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Bufalin inhibits glioblastoma growth by promoting proteasomal degradation of the Na^+/K^+-ATPase α1 subunit

Yu-Long Lan[a,b,c,d,1], Xun Wang[a,1], Jia-Cheng Lou[a,1], Jin-Shan Xing[a], Zhen-Long Yu[c], Hongjin Wang[b], Shuang Zou[d], Xiaochi Ma[c,*], Bo Zhang[a,*]

[a] Department of Neurosurgery, The Second Affiliated Hospital of Dalian Medical University, 467 Zhong Shan Road, Dalian, 116023, China
[b] Department of Neurology, The Second Affiliated Hospital of Dalian Medical University, Dalian, 116023, China
[c] Department of Pharmacy, Dalian Medical University, Dalian, 116044, China
[d] Department of Physiology, Dalian Medical University, Dalian, 116044, China

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ABSTRACT

Chansu is a traditional Chinese medicine that is generally recognized as a specific inhibitor of Na^+/K^+-ATPase. Bufalin, an active component of Chansu, is an endogenous steroid hormone with great potential as a cancer treatment. However, the mechanism by which it exerts its antitumor activity requires further research. Currently, the α1 subunit of Na^+/K^+-ATPase (ATP1A1) is known to exert important roles in tumorigenesis, and the precise mechanisms underlying the effect of Bufalin on the Na^+/K^+-ATPase α1 subunit was therefore investigated in this study to determine its role in glioblastoma treatments. The effect of ATP1A1 on the sensitivity of glioblastoma cells to Bufalin was investigated using MTT assays, RT-PCR and siRNA. Western blot was also used to explore the important roles of the ubiquitin-proteasome pathway in the Bufalin-mediated inhibition of ATP1A1. Xenografted mice were used to examine the anti-tumor activity of Bufalin *in vivo*. LC–MS/MS analysis was performed to determine the ability of Bufalin to traverse the blood-brain barrier (BBB). The results indicated that Bufalin inhibited the expression of ATP1A1 in glioblastoma by promoting the activation of proteasomes and the subsequent protein degradation of ATP1A1, while Bufalin had no effect on ATP1A1 protein synthesis. Bufalin also inhibited the expression of ATP1A1 in xenografted mice and significantly suppressed tumor growth. These data should contribute to future basic and clinical investigations of Bufalin. In conclusion, Bufalin significantly inhibited the expression of ATP1A1 in glioblastoma cells by activating the ubiquitin-proteasome signaling pathway. Bufalin may therefore have the potential to be an effective anti-glioma drug for human glioblastoma in the future.

1. Introduction

Glioma is the most common malignant tumor in neurosurgery. It has a poor prognosis, a high recurrence rate, a high mortality rate and a low 5-year survival rate. Glioblastoma (GBM) is the type of glioma with the highest degree of malignancy. It is difficult to completely remove these tumors *via* surgery, and the recurrence rate is high. Chemotherapy can be effective, but its curative effect is not satisfactory. Even when using surgical resection combined with radiotherapy and chemotherapy, the median survival time in patients with glioblastoma is only approximately 14 months, and this duration has not been improved in the last decade [1,2]. Chemotherapy could be key to future breakthroughs in the treatment of glioblastoma. There is therefore an urgent need to explore the mechanism underlying glioma cell death and identify useful chemotherapeutic drugs. This has become an important research direction with great translational value. Therefore, to improve the survival rate of glioma patients, it is particular important that new treatment strategies or treatment drugs are developed.

The ubiquitin-proteasome system is the most important protein selective degradation system in eukaryotic cells. The ubiquitin-proteasome system is widely involved in regulating cell metabolism, differentiation and proliferation, and abnormalities in this system are closely related to the etiology, development and prognosis of malignant tumors [3]. Proteasome inhibitors can bind to proteasome enzymatic active sites, block the hydrolysis of ubiquitin target proteins, and induce apoptosis in various tumor cells, and they therefore provide a new strategy for treatments for cancer. Studies have shown that the ubiquitin proteasome pathway is involved in the hydrolysis of most

* Corresponding authors.
E-mail addresses: maxc1978@163.com (X. Ma), zhangbodl@126.com (B. Zhang).
[1] These authors contributed equally to this work.

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cellular nuclear and cytoplasmic proteins, especially the short-term regulatory proteins that control cell cycle progression, transcription factor activity, and signal transduction [4,5]. Thus, the proteasome could also be a new potential target for cancer therapies [6]. Recently, researchers using siRNA screening technology found that among the important genes that may affect the survival of glioblastoma cells, nearly 22% were involved in the formation of the 26S and 20S proteasome subunits, indicating that proteasomes could be a molecular target for glioblastoma therapies [7]. Proteasome inhibitors inhibit cell proliferation in a variety of tumors, including glioblastoma cells, but the mechanism underlying this activity is unclear. Currently, proteasome inhibitors, including MG132, lactacystin, bortezomib (PS341), epoxomicin and SC68896, are being gradually investigated and used in clinical practice, and they have exhibited obvious effects in cancer treatments [8,9]. However, their anti-tumor potential needs to be further confirmed, and their specificity for different types of tumors requires further research.

In recent years, many traditional Chinese medicines have attracted an increasing amount of attention because of their potential anti-tumor activity [10]. Toad venom, known as “Chansu” in China, is secreted from the glands behind the auricle and skin of toads [11,12] and has been widely used as a drug, either alone or in combination with other herbs, for centuries as an analgesic, a cardiopedic, an anti-microbial agent, a local anesthetic, and an anti-inflammatory and anti-tumor drug, and it shows good pharmacological activity [13]. Bufalin, one of the main active components of Chansu, is one of the few endogenous traditional Chinese medicines, and modern pharmacological studies have found that it exerts substantial anti-tumor activity [14,15]. In our previous study, we showed that Bufalin has better stability and causes fewer side effects than other compounds extracted from Chansu. In addition, an increasing amount of research has, in recent years, further indicated the importance of this potential agent in the treatment of glioblastoma [16–18]. However, the exact anti-glioma mechanisms by which Bufalin exerts its activity needs further research.

The role of Na^+/K^+-ATPase in cancer [19] and the use of Na^+/K^+-ATPase inhibitors in anti-tumor therapies [20] are currently being extensively explored. Specifically, the α1 subunit, as the main functional unit of Na^+/K^+-ATPase, plays an important role in glioma. The Na^+/K^+-ATPase α1 subunit resides on the membranes of cancer cells and may be a new target for various anti-cancer agents. In addition, in prostate cancer [21], non-small cell lung cancer [22], glioma [23] and breast cancer [24], the expression level of the α1 subunit is increased, and cardiac glycosides have all been demonstrated to have better therapeutic effects. It has been reported that the protein expression of the α1 subunit is higher in glioblastoma cell lines and tissue samples than in normal tissues [25]. Interestingly, as the main active component of Chansu, Bufadienolides (including bufalin, cinobufagin, resibufogenin, *etc.*), a group of steroid hormones, have generally been shown to specifically inhibit Na^+/K^+-ATPase [26–29]. Thus, in this study, we further investigated the mechanisms underlying the inhibitory effects of Bufalin on glioblastoma growth that are mediated by the α1 subunit.

In this study, we explored the anti-cancer effect of Bufalin on glioblastoma cells, including U87, U251 and LN229 cells, in order to identify the inhibitory and apoptosis-inducing effects of Bufalin on glioblastoma cells and confirm the important role of the ubiquitin-proteasome system in this process. Furthermore, the effect of ATP1A1 inhibition on glioblastoma growth is also discussed to provide theoretical and experimental support for using Bufalin and various other potential ATP1A1 inhibitors to treat glioma.

2. Materials and methods

2.1. Antibodies and other materials

The primary antibodies for ATP1A1, ubiquitinated proteins, β-actin and all the secondary antibodies were obtained from Cell Signaling Technology (Cell Signaling Technology, Inc, Danvers, Massachusetts, USA). Trypsin, Dulbecco’s Modified Eagle’s Medium (DMEM) and fetal bovine serum (FBS) were obtained from HyClone Laboratories (HyClone Laboratories Inc., Waltham, Massachusetts, USA). Bufalin was purchased from Yuanye Biotech. (Jinan, Shandong, China), and was solutioned in DMSO and kept at −20 °C, as the stock solution. Bufalin was diluted in culture medium to obtain the desired concentration, which was stable in DMSO. The Phosphate Buffered Saline (PBS), protease inhibitor cocktail and 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co (St. Louis, MO, USA). All other chemicals were purchased from Sigma Chemical Co. unless otherwise specified.

2.2. Cell culture

Human U87MG, U251 and LN229 cell lines were obtained from ATCC (Manassas, VA, USA). Cells were maintained in either DMEM medium supplemented with 10% fetal bovine serum. All cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO2.

2.3. Cell viability assay

Cell viability was explored in MTT assays (Roche Diagnosis, Indianapolis, IN). Briefly, lung cancer cell lines were seeded at 6×10^3 cells/well in 96-well plates. The cells were allowed to adhere overnight, and the medium was then changed to fresh medium containing various concentrations of Bufalin dissolved in DMSO (final concentration, 0.1%). After 48 h of incubation, the growth of the cells was measured. The effect on cell viability was assessed by comparing the percent cell viability between the treated groups and the untreated control group, which was arbitrarily assigned to represent 100% viability. The Bufalin concentration required to cause 50% cell growth inhibition (IC_{50}) was determined by interpolation from dose-response curves. The OD values were determined. All experiments were performed in triplicate.

2.4. Colony formation assay

To analyze cell sensitivity to Bufalin, we used *in vitro* colony formation assays. Briefly, U87 cells (0.8×10^3 per well) were seeded in 6-well plates containing 2 ml of growth medium with 10% FBS and then cultured for 24 h. The medium was then removed, and the cells were exposed to various concentrations of Bufalin. After 24 h, the cells were washed with PBS, and fresh medium containing 10% FBS was added to the wells. The cultures were maintained in a 37 °C, 5% CO_2 incubator for 14 days to allow the viable cells to grow into macroscopic colonies. The medium was then removed, and the colonies were counted after they were stained with 0.1% crystal violet.

2.5. Apoptosis assay

Apoptosis was measured by a fluorescence-activated cell sorter (FACS) using an Annexin V- FITC Apoptosis Detection Kit (Nanjing KeyGEN Biotech. CO., LTD., Nanjing, Jiangsu, China). Briefly, the cells were plated in 6-well plates and treated with Bufalin. After treatment for 12 h, the cells were collected and washed once with cold PBS. They were subsequently simultaneously stained with FITC-labeled Annexin V and PI. The stained cells were analyzed using FACS Accuri C6 (Genetimes Technology Inc., Shanghai, China).

2.6. Cell cycle analysis by flow cytometry

U87MG cells were incubated with different concentrations of Bufalin for 24 h. Then, the cells were trypsinized into single cells and