

Original Article

Unveiling the cytotoxicity of a new gold(I) complex towards hepatocellular carcinoma by inhibiting TrxR activity

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Abstract

Hepatocellular carcinoma (HCC), the predominant type of liver cancer, is an aggressive malignancy with limited therapeutic options. In this study, we assess a collection of newly designed gold(I) phosphine complexes. Remarkably, the compound GC002 exhibits the greatest toxicity to HCC cells and outperforms established medications, such as sorafenib and auranofin, in terms of antitumor efficacy. GC002 triggers irreversible necroptosis in HCC cells by increasing the intracellular accumulation of reactive oxygen species (ROS). Mechanistically, GC002 significantly suppresses the activity of thioredoxin reductase (TrxR), which plays a crucial role in regulating redox homeostasis and is often overexpressed in HCC by binding directly to the enzyme. Our *in vivo* xenograft study confirms that GC002 possesses remarkable antitumor activity against HCC without severe side effects. These findings not only highlight the novel mechanism of controlling necroptosis via TrxR and ROS but also identify GC002 as a promising candidate for the further development of antitumor agents targeting HCC.

Key words gold complex, hepatocellular carcinoma, necroptosis, thioredoxin reductase, reactive oxygen species

Introduction

Hepatocellular carcinoma (HCC), the primary malignant tumor of the liver, has been recognized as the fifth most diagnosed and the second most lethal cancer worldwide [1]. Currently, the most effective treatments for HCC are surgical resection and liver transplantation [2]. However, patients are often diagnosed with HCC in an advanced state and miss the opportunity for curative resection. Although immune checkpoint inhibitors and molecularly targeted drugs, such as sorafenib and lenvatinib, have been used in the clinical management of HCC in recent years, HCC remains as one of the cancers with the poorest prognosis due to challenges such as drug resistance and frequent tumor metastasis and recurrence [3,4]. Therefore, identifying more effective HCC treatments remains a crucial and emerging field of research.

Redox control systems are essential for cellular homeostasis. They manage the tightly regulated balance between the production

and elimination of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [5]. At the core of this system is the thioredoxin (Trx) system, which consists of NADPH, Trx, and TrxR [6,7]. TrxR, a member of the pyridine nucleotide disulfide oxidoreductase family, is known for its ability to reduce disulfide bonds (-S-S-) to dithiol (-SH) groups, a process that is crucial for oxidative stress defense [8]. By reducing Trx, TrxR facilitates the transfer of electrons to peroxiredoxin (Prx), subsequently eliminating intracellular harmful ROS. Consequently, the thioredoxin system plays a pivotal role in maintaining a balanced state of oxidation and reduction within cells.

Increasing evidence suggests that the thioredoxin system significantly influences tumor growth, including in HCC tumors, which are characterized by the overexpression of TrxR [9–11]. A nude mouse cancer xenograft model demonstrated that tumors with low TrxR levels progress more slowly and are smaller than those

with normal TrxR expression [12,13]. These findings suggest the potential therapeutic benefit of targeting TrxR. Recent studies have identified various TrxR inhibitors, such as metal-containing inhibitors (e.g., gold(I) NHC complexes), that exhibit antiproliferative effects on various tumor cells [14–16]. Therefore, targeting and inhibiting the thioredoxin system is a promising strategy for cancer treatment.

Gold has long been recognized for its medicinal value due to its high stability, acceptable side effect profile, and biocompatibility [17]. The therapeutic potential of gold can be realized when gold complexes enter the body and their ions participate in redox reactions with sulfur or selenium atoms within intracellular reducing biomolecules, which leads to stable cytotoxic effects [17]. TrxR, which contains a selenocysteine residue at its active site, is an important intracellular target for these gold complexes [18]. *In vitro* studies have shown that gold complexes can inhibit the growth of various tumor cells, including those from HCC, liver, ovarian, lung, leukemia, and cervical cancers [18–25]. For instance, auranofin, an FDA-approved gold complex for the treatment of rheumatoid arthritis, has received increasing attention for its ability to inhibit tumor cell proliferation by targeting TrxR [26,27]. Moreover, studies have confirmed that auranofin is effective against sorafenib-resistant acute myelocytic leukemia (AML) cells [27], suggesting its potential as an alternative to sorafenib in tumor therapy.

TrxR overexpression is commonly observed in HCC and plays a role in the proliferation of HCC cells [9–11]. Therefore, targeting TrxR may be an effective strategy for inhibiting HCC growth. In light of this, we present a series of gold(I) complexes containing diphenyl-2-pyridylphosphine groups. Among these, GC002 has shown the most promise. This complex not only directly inhibits TrxR but also triggers necroptosis in HCC cells by increasing the accumulation of intracellular ROS. Furthermore, GC002 successfully inhibited tumor growth in an HCC nude mouse model. Our research not only revealed how interference with the thioredoxin system is connected to necroptosis via ROS accumulation but also identified GC002 as an innovative leading compound, suggesting a new avenue for the development of treatments for HCC.

Materials and Methods

Cells and cell culture

HCC Huh7 cells were obtained from Xiamen Immocell Biotechnology Co., Ltd. (Xiamen, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, St Louis, USA) according to the supplier's specifications. All media were supplemented with 10% fetal bovine serum (FBS, cat# G11-70500; Genial Biologicals, Inc., Brighton, USA), 100 IU of penicillin (cat# A600135; Sangon Biotech, Shanghai, China), and 100 mg/mL streptomycin (cat# A610494; Sangon Biotech). The cells routinely tested negative for mycoplasma.

Antibodies and reagents

Anti-MLKL (cat# ab243142) and anti-Trx (cat# ab26320) antibodies were purchased from Abcam (Cambridge, UK). The anti-TrxR1 (cat# 11117-1-AP) antibody was purchased from Proteintech (Wuhan, China). Anti-RIP1 (cat# 3493), anti-RIP3 (cat# 13526), anti-pMLKL Ser-358 (cat# 91689) and anti-actin (cat# 4970) antibodies were purchased from Cell Signaling Technology (Boston, USA). Goat anti-rabbit (cat# 31210) and anti-mouse (cat# 31160)

secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, USA).

The following reagents were also used in this study: N-acetylcysteine (NAC) (cat# A9165), JC-1 (cat# T4069), necrostatin-1 (Nec-1) (cat# N9037), recombinant TrxR1 (cat# T9698) and glutathione reductase (GR) (cat# G3664), purchased from Sigma Aldrich; propidium iodide (PI) (cat# A601112), purchased from Sangon Biotech; CQ (cat# HY-17589A), ferrostatin-1 (cat# HY-100579), necrosulfonamide (NSA) (cat# HY-100573), auranofin (cat# A6733), Q-VD-OPh (cat# HY-12305), sorafenib (cat# HY-10201) and 3-methyladenine (3-MA) (cat# HY-19312), purchased from MedChemExpress (Monmouth Junction, USA); liproxstatin-1 (cat# S7699), purchased from Selleck Chemicals (Houston, USA); and a protease inhibitor cocktail (cat# K1007), a phosphatase inhibitor cocktail (cat# K1015 and Z-VAD-FMK (cat# A1902), purchased from ApexBio (Houston, USA). The recombinant U498C TrxR1 mutant (Sec→Cys) was prepared according to a previous report [28].

Synthesis of Gold(I) phosphine complexes

At room temperature, solvent A with a volume ratio of 5:1 (ethanol: deionized water) was prepared, and 2 g of HAuCl₄ (Shanghai Fine Chemical Materials Research Institute, Shanghai, China) was dissolved in 10 mL of solvent A to obtain yellow solution B. While stirring, dimethyl sulfide (Me₂S) (Energy Chemical Inc., Shanghai, China) was slowly added to yellow solution B. The addition was stopped when the yellow solution became colorless, and stirring was continued for 2 h. The white Au(Me₂S)Cl was obtained by filtration and vacuum drying [29,30]. A solution containing 1 mmol of Au(Me₂S)Cl dissolved in 10 mL of dichloromethane (CH₂Cl₂) was added with 1.05 mmol of phosphorus ligands (Energy Chemical Inc., Shanghai, China) at room temperature while stirring for 2 h [31]. After the reaction, the white product 1 was obtained by filtration and dried under reduced pressure. Then, 0.5 mmol of product 1 and 0.55 mmol of alkyne ligand (Energy Chemical Inc., Shanghai, China) were dissolved in 5 mL of ethanol. After stirring for 10 min, 0.6 mmol of KOH dissolved in 0.5 mL of deionized water was added, and the reaction was carried out for 12 h at room temperature. After the reaction, product 2 (using GC002 as an example) was obtained by filtration and recrystallization. A saturated solution of compound GC002 was prepared in dichloromethane and stirred well for 20 min. The solution was then filtered to remove impurities. The clear filtrate was collected in 15 mL Schering bottles, and the bottles were placed in an ether gas phase at 4°C. After 6 days, a colorless, transparent crystal was precipitated, and a suitable crystal for structure analysis were isolated. A single crystal of title compound GC002 was mounted on a glass fiber for a SMART APEX CCD diffractometer at 293(2) K using Mo K α radiation ($\lambda = 0.71073 \text{ \AA}$). The program SAINT was used for the integration of the diffraction profiles, and the SADABS program was used for semiempirical absorption corrections [32,33]. The structure was solved by direct methods using the program SHELXS-97 and refined by full-matrix least-squares techniques on F² with SHELXL-97 [34,35].

Cell survival rate

The cell survival rate was determined by staining the cells with propidium iodide (PI). In brief, the cells were harvested and resuspended in 1 mL of PBS containing 5 μ g of PI. The assimilation

of PI was assessed by flow cytometric analysis. Cells that did not absorb PI and remained within the standard size range were deemed to be alive.

MTT assay

Briefly, 6×10^3 Huh7 cells were seeded in triplicate in a 96-well plate and incubated with the indicated reagents for 72 h at 37°C in a final volume of 200 μ L in each well. Cells treated with DMSO served as the control group. Following the treatment period, 10 μ L of MTT (5 mg/mL) was added to each well, and the plates were incubated for an additional 4 h at 37°C. Subsequently, the MTT-containing medium was removed and 150 μ L of DMSO was added to each well to dissolve the formazan crystals. After 10 min of incubation to ensure complete dissolution, the absorbance at 490 nm was measured using a microplate reader.

Lactate dehydrogenase (LDH) release assay

LDH release in the cell culture supernatants was analyzed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (cat# G1780; Promega, Madison, USA) in accordance with the manufacturer's instructions. The cell culture media were collected and subjected to centrifugation for 5 min at 200 *g* at room temperature. The supernatants (50 μ L) were then transferred to 96-well plates, combined with the LDH assay reagent, and incubated at room temperature for 30 min. The absorbance values were subsequently measured at 490 nm with a microplate reader.

Real-time PCR

Total RNA was extracted from Huh7 cells utilizing TRIzol reagent (cat# 15596026; Thermo Fisher Scientific) in compliance with the protocol provided by the manufacturer. One microgram of total RNA from each sample was reverse transcribed with SuperScript® II Reverse Transcriptase (cat# 18064014; Thermo Fisher Scientific). The resulting complementary DNA (cDNA) was subjected to real-time PCR analysis with SYBR Green dye (cat# S7563; Thermo Fisher Scientific). Gene expression was quantified as arbitrary units and normalized to the expression of *actin*. The specific real-time PCR primers used for human *TrxR1* were as follows: forward primer, 5'-TCATCATTTGGAGTGGCTCAG-3' and reverse primer, 5'-CA CATGTTCTCCGAGACCC-3'.

Western blot analysis

The cells were lysed with ELB lysis buffer (NaCl 150 mM, Tris 50 mM, NP-40 0.5%, NaF 100 mM, pH 7.6) that included both a protease inhibitor cocktail and a phosphatase inhibitor cocktail. Following lysis, the samples were centrifuged at 14,000 *g* for 15 min at 4°C. The resulting supernatants were then combined with 2 $\times$ SDS sample buffer and heated to 95–100°C for 5–10 min. After denaturation, the samples were loaded onto SDS-PAGE gels for electrophoresis, transferred to PVDF membranes, and subjected to immunoblot analysis with specific antibodies.

Cellular TrxR activity assay

Huh7 cells were treated with various concentrations of GC002 (synthesized by our laboratory according to the method) and incubated for 3 h. Subsequently, total cellular protein was extracted from the cells using RIPA buffer on ice and the protein concentration was quantified by the Bradford assay. TrxR activity within the extracted proteins was then determined using a TrxR activity

detection kit according to the manufacturer's instructions (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China).

In vitro TrxR activity assays

A DTNB assay, as previously described [36], was employed to assess the activity of TrxR. The assay was conducted by incubating TrxR, either wild-type (80 nM) or U498C (300 nM), in TE buffer (50 mM Tris-HCl, 1 mM EDTA, pH 7.5) with varying concentrations of the inhibitor GC002 in a 96-well plate. The final volume of each reaction mixture was adjusted to 100 μ L, and each mixture was incubated at 37°C for 2 h. Subsequently, 100 μ L of master mix containing 200 μ M NADPH and 2.5 mM DTNB in TE buffer was added to reach a final reaction volume of 200 μ L in each well. The increase in absorbance at 412 nm was monitored for 3 min using a microplate reader to ensure the linearity of the reaction. TrxR activity was calculated relative to that of the vehicle control dimethyl sulfoxide (DMSO) and is expressed as a percentage.

In vitro glutathione reductase (GR) activity assay

Samples of TE buffer containing GR prerduced by NADPH (0.23 U) and various concentrations of GC002 were incubated in a 96-well plate for the specified durations at 37°C. Each reaction mixture had a final volume of 100 μ L. Reactions were initiated by the addition of 50 μ L of a solution containing 1.2 mM NADPH and 3 mM oxidized glutathione (GSSG) prepared in TE buffer to each well, resulting in a total volume of 150 μ L in each well. The change in absorbance at 340 nm, which correlates with the consumption of NADPH and thus GR activity, was continuously monitored for 3 min using a microplate reader to ensure a linear response.

Molecular docking

Protein–ligand docking was performed using AutoDock [37]. The crystal structure of the selenoprotein thioredoxin reductase 1 (PDB ID: 3EAN) was used as the receptor, and the compound GC002 was used as the ligand. To prepare the receptor, ligands and solvent molecules were removed, and hydrogens were added to all residues. For docking, the region comprising chain A and chain B of the receptor was chosen as the binding site. After docking, the TrxR1-GC002 complex with the best complementarity between the receptor and ligand was chosen for analysis using AutoDock and PyMOL [38].

Fluorescence quenching

Fluorescence spectra were acquired via a fluorescence spectrophotometer (model F-4500; HITACHI, Tokyo, Japan). Briefly, TrxR protein at a concentration of 5 μ M was incubated with different concentrations of GC002 ranging from 1 to 20 μ M. Then, fluorescence quenching was monitored at 25°C. The slit widths were 5 nm for excitation and 2.5 nm for emission. The excitation wavelength was fixed at 280 nm, while emission spectra were recorded from 285 to 430 nm. To estimate the binding affinity, the fluorescence intensities at various concentrations of quencher were collected, and the K_d values were calculated using the standard formula.

Trx redox state determination

Trx redox state experiments were performed as described previously [39]. Briefly, Huh7 cells were treated with GC002 for 12 h and then collected and washed twice with PBS. Total cellular protein was extracted using RIPA buffer on ice, and protein

concentration was quantified via the Bradford assay. The samples were then added to phenylarsenoxide (PAO) Sepharose and incubated at room temperature for 30 min with intermittent vortexing every 5 min. The supernatant and beads were subsequently separated to isolate the oxidized and reduced Trx fractions. Total Trx protein expression was directly assessed in untreated samples. SDS-PAGE was employed to analyze all the samples, followed by Trx detection by western blot analysis.

Intracellular GSH/GSSG detection

A GSH/GSSG quantification kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to measure reduced and oxidized glutathione in Huh7 cells. Briefly, Huh7 cells were treated with GC002 for 12 h. The cells were subsequently washed with PBS and subjected to rapid freezing and thawing twice by alternately immersion in liquid nitrogen and 37°C water. Following cell lysis, the supernatants were collected and combined with the detection reagents following the manufacturer's instructions. The changes in the reaction were monitored by measuring the absorbance at 415 nm.

Intracellular ROS detection

CellROX Green (Molecular Probes; Life Technologies, Carlsbad, USA) was used to detect ROS production. After being washed with phosphate-buffered saline (PBS), the cells were incubated with CellROX Green reagent at 37°C for 30 min. The cells were subsequently trypsinized and then washed twice with ice-cold PBS to remove any excess dye. Finally, the ROS levels were quantified by flow cytometry.

Generation of the lentiviral system

The lentiviral vector pLL3.7 was used to express short hairpin RNAs (shRNAs) in Huh7 cells. The oligonucleotides used for shRNA expression were subcloned and inserted into the lentiviral vector pLL3.7. Lentiviruses were produced in HEK293T cells by cotransfection with the pLL3.7 vector containing shRNA sequences and packaging plasmids, and polyethylenimine (PEI) was used for transfection. Forty-eight hours post-transfection, the viral supernatants were collected, centrifuged at 75,000 *g* for 90 min to concentrate the viruses, and passed through 0.45 µm pore size filters (Millipore, Billerica, USA) to remove cell debris. Freshly plated Huh7 cells were transduced with lentiviruses and incubated for an additional 48 h. The knockdown efficiency was evaluated via reverse transcription-PCR (RT-PCR). The oligonucleotide sequences for the shRNA-targeted mRNAs were as follows: negative control (NTC) shRNA, 5'-GCGCGCTTTGTAGGATTCG-3'; shRNA-Caspase-3, 5'-GCAAACCTCAGGGAAACAT-3'; shRNA-RIP1, 5'-GCTGCTAAGTACCAAGCTA-3'; shRNA-Atg7, 5'-GCTGGTCATCAATGCTGCT-3'; and shRNA-GSDMD, 5'-GCAGGAGCTTCACCTTCTA-3'.

Animal studies

All the mice utilized in this study were housed under standard specific pathogen-free (SPF) conditions. The mice were maintained on a 12-hour light/12-hour dark cycle and had unrestricted access to both food and water. The animal studies were carried out in accordance with the Institutional Animal Care and Use Committee of the University of Electronic Science and Technology of China,

and the approval of the Animal Experimental Ethics Committee (No. 28139) was obtained.

Xenograft formation

Athymic nude mice (BALB/c, male, 18–20 g, 7–8 weeks old) were subcutaneously injected with Huh7 cells (5×10^6) in the right flank to establish tumors. The initial tumor volumes ranged from 130 to 170 mm³, ensuring successful model development. After tumor establishment, the mice were randomly divided into three groups (*n* = 6 each): (1) a vehicle-treated control group, (2) a group treated with 10 mg/kg GC002, and (3) a group treated with 10 mg/kg sorafenib. Vehicle, GC002 and sorafenib were administered to Huh7 tumor-bearing mice via intratumoral injection every 4 days for two weeks. At the end of the study, the mice were euthanized, and the tumor weights were recorded.

Statistical analysis

Most of the data are expressed as the mean $\pm$ standard deviation (SD) from multiple independent experiments or replicates of representative experiments, with each having at least two or three independent determinations. Differences among multiple groups were analyzed using one-way or two-way ANOVA, followed by *post hoc* test with either Tukey's test or Sidak's multiple-comparisons test. Enzyme activities and cell viabilities are expressed as the mean $IC_{50} \pm$ standard error (SE). All the statistical analyses were performed using GraphPad Prism 8 software (La Jolla, USA). *P* < 0.05 indicates a statistically significant difference.

Results

Gold(I) phosphine complexes demonstrate antitumor activity in HCC cells.

We synthesized a series of gold(I) complexes adorned with diphenyl-2-pyridylphosphine (denoted as R1) and alkyne (denoted as R2) units inspired by the parent compound auranofin and following the synthetic blueprint established by the known precursor [(PPh₂py)AuCl]. Our synthesis yielded six unique gold complexes featuring a variety of units, as depicted in Figure 1A. To investigate the potential of these complexes to combat HCC, we exposed Huh7 HCC cells to the complexes at a concentration of 1 µM for 24 h. Most of the synthesized complexes effectively suppressed cell viability, with GC002 exerting the most pronounced cytotoxic effect on HCC cells (Figure 1B). Notably, GC002 uniquely induced cell lysis, characterized by cellular swelling and the emergence of large bubbles from the cell membrane, whereas most of the other compounds induced apoptotic morphological changes (Figure 1C).

To assess the inhibitory impact of GC002 on HCC, Huh7 cells were treated with GC002, and the effects of GC002 were compared to those of auranofin and sorafenib (the standard first-line drug medication for advanced HCC). The IC_{50} of GC002 in Huh7 cells was 0.49 ± 0.01 µM, which was lower than those of auranofin and sorafenib (2.17 ± 0.04 µM and 10.46 ± 0.19 µM, respectively) (Figure 1D). These results demonstrate that gold(I) phosphine complexes, particularly GC002, exhibit potent antitumor effects on HCC cells.

GC002 induces necroptosis in HCC cells

To elucidate the type of cell death induced by GC002 in HCC, we pretreated Huh7 cells with a variety of cell death inhibitors before

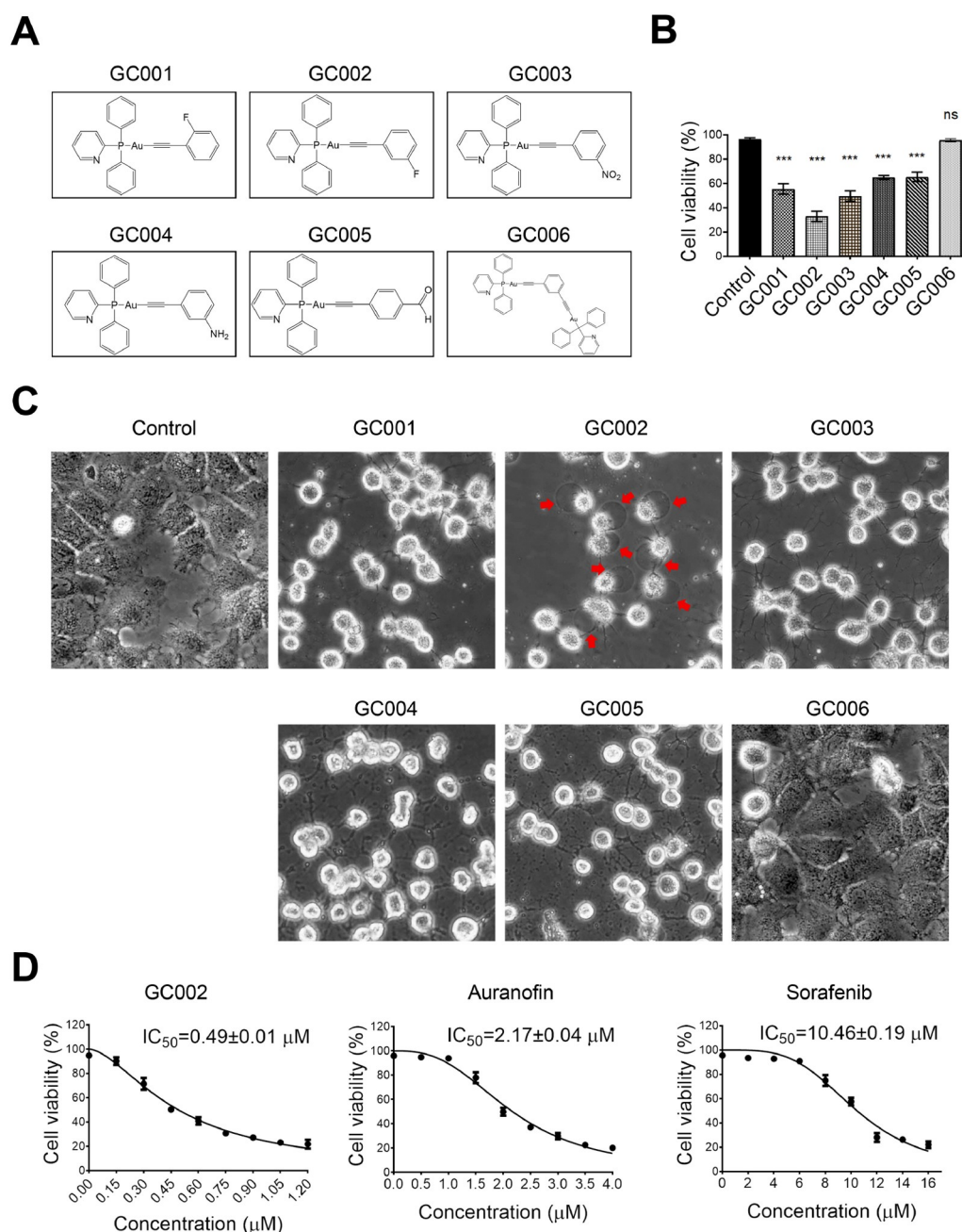


Figure 1. Gold(I) phosphine complexes exhibit cytotoxicity to HCC cells (A) The structures of the synthesized gold(I) complexes. (B,C) Huh7 cells were treated with the indicated phosphine gold(I) complexes at a concentration of 1 μM for 24 h. Cell survival was determined by flow cytometry after PI staining (B), and the morphology examination (C, red arrows indicate cells undergoing lysis). (D) Huh7 cells were treated with chemicals at the indicated concentrations for 72 h, and then the cell viability was determined by MTT assay. The IC_{50} values $\pm$ SE of GC002, auranofin and sorafenib are presented. Most data (except Figure 1D) are presented as the mean $\pm$ SD of three independent experiments. *** $P < 0.001$, ns, not significant.

administering GC002. The results from the cell viability assay revealed that only the necroptosis inhibitors Nec-1 and NSA effectively decreased GC002-induced cell death, while inhibitors targeting apoptosis (zVAD and Q-VD-OPh), autophagy (chloroquine and 3-MA), and ferroptosis (Lipo-1 and Fer-1) had no significant effect (Figure 2A). Moreover, we observed a time-dependent increase in necroptosis following GC002 exposure, as indicated by compromised cell membrane integrity, as determined

by measuring lactate dehydrogenase (LDH) release (Figure 2B). Consistent with cell survival, LDH release was impeded only by the necroptosis inhibitors Nec-1 and NSA and remained unaffected by all other inhibitors tested (Figure 2C).

To gain further insight into the necroptotic pathway triggered by GC002, western blot analysis was performed to measure the levels of key proteins involved in necroptosis. Treatment with GC002 resulted in a significant increase in the levels of RIP1, RIP3, and

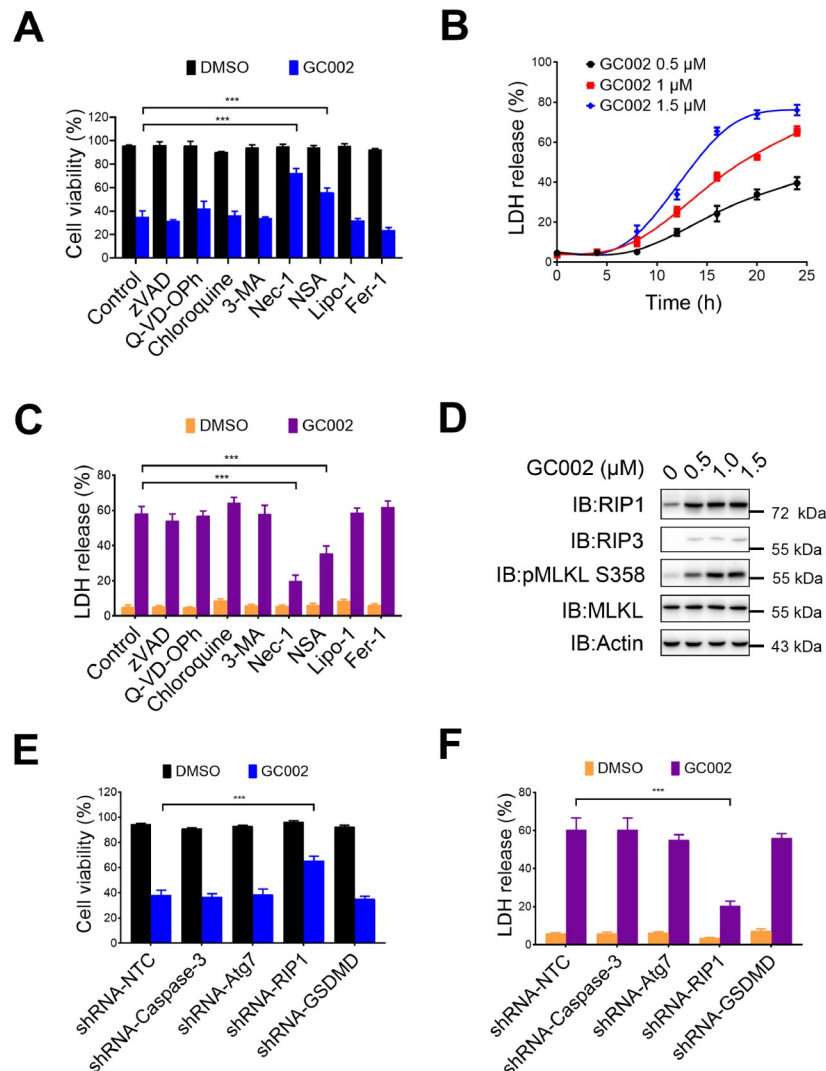


Figure 2. GC002 induces necroptosis in HCC cells (A) Huh7 cells were pretreated with the apoptotic inhibitors zVAD (20 μ M) and Q-VD-OPh (40 μ M), the autophagic inhibitors 3-MA (5 mM) and chloroquine (20 μ M), the necrotic inhibitors NSA (0.5 μ M) and Nec-1 (20 μ M), and the ferroptotic inhibitors Lipo-1 (0.5 μ M) and Fer-1 (0.5 μ M) separately for 2 h before GC002 treatment (1 μ M, 24 h). Cell survival was then determined using flow cytometry with PI staining. (B) Huh7 cells were treated with GC002 at different concentrations for the indicated durations, and cell lysis was determined by LDH release assay. (C) Huh7 cells were incubated with the indicated inhibitors separately for 2 h before GC002 treatment (1 μ M, 24 h), after which LDH release was detected. (D) Huh7 cells were treated with GC002 at the indicated concentrations for 24 h, and the protein levels of RIP1, RIP3, MLKL, and phosphorylated MLKL (Ser358) were detected by western blot analysis. Actin was used as the loading control. (E,F) *Caspase-3*, *Atg7*, *RIP1*, or *GSDMD* in Huh7 cells were separately knocked down using the corresponding shRNA, followed by treatment with GC002 (1 μ M) for 24 h. Cell survival (E) and LDH release (F) were determined. All the data are presented as the mean $\pm$ SD of three independent experiments. *** $P < 0.001$.

phosphorylated MLKL at Ser-358 (Figure 2D), indicating the activation of the necroptotic cell death pathway. To further validate the role of necroptosis in the cellular response to GC002, we conducted gene knockdown experiments targeting key regulators of various cell death pathways. Remarkably, decreasing the expression of RIP1, a key component of necroptosis, allowed cells to resist death and LDH release after exposure to GC002. Conversely, the suppression of genes involved in apoptosis (Caspase-3), autophagy (Atg7), or pyroptosis (GSDMD) did not dramatically influence cell death or LDH release (Figure 2E,F). In summary, these findings indicate that GC002 predominantly induces necroptosis in HCC cells via activation of the RIP1/RIP3/MLKL signaling axis.

GC002 inhibits TrxR activity in HCC cells

Accumulating evidence has revealed that gold complexes, particularly gold(I) phosphine complexes, can effectively attack the selenocysteine residue in the active site of TrxR [18,40]. We thus explored the influence of GC002 on TrxR activity in HCC cells. After the administration of various concentrations of GC002 and auranofin to Huh7 cells and 3 h of incubation, we assessed TrxR activity using TrxR assay kits. Our findings demonstrated robust dose-dependent inhibition of TrxR by GC002, in contrast with the comparatively modest effect of auranofin (Figure 3A). To confirm that the effect of GC002 on TrxR is not attributable to transcriptional modifications or alterations in protein stability, we performed western blot analysis and real-time PCR. These experiments

confirmed that neither the mRNA level (Figure 3B) nor the protein level (Figure 3C) of TrxR was disturbed within the cells, underscoring that GC002 specifically inhibits the enzymatic activity of TrxR as its target in HCC cells.

Subsequently, the ability of GC002 to inhibit wild-type cytosolic TrxR1, a TrxR1 mutant, and glutathione reductase (GR), which is structurally similar to TrxR1 but lacks selenocysteine residues, was evaluated *in vitro*. The TrxR1 mutant is a variant in which

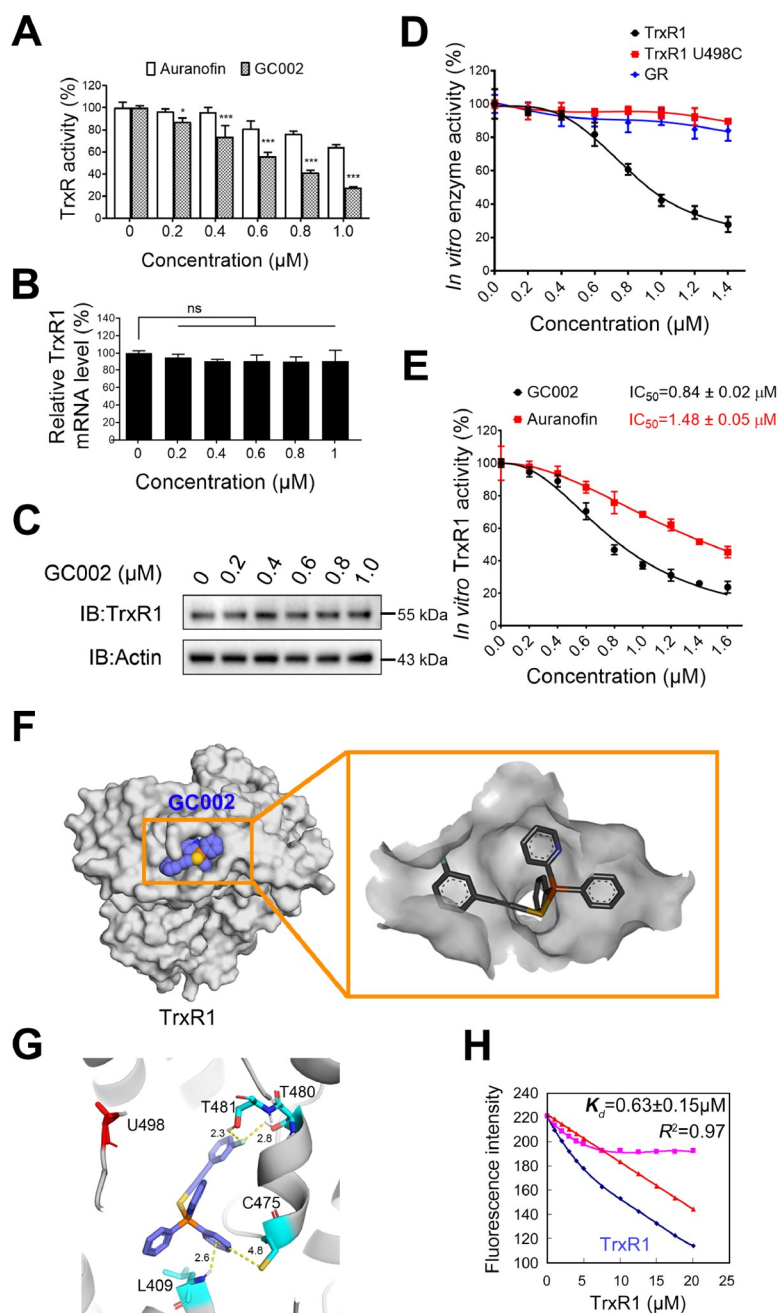


Figure 3. GC002 inhibits TrxR activity in HCC cells (A) After treatment with GC002 at 37°C for 3 h, the TrxR activity were measured in Huh7 cells. (B,C) Huh7 cells were treated with GC002 at the indicated concentration for 3 h. Subsequently, the mRNA level (B) and protein level (C) of TrxR1 were assessed by real-time PCR and western blot analysis, respectively. Actin was used as the loading control. (D) The purified proteins of NADPH-reduced GR, recombinant TrxR1, and TrxR1 U498C were separately incubated with GC002 at the indicated concentrations for 2 h, and the activities were measured by DTNB assay. (E) TrxR1 was incubated with GC002 or auranofin for 2 h at the indicated concentrations, and the activities were measured using DTNB assay. The IC₅₀ values of auranofin and GC002 are presented. (F) GC002 was docked into the binding site of TrxR1 (PDB ID: 3EAN) (overall view). (G) GC002 and TrxR1 binding site (PDB ID: 3EAN) (detailed view). GC002 is represented with slate sticks, the hydrogen and Pi-Sulfur bonds are shown as yellow dotted lines, and representative residues in TrxR1 are indicated in light blue or red color. (H) The K_d value for GC002 on TrxR1 was determined by fluorescence quenching. TrxR1 was incubated with increasing amounts of GC002 as indicated, with GST used as the internal filter control. K_d is presented as mean ± SEM of three independent reactions. R² is the coefficient of determination. Most data are presented as the mean ± SD, but the enzyme activities are expressed as the mean IC₅₀ ± SE. *P < 0.05, ***P < 0.001, ns, not significant.

selenocysteine at position 498 is substituted with alanine. Treating reduced recombinant TrxR1 with GC002 led to a dose-dependent reduction in enzymatic activity, as shown in Figure 3D. In contrast, GC002 had negligible effects on the activity of both GR and the TrxR1 mutant (Figure 3D), suggesting that the selenocysteine residue is crucial for the inhibition of TrxR by GC002. Next, the activity of GC002 was compared with that of auranofin. The IC_{50} value of GC002 for the recombinant TrxR1 protein was determined to be $0.84 \pm 0.02 \mu\text{M}$ (Figure 3E), which was significantly lower than that of auranofin ($1.48 \pm 0.05 \mu\text{M}$) under identical conditions. On the basis of these observations, we concluded that GC002 directly and selectively inhibits TrxR1 in HCC cells.

To identify the binding site of GC002 on TrxR1, we utilized molecular docking methods and determined that the estimated binding energy was -8.81 kcal/mol . Subsequently, the hypothetical binding mode of GC002 to TrxR1 (PDB ID: 3EAN) was established. In this model, we observed that GC002 adopted a compact conformation to interact with a pocket in TrxR1 (Figure 3F), which is composed of residues His-108, Ile-347, Phe-406, Thr-412, Gly-470, Pro-473, Val-474, and Gln-494. Notably, three crucial hydrogen bonds were formed between GC002 and TrxR1 residues Leu-409, Thr-480, and Thr-481, as well as a pi-sulfur bond measuring $4.8 \text{ \AA}$ between GC002 and Cys-475 of TrxR1 (Figure 3G). All of these interactions facilitate the stable interaction between GC002 and the TrxR1 binding site. Moreover, the selenocysteine residue (U498) at the C-terminus of TrxR1 is in close proximity to GC002. Considering the flexibility of the C-terminus [41], particularly when the enzyme is in its reduced form, the selenocysteine residue is prone to react with gold ions. In addition, we assessed the interaction between GC002 and TrxR1 using a fluorescence quenching assay and determined an equilibrium dissociation constant (K_d) of $0.63 \pm 0.15 \mu\text{M}$ (Figure 3H) for the GC002-TrxR1 complex, which indicates that GC002 has high affinity for TrxR1. In summary, GC002 directly binds to TrxR1 and inhibits the enzyme's activity.

GC002 stimulates intracellular ROS accumulation in HCC cells

TrxR plays a key role in reducing oxidized Trx back to its reduced state, which is crucial for the clearance of intracellular ROS [8] (Figure 4A). To investigate the impact of GC002-mediated TrxR inhibition on the redox state of Trx, Huh7 cells were incubated with GC002 at the indicated concentrations for 12 h. Subsequently, reduced Trx was separated from oxidized Trx using phenylarsenoxide (PAO) Sepharose and assessed by western blot analysis [39]. Our findings indicated that Trx primarily exists in a reduced state under normal conditions. However, the ratio of reduced Trx to oxidized Trx significantly decreased after the treatment of Huh7 cells with increasing concentrations of GC002 (Figure 4B). These results suggest that GC002 hinders intracellular TrxR function, preventing Trx reduction and consequently decreasing the ratio of reduced Trx to oxidized Trx.

Oxidized Trx loses the ability to couple with Trx-dependent peroxiredoxin (Prx) to scavenge hydrogen peroxide (H_2O_2), leading to ROS accumulation [42]. Therefore, the cellular ROS levels after GC002 administration were measured via fluorescence-based analysis. As shown in Figure 4C,D, the ROS levels were elevated in Huh7 cells in a dose-dependent manner upon GC002 treatment. Consistent with these findings, GC002 also impaired the intracellular GSH/GSSG ratio, which is an important indicator of the

cellular redox status (Figure 4E). In light of these data, we focused on the protective effects of NAC and glutathione (GSH), which are antioxidants known to neutralize excessive ROS (Figure 4F) [43]. Our results revealed that the impact of GC002 on Huh7 cells, notably the increases in cell death and LDH release, was significantly blunted when the cells were pretreated with NAC and GSH (Figure 4G,H). Taken together, these findings indicate that GC002 application effectively induces HCC necroptosis, which is dependent on the accumulation of intracellular ROS.

Previous reports have suggested that necroptosis may contribute to intracellular ROS accumulation [44,45]. Our experimental data demonstrated that necroptosis inhibitors slightly decreased the intracellular ROS levels (Figure 4I) and compromised the GSH/GSSG ratio (Figure 4J), suggesting that GC002 can enhance ROS accumulation via necroptosis, albeit marginally, in addition to its direct inhibition of TrxR. These results point to a synergistic feedback loop in which necroptosis and ROS accumulation reinforce each other. In conclusion, the collected evidence suggests that ROS accumulation is crucial for GC002-induced necroptosis in HCC cells, indicating a new mechanism of necroptosis regulation involving TrxR and ROS.

The physiological role of GC002 in inhibiting the growth of HCC in mouse models

To evaluate the effectiveness of GC002-mediated inhibition of HCC growth *in vivo*, we established Huh7 tumor xenograft models in nude mice. We subcutaneously injected Huh7 cells into the right flanks, and then the mice were randomly divided into three groups: (1) vehicle-treated control group, (2) GC002-treated group, and (3) sorafenib-treated group.

During the two-week treatment period, GC002 significantly inhibited the growth of Huh7 tumors compared with that in the control group (Figure 5A). Mice treated with GC002 showed a marked reduction in tumor weight, with a tumor growth inhibition rate of 70.3% ($P < 0.01$), which was much greater than the 48.8% inhibition rate in the sorafenib-treated group ($P < 0.01$), although the difference was not significant ($P = 0.27$) (Figure 5B,C). Additionally, no obvious side effects, including effects on body weight (Figure 5D), renal biochemical parameters (blood urea nitrogen (BUN) and serum creatinine) (Figure 5E,F), or liver function parameters (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) (Figure 5G,H), were observed in the GC002-treated group. Taken together, these findings indicate that GC002 effectively suppresses tumor growth *in vivo* without causing severe side effects and has a superior effect to that of sorafenib.

Moreover, western blot analysis revealed increased levels of RIP1, RIP3, and phosphorylated MLKL at Ser-358 in the GC002-treated group (Figure 5I), indicating the activation of the necroptosis pathway in tumor tissue. In summary, these findings suggest that treatment with GC002 can effectively inhibit tumor growth *in vivo* by promoting necroptosis, suggesting a novel strategy for the development of therapeutics to treat HCC.

Discussion

Clinical applications of platinum-based chemotherapeutics have shown substantial antitumor effects, but their effectiveness against some cancer types is hampered by side effects and the emergence of resistance [46]. To address this issue, medicinal chemists are concentrating on creating innovative metal-based lead compounds

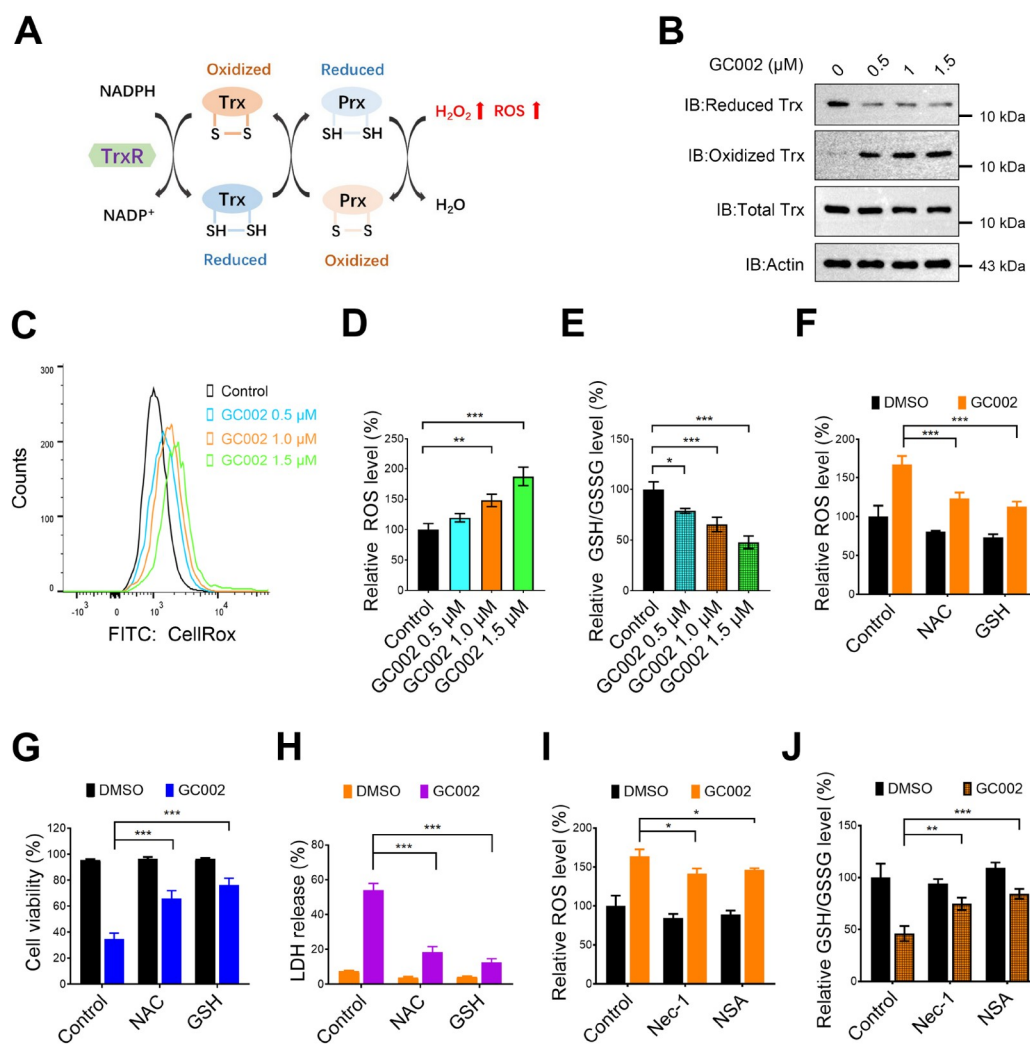


Figure 4. GC002 stimulates ROS accumulation in HCC cells (A) A schematic view of the thioredoxin system. (B) Huh7 cells were treated with GC002 at the indicated concentrations for 12 h, and the redox state of Trx was determined by western blot analysis. (C,D) Huh7 cells were stained with CellRox Green after treatment with GC002 at the indicated concentrations for 12 h, and the ROS level was determined by flow cytometry (C) and quantified (D). (E) The intracellular GSH/GSSG ratio in Huh7 cells was measured after treatment with GC002 at the indicated concentrations for 12 h. (F) Huh7 cells were pretreated with NAC (5 mM) or GSH (1 mM) for 2 h separately, and then, GC002 (1 μM) was applied for another 12 h. Subsequently, the cells were stained with CellRox Green, and the ROS levels were analyzed by flow cytometry. (G,H) Huh7 cells were pretreated with NAC (5 mM) or GSH (1 mM) for 2 h separately before the GC002 treatment (1 μM, 24 h). Cell survival (G) and LDH release (H) were determined. (I,J) The conditions are the same as those in (F), and the ROS level (I) and GSH/GSSG ratio (J) were analyzed separately. All the data are presented as the mean $\pm$ SE of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

that operate via unique biochemical pathways and offer distinctive pharmacological characteristics. Among various nonplatinum anticancer compounds, gold-based anticancer complexes have gained attention because of their strong antigrowth capabilities and generally favorable side effect profile [47]. Our study evaluated a series of novel gold(I) phosphine compounds, and GC002 emerged as a lead compound. Compared with established clinical agents such as sorafenib and auranofin, GC002 exhibited more pronounced cytotoxic effects on HCC cells, indicating its antitumor activity. Crucially, our research revealed that GC002 effectively targets the TrxR system, causing the accumulation of ROS and ultimately leading to irreversible necroptosis in HCC cells. Furthermore, *in vivo* experiments confirmed that GC002 formidably suppressed the growth of HCC with endurable side effects, underscoring its

potential as a promising new therapeutic option for HCC.

In our study, we focused on the ability of GC002 to inhibit TrxR in Huh7 HCC cells. GC002 exhibited significant dose-dependent TrxR inhibition, which was markedly more potent than the effect observed with auranofin in the same cell line. Additionally, our *in vitro* data demonstrated that GC002 exerted a potent and direct inhibitory effect on the TrxR enzyme. Gold complexes typically inhibit enzymes, particularly those containing thiol or selenocysteine groups, as reported in the literature [17,47]. These complexes have a greater affinity for sulfur and selenium and can rapidly react with activated cysteine or selenocysteine residues in enzymes, leading to the formation of gold–thiolate or gold–selenonate complexes. The distinctive mechanism of action employed by gold compounds offers prospective benefits for counteracting the

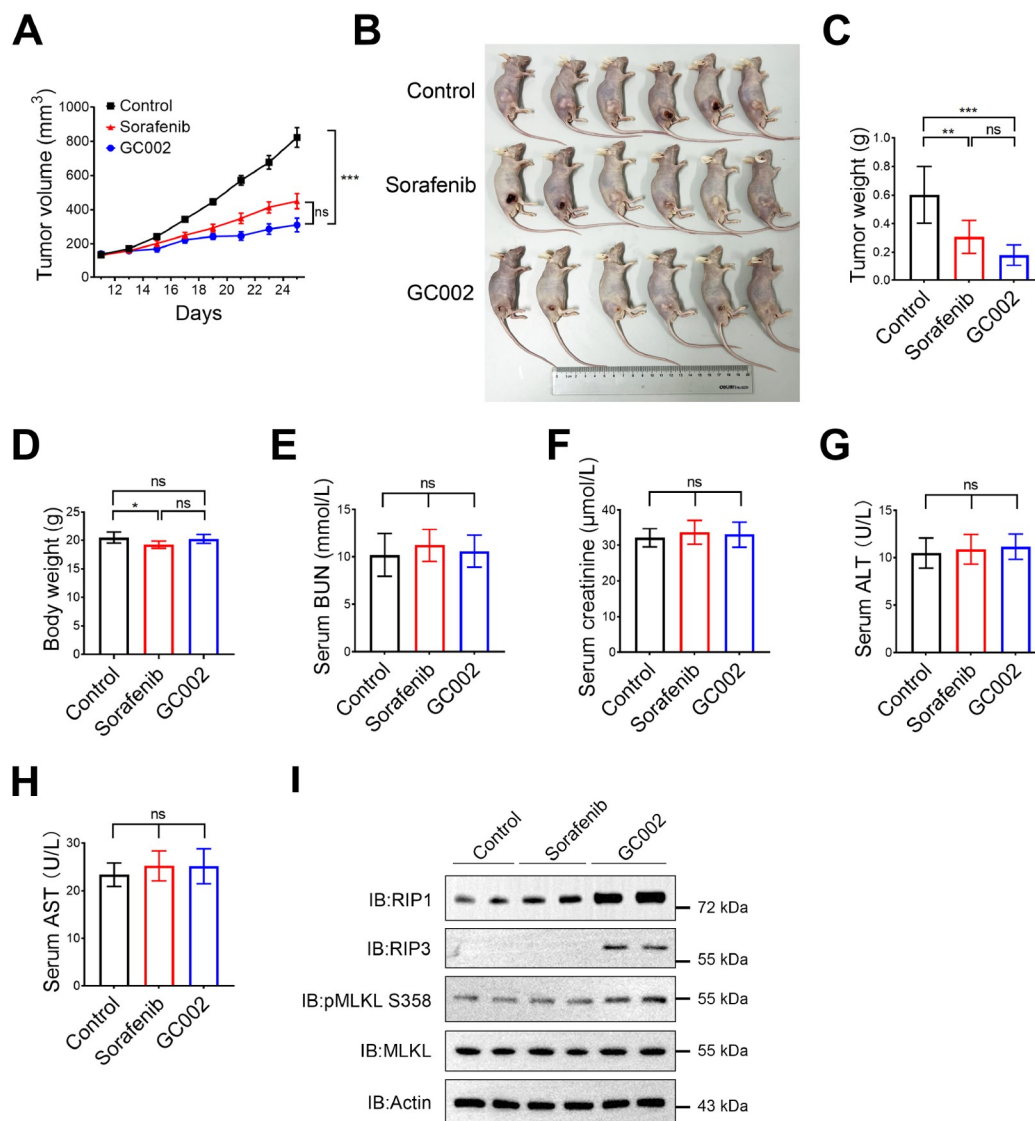


Figure 5. Physiological role of GC002 in suppressing HCC growth in mouse models (A–I) Huh7 cells were injected into nude mice to form subcutaneous xenografts ($n=6$). GC002 (10 mg/kg) and sorafenib (10 mg/kg) were intratumorally injected every 4 days for two weeks. (A) Tumor growth curves of mice receiving the GC002 or sorafenib treatment schedule. (B) Nude mice with xenograft tumors. (C) Tumor weights. (D) Body weights of the mice. (E) The level of serum BUN. (F) The level of serum creatinine. (G) The level of serum ALT. (H) Serum AST levels. (I) Phosphorylated MLKL, RIP1 and RIP3 in tumors were detected by western blot analysis. Actin was used as the loading control. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ns, not significant.

detrimental effects and resistance issues characteristically linked to traditional platinum-based cancer therapies, thus guiding the development of novel metal-based cancer treatments.

Necroptosis was originally believed to be a random occurrence; however, accumulating evidence suggests that it is actually a form of programmed cell death regulated by specific signals [48]. Two methods are commonly utilized to assess cell death by measuring changes in plasma membrane permeability. The first method measures the release of intracellular molecules, such as LDH [49]. The second method involves examining the uptake of DNA-binding dyes, such as PI, which do not penetrate the plasma membrane of viable cells. In this study, we evaluated HCC cell death via an LDH release assay and flow cytometry analysis with PI staining. We

found that GC002 triggered dose-dependent cell death. Moreover, cells treated with GC002 displayed signs of lysis, including cellular bloating and the emergence of sizable vesicles from the cell membrane. During necroptosis, RIP1 associates with RIP3 to form an amyloid complex that stimulates kinase activity [50,51]. Once RIP3 is activated, it attaches to and phosphorylates MLKL (at Ser-345 in mouse MLKL and at Thr-357/Ser-358 in human MLKL), permitting it to form oligomers via its brace region [51,52]. Upon phosphorylation and oligomerization, the N-terminal domain of MLKL directly binds to phosphatidylinositol phosphates (PIPs), permitting their relocation to cellular membranes and resulting in cell rupture and the release of cellular components [52,53]. Our analysis revealed that GC002 administration significantly increased

the levels of RIP1 and RIP3, along with phosphorylation at the Ser-358 residue of MLKL. These results indicate that the activation of the RIP1/RIP3/MLKL pathway leads to cell death. Furthermore, GC002-induced cell death was reduced by pretreatment with necroptosis inhibitors (Nec-1 and NSA). Notably, when *RIP1* was silenced, the cells were resilient to death, and LDH release decreased after GC002 treatment. Overall, these findings demonstrate that GC002 predominantly induces necroptosis in HCC cells by triggering the RIP1/RIP3/MLKL signaling cascade.

ROS are upstream regulators of necroptosis. Our results demonstrated that GC002 triggered irreversible necroptosis in HCC cells by increasing intracellular ROS accumulation, as evidenced by the significant attenuation of cell death and LDH release induced by GC002 when the cells were pretreated with antioxidants (NAC and GSH). Consistent with this finding, recent studies have indicated that ROS influence the regulation of RIP1 and RIP3 expressions and their interaction [54,55]. For example, ROS have been reported to enhance the interaction between RIP1 and RIP3 in glioma cells under stress from photodynamic therapy [56]. Interestingly, ROS also serve as executors of necroptosis [55,57]. ROS can damage intracellular macromolecules, including proteins, lipids, and nucleic acids [58]. Accumulating evidence suggests that RIP1 and RIP3 can modulate intracellular ROS levels through distinct mechanisms. RIP1 increases ROS production at the plasma membrane by targeting NOX1 and the small GTPase RAC1 [59], while RIP3 increases the levels of mitochondrial superoxide and intracellular ROS by interacting with and activating the mitochondrial protein GLUD1 [60]. In our study, the necroptosis inhibitors Nec-1 and NSA hindered the increase in ROS levels provoked by GC002 in HCC cells. These findings indicate that GC002 can increase ROS accumulation through the RIP1/RIP3/MLKL signaling axis, albeit to a minor extent, in addition to its direct inhibition of TrxR. Therefore, our findings suggest that GC002 may induce a positive feedback loop between ROS production and necroptosis signaling.

In summary, we evaluated a collection of innovatively engineered gold(I) phosphine complexes for their potential to combat HCC. Among these candidates, GC002 exhibited greater cytotoxicity and stronger antitumor effects than commonly used drugs, such as sorafenib and auranofin. Mechanistically, GC002 effectively disrupts the TrxR system and induces the accumulation of ROS, which leads to irreversible necroptosis via activation of the RIP1/RIP3/MLKL signaling axis in HCC cells. Furthermore, the activation of necroptosis can also reinforce the synergistic accumulation of ROS, thereby forming a positive feedback loop. Importantly, GC002 also showed outstanding antitumor activity *in vivo* without causing severe side effects. These findings identify GC002 as a promising lead compound for the development of novel antitumor agents targeting HCC and provide a valuable tool for investigating TrxR and ROS modulation in the necroptosis signaling pathway.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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