Induction of Mitosis Delay And Apoptosis By RTA 404 In Glioblastoma Multiforme

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Research

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Abstract

Background

Glioblastoma multiforme (GBM) is the vicious malignant brain tumor in adults. Despite advances multi-disciplinary treatment, GBM continues to have a poor overall survival. CDDO-trifluoroethyl-amide, a trifluoroethylamide derivative of CDDO, is an Nrf2/ARE pathway activator. RTA 404 is used to inhibit proliferation and induce differentiation and apoptosis in glioma cells. However, it is not clear what effect it may have on tumorigenesis in GBM.

Methods

This *in vitro* study evaluated the effects of RTA 404 on GBM cells. To do this, we treated GBM8401 cell lines with RTA 404 and assessed apoptosis, cell cycle. DNA content and induction of apoptosis were analyzed by flow cytometry and protein expression by Western blot analysis.

Results

RTA 404 significantly inhibited the proliferation induced cell apoptosis on GBM 8401 cell line. Typical plasma membrane undergoes structural changes that cause translocation of phosphatidylserine from the inside to outside. Due to cell external pressure cause mitochondrial membrane potential change lead to cell apoptosis. Caspase-3 active respond to apoptosis phenomenon, continuous progression of apoptosis. In addition, treatment with RTA 404 led to an accumulation of G₂/M-phase cells. An analysis of Cyclin B1, CDK1 and Cyclin B1/CDK1 complex association suggested that cell cycle progression seems also to be regulated by RTA 404. Therefore, RTA 404 may not only induced cell cycle G₂/M arrest, it may also exert apoptosis in established GBM cells.

Conclusion

RTA 404 can inhibit proliferation, cell cycle progression and induce apoptosis in GBM cells *in vitro*, possibly through its inhibition of Cyclin B1, CDK1 expression, and Cyclin B1/CDK1 association.

Introduction

Glioma, the most common brain tumor is classified by The World Health Organization (WHO) into four grades based on histologic features [1, 2]. WHO grade IV, also called glioblastoma multiforme (GBM), is angiogenic and can cause necrosis. Glioblastoma multiforme are aggressive tumors of the central nervous system. Despite advances in multi-disciplinary treatment, GBM continues to have poor overall survival. Patients with glioblastoma have a median survival rate of about twelve months [3]. Therefore, it is very important to identify new and effective anticancer drugs and understand the pharmacological mechanism of the drugs to help treat malignant tumors.

One component found in Chinese herbal medicine for hepatitis, oleanolic acid, has been modified chemically creating an oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO), a plant synthesized drug [4]. The therapeutic effect of CDDO results from its ability to up-regulate Nrf2 by changing the conformation of the Nrf2-repressing, Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1 (Keap1) [5]. Antioxidant Response Element (ARE) has been found by several animal and human studies to activate Nrf2-controlled antioxidant genes upstream [5]. Much research has been devoted to manipulating these compounds to generate new derivatives with higher sought-after activities, increasing for example their anti-inflammatory activity and creating additional functional groups with the potential in the treatment of conditions. At present, CDDO derivatives have been used to treat lung injury [5], inflammation [6], and chronic kidney disease [7]. More and more evidence confirms that CDDO and its derivatives have anti-cancer effects. CDDO and its derivatives, CDDO-Me and CDDO-Im have been extensively investigated for their strong antiproliferative, antiangiogenic, antimetastatic activities, and for their capability to induce apoptosis and differentiation in cancer cells [8].

RTA 404, a trifluoroethylamide derivative of CDDO, has been found to have anti-oxidation, anti-inflammatory, and anti-proliferative effects, and also have enhanced ability to cross the blood-brain barrier.[9].Currently, CDDOTEFA has been used in neurodegenerative diseases, neuroinflammatory diseases, and neurooncology tumors.It has also been shown to enhance Nrf2 expression and signaling in various models of neurodegeneration [10], including those that simulate multiple sclerosis [11], amyotrophic lateral sclerosis [12], and Huntington's disease [13]. RTA 404 has been found to have neuroprotective effects in some animal models of degenerative diseases, including ischemic stroke [9] and autoimmune encephalomyelitis [11].It has also been found to induce apoptosis of neuroblastoma cells [14]. Furthermore, RTA 404 can inhibit proliferation, cell locomotion, cell cycle progression and induce apoptosis in GBM cells[15].

Although RTA 404 has been found to inhibit some cancers [16], it is unclear what effect RTA 404 may have on GBM and how it may achieve its effect. This study aims at focusing especially on RTA 404 and its chemo-preventive activities against glioma cells GBM8401. Efforts have been initiated to inspect the effects of RTA 404 on cell growth and cell cycle regulation, and expression levels of downstream molecules were additionally evaluated. The results illustrated that RTA 404 reduced glioma cell proliferation and induced glioma cell apoptosis in GBM8401 cells. This study demonstrated also that the increase in the G2 and M population of GBM8401 cells can be attributed to the repression of Cyclin B and CDK1 after exposure to RTA 404.

Result

RTA 404 decrease cell viability on GBM8401 cell line

We wanted to know whether RTA 404 could mediate the survival of human brain malignant glioma cells (GBM 8401) and mitigate their proliferation. To find out, we performed an *in vitro* study in which we

treated each of the glioma cell lines with increasing doses of RTA 404 (0, 1, 1.5, 2 μ M) for 24 hours and then measured proliferation by PB. As can be seen in Figure 1, there was a significant decrease in survival and proliferation of the GBM 8401 cells, both dose-dependently decreased by increased doses of RTA 404 (GBM8401; 24 hrs: $y = -24.339x + 111.61$, $R^2 = 0.841$, 48 hrs: $y = -30.959x + 107.27$ $R^2 = 0.7175$, 72 hrs: $y = -30.813x + 105.13$ $R^2 = 0.6821$) 24 to 72 h after treatment.

RTA 404 induced cell apoptosis on GBM8401 cell line

To clarify the role of RTA 404 in the apoptosis of GBM8401 cells, the cells were treated with RTA 404 for 4 h followed by detecting the Annexin V-FITC and PI staining, caspase 3 assay, and MMP2 assay. Cell population and apoptotic ratios were analyzed by flow cytometry. Compared with the untreated (control) cells, an Annexin-FITC/PI assay exhibited significant changes in the percentage of apoptosis on RTA 404-treated cells (Fig. 2A). RTA 404 treated cells had significant increase in the percentage of apoptotic cells compared with the untreated (control) cells (Fig 2B, $y = 1.0137x + 0.187$ $R^2 = 0.9202$). The loss of mitochondrial membrane potential ($\Delta\Psi_m$), an early cellular metabolism event coinciding with caspase activation, is a hallmark of apoptosis. In non-apoptotic cells, JC-1 is present as a green monomer in the cytosol, but it accumulates as red aggregates in the mitochondria. In apoptotic and necrotic cells, although JC-1 is present in the monomeric form, it stains the cytosol green. We wanted to explore the possible effect of RTA 404 on $\Delta\Psi_m$ in the GBM cells. To do this, we used JC-1 as a dye to assess to $\Delta\Psi_m$ loss in the RTA 404 treated cancer cells. As shown in Fig 3A (left), $\Delta\Psi_m$ of the cancer cells was reduced after treatment with RTA 404. Fig 3A (right) illustrates typical FL-1/FL-2 dot plots for apoptotic and nonapoptotic GBM8401 cells stained by JC-1. Non-RTA 404-treated cancer cells did not undergo apoptosis, resulting in red fluorescing JC-1 aggregates. The green fluorescing monomers in the lower part of the figure indicate apoptotic cells. Taken together, these observations suggest that treatment with RTA 404 led to a significant reduction in the $\Delta\Psi_m$ of the GBM8401 cells (Fig 3B, $y = 11.482x - 4.7167$ $R^2 = 0.6904$). The results, summarized in Fig 2 and 3, suggest that RTA 404 may mediate GBM8401 cell line survival. Thus, we hypothesized that the proliferation of these cells was inhibited by pathways of apoptosis.

Moreover, loss of mitochondria membranes potential was detected also due to RTA 404 treatment. Finally, a significant rise in the percentage of caspase-3 activity was detected in RTA 404-treated cancer cells (Fig.4 A and B, $y = 2.863x - 2.2267$ $R^2 = 0.8766$). Therefore, these results indicated that incubation with RTA 404 inhibited cell survival and proliferation and underwent cell apoptosis.

RTA 404 induced cell cycle arrest in G2/M phase in GBM 8401 cell line.

To further investigate the effect of RTA 404 on glioma cell growth, the cell cycle distribution among RTA 404 treated cells was analyzed and quantified by flow cytometry. Cells were treated with RTA 404 for 24 h followed by processing and analysis. As shown in Figure 5(A), treatment with RTA 404 led to an increment in the G2/M phase cell population, implying that GBM 8401 cells underwent a delay in the

G2/M phase checkpoint. These results suggested that exposure to RTA 404 enhanced cell populations in the G2/M phase (Figure 5B).

Mitosis protein synthesis inhibited by RTA 404 treatment on GBM 8401 cell line.

We were interested in what role RTA 404 might play in mitosis in GBM 8401. Mitosis protein synthesis of RTA 404-treated cells was analyzed using MPM-2 Staining. Figure 6 shows cells exposed to RTA 404 at different concentrations (0, 1, 1.5 and 2 μ M) before processing and analysis. Figure 6(A) shows that exposure to RTA 404 resulted in an increase in the mitosis protein. These results indicated that RTA 404 increased the mitosis protein synthesis (Figure 6B, $y = 16.635x - 1.955$ $R^2 = 0.8103$).

RTA 404 inhibited glioma cell cycle arrest through the cell cycle relates protein and induced apoptosis through the cyclinB1/CDK1 complex dissociation and p21 signaling pathway

As shown above, we found that RTA 404 induced apoptosis and inhibited *cell cycle* in the GBM cells. Figure 7 (A and B) shows the results of our Western blot analysis of cellular proteins extracted from the brain cancer cell lines treated with RTA 404. We measured the relative intensities of cyclin A2, cyclin B1, CDK1, NRF2, CHK1/2 and p21 gene expression. We found the protein expressions of cyclin A2, cyclin B1, CDK1, NRF2, CHK2 and to be significantly down-regulated, but the p-CHK2, CHK1 and tumor suppressor gene p21 up-regulated in RTA 404-treated GBM8401 cells.

Therefore, we wanted to investigate whether RTA 404 exerted its effects on cell cycle arrest through Cyclin B1 and CDK1. Co-IP is an effective means of quantifying protein-protein interactions in cells (Figure 8A). They were found to have significant decreases in cyclinB1/CDK1 complex (Figure 8B) in COOD-TREA treatment. These results suggest that the increased mitosis protein synthesis, G₂/M phase arrest may occur via Cyclin B1, CDK1, and Cyclin B1/CDK1; and apoptosis may occur via the p21 signaling pathway.

Discussion

This study demonstrated that RTA 404 reduced glioma cell proliferation and induced glioma cell apoptosis in GBM8401 cells. In addition, treatment with RTA 404 led to an accumulation of G₂/M-phase cells. Cell cycle progression seems also to be regulated by RTA 404 through inhibition of Cyclin B1, CDK1, and Cyclin B1/CDK1 complex. Therefore, RTA 404 may not only induce cell cycle G2/M arrest, but it may also exert apoptosis in established GBM cells.

Induction of apoptosis and arrest of the cell cycle are two of the best approaches to suppressing cancerous tumors [17]. Apoptosis is the one of programmed cell death that is involved in intrinsic and extrinsic pathways that activate the caspase family of cysteine proteases. The apoptotic markers such as Bax induce apoptosis via mitochondrial membrane potential alteration that results in the translocation of cytochrome c from the mitochondrial inner membrane to the cytosol that triggers signaling of caspase cascades [18, 19]. Cytochrome c activates caspase-9, thereby caspase-9 cleaves and activates caspase-3 [20]. Caspase-9 activates downstream caspase-3 [20], which is well known as one of the apoptotic

mediators leading to DNA damage and eventually apoptosis. In the extrinsic pathway, stimulation of the TNF family of receptors results in the activation of caspase-8[21]. Caspase-8 can directly activate caspase-3[22]. Finally, there is an activation of the same target apoptotic molecules such as caspase-3[23]. Many anticancer drugs induce apoptosis by activation of the intrinsic pathway[24]. The available evidence indicates that CDDO induces apoptosis by activation of the extrinsic caspase-8 pathway [25–29]. In the present study, it was revealed that RTA 404 induced cytotoxicity in GBM8401 cells, while there was no change in normal skin fibroblasts Hs-68 cells and normal lung fibroblasts MRC-5 cells (data not shown). RTA 404 decreased cell viability of GBM8401 cells by apoptotic induction as seen in the increasing number of Annexin V positive cells with nuclear chromatin condensation and DNA fragmentation after incubation with RTA 404 in a dose-dependent manner. (Fig. 2). Moreover, a significant rise in the percentage of caspase-3 activity was detected in RTA 404-treated cancer cells (Fig. 3). RTA 404 also decreased the aggregate to monomer ratio of JC-1 corresponding with the decreased number of cells with intact mitochondrial membrane potential evaluated by fluorescent microscope (Fig. 4). Therefore, according to the effects of RTA 404 in the up-regulation of caspase-3 and alteration of mitochondrial membrane potential, it could be implied that RTA 404 has potential in apoptotic induction via an intrinsic pathway on GBM8401 cell.

Cell cycle deregulation is a unique feature of human cancer. The mechanism of this characteristic is involved with the mutation of cell cycle regulatory genes which allows for a cell to avoid the checkpoint control systems[30]. It has been revealed that treatment with RTA 404 in various types of cell lines could induce cell cycle arrest in different phases. Alabran et al observed an increase in sub-G1/G0 and severe depletion in the S-phase populations following treatment with RTA 404. Severe depletion in S-phase indicates that DNA synthesis is extremely sensitive to RTA 404. However, the author also cannot rule out G2/M block in addition to the S-phase depletion, since cells trapped in the G2/M at the time of treatment by triterpenoids did not progress into G1/G0 phase[14]. In the human brain, malignant glioma cell lines incubated with RTA 404 resulted in cell cycle arrest in the G2/M phase [15]. As shown in Fig. 5, treatment with RTA 404 led to an increment in the G2/M phase cell population, implying that GBM 8401 cells underwent a delay in the G2/M phase checkpoint. These results suggested that exposure to RTA 404 enhanced cell populations in the G2/M phase.

Cancer cells frequently display unscheduled division. Cell cycle progression is dependent on highly ordered events controlled by a subfamily of cyclin-dependent kinases (CDKs), the activity of which is modulated by cyclins[31]. The cyclin B1/Cdk1 complex is shown to specifically regulate the entry into mitosis [32]. A large number of proteins are phosphorylated by the cyclin B1–Cdk1 complex prior to mitotic entry. Through its cytoplasmic, nuclear, and centrosomal localization, cyclin B1/Cdk1 is able to synchronize different events in mitosis such as nuclear envelope breakdown and centrosome separation [33]. Regulation of the mitotic events is linked to the control of the activity of the cyclin B1–Cdk1 complex to make cells enter mitosis, arrest at G2-phase, or skip mitosis [34]. In our study showed that treatment with RTA 404 led to an increment in the G2/M phase cell population, implying that GBM 8401 cells underwent a delay in the G2/M phase checkpoint. Exposure to RTA 404 resulted in an increase in the mitosis protein. These results indicated that RTA 404 increased mitosis protein synthesis. We found the

protein expressions of cyclin A2, cyclin B1 and CDK1 to be significantly down-regulated in RTA 404-treated GBM8401. Co-IP is an effective means of quantifying protein-protein interactions in cells (Fig. 9). They were found to have significant decreases in Cyclin B1, CDK1, and cyclinB1/CDK1 complex. These results suggest that the increased mitosis protein synthesis, G₂/M phase arrest may occur via Cyclin B1, CDK1, and Cyclin B1/CDK1.

Conclusions

In summary, RTA 404 significantly inhibited the proliferation and induced cell apoptosis on GBM 8401 cell line; it induced glioma cell cycle arrest and accumulation of G₂/M-phase via inhibiting the Cyclin B1, CDK1, and Cyclin B1/CDK1. These findings suggest that CDDDO-TFEA can inhibit proliferation, cell cycle progression and induce apoptosis in GBM cells *in vitro*, possibly through its inhibition of Cyclin B1, CDK1, and Cyclin B1/CDK1. Therefore, RTA 404 may not only induce cell cycle G₂/M arrest, but it may also exert apoptosis in established GBM cells.

Materials And Methods

Materials

2-cyano-3,12-dioxo-N-(2,2,2-trifluoroethyl)-oleana-1,9(11)-dien-28-amide, RTA 404, was purchased from Cayman Chemical, DMSO (dimethyl sulfoxide) and PrestoBlue™ Cell Viability Reagent (PB) were purchased from ThermoFisher (USA). Cell culture medium (DMEM), fetal bovine serum, antibiotics, sodium pyruvate, trypsin, and phosphate-buffered saline (PBS) were obtained from Gibco, BRL (Grand Island, NY), and polyvinylidene fluoride membrane (PVDF) (Millipore), and molecular weight markers from Bio-Rad (USA). All other reagents and compounds were of analytical grade.

Cell culture

Human brain malignant glioma GBM 8401 cells were obtained from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). All cell lines were incubated in an atmosphere containing 5% CO₂ at 37°C. GBM8401 cells were cultured in an RPMI1640 medium with supplemental 10% fetal bovine serum (FBS) and U87 MG cells in modified Eagle's medium (MEM) with supplemental 10% FBS.

Cell viability

A density of 5000 cells was suspended in a culture medium containing 10% FBS and placed in a 96-well plate (0.1 ml of medium per each well) and incubated in an atmosphere containing 5% CO₂, saturated humidity, and 37°C for 24 h. The cells were added with 0, 1, 1.5, 2 µM RTA 404 and incubated with PrestoBlue™ Cell Viability Reagent for 10 mins. The reaction was measured at 570 nm using a multiwell plate reader (IQUANT; BioTek Instruments, Inc., USA). When there were no cells, we subtracted the background absorbance of the medium. All samples were assayed at least in triplicate, and the mean

was calculated for each experiment. Results were expressed as a percent of control, control being 100%. Each experiment was performed in triplicate with a mean (+/-SEM) used to express results.

Cell cycle analysis

The GBM8401 cells were plated in 6-well plates (1×10^6) in a cell incubator and cultured overnight. The next day, the cells were centrifuged in a 10ml centrifuge tube and the supernatant was collected. They were washed two times in PBS and added with 1x Trypsin. They were then placed in a 37 ° C oven for 1–2 minutes. Once the cells fell off, they were collected in a centrifuge tube run at 2500 rpm for 5 minutes to remove the supernatant. Then, 1 ml of PBS was added to wash the remaining culture solution and the cells were centrifuged again at 2500 rpm for five minutes. 500 µl of PBS was added to break up the cell pellet and then 500 µl of 70% ethanol was slowly added to the cells for fixation. They were placed in a refrigerator and left there overnight. The next day those cells were centrifuged at 2500 rpm for 5 min, and the supernatant containing Ethanol was removed. The cells were washed in one ml PBS. 5 µl of RNase A 100 mg /ml was added to PBS and placed in an oven at 37 ° C.

To facilitate cell cycle analysis, a fluorescent nucleic acid dye PI was used to identify the proportion of cells in each of the three inter-phase stages. After a 30-minute reaction time, 20 µl of propidium iodide 2mg/ml (final concentration 40 µg/ml) was added and the cells were placed an oven at 37 ° C for 15 minutes. The cells were treated with RTA 404 for 24 h followed by harvesting and fixing in 1mL of ice-cold ethanol (70%) at -20° C for at least 8 h. DNA was stained with PI/RNaseA staining buffer, and the cell cycle was analyzed using a FACSCalibur flow cytometer. Data were interpreted using WinMDI 2.9 software.

Apoptosis measurement

The cells were cultured in 6 well culture plates (Orange Scientific, EU). After exposure to RTA 404 for 4 h, the cells were harvested by centrifugation, resuspended in, and incubated with 1 x annexin-binding buffer containing 5 IL of annexin V-FITC and 1 IL of propidium iodide (PI) (100 mg/mL), and incubated at room temperature for 15min. The stained cells were analyzed on a FACSCalibur flow cytometer (BD Pharmingen) using WinMDI 2.9 free software (BD Pharmingen).

Evaluation of mitochondrial membrane potential

The cells were seeded into 24-well plates (Orange, United Kingdom). Following treatment with RTA 404 for 6 h, we added 10 µg/mL JC-1 (Sigma, USA) to the culture medium at 50 µL/well, which was then incubated at 37°C for 20 min for mitochondrial staining. After being washed twice with warm PBS, the cells were fixed with 2% paraformaldehyde and detected by FACS Calibur flow cytometer (JC-1). Data were analyzed using WinMDI 2.9. JC-1 was also *detected by using* fluorescence microscopy (Olympus CKX41 and U-RFLT 50, Japan).

Western blotting

All samples were lysed in 200 µl of lysis buffer. A total of 50–75µg of protein per sample were loaded onto 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis membranes for electrophoretic separation and then transferred to PVDF membranes and subjected to electrophoresis at 50 V for 4 h. After blocking overnight with Odyssey blocking buffer (USA), the membranes were incubated with primary antibodies [Cyclin A2 (1:1000; proteintech; 18202-1-AP), Cyclin B1 (1:1000; proteintech; 55004-1-AP), CDK1 (1:1000; cell signaling; E1Z6R), NRF2 (1:1000; proteintech; 16396-1-AP), CHK2 (1:1000; abgent.com; AP4999a), p-CHK2 (1:1000; abgent; AP50241), CHK1 (1:1000; proteintech; 22018-1-AP), p21 (1:1000; Cell Signaling; #2947), and β-actin (1:20000; Sigma; A5441)] for 2 h at room temperature. Subsequently, the membranes were washed several times and then incubated with a corresponding secondary antibody (IRDye Li-COR, USA) at a dilution of 1:20,000 for 30–45 min. Antigens were then visualized using a near-infrared fluorescence imaging system (Odyssey LICOR, USA), and the data were interpreted using the Odyssey 2.1 software or a chemiluminescence detection kit (ECL; Amersham Corp., Arlington Heights, IL, USA).

Mitotic index analysis

Mitotic index was assessed based on MPM-2 (anti-phospho-Ser/Thr-Pro) expression. After treatment with CDDOTFEA, cells were harvested and fixed in 70% ethanol overnight. The cells were then washed and suspended in 100 µL of IFA-Tx buffer (4% FCS, 150 nM NaCl, 10 nM HEPES, 0.1% sodium azide, and 0.1% Triton X-100) with the MPM-2 antibody at room temperature for 1 h. The cells were then washed and resuspended in IFA-Tx buffer with a rabbit anti-mouse FITC-conjugated secondary antibody (1:50 dilution; Serotec) for 1 h at room temperature in the dark. Finally, the cells were washed and resuspended in 500 µL of PBS with 20 µg/mL PI (Sigma) for 30 min in the dark. MPM-2 expression was measured by FACSCalibur flow cytometer. Data were analyzed using WinMDI 2.9.

Co-immunoprecipitation (Co-IP)

Co-IP is an effective means of quantifying protein-protein interactions in cells. Briefly, after incubation at room temperature overnight, 500 mg of cellular proteins were labeled using anti-CDK1 antibody. The protein–antibody immunoprecipitates were collected using protein A/G plus-agarose beads (SC-2003 Santa Cruz BioTechnology). Following the final wash, the samples were boiled and centrifuged to transform the agarose beads into pellets. Finally, cyclin B1 proteins were performed using western blot analysis. Antigens were visualized and data were analyzed using Odyssey 2.1 software.

Data analysis

Data are expressed as the mean ± standard error of the mean of at least three independent experiments. Student's t-test or one-way analysis of variance with Scheffé's posthoc test was used for statistical analysis. A P-value of < .05 was considered statistically significant.

Abbreviations

Antioxidant Response Element (ARE)

2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO)

CDDO imidazolid (CDDO-Im)

CDDO methyl ester (CDDO-Me)

CDDOtrifluoroethyl amide(RTA 404)

Glioblastoma multiforme (GBM)

Kelch-like ECH-associated protein 1 (Keap1)

Nuclear factor erythroid 2-related factor 2 (Nrf2)

Reactive nitrogen species (RNS)

World Health Organization (WHO)

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The data used to support the findings of this study are available in article supplementary material.

Competing interests

The Authors declare that there is no conflict of interest.

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Authors' contributions

All authors contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

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Figures

Figure 1

RTA 404 mediates the survival of GBM8401 cell by inhibiting proliferation: Cells were treated with increasing doses of RTA 404 for 24-72h in vitro. The survival of RTA 404-treated cancer cells was measured using the PB assay. Results are expressed as percentage of the control, control being 100%. All data are reported as the mean ($\pm$ SEM) based on results of at least three separate experiments. Statistical analysis was performed using the t test, with differences between the treatment and control groups (0 μM RTA 404, # vs 24 hrs treatment) considered significant at $p < 0.05$, delineated by * and # SEM, standard error of the mean; RTA 404, CDDO trifluoroethyl amide.

Figure 2

RTA 404 induced cell apoptosis on GBM 8401 cells. (A) Annexin V-FITC and PI staining (B) Results are expressed as percentage of the control, control being 100%. All data are reported as the mean ($\pm$ SEM) based on results of at least three separate experiments. Statistical analysis was performed using the t test, with differences between the treatment and control groups (0 μ M RTA 404) considered significant at $p < 0.05$, delineated by *.

Figure 3

The disruption of mitochondrial membrane potential due to RTA 404 treatment. (A) JC-1 was also detected by using fluorescence microscopy and flow cytometry. (B) Results are expressed as percentage of the control, control being 100%. All data are reported as the mean ($\pm$ SEM) based on results of at least three separate experiments. Statistical analysis was performed using the t test, with differences between the treatment and control groups (0 μM RTA 404) considered significant at $p < 0.05$, delineated by *.

Figure 4

(A) Numbers of active caspase-3 induced by RTA 404 treatment on GBM 8401 cell line. (B) Results are expressed as percentage of the control, control being 100%. All data are reported as the mean ($\pm$ SEM) based on results of at least three separate experiments. Statistical analysis was performed using the t test, with differences between the treatment and control groups considered significant at $p < 0.05$, delineated by *.

Figure 5

(A) Cell cycle arrest in G2/M phase because of RTA 404 treatment on GBM 8401 cell line. The cell cycle analysis of the cancer cells after being cultured with RTA 404 for 24 h. RTA 404 induced an increase in the G2/M phase cell percentage (%). Cells underwent staining with propidium iodide to analyze DNA content, which was then quantified through flow cytometry (Figure 5B). In each group of bars, * indicates

that the number of G2/M cells in the RTA 404 treatment group was significantly higher than that of the control group ($P < .05$).

Figure 6

Mitosis protein synthesis inhibited by RTA 404 treatment on GBM 8401 cell line. Flow cytometry analysis of MPM-2 expression in RTA 404-treated GBM 8401 cells. (A) Cells were untreated or treated with 0, 1, 1.5 and 2 μ M RTA 404. After 24 h of treatment, cells were fixed with 70% ethanol, stained with MPM-2 and PI, and analyzed by FACSscan. (B) Results are expressed as percentage of the control, control being 100%. All data are reported as the mean ($\pm$ SEM) based on results of at least three separate experiments. Statistical analysis was performed using the t test, with differences between the treatment and control groups (0 μ M RTA 404) considered significant at $p < 0.05$, delineated by *.

Figure 7

(A) RTA 404 regulated cell cycle related gene expression (Cyclin A2, Cyclin B1, CDK1) and apoptosis (p21) related gene expression in GBM8401 cells. Cells were treated with RTA 404 for 24 h. Gene and protein expressions were subsequently detected using Western blot analysis. (B) Results are expressed as percentage of the control, control being 100%. All data are reported as the mean ($\pm$ SEM) based on results of at least three separate experiments. Statistical analysis was performed using the t test, with differences between the treatment and control groups considered significant at $p < 0.05$, delineated by *

Figure 8

Co-immunoprecipitation (Co-IP) is an effective means of quantifying protein–protein interactions in cells (Primary target CDK1, secondary target Cyclin B1). Cells were treated with RTA 404 for 24 h. Gene and protein expressions were subsequently detected using Western blot analysis. (B) Results are expressed as percentage of the control, control being 100%.

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