Title: Ursodeoxycholic acid inhibits glioblastoma progression via endoplasmic reticulum stress related apoptosis and synergizes with the proteasome inhibitor bortezomib

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Abstract

Ursodeoxycholic acid (UDCA) has demonstrated cancer suppressive potential in several tumors. Here, we investigated the antitumor potential and biochemical mechanism of UDCA on glioblastoma multiforme (GBM), the deadliest form of brain cancer with a median survival of 15 months. Cell viability was assessed using the CCK-8 and colony forming assays. Expression profiles were obtained using RNA sequencing, PCR and western blot were used to validate changes in related markers at the RNA and protein levels. Flow cytometry was used to examine cell cycle, apoptosis, mitochondrial membrane potential (MMP) and reactive oxygen species (ROS). UDCA inhibited GBM cell viability in a dose- and time-dependent manner. Flow cytometry demonstrated that cells were arrested in the G1 phase and underwent apoptosis. The RNA sequencing results showed UDCA treatment in part targeted gene expression related to mitochondria and endoplasmic reticulum (ER). UDCA indeed led to decreased MMP, overproduction of ROS and ER stress. Three critical ER stress sensors ATF6, IRE1α and PERK were increased in the acute phase. Additionally, combining UDCA with the proteasome inhibitor bortezomib (BTZ) achieved a synergistic effect through enhancing the PERK/ATF4/CHOP pathway and protracting ER stress. UDCA inhibited GBM progression and the combination with BTZ achieved a synergistic effect via protracted ER stress. Thus, UDCA, alone or with combination of BTZ, shows promise as a possible therapeutic agent for the treatment of GBM.

Keywords: ursodeoxycholic acid; glioblastoma; ER stress; bortezomib; synergism

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive adult human brain cancer. Despite aggressive treatment protocols including surgery, radiation and chemotherapy, prognosis of GBM patients remains dismal with a median survival of 15 months.[1] Intertumor heterogeneity in GBM, in particular, underlies drug resistance and recurrence. Thus, novel and diverse treatment strategies are urgently needed.

In addition to targeting tumor progression related oncogenes, interfering with cancer cell metabolism is a promising approach. GBM cell survival relies heavily on exogenous cholesterol, [2] and the levels of cholesterol are modulated by many factors, including its metabolites and bile acids, the function of which has been studied in GBM. Among the secondary bile acids, ursodeoxycholic acid (UDCA) is an endogenous bile acid existing in human bile, though in a low proportion of about 4% of total bile acids.[3, 4] Due to favorable safety and tolerance levels, the use of UDCA was extensively studied in recent years in the treatment of cholestasis diseases and won FDA approval for clinical use. No severe side effects of UDCA have been reported, even in children or women during pregnancy.[3] To date, over 60 clinical trials have been registered in the International Clinical Trials Registry Platform, studying the efficacy of UDCA treatment for infection, inflammation and cancers.

Unlike hydrophobic bile acids deemed as carcinogens, UDCA, with hydrophilic properties, tends to suppress tumor progression. Previous study demonstrated UDCA to be chemopreventive in colon cancer development.[5] UDCA has also been shown to induce apoptosis of several cancer cell types but through various mechanisms. In gastric cancer cells, cell death occurrence was accompanied with an increase in lipid raft dependent death receptor 5 expression.[4] In oral squamous carcinoma, UDCA inhibited proliferation via caspase mediated apoptosis,[6] whereas in hepatocellular carcinoma, UDCA induced apoptosis through both caspase-dependent and independent mechanisms.[7]

Though as a hydrophilic bile acid, UDCA possesses the ability to cross blood brain barrier (BBB) and thus the possible application in central nervous system diseases. In a stroke model in rats, more than half of the UDCA appeared in the brain after intravenous administration.[8] Also in a vitro model of the human blood brain barrier, UDCA crossed the human brain microvascular endothelial cell monolayer in a time-dependent manner.[9] Recent studies have demonstrated that treatment with UDCA effectively alleviated Parkinson's and Alzheimer's disease related features in corresponding models, but its effect on brain malignancies remains uncertain.[10, 11] In this study, we investigated the antitumor potential of UDCA in GBM, and explored underlying molecular and biochemical mechanisms. We found that UDCA inhibited GBM cell proliferation mainly through apoptosis, and that combination treatment with the proteasome inhibitor bortezomib (BTZ) enhanced the efficacy

of UDCA. These results highlight UDCA as a possible therapeutic agent in the treatment of GBM.

Results and Discussion

As an endogenous bile acid, UDCA participated in multiple biochemistry processes and modulated cholesterol metabolism. Meanwhile, UDCA was deemed as an effective tumor-suppressive agent and possessed permeability into BBB that was essential for its application in the central nervous system. Here, we demonstrated UDCA effectively inhibited GBM progression through affecting the homeostasis of intracellular membrane components and inducing cell death.

UDCA decreases GBM cell viability

To begin to determine whether UDCA had anti-tumor properties against GBM, growth curves were first generated for GBM cell lines treated with different concentrations of UDCA using the CCK-8 assay. Cell viability of 4 different GBM cell lines decreased in a dose and time-dependent manner after UDCA treatment (**Fig. 1A**). Vehicle control (ethanol) at the corresponding concentrations did not significantly affect cell viability (**supplementary Fig. S1**). The antitumor properties of UDCA were also evaluated in the colony forming assay. Colony formation numbers were decreased for both A172 and LN229 cells (~60%, $p < 0.01$, and $p < 0.001$, respectively) under treatment with UDCA in a dose-dependent manner (**Fig. 1B and 1C**).

Expression of genes associated with cell cycle arrest and apoptosis are increased in UDCA treated cells

To understand the molecular mechanisms underlying effects of UDCA, we performed RNA sequencing on RNA isolated from treated LN229 cells and controls. A total of 7118 genes matching the cutoff of $|\log_2 FC| \geq 1$ and $q \leq 0.05$ were obtained, and a heatmap was generated to show patterns of gene expression in the samples tested. The heatmap demonstrated that gene expression patterns were similar among samples within each group but differed between groups (NC1, NC2, and NC3 vs U1, U2, and U3; **Fig. 2A**). A Volcano plot was used to distinguish upregulated (n = 3212) and downregulated genes (n = 3906; **Fig. 2B**). The top 10 up- and down-regulated genes were selected for further analysis, and their function was found to be mainly related to pathways in cancers, apoptosis, aerobic respiration and mitochondrial function (**supplementary Table S1**).

KEGG pathway analysis demonstrated that the genes altered under UDCA treatment were associated with glioma progression and furthermore highly related to pathways in cancer and cell cycle processes (**Fig. 2C**). The directed acyclic graph (DAG) based on results from GO analysis illustrated that putative functions of the genes involved were associated with endoplasmic reticulum (ER), regulation of multicellular organismal development and metal ion binding under the categories of cellular component, biological process and molecular function respectively (**Fig. 2D**).

Based on the sequencing results, we focused on genes involved in the cell cycle and apoptosis, located in mitochondria and ER.

UDCA induces cell cycle arrest and apoptosis in GBM

To determine the biological processes contributing to the decreased viability of GBM under treatment, cell cycling parameters of cells exposed to UDCA were assessed using FACS. Both A172 and LN229 cells exposed to UDCA were arrested in the G1 phase of the cell cycle, and the percentage of cells in G1 arrest increased with UDCA treatment (**Fig. 3A and 3B**). Expression of cell cycle related markers, including CDK2, CDK4, CDK6, and p-Rb were decreased, whereas p53 and p21 were increased as assessed on western blot (**Fig. 3C**).

Our results also demonstrated that UDCA induced apoptosis in a dose dependent manner. The percentage of Annexin-V positive cells was increased in both A172 and LN229 cells with a greater percentage of A172 cells undergoing apoptosis (**Fig. 4A and 4B**). On western blot analysis, although the expression of BCL2 family members decreased, caspase-3 and PARP were not correspondingly activated, indicating the involvement of other types of cell deaths (**Fig. 4C**). Several caspase-independent apoptosis pathways were also examined, such as the AIF/EndoG pathway and necroptosis, but associated markers were not activated during UDCA induced cell death. For example, the protein levels of AIF did not increase, and furthermore translocation to the nucleus was not observed (**supplementary Fig. S2**). To further investigate pathways that might mediate apoptosis, we introduced several inhibitors, such as pan-caspase inhibitor z-VAD-fmk, p53 inhibitor PFTβ, the necroptosis inhibitor necrostain-1 and Erk inhibitor U0126, into UDCA treated cells. However, none of these inhibitors reversed the cytotoxic effects of UDCA as assessed in CCK-8 viability assays (**Fig. 4D**). The results indicated that UDCA induced G1 phase cell cycle arrest and apoptosis.

UDCA induces mitochondrial damage

The BCL-2 family proteins are mainly localized on the outer mitochondrial membrane and ER, the biogenesis of which is closely related to apoptosis. We therefore assessed whether UDCA caused mitochondrial damage in GBM in vitro using JC-1 dye to detect changes in the mitochondrial membrane potential (MMP). In A172 and LN229 cells treated with UDCA, the ratio of red/green fluorescence decreased by ~ 50% indicating a decreased MMP (**Fig. 5A and 5B**). In addition, the production of reactive oxygen species (ROS), observed through the fluorescence intensity of DCFH, was also increased over controls (**Fig. 5C and 5D**). These results were consistent with the presence of damaged mitochondria.

ROS production has been shown to be closely related to activation of the MAPK pathway. We therefore examined proteins in the pathway by western blot. The phosphorylation status of Erk was

enhanced after treatment with different concentrations of UDCA, while the phosphorylation of JNK remained unchanged. However, while p-p38 was increased in A172, it was decreased in LN229 cells (**Fig. 5E**). Hence, UDCA treatment resulted in mitochondrial damage and enhanced ROS production which was possibly mediated through the MAPK pathway, i.e. Erk activation. As mitochondrial-endoplasmic reticulum system performed as a calcium hemostasis regulator. Our results showed UDCA also induced enhanced calcium release into the cytosol, where the disruption of calcium level increase the burden of ER (**Fig. 5F and 5G**)

UDCA induces ER stress

Changes in genes involved in ER protein processing can be a marker of ER stress. We therefore examined the expression levels of a set of mRNAs in GBM cell lines after UDCA treatment. A dose-dependent increase was observed in the mRNA levels of *IRE1α*, *BIP*, *ATF4*, *ATF6*, *CHOP, XBP1s* and the differences were statistically significant (**Fig. 6A**). In general, ER stress is followed by the unfold protein response that leads to reduced protein synthesis and enhanced protein degradation. We further found the total ubiquitination of protein was augmented after UDCA treatment (**Fig. 6B**). The protein levels of BIP, IRE1α and ATF6 also increased dose-dependently. However, despite a significant increase at the transcriptional level, CHOP protein levels increased only slightly. Even its upstream proteins, such as p-PERK and ATF4, showed a tendency to decrease (**Fig. 6C**). Thus, we examined protein levels over a 48H time course. The protein levels of p-PERK, ATF4 and CHOP reached peak expression at approximately 24H and then decreased, while the protein levels of ATF6 and IRE1α increased continuously in both cell lines (**Fig. 6D**). UDCA induced ER stress and the PERK/ATF4/CHOP pathway was activated in the acute phase.

UDCA synergizes with bortezomib (BTZ) in GBM

Since the overload of unfold protein could be alternatively degraded via a proteasome pathway, acute and short-term ER stress with UDCA was not enough to generate a continuous proapoptotic effect. We therefore postulated that enhancing ER stress with a proteasome inhibitor might achieve a synergistic effect. When combined with the proteasome inhibitor BTZ, UDCA exhibited an enhanced cytotoxic effect in A172 and LN229 cells as assessed in the cell viability assay (**Fig. 7A**). When UDCA at 600uM concentration was combined with BTZ, combination index (CI) was 0.69 and 0.55 for A172 and LN229 respectively, indicating a synergistic effect according to the recommended description for CI (**Fig. 7B and supplementary Table S2**). Furthermore, the number of apoptotic cells increased in the UDCA+BTZ group compared to the UDCA group (**Fig. 7C and 7D**). To identify pathways underlying this synergistic effect, we examined protein levels of p-PERK, ATF4 and CHOP under combined treatment. We found that UDCA+BTZ enhanced p-PERK, ATF4 and CHOP expression which contribute to chronic and protracted ER stress. However, the increased expression of IRE1α was partially alleviated in cells under UDCA+BTZ treatment (**Fig. 7E and 7F**). These results indicated that UDCA+BTZ augmented cell death through further enhancing

PERK pathway.

Study summary

Our study reveals an anticancer role for UDCA in GBM. Expression profiles obtained through RNA sequencing revealed a relationship between UDCA and glioma, and furthermore led us to find UDCA induced cell cycle arrest and apoptosis. GO and KEGG pathway analysis of differentially expressed genes indicated involvement of the mitochondrial respiratory chain and ER components in the UDCA killing effect. Cell death appeared to be apoptosis, and mitochondria was found to be dysfunctional as indicated by the decreased MMP and over production of ROS after UDCA treatment. Although UDCA induced excessive ROS and initiated ER stress, transient ER stress was not sufficient to effectively kill GBM. The combination of UDCA with the proteasome inhibitor BTZ achieved a synergistic effect through enhancing expression of PERK, ATF4 and CHOP and attenuated IRE1α increase. Combination treatment may therefore augment therapeutic effect while simultaneously reducing the doses of the respective drugs. Nevertheless, previous studies have demonstrated UDCA and its taurine-conjugated form played anti-apoptotic and neuroprotective roles in neurodegenerative disease models,[12] which seemed inconsistent with our results. Actually, unlike this study focusing on cancer cells, neurodegenerative disease models targeted normal neurons and astrocytes and their responses to UDCA treatment. Nearly all cholesterol in brain is synthesized de novo in normal astrocytes, while astrocytoma mainly relies on uptake of cholesterol from surrounding environment.[2] Since UDCA exerted its roles through affecting cholesterol-containing membrane components, the different mechanisms of cholesterol metabolism between normal and cancer cells may be responsible for the paradoxical results. In line with our work, previous literatures also showed anticancer activity of UDCA in various malignances. Moreover, the dual proapoptotic and antiapoptotic properties of UDCA represented a characteristic trait of bile acids.[13]

The intractability in curing GBM is due to its genetic diversity and drug resistance, as tumor cells fundamentally possess multiple mechanisms to oppose cell death. Classical apoptosis, via caspase activation, is the most well-characterized and possibly powerful mechanism available to kill tumor cells. However, a recent study demonstrated that activated caspase 3 could also stimulate the growth of surviving tumor cells, which might contribute to the recurrence of glioma.[14] Other caspase-independent cell death processes, such as necroptosis, ferroptosis, and pyroptosis, have been proposed as more efficient cytocidal mechanisms to activate for the treatment of human tumors.[15] Here, we found that UDCA inhibited glioma cell growth through inducing apoptosis. These results corroborate the results of a previous study, that caspase activation was not always related to increased apoptosis.[7] Furthermore, cell death initiated by UDCA treatment proceeded even in the presence of several inhibitors of apoptosis, including the pan-caspase inhibitor z-VAD-fmk.

The dysfunction of membranous organelle mitochondria, resulting in ROS overproduction and the activation of downstream pathways as MAPK, has been previously implicated as a critical mechanism in cancer cells death.[16] Baseline levels of ROS are higher in tumors than in paired non-neoplastic tissues, which stimulats protumorigenic signaling partially through MAPK pathways activation.[17] Nevertheless, overproduction of ROS could induce hyperactivation of Erk/MAPK that exerts proapoptosis and anticancer effects. Hence properly increasing ROS production would induce cancer cells apoptosis while sparing normal cells with low baseline levels of ROS and tolerance to slight increase of ROS. Extensive reports have shown the anticancer role of UDCA was closely related with MAPK pathway, and ROS also plays an important role in regulating MAPK activation.[5, 18, 19] In this study, we observed that UDCA induced ROS over production and activated Erk, but the phosphorylation level of JNK remained unchanged. These results were further supported by the finding that the proapoptotic effect of bile acids is partially counteracted by MAPK pathway activation, while inhibition of MAPK sensitized cancer cells to apoptosis.[18] Additionally, hyperactivation of Erk has been proposed to induce cell cycle arrest through increasing expression of CDK inhibitors p21 and p27.[20] The extent of activation and cell context might decide which functions Erk exhibits. This opposed two-way effect, known as the double-edged sword effect, is common in tumor progression as ROS production and Erk activation.

Just as the baseline production of ROS, ER stress is widely present in cancers maintaining homeostasis, but unsolved and prolonged ER stress could induce cell death. Three main sensors, PERK, ATF6 and IRE1α, orchestrate ER stress.[21] The ATF6 and IRE1α pathways are generally associated with adaption and survival of cancer cells, while PERK has been shown to induce ATF4/CHOP activation which leads to cell death.[22, 23] Despite the accumulation of CHOP correlates with cell death, both the mRNA and protein of ATF4 and CHOP exhibit short half-lives; hence, continuous and chronic PERK activation is needed to generate steady levels of CHOP sufficient to induce death.[24] Under condition of chronic stress, the PERK-mediated eIF2α/ATF4/CHOP axis leads to apoptosis, while IRE1α and ATF6 pathways are attenuated.[23] In our study, we found that UDCA promoted baseline ER stress in GBM, but it was acute and short-lived as p-PERK protein decreased at 48 h after UDCA treatment. In addition, IRE1α and ATF6 continued to increase over a time course of 48 h. Thus, to enhance ER stress, we introduced the proteasome inhibitor BTZ in combination with UDCA.

BTZ is a selective inhibitor of the 26S proteasome and commonly used to treat multiple myeloma clinically. Despite the efficacy of BTZ in hematological disease, treatment of GBM with this drug failed to significantly benefit patients.[25] Moreover, the efficacy of BTZ was directly proportional to the baseline proteasome activity, indicating that tumor cells with activated unfolded protein response and high proteasome activity were more susceptible to BTZ.[26] We therefore investigated whether UDCA induced ER stress predisposed GBM to BTZ treatment. The combination therapy achieved

a CI representing a synergistic effect in GBM cell lines. Enhanced expression of PERK/ATF4/CHOP also occurred in parallel with prolonged ER stress under combination treatment of UDCA with BTZ. Given that both UDCA and BTZ have been approved by the FDA for clinical use, combination therapy may enable treatment with reduced doses and therefore diminish corresponding side effects.

Conclusions

In GBM, UDCA played cytocidal effect through cell cycle arrest and apoptosis mediated by mitochondria dysfunction and ER stress. Although UDCA-induced ROS production and short-term ER stress were accompanied with tumor adaption process as IRE1α/XBP1 pathway, combination with proteasome inhibitor BTZ achieved a synergistic effect via enhancing PERK/ATF4/CHOP pathways and protracted ER stress. UDCA, alone or with combination of BTZ, presented a promising drug to treat GBM.

Materials and Methods

Reagents

UDCA purchased from Sigma-Aldrich (St. Louis, MO, USA) was dissolved in ethanol as a stock solution of 100 mg/mL and diluted for working concentrations. The manufacturers and catalog information of antibodies are summarized in **supplementary Table S3**.

Cell culture

The human GBM cell lines A172, LN229, U87 and U251 were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). The cell lines had been recently authenticated based on DNA fingerprinting, isozyme detection and cross species checks. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1% penicillin and streptomycin, at 37°C in a humidified chamber containing 5% CO_2.

Cell viability assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8, C0040, Beyotime; Shanghai, China). GBM cell lines were seeded into 96-well, flat-bottomed plates at 4000 cells/well and incubated overnight. After the indicated treatment, cells were incubated for an additional 1 h in the medium with 10% (v/v) CCK-8. The absorbance at 450 nm was measured in a microplate reader (Bio-Rad; Hercules, CA, USA).

Colony formation assay

Cells at 1000 cells/well were seeded onto six-well plates and exposed to the indicated treatment. The medium was changed twice per week until single colonies contained more than 50 cells. Cells were fixed in methanol and stained with 5% crystal violet. The number of colonies with > 50 cells was counted manually, and results were repeated in triplicate.

RNA-Seq and data analysis

Three paired groups of LN229 cells treated with or without UDCA were sent to Annoroad Corporation (Beijing, China) for RNA-sequencing. The raw data have been deposited in NCBI's Gene Expression Omnibus, accessible through accession number GSE143798 (https://www.ncbi.nlm.nih.gov/geo/). After quality assessment, RNA was sequenced using the Illumina platform PE150 (Illumina; San Diego, CA, USA). Expression of each transcript was estimated as a value of fragments per kilobase of transcript per million mapped reads. Differentially expressed genes were measured using DESeq2, and the cutoff of $|\log_2 FC| \geq 1$ and $q \leq 0.05$ indicated significant difference. Gene annotation and functional enrichment analyses were conducted using the KEGG pathway and Gene Ontology (GO).

Cell cycle and apoptosis

For cell cycle detection, cells were dehydrated with 70% ethanol overnight at 4°C, stained with PI/Rnase (550825, BD Biosciences; San Jose, CA, USA) for 15 min in the dark, and analyzed with flow cytometry (Accuri C6, BD Biosciences). To detect apoptosis, cells were harvested, resuspended in 300 μL binding buffer, stained with Annexin V-FITC (BD Biosciences) according to the manufacturer's instructions, and analyzed with flow cytometry.

Quantitative reverse transcription PCR

RNA was isolated with the Total RNA Extraction Kit (R1200, Solarbio; Beijing, China), and cDNA was synthesized using the ReverTra Ace qPCR RT Kit (FSQ-101, Toyobo; Osaka, Japan) according to the manufacturers' instructions. The standard SYBR premix Ex Taq (Takara; Kusatsu, Japan) was used for qRT-PCR amplification on the Real-Time PCR Detection System (Roche, 480II; Indianapolis, IN, USA). Target gene expression levels were normalized to GAPDH and experiments were conducted in triplicate. The primer sequences are listed in **supplementary Table S4**.

Western blot

Total protein was extracted with cold RIPA buffer containing protease and phosphatase inhibitors. Cell lysate (20 μg) was loaded on SDS-PAGE and blotted onto PVDF membranes. Membranes were blocked with 5% skim milk in TBST (10 mM Tris, 0.9% NaCl and 0.1% Tween-20), incubated with the indicated primary antibody overnight at 4°C, rinsed 3 times for 10 min with TBST, probed with horseradish peroxidase conjugated secondary antibody, and rinsed again 3 times for 10 min in TBST. The proteins were visualized using Millipore's enhanced chemiluminescence and detection system (ChemiDoc Touch, BioRad). Each blot was quantified relative to loading control and the values above corresponding blots represent at least three experiment results.

Immunofluorescence

Immunofluorescence was performed to monitor nuclear translocation of AIF. Cells cultured on coverslips were treated with or without 800 μM of UDCA for 48 hours. Then the cells were rinsed with PBS for three times and fixed in 4% paraformaldehyde for 20 min. After permeabilization with 0.3% Triton X-100, cells were blocked with 5% BSA followed by incubation with AIF antibody overnight at 4 °C. Stained with Fluorescein-conjugated goat anti-rabbit IgG, the cells were counterstained with DAPI and observed under an Olympus BX61 fluorescence microscope.

Calcium release assay

A172 and LN229 were seeded onto six-well plates. After 24 hours, cells were harvested with 2 μM of Fluo-4 AM for 30min at 37℃ according to manufacturer's recommendations (Beyotime, China). Washed with PBS without calcium three times, cells were treated with or without UDCA for 30min followed by fluorescence imaging. Mean optical intensity was calculated by dividing the integrated intensity by the area of the measured spot with NIH imageJ software.

Reactive oxygen species (ROS) assay

Intracellular ROS production was assayed using DCFH (D6470, Solarbio). Cells were harvested with trypsin-ethylenediaminetetraacetic acid and incubated with 10 μM DCFH at 37˚C for 30 min. Harvested cells were divided into 3 different groups and treated separately with DMEM, 800 μM UDCA or Rosup for another 30 min. Cells that had not been incubated with DCFH were used as the negative control. Fluorescence intensity was detected by flow cytometry.

Mitochondrial membrane potential

The mitochondrial membrane potential (MMP) change △Ψm was measured with a JC-1 Kit according to the manufacturer's instructions (M8650, Solarbio). Cells were exposed to JC-1 (1×) for 30 min, washed 2 times with buffer, and collected for detection of fluorescence with flow cytometry. The red/green fluorescence ratio was calculated to reflect △Ψm.

Statistical analysis

All data are presented as the mean ± the standard error from at least 3 independent experiments. The unpaired t-test was used for comparison of 2 groups of normal distribution data. The F test was used to compare variances, and Welch's correction was applied in the presence of variance nonhomogeneity. The one-way ANOVA test used when there were more than 2 groups of data. All statistical analysis was conducted with GraphPad Prism 5 software (La Jolla, CA, USA). *, **, and *** indicated statistical significance of $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively.

Supporting Information

Supplementary Table S1: top 10 up- and down-regulated genes in RNA-sequencing; Supplementary Table S2: recommended symbols for describing synergism; Supplementary Table S3: details of

antibodies used; Supplementary Table S4: quantitative PCR primer sequences; Fig. S1: cells viability treated with vehicle control ethanol; Fig. S2: fluorescence imaging showing AIF translocation in LN229

Conflicts of Interest

The authors declare no conflicts of interest.

Author contributions

Zhong Yao and Xun Zhang conceived and designed the study; Feihu Zhao and Shuai Wang performed the experiments and analyzed the data; Anjing Chen and Bin Huang contributed materials and data and assisted in data analysis; Zhong Yao wrote the paper; and Jian Wang and Xingang Li revised the manuscript.

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Fig. 1. UDCA inhibits GBM cell viability. (A) Cell viability of 4 GBM cell lines treated with different concentrations of UDCA and measured with CCK-8 at indicated time points. (B) Images of colony forming assays performed with A172 and LN229 treated with UDCA, fixed and stained with crystal violet. (C) Quantification of the number of colonies forming for each cell line performed in 3 independent experiments. *, **, and *** indicated statistical significance of $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively.

Fig. 2. RNA sequencing and enrichment analysis of UDCA treated cells reveals genes involved in mitochondria and ER. (A) Heatmap showing differences in gene expression between control and UDCA treated groups (NC1, NC2, and NC3 vs U1, U2, and U3). (B) Volcano plot exhibiting differentially expressed genes between UDCA treated cells and controls. (C) KEGG analysis showing related pathways and possible function. (D) Directed acyclic graph showing the relationship between the most significant enrichment GO terms associated with the differentially expressed genes.

Fig. 3. UDCA induces cell cycle arrest in the G1 phase of the cell cycle. (A) Flow cytometry showing cell cycle parameters in A172 and LN229 cells following UDCA treatment for 48 h. (B) Graphs representing proportions of UDCA-treated or cells in G0/G1, S, and G2 phases of the cell cycle. (C) Western blot analysis showing expression of cell cycle related protein markers in A172 and LN229 cells following UDCA treatment. GAPDH and β-actin are used as the loading controls.

Fig. 4. UDCA induces caspase-independent apoptosis. (A) Flow cytometry showing Annexin V-FITC stain to detect apoptosis in A172 and LN229 cells treated with UDCA for 48 h. (B) Graphs representing quantification of Annexin V-FITC positive staining for treated cells and controls. (C) Western blot analysis of apoptosis related protein markers from A172 and LN229 cells. (D) Cell viability measured using CCK-8 for A172 and LN229 cells pretreated with inhibitors for 1 h (z-VAD-fmk 50 μM, PFTβ 10 μM, necrostatin-1 20 μM and U0126 10 μM) followed by exposure to UDCA for 48 h.

Fig. 5. UDCA leads to decreased MMP and overproduction of ROS. (A) Flow cytometry for fluorescence detection of JC-1 staining in UDCA treated A172 and LN229 cells. (B) Graphs showing quantitation of the red/green fluorescence ratio in control and treated A172 and LN229 cells. (C) Flow cytometry for fluorescence detection of DCFH staining of UDCA treated A172 and LN229 cells. (D) Graphs representing mean DCFH fluorescence intensity compared among groups: mock, negative control; Rosup, positive control; NC and UDCA were treated with or without UDCA. (E) Western blot showing the phosphorylation levels of MAPKs, JNK, ERK and p38, in cells under UDCA treatment relative to controls. (F) After incubated with Fluo-4 AM, cells were treated with or without UDCA followed by fluorescence imaging. Scale bar was 100μm; (G) Quantification analysis of optical intensity with at least five fields for each condition.

Fig. 6. UDCA enhances baseline ER stress in the acute phase. (A) RT-qPCR showing the relative fold changes in mRNA level of ER stress related markers in A172 and LN229 after UDCA treatment, *** indicates $p < 0.001$ compared with mock control. (B) Immunoblot of ubiquitinated protein in A172 after UDCA treatment. (C) Western blot showing dose-dependent changes in the protein levels of ER stress related markers in A172 and LN229 after UDCA treatment for 48 h. (D) Western blot showing protein levels of ER stress related markers at indicated time points after 800μM UDCA treatment.

Fig. 7. UDCA synergizes with BTZ resulting in protracted ER stress. (A) Cell viability assessed with CCK-8 in cells under combined treatment of UDCA with BTZ. (B) CI calculated based on the Chou-Talalay method in cells under combination treatment of UDCA and BTZ. UDCA and BTZ were mixed in a constant ratio of 100:1 (μM:nM). (C) Flow cytometry showing Annexin V-FITC staining to detect apoptosis in A172 and LN229 cells under combination treatment with UDCA and BTZ. (D) Graphs representing quantitation of apoptosis in (C). (E) Western blot analysis showing protein levels of ER stress protein markers in A172 and LN229 after UDCA or UDCA+BTZ treatment for 48 h. (F) Schematic diagram depicting the anticancer effect of UDCA and the pathways mediating the response in GBM cell lines in culture. UDCA induces ER stress in acute phase, and the combination with BTZ achieves a synergistic effect resulting in protracted ER stress and tipping the balance to cell death.