorimetric assay.

Table 1

Human primers used to determine the levels of mRNA expression of specific genes

Gene	Primer sequence	Product size (bp)
β -actin	Sense: 5'-TAT CCC TGT ACG CCT CT-3'	460
	Antisense: 5'-AGG TCT TTG CGG ATG T-3'	
bax α	Sense: 5'-AAG AAG CTG AGC GAG TGT-3'	265
	Antisense: 5'-GGA GGA AGT CCA ATG TC-3'	
bcl-2 α	Sense: 5'-CTT CTC CCG CCG CTA C-3'	306
	Antisense: 5'-CTG GGG CCG TAC AGT TC-3'	
BIRC-2	Sense: 5'-CAG AAA GGA GTC TTG CTC GTG-3'	536
	Antisense: 5'-CCG GTG TTC TGA CAT AGC ATC-3'	
BIRC-3	Sense: 5'-GGG AAC CGA AGG ATA ATG CT-3'	368
	Antisense: 5'-ACT GGC TTG AAC TTG ACG GAT-3'	
BIRC-4	Sense: 5'-AAT GCT GCT TTG GAT GAC CTG-3'	470
	Antisense: 5'-ACC TGT ACT CAG CAG GTA CTG-3'	
BIRC-5	Sense: 5'-GCC CCA CTG AGA ACG-3'	302
	Antisense: 5'-CCA GAG GCC TCA ATC C-3'	
BIRC-6	Sense: 5'-AGC CGA AGG ATA GCG A-3'	385
	Antisense: 5'-GCC ATC CGC CTT AGA A-3'	
BIRC-7	Sense: 5'-GCC TCC TTC TAT GAC T-3'	283
	Antisense: 5'-CGT CTT CCG GTT CT-3'	
BIRC-8	Sense: 5'-GTG AGC GCT CAG AAA GAC ACT AC-3'	209
	Antisense: 5'-CAC ATG GGA CAT CTG TCA ACT G-3'	
GFAP	Sense: 5'- CGG CTC GAT CAA CTC A -3'	210
	Antisense: 5'- CTC CTC CAG CGA CTC AAT -3'	
hTERT	Sense: 5'- GTA CAT GCG ACA GTT C -3'	418
	Antisense: 5'- TTC TAC AGG GAA GTT CAC -3'	