Autophagy Involvement in Olanzapine-Mediated Cytotoxic Effects in Human Glioma Cells

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Abstract

The aim of this study was to investigate the effects of olanzapine on growth inhibition as well as autophagy in glioma cells in vitro and in vivo. The proliferation of both LN229 and T98 glioma cells, measured by MTT assay, was suppressed in a concentration-dependent and time-dependent manner. Moreover, apoptosis of both cells was significantly increased with the treatment of olanzapine as evidenced by increased Bcl-2 expression, Hoechst 33258 staining and annexinV-FITC/PI staining. Olanzapine treatment also enhanced activation of autophagy with increased expression of LC3-II, expression of protein p62, a substrate of autophagy, being decreased. The growth inhibition by olanzapine in both glioma cell lines could be blocked by co-treatment with 3-MA, an autophagy inhibitor. Furthermore, olanzapine effectively blocked the growth of subcutaneous xenografts of LN229 glioma cells in vivo. The increased level of protein LC3-II and decreased level of p62 followed by a decreased level of Bcl-2, suggesting that autophagy may contribute to apoptosis. In addition, reduced proliferation of glioma cells was shown by a decrease of Ki-67 staining and increased caspase-3 staining indicative of apoptosis in mouse xenografts. These results indicated that olanzapine inhibited the growth of glioma cells accompanied by induction of autophagy and apoptosis both in vitro and in vivo. Olanzapine-induced autophagy plays a tumor-suppressing role in glioma cells.

Keywords: Olanzapine - autophagy - apoptosis - glioma - xenograft

Introduction

Malignant glioma is one of the most common primary brain tumors in humans (Katsetos et al., 2007). Although multi-modality treatments, including surgery, radiation and chemotherapy, exist for this aggressive tumor, the prognosis of patients remains unfavorable (Stupp et al., 2009). The majority of glioblastoma patients survive less than 1 year from the time of diagnosis (Ryu et al., 2012). Moreover, most patients die within 2 years even under the standard combined therapy of radiotherapy and the alkylating agent temozolomide (TMZ), which is a standard chemotherapeutic drug for the firstline treatment of glioblastoma (Stupp et al., 2009). However, the resistance to TMZ becomes an obstacle for the success of glioblastoma therapy such as IDH1 overexpression occurred in glioma parents displayed chemotheraphy resistance characteristic in TMZ induced invasion and apoptosis inhibition (Wang et al., 2014). Thus, an effective anti-glioblastoma drug is urgently needed in order to create life-changing therapeutics for brain cancer patients, especially for those resistant to TMZ.

Autophagy is a cellular self-digestive process that is essential for survival, differentiation, development and homeostasis (Rabinowitz and White, 2010; Yang and Klionsky, 2010). Although autophagy is primarily a process for the cell protection, it can also play a role in cell death (Mizushima et al., 2008). It has been shown that a number of tumor suppressors can promote autophagy, such as LKB1 (Liang et al., 2007) , TSC (Zhou et al., 2009), DAP kinase (Bialik and Kimchi, 2010), PTEN (Errafiy et al., 2013), UVRAG (ultraviolet radiation resistance-associated gene)(Liang et al., 2006) and AMPK (Luo et al., 2010). Interestingly, autophagy in TMZ-resistant glioblastoma cells cannot be induced by TMZ while the combination of VPA and TMZ can enhance autophagic cell death in TMZ-resistant glioma cells (Ryu et al., 2012). Collectively, these results support that autophagy plays a tumor-suppressing role in cancer cells (Hanahan and Weinberg, 2011).

Olanzapine is widely used as an atypical antipsychotic drug due to its lower incidence of extrapyramidal symptoms (Zhang et al., 2013). Olanzapine may have the ability to induce autophagy in glioma cells through two possible mechanisms. First, olanzapine is a potent antagonist of the histamine H1 receptor (Kirk et al., 2009). Evidence indicates that H1 receptors are commonly expressed in glioblastoma cells lines and even in patient biopsies (Li et al., 2003; Fioretti et al., 2009). Nicolaou-Galmes et al. (2011) demonstrated that H1-antihistamines...
induced autophagy in melanoma cells and this contributes to cell death of melanoma cells. Secondly, olanzapine has also been shown to enhance AMPK activation in mammalian cells in particular that it activates AMPK in the brain of mice and rats (Schmidt et al., 2013; Skrede et al., 2014). When activated, AMPK positively regulated autophagy induction through inhibition of mTORC1 indirectly or phosphorylated Ulk1 kinase activity directly (Alers et al., 2012). Activation of AMPK is also involved in glioma cell death (Zhang et al., 2010). Therefore, these evidence supports the notion that olanzapine has the potential to induce autophagy, which in turn suppresses the growth of glioma cells.

In this study, we have investigated the antitumor activity of olanzapine on malignant glioma cells and examined the induction of autophagy by olanzapine. Based on the results, we propose that olanzapine-induced autophagy may contribute to the antitumor effect.

Materials and Methods

Cell culture

Glioma cell lines LN229 and T98 were purchased from American Type Culture Collection (ATCC). LN229 and T98 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen), supplemented with a 10% fetal bovine serum (FBS, Invitrogen) and incubated at 37°C in a humified atmosphere of 5% CO2. The glioma cell line T98 was resistant to TMZ.

Reagents

Olanzapine was purchased from Selleckchem (USA) and dissolved in DMSO as a stock solution of 200 mM stored in -20°C. The autophagy inhibitor 3-methyladenine (3MA) was obtained from Sigma Chemical Company (St. Louis, MO, USA). Olanzapine was purchased from Selleckchem (USA) and dissolved in DMSO as a stock solution of 200 mM stored in -20°C. The autophagy inhibitor 3-methyladenine (3MA) was obtained from Sigma Chemical Company (St. Louis, MO, USA), dissolved in DMEM at the concentration of 4 mM and stored in 4°C until use. Antibodies specific for LC3 (1:1000), Beclin1 (1:200), caspase-3 (1:200) and Ki-67 (1:200) respectively were purchased from Abcam (Cambridge, Mass, USA). P62 (1:2000) specific antibody was purchased from Cell Signaling Technology (Beverly, MA). Anti-β-actin antibody (1:1000) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell viability assay

Cell viability was measured by MTT assay. Five thousand T98 or LN229 cells were seeded in a well of 96-well plates and then cultured in a CO2 incubator overnight. Olanzapine at various concentrations were then added for 24, 48, 72 h before 10 μL of MTT solution (Sigma), which was dissolved in autoclaved phosphate-buffered saline (PBS) at a concentration of 5 mg/mL. After 4 hours, 100 μL of 10% SDS-HCL were added to dissolve formazan crystals. The absorbance of each well was obtained by a automatic fluorescence microplate reader using the wavelength of 570 nm. Each control and experimental group was performed in six wells and each experiment was repeated for three times. Cell viability results were presented as a percentage as follows: cell viability %=(OD<sub>con blank</sub>−OD<sub>blank</sub>) / (OD<sub>con</sub>−OD<sub>blank</sub>) x 100.

Western blot

Cells were rinsed with phosphate-buffered saline (PBS) followed by lysis with ice-cold lysis buffer containing protease inhibitor cocktail (Roche, Munich, Germany). The insoluble materials were removed by centrifugation at 12,000 rpm for 15 min. Protein concentration of the lysates was quantified by a protein assay kit (Thermo). Equal amounts of proteins of each sample were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane. The membranes were first blocked with 5% non-fat dried milk in TBST for 1 h at room temperature before specific primary antibodies were added for incubation overnight at 4°C. Subsequently the membranes were washed with TBST for three times and incubated with secondary antibodies at room temperature for 1 h. The blots were detected using the Odyssey Infrared Imaging System (LiCor) and digitized data were quantified as integrated optical density (IOD) by Image J software.

Hoechst 33258 staining

To detect apoptotic cells, cells were stained with the DNA dye Hoechst 33258. Cells with the indicated treatment were fixed with methanol for 10 min at 4°C before incubation with Hoechst 33258 for 10 min at room temperature. After washes with PBS, the apoptotic cells were mounted onto slides and observed under the fluorescence microscope BX61 (Olympus, Japan). Images were captured using DP71 CCD digital camera (Olympus). Cells exhibiting abnormal nuclei (crenation, condensation, and fractionation) were scored as apoptotic cells.

Detection of cell death by flow cytometry

Cellular death was evaluated by Annexin V-FITC apoptosis detection kit (Keygene) following the manufacture’s instruction. Glioma cells were collected 72 h after treatment, washed three times with PBS, and then resuspended in 400 μL binding buffer. Subsequently cells resuspended in 100 μL were incubated with 5 μL Annexin V-FITC and 5 μL propidium iodide (PI) for 15 min at room temperature in the dark. Data acquisition was conducted by collecting 10,000 cells per tube and the percentage of cell death was analyzed using the LSRII flow cytometer (BD Biosciences, San Jose, CA) and the FACSDiva software.

Immunocytochemistry

Glioma cells were plated on cover slips and treated as indicated for 48 h. Cells were then fixed at 4°C for 10 min in pre-cooled 100% methanol (-20°C), rinsed with phosphate-buffered saline (PBS) three times, permeabilized at room temperature in 0.2% Triton X-100 for 5 min and blocked at room temperature for 30 min in 5% BSA and 0.1% Triton X-100. Samples were incubated with primary antibodies at 4°C overnight followed by a secondary antibody conjugated to rabbit Alexa Fluor 488 (1:500, Molecular Probes, Eugene) for 1 h. After rinsing three times with PBS, cells were incubated with 0.5 ng/ml of DAPI for 15 min at room temperature before mounting. All coverslips were examined under a
Spectral laser scanning confocal microscope (Nikon C1-Si, Japan). Images were analysed by EZ-C1 3.20 FreeViewer software.

**Colony formation assay**

A thousand T98 or LN229 cells were seeded into 6-well plates for 24h then treated with 3MA in the absence or presence of olanzapine. Cells were subsequently stained with crystal violet staining solution (Beyotime, Nantong, China) after 10 days-incubation. The visible colonies (≥50 cells) were counted and typical images were captured using a common Nikon camera.

**Animal experiments**

Human glioma cell line LN229 was used in this experiment. LN229 cells were injected into BALB/c nude mice (male, 6 weeks olds) subcutaneously. Nude mice bearing tumors were divided into four groups (n = 5 in each group) and treated when the volume of tumor reached about 100 mm³. Four groups were control (saline solution), olanzapine 0.75mg/kg, olanzapine 1.5mg/kg, and olanzapine 3mg/kg. Drugs were administered by oral gavage once a day for 3 weeks. Tumor length (a) and width (b) were measured in situ every other day using digital calipers, and tumor volumes were calculated according to the following equation: V(mm³) = a × b² / 2. The significance of differences between control and treated groups was evaluated by ANOVA. The doses of olanzapine used correspond to those used in humans (commonly 400 mg daily at bedtime).

**Immunohistochemistry**

Tumors were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections of 4-µm thickness were used for immunohistochemical staining by DAB reagents (Abcam) according to the instructions of supplier. Mounted tissue sections were baked at 60℃ for 30 min, deparaffinized in xylene and rehydrated through graded alcohols. Sections were incubated in 3% H₂O₂ for 15 min at room temperature, followed by antigen retrieval by incubation in 10mM sodium citrate (PH 6.0) at 100℃ for 15 min, then the sections were blocked with 1% BSA in PBS and 0.5% Triton X-100 for 1 h. According to the manufacturer’s instruction, antibodies specific for Ki-67 (1:200, Abcam, Cambridge, Mass, USA) and caspase-3 (1:200, Abcam, Cambridge, Mass, USA) were incubated on the sections overnight at 4°C, followed by incubation with a horseradish peroxidase conjugated secondary antibody. Staining was visualized using DAB chromogen substrate and specimens were analyzed using microscope (Olympus, Japan). Representative images of Ki-67 and caspase-3 staining were shown.

**Statistical analyses**

The values in the figures were expressed as means±SEMs. The figures illustrated in this study were representatives of at least three independent experiments. The significance of differences between different test conditions was determined using one-way ANOVA and Student’s t-test. P<0.05 were considered statistically significant.

**Results**

**Olanzapine inhibited the growth of human malignant glioma cells**

Using the MTT assay, we determined the effects of olanzapine on malignant glioma cells. We treated two malignant glioma cell lines T98 and LN229 with 0-1.0mM olanzapine for 24h, 48h, 72h, respectively, and assessed the viability of the two glioma cell lines. As shown in Figure 1, olanzapine had a significant growth inhibitory effect on LN229 as well as TMZ-resistant glioma cells in a dose-dependent and time-dependent manner, indicating that olanzapine has antitumor effects on the malignant glioma cell lines.

**Olanzapine induced apoptosis in glioma cells**

Using western blot analysis, apoptosis was indicated by the decreased expression of Bcl-2 in glioma cells treated with olanzapine (Figure 2A and 2B). Consistent with this finding, Hoechst 33258 staining demonstrated an increase of apoptotic glioma cells exposed to olanzapine (0, 0.2, 0.3, 0.4mM) for 72h. Representative microphotographs showed that morphological characteristics of apoptosis, such as nuclear fragmentation and chromatin condensation. Apoptotic cells with abnormal nuclear increased in LN229 and T98 cells treated with olanzapine (Figure 2C). In addition, we used Annexin V-FITC/PI staining to measure the percentage of apoptosis in each treatment groups. As figure 2D and 2E showed, there was a dose-dependent increase in apoptosis levels compared to those of untreated group in both glioma cell lines.

**Olanzapine activates autophagy in glioma cells**

To evaluate the activation of autophagy by olanzapine, we measured the expression levels of autophagy-related LC3 and p62 by western blot analysis. During autophagosome formation, the microtubule-associated protein light chain 3 (LC3-I) is converted to the membrane-bound form LC3-II. P62 binds the ubiquitinated targets and transports their aggregates to the autophagosome.
through its interaction with LC3-II. In LN229 and T98 cells, the expression level of LC3 increased accompanied by a decrease of p62 in a dose-dependent and time-dependent manner after treatment with olanzapine (Figure 3A). Quantification of the proteins expression levels showed an apparent changes in cells treated with olanzapine (Figure 3B and 3C). These results indicate that autophagy is activated by olanzapine in T98 and LN229 glioma cells.

**Autophagy is crucial for olanzapine-induced cell death**

As autophagy can play either a tumor-protective or tumor-suppressive role under various stressful conditions, we wanted to understand how activation of autophagy affected viability of glioma cells in response to olanzapine. As the results were shown by MTT assays, the reduced cells viability seen in Figure 1 were reversed by autophagy inhibitor 3-MA (2 mM) (Figure 4A and 4B), suggesting that olanzapine-induced autophagy is likely to serve as a pro-death signal in glioma cells. Colony formation assays were further performed to investigate the effects of 3MA in the absence or presence of olanzapine on T98 and LN229 cells (Figure 4C). As a result, the survival rate in glioma cells treated with 3MA and olanzapine was significantly increased in comparison to the group treated with olanzapine alone (Figure 4D and 4E). This finding further supported that autophagy induced by olanzapine has a pro-death role in LN229 and T98 glioma cells.
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Olanzapine supressed the growth of subcutaneous glioma xenograft

To determine the importance of our observation in vivo, we examined the therapeutic benefits of the treatment with olanzapine in a mouse glioma model, namely the LN229 human glioma cells were injected into BALB/c nude mice subcutaneously. Mice were separated into four groups: control (C: saline solution), L, low dose of olanzapine (0.75 mg/kg), M, middle dose of olanzapine (1.5 mg/kg), and H, high dose of olanzapine (3 mg/kg). (A) Olanzapine significantly diminished xenograft tumor size at high dose of olanzapine (3 mg/kg). Points, mean; bars, SE. **p<0.01, ANOVA. (B) The expression of LC3 in xenograft was detected by western blots. (C) The quantification of LC3 protein expression after normalization to the level of β-actin. Mean±SEM, n=3. ***P<0.001 vs the control group. (D) The expression of Bcl-2 proteins in xenograft was examined by western blots. (E) The quantification of Bcl-2 protein expression after normalization to the level of β-actin. Mean±SEM, n=3. ***P<0.001, compared with the control group. (F) Representative images of IHC staining of Ki-67 and caspase-3 expression in subcutaneous xenograft of LN229 cells

Discussion

The overall goals of this study were to investigate the anti-tumor effect of olanzapine in glioma cells, and to unravel the possible mechanisms of its action. We demonstrated for the first time that the cumulative

Figure 4. Autophagy Inhibitor 3-Methyladenine Abrogated Glioma Cell Growth Inhibition Induced by Olanzapine. (A) MTT assays were performed in T98 glioma cell. Cells treated with olanzapine alone (0.6mM), 3MA (2mM) or olanzapine plus 3MA. Data are represented as the mean±SEM, n = 3. **p<0.01 vs olanzapine group. (B) MTT assays were performed in LN229 glioma cells, ***p<0.001 vs olanzapine group. (C) Colony formation assay in LN229 or T98 cells treated with 3MA (0.5mM) in the absence or presence of olanzapine (0.05mM). (D) The plot depicts the survival rate of the olanzapine-treated cells compared with that of the control LN229 cells. (E) The survival rate of T98 cell resulted from colony formation assay. Data are represented as the mean±SEM, n = 3. ***P<0.001 vs olanzapine group

Figure 5. The Effect of Olanzapine in Human Glioma Xenograft Models. This study included four groups: C, control (saline solution), L, low dose of olanzapine (0.75 mg/kg), M, middle dose of olanzapine (1.5 mg/kg), and H, high dose of olanzapine (3 mg/kg). (A) Olanzapine significantly diminished xenograft tumor size at high dose of olanzapine (3 mg/kg). Points, mean; bars, SE. **p<0.01, ANOVA. (B) The expression of LC3 in xenograft was detected by western blots. (C) The quantification of LC3 protein expression after normalization to the level of β-actin. Mean±SEM, n=3. ***P<0.001 vs the control group. (D) The expression of Bcl-2 proteins in xenograft was examined by western blots. (E) The quantification of Bcl-2 protein expression after normalization to the level of β-actin. Mean±SEM, n=3. ***P<0.001, compared with the control group. (F) Representative images of IHC staining of Ki-67 and caspase-3 expression in subcutaneous xenograft of LN229 cells

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p62 in high-dose olanzapine group (Figure 5B and 5C), suggesting that olanzapine indeed could induce autophagy in vivo. Likewise western blot analysis showed a decreased expression level of apoptosis-related protein Bcl-2 in olanzapine treated group (Figure 5D and 5E). Based on the IHC results from the resection specimens, the expression of caspase-3 proteins increased in olanzapine treatment groups (Figure 5F, bottom) and this further confirms that apoptosis was induced in the subcutaneous xenograft of LN229 glioma cells. Moreover, the decreased tumor size following olanzapine treatment was also attributed to the reduced proliferation status of tumor cells as evidenced by decreased Ki-67 staining on mouse tumor sections specially in high-dose group (Figure 5F, top).
The antitumor effect of olanzapine in glioma cells is attributed to its induction of autophagy. In this study, olanzapine inhibited the growth of glioma cells by MTT assay. As a result, olanzapine had a significant growth inhibitory effect on both glioma cells in a dose-dependent and time-dependent manner.

There are not any studies about the relationship between olanzapine and glioma cells yet. We wonder how olanzapine fulfills its tumor-suppressing role in glioma cells. First of all, we assessed whether olanzapine could induce apoptosis in glioma cells. Using western blotting, we detected the protein level of Bcl-2 after treatment with olanzapine for 72h (Figure 2A-2B). The decreased levels of Bcl-2 indicated that olanzapine induced apoptosis in glioma cells as evidenced by measurements of annexin V-FITC/PI and Hoechst 33258 staining, which showed a concurrent increase of apoptosis (Figure 2C-2E). All these results demonstrated that apoptosis was induced at a later time point than that of autophagy, which was apparent at 48 h (Figure 3A-3C).

Despite that autophagy and apoptosis were different cell death processes, there is a crosstalk between them. Antagonists of H1 receptor and activators of AMPK can induce autophagy (Nicolaou-Galmes et al., 2011; Alers et al., 2012), thereby making olanzapine a promising target for glioma treatment. Evidences in recent years indicate that autophagy played a dual role in cancer cells depending on cell type, context or stage of tumor development (Mathew et al., 2007). Under unfavorable growth conditions, autophagy is induced to meet energy demands in the early stages of tumor growth (Levine and Kroemer, 2008). What’s more, a complex interplay between cell death and/or survival including necrosis, apoptosis and autophagy may in turn govern tumor metastasis, and subsequent carcinogenesis (Pandey and Chandravati, 2012). However, if unfavorable conditions continue beyond a crucial point, unrestrained autophagy might result in autophagic cell death (Lum et al., 2005). Since our laboratory early studies have shown that autophagy was induced in glioma cells and contributed to the differentiation of glioma-initiating cells (Zhuang et al., 2011). So we proposed that autophagy might relate to olanzapine-induced cell death. We then determined the expression of autophagy related proteins LC3II (an autophagy-specific marker that specifically localizes to the autophagosomal membranes) and p62 (a substrate that is degraded by autophagy) in olanzapine-treated glioma cells. The findings suggest that olanzapine induced increased autophagy continuously in glioma cells even in T98 cells, a TMZ resistant glioma cell line.

We have known induction of autophagy in response to therapeutics fulfills a dual role by having tumor-promoting and tumor-suppressing properties. Moreover, some strategies have aimed at modulating autophagy to sensitize glioma cells to TMZ. We next want to know the role of autophagy in cytotoxic effects of olanzapine in human glioma cells. Thus, we combined olanzapine and 3-MA, an autophagy inhibitor, to treat glioma cells. The MTT assay and the clonogenic assay both showed that 3-MA significantly rescued glioma cells from the inhibition of olanzapine treatment, suggesting that autophagy induced by olanzapine indeed plays a tumor suppressive role.

To confirm if the antitumor effects of olanzapine in vitro could fulfill the function in vivo, we also investigated the anti-glioblastoma effects of olanzapine in a human glioma LN229 xenograft model in nude mice. Compared with the control group, high dose of olanzapine significantly decreased the growth of subcutaneous glioma xenograft. Interestingly, only in high-dose group of olanzapine treatment the autophagy was induced as shown by western blots. Concomitantly a significant decrease of Bcl-2 levels in olanzapine-treated groups compared to particularly high protein level of Bcl-2 in control group were shown by western blots while an increase of active caspase-3 treated with olanzapine were measured by immunostaining of tissues, suggesting that olanzapine induces apoptosis in glioma xenograft. Moreover, olanzapine also inhibited the proliferation of glioma xenograft as the number of Ki-67 positive cells decreased in olanzapine-treated tissues. Collectively, treatment of olanzapine inhibited cell growth of subcutaneous xenograft of LN229 glioma cells and this appears to associate with the induction of autophagy followed by apoptosis.

In summary, in the present study we have demonstrated for the first time that olanzapine inhibited the glioma growth both in vitro and in vivo. Moreover, our data suggested that autophagy and apoptosis were sequentially induced by olanzapine in malignant glioma cells. Under this circumstance, autophagy is favored to play a pro-death role in olanzapine-treated glioma cells. In addition to potential anti-tumor activity, studies and clinical trials of olanzapine have demonstrated its efficacy in the prevention of chemotherapy-induced nausea and emesis (Navari, 2014). Some subjects without psychiatric disorders also experience sleep continuity and efficiency as well as increased stage III/IV sleep in the olanzapine treated groups (Cohrs, 2008). All these attributes would be beneficial to glioblastoma patients as well. Our experimental results provide a basis for the possible clinical use of olanzapine in the treatment for patients with malignant glioma.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 81072656, 81373430).

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DOI:http://dx.doi.org/10.7314/APJCP.2014.15.19.8107

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