



Research Article

Ursolic acid sensitizes hepatoma cells to sulfasalazine-induced ferroptosis *via* AMPK/BECN1/SLC7A11-mediated redox disruption *in vitro* and *in vivo*

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Abstract: Targeting ferroptosis has emerged as a promising anti-cancer strategy. A Food and Drug Administration (FDA)-approved drug, sulfasalazine (SAS), also a ferroptosis inducer, is being explored as a potential treatment drug for cancer. However, long-term high-dose SAS treatment may induce several adverse effects in the liver. Our current study found that ursolic acid (UA), derived from traditional medicinal plants, fruits, and vegetables, enhanced the anti-tumor effects of low-dose SAS both *in vitro* (SMMC-7721 liver cells) and *in vivo* (a mouse tumor xenograft model). Treatment with a combination of UA and SAS reduced the glutathione (GSH), glutathione peroxidase 4 (GPX4), and promoted a substantial accumulation of lipid reactive oxygen species (ROS), leading to oxidative stress and ferroptosis. Mechanistically, the UA/SAS treatment significantly reduced the system xc^- activity and cystine uptake, attributed to the AMPK activation-induced formation of the SLC7A11-BECN1 complex. Moreover, the induction of NCOA4-mediated ferritinophagy also played a role in this process. Besides, UA sensitized hepatoma cells to erastin as well, another SLC7A11-targeting ferroptosis inducer, implying a potential use of UA as a SLC7A11-targeting enhancer to combat hepatocellular carcinoma (HCC).

Keywords: ursolic acid; sulfasalazine; ferroptosis; SLC7A11; HCC

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1 Introduction

Ferroptosis, a novel form of programmed cell death, plays a crucial role in the development and progression of various cancers^[1]. Ferroptosis is characterized by cell death driven by the accumulation of iron-dependent lipid peroxidation, distinguishing it from apoptosis, autophagy, and necroptosis in terms of both morphology and biochemistry. The regulation of ferroptosis reflects an antagonism between ferroptosis-driving and ferroptosis-defence systems^[2]. The abnormal metabolism of tumor cells leads to distinct oxidative stress characteristics and an increased demand for iron, thereby enhancing their sensitivity to ferroptosis. The induction of ferroptosis has emerged as a potentially effective strategy for the prevention and treatment of cancer^[3]. Sulfasalazine (SAS), is a classic ferroptosis inducer which target and inhibit system xc^- , resulting in cysteine starvation, glutathione (GSH) depletion, and consequently ferroptotic cell death^[4]. Previous studies have shown that SAS can inhibit the growth of some cancer cells, but long-term high-dose SAS treatment may induce

several adverse effects in the gastrointestinal tract, liver, and kidney^[5].

Natural products have attracted increased attention in cancer prevention and chemotherapy owing to their biologically friendly and good therapeutic effects. Ursolic acid (UA) is a type of pentacyclic triterpenoid, which can be found in various medicinal plants and most fruits and vegetables, exhibiting promising therapeutic potential in the treatment of different diseases^[6]. Numerous *in vivo* and *in vitro* studies have reported that UA can effectively inhibit the growth, proliferation, and metastasis of cancer cells while promoting cell death, thus demonstrating strong anticancer properties^[7]. Moreover, UA has shown a remarkable synergistic effect when used in combination with other cancer treatments, which can enhance the sensitivity of cancer cells to chemotherapy and radiotherapy^[8,9]. This suggests that ursolic acid could potentially be used as an adjunct therapy to improve the effectiveness of existing cancer treatments.

Hepatocellular carcinoma (HCC), which constitutes 75%-80% of primary liver malignancies, is one of the most lethal cancers

worldwide. The late-stage presentation is a typical characteristic of HCC, which severely limits the options and effectiveness of treatment methods. Furthermore, the development of drug resistance further constrains the use of chemotherapy drugs^[10]. Therefore, combination chemotherapy strategies have been explored to advance the development of a more potent treatment for liver cancer.

In this work, we aim to investigate the synergistic anticancer effects of UA and SAS in both the SMMC-7721 cell model and the BALB/c nude mouse model, delving deep into the specific mechanisms involved. The results revealed that UA dramatically facilitates SAS-induced ferroptosis by targeting AMPK-BECN1 pathway to inhibit the activity of SLC7A11. Our findings may provide a novel therapeutic strategy of combined UA and SAS against liver cancer.

2 Materials and methods

2.1 Chemicals and reagents

UA, ferostatin-1 (Fer-1), liproxstatin-1 (Lip-1), deferoxamine mesylate (DFO), chloroquine (CQ), erastin, and compound C were purchased from MedChem Express (Monmouth Junction, NJ, USA). SAS was purchased from Macklin (Shanghai, China). 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), N-acetyl-L-cysteine (NAC), and α -tocotrienol (α -TT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). FerroOrange and Liperfluo were purchased from Dojindo (Kyushu, Japan). The antibodies against FTH1 (4393), SLC7A11 (12691), p-BECN1 (14717), p-AMPK (2535), and Rabbit (DA1E) mAb IgG XP® Isotype Control (3900) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific for transferrin receptor (TFR1) (ab214039) and SLC7A11 (ab307601) were purchased from Abcam (Cambridge, MA, USA). The antibody against nuclear receptor coactivator 4 (NCOA4) (sc-373739) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against iron export (FPN1) (TD13561) was purchased from Abmart (Shanghai, China). The antibody against β -actin was purchased from ABClonal (Wuhan, China). The secondary antibodies for HRP-linked goat anti-rabbit IgG and HRP-linked goat anti-mouse IgG were obtained from MBL (Beijing, China). HRP-conjugated Fraction Monoclonal mouse anti-rabbit IgG and Light Chain Specific and CoraLite488-conjugated goat anti-rabbit IgG (H+L) were obtained from Proteintech Group (Wuhan, China).

2.2 Cell culture and treatment

The liver cancer cell line SMMC-7721 was obtained from American Type Culture Collection (ATCC) and grown in RPMI-1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS). The normal mouse liver cell line AML12 (ATCC) was grown in DMEM/F12 medium (Hyclone, Logan, UT, USA) supplemented with 10% FBS and 1% ITS. All cell lines were incubated at 37 °C in a 5% CO₂ incubator. Cells were treated with various concentrations of UA, SAS, Fer-1 and/or other agents for the time indicated.

2.3 Cell viability assay

Cells (1×10^5 cells/well) were seeded in a 12-well plate. After 24 h, the culture medium was replaced with fresh medium containing the specified reagents treatment. Following the

designated treatment duration, the culture medium was removed, and the cells were fixed using a 1% glutaraldehyde solution for 15 min. Then, the cells were stained with a 0.02% crystal violet solution for a duration of 30 min. After that, the cells were solubilized using 75% ethanol. The absorbance at 570 nm was measured using a microplate reader. Cell viability was calculated from the ratio of cells treated with each of the drugs to that of untreated cells, which was set as 100%. Untreated cells were used as a control.

2.4 Measurement of ROS

The intracellular reactive oxygen species (ROS) was detected with a flow cytometry using the DCFH-DA fluorescent probe. Cells (2×10^5 cells/well) were seeded into 6-well plates and after 24 h of growth, the medium was replaced with fresh medium supplemented with the indicated treatments. Cells were harvested after being treated with the indicated time, washed with PBS, then incubated with DCFH-DA (20 μ mol/L) in an FBS-free medium at 37 °C for 30 min in the darkness. Subsequently, the cells were washed with serum-free medium and resuspended in the same medium intended for fluorescence analysis using a flow cytometry. The increase in fluorescence was assessed utilizing the excitation wavelength of 485 nm and the emission wavelength of 535 nm.

2.5 Measurement of intracellular Fe²⁺

The total cellular Fe²⁺ was detected with a flow cytometry using the FerroOrange fluorescent probe. Cells (2×10^5 cells/well) were seeded into 6-well plates and after 24 h, the medium was replaced with fresh medium supplemented with the indicated treatments. After being treated for the indicated times, cells were trypsinized and collected, washed with PBS, and then incubated with FerroOrange (1 μ mol/L) in an FBS-free medium for 30 min at 37 °C under dark conditions. Fluorescence analysis was then performed using a flow cytometry. The increase in fluorescence was assessed using an excitation wavelength of 532 nm and an emission wavelength of 580 nm.

2.6 Measurement of LPO and MDA

The total cellular lipid peroxides (LPO) were measured with a flow cytometry using the Liperfluo fluorescent probe. Cells (2×10^5 cells/well) were seeded into 6-well plates and after 24 h, the medium was replaced with fresh medium supplemented with the indicated treatments. After being treated for the indicated times, cells were trypsinized and collected, washed with PBS, and then incubated with Liperfluo (5 μ mol/L) in an FBS-free medium for 30 min at 37 °C under dark conditions. The increase in fluorescence was assessed using an excitation wavelength of 488 nm and an emission wavelength of 535 nm. The cell samples or tissue samples were processed according to the instructions, then the levels of malondialdehyde (MDA) were measured using the detection kit (A003-1) purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) based on the protocol of the manufacturer.

2.7 Measurement of cysteine and GSH

Cells (1.5×10^6 cells/dish) were seeded in 60 mm dishes and after 24 h of growth, they were treated with SAS, UA, or a combination for the indicated periods. The cells were collected by trypsinization, washed in PBS and extracted. The supernatant obtained from cell extracts after sonication and centrifugation (at

12,000 r/min for 10 min) was used for the determination of cysteine levels according to the instructions of the kit (G0428W) provided by Suzhou Grace Biotechnology Co., Ltd. (Suzhou, China). For GSH measurement, cells (2×10^5 cells/well) were seeded in 6-well plates. After a period of drug treatment, the medium was discarded and the cells were washed with PBS then resuspended in PBS. After sonication and centrifugation (at 5,000 r/min for 10 min), the supernatant was obtained and GSH levels were measured using a commercial kit (A006-2) provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions. Tumor tissues were made into a 10% tissue homogenate with saline and the intermediate layer of clear liquid was obtained by centrifugation for detection.

2.8 Cystine uptake and GPX activity assay

Cystine uptake capacity was detected by the assay kit (UP05) according to the protocol of manufacturer from Dojindo (Kyushu, Japan) to analyze the system xc^- activity. Cells (1.5×10^4 cells/well) were seeded in black 96-well plates and incubated for 24 h at 37 °C. The culture medium was aspirated, and the cells were washed three times with pre-warmed DMEM medium (cystine-free, serum-free, with 2 mmol/L glutamine) at 37 °C. Subsequently, they were incubated with this DMEM medium containing the indicated treatment in an incubator for 5 min. Cystine analog was added to the medium and incubated at 37 °C for 30 min. Following this, the cells underwent three PBS washes before being treated with methanol. Next, the working solution containing fluorescent probe was added, and incubated at 37 °C for 30 min in a dark condition for fluorescence measurement. The transport of cystine analogs into the cells *via* system xc^- was evaluated using the fluorescent probe. Fluorescence intensity was determined using a fluorescent microplate reader with a blank cell suspension that did not contain cystine analogs. The excitation wavelength was set at 485 nm, and the emission wavelength at 535 nm. Finally, the concentration of cellular proteins in each treatment group was determined to calibrate the corresponding fluorescence intensity. For the glutathione peroxidase (GPX) activity determination, cells (1.5×10^6 cells/dish) were seeded in 60 mm dishes. After a period of drug treatment, the medium was discarded and the cells were resuspended in PBS. Following ultrasonic fragmentation and oscillatory mixing, cell suspensions were obtained and GPX activity was measured using a commercial kit (A005-1) provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions.

2.9 Western blotting analysis

Cells (2×10^5 cells/well) were seeded in 6-well plates and after 24 h of growth, they were treated with SAS (or erastin), UA, or a combination for the indicated periods, and then harvested. The cell lysates were prepared in ice-cold radioimmunoprecipitation assay (RIPA) buffer. Tumor tissue was lysed with RIPA buffer for 30 min, then homogenized using a tissue homogenizer and centrifuged to collect the intermediate layer of liquid. The lysates were subsequently sonicated and centrifuged (at 14,000 r/min for 30 min), and the supernatant was collected. Protein concentration was measured using the BCA Protein Assay Kit (Solarbio, Beijing, China). Then, the supernatant adjusted to the same concentration was mixed well with loading buffer and boiled at 95 °C for 5 min. The protein samples were resolved by sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA; IPVH00010). After blocked with 5% non-fat milk for 1 h at room temperature, the membranes were incubated with specific primary antibodies overnight at 4 °C, followed by the incubation with corresponding HRP-conjugated secondary antibodies at room temperature for 1 h. Finally, the immunoblots was detected by enhanced chemiluminescence (Fisher/Pierce, Rockford, IL, USA) and recorded on an X-ray film (Eastman Kodak Company, Rochester, NY, USA). ImageJ version 1.8.0 software was used for the analysis of density.

2.10 Immunoprecipitation analysis

Cells (2×10^5 cells/well) were seeded in 6-well plates and after 24 h of growth, they were treated with drugs for the indicated periods. Cells were lysed at 4 °C in nondenaturing lysis buffer (R0030, Solarbio, Beijing, China) and cell lysates were sonicated and centrifuged (at 14,000 r/min for 30 min). Concentrations of proteins in the supernatant were determined and then adjusted to 1 mg/mL. Samples were pre-cleared with protein A/G magnetic beads (HY-K0202, MedChem Express, Monmouth Junction, NJ, USA), and following incubated with IgG or specific antibody against SLC7A11 (5 µg/mL) in the company of protein A/G magnetic beads at 4 °C overnight. Afterward, magnetic beads were washed thoroughly with PBS, and proteins were eluted by boiling in 1 × SDS sample buffer before SDS-PAGE.

2.11 Immunofluorescence assay

Cells (2×10^5 cells/dish) were seeded in 35 mm confocal dishes. After 24 h, the cells were treated with the indicated drugs for 8 h. Then, the cells were fixed in 4% paraformaldehyde for 15 min. Next, cells were permeabilized by 0.25% Triton X-100 buffer for 10 min. Then, cells were blocked by 1% bovine serum albumin (BSA) solution at room temperature for 30 min and incubated with a primary antibody against SLC7A11 (ab307601) at 4 °C overnight in the dark. The cells were washed with PBS three times for 5 min each and then incubated with fluorochrome-conjugated secondary antibody at room temperature for 1 h under dark conditions. Mounting medium, antifading (with DAPI) (S2110, Solarbio, Beijing, China) was added to mount and stain the nuclei. The samples were observed under a laser scanning confocal microscope.

2.12 Tumor xenograft model

5-week-old male BALB/c nude mice supplied by Charles River (Beijing, China) were kept in specific pathogen-free conditions at (22 ± 2) °C with $55 \pm 10\%$ relative humidity and with 12 h light/dark cycles for a week to acclimatize. 5×10^6 SMMC-7721 cells in 100 µL PBS combined with 100 µL matrigel (BD, CA, USA.) were subcutaneously injected into the right axilla of nude mice. Once tumor reached about 75 mm³ after 7 days, the mice were randomly divided into four groups ($n = 6$ for each group): Control, SAS, UA and SAS+UA. For the control group, the mice were administrated with saline solution containing 2% dimethyl sulfoxide (DMSO) and 5% Tween-80 by intraperitoneal injection. For the SAS group, the mice were daily administrated with SAS (200 mg/kg, i.p.). For the UA group, the mice were daily administrated with UA (30 mg/kg, i.p.). For the SAS+UA group, the mice were daily administrated with SAS plus UA. Tumor volume and body weight were measured and recorded every 4 days. Tumor volume was calculated as the formula:

$Volume (mm^3) = length (mm) \times width (mm)^2/2$. After treatment, the mice were sacrificed by the cervical dislocation method after blood collection, and the tumors were excised and weighed. The tumors were further used for western blot analysis, GSH and MDA assay. All experiments were approved by the China Agricultural University Institutional Animal Care and Use Committee (Aw41405202-4-1).

2.13 Biochemical analysis of the liver function

Serum samples were obtained through two times of centrifugation at 3,500 r/min for 15 min. Then, the biochemical indicators, including aspartate aminotransferase (AST, C010-2-1) and alanine aminotransferase (ALT, C009-2-1) were detected by the assay kits based on the protocol of the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.14 Statistical analysis

All statistical analyses were performed using Microsoft Excel and GraphPad Prism version 9.0. The results were presented as mean $\pm$ standard deviations (SD) and were analyzed using either one-way or two-way ANOVA, with subsequent appropriate post hoc tests to compare the means. $P < 0.05$ was considered a statistically significant.

3 Results

3.1 Combined treatment with UA and SAS synergistically enhanced ferroptosis in SMMC-7721 liver cancer cells

The synergic effects of UA combined with SAS in suppressing viability in SMMC-7721 cells were first evaluated by crystal violet

staining. As shown in Figs. 1A and B, low concentrations of UA (6 and 12 μ mol/L) or SAS (0.75 mmol/L) had no significant effect on cell viability, while high concentration led to proliferation inhibition in a dose-dependent pattern. Compared with the single group, treatment with UA (12 μ mol/L) and SAS (0.75 mmol/L) combination significantly increased the inhibition of cell viability (Fig. 1C). These results showed that synergistic antitumor activity was observed with UA and SAS *in vitro*. Given that SAS is a known inducer of ferroptosis, we further tested whether the synergistic effect is associated with ferroptosis^[11]. SMMC-7721 cells were treated with UA and SAS in the presence or absence of ferroptosis inhibitors (10 μ mol/L Fer-1 or 5 μ mol/L Lip-1). As shown in Fig. 1C, the inhibition by combinative treatment of UA and SAS was notably relieved in the presence of Fer-1 and Lip-1. Ferroptosis is characterized by iron-dependent lipid peroxidation^[12]. Flow cytometry was used to analyze the accumulation of lipid peroxides (LPO) and ferrous ions (Fe^{2+}). As a result, in contrast to UA or SAS alone, the combination of UA and SAS remarkably increased LPO levels (Fig. 1D). As for Fe^{2+} levels, great elevations were observed in both SAS and combined groups, but there was no significant difference in elevation in the combined group to the SAS group (Fig. 1E). It may suggest that the mechanism by which UA enhances ferroptosis is not strongly associated with increased Fe^{2+} . In contrast, normal mouse hepatocytes cells, AML12 cells displayed resistance to this combined treatment of UA and SAS, suggesting that the synergistic effect of UA and SAS in inducing ferroptosis was selective for SMMC-7721 cancer cells (Fig. 1F).

3.2 The combination of UA and SAS induces autophagy in SMMC-7721 liver cancer cells

Ferritinophagy plays an important role in driving ferroptosis^[13].

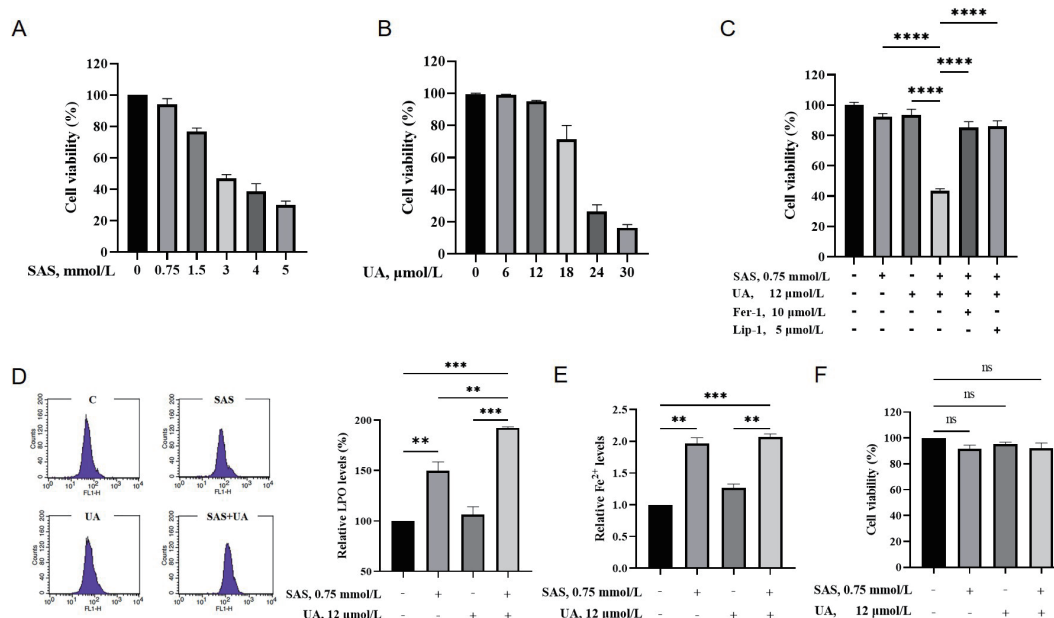


Figure 1 Combined treatment with UA and SAS showed synergistic anti-tumor effects in SMMC-7721 cells by inducing ferroptosis. (A) The effect of SAS (0–5 mmol/L) on cell viability. Cancer cells were treated with the indicated concentrations of SAS for 48 h, and cell viability was then analyzed by crystal violet staining. (B) The effect of UA (0–30 μ mol/L) on cell viability. (C) Cell viability after pretreatment with Fer-1 or Lip-1 for 3 h, followed by treatment with SAS and UA for an additional 48 h. (D) The intracellular LPO levels of SMMC-7721 cells were detected by flow cytometry after treatment with SAS and UA. The histogram presents the results of the statistical analysis of the intracellular LPO levels. The data are given as the relative values. (E) The intracellular Fe^{2+} levels of SMMC-7721 cells after treatment with SAS and UA. (F) Cell viability of AML12 cells after 48 h of combination treatment. Data are expressed as the mean $\pm$ SD ($n = 3$), $^{*}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

In our study, pretreatment of cells with the autophagy inhibitor CQ for 1 h partially rescued UA/SAS-induced cell death (Fig. 2A). Western blot analysis in Fig. 2B revealed that the combination of UA and SAS augmented the expression of the autophagosome marker LC3B-II and the ferritinophagy substrate nuclear receptor coactivator NCOA4, accompanied by decrease in FTH1, suggesting the increase of free iron release^[14]. To further evaluate the free iron level in cells, we detected the transferrin receptor TFR1 and FPN1 expression. As shown in Fig. 2B, TFR1 expression significantly increased in the UA/SAS group compared to the SAS group. However, in the combined treatment group, we also observed a further elevation of the expression of FPN1, which plays a crucial role in iron export from the cells (Fig. 2B). This could be the reason for the lack of further increase in ferrous ions, which is consistent with the previous determinations (Fig. 1E). These results suggested that UA/SAS-induced ferritinophagy may not play a predominant role in UA-sensitized ferroptosis.

3.3 The combination of UA and SAS induces oxidative stress in SMMC-7721 liver cancer cells

Oxidative stress, induced by ROS, is a critical factor in the development of ferroptosis. Oxidative stress damage was quantified by measuring total ROS production in the cells with DCFH-DA and the content of secondary products of lipid peroxidation, MDA. In this study, ROS and lipid peroxidation production were significantly increased by the UA/SAS combination treatment in SMMC-7721 cells (Figs. 3A and B). The GSH/glutathione peroxidase 4 (GPX4) system plays an important role in protecting cells from oxidative damage, which makes it a negative regulator of ferroptosis^[15]. Correspondingly, we found that GSH content and GPX enzyme activity were substantially suppressed in the combination therapy group (Figs. 3C and D). Whereas, after pretreatment with two antioxidants, NAC and α -TT, especially α -TT with its lipid peroxidation inhibitory effect, significantly rescued the decline in cell viability in the co-treatment group (Fig. 3E). Our results suggested that the combination therapy caused intense intracellular oxidative stress, leading to ferroptosis.

3.4 SLC7A11 plays a critical role in UA/SAS-induced cell ferroptosis

Solute carrier family 7 member 11 (SLC7A11), also known as xCT, is a vital component of the system xc⁻, plays a pivotal role in system xc⁻ mediated antioxidant capacity. Therefore, we examined influence of the synergistic effects of UA and SAS on SLC7A11 expression. Interestingly, treatment with SAS induced a significantly increased expression of SLC7A11, which was further increased in the combined treatment group in SMMC-7721 cells (Fig. 4A). SLC7A11 is responsible for the transport of cystine into the cell, which is essential for the synthesis of GSH. To investigate the effect of the combination on system xc⁻ activity more directly, the changes of cellular cystine import were measured by a cystine uptake assay kit. As shown in Fig. 4B, combined treatment with UA and SAS caused markedly diminished cystine compared with their individual treatments. Absorbed cystine *via* system xc⁻ is rapidly reduced to cystine, which serves as the rate-limiting precursor for GSH biosynthesis^[16]. We further tested the cysteine, and results in Fig. 4C showed that cysteine was decreased in response to this combination in SMMC-7721 cells. These results suggest that the co-treatment of UA and SAS further inhibits the transporter activity of SLC7A11, which leads to a compensatory rise in the expression of SLC7A11.

To further verify the mechanism of inhibition of SLC7A11 activity, we detected the membrane localization of SLC7A11 through immunofluorescence staining. In Fig. 4D, we observed a notable increase in green fluorescence on the cell membrane in the co-treated group, implying the up-regulation of SLC7A11 expression instead of translocation from membrane to cytosol.

It has been reported that AMPK-BECN1 pathway can block system xc⁻ activity *via* directly formatted of BECN1-SLC7A11 complexes^[17]. We then examined the changes of p-AMPK α and p-BECN1 in SMMC-7721 cells. Results showed that phosphorylation levels of these two proteins were up-regulated by UA/SAS (Fig. 4E). In subsequent inquiry, we asked whether the elevation in BECN1 phosphorylation levels is accompanied by an increase in the quantity of the BECN1-SLC7A11 complex. Immunoprecipitation (IP) of cellular lysates by means of an anti-

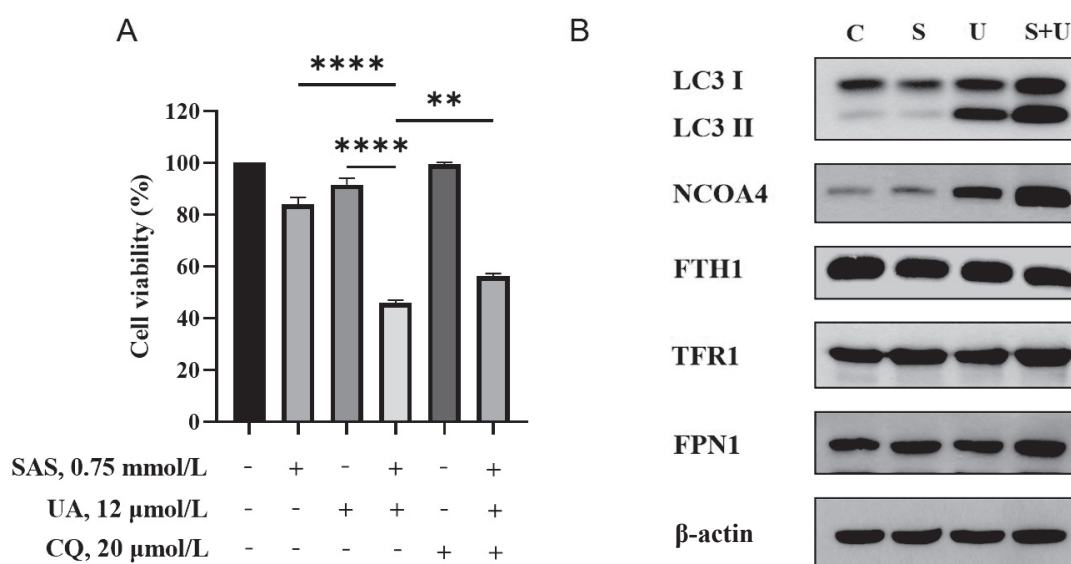


Figure 2 Combined treatment of UA and SAS induced autophagy in SMMC-7721 cells. (A) Cell viability after pretreatment with CQ for 1 h, followed by treatment with SAS and UA for an additional 48 h. (B) Western blot analysis of the levels of autophagy and iron metabolism related proteins. Data are expressed as the mean $\pm$ SD ($n = 3$), ** $P < 0.01$, **** $P < 0.0001$.

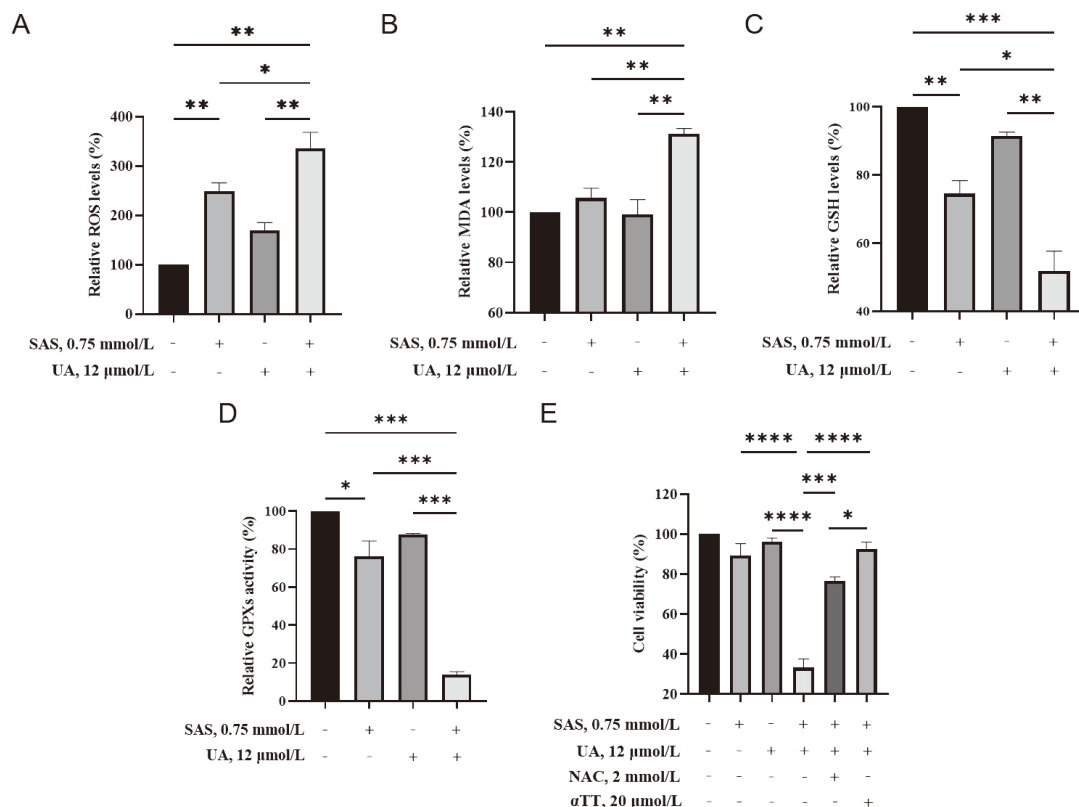


Figure 3 The combination of UA and SAS induces oxidative stress in SMMC-7721 cells. (A) The intracellular ROS levels of SMMC-7721 cells were detected by flow cytometry after treatment with SAS and UA. (B) The relative MDA levels of SMMC-7721 cells after treatment with SAS and UA. (C) The relative GSH levels of SMMC-7721 cells after treatment with SAS and UA. (D) The relative GPX activity levels of SMMC-7721 cells after treatment with SAS and UA. (E) Cell viability of SMMC-7721 cells after pretreatment with NAC and α -TT for 3 h, followed by treatment with SAS and UA for an additional 48 h. Data are expressed as the mean $\pm$ SD ($n = 3$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

SLC7A11 antibody followed by immunoblot detection of SLC7A11 or p-BECN1 revealed a significant BECN1-SLC7A11 interaction in SMMC-7721 cells following the combinative treatment (Fig. 4F). To clarify the role of AMPK in this process, we further tested the effects of the AMPK inhibitor, compound C on cell viability. As shown in Fig. 4G, the administration of compound C significantly protected the cytotoxicity induced by UA and SAS co-treatment. Collectively, the formation of the AMPK-mediated BECN1-SLC7A11 complex emerges as the primary mechanism through which UA enhances the suppression of SLC7A11 activity, consequently inducing ferroptosis.

3.5 UA enhances erastin-induced ferroptosis via SLC7A11 in SMMC-7721 liver cancer cells

To validate the UA-sensitized ferroptosis pattern, our attention was directed toward another ferroptosis inducer that targets SLC7A11, erastin. The synergic effects of erastin combined with UA in the suppression of viability in SMMC-7721 cells was evaluated by crystal violet staining. As shown in Fig. 5A, the inhibition of combination treatment of UA and erastin was remarkably relieved in the presence of ferroptosis inhibitor, DFO, Fer-1 and Lip-1, especially Fer-1 and Lip-1, which have antioxidant properties by scavenging free radicals. Likewise, the antioxidants NAC and α -TT mitigated the synergistic impact of UA and erastin on inducing cancer cell death (Fig. 5B). These findings implied that UA amplifies erastin-induced ferroptosis, with oxidative stress playing a pivotal role in this mechanism.

Next, we aim to elucidate the role of SLC7A11 in this process. Consistent with the findings from the SAS and UA co-treatment experiments, immunoblotting results demonstrated a substantial increase in SLC7A11 expression with the co-administration of UA and erastin. Additionally, upregulation of AMPK expression and the level of BECN1 phosphorylation mediated by AMPK was observed (Fig. 5C). A significant increase in the content of the BECN1-SLC7A11 complex also occurred (Fig. 5D). Similarly, the use of AMPK inhibitor, compound C, also reduced cell death induced by UA in combination with erastin (Fig. 5E). Immunofluorescence images of SMMC-7721 cells revealed a pronounced increase in the green fluorescence associated with SLC7A11 protein on the cell membrane in the UA + erastin-treated group compared to both the control and single-treatment groups. This suggests an augmentation in the protein content of the cell membrane (Fig. 5F). All these results shown that UA intensifies ferroptosis induced by the inducer, erastin, which likewise targets SLC7A11. The main mechanism of this synergistic effect also involves the inhibition of SLC7A11 and the disruption of antioxidant capacity due to the activation of the AMPK-BECN1 pathway.

3.6 The combination of UA and SAS exhibited synergistic therapeutic effects *in vivo*

To evaluate the synergistic therapeutic effects and the safety of UA and SAS combination treatment *in vivo*, we established a subcutaneous tumor model, and tumor growth was observed

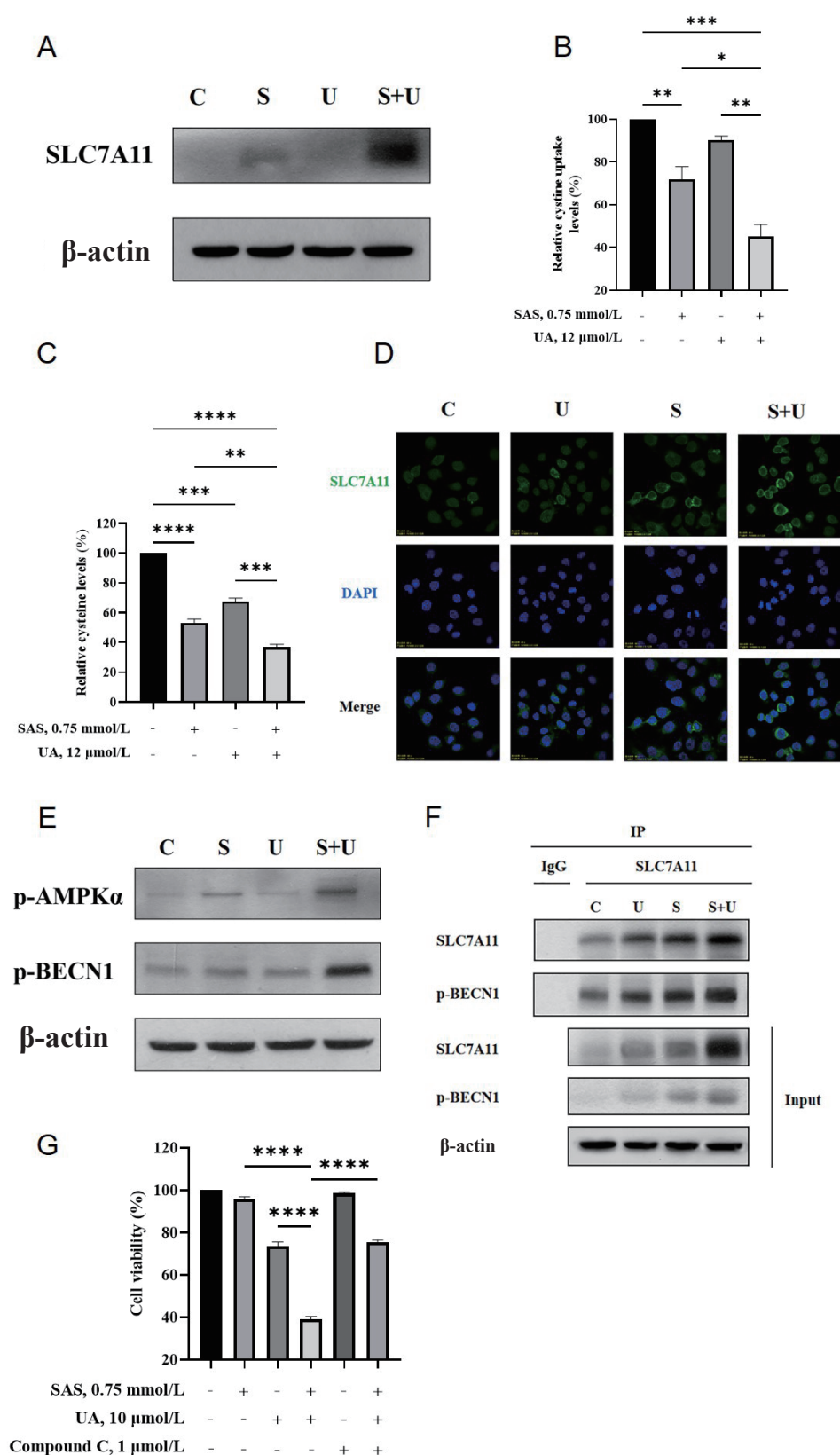


Figure 4 The combined treatment of UA and SAS intensifies the suppression of SLC7A11 activity. (A) SLC7A11 protein levels of SMMC-7721 cells after treatment with UA and SAS for 48 h. (B) The relative cystine uptake levels with treatment of UA and SAS for 4 h. (C) The relative cystine levels with treatment of UA and SAS for 8 h. (D) Immunofluorescence staining of SLC7A11 formation (green fluorescence) in SMMC-7721 cells after treatment with UA and SAS for 8 h. Nuclei were stained with DAPI (blue fluorescence), magnification 60×. (E) The protein levels of p-AMPKα and p-BECN1 after treatment with UA and SAS for 48 h. (F) Immunoprecipitation analysis of SLC7A11 to study interaction of SLC7A11 with p-BECN1 in SMMC-7721 cells after treatment of UA and SAS for 48 h. (G) Cell viability of SMMC-7721 cells after pretreatment with compound C for 1 h, followed by treatment with SAS and UA for an additional 48 h. Data are expressed as the mean ± SD ($n = 3$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

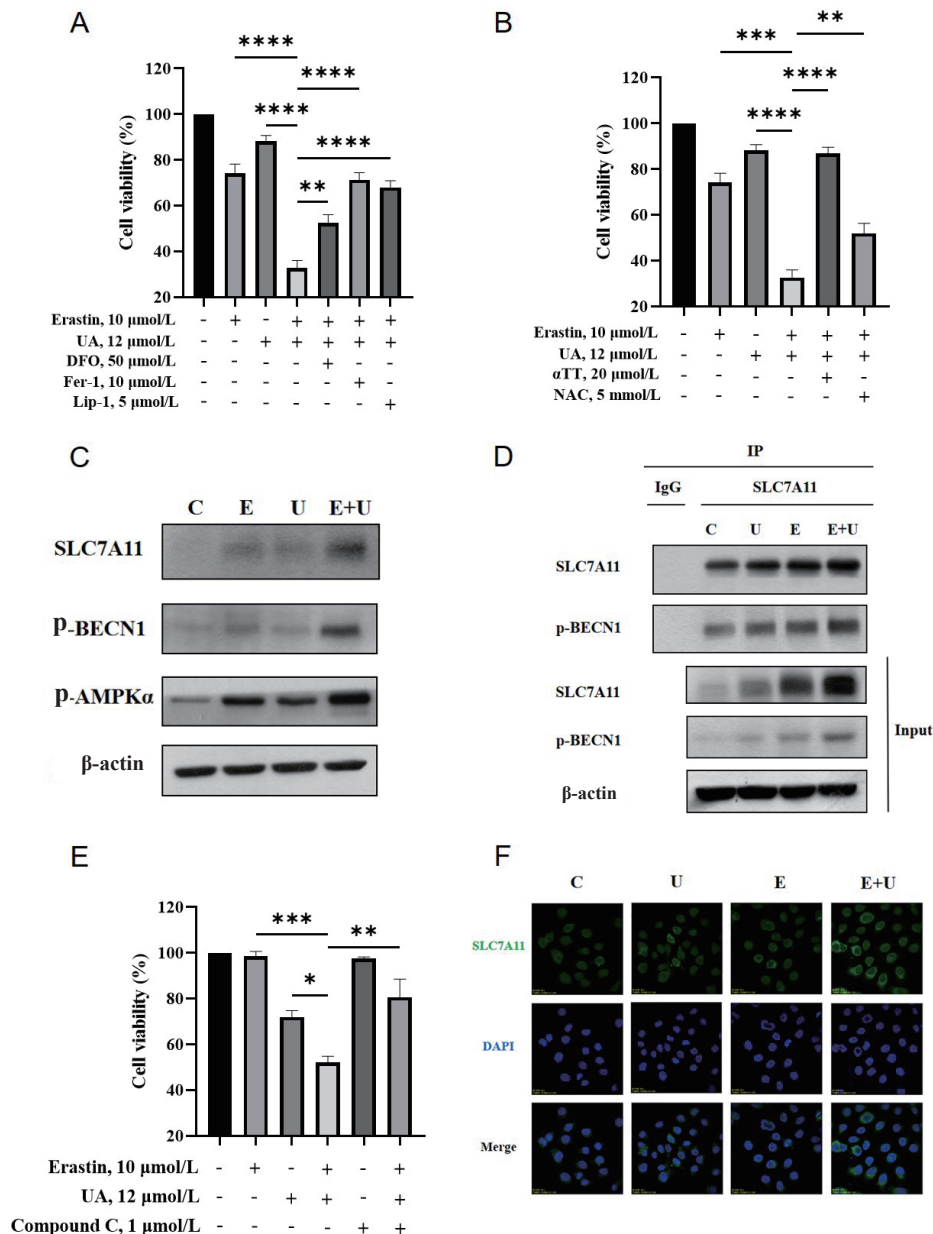


Figure 5 UA enhances erastin-induced ferroptosis *via* the suppression of SLC7A11 activity. (A) Cell viability of SMMC-7721 cells after pretreatment with DFO, Fer-1 and Lip-1 for 3 h, followed by treatment with erastin and UA for an additional 48 h. (B) Cell viability of SMMC-7721 cells after pretreatment with NAC and α-TT for 3 h, followed by treatment with erastin and UA for an additional 48 h. (C) The protein levels of SLC7A11, p-BECN1 and p-AMPKα after treatment with UA and erastin for 48 h. (D) Immunoprecipitation analysis of SLC7A11 to study interaction of SLC7A11 with p-BECN1 in SMMC-7721 cells after treatment of UA and erastin for 48 h. (E) Cell viability of SMMC-7721 cells after pretreatment with compound C for 1 h, followed by treatment with erastin and UA for an additional 48 h. (F) Immunofluorescence staining of SLC7A11 formation (green fluorescence) in SMMC-7721 cells after treatment with UA and SAS for 8 h. Nuclei were stained with DAPI (blue fluorescence), magnification 60×. Data are expressed as the mean ± SD ($n = 3$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

periodically. The results showed that although UA and SAS administrated alone inhibited tumor growth to a certain extent, the combination of UA and SAS showed more effective treatment than monotherapy at inhibiting the tumor volume and weight growth (Figs. 6A-C). Recording of body weight showed that animal body weights remained essentially unchanged during the combined treatment of UA and SAS, making it safer for clinical use (Fig. 6D). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) serve as markers for assessing liver function and detecting liver damage. As shown in Figs. 6E and F, the treatment with UA and SAS not only suppressed tumor growth but also led to decreased serum levels of ALT and AST,

indicative of diminished liver injury. Similarly, the levels of the lipid peroxidation product MDA were found to be markedly elevated in xenograft tissue, while GSH, a crucial antioxidant factor, was reduced (Figs. 6G and H). These observations strongly indicated that the combined treatment suppressed tumor growth by inducing ferroptosis. Next, we examined the effect of SLC7A11 during combination treatment *in vivo* by western blot. As shown in Fig. 6I, consistent with the outcomes of *in vitro* experiments, the expression level of SLC7A11 exhibited an increase in tumor tissue following co-treatment, concomitant with the upregulation of the AMPK-BECN1 pathway. This confirms the critical role of SLC7A11 in the *in vivo* synergistic anti-cancer mechanism.

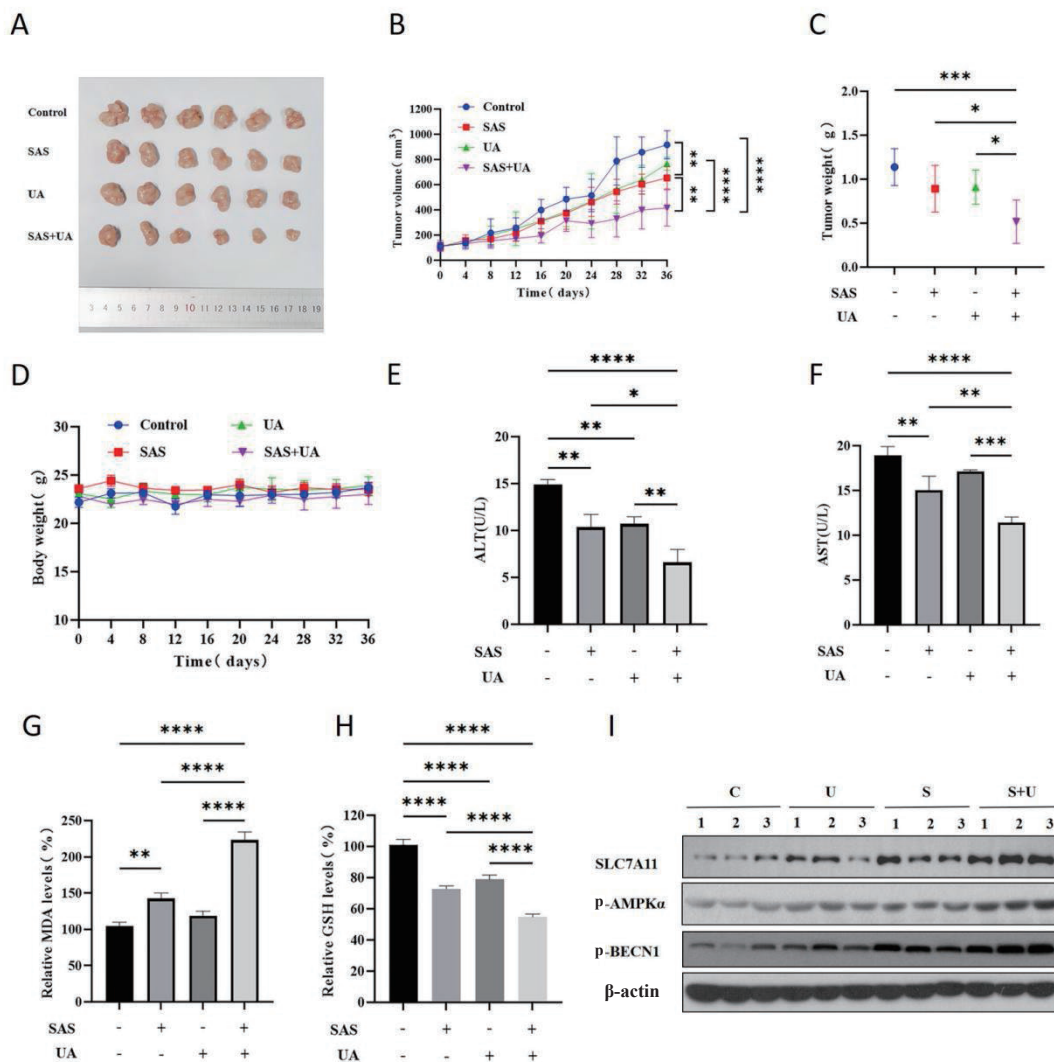


Figure 6 The combination of UA and SAS inhibited the growth of xenografts *in vivo*. (A) Macroscopic view of xenograft tumors at the endpoint of the experiment. (B) The average volumes of xenograft tumors were measured every 4 days and plotted. (C) The average tumor weight of the different treatment groups at the end of the experiment. (D) The average body weight was measured every 4 days and plotted. (E) Serum ALT levels in different treatment groups. (F) Serum AST levels in different treatment groups. (G) The relative MDA levels of tumor tissue in different treatment groups. (H) The relative GSH levels of tumor tissues in different treatment groups. (I) Western blot of tumor lysates from mice with indicated treatment, including SLC7A11, p-AMPKα and p-BECN1. Data are expressed as the mean $\pm$ SD ($n = 6$ per group), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

4 Discussion

The challenges of treating advanced HCC include the difficulty in finding effective treatments and the grim prognosis associated with drug resistance^[18]. The combination of two or more agents is the ideal treatment strategy which can improve the therapeutic effects, avoid toxic side effect, and minimize or slow down the development of drug resistance^[19]. Increasingly, compelling evidence supports the idea that triggering ferroptosis holds significant promise as a cancer treatment strategy, particularly for effectively targeting and eliminating aggressive malignancies that exhibit resistance to traditional therapeutic modalities. SAS is an FDA-approved drug commonly used for the treatment of rheumatoid arthritis. It is also a well-recognized inducer of ferroptosis by inhibiting the cystine transport and has been shown to inhibit the growth and metastasis of several cancer types^[20,21]. UA, a pentacyclic triterpenoid compound found in plants, exerts anticancer activity by targeting multiple signaling pathways in cancer cells and may be used as an ideal adjuvant in cancer therapy. More notably, it has been demonstrated in several studies

to enhance the antitumor activity of anticancer drugs such as cisplatin and sorafenib by synergistically inducing ferroptosis^[22,23].

In the present study, we found that combination treatment of UA and SAS could synergistically suppress the progression of liver cancer *via* ferroptosis induction. *In vitro*, the combination of UA and SAS significantly reduced the cell viability of SMMC-7721 cells compared to the treatment alone, and this synergistic effect was eliminated by the dedicated ferroptosis inhibitor Fer-1, Lip-1 (Figs. 1A-C). In addition to *in vitro* studies, a xenograft tumor of liver cancer in nude mice was established. The co-treatment of UA and SAS significantly reduced tumor volume and tumor weight (Figs. 6A-C). Furthermore, this combination did not show significant inhibitory effect on normal hepatocytes cells, AML12 cells (Fig. 1F). Moreover, there were no significant body weight differences among the groups during the experiment, implying the treatment with UA and SAS does not cause severe systemic toxicity (Fig. 6D). We also examined the key events of ferroptosis. LPO, a hallmark of ferroptosis that directly damages cellular membranes, and MDA, a final product of lipid peroxidation, were significantly elevated when UA and SAS were

co-administered, both *in vitro* and *in vivo* (Fig. 1D and Fig. 6G). These results indicated that combination treatment of UA and SAS could markedly induce ferroptosis-mediated cell death in liver cancer.

Autophagy is a cellular mechanism that maintains homeostasis and adapts to various stress conditions. It is also a double-edged sword in tumor tumorigenesis and anticancer therapies^[24]. More importantly, selective autophagy, notably NCOA4-mediated ferritinophagy, possesses the capability to degrade ferritin proteins, liberate iron ions, amplify the Fenton reaction, and intensify oxidative stress. It assumes a pivotal role in the progression of ferroptosis^[13]. Previous studies have found that UA can target AMPK/mTOR, PI3K/AKT and other pathways to induce autophagy in a variety of cancer cells^[25,26]. Therefore, we tested the contribution of autophagy in the synergy of the UA and SAS. The autophagy inhibitor CQ disrupts the activity of acid hydrolases within lysosomes, consequently impeding the fusion and degradation processes of autophagic lysosomes in cells. Our results showed that CQ only slightly rescued cell death caused by the combination treatment of UA and SAS (Fig. 2A). Analysis of changes in the levels of proteins associated with ferritinophagy showed that the NCOA4 was significantly higher, accompany by the FTH1 was further degraded in the combined group (Fig. 2B). The co-treated group exhibited a further increase in the expression of TFR1, which is responsible for iron ion uptake into cells, in comparison to the individually treated group. However, the levels of the iron ion output protein, FPN1, followed a similar pattern. This is consistent with the detection of intracellular Fe²⁺ levels (Fig. 1E). All these results suggest that autophagy does not play a key role in the synergistic treatment of UA and SAS, and that UA does not significantly increase Fe²⁺ levels on the basis of SAS treatment.

The initiation of oxidative stress seems to be essential in the process of ferroptosis. Massive accumulation of ROS was observed in various cancer cell death induced by UA^[27,28]. Consistent with these reports, ROS regulates ferroptosis when SMMC-7721 cells are undergoing treatment with UA and SAS. According to the results, ROS and MDA were significantly elevated in the co-treated group, whereas GSH and GPX4, which play an antioxidant role, were greatly inhibited (Figs. 3A-D). Pretreatment with antioxidants NAC and α -TT dramatically rescues ferroptosis synergistically induced by UA and SAS (Fig. 3E). Especially, the rescue effect of the lipophilic ROS scavenger α -TT is better than that of the broad-spectrum ROS scavenger NAC, and we speculate that intracellular lipid ROS were involved in UA/SAS combined therapy-induced ferroptosis. This suggests that UA promotes ferroptosis primarily by further enhancing lipid peroxidation. Polyunsaturated fatty acids (PUFAs), especially arachidonic acid (AA) and adrenic acid (AdA), are important substrates for lipid peroxidation^[29]. The intracellular lipid metabolism can play a crucial role in regulating lipid peroxidation and ferroptosis. UA has been reported to enhances degradation of lipid droplets and promote the release of fatty acids by regulate lipolysis-related genes, HSL and ATGL^[30]. Additionally, UA has been found to promote the production of AA in colon and prostate cancer cells, which involves the overexpression of phospholipase A22 (PLA) family members, iPLA2 and sPLA2^[31]. Oleanolic acid (OA), an isomer of UA, which is always found together with UA. It has been reported that OA could stimulate oxidative stress and promote lipid peroxidation by targeting ACSL4, which is involved in the formation of lipid peroxides^[32]. This suggests that UA may have more to explore in promoting lipid peroxidation and

synergistically inducing ferroptosis by modulating lipid metabolism.

SLC7A11-GSH-GPX4 is one of the most important antioxidant axes and a major defence system against ferroptosis. SAS, as a ferroptosis inducer, has primarily demonstrated its action through the inhibition of SLC7A11 function. Likewise, in a synergistic anticancer study combining UA with cisplatin and sorafenib, notable alterations in SLC7A11 expression were observed, but the exact mechanism involved was not elucidated^[22,23]. In our study, the decrease in GSH and GPX4 levels seems to corroborate this finding. Interestingly, SLC7A11 protein content was instead elevated (Fig. 4A). To better assess the functional activity of the SLC7A11 protein, we directly measured the cells cystine uptake capacity using a specialized kit. It was found that a significant reduction in cystine uptake capacity as well as cysteine levels occurred in the co-treated group (Figs. 4B and C). This suggests that the increase in protein levels may be a compensation for the decrease in its activity. In addition to protein abundance, the protein membrane localization, phosphorylation status and binding to other proteins affect the activity of SLC7A11^[33-36]. Autophagy inhibition could lead to loss of membrane localization of SLC7A11 thus making it difficult to perform its translocation function^[34]. Under laser confocal microscopy, the green fluorescence on the cell membrane of the combinative treatment group was significantly enhanced, indicating that the inhibition of protein activity was independent of membrane localization (Fig. 4D).

Although previous research has demonstrated that UA enhances the anticancer efficacy of sorafenib by inhibiting SLC7A11^[23], the mechanism by which UA acts as a sensitizer for SLC7A11 inhibitors is not yet clear. It has been previously reported that AMPK-mediated activation of BECN1 followed by direct binding to SLC7A11 also leads to activity inhibition^[17]. AMPK is a critical energy sensor in healthy cells, regulating cellular energy balance by maintaining and modulating various cellular metabolic pathways, making it a key factor in tumor development^[37]. UA has been shown to regulate multiple signaling pathways related to apoptosis and autophagy in cancer cells by activating AMPK^[25,28]. Our data demonstrated that combined UA and SAS treatment increased the phosphorylation levels of AMPK and BECN1, concomitant with elevated formation of the BECN1-SLC7A11 complex in SMMC-7721 cells (Figs. 4E and F). Chemical inhibition of AMPK also attenuated the synergistic inhibition of cancer cell viability (Fig. 4G). These results indicated that AMPK-BECN1 axis played a critical role in inhibiting SLC7A11 activity by UA and SAS. To confirm the significant role of this mechanism in the enhanced ferroptosis effects of UA, we conducted a study to explore the synergistic anticancer effects of UA when combined with erastin, another ferroptosis inducer that targets SLC7A11. In line with the previously findings, UA/erastin effectively suppressed SLC7A11 activity through AMPK activation and the formation of the BECN1-SLC7A11 complex (Fig. 5). Furthermore, the combination of UA and SAS treatment significantly reduced serum ALT and AST levels (Fig. 6), indicating that UA not only acts as an effective anticancer sensitizer but may also exhibit reduced hepatotoxicity. However, the underlying mechanisms contributing to UA's favorable safety profile remain to be elucidated.

This study has several limitations that warrant further investigation. First, it remains unclear whether UA directly interacts with SLC7A11 and modulates its protein conformation, thereby promoting ferroptosis. Second, whether structurally analogous pentacyclic triterpenoids exhibit comparable biological

activities remains to be elucidated. Addressing these questions will provide critical mechanistic insights into the potential application of UA and its derivatives in sensitizing SLC7A11-targeted anticancer therapies.

5 Conclusion

In summary, the present study provides strong evidence that UA and SAS have a synergistic effect in inducing liver cancer cells ferroptosis *in vitro* and *in vivo*. The major mechanism involves the AMPK-mediated formation of the SLC7A11-BECN1 complex and the inhibition of the SLC7A11-GSH-GPX4 antioxidant system. Consequently, the combined administration of UA and SAS may be a promising strategy to sensitize liver cancer cells to ferroptosis. It also provides additional evidence to investigate the potential role of UA in inducing ferroptosis for exerting anticancer activity.

Data availability

All data needed to support the conclusions in the paper are presented in the manuscript. Additional data related to this paper may be requested from the corresponding author upon request.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work.

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Author contribution statement

Kai Han: Experimental design, data curation, writing manuscript. Jia-Yuan Li: Data curation, validation. Bing-Ying Shuai: Project administration, validation. Chong Zhao: Writing-review & editing, funding acquisition. All the authors have approved the final manuscript.

Informed consent

Not applicable.

Ethics statement

The animal experiments were carried out following the guidelines of the China Agricultural University Institutional Committee for the Care and Use of Laboratory Animals and were approved by the Committee on Ethical Use of Animals of China Agricultural University (Aw41405202-4-1).

Use of AI statement

None.

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