Original article

Inhibition of tumor growth and angiogenesis by 2-(4-aminophenyl) benzothiazole in orthotopic glioma C6 rat model

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A B S T R A C T

In the present study antitumor effect of 2-(4-aminophenyl) benzothiazole (BTZ) was evaluated against human U251 and rat C6 glioma cell lines using MTT assay. It was observed that BTZ exhibited significant antitumor effect with IC50 of 3.5 and 4 μM against human U251 and rat C6 glioma cells respectively. To gain in-depth insights about the antitumor effect of BTZ, glioma xenograft rat model was prepared. The rats were treated with 10 mg and 15 mg/kg body weight doses of BTZ daily for 21 days after C6 cell administration. Treatment of the rats with BTZ reduced the tumor volume to 12% compared to 100% in the untreated rats. TUNEL assay showed a remarkable increase in the proportion of apoptotic cells in the BTZ treated rats than those in the untreated rats. The increase in the population of apoptotic cells was 23-fold compared to control. Immuno-histological staining revealed marked reduction (16%) in the proportion of CD31-stained vessels in the BTZ treated rats than those of the untreated rats. These changes were accompanied with decreased transcript levels of vascular endothelial growth factor (VEGF) and the VEGF receptor Flt1 as well as ERK1/2 and matrix metalloproteinase-2 (MMP2). Moreover, BTZ altered the expression of several cell cycle control proteins. While as pRb protein expression decreased, E2F1 remained unaltered and cyclin D1 protein and p53 expression was enhanced. Taken together, the results indicate that BTZ is a potent inhibitor of glioma cell proliferation in vivo and exerts its effects on cell cycle control and angiogenesis related proteins.

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1. Introduction

Malignant glioma constitutes the commonly detected primary tumor of brain in adults throughout the globe and leads to very high of mortality (Wen and Kesari, 2008). Although numerous efforts have been performed to investigate the treatment for glioma but the rate of prognosis remains poor. Studies have demonstrated that gliomas do not metastasize to distant organs but undergo local infiltration and are considered to be the highly invasive type tumors (Maher et al., 2001). Currently gliomas are treated using radiotherapy, chemotherapy and surgery either alone or in combination (Chang et al., 2004). In spite of the currently available strategies treatment of the gliomas is very difficult. Taken into the consideration, the current status of gliomas, studies are constantly being performed to develop new treatment strategies (Marx et al., 2001; Fine et al., 2003). These studies have led to the identification of some molecules which are in the clinical trials because of their promising preliminary results.

Benzothiazoles because of the diverse range of promising biological activities constitute interesting molecules for chemists and clinicians worldwide. The various biological activities exhibited by the benzothiazoles include antimicrobial (Gupta et al., 2009; Kumbhare and Ingle, 2009), anticancer (Stanton et al., 2008), anthelmintic (Sreenivasa et al., 2009), anti-diabetic (Pattan et al., 2005) scaffolds. Not only benzothiazole scaffold but even the natural products obtained from marine origin embodying benzothiazole system possess impressive bioactive profile. One of the members of benzothiazole family, 2-aryl benzothiazole has been used for radioactive imagining (Reddy et al., 2007) and anticancer agent (Heo et al., 2006). Biological evaluation of 2-aminobenzothiazoles led to the identification of its prominent
cytotoxic activity against several types of carcinoma cells (Piscitelli et al., 2010). This compound has also been found to act as potent and selective antitumor agents. In the present study effect of 2-[(4-amino phenyl) benzothiazole (benzothiazole) on the tumor growth in glioma rat model was investigated. The results revealed that benzothiazole inhibits tumor growth through inhibition of cell proliferation and angiogenesis by exerting its effects on angiogenesis related and cell cycle control proteins.

2. Materials and methods

2.1. Cell culture

Human U251 and glioma C6 rat cells were purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China. The cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (Life Technologies).

2.2. MTT assay

The cytotoxic effect of caffeic acid n-butyl ester was measured against human U251 and rat C6 glioma rat cells using the MTT assay (Mosmann, 1983). The cytotoxic effect of caffeic acid n-butyl ester against all the cancer cell lines was expressed as IC50 values.

2.3. Preparation of orthotopic rat model

Sprague Dawley rats were purchased from the Shanghai Laboratory Animal Center (SLAC, Shanghai, China). The animals were housed in an environment free of pathogens in the animal care facility center of the Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The approval for present study was obtained from the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). For the administration of C6 glioma cells, the animal after anesthetization was shaved. The rats were put into the stereotactic frame. A small screw was used for the administration of 2 × 106 cells into the brain white matter of the rats slightly deep.

2.4. Treatment strategy

Benzothiazole was purchased from Sigma-Aldrich (St. Louis, MO, USA) and its stock solution was prepared in DMSO. The animals were divided into four groups of 5 each randomly. The control group were given normal saline (0.9% NaCl) while as those in the treatment groups received intraperitoneally 10 and 15 mg/kg doses of benzothiazole daily for 21 days after administration of C6 glioma cells.

2.5. Extraction of rat brain

On the day 16 following treatment, the rats were sacrificed to extract the brains. The brain from each rat was divided into two halves, one half was embedded in paraffin after treatment with formalin. The other half was stored under cold conditions of liquid nitrogen. Volume of the tumor in each animal was calculated after measurement of the tumor dimensions.

2.6. Immunohistochemistry

The right half of the rat brain was fixed in 4% paraformaldehyde solution and paraffin embedded. The tissue was sliced into 5 μm sections for examining the tumor cell proliferation and micro vessel density quantification by proliferating cell nuclear antigen (PCNA) and CD31 immuno histo-chemistry respectively as described previously (Weidner et al., 1991). PCNA stained was carried out to investigate tumor cell proliferation. PCNA specifically exhibited its expression in the proliferating cells and therefore, positive cells are depicted as brown. CD31 exhibits high affinity for vascular endothelial cell after brown-staining by biotinylination. CD31 vessel immunostaining was carried out as described previously (Peng et al., 2008) to examine the angiogenesis in tumor tissues. Microvessel that showed brown-staining was considered as a countable microvessel. Briefly, Sections were deparaffinized and rehydrated. Afterwards antigen retrieval was carried out with retrieval buffer. The peroxidase activity was repressed by 3% H2O2. The sections were then incubated with 10% normal goat serum to block the non-specific binding of reagents. Rat antimonious CD31 antibody (1:100, Santa Cruz Biotechnology) and mouse anti-human PCNA antibody (1:100, Santa Cruz Biotechnology) were applied overnight as primary antibody in a moist chamber at 4 °C. Goat anti-rat immunoglobulin (1:100, Santa Cruz Biotechnology) and goat anti-mouse immunoglobulin (1:100, Santa Cruz Biotech).

2.7. Tunnel assay

The TUNEL assay was carried out by the in situ Cell Death Detection Kit (Roche, Switzerland), as per manufactures protocol. Briefly, 5 μm sections from paraffin-embedded tumor tissues were de-waxed and hydrated and incubated with proteinase K (200 μg/ml) at 21 °C for 30 min. Afterwards, the TUNEL reaction mix containing DIG-dUTP, terminal deoxynucleotidyl transferase, and reaction buffer was added to the slides. The slides were then incubated in a humidified chamber for 2 h at 37 °C. This was followed by washing and incubation. The slides were then incubated with biotin anti-digoxin-specific antibody (1:100; Abcam Inc, Cambridge, UK). Diaminobenzidine staining was used to visualize the TUNEL-positive cells (brown color) by microscopy (Olympus, Tokyo, Japan).

2.8. Determination of mRNA expression by RT-PCR

To examine the mRNA expression, samples were dissected with the aid of a surgical microscope and used for total RNA extraction with Trizol (Life Technologies, USA). The cDNA synthesis was carried out using 1 μg RNA as previously described (Ribeiro et al., 2008). PCR amplification conditions were; 1 min at 94 °C. 30 s at primer-specific annealing temperature, 1 min extension at 72 °C and final extension of 10 min at 72 °C. To ensure the exponential phase of amplification, the number of PCR cycles was determined and optimized for each of the genes.

2.9. Western blotting

About 20-μg of proteins from each sample were electrophoresed through, 7.5% SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL membrane, Amersham Pharmacia Biotech). Immunoblotting was carried out as described previously (Benadiba et al., 2009) and the fluorescent bands (Alexa-488 label) were visualized in an image system (Molecular Dynamics Typhoon 8600 Variable Mode Imager). The secondary antibodies conjugated with Alexa-488 (anti-goat, anti-mouse and anti-rabbit) used were produced in donkey (Invitrogen –Molecular Probes).
2.10. Statistical analysis

The data presented are the means ± SD. The groups were compared using Student’s t-test and ANOVA. The values were considered statistically significant at p < 0.05.

3. Results

3.1. Cytotoxic activity of BTZ against human U251 and rat C6 glioma cell lines

To evaluate the anti-proliferative role of BTZ, U251 were treated with BTZ concentration range 0.01–20 μM for 48 h. The BTZ showed a dose-dependent inhibition of human U251 rat C6 glioma cell lines with IC50 of 3.5 and 4 μM respectively.

3.2. BTZ treatment inhibits tumor growth in an orthotopic rat glioma model

Treatment of the rats bearing glioma with BTZ daily for 21 days caused a significant reduction in the tumor volume compared to the control group (Fig. 1). The tumor volume in BTZ treated rats was reduced to 21 and 12%, respectively on treatment with 10 and 15 mg/kg doses of benzothiazole compared to 100% in the untreated rats.

3.3. BTZ treatment inhibits cell proliferation and induces apoptosis

Treatment of the glioma cell bearing rats with BTZ daily for 21 days led to a significant decrease in the proportion of PCNA positive cells as revealed by immuno staining (Fig. 2). The population of PCNA positive cells was reduced to 23% in the BTZ treatment group compared to the untreated rats. TUNEL assay showed a remarkable increase in the proportion of apoptotic cells in the BTZ treated rats than those in the untreated rats after 21 days (Fig. 3).

3.4. BTZ treatment inhibits angiogenesis

Immunohistological staining revealed a marked reduction in the proportion of CD31-stained vessels in the BTZ treated rats compared to the control group. The proportion of CD31-stained vessels was reduced to 16% than those of the untreated rats (Fig. 4).

3.5. BTZ treatment effects the expression of angiogenesis proteins

The expression of angiogenesis related proteins was examined by mRNA and/or protein expression levels. The mRNA expression of VEGF and its receptor Flt1 were compared in control and 10 and 15 mg/kg BTZ treated animals (Fig. 5). The mRNA expression of VEGF was reduced up to 27 and 48% at 10 and 15 mg/kg body weight doses of BTZ respectively. The mRNA expression of Flt1 was reduced by 24 and 43% at 10 and 15 mg/kg body weight doses of BTZ respectively (Fig. 5). Similarly BTZ administration was also...
associated with a reduction in MMP2 (35 ± 6.8%) mRNA expression. BTZ treatment caused a significant decrease in both ERK1 and ERK2 protein expression (Fig. 6).

3.6. BTZ treatment alters the expression of proteins involved in cell cycle control

GLA was found to alter the expression of several proteins involved in cell cycle control (Fig. 7). pRb protein expression was decreased (up to 72%) while E2F1 expression was not consistent. Cyclin D1 protein expression was increased up to 23% in the presence of BTZ. The expression of p53 was increased (up to 29%) by BTZ treatment.

4. Discussion

The present study revealed the effect of BTZ on the growth of tumor in vitro on glioma rat C6 cells as well as in vivo the glioma rat model. To elucidate the anti-tumor mechanism of BTZ in vivo, proliferation, apoptosis and angiogenesis were systematically analyzed. Immunohistochemistry staining with PCNA and TUNEL assay of tumor tissue were performed. The current study showed a significant reduction in the population of proliferating cells in the BTZ-treated rats. The proportion of apoptotic cells was increased markedly on treatment of the glioma rat model with BTZ for 21 days. The quantitative analysis showed that BTZ-treated groups had a significant reduction of PCNA-positive cells and increment of apoptotic index compared to control. There are concrete evidence indicating that a nascent tumor can stimulate angiogenesis. Angiogenesis plays a key role in tumor growth as the newborn vessels supply oxygen and nutrients to the growing tumor. Treatment with BTZ greatly inhibited angiogenesis in vivo. As reported in literature, antiangiogenic therapy has been considered as an essential direction to fight cancers (Folkman, 1971; Hanahan and Folkman, 1996). As angiogenesis is inhibited, the supported tumor cells by those vessels subsequently suffer apoptosis due to lack of oxygen and nutrients (Dixelius et al., 2000). To gain an in-depth understanding about the underlying mechanism we analyzed the expression of several angiogenesis and cell cycle related proteins. The administration of BTZ reduced the transcript levels of VEGF, Flt1, ERK1/2 and MMP2. Moreover, the ERK1 and ERK2 protein expression was significantly decreased as well. The status of VEGF and its receptors Flt1, along with ERK1/2 and MMP2 in glioma angiogenesis and the inhibitory effects of BTZ on expression of these proteins indicated that BTZ could prove effective in inhibiting angiogenesis of gliomas. After determining the micro vessel density (MVD) it was evident that BTZ had an inhibitory effect on vessel number causing up to 84% reduction in MVD. This in itself is considered as an important mechanism of tumor growth inhibition (Ferrara, 2004; Shibuya, 2006). It has been suggested that Flt1 may protect VEGF from proteolytic degradation (Lamszus et al., 2003) and decrease in Flt1 expression observed in the presence of BTZ may reduce the degree of protection of VEGF from degradation and therefore augmenting the effect of reduced VEGF expression. Therefore, the current study indicated that the marked reduction in Flt1 and VEGF expression caused by BTZ treatment could cause a significant reduction in angiogenesis. Moreover, the significant reduction in the expression of ERK1 and ERK2 may also cause decrease in the expression of VEGF and the reduction in the expression of ERK1 and ERK2 may be directly related to decreased MMP2 expression in the BTZ-treated tumors. Previous studies have also reported that ERK-specific inhibitors cause a decrease in the expression of MMP2 in breast cancer brain metastases (Strauss et al., 2002). It is therefore quite possible that BTZ may affect C6 glioma cells in a similar manner however, this requires further confirmation. Alterations in the expression of angiogenesis related proteins, BTZ also induced important changes in the expression of proteins involved in in cell cycle control. The decrease in the expression of pRb may cause the cell to lose control over the pRb pathway which is reinforced by the elevation in cyclin D1 expression. Increased expression of p53 together with changes in pRb and cyclin D1 can considerably increase cell apoptosis (Godefroy et al., 2006; Maddika et al., 2007).

Taken together present indicates the potential of BTZ as an inhibitor of glioma cell proliferation and angiogenesis in vivo by exerting its effects upon cell cycle control and angiogenesis. We strongly believe that advancements in cancer research, BTZ may prove beneficial in the treatment of notoriously difficult to treat tumors.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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