Arsenic sulfide Triggers Ferroptosis in Hepatocellular Carcinoma Cells via TRPC6/GPX4 Signaling

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Abstract

Ferroptosis plays a vital role in the pathological process of numerous human diseases, including cancer. It is possible that ferroptosis stimulation could be used as a cancer treatment strategy. Due to this, ferroptosis-inducing drugs are gaining more attention for the clinical treatment of tumors. For the first time, we demonstrated that arsenic sulfide (As\textsubscript{4}S\textsubscript{4}) initiated ferroptotic cell death in hepatocellular carcinoma (HCC) cells, which was concomitant with ROS accumulation, lipid peroxidation, and GSH depletion. Arsenic sulfide-mediated cell death in HCC cells was blocked by ferroptosis inhibitors ferrostatin-1 (Fer-1) and deferoxamine (DFO, an iron chelator), but not Z-VAD-FMK, necrosulfonamide, or chloroquine, suggesting that ferroptosis participated in arsenic sulfide-induced cell death. Transient receptor potential channel 6 (TRPC6) expression was notably inhibited under arsenic sulfide intervention and the overexpression of TRPC6 rescued the effects of arsenic sulfide on ferroptosis. Furthermore, glutathione peroxidase 4 (GPX4), was identified to interact with TRPC6 through confocal microscopy images and co-immunoprecipitation assay. In summary, arsenic sulfide exerts anticancer effects on HCC in vitro and in vivo by inducing ferroptosis via inhibiting TRPC6/GPX4 pathway. Our findings led us to conclude that arsenic sulfide could be considered as a prospective drug for liver cancer treatment.

1. Introduction

Worldwide, liver cancer accounts for more than 850,000 new cancer cases annually which ranks among the top causes of death from cancer (Sung et al., 2021). In primary liver cancer, hepatocellular carcinoma (HCC) occupies the majority of the histological spectrum and is often diagnosed at advanced stages of disease, resulting in a dismal prognosis (M. D. Huang et al., 2015; McGlynn, Petrick, & London, 2015). The past decades have witnessed dramatic advancements in survival for patients with HCC. Despite recent advances in therapeutic regimens including transplantation, ablation, transarterial chemoembolization, targeted therapies and immunotherapies, many obstacles remain (Yang et al., 2019). Consequently, it is of great importance to identify new anticancer therapies for HCC.

Unlike apoptosis, necrosis, and autophagy, ferroptosis is a novel form of programmed cell death with distinct biochemical, morphological, and genetic characteristics (Dixon et al., 2012; Stockwell et al., 2017). It is characterized by iron-dependent lipid peroxidation and intracellular accumulation of reactive oxygen species (ROS) (Stockwell, 2022). GPX4, a phospholipid hydroperoxidase that mitigates lipid peroxidation by using reduced glutathione (GSH), inhibits ferroptosis (Ma et al., 2022; Seibt, Proneth, & Conrad, 2019). It has been reported that ferroptosis is modulated by STAT3/GPX4 signaling (W. Zhang et al., 2022; Z. Zhang et al., 2022). Ferroptosis has drawn broad attention and prompts its use in therapy. However, the study concerning ferroptosis in HCC is elusive.

In acute promyelocytic leukemia (APL), arsenic trioxide (As\textsubscript{2}O\textsubscript{3}, ATO) is highly effective (Shen et al., 1997; Wang & Chen, 2008; Zhu et al., 2016). Researchers have also reported that ATO inhibited the proliferation and promoted ferroptosis in solid tumors (Feng et al., 2022; Tang et al., 2022). Arsenic sulfide (As\textsubscript{4}S\textsubscript{4}) offers a multitude of advantages over ATO such as relative safety, oral administration and abundant
resources, which is of greater acceptability and convenience for patients. Previous studies indicated that arsenic sulfide is expected to suppress tumor progression in solid tumor cells (Ding et al., 2015; Tan, Zhang, Kang, Zhang, & Chen, 2018; L. Zhang, Kim, et al., 2015; X. Zhang et al., 2017). Nuclear Factor Of Activated T Cells 3 (NFATc3) is targeted by arsenic sulfide to evoke double strand DNA breaks (DSB) via activation of recombination activating gene 1 (RAG1) (Kang et al., 2019). In our previous study, arsenic sulfide inhibited the proliferation and evoked apoptosis of HCC in a dose and timedependent pattern. However, it had less of an effect on L02 cells (Ding et al., 2015). Thus, it's worthy of further investigation in the role of arsenic sulfide on liver cancer.

In this research, we report for the first time that ferroptosis is both in vitro and in vivo involved in arsenic sulfide -induced cell death, in conjunction with accumulation of ROS, the depletion of GSH, and lipid peroxidation. Moreover, TRPC6 plays essential roles in arsenic sulfide -induced ferroptosis. Taken collectively, the data showed that arsenic sulfide triggers its anticancer effects by triggering ferroptosis in hepatocellular carcinoma.

2. MATERIALS AND METHODS

2.1. Cell culture and reagents

Human hepatocellular carcinoma cell lines (HepG2 and Huh7) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Institute of Biochemistry and Cell Biology, Shanghai, China). DMEM (Hyclone, USA) containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin streptomycin (Gibco, USA) was used to culture cells. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. We prepared highly purified arsenic sulfide as previously described supplied by the Shanghai Institute of Hematology (Shanghai, China) (L. Zhang, Tian, et al., 2015). Antibodies for TRPC6 was obtained from Novus Biologicals (Littleton, USA). GPX4 was obtained from Santa Cruz (CA, USA). STAT3, p-STAT3 antibody were obtained from Abclonal (Wuhan, China), and antibodies against HO-1, SLC40A1, SLC7A11, and GAPDH were purchased from CST (Boston, USA). Deferoxamine, ferrostain-1, Z-VAD-FMK, necrosulfonamide, CQ, N-acetyl-l-cysteine (NAC) were obtained from MCE (Shanghai, China).

2.2. Cell Counting Kit-8 (CCK8) Assay

CCK8 assay was conducted to measure the cell inhibition. HepG2 and Huh7 cells were suspended and seeded into 96-well culture plates and routinely cultured overnight. HCC cells were then incubated with a variety of concentrations of arsenic sulfide for 24 h. The CCK-8 reagent was then added and incubated in the dark for 2 h, followed by the use of a microplate reader (Bio-TEK) in order to measure the absorbance at 450 nm. Cells incubated without any treatment were used as the control.

2.3. GSH assay measurement
As per the manufacturer's instructions, the relative glutathione content of cell lysates was detected using the GSH Assay Kit (Nanjing Jiancheng, China).

2.4. Malondialdehyde (MDA) measurement

Using a Lipid Peroxidation (MDA) Assay Kit from Abcam, USA, relative MDA concentrations were measured in cell lysates.

2.5. Detection of Intracellular Reactive Oxygen Species (ROS)

The ROS assay kit (Abcam, USA) was used to assess intracellular ROS levels according to the manufacturer's instructions.

2.6. Western blot analysis

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer from Yeasen, Shanghai, China. 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the protein samples, which were then transferred to PVDF membranes from Millipore (Billerica, MA, USA). A 5% skim milk powder solution in phosphate-buffered saline with 0.1% Tween-20 was used first to block the membranes for two hours. After incubating overnight with primary antibodies at 4°C, they were washed with TBST. Corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies were incubated at room temperature for 1 h. Specific proteins were visualized using the enhanced chemiluminescence detection system (Millipore, USA) and photographed them using G-BOX (Gene Company Ltd, Beijing, China).

2.7. Iron Assay

We used an Iron Assay Kit (Abcam, USA) to measure the relative iron concentration in cell lysates.

2.8. Immunofluorescence (IF)

HCC cells were seeded onto glass coverslips and treated with As$_4$S$_4$ for 24 h. Afterward, we washed the cells twice with PBS and fixed them for 20 minutes at room temperature in 4% paraformaldehyde (PFA). We blocked cells for 1 hour with PBST containing 3% goat serum at room temperature. We then incubated the cells with primary antibodies to TRPC6 (1:200 dilution) and GPX4 (1:200 dilution) overnight at 4°C, followed by incubation with corresponding fluorescent secondary antibodies (1:1000) at room temperature for 2 h. DAPI (Sigma-Aldrich) was used for nuclear staining. Finally, all images were photographed using a fluorescence microscope.

2.9. Quantitative Polymerase Chain Reaction (RT-qPCR)

Based on the manufacturer's instructions, cellular ribonucleic acid (RNA) was extracted from HepG2 and Huh7 cells using TRIzol reagent (Invitrogen, CA, USA). The quantity was determined utilizing a NanoDrop ND-1100 spectrophotometer (NanoDrop Technologies). The A260/A280 ratio between 1.8 and 2.0 were used for further experiments. RNA was reverse-transcribed into complementary DNA (cDNA) using
PrimeScript™ kit (TaKaRa, Japan). cDNA was amplified using SYBR-Green PCR Master Mix and measured utilizing a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 45 seconds were the conditions for thermocycling. GAPDH was used as an internal standard for measuring cellular RNA expression. Quantification of target gene mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences for PCR were as follows: GPX4 forward, 5′- GAGGCAAGACCGAAGTAAACTAC - 3′ and reverse, 5′- CCGAACTGGTTACACGGGAA - 3′; TRPC6 forward, 5′- CCTTGCTGGTGCATTGGA - 3′ and reverse, 5′- TCTTCCCCATCTTGGCTGCAT - 3′; GAPDH forward, 5′- GGAGGAGTGGGTGTCGCTGT - 3′ and reverse, 5′- GTGGACCTGACCTGCGCCTGTC - 3′. All samples were run in triplicate.

2.10. Immunoprecipitation Experiment

Two washes of PBS buffer were performed on the cells and then they were lysed with RIPA (Yeasen, Shanghai, China). Protein A/G plus agarose beads were incubated with the lysates overnight at 4°C with specific primary antibodies. Western blotting was used to analyze proteins bound on beads after three washes with lysis buffer.

2.11. In vivo Xenograft Experiment

We divided ten BALB/c nude mice (Institute of Zoology, China Academy of Sciences) into two groups of five mice each. We injected the mice with either vehicle or arsenic sulfide at a dose of 2 mg/kg intraperitoneally. Tumor volume was measured using calipers every week and calculated using formula: tumorous volume = (width$^2 \times$ length)/2. Nude mice received humane care. We followed protocols approved by the Institutional Animal Care and Ethics Committee at Shanghai Jiao Tong University for all animal procedures.

2.12. Statistical Analysis

Statistical Product and Service Solutions (SPSS) software version 23.0 (IBM Corporation., Armonk, NY, USA) was used to analyze the data. The data are presented as mean ± standard deviation. $p < 0.05$ was considered significant for all tests.

3. Results

3.1. Ferroptosis contributes to arsenic sulfide-induced cell death in HCC

To validate the approach of arsenic sulfide-triggered cell death, a number of cell death inhibitors were employed. Z-VAD-FMK (a pancaspase inhibitor), necrosulfonamide (a potent inhibitor of necroptosis) or chloroquine (CQ, a potent inhibitor of autophagy), were not effective in protecting HCC from arsenic sulfide-induced apoptotic death (Fig. 1A–C), indicating that other kinds of cell death may have taken place.
As a next step, RNA-seq analysis was conducted in untreated and arsenic sulfide-treated HepG2 cells to identify differentially expressed genes. Based on a heatmap analysis of HepG2 cells, GPX4 (glutathione peroxidase 4), a negative target of ferroptosis, was consistently downregulated (Fig. 1D). In addition, KEGG pathway enrichment analyses showed that p53 and STAT pathway were enriched, which has been reported to be functionally associated with ferroptosis (Fig. 1E) (Li et al., 2022; Yu et al., 2022). Thus, we speculated that ferroptosis could contribute to arsenic sulfide-provoked cell death.

The accumulation of redox-active iron, the depletion of glutathione (GSH) and lipid peroxidation are known to the key events of ferroptosis (Z. Zhang et al., 2020). Our study examined intracellular Fe²⁺ levels, ROS levels, GSH levels, and the oxidative stress marker malondialdehyde (MDA) in HCC cells. Expectedly, levels of Fe²⁺ (Fig. 2A), ROS (Fig. 2B) and MDA (Fig. 2D) were notably enhanced after the treatment of arsenic sulfide, while the level of GSH (Fig. 2C) was simultaneously decreased. Lastly, the ROS inhibitors N-acetyl-l-cysteine (NAC) can rescue cell death triggered by arsenic sulfide in HCC cells (Fig. 2E). Furthermore, ferroptosis inhibitor ferrostatin-1 (Fer-1) and deferoxamine (DFO, an iron chelator) almost prevented the death of HCC cells induced by arsenic sulfide (Fig. 2F). Altogether, these results indicated that arsenic sulfide triggered ferroptotic cell death in HCC cells. Further confirmation was obtained through transmission electron microscopy (TEM). In HCC cells, mitochondrial matrix condensation and enlarged cristae were observed (Fig. 2G).

### 3.2. Arsenic sulfide Regulates the Expression of Ferroptosis-Associated Proteins are in HCC cells

We further checked regulatory proteins in HCC cells by western blot, which is established markers of ferroptosis. As described in Fig. 3A, the expression of heme oxygenase 1 (HO-1) was considerably elevated after arsenic sulfide treatment, whilst the protein expression of GPX4, solute carrier family 7 member 11 (SLC7A11) and solute carrier family 40 member 1 (SLC40A1) decreased. In line with these results, arsenic sulfide downregulated the mRNA expression of GPX4 (Fig. 3B). Previous studies have demonstrated that that GPX4 is controlled by various molecules, such as STAT3. Protein expression level of STAT3 were verified by western blot as indicated (Fig. 3A). Arsenic sulfide remarkably suppressed the phosphorylation of STAT3 at Ser705 in a manner that varies with the dosage. The results implied that arsenic sulfide provoked ferroptosis via STAT3–GPX4 signaling pathway.

### 3.3. Arsenic sulfide Inhibits the TRPC6 Expression in HCC Cells

Next, we embarked to decipher the potential molecular mechanism that may account for the activation of ferroptosis by arsenic sulfide. Following up on transcriptome analysis, TRPC6 was noticed as a target of arsenic sulfide, which is involved in tumor development and malignant growth. After arsenic sulfide treatment, TRPC6 protein and mRNA expression were both downregulated in a dose-dependent manner,
as measured by western blot (Fig. 3C) and RT-qPCR (Fig. 3D), respectively. Meanwhile, the immunofluorescence staining results showed an obviously suppressed expression of TRPC6 and GPX4 protein, which was consistent with the western blot and RT-qPCR results (Fig. 3E). Upon the treatment of arsenic sulfide, we found a decrease of TRPC6 in the nucleus. We also use TCGA database to detect the expression of TRPC6 in patients of liver cancer and normal tissues. There was a higher expression of TRPC6 in tumor tissues compared with paracancerous tissues in 50 paired samples of tumor and paracancerous tissues ($p = 9.3e-10$) (Fig. 3F). And there was an apparent increase of TRPC6 expression in the 321 samples of cancer tissues, compared with the 50 samples normal tissues ($p = 4.3e-25$) (Fig. 3G).

3.4. TRPC6 contributes to arsenic sulfide-induced ferroptosis in HCC cells

In order to discover the specific function of TRPC6, TRPC6 was overexpressed by transfecting into HCC cells with TRPC6 expression vector. The level of TRPC6 expression significantly increased after transfection for 24 hours. Afterwards, HCC cells were significantly more viable when TRPC6 was overexpressed in the CCK-8 assay than when it was not overexpressed (Fig. 4A). Altogether, the results demonstrate that overexpression of TRPC6 counteracts the effects of arsenic sulfide on cell viability (Fig. 4A). Based on the abovementioned results, we assumed that arsenic sulfide promotes ferroptosis by regulating TRPC6-GPX4 signaling. In the following section, we overexpressed TRPC6 in HCC cells treated with arsenic sulfide to further test this hypothesis. The reduction of GPX4 protein and mRNA expression induced by arsenic sulfide was partially rescued by TRPC6 overexpression (Fig. 4B, C). The results revealed that enhanced expression of TRPC6 partially reversed the suppressive effects of arsenic sulfide on Fe$^{2+}$ in HCC cells (Fig. 4D). Consistently, the abnormal levels of GSH and MDA triggered by arsenic sulfide were also reversed by TRPC6 overexpression (Fig. 4E, F). By querying Cancer Genome Atlas (TCGA) database, an analysis of TRPC6 and GPX4 in HCC patients was conducted. We discovered that the TRPC6 and GPX4 expression were correlated in HCC patients through TCGA database analysis (Fig. 4G), which demonstrated that GPX4, a gene crucial to ferroptosis, was significantly associated with TRPC6. Co-immunoprecipitation (Co-IP) identified the relationship between TRPC6 and GPX4, and arsenic sulfide attenuated the TRPC6-GPX4 complex formation (Fig. 4H). Based on these results, we conclude that arsenic sulfide triggers ferroptosis via the TRPC6–GPX4 signaling pathway.

3.5. Arsenic sulfide benefits to treating liver cancer in vivo

An *in vivo* investigation of arsenic sulfide's therapeutic potential was conducted. HepG2 cells were inoculated into mice through subcutaneous injection to construct xenograft model. Two groups of mice were divided at random which was intraperitoneally injected with either vehicle or arsenic sulfide at a dosage of 2 mg/kg for a period of two weeks. Arsenic sulfide remarkably reduced the size of the mice's tumors, compared with the control group (Fig. 5A). As expected, tumor growth curves indicated that arsenic sulfide slowed down tumor growth significantly ($p < 0.05$, Fig. 5B). Meanwhile, an obvious
decrease in the tumor weight were also observed ($p < 0.05$, Fig. 5C). Western blot analysis was performed on tumor cell lysates from control (M1, M2) and arsenic sulfide-treated mice (M3, M4) to detect expression of TRPC6 and GPX4. When mice were treated with arsenic sulfide, TRPC6 and GPX4 expression was evidently reduced (Fig. 5D).

4. Discussion

Although previous reports have suggested that arsenic sulfide exhibited anticancer activity in some tumors, its effect against HCC has not been reported yet. In our study, we found that arsenic sulfide trigger ferroptosis both in vitro and in vivo. It is now recognized that ferroptosis is a method of controlling cell death in cancer treatment (Astudillo, Balboa, & Balsinde, 2022). Lipid peroxidation and iron accumulation are key components associated with the occurrence of ferroptosis (Z. Huang, Xia, Cui, Yam, & Xu, 2023). Depleted GSH leads to decreased GPX4, and lipid oxides cannot be hydrolyzed by GSH reductase catalyzed by GPX4, ultimately causing ferroptosis (Pan et al., 2021). Currently, ferroptosis is attracting increasing attention as a target of cancer therapy.

This study was conducted to investigate the therapeutic effects of arsenic sulfide on hepatocellular carcinoma and its underlying mechanisms. In vitro, we demonstrated that arsenic sulfide induced ferroptosis in hepatocellular carcinoma cells for the first time. First, treatment with CQ, necrosulfonamide, or Z-VAD-FMK did not protect cells from arsenic sulfide-induced death in hepatocellular carcinoma, implying that other cell death mechanisms may have taken place. The transcriptome analysis indicated that arsenic sulfide-induced cell death might be mediated by ferroptosis By accumulating lipid peroxidation products in a cellular-iron dependent manner, ferroptosis is initiated by cytoplasmic ROS (H. Zhang et al., 2020). As expected, we confirmed that arsenic sulfide caused GSH depletion, ROS accumulation, and lipid peroxidation. Furthermore, arsenic sulfide-induced cell death was reversed by treatment with the ferroptosis inhibitor Fer-1 and DFO. The morphological characteristic of ferroptosis is smaller mitochondria with condensed mitochondrial membranes densities, reduced or disappearance mitochondrial crista and ruptured outer mitochondrial membranes (Xie et al., 2016). Clearly, we found by transmission electron microscopy that the morphology of the arsenic sulfide-treated HepG2 cells was virtually similar to the ferroptotic mitochondria. We hypothesized the following based on our results: ferroptosis was partially involved in hepatocellular carcinoma cell death induced by arsenic sulfide.

We next set out to identify the underlying mechanism by which arsenic sulfide induces ferroptosis of HCC. Transcriptome analysis suggested that TRPC6 might be targeted by arsenic sulfide. TRPC6 is a member of TRPC family which is nonselective Na$^+$ and Ca$^{2+}$-permeable cation channel and expressed in numerous mammalian tissues. TRPC6 plays vital roles in cellular processes and is implicated in the genesis of numerous diseases. Clinically, TRPC6 was upregulated in glioblastoma multiforme compared with normal brain and the suppression of TRPC6 inhibited glioma growth, invasion and angiogenesis (Chigurupati et al., 2010). The repression of WNK1-mediated TRPC6 expression leads to the deactivation of NFATc1 and the subsequent proliferation and migration of clear-cell renal-cell carcinoma (Kim et al., 2019). Studies conducted in the past have shown that TRPC6 was very weakly
expressed in normal hepatocytes and expressed more strongly in human HCC tissues (El Boustany et al., 2008). TGFβ resulted in the formation of TRPC6/NCX1 complex, which upregulated the migration, invasion, and intrahepatic metastasis of human hepatocellular carcinoma cells (Xu et al., 2018). Collectively, these studies represent that TRPC6 are crucial to tumor initiation and progression. In the current study, arsenic sulfide inhibited the mRNA and protein level of TRPC6 in HepG2 and Huh7 cells. Additionally, overexpression of TRPC6 reversed the impacts of arsenic sulfide on ferroptosis of HepG2 and Huh7 cells. To further excavate the molecular mechanisms underlying TRPC6-mediated ferroptosis, we observed a correlation between TRPC6 and GPX4 by analyzing TCGA data sets. By using co-IP assays, TRPC6 and GPX4 were confirmed to interact. Further, arsenic sulfide inhibited TRPC6 and GPX4 crosstalk by decreasing their expression and the nucleus location of TRPC6. Thus, these findings suggest that arsenic sulfide triggered ferroptosis in HepG2 and Huh7 cells partly via targeting TRPC6/GPX4 signaling. Ultimately, our research demonstrated that arsenic sulfide might be capable of inducing ferroptosis in HCC cells. Hopefully, ongoing studies will prove arsenic sulfide to be a viable treatment choice for liver cancer in the future.

**Abbreviations**

A₄S₄: arsenic sulfide; Z-VAD: Z-VAD-FMK; ROS: reactive oxygen species; GSH: glutathione; MDA: malondialdehyde; NAC: N-acetyl-l-cysteine; Fer-1: ferrostatin-1; DFO: deferoxamine; HO-1: heme oxygenase 1; SLC7A11: solute carrier family 7 member 11; SLC40A1: solute carrier family 40 member 1; GPX4: glutathione peroxidase 4; TRPC6: transient receptor potential channel 6.

**Declarations**

**Ethical Approval**

This study was conducted in accordance with the Declaration of Helsinki principles. It was approved by the Medical Research Ethics Committee of Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University.

**Consent for publication**

Not applicable.

**Conflict of interest**

The authors declare no competing interests.

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Availability of data and materials

The data underlying this article will be shared on reasonable request to the corresponding author.

Author Contributions:

L.S.M. and C.Y. designed and performed most of the experiments, analyzed data, and prepared the manuscript as leading authors. K.T. and Z.C.Y. contributed to editing and commented on the article. F.Z.W. and C.S.X. performed cell Culture. C.S.Y. supervised the project. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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References


**Figures**

**Figure 1**

The effect of arsenic sulfide in combination with other cell death inhibitors on the growth of HepG2 and Huh7 cells.

(A) Arsenic sulfide was applied to HepG2 and Huh7 cells for 24 hours with or without Z-VAD-FMK, and growth inhibition was measured. (B) Inhibition rates of HepG2 and Huh7 cells were determined by treating them with arsenic sulfide with or without necrosulfonamide for 24 h. (C) Arsenic sulfide with or
without CQ was applied to Huh7 and HepG2 cells for 24 hours, and the inhibition rate was determined. 
(D) Heatmap analysis showing the differentially regulated genes by mRNA sequencing in HepG2 cells. (E) 
KEGG pathway enrichment analysis in HepG2 cells.

**Figure 2**

Arsenic sulfide induces ferroptosis in HepG2 and Huh7 cells.
A variety of concentrations of arsenic sulfide were applied to HepG2 and Huh7 cells for 24 hours, and the level of Fe$^{2+}$ (A), ROS (B), GSH (C), MDA (D) was assayed. (E) Cell viability was calculated by treating with arsenic sulfide with or without the ROS scavenger NAC for 24 h in HepG2 and Huh7 cells. (F) Inhibition rate of the cells was calculated after 24 hours of treatment with arsenic sulfide with or without ferroptosis inhibitors in HepG2 and Huh7 cells *$p < 0.05$, **$p < 0.01$. (G) The ferroptosis of HepG2 and Huh7 cells was observed by transmission electron microscopy (TEM) (original magnification: x100).
Figure 3

Arsenic sulfide regulates the expression of ferroptosis-associated proteins in HCC cells

(A) By using Western blot analysis, several key ferroptosis regulators were identified. (B) RT-qPCR was used to measure the mRNA level of GPX4. Using (C) Western blot analysis and (D) RT-qPCR, the expression of TRPC6 was detected in HepG2 and Huh7 cells (*p < 0.05, **p < 0.01). (E) Immunofluorescence assays for the expression of TRPC6 and GPX4 proteins in HepG2 and Huh7 cells treated with or without 5 μM arsenic sulfide for 24 h. (F) By using qPCR, TRPC6 expression was detected in HCC and paired paraneoplastic tissues from 50 patients. (G) Expression of TRPC6 was detected by qPCR in 321 cancer tissues and 50 normal tissues.
Figure 4

The increasing of TRPC6 reverses arsenic sulfide's effect on cell viability and ferroptosis.

(A) The effect of overexpression of TRPC6 on arsenic sulfide-induced growth in HepG2 and Huh7 cells. Level of GPX4 detected by (B) Western blot and (C) RT-PCR analysis. D-F Levels of Fe^{2+} (D), MDA (E), and GSH (F) were assayed in TRPC6 overexpression cells treated with arsenic sulfide. (G) The correlation

\[ r = -0.162, \ P < 0.001 \]
between TRPC6 and GPX4 in patients with hepatocellular carcinoma was analyzed from TCGA database. (H) Co-IP of TRPC6 and GPX4 in HCC cells. The immunocomplexes were subjected to Western blot assay.

Figure 5

Arsenic sulfide exerts its antitumor effects in vivo.

(A) Images of HepG2 xenograft tumors treated with PBS or arsenic sulfide. (B) Growth curve shows the changes in the tumor volume in mice after arsenic sulfide (*p < 0.05). (C) The tumor weights of xenograft mice (*p < 0.05). D The expression of TRPC6 and GPX4 proteins in excised tumors was analyzed by immunoblotting.