Betulinic acid inhibits glioma cell viability by down-regulation of NF-κB and enhancement of apoptosis

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INTRODUCTION

Glioblastoma multiforme accounts for almost 30% of primary tumors in the brain, and it is a major cause of carcinoma deaths associated with intracranial malignant tumors [1]. Sustained and effective efforts have led to the development of the novel therapeutic strategy of NovoTTF-100A System for glioblastoma [2]. Moreover, the chemotherapeutic drug temozolomide has been found effective in the treatment of glioblastoma [3]. However, the...
clinical benefits of these therapeutic strategies are limited by the presence of genetic heterogeneity in glioblastoma patients [4]. The potential of therapeutic agents to target a single factor in a molecular pathway is nullified by compensation from another pro-survival signaling pathway [4]. Thus, the requirement for an effective therapeutic candidate that can inhibit glioblastoma cell growth by down-regulation of survival pathways through multiple targets, is yet unmet. Many bioactive molecules have produced effective radio-sensitization effects in the treatment of various carcinomas [5-7]. Several types of carcinomas such as brain tumor, breast cancer and ovary cancer are effectively inhibited by natural compounds isolated from plants [5-7]. It has been reported that anti-cancer compounds inhibit cancer cell growth through diverse mechanisms [8]. Some compounds inactivate enzymes associated with pro-survival signaling pathways, or target major apoptosis mediators and Bcl-2 family members [8]. The frequently influenced pro-survival pathways are NF-κB and phosphoinositide 3 kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathways [8]. The targeting of these pathways with therapeutic agents has produced very successful results [8]. Betulinic acid is a lipophilic molecule belonging to the class of triterpenoid compounds encompassing various structural features suitable for diverse chemical transformations [9,10]. Structural modification and fine tuning of betulinic acid have resulted in many compounds with broad range of activities [9,10]. The present study was carried out to determine the inhibitory potential of betulinic acid on pro-survival signaling pathways in glioblastoma cells so as to develop an effective treatment for the disease.

EXPERIMENTAL

Cell lines and cell culture

The U87MG and A172 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in plates in DMEM at 37 °C in an incubator with humid atmosphere containing 5 % CO₂ and 95 % oxygen. The incubation medium contained 10 % newborn calf serum and antibiotics. The primary cultured astrocytes were a gift from Dr. Zhang, while betulinic acid was purchased from Sigma-Aldrich.

Cell viability assay

Changes in the viabilities of the glioma cells (U87MG and A172) and primary astrocytes were measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The cells were seeded in 96-well plates in DMEM, each at a density of 2 x 10⁶ cells per well, and cultured for 24 h. Then, gradient concentrations betulinic acid (1.25, 2.5, 5.0, 10, 20 and 40 µg/mL) were added to the plates. After 24 and 48 h, 20 µL MTT (5 mg/ mL) was added to each well, followed by incubation for 4 h. Thereafter, the medium was replaced with DMSO to solubilize the resultant formazan crystals. The absorbance of each plate was read at 487 nm using a microplate reader (BioRad Laboratories, Hercules, CA).

Hoechst 33342 staining

The apoptotic nuclei in glioma cells (U87MG and A172) and primary astrocytes were detected using Hoechst 33342 staining. Cells grown for 24 h in 6-well plates were treated with betulinic acid (8.75 µg/mL) for 24 h. Following washing with PBS (two times), the cells were fixed for 2.5 h in 70 % ethyl alcohol at 4 °C. Then, the cells were incubated with Hoechst 33342 stain for 5 min, followed by washing in PBS, after which the cell nuclei were examined for morphological changes under a fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Apoptosis assay using Annexin V/propidium iodide

Apoptosis of glioma cells (U87MG and A172) and primary astrocytes was analyzed using Annexin V-FITC/PI Kit (ImmunoTech Co. Marseille, France). The cells were plated in 60-mm dishes at a density of 2 x 10⁶ cells per dish, and were treated with betulinic acid (8.75 µg/mL) for 24 h. The harvested cells were rinsed twice in PBS, followed by incubation for 20 min with AnnexinV and PI conjugated to fluorescein. Then, flow cytometry was used for apoptosis determination, and the data were analyzed using Modfit software.

Immunocytochemical analysis

Immunocytochemical analysis of NF-κB p65 located in the nucleus and determination of immunoreactivity scores were carried out using methodology reported previously [11]. The glioblastoma cells (U87MG and A172) and primary astrocytes were treated with betulinic acid (8.75 µg/mL) and cultured on glass slides. After 24 h, the cells were incubated with mouse monoclonal primary antibody against NFκB p65, followed by incubation with biotinylated goat anti-mouse IgG secondary antibody.

Western blot analysis

The glioblastoma cells (U87MG and A172) and primary astrocytes were treated with betulinic acid (8.75 µg/mL) for 24 h, and were lysed using RIPA buffer (150 mM sodium chloride, 1.2 % NP-40, 0.6 % sodium deoxycholate, 0.2% SDS, 50 mM Tris-hydrochloric acid, pH 8.0; 10 mM EDTA and 1 mM PMSF) for 40 min at 4°C. The lysate was subjected to centrifugation for 25 min at 12000 x g, and protein concentration was measured with BCA Assay Kit. Following resolution on 10 % SDS-PAGE, the proteins were transferred to PVDF membranes which were treated with a mixture of TBS, Tween-20 (0.1%) and milk (5%; w/v) for 2 h to block non-specific binding sites. Then, the membranes were incubated overnight at 4°C with primary antibodies against p65, Survivin, XIAP, Bax, Bcl-2, cleaved caspase-9, cleaved caspase-3 p11, followed by washing in PBS and incubation for 1 h with HRP-conjugated anti-rabbit IgG secondary antibody at room temperature. Quantification of band intensities was done using image analysis software ImageJ.

Determination of nuclear NF-kappaB level

The glioma cells (U87MG and A172) and primary astrocytes were fractionated after betulinic acid treatment, using Qproteome Nuclear Protein Kit (No. 37582). The nuclear and cytosolic fractions were generated according to the instructions from the kit manufacturer. Equal amounts of lysates from nuclear and cytosolic fractions were subjected to assay of expression of NF-kappa B using western blotting. The primary antibodies used for incubation were anti-p65 and anti-Lamin B.

Statistical analysis

Data are presented as mean ± SD. Statistical analysis was carried out with One-Way Analysis of Variance (ANOVA), followed by Friedman test. The statistical analysis of data was performed using SPSS 16.0 software (SPSS, Chicago, IL). A value of \( p < 0.05 \) was taken as indicative of statistically significant difference.

RESULTS

Suppressive effect of betulinic acid on glioblastoma cell viability

The U87MG and A172 cells were treated with gradient concentrations of betulinic acid (1.25, 2.5, 5.0, 10, 20 and 40 µg/mL), and changes in viability were measured at 24 and 48 h (Figure 1). The proliferative potential of U87MG and A172 cells were significantly reduced on treatment with betulinic acid in a concentration-and time-dependent manner. The reduction of proliferative potential by betulinic acid was more prominent in A172 cells than in U87MG cells. At 24 h, the proliferative potential of U87MG and A172 cell were suppressed to 31.5 ± 0.7 and 26.6 ± 0.5%, respectively, relative to control cells, on treatment with betulinic acid at a dose of 20 µg/mL betulinic acid. The concentrations of betulinic acid required to bring about 50% growth inhibition at 24 h were 8.75 and 7.6 µg/mL for U87MG and A172 cells, respectively. At 48 h, the 50 % growth inhibitory concentrations of betulinic acid for U87MG and A172 cells were 4.3 and 3.75 µg/mL, respectively. However, the proliferative potential of primary astrocytes was not affected by betulinic acid at a dose of 40 µg/mL for 48 h.

Figure 1: Effect of betulinic acid on proliferations of A: U87MG cells, B: A172 cells, and C: astrocytes. Following 24 and 48 h exposure to gradient concentrations of betulinic acid (1.25, 2.5, 5.0, 10, 20 and 40 µg/mL), cell proliferation was measured with MTT assay; \( *p < 0.05 \); \( **p < 0.02 \), vs. untreated cells

Betulinic acid induced apoptosis of U87MG and A172 cells

Shrinkage and detachment from culture flask surface were prominent in U87MG and A172 cells on treatment with 8.75 µg/mL betulinic acid at 24 h (Figure 2 A). In contrast, the astrocytes neither showed shrinkage in size nor any detachment from the flask surface on treatment
with 8.75 µg/mL betulinic acid at 24 h. Hoechst 33342 staining of U87MG and A172 cells also confirmed condensation of nuclei on treatment with betulinic acid (Figure 2 B). Again, there were no apoptotic changes in astrocytes treated with betulinic acid for 24 h.

**Figure 2**: Effect of betulinic acid on morphologies of U87MG and A172 cells. (A) The U87MG, A172 and astrocyte cells were observed under inverted microscope after 24 h treatment with betulinic acid (8.75 µg/mL). (B) Hoechst 33342-stained U87MG cells, A172 cells and astrocytes, as observed using fluorescent microscopy.

Apoptotic changes in U87MG cells, A172 cells and astrocytes were also determined using Annexin V/PI assay (Figure 3). Treatment with betulinic acid at a dose of 8.75µg/mL for 24 h increased apoptosis in U87MG cells to 41.8 ± 0.5%, relative to 1.86 ± 0.2 % in untreated cells. Apoptosis in A172 cells was increased to 48.8 ± 0.5% on treatment with 8.75 µg/mL betulinic acid for 24 h, relative to 2.89 ± 0.2% in control cells. In astrocytes, betulinic acid treatment for 24 h had no effect on apoptosis.

**Figure 3**: Effect of betulinic acid on cell apoptosis. (A) U87MG cells, A172 cells and astrocytes after 24 h of betulinic acid treatment (8.75 µg/mL), as analyzed using Annexin V/PI assay. (B) Semi-quantified data from Annexin V/PI assay; *p < 0.05; **p < 0.02, vs. untreated cells

Betulinic acid suppressed intracellular NF-κB level and its nuclear translocation

Immunocytochemistry showed significant decreases in the levels of intracellular NF-κB p65 in U87MG and A172 cells on treatment with betulinic acid (Figure 4 A). The immunoreactivity score for intracellular NF-κB p65 was decreased to 3.6 ± 0.3% in U87MG cells on treatment with betulinic acid, relative to 8.9 ± 0.6 % in control cells. In A172 cells, the immunoreactivity score for intracellular NF-κB p65 was reduced to 2.1 ± 0.2 % by betulinic acid, relative to 7.8 ± 0.5 % in control cells. Betulinic acid treatment of U87MG cells decreased nuclear NF-κB p65/total NF-κB p65 from 39.5 ± 1.8 to 10.8 ± 0.4% at 24 h (Figure 4 B). In A172 cells, the level of nuclear NF-κB p65/total NF-κB p65 was reduced from 49.5 ± 2.0 to 8.32 ± 0.4 % on treatment with betulinic acid. However, betulinic acid treatment caused no significant change in immunoreactivity score for NF-κB p65 in astrocytes.

Betulinic acid suppressed IAPs

Treatment with betulinic acid suppressed levels of survivin and XIAP in U87MG and A172 cells at 24 h, relative to control cells (Figure 5). The Bcl-2 levels in betulinic acid-treated U87MG and A172 cells were also markedly lower than the corresponding levels in the control cells. In contrast, Bax levels in U87MG and A172 cells was markedly enhanced, relative to control, on treatment with betulinic acid. However, there were no significant alterations in Bcl-2/Bax ratio in astrocytes treated with betulinic acid for 24 h.

Betulinic acid stimulated activation of caspase-9 and caspase-3

Changes in caspase-9 and caspase-3 activation by betulinic acid treatment in U87MG, A172 cells and astrocytes were determined using western blot (Figure 6). In astrocytes, betulinic acid did not affect the activation of caspase-9 and caspase-3. However, activation of caspase-9 and caspase-3 in U87MG and A172 cells were markedly increased on treatment with betulinic acid for 24 h.
DISCUSSION

Glioblastoma is considered a major challenge to clinicians because of complications associated with its resection [12]. The treatments used for glioblastoma are comprised of surgical resection, administration of chemotherapy and exposure to radiotherapy [12]. The discovery of irinotecan and temozolomide has, to some extent improved the quality of life of some glioblastoma patients, although about 50% of the patients display resistance to chemotherapeutic agents [13].

The present study determined the growth-inhibitory effect of betulinic acid on U87MG and A172 glioblastoma cells. The study demonstrated a significant suppression of the viabilities of U87MG and A172 cells by betulinic acid via apoptosis induction. Betulinic acid-treated U87MG and A172 cells showed detachment from flask surface, shrinking in shape and condensed nuclei. On the other hand, primary astrocytes were not affected by betulinic acid. The NF-κB levels in cells, and their nuclear translocation were markedly suppressed by betulinic acid in U87MG and A172 cells. The survival signals induced by NF-κB were regulated differentially by betulinic acid in glioblastoma cells and astrocytes.

Genetic studies have shown that proteins of the NF-κB signaling pathway are constitutively activated in glioblastoma cells [14]. It is known that NF-κB mediates cell survival signals, activates several target genes involved in survival, and suppresses apoptosis [15]. The phenotypic malignant behavior of glioblastoma cells is regulated by NF-κB which is present in the nucleus [16]. It has been reported that bortezomib increases anti-tumor efficiency of docetaxel through downregulation of NF-κB [17]. In the present study, betulinic acid suppressed the levels of intracellular NF-κB as well as their nuclear translocation in U87MG and A172 cells. This suggests that betulinic acid-mediated apoptosis of U87MG and A172 cells is associated with suppression of NF-κB activation. Two members of apoptosis inhibitor family i.e. survivin and XIAP are part of pro-survival signaling pathway [18]. The pathway functions by generating resistance towards pro-apoptotic signals initiated by therapeutic compounds in cells [18].

Studies have found that suppression of these apoptosis inhibitors by therapeutic compounds leads to glioblastoma cell death [19]. The expression of survivin is markedly enhanced as glioma deteriorates from low to high grade [20]. An inverse relationship between cellular apoptosis and expression of survivin have been reported in glioblastoma cells [21]. Thus, surviving is believed to be the key target for therapeutic agents for glioblastoma [21]. The resistance of glioblastomas to therapeutic agents is attributed to the presence of survivin [22]. Suppression of survivin results in activation of apoptosis by promoting pro-apoptotic proteins and catalyzing cleavage of caspases [22]. In the present study, betulinic acid down-regulated survivin and XIAP in U87MG and A172 cells. Moreover, Bcl-2 was suppressed, while Bax, caspase-9 and -3 were enhanced in U87MG and A172 cells on treatment with betulinic acid.

CONCLUSION

Betulinic acid acts as an inhibitory agent against the proliferation of U87MG and A172 glioblastoma cells, and mediates their apoptosis. It exerts anti-proliferative effect via downregulation of NF-κB activation and suppression of apoptosis inhibitors. Moreover, caspase-9 and caspase-3 are activated, while Bcl-2 is reduced by betulinic acid in glioblastoma cells. These findings point to the need for in vivo studies aimed at validation of the potential of
betulinic acid as a therapeutic agent for glioblastoma.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Zhu Yaozu and Yang Liu contributed to this work equally. Hong Jiang, Yan Chen- conceived and designed the study; Wei Zhang, Jing Yu, Manli Feng, Xiaoyu Wang, Xiaopeng Wang, Yanan Jiao, Chengcheng Wang- collected and analyzed the data; Hong Jiang, Wen li Guo, while Dongdong Zhu -wrote the manuscript. Yan Chen-Approved final version of the manuscript. All authors read and approved the manuscript for publication.

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REFERENCES


