Ibuprofen induces ferroptosis of glioblastoma cells via downregulation of nuclear factor erythroid 2-related factor 2 signaling pathway

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Ferroptosis is a newly discovered type of cell death decided by iron-dependent lipid peroxidation, but its role in glioblastoma cell death remains unclear. Ibuprofen, a nonsteroidal anti-inflammatory drug (NSAID), has been associated with antitumorigenic effects in many cancers. In this study, we first found that ibuprofen inhibited the viabilities of glioblastoma cells *in vitro* and *in vivo*, accompanied by abnormal increase in intracellular lipid peroxidation. Further study showed that the cell growth inhibition caused by ibuprofen could be rescued by the ferroptosis inhibitors deferoxamine (DFO), ferrostatin-1 and Liproxstatin-1. Nuclear factor erythroid 2-related factor 2 (Nrf2), glutathione peroxidase 4 (GPX4) and solute carrier family 7 member 11 (SLC7A11) are key regulators of ferroptosis. Our data showed that Nrf2, GPX4 and SLC7A11 were downregulated in glioblastoma cells under ibuprofen treatment. Interestingly, we found that decreased mRNA expression of GPX4 and SLC7A11 was accompanied with reduced Nrf2, which is a redox sensitive transcription factor that controls the expression of intracellular redox-balancing proteins such as GPX4 and SLC7A11. All the data suggested that Nrf2 could regulate the expression of GPX4 and SLC7A11 in glioma cells. Taken together, our findings reveal that ibuprofen could induce ferroptosis of glioblastoma cells via downregulation of Nrf2 signaling pathway and is a potential drug for glioma treatment. *Anti-Cancer Drugs* 31:27–34

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Introduction

Glioblastoma (GBM), also known as astrocytoma grade IV, is one of the most common primary central nervous system tumors. They are characterized by rapid proliferation, migration and invasion. Surgery combined with radiotherapy and chemotherapy is effective therapy for gliomas. However, the median survival of the patients with GBM is approximately 1–2 years [1,2]. Thus, there is an urgent need to illuminate the molecular mechanism of GBM and develop new compounds against GBM.

Recently, many studies showed that the treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with reduced incidence of various human cancers [3,4]. Ibuprofen, 2-(4-isobutylphenyl) propionic acid, is the most commonly used over-the-counter NASAID [5]. Furthermore, ibuprofen has anti-inflammatory, analgesic and antipyretic properties due to inhibition of the activity of cyclooxygenases-1 and -2 and has been shown to exert antitumor effects in many different tumor cells including GBM. There is an inverse association between NSAIDs use and tumors, including ibuprofen and GBM in a case-control study [6]. Furthermore, ibuprofen can significantly reduce tumor growth in rat models of glioma and restrict migration and proliferation of GBM cells [7,8]. All these suggest that ibuprofen might be a potential therapeutic option for GBM treatment, but the molecular mechanism needs to be further investigated.

Ferroptosis is a newly discovered type of cell death which is caused by iron accumulation and oxidative injury. Ferroptosis is implicated in a wide array of diseases including inflammation, neurodegeneration and cancer [9]. A lot of studies showed that the selenoenzyme glutathione (GSH) peroxidase 4 (GPX4) is a key regulator of ferroptosis. GPX4 catalyzes the reduction of organic hydroperoxides and lipid peroxides at the expense of GSH [10]. Inhibition of GPX4 could lead to increased levels of uncontrolled lipid peroxidation and result in ferroptosis [11], whereas high levels of GPX4 conferred resistance to ferroptosis activation [12]. Zhao *et al.* [13]

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found that the level of GPX4 was higher in glioma tissues and cell lines and there was statistical significance between the expression of GPX4 and the WHO grade. The glutamate exchanger system Xc- is a key player in glutamate, cystine and GSH metabolism, which is mainly required for GSH production [14], thus it plays an important role in ferroptosis. Erastin, a ferroptosis inducer, triggers ferroptosis by directly inhibiting system Xc- activity [15]. Solute carrier family 7 member 11 (SLC7A11) is a core component of system Xc-, which is known as the catalytic subunit. Moreover, many studies indicate that inhibiting SLC7A11 can induce ferroptosis *in vivo* and *in vitro* [16–18].

Many research show that ferroptosis plays a role in GBM development. Fan *et al.* [19] showed that nuclear factor erythroid 2-related factor 2 (Nrf2)-Keap1 pathway was critical for malignancy in gliomas via promoting cell proliferation and resistance to ferroptosis. Chen *et al.* [20] proved that sensitizing GBM cells to ferroptosis by inhibition of ATF4 was an effective way for reducing tumor growth and vasculature. Sehm *et al.* [21] found that ferroptosis inducers such as erastin and sorafenib could increase efficacy of temozolomide on GBM cells. Therefore, triggering ferroptosis is emerging to be an effective approach to eliminate GBM cells.

In the present study, we investigated the influence of ibuprofen on the viability and ferroptosis of GBM cells and explored the potential mechanisms. We found that ibuprofen induced GBM ferroptosis by inhibiting the Nrf2/GPX4/SLC7A11 signaling pathway. Thus, these results may be beneficial for developing improved therapies for glioma.

Materials and methods

Cell lines

Human U87MG and U251MG glioblastoma cell lines were purchased from the Chinese Academy of Sciences Cell Bank in 2015. The authenticity of cancer cell lines was tested by short tandem repeat (STR) profiling. All cell lines were grown in DMEM medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, California, USA) and 1% Non-essential amino acid (Gibco, Carlsbad, California, USA).

Chemicals

Ibuprofen, Erastin and Liproxstatin-1 (Lip-1) were purchased from Selleck Chemicals (Houston, Texas, USA). DMSO, Desferoxamine (DFO) and Ferrostatin-1 (Fer-1) were bought from Sigma-Aldrich (Taufkirchen, Germany). BODIPY C11 (581/591) was purchased from Life Technologies (Darmstadt, Germany).

Three-dimensional tumor cell culture

Two hundred microliters per well of cell suspensions at 0.5×10^4 cells/ml densities for U87 MG was dispensed into ULA 96-well round-bottomed plates (Corning B.V. Life Sciences, Amsterdam, The Netherlands) using a multichannel pipette. Plates were incubated at 37°C, 5% CO_2 and 95% humidity. Cultures were maintained by replacing 50% of the medium on days 4, 7, 10, 12 and 14 [22]. Images were captured by a Nikon DS-5M Camera System mounted on a phase-contrast Leitz microscope on days 4, 7, 10, 12 and 14. The radius of each tumor spheroid was used to calculate the volume (μm^3): $V=4/3\ \pi\ r^3$.

Cell viability assay

Cells were seeded at a density of 0.5×10^4 cells per well in 96-well plates and allowed to attach overnight. For inhibitor studies, cells were treated with indicated concentration for DMSO, ibuprofen for 24, 48 and 72 hours, respectively. An aliquot of 10 µl of CCK-8 was added to the wells and incubated for 1 hour (Beyotime, Shanghai, China). The absorbance was measured at 450 nm to calculate the numbers of viable cells in each well. Each measurement was performed in triplicate and the experiments were repeated three times.

Animal studies

All animal protocols were approved by the Institutional Animal Care and Use Committee of Xi'an Medical University. In the intracranial glioma model, U87MG cells (5×10^5) were intracerebrally injected into the left side (bregma: 1 mm; lateral: 2 mm; ventral: 3 mm) of the brains of nude mice. Two weeks after tumor cell transplantation, mouse brains were scanned to detect tumor formation using a 3.0-T scanner (GE Signa HD MRI Systems). Then, mice were divided randomly into two groups ($n=6$/group) and treated with vehicle control (PBS), or ibuprofen (20 mg/kg), in 100 µl of PBS given i.p. 1×/day, 5 days/week.

Real time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated by the Trizol method following the manufacturer's protocol (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Total RNA was isolated using Trizol reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and reverse-transcribed into cDNA using BcaBest RNA PCR kit from TaKaRa according to the manufacturer's instructions. Quantitative real-time PCR was performed using a Peltier Thermal Cycler (BioRad) plus Realtime PCR Master Mix (SYBR Green, Toyobo, Osaka, Japan). The specific primers used for PCR are listed in Supplementary Table S1, Supplemental digital content 1, *http://links.lww.com/ACD/A309*. Glyceraldehyde-3-phosphate dehydrogenase was chosen as the endogenous control in the assay.

Western blotting

Cells were collected and lysed in high KCl lysis buffer with complete protease inhibitor cocktail (Roche). The protein concentration was determined using a BCA

protein assay kit (Pierce). The samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Roche). The membranes were treated with 5% nonfat dry milk in tris buffered saline, followed by incubation with primary antibodies and then horseradish peroxidase-labeled secondary antibodies (Roche). The immunolabeled proteins were detected using ECL detection system (Boster, Wuhan, China). Densitometry quantification was acquired with Gel Doc 1000 system and was analyzed using the Quantity One software. The following primary antibodies were used: GPX4 (abcam; 1:5000), β-tubulin (Santa Cruz:1:2000), Nrf2 (abcam, 1:2000) and SLC7A11 (abcam, 1:2000).

Staining for lipid peroxidation in cells by flow cytometry

In six-well plate, 2×10^5 cells/well were seeded. The next day, culture media was replaced with media containing sorafenib (10 μM), erastin (10 μM) or RSL3 (0.1 μM) ± DFO (100 μM) or ±Fer-1 (0.5 μM) for 18 hours (RSL3, 8 hours). After incubation, cells were harvested by trypsinization, washed and resuspended in 500 μl of PBS containing BODIPY C11 (2 μM) in fluorescence activating cell sorter (FACS) tubes. Flow Cytometer BD FACSCanto II was used for the follow cytometer analysis. A minimum of 1×10^4 cells were counted and analyzed per condition. Analyses were carried out with FCS Express 5 Demo Software.

Statistics

Data were analyzed using the Student's *t*-test. $P<0.05$ was considered statistically significant.

Results

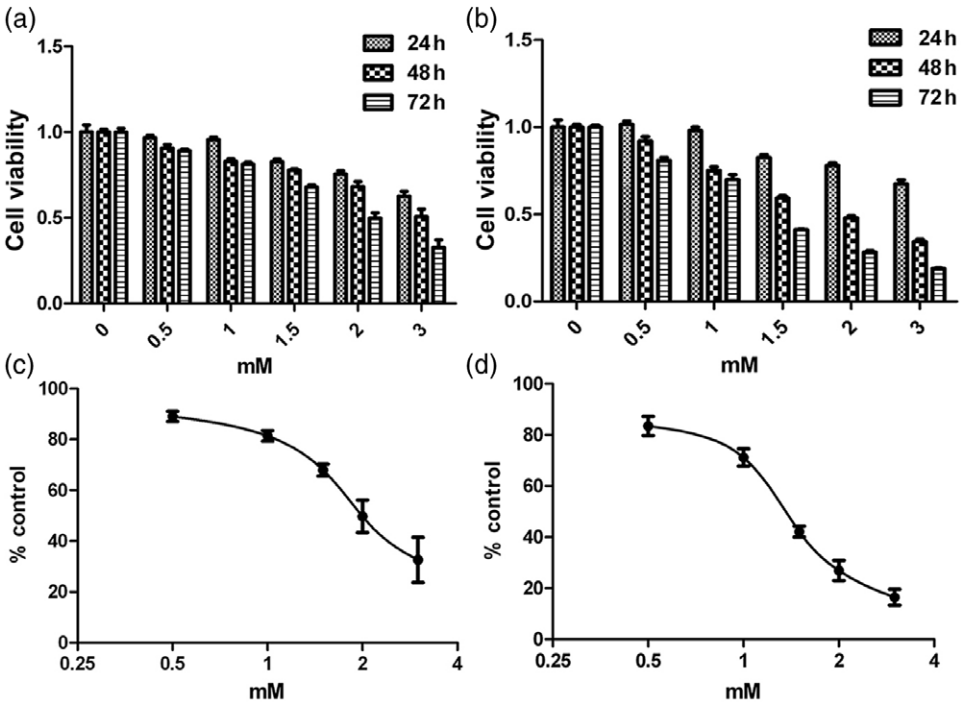
Ibuprofen inhibits glioblastoma cell viability in a dose- and time-dependant manner

To investigate the toxic effect of ibuprofen on glioma cells, we examined ibuprofen-induced changes in the viability of U87MG and U251MG glioma cells by using CCK-8 assay. As shown in Fig. 1a and b, in comparison with control cells, the viabilities of U87MG and U251MG cells decreased drastically by ibuprofen in a concentration- and incubation time-dependent manner. IC50 values were 2.51 mM for U87MG cell and 1.342 mM for U251MG cell (Fig. 1c and d).

Ibuprofen inhibits glioblastoma cell viability in three-dimensional tumor cell cultures

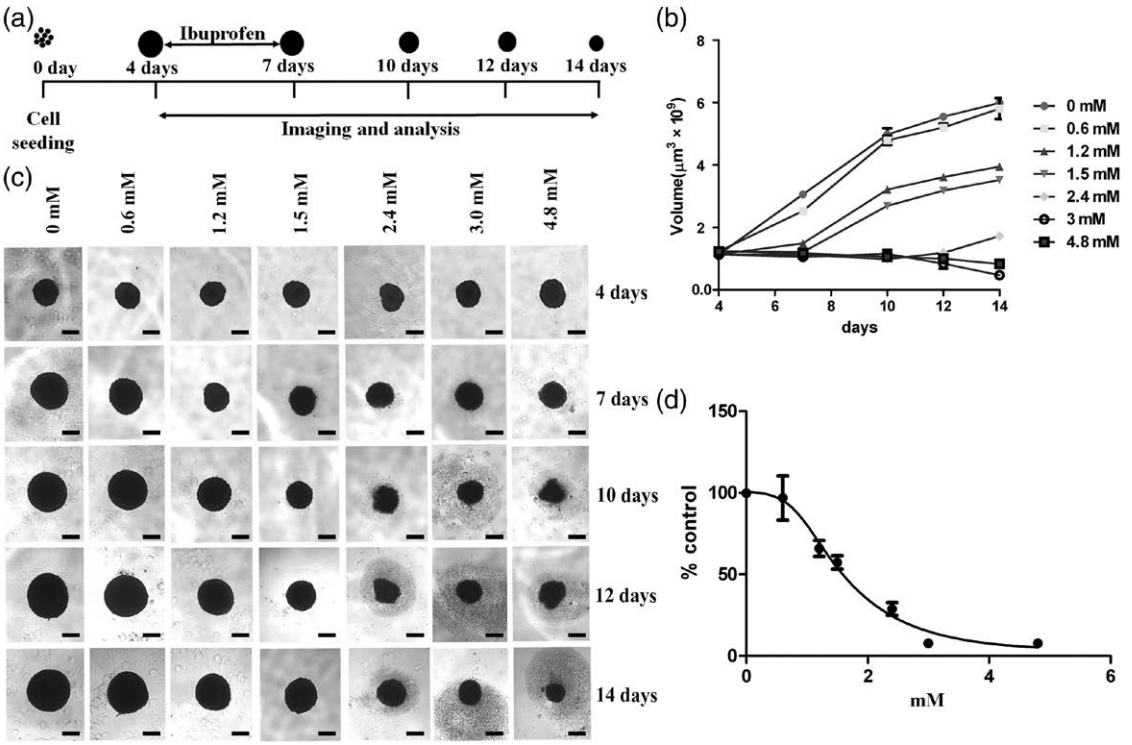
As we know, monolayer (two-dimensional) cell cultures are unable to mimic cellular functions and responses that occur in tissues, limiting the predictive capability of drug sensitivity. So, we first tested toxic effect of ibuprofen on glioma cells in three-dimensional cell cultures. Media supplementation and imaging were shown in the schematic protocol in Fig. 2a, and the ibuprofen induced concentration-dependent growth inhibition of U87MG

Fig. 1



Concentration-dependent cytotoxicity of ibuprofen was investigated by treating the GBM cell lines. (a) and (b) CCK-8 showed that ibuprofen inhibited the viabilities of U87MG and U251MG in a dosage- and time-dependent manner. (c) and (d) The IC50 values of U87MG and U251MG cells treated with ibuprofen for 72 hours were calculated. The data are presented as mean ± standard error of three tests. *$P<0.05$, **$P<0.01$, ***$P<0.001$, significant differences between treatment group and DMSO control group. GBM, glioblastoma.

Fig. 2



Ibuprofen inhibits GBM cell viability in a dose-dependent manner in 3D culture. (a) Schematic illustration of tumor spheroid growth kinetics and ibuprofen treatment procedures. Spheroids were initiated on day 0, treated with ibuprofen on day 4, and 50% medium replenishment was performed on days 7, 10 and 12. (b) The U87MG spheroids were treated with ibuprofen on day 4 and the volume of the spheroids was inhibited in a dose-dependent manner. (c) The morphology of tumor spheres. (d) The IC50 values of ibuprofen on U87MG in 3D culture were calculated. Values are means ± standard error (n=6), and a representative of three separate experiments for each agent is shown. Scale bar: 200 μm. GBM, glioblastoma.

spheroids as shown in Fig. 2b and c. The IC50 value was 1.611 mM for U87MG spheroid in Fig. 2d.

Ibuprofen inhibits glioblastoma growth *in vivo*

To analyze the role of ibuprofen in glioma carcinogenesis, we further assessed the effects of ibuprofen effort on tumor growth *in vivo*. The U87MG cells were intracerebrally injected into the left side of the brains of nude mice. Two weeks after tumor cell transplantation, mouse brains were scanned to detect tumor formation by MRI. Then mice were divided randomly into two groups and treated with vehicle control (PBS), or ibuprofen (20 mg/kg). We found a significant improvement in the survival ibuprofen-treated xenogeneic GBM model (Fig. 3); these supported that ibuprofen inhibits GBM growth *in vivo*.

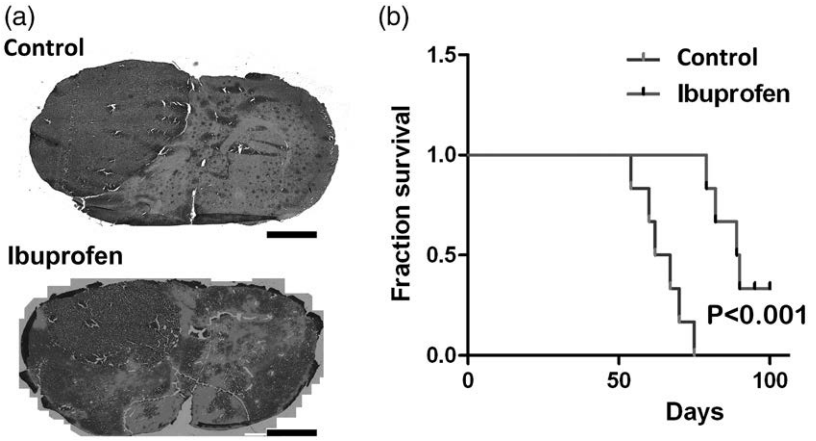
Ibuprofen inhibits glioblastoma cell viability by increasing ferroptosis

In order to investigate whether ibuprofen affects the induction of ferroptosis, we analyzed the cell death mechanisms induced by ibuprofen or erastin with and without iron chelation and ferroptosis inhibitor (Fig. 4). After 72 hours of U87MG cells exposed to 2 mM ibuprofen, we found ibuprofen robustly inhibited cell viability, and this effect could be rescued by the ferroptosis inhibitors DFO (100 μM), Fer-1 (0.5 μM) or Lip-1 (0.1 μM) (Fig. 4a and b). Flow cytometric analysis using the fluorescent probes C11-BODIPY demonstrated that ibuprofen-treated cells showed increased lipid peroxidation, which was also suppressed by cotreatment with the ferroptosis inhibitors (Fig. 4c). These data show that ibuprofen-induced cell growth inhibition is associated with increased ferroptosis.

Ibuprofen treatment decreases expression of Nrf2, GPX4 and SLC7A11

The Nrf2, GPX4 and SLC7A11 are important genes to regulate ferroptosis, so we examined the expression level of Nrf2, GPX4 and SLC7A11 by western blotting in glioma cells. The results showed that the expression of Nrf2, GPX4 and SLC7A11 decreased drastically by ibuprofen in a concentration-dependent manner (Fig. 5a). These data suggest that ibuprofen-induced ferroptosis may be due to inhibition of Nrf2, GPX4 and SLC7A11 expression. Furthermore, we detected the mRNA expression of GPX4 and SLC7A11, and found the mRNA expression of GPX4 and SLC7A11 was decreased treated with increasing concentrations of ibuprofen (Fig. 5b).

Fig. 3



Ibuprofen inhibits GBM growth *in vivo*. (a) Immunohistochemistry of coronal brain sections illustrating tumor growth. (b) Survival was significantly longer in ibuprofen treated mice compared with nontreated mice ($P<0.001$). Scale bar: 2 mm. GBM, glioblastoma.

Discussion

Many studies have suggested that NSAIDs can prevent tumors and ibuprofen is the most commonly used NSAID [5,6,23]. In this work, ibuprofen was studied for their antitumor effect on U87MG and U251MG glioma cell lines, and the molecular mechanisms were explored. Our data first indicate that ibuprofen inhibits the glioma cell viability through increasing ferroptosis.

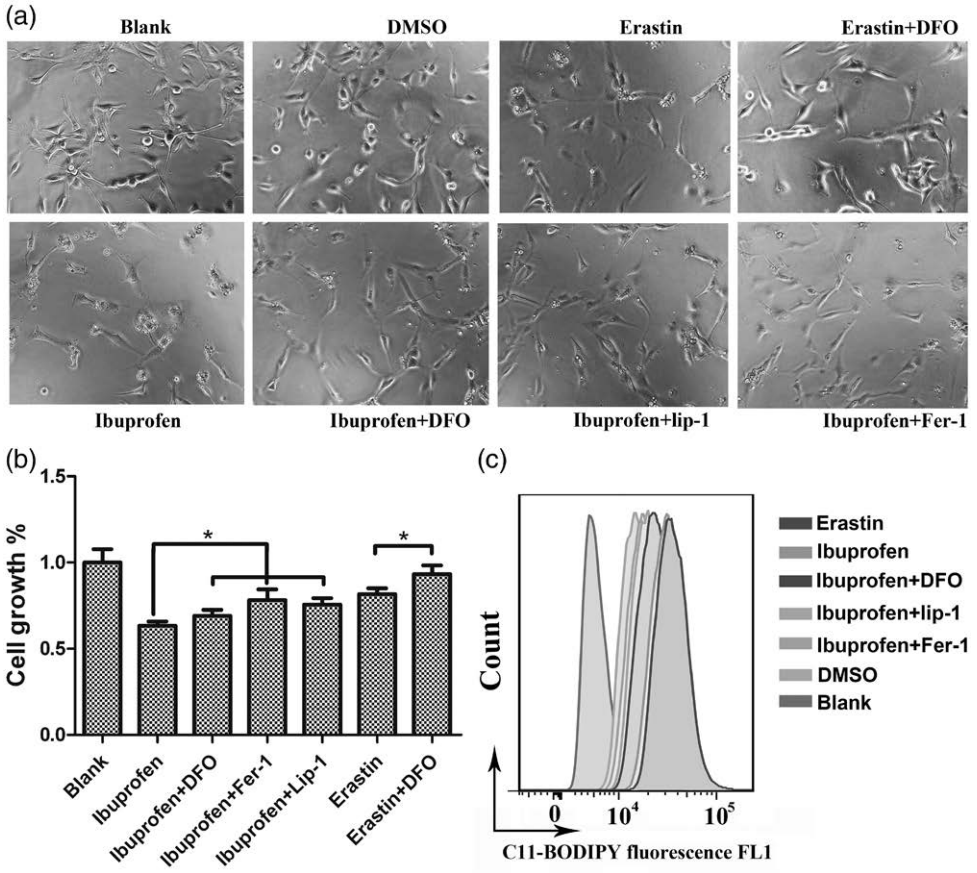
We found that the ibuprofen inhibited GBM cell growth in a dose- and time-dependant manner in 2D cell culture models (Fig. 1). But the monolayers cell obtained from 2D culture were unable to reproduce the real complexity and 3D structure found in the human body [24]. So, we used the 3D spheroids-based assays to detect the inhibitory effect of ibuprofen on GBM cell viability. We found the ibuprofen could also inhibit GBM cell growth in a dose- and time-dependant manner *in vitro* (Fig. 2), and further research proved that ibuprofen also inhibit GBM growth *in vivo* (Fig. 3). These results showed that the ibuprofen could suppress the GBM cell growth both in 2D or 3D cell culture, suggesting that the 3D tumor spheroids assay can be employed to characterize and evaluate the efficacy of anticancer therapeutics.

Ferroptosis is a new type of regulated cell death, which plays a pivotal role in killing cancer cells and suppressing cancer growth. A lot of studies showed that many drugs could induce ferroptosis of cancer cell, such as that sulfasalazine induced ferroptotic cell death in glioma cell [23]. But the relationship between ibuprofen and ferroptosis has not been reported. We found that the cell growth inhibition caused by the ibuprofen and ferroptosis inducer erastin could be rescued by the ferroptosis inhibitors DFO, Fer-1 and Lip-1(Fig. 4a and b); these data suggested that the ibuprofen could induce ferroptosis in the GBM cells. Furthermore, ferroptosis is characterized by the overwhelming, iron-dependent accumulation of lethal lipid reactive oxygen species (ROS). Using the general lipid ROS probe C11-BODIPY (581/591) [25], we found that the lipid ROS induced by treatment with ibuprofen could be reduced by cotreatment with the ferroptosis inhibitors (Fig. 4c). All these data suggested that the ibuprofen could trigger ferroptosis in glioma cells, but the mechanism needs to be further studied.

Recently, people found that one of the key pathways contributing to carcinogenesis and ferroptosis is Nrf2 pathway [26]. Nrf2 is a redox sensitive transcription factor which interacted with antioxidant response elements (AREs) in the promoter region of target genes [27], so dysregulation of the Nrf2 signaling pathway would influence the transcriptional activities of downstream target genes including the encoding intracellular redox-balancing proteins involved in GSH synthesis [28]. We found that ibuprofen inhibited the Nrf2 expression in a dose-dependent manner (Fig. 5a), indicating that ibuprofen induces ferroptosis at least partly due to inhibition of Nrf2.

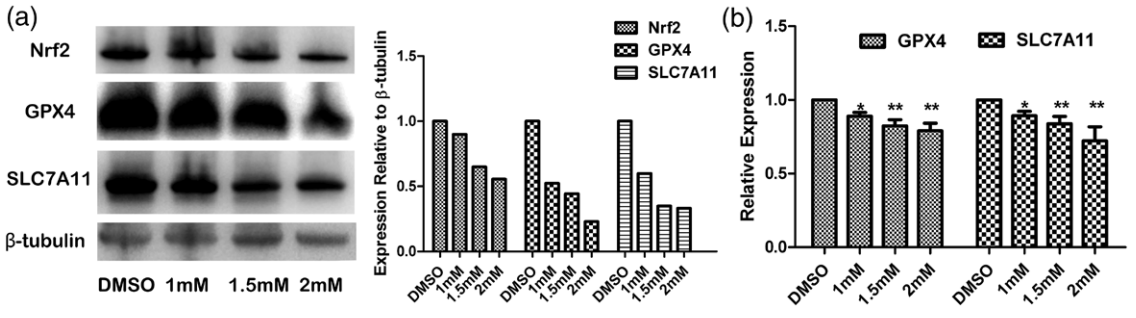
Many studies proved that the GPX4 and SLC7A11 are key regulators of ferroptosis. Consistently, we found that ibuprofen downregulated the protein levels of GPX4 and SLC7A11 in this study (Fig. 5a). Interestingly, preexisting evidences showed that GPX4 and SLC7A11 are downstream targets of Nrf2 [26]. GPX4 catalyzes the reduction of organic hydroperoxides, lipid peroxides and hydrogen peroxide at the expense of reduced GSH for preventing ferroptosis [10,29]. Osburn *et al.* [30] has been proved that the GPX4 was downregulated in Nrf2-deficient mice. Shin *et al.* [31] proved that activation of the Nrf2-ARE pathway contributed to the

Fig. 4



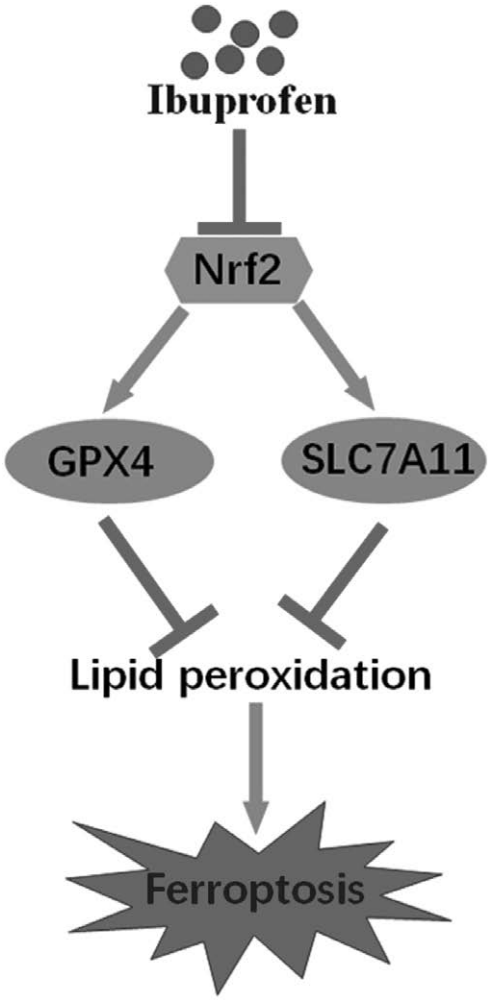
Ibuprofen induces GBM cells ferroptosis. (a) Representative images of U87MG cells treated with 10 μM erastin(E) ±100 μM DFO or 2 mM ibuprofen with or without DFO, ferrostatin-1 (Fer-1, 0.5 μM), (Lip-1, 0.1 μM) for 72 hours. (b) Quantification of cell viability was measured by CCK-8 in U87MG cells. Statistical significance was calculated with Student's *t*-test (shown as mean ± SD, $n=6$, *$P<0.05$). (c) Lipid ROS production in GBM cells treated with Erastin, ibuprofen ± DFO, Fer-1 and lip-1 for 72 hours was assayed by flow cytometry using C11-BODIPY. DFO, deferoxamine; GBM, glioblastoma.

Fig. 5



Ibuprofen affects Nrf2, GPX4 and SLC7A11 expression. (a) After incubation with increasing concentrations of ibuprofen (1, 1.5 and 2 mM), the protein expression levels of Nrf2, GPX4 and SLC7A11 were investigated by Western blotting. The expression level of Nrf2, GPX4 and SLC7A11 protein in each group was quantified with IPP6.0 software. (b) qRT-PCR analysis of GPX4 and SLC7A11 expression in glioma cells treated with increasing concentrations of ibuprofen (1, 1.5, 2 mM), (*$P<0.05$, **$P<0.01$). GPX4, glutathione peroxidase 4; Nrf2, nuclear factor erythroid 2-related factor 2; SLC7A11, solute carrier family 7 member 11.

Fig. 6



Schematic diagram for ibuprofen-induced ferroptosis in glioma cells. In conclusion, we demonstrated that ferroptosis was a pivotal pathway mediating ibuprofen-induced glioma cell growth inhibition in this study. Moreover, ibuprofen could increase lipid peroxidation by inhibiting the expression of GPX4 and SLC7A11 via regulating Nrf2 transcriptional activity. This is the first time showed that ibuprofen could induce the tumor cell ferroptosis, but the molecular mechanism needs to be further studied. GPX4, glutathione peroxidase 4; Nrf2, nuclear factor erythroid 2-related factor 2; SLC7A11, solute carrier family 7 member 11.

resistance of HNC cells to GPX4 inhibition. Kitaoka *et al.* [32] showed that the Nrf2 downstream target genes such as NQO1, HMOX1, GPX4 were higher in mcardle disease patients who have higher nuclear Nrf2 protein content and Nrf2-ARE binding. We also found the levels of GPX4 mRNA and protein were decreased (Fig. 5); these suggest that ibuprofen maybe reduce the GPX4 expression through via inhibition of Nrf2 signaling pathway.

Nandar *et al.* [33] showed a correlation between Nrf2 and SLC7A11 in H67D mice in response to oxidative stress. Electrophilic agents and other Nrf2 activators have been correlated with increases in SLC7A11 expression in glioma stem cell [34], RGC-5 cells [35] and human bronchial epithelial cells [36]. More directly, Ye *et al.* [37] showed that the human SLC7A11 promoter contains an ARE/AP1 site which is bound by Nrf2 in bladder carcinoma cells, and Habib *et al.* [28] proved that overexpression of Nrf2 upregulated the activity of the SLC7A11 promoter and increasing expression of SLC7A11 in human breast cancer cells. Consistently, we found that ibuprofen downregulated the protein levels of Nrf2 and SLC7A11 in this study (Fig. 5a) and found that the levels of SLC7A11 mRNA were also decreased (Fig. 5b); these results suggest that the expression of SLC7A11 maybe regulated by Nrf2 in glioma cells. Thus, ibuprofen could induce ferroptosis via inhibition of Nrf2 signaling pathway. Furthermore, we summarize the mechanism of ibuprofen induced ferroptosis in Fig. 6.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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