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Therapeutic Potential of Trilobatin in Cisplatin-Induced Kidney Injury: Targeting Ferroptosis and Inflammation

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ABSTRACT: Cisplatin-induced acute kidney injury (CI-AKI) is a common and severe complication in cancer patients undergoing chemotherapy, which can progress to chronic kidney disease (CKD) and end-stage renal disease (ESRD). Ferroptosis and inflammation are critical pathological mechanisms underlying CI-AKI. Trilobatin (TLB), a natural product, exhibits significant anticancer, antioxidant, and anti-inflammatory activities. In this study, we investigated the protective effect and molecular mechanism of TLB against CI-AKI through in vitro, in vivo, and network pharmacology experiments. Results from both in vitro and in vivo experiments demonstrated that TLB reversed Erastin-induced ferroptosis and inflammatory responses in human kidney-2 (HK-2) cells, a human renal cortical proximal tubule epithelial cell line. Moreover, TLB significantly ameliorated CI-AKI in model mice in a dose-dependent manner. Mechanistic studies revealed that TLB activated Nrf2 to upregulate the expression of GPX4, thereby alleviating ferroptosis in both HK-2 cells and renal cells of CI-AKI mice. Additionally, TLB effectively reduced the mRNA and protein levels of pro-inflammatory cytokines TNF- α and IL-6 in Erastin-induced HK-2 cells and renal tissues of CI-AKI mice, thus mitigating inflammatory responses. These findings provide a theoretical basis for the clinical application of TLB in the prevention and treatment of CI-AKI.

Keywords: Cisplatin-induced acute kidney injury, Trilobatin, Ferroptosis, Inflammatory

1. Introduction

Acute kidney injury (AKI) is a prevalent critical condition globally, characterized by an increasing incidence rate¹. The pathological features of AKI encompass a sudden decline in renal function, accompanied by the injury and death of renal tubular cells, particularly in the proximal tubules. These alterations can progress to chronic kidney disease (CKD) and end-stage renal disease (ESRD). The primary triggers of AKI include nephrotoxic drugs, sepsis, and ischemia-reperfusion (I/R) injury. Cisplatin, a commonly used antitumor agent in clinical settings, tends to accumulate in proximal tubular epithelial cells, leading to significant nephrotoxicity and establishing it as a major contributor to drug-induced AKI². Nevertheless, the

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precise molecular mechanisms underlying cisplatin-induced acute kidney injury (CI-AKI) remain incompletely understood, and current strategies for its prevention and treatment are still quite limited.

The mechanism underlying the development of CI-AKI is complex and involve multiple pathological processes, including oxidative stress³, inflammatory response⁴, cell necrosis, cell apoptosis⁵, and vascular injury⁶. Previous studies have predominantly concentrated on cisplatin (CDDP)-induced apoptosis and necrosis of renal tubular cells. However, experimental evidence indicates that even with the use of specific inhibitors of apoptosis and necrosis, the onset of CI-AKI cannot be completely prevented or inhibited⁷. This observation implies that there may be additional modes of cell death in CI-AKI that are distinct from apoptosis and necrosis⁸.

Ferroptosis is a recently identified form of regulated cell death, characterized by the lethal accumulation of lipid peroxides in an iron- and reactive oxygen species (ROS)-dependent manner⁹. Existing studies have confirmed that ferroptosis plays a key role in the pathogenesis of CI-AKI¹⁰, and may serve as an important therapeutic target for alleviating renal tissue damage in CI-AKI. For instance, in mouse models of AKI induced by ischemia/reperfusion (I/R) or cecal ligation and puncture (CLP), ferroptosis can directly induce renal tubular injury¹¹; it also plays a critical role in nephrotoxic folic acid-induced AKI¹².

Nuclear factor erythroid 2-related factor 2 (Nrf2) is crucial nuclear transcription factor that regulates antioxidant stress. It modulates the expression of various signaling proteins and enzymes that maintain cellular redox homeostasis and regulates key genes involved in iron storage and transport, establishing it as a critical regulatory pathway for ferroptosis¹³. Activation of Nrf2 can upregulate the expression of glutathione peroxidase 4 (GPX4), thereby enhancing the antioxidant capacity of cells¹⁴. GPX4 is one of the most important antioxidant enzymes in mammals, functioning by degrading lipid peroxides, and serves as a core regulator of ferroptosis¹⁵. Research has demonstrated that GPX4 deficiency can spontaneously induce renal tubular injury and AKI, while upregulation of GPX4 can mitigate renal tubular damage¹⁰. Nrf2 plays a pivotal role in CI-AKI, particularly in regulating ferroptosis^{16, 17}. The Nrf2 activator ferrostatin-1 (Fer-1) has been shown to significantly reduce CDDP-induced death of HK-2 cells and alleviate CI-AKI in mice¹⁸. In addition, CDDP can induce ferroptosis in renal tubular cells, leading to substantial production of ROS, which subsequently activates members of the mitogen-activated protein kinase (MAPK) family, including extracellular regulated protein kinases (ERK), p38, and c-Jun N-terminal kinase (JNK)^{19, 20}. The phosphorylation of ERK, p38, and JNK exacerbates renal injury and activates nuclear factor kappa-B (NF- κ B), the Nod-like Receptor Pyrin-containing Antifibrinolytic Protein 3 (NLRP3) inflammasome, and NF- κ B downstream genes such as interleukin 1 β (IL-1 β), thereby promoting the progression of CI-AKI. Therefore, inhibiting ferroptosis and improving inflammatory responses represent potential targets for the prevention of CI-AKI.

Trilobatin (TLB) is a glycosylated dihydrochalcone extracted from the leaves of *Lithocarpus polystachyus* Rehd. (also known as Chinese sweet tea)^{21, 22}. As a potent natural sweetener, TLB not only exhibits low cytotoxicity and good solubility but also demonstrates various biological properties, including hypoglycemic²³, anti-inflammatory, anticancer, and antioxidant effects^{24, 25}. Consequently, it has emerged as a

significant research focus in the pharmaceutical field. Studies have indicated that in a mouse model of type II diabetes induced by a long-term high-fat and high-sugar diet combined with streptozotocin, TLB treatment promotes hepatic glycogen synthesis, reduces oxidative stress, and enhances glucokinase expression²⁶. In addition, TLB exhibits notable anticancer activity, as it can inhibit the proliferation of lung, liver, ileocecal, and colon cancer cells²⁷. Another study indicates that TLB can alleviate aging and ischemic stroke in mice by activating the Nrf2 pathway²⁸. Meanwhile, the role of TLB in AKI has also been gradually elucidated: it alleviates CDDP-induced nephrotoxicity by inhibiting the nuclear transcription factor NF- κ B and improving mitochondrial function²⁹. Moreover, TLB can attenuate LPS-mediated inflammatory responses by inhibiting the NF- κ B signaling pathway²⁴.

Despite existing research, the protective effect of TLB against CI-AKI through the inhibition of ferroptosis and the improvement of inflammatory responses, as well as its underlying mechanisms, has not been thoroughly investigated. This study aims to investigate whether TLB alleviates CI-AKI by inhibiting ferroptosis and inflammation, and to further clarify the impact of TLB on cisplatin-induced ferroptosis and inflammation, as well as its mechanisms of action, thereby. This research seeks to provide new insights into the prevention and treatment of CI-AKI.

2. Materials and methods

2.1 Reagents and Materials

TLB with a purity of $\geq 98\%$ was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). DMSO was obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). CDDP was provided by Shanghai Bide Pharmaceutical Technology Co., Ltd. (Shanghai, China). Erastin and ML385 (purity 99.96%) were purchased from MCE. Antibodies against TNF- α and IL-6 were obtained from Abcam (Cambridge, USA); while Nrf2, GPX4, Cy3 fluorescent goat anti-rabbit IgG, β -actin, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were purchased from Proteintech (Chicago, USA). In addition, detection kits for serum creatinine (SCr) and blood urea nitrogen (BUN) were obtained from Milbio (Shanghai, China); GSH assay kit and BCA protein quantification kit were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Reverse transcription kit and real-time fluorescent quantitative PCR kit were provided by GenStar (Beijing, China). All other reagents were purchased from Solarbio unless specified otherwise.

2.2 Animals

All animal experiments were conducted at Qingdao Haihua Biological Group Co., Ltd. The C57BL/6J male mice used in the experiment were 6-8 weeks old and weighed approximately 20-22 g, all provided by Qingdao Haihua Biological Group Co., Ltd. All procedures involving in the animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (Approval No.: AUP-QY-C-S-2024-021), ensuring that the experimental process adhered to ethical standards and animal welfare

requirements. Mice were housed in an environment with a 12-hour light/dark cycle and a temperature maintained at 20–24 °C, with free access to adequate food and water.

2.3 Establishment of AKI Model

After 1 week of adaptive feeding, mice were randomly divided into 5 groups ($n = 6$). Based on published literature, the doses of TLB used in the experiment were 10 and 40 mg/kg, while the dose of cisplatin was 20 mg/kg^{30–32}. The experimental groups were categorized as follows: (1) Normal group; (2) CDDP group: intraperitoneal injection of 0.9% normal saline for 6 consecutive days, followed by a single intraperitoneal injection of cisplatin (20 mg/kg) at the end of the 6th day³³; (3) CDDP+TLB (10 mg/kg) group: intraperitoneal injection of TLB for 6 consecutive days, with a single intraperitoneal injection of CDDP at the end of the 6th day; (4) CDDP+TLB (40 mg/kg) group: intraperitoneal injection of TLB for 6 consecutive days, with a single intraperitoneal injection of cisplatin at the end of the 6th day. On the 7th day, blood was collected from the orbital sinus of mice, and perfusion was performed. Subsequently, serum was prepared, and SCr and BUN levels were measured to evaluate renal function. Meanwhile, kidney samples were promptly collected for further analysis. A significant increase in serum creatinine (Scr) and BUN (BUN) levels, along with severe degeneration and necrosis of renal tubular epithelial cells, luminal dilation, and formation of intraluminal casts in mice, indicated the successful establishment of the model^{34, 35}.

2.4 Analysis of Scr and BUN

Serum samples were centrifuged at 12,000 r/min for 15 minutes at 4 °C. Subsequently, the supernatant was transferred to a new EP tube. The procedures were performed according to the instructions of the SCr and BUN detection kits. After sample loading was completed, a microplate reader was used for detection and analysis.

2.5 Cell Culture and Grouping for Cell Experiments

HK-2 cells were cultured in MEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), 50 µg/mL penicillin (Gibco), and 50 µg/mL streptomycin (Gibco). The cultures were maintained in an incubator at 37 °C with 5% carbon dioxide. Cell experiments were divided into the following groups: control group, Erastin (4.6 µmol/L) group, Erastin (4.6 µmol/L) + DMSO (3 µmol/L) group, Erastin (4.6 µmol/L) + TLB (1 µmol/L) group, Erastin (4.6 µmol/L) + TLB (3 µmol/L) group, Erastin (4.6 µmol/L) + ML385 (2.73 µmol/L) group, and Erastin (4.6 µmol/L) + ML385 (2.73 µmol/L) + TLB (3 µmol/L) group. After 24 hours of culture, cells were collected for the detection of cell viability, ferroptosis, oxidative stress, and inflammatory responses, including Western blot analysis, real-time quantitative polymerase chain reaction, and other experimental methods.

2.6 Cell Viability Assay

Cell viability was measured using the SolarBio Cell Counting Kit-8 (CCK-8). First, HK-2 cells were seeded in 96-well plates at a density of 3×10^3 cells per well, with 100 μL of culture medium added to each well, and cultured overnight. Subsequently, cells were treated with different concentrations of Erastin, TLB, or ML385 either alone or in combination for 24 hours. After treatment, the original medium was replaced with 100 μL of fresh medium containing 10 μL of CCK-8 solution, and the cells were incubated at 37 °C for 1 h. Finally, a microplate reader was used to measure the absorbance at 450 nm, and the relative cell survival rate was expressed as a percentage of the control group.

2.7 Cell White Light Observation

HK-2 cells were seeded in 24-well plates at a density of 5×10^4 cells per well and cultured overnight in normal medium to allow adherence. After co-culturing the cells with drugs in the medium for 24 hours, the original medium was aspirated and discarded, and 500 μL of PBS was added to each well. Then, observations were made under a fluorescence microscope (Nikon, Japan) at a magnification of $\times 100$.

2.8 Cytotoxicity Assay

The toxic effect of TLB on HK-2 cells was detected using the SolarBio CCK-8. Cells were seeded in 96-well plates at a density of 3×10^3 cells per well, cultured overnight in 100 μL of culture medium per well, and then co-incubated with TLB at a series of concentrations for 12 h. After that, the original medium was replaced with 100 μL of fresh medium containing 10 μL of CCK-8 solution, and the cells were incubated at 37 °C for 1 h. Finally, the absorbance at 450 nm was recorded using a microplate reader, and the relative cell survival rate was expressed as a percentage of the control group.

2.9 Determination of Malondialdehyde, Glutathione, and Iron Levels

Lipid peroxidation in renal tissues and HK-2 cells was measured using malondialdehyde, glutathione, and iron ion detection kits, respectively, according to the manufacturers' instructions.

2.10 ROS Measurement

The production of ROS was observed under dark conditions using the 2',7'-dichlorofluorescein diacetate (DCFH-DA) cellular ROS kit (Nanjing Jiancheng Bioengineering Institute). According to the manufacturer's instructions, HK-2 cells were seeded in 24-well plates at a density of 1×10^4 cells per well and cultured overnight in normal medium to allow adherence. After treatment with drugs in different groups, the cells were stained with DCFH-DA for 45 min, and then ROS levels were detected using a fluorescence microscope (Nikon, Japan) at a magnification of $\times 100$.

2.11 Western Blot Analysis

Equal amounts of disrupted renal tissue or HK-2 cells were lysed in pre-cooled cell lysis buffer (RIPA lysis buffer and PMSF) at 4 °C for 40 minutes. Subsequently, the mixture was centrifuged at 12,000 r/min at

4 °C for 10 min, and the supernatant was collected. The protein concentration was determined using the BCA Protein Assay Kit (Beyotime Biotech). Samples were mixed with 4× loading buffer and heated at 100 °C for 10 min to obtain fully denatured proteins. Equal amounts of protein (15 µg) were separated by 10% SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked with 10% non-fat milk for 1 hour and incubated with primary antibodies overnight at 4 °C. Thereafter, the membranes were washed 3 times with TBST, incubated with rabbit HRP-conjugated secondary antibodies at room temperature for 2 h, and finally washed 3 times with TBST. Visualization was performed using ECL chemiluminescent substrate (BioSharp). Band intensities were quantified using Image J software and normalized to the band intensity of β -actin.

2.12 RNA Extraction and Real-Time Fluorescent Quantitative Polymerase Chain Reaction (qRT-PCR)

1 mL of pre-cooled Trizol reagent was added to equal amounts of renal tissue or cells, followed by cell disruption and tissue homogenization using a cell disrupter or tissue grinder, respectively. Subsequently, centrifugation was performed at 12,000 r/min for 5 min at 4 °C. The supernatant was transferred to a new EP tube, incubated on ice for 5 min, and then 200 µL of chloroform was added. The supernatant was collected, centrifuged at 12,000 r/min for 30 min, mixed with an equal volume of isopropanol, and incubated at -20 °C for 40 min. After another centrifugation at 12,000 r/min for 30 min, the supernatant was discarded. 1 mL of 75% ethanol was added, and centrifugation was conducted at 7,500 r/min for 20 min. The supernatant was discarded, and the precipitate was air-dried.

Table 1 The primer sequences involved in the experiment.

		Forward	Reverse
Human	Nrf2	ACTCCCTGCAGCAAACAAGAG	TTTTTCTTAACATCTGGCTTCTTACTTTT
	GPX4	GGCTTCGTGTGCATCGTCACC	TTCACCACGCAGCCGTTCTTG
	β -actin	TGAAGATCAAGATCATTGCTCCTCCT	GACTCGTCATACTCCTGCTTGCTG
	Nrf2	GCATAGAGCAGGACATGGAGCAAG	ACTGATGGCAGCGGAGGAAGG
	GPX4	CATGCCCCGATATGCTGAGTGTGG	TAGCACGGCAGGTCCTTCTCTATC
Mouse	β -actin	AACAGTCCGCCTAGAAGCAC	CGTTGACATCCGTAAAGACC
	IL-1 β	GCAACTGTTCTTGAAGTCAACT	ATCTTTTGGGGTCCGTCAACT
	IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTTCATACA
	TNF- α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC

The mRNA levels of Nrf2, GPX4, IL-1 β , TNF- α , and IL-6 were detected using Takara (Kyoto, Japan) reverse transcription polymerase chain reaction. Real-time quantitative polymerase chain reaction was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad, California, USA). The $2^{-\Delta\Delta Ct}$ method was used to normalize the relative gene expression, with β -actin serving as the internal reference gene. The primer sequences are shown in the table below.

2.13 Immunofluorescence Staining

HK-2 cells were seeded onto cell climbing slices in 24-well plates at a density of 1×10^4 cells per well and cultured overnight in normal medium to allow adherence. Subsequently, the cells were fixed with 4% paraformaldehyde at 37 °C for 10 min and blocked with 10% bovine serum albumin (BSA). Then, the cells were incubated with rabbit anti-Nrf2 and rabbit anti-GPX4 antibodies overnight at 4 °C. After that, incubation

with fluorescent secondary antibodies was performed. Finally, the slices were mounted with anti-fluorescence quencher containing DAPI and observed under a fluorescence microscope (Nikon, Japan, magnification $\times 100$).

2.14 Histology

Renal samples were cut coronally, fixed with 4% paraformaldehyde for 48 h, then subjected to gradient dehydration and paraffin embedding to prepare 4- $\mu\text{mol/L}$ -thick sections. The sections were deparaffinized with xylene and hydrated in gradient concentrations of alcohol. Histological observation was performed using hematoxylin-eosin (HE) staining according to the instructions of the hematoxylin-eosin staining kit. After staining, the sections were dehydrated in gradient alcohol, cleared with xylene, and finally mounted with neutral mounting medium. Stained images were observed under a Nikon fluorescence microscope (Japan) at a magnification of 100 $\times$.

2.15 Network Pharmacology Prediction

First, the chemical structure of TLB was searched using the PubChem platform (<https://pubchem.ncbi.nlm.nih.gov>) and saved as an SDF format file. Subsequently, this file was imported into the PharmMapper Server platform (<https://lilab-ecust.cn/pharmmapper/index.html>) for target prediction, with human protein targets set as the pharmacophore map and the number of returned targets limited to 300. Next, the UniProt database (<https://www.uniprot.org/>) was used to search and select the species "Homo sapiens," and the results were converted into TLB targets and potential target genes. The GeneCards database (<https://www.genecards.org/>) was utilized to obtain targets related to AKI and ferroptosis, and the names of these targets were standardized using the UniProt database (UniProt Consortium, 2018). Then, the Venny online tool (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>) was used to analyze ferroptosis-related genes and common targets between TLB and AKI. Finally, the common target genes were input into the STRING database to construct a PPI network, and Cytoscape (version 3.9.1) was used to analyze these data to build a visual compound-target network.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses are important bioinformatics tools. GO is used for gene annotation and to analyze the roles of these genes in biological processes (BP), cellular components (CC), and molecular functions (MF). KEGG is a database for systematic analysis of gene functions, which links genomic information with higher-order functional information and assigns functional significance to genes and genomes at the molecular and higher levels. The Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8; <http://david.ncifcrf.gov>) was used for functional annotation and pathway enrichment analysis of shared genes. A P -value < 0.05 indicated a statistically significant difference.

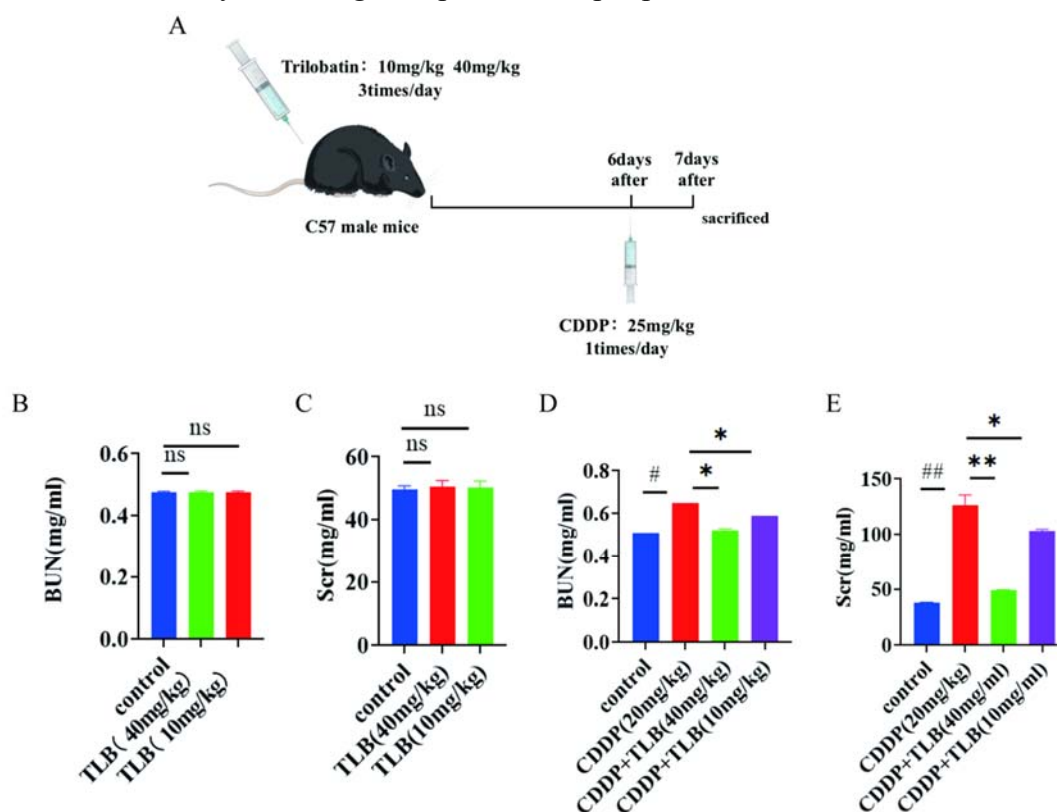
2.16 Statistical Analysis

All data were expressed as mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) was used for analysis, and GraphPad Prism software was applied to perform T-tests on the experimental data and visualize the results. A P -value < 0.05 was considered statistically significant.

3.Result

3.1 Protective Effect of TLB on CI-AKI in Mice

To investigate the protective effect of TLB against CI-AKI, we established a mouse model of CI-AKI. The experimental design workflow is detailed in Figure 1A. Briefly, mice were intraperitoneally injected with TLB (10 or 40 mg/kg) for 6 consecutive days, followed by a single intraperitoneal injection of cisplatin at the end of the 6th day to induce CI-AKI. Mice were euthanized at the end of the 7th day, and serum and kidney samples were collected. Serum creatinine (Scr) and blood urea nitrogen (BUN) levels were used as indicators to evaluate CI-AKI. As shown in Figure 1B, TLB had no significant effect on BUN and Scr levels in normal mice; however, following CDDP administration, the levels of BUN and Scr in the model group increased significantly ($P < 0.01$). Notably, TLB treatment reversed this change in a dose-dependent manner (Figure 1C). HE staining of renal tissues revealed that TLB significantly alleviated pathological damages in CI-AKI mice, including renal tubular necrosis, cast formation, renal tubular degeneration, and vacuolation (Figure 1D). In addition, compared with the cisplatin group, TLB treatment significantly improved the alterations in GSH, Fe^{2+} , and MDA levels in mouse renal tissues, as well as serum MDA content (Figure 1E-H). In summary, TLB can alleviate CI-AKI by inhibiting ferroptosis and lipid peroxidation.



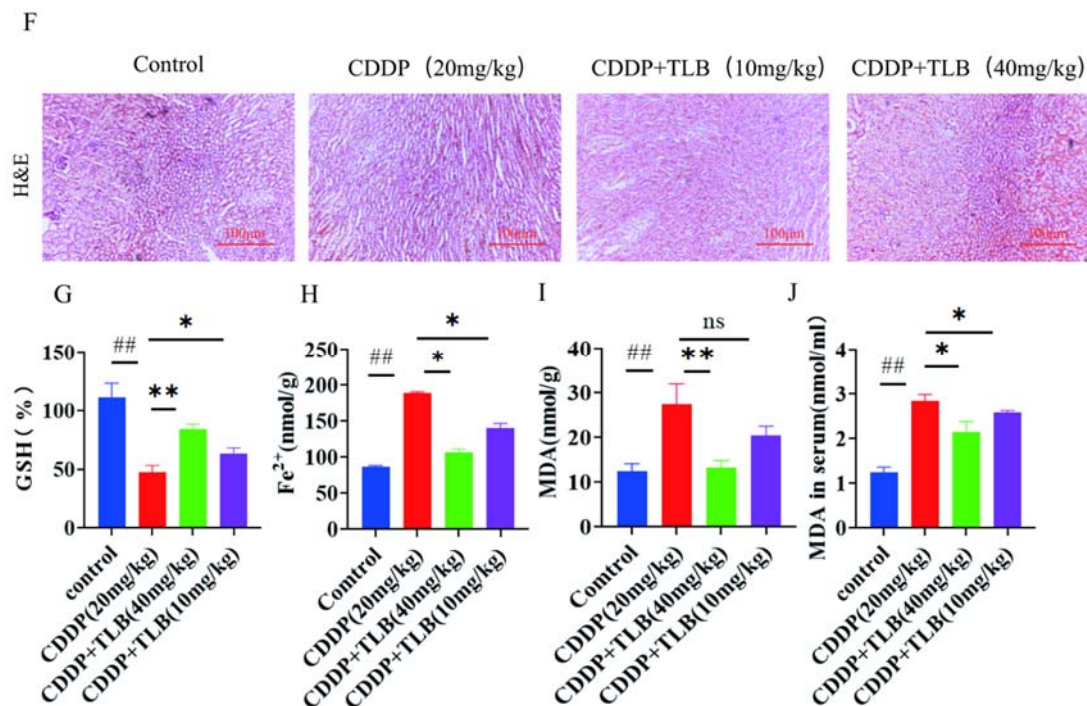


Fig.1 TLB demonstrates a protective effect against CI-AKI. (A) Experimental process of pre-protective administration in mice and CI-AKI. (B-E) Serum BUN and SCr levels in mice (n = 3). (F) Images of HE-stained mouse kidney sections (n = 3), with a scale bar of 100 μmol/L. (G-J) Contents of GSH, Fe²⁺, and MDA in renal tissues and Serum (n = 3). Compared with the control group, [#]*P* < 0.05, ^{##}*P* < 0.01; compared with the CDDP group, **P* < 0.05, ***P* < 0.01; ns, not significant.

3.2 Study on TLB Inhibiting Ferroptosis and Lipid Peroxidation in Renal Tubular Cells

Figure 2 illustrates the inhibitory effect of TLB on Erastin-induced ferroptosis and lipid peroxidation in HK-2 cells *in vitro*. First, we established an Erastin-induced ferroptosis model in HK-2 cells (IC₅₀ = 4.6 μmol/L, Figure 2A). As shown in Figure 2B, TLB effectively inhibited Erastin-induced ferroptosis in HK-2 cells with an EC₅₀ value of 352.1 nmol/L. Cell white light imaging results revealed that, compared to the control group, both the Erastin-treated group and the solvent group exhibited characteristic changes such as cell shrinkage, abnormal morphology, and a reduced cell count. In contrast, TLB treatment significantly improved cell morphology and increased cell count (Figure 2C). Given that ferroptosis is associated with a substantial production of ROS, we evaluated intracellular ROS levels using the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) probe. The results indicated that TLB treatment significantly reduced ROS accumulation in HK-2 cells stimulated by Erastin (Figure 2D-E).

Furthermore, to further investigate the relationship between TLB-inhibited ferroptosis and lipid peroxidation, we measured intracellular levels of GSH, Fe²⁺, and MDA. The results demonstrated that Erastin treatment significantly decreased GSH levels while increasing MDA and Fe²⁺ levels; conversely, TLB treatment reversed these trends (Figure 2F-H). Notably, TLB did not significantly improve cell viability in the H₂O₂-induced apoptosis model and nigericin-induced pyroptosis model of HK-2 cells (Figure 2I). In summary, TLB selectively inhibit ferroptosis in HK-2 cells and alleviate lipid peroxidation damage.

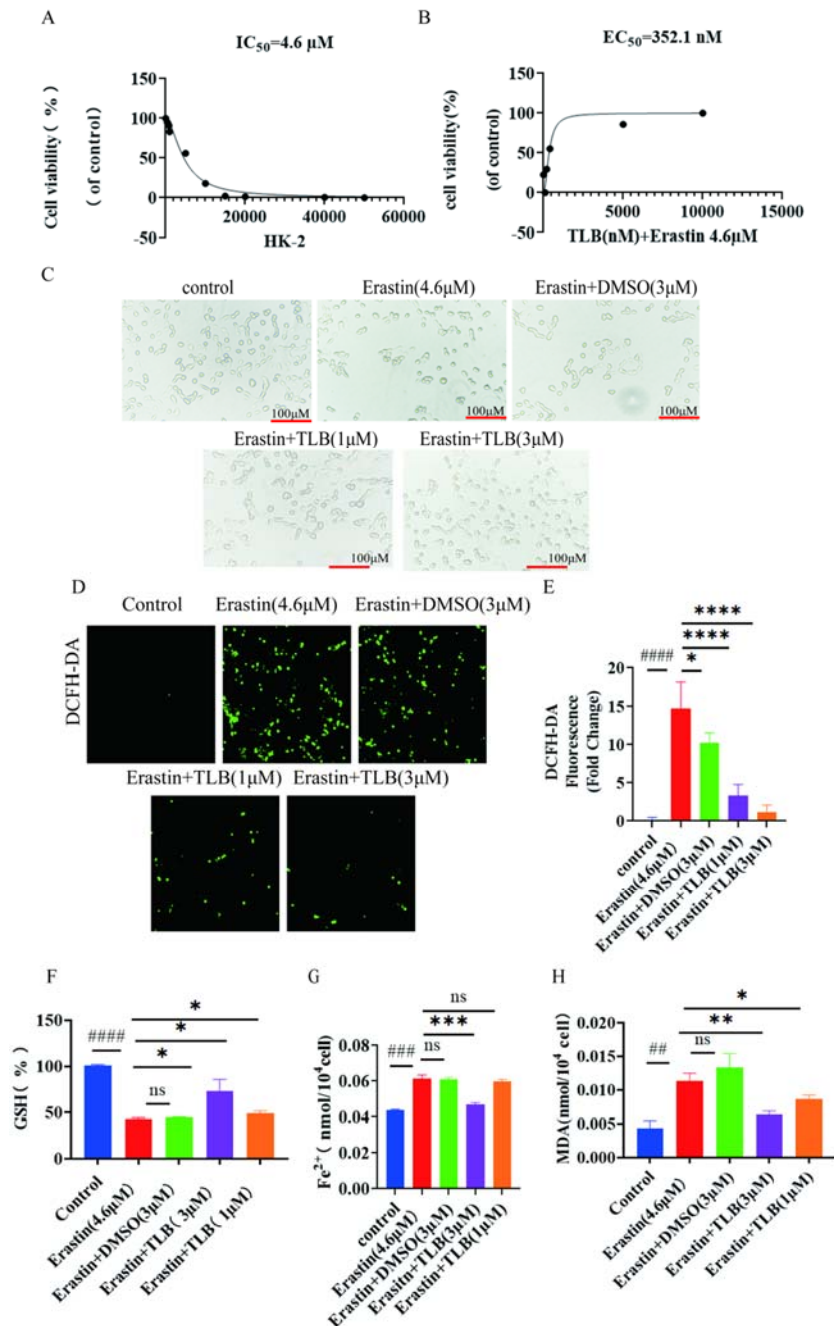


Fig. 2 TLB inhibits ferroptosis and lipid peroxidation in renal tubular epithelial cells. (A) Cell viability measured by CCK-8 assay after 24-hour Erastin treatment ($n = 5$). (B) Cell viability detected by CCK-8 assay: cells were pre-treated with TLB for 1 hour followed by co-treatment with Erastin for 24 hours ($n = 5$). (C) White light images of HK-2 cells ($n = 3$). (D-E) Images and quantitative data of DCFH-DA staining in HK-2 cells ($n = 3$). (F-H) Intracellular contents of GSH, Fe^{2+} , and MDA ($n=3$). Compared with the control group: $###P < 0.01$, $####P < 0.001$, $#####P < 0.0001$; compared with the Erastin group: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$; ns, not significant.

3.3 Network Pharmacology Target Prediction: *Nrf2* as a Key Target for TLB in Treating CI-AKI

To further explore the relationship between TLB, ferroptosis, and AKI, we identified 26 intersecting genes through screening (Figure 3A). The STRING database was used to analyze the interactions of these common targets, constructing a protein-protein interaction (PPI) network (Figure 3B). Core target genes were screened using the degree algorithm in Cytoscape software, and the results revealing that genes such as *Nrf2* (*NFE2L2*) ranked high in degree value, suggesting that these genes may be key hub genes of TLB, exerting anti-AKI effects by regulating ferroptosis.

The main biological processes (BP) predicted by GO analysis include: cellular response to H₂O₂, positive regulation of apoptotic processes, negative regulation of ferroptosis, and response to oxidative stress. Cellular components (CC) were mainly enriched in the endoplasmic reticulum, mitochondria, nuclear membrane, and cytoplasm. Enrichment results for molecular functions (MF) showed involvement in damaged DNA binding, glutathione peroxidase activity, DNA binding, and transcription factor binding (Figure 3C).

KEGG pathway enrichment analysis indicated that the potential mechanisms of TLB in preventing and treating AKI may be related to the following pathways: lipid and oxidative stress, glutathione metabolism, metabolic pathways, endoplasmic reticulum protein processing, FoxO signaling pathway, NF- κ B signaling pathway, and NOD-like receptor signaling pathway. These findings imply that TLB may alleviate acute kidney injury by regulating oxidative stress, inflammatory responses, and metabolic pathways (Figure 3D).

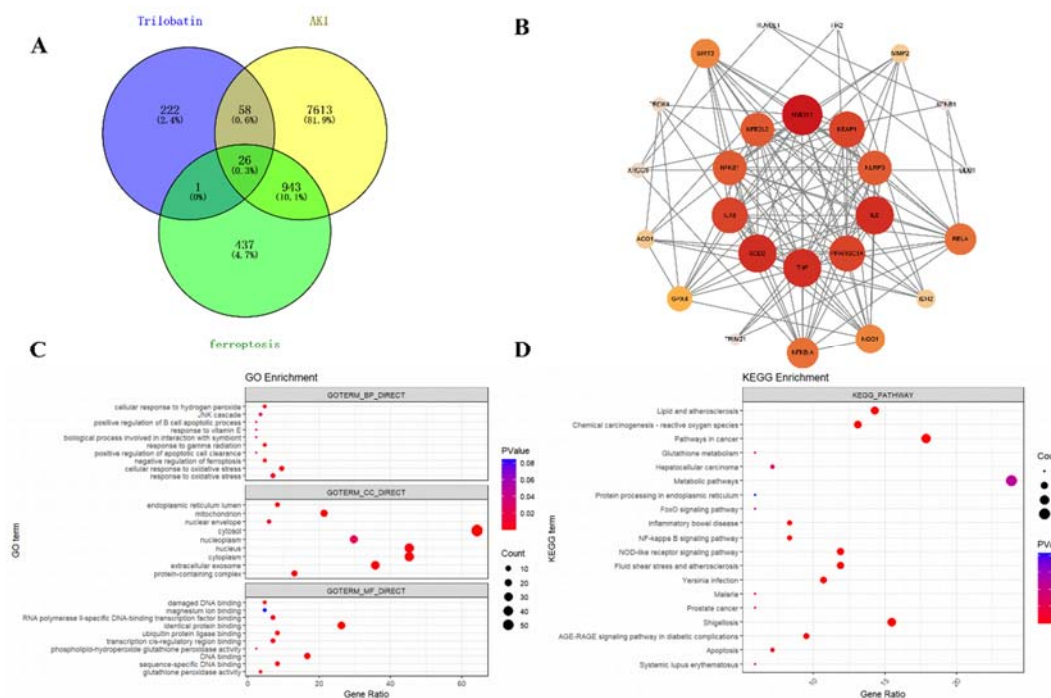


Fig.3 Network pharmacology analysis and molecular docking results of acute kidney injury. (A) Venny diagram showing the number of overlapping genes among AKI, TLB, and ferroptosis. (B) Protein-protein interaction network among AKI, ferroptosis, and TLB. (C-D) GO and KEGG enrichment analyses of intersecting genes among AKI, TLB, and ferroptosis using DAVID bioinformatics resources.

3.4 TLB Inhibits CI-AKI and Ferroptosis in HK-2 Cells by Regulating the Nrf2/GPX4 Pathway

To further evaluate whether Nrf2 is a pharmacological target of TLB, we conducted in vivo experiments in a mouse model of CI-AKI. Based on the previously mentioned pharmacodynamic results, we administered 25 mg/kg of TLB for in vivo experimental validation. Western blot analysis showed that, compared to the control group, CDDP treatment significantly decreased the protein levels of Nrf2 and GPX4 in mouse renal tissues, whereas TLB treatment effectively restored these levels (Figure 4A-C). In line with the observed changes in protein levels, the mRNA levels of Nrf2 and GPX4 in the renal tissues of mice treated with CDDP also decreased; however, TLB treatment significantly increased the transcriptional levels of these two genes

(Figure 4D-E). These findings suggest that the mechanism by which TLB alleviates CI-AKI is closely associated with its activation of the Nrf2/GPX4 signaling pathway.

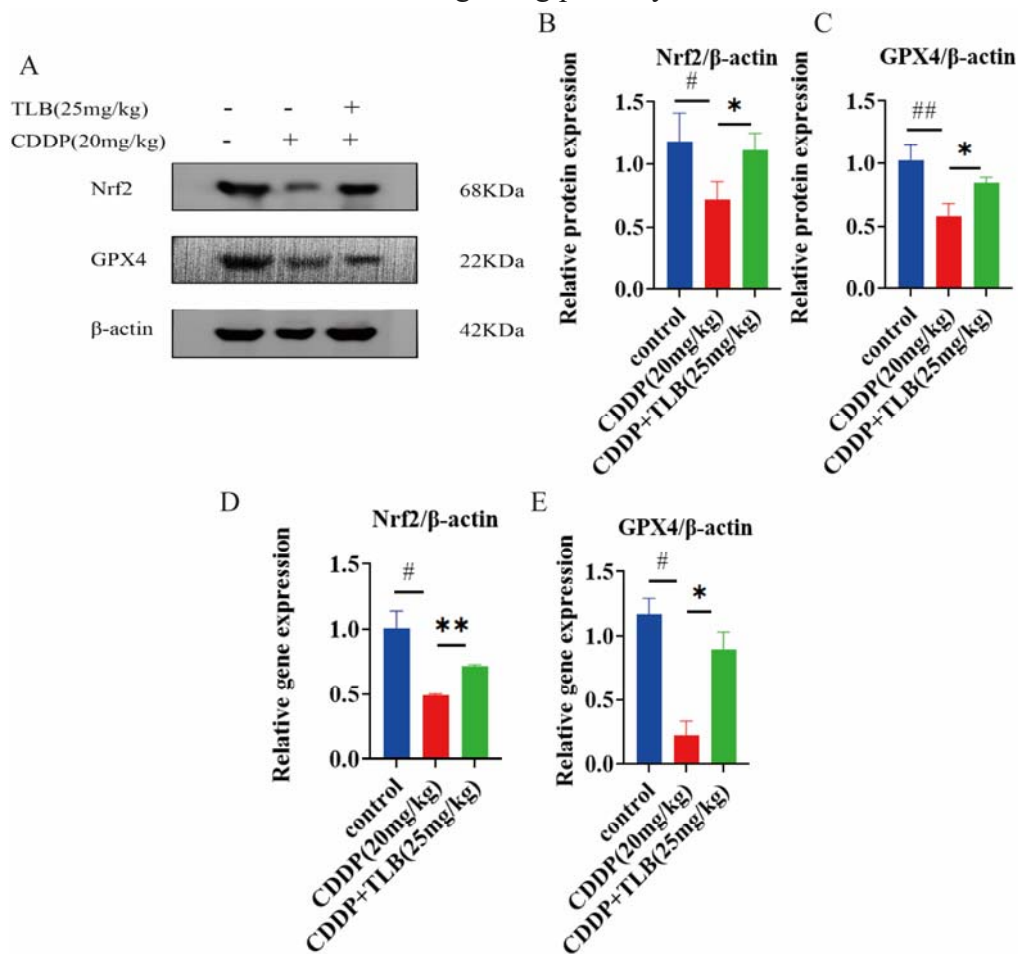


Fig. 4 TLB inhibits CI-AKI by regulating the Nrf2/GPX4 pathway. (A-C) Western blot images of Nrf2 and GPX4 proteins in tissues, and analysis graphs of Nrf2 and GPX4 protein expression levels ($n = 3$). (D-E) Graphs of Nrf2 and GPX4 gene expression levels in tissues ($n = 3$). Compared with the control group, $^{\#}P < 0.05$, $^{##}P < 0.01$; compared with the CDDP group, $^*P < 0.05$, $^{**}P < 0.01$; ns, not significant.

The in vitro experimental results demonstrate that TLB (3 $\mu\text{mol/L}$) inhibits ferroptosis in HK-2 cells by regulating the Nrf2/GPX4 pathway are shown in Figure 5. Western blot analysis revealed that Erastin treatment significantly downregulated the protein levels of Nrf2 and GPX4, while TLB treatment effectively restored them (Figure 5A-C). Correspondingly, Erastin treatment resulted in a decrease in the mRNA levels of Nrf2 and GPX4 in HK-2 cells, whereas TLB treatment significantly enhanced the transcriptional levels of both Nrf2 and GPX4 (Figure 5D-E). These results indicate that the mechanism by which TLB alleviates Erastin-induced ferroptosis in HK-2 cells is associated with the Nrf2/GPX4 pathway.

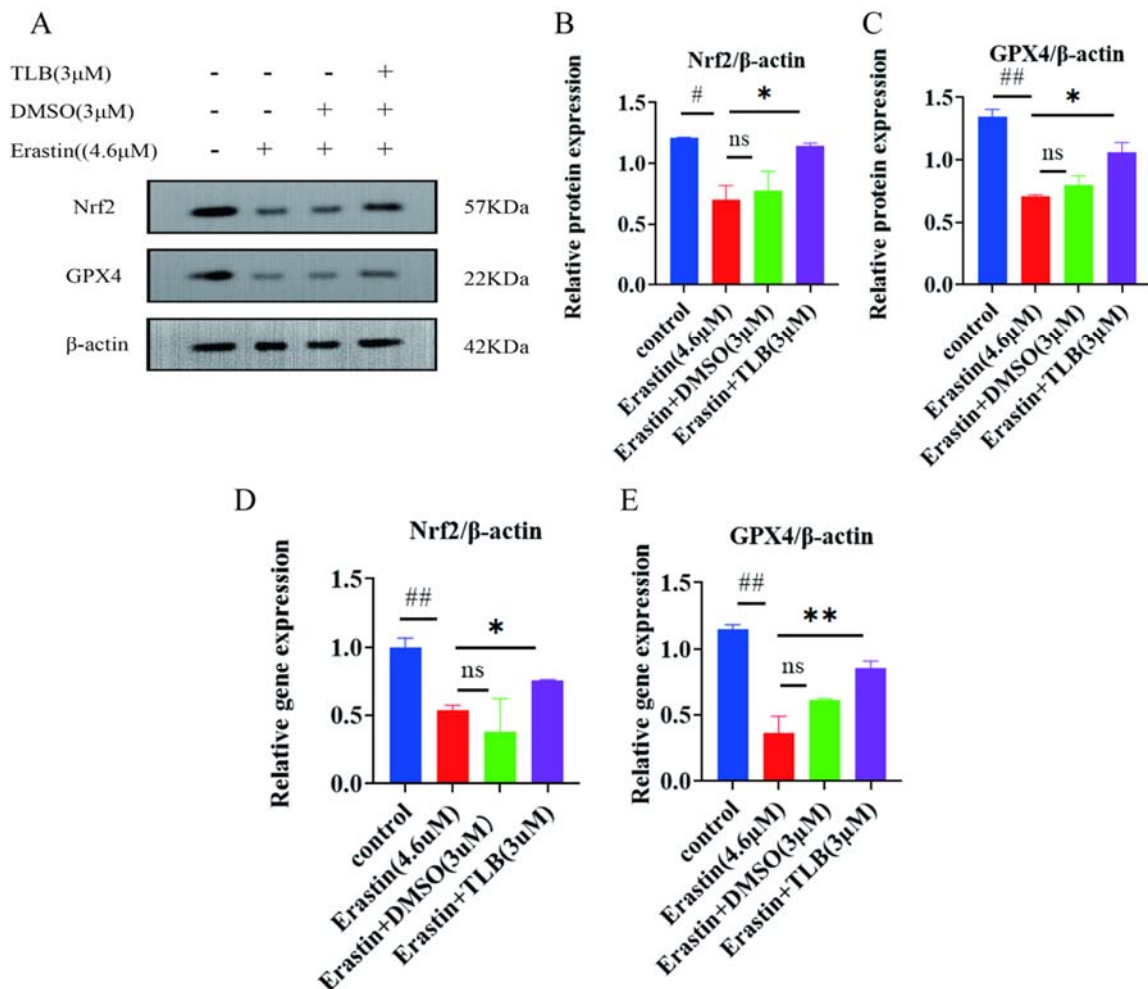


Fig.5 TLB inhibits ferroptosis in HK-2 cells by regulating the Nrf2/GPX4 pathway. (A-C) Western blot images of Nrf2 and GPX4 proteins in HK-2 cells, and analysis graphs of Nrf2 and GPX4 protein expression levels (n = 3). (D-E) Graphs of Nrf2 and GPX4 gene expression levels in cells (n = 3). Compared with the control group, [#]*P* < 0.05, ^{##}*P* < 0.01; compared with the Erastin group, ^{*}*P* < 0.05, ^{**}*P* < 0.01; ns, not significant.

3.5 Inhibition of Nrf2 Abolishes the Effects of TLB on ROS Production, Lipid Peroxidation, and Ferroptosis

To further elucidate the critical role of Nrf2 in TLB-mediated inhibition of Erastin-induced ferroptosis in HK-2 cells, we conducted verification using the specific Nrf2 inhibitor ML385, with results presented in Figures 6 and Figure 7. In alignment with the findings shown in Figure 2B, TLB pretreatment significantly increased the survival rate of HK-2 cells subjected to Erastin. However, the co-treatment with the Nrf2 inhibitor ML385 markedly diminished or completely negated the protective effect of TLB against Erastin-induced ferroptosis in HK-2 cells (Figure 6A-B). Furthermore, in the presence of ML385, the TLB-mediated increase in intracellular GSH levels was counteracted, and its inhibitory effect on the elevation of Fe²⁺ levels was also weakened (Figure 6C-D). Similarly, the effects of TLB in reducing intracellular MDA content and ROS levels was reversed by ML385 (Figure 6E-G).

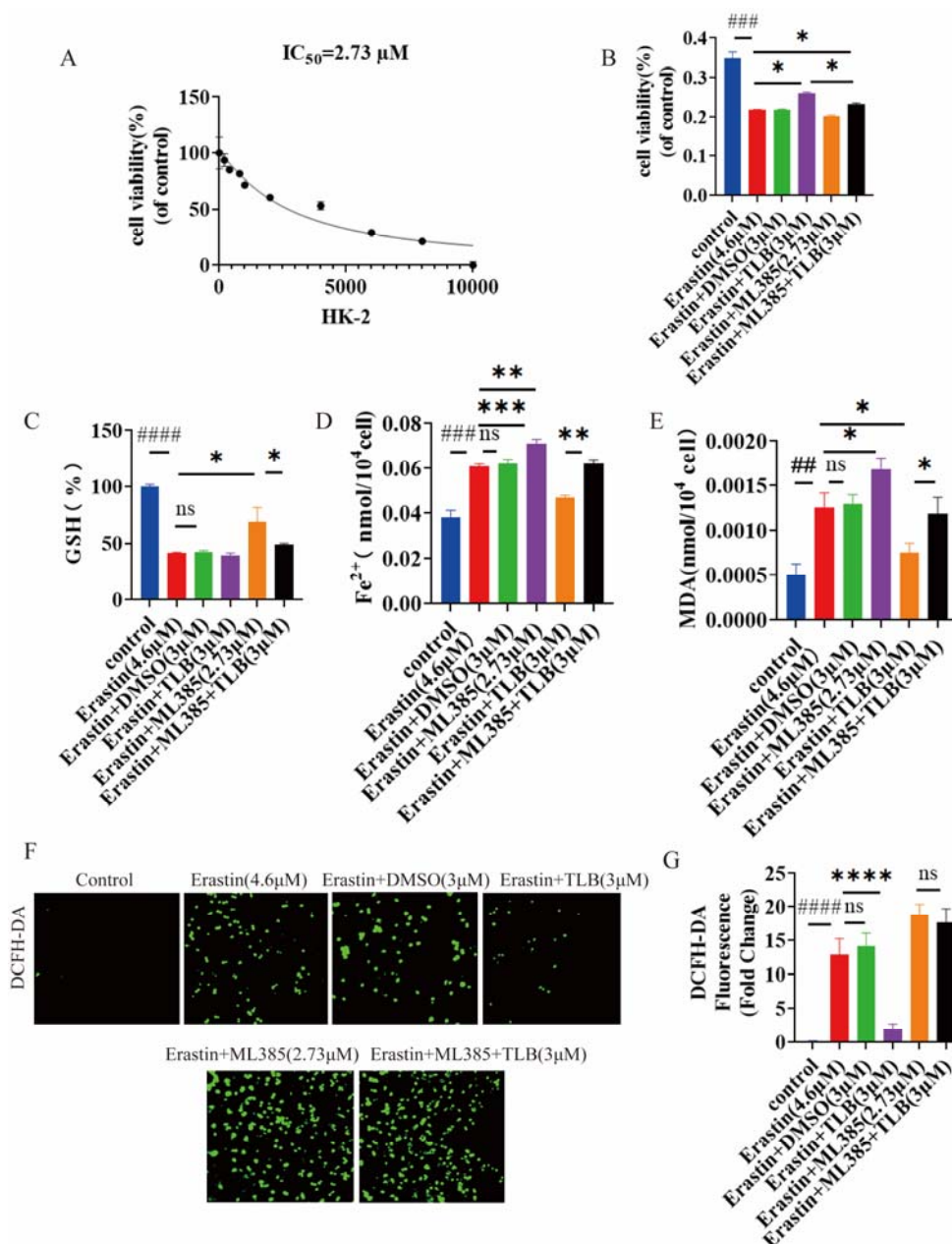


Fig. 6. Inhibition of Nrf2 abolishes the effects of TLB on ROS production, lipid peroxidation, and ferroptosis in HK-2 cells. (A-B) HK-2 cell viability detected by CCK-8 assay ($n = 3$). (C-E) Intracellular contents of GSH, Fe^{2+} , and MDA ($n = 3$). (F-G) DCFH-DA staining images and quantitative analysis graphs ($n = 3$). Compared with the control group: $^{##}P < 0.01$, $^{###}P < 0.001$, $^{####}P < 0.0001$; compared with the Erastin group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; ns, not significant.

Western blot (WB) analysis and qRT-PCR results demonstrated that, compared to Erastin treatment alone, TLB treatment significantly upregulated both the protein (Figure 7A-C) and mRNA expression levels of Nrf2 and GPX4 in HK-2 cells (Figure 7D-E). Notably, co-treatment with ML385 reversed the TLB-mediated increases in the protein and gene expression levels of Nrf2 and GPX4 (Figure 7A-E). In addition, immunofluorescence results further corroborated the fluorescence intensity of Nrf2 and GPX4 in the Erastin-treated group was significantly reduced, while TLB treatment markedly enhanced the fluorescence signals of these two proteins. However, in the ML385 co-treatment group, the TLB-induced enhancement of Nrf2 and GPX4 fluorescence intensity was significantly inhibited (Figure 7D-G). In summary, these results strongly

demonstrate that Nrf2 is an essential core factor mediating TLB resistance to Erastin-induced ferroptosis in HK-2 cells.

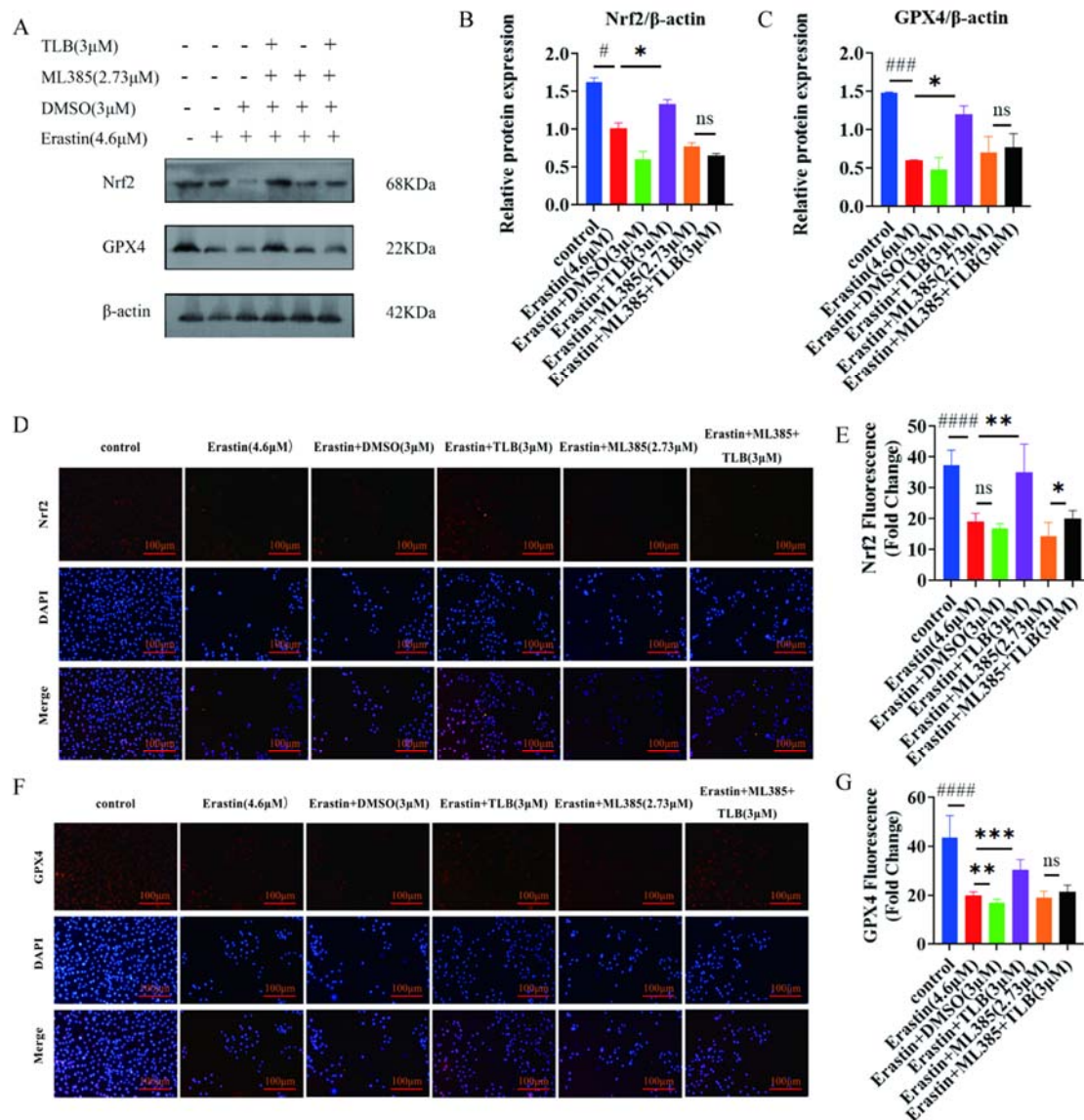


Fig. 7 Inhibition of Nrf2 abolishes the effects of TLB on ROS production, lipid peroxidation, and ferroptosis in HK-2 cells.

(A-C) Western blot images of Nrf2 and GPX4 proteins in HK-2 cells, and analysis graphs of Nrf2 and GPX4 protein expression levels (n = 3). (D-E) Immunofluorescence images and expression level analysis graphs of Nrf2 in HK-2 cells (n = 3). (F-G) Immunofluorescence images and expression level analysis graphs of GPX4 in HK-2 cells (n = 3). Compared with the control group: # $P < 0.05$, ## $P < 0.01$, ### $P < 0.0001$; compared with the Erastin group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; ns, not significant.

3.6 TLB Exerts Anti-Inflammatory Effects In Vivo and In Vitro

Inflammation plays a crucial role in the progression of CI-AKI [36,37]. We evaluated the effect of TLB on the anti-inflammatory response in CI-AKI mice and ferroptosis HK-2 cells, with the experimental results presented in Figure 8. In in vivo experiments, Western blot analyses indicated that, compared to the control group, the levels of TNF- α and IL-6 in the renal tissues of the CDDP group were significantly increased. TLB treatment markedly reduced the expression of these two pro-inflammatory factors (Figure 8A-C). qRT-PCR results were consistent with the Western blot finding, confirming the corresponding changes in TNF- α and IL-6 mRNA levels (Figure 8D-E).

In in vitro experiments, verification using HK-2 cells demonstrated that, relative to the control group, intracellular TNF- α and IL-6 expression increased following Erastin induction, while TLB treatment significantly inhibited the protein expression of TNF- α and IL-6 in HK-2 cells. In conclusion, these results indicate that TLB significantly alleviates the inflammatory response in CI-AKI mice and HK-2 cells by inhibiting the expression of the pro-inflammatory factors TNF- α and IL-6.

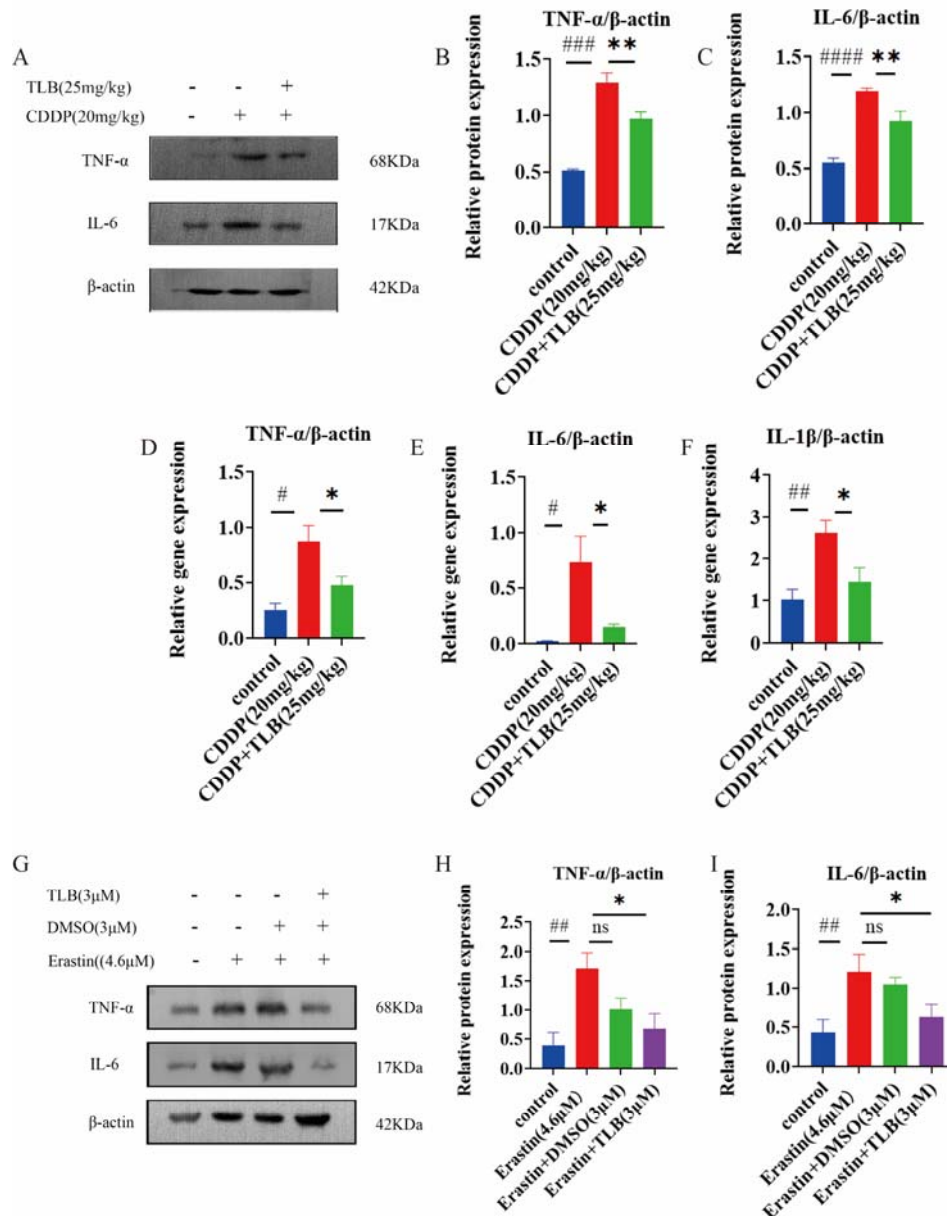


Fig. 8. TLB exerts anti-inflammatory effects in vivo and in vitro. (A-C) Western blot images of Nrf2 and GPX4 proteins in tissues, and analysis graphs of Nrf2 and GPX4 protein expression levels ($n = 3$). (D-F) Graphs of Nrf2 and GPX4 gene expression levels in tissues ($n = 3$). (G-I) Western blot images of Nrf2 and GPX4 proteins in cells, and analysis graphs of Nrf2 and GPX4 protein expression levels ($n = 3$). Compared with the control group, $^{\#}P < 0.05$, $^{##}P < 0.01$, $^{###}P < 0.001$, $^{####}P < 0.0001$; compared with the Erastin group, $^{*}P < 0.05$, $^{**}P < 0.01$; ns, not significant.

4. Discussion

As mentioned earlier, CDDP, as a highly effective chemotherapeutic agent, is frequently associated with significant nephrotoxicity, and there is currently, a lack of effective preventive and therapeutic strategies in clinical practice. The pathological mechanisms of CI-AKI have been extensively studied and involve multiple

processes, including ferroptosis, oxidative stress³, inflammatory response⁴, cell necrosis, cell apoptosis⁵, and vascular injury⁶. Among these processes, ferroptosis, a novel form of cell death closely linked to oxidative stress and inflammation⁹, may play a crucial role in CI-AKI. Consequently, intervention strategies targeting ferroptosis are considered promising approaches for the treatment of CI-AKI.

To date, various synthetic compounds and natural products have been confirmed to effectively ameliorate CI-AKI by inhibiting ferroptosis. For instance, Fer-1 significantly improves renal tubular damage in CI-AKI and regulates the levels of GPX4, NADPH, and GSH in CDDP-treated HK-2 cells¹⁸. Additionally, Celastrol has been shown to reduce Scr and BUN levels, while inhibiting renal dysfunction, inflammation, and oxidative stress³⁹. Recent studies indicate that alliin significantly increases survival rate, MDA levels, and SOD activity in CI-AKI rats, enhancing HK-2 cell viability and reduce ROS production⁴⁰. Although, current research on TLB has mainly focused on its anticancer effects⁴¹, regulation of glucose metabolism²² and promotion of angiogenesis⁴², there are limited reports addressing its antioxidant and anti-inflammatory properties⁴³. Notably, the role of TLB in ameliorating CI-AKI through ferroptosis inhibition remains unexplored. Previous studies have confirmed that TLB contains a flavonoid structure⁴⁴, which is closely related to its significant antioxidant capacity; multiple colorimetric and DPPH-HPLC analyses have demonstrated that TLB exhibits strong antioxidant activity. Furthermore, another study reported that TLB can alleviate oxidative damage in PC12 cells by maintaining ROS homeostasis and reduce the levels of pro-inflammatory factors such as TNF- α and IL-6 in the serum of obese hyperglycemic mice²⁶. Consistent with these findings, this study is the first to reveal that TLB exhibits significant antioxidant effects in both in vivo and in vitro models, effectively inhibiting ferroptosis in HK-2 cells, reducing lipid peroxidation, alleviating inflammatory responses, and thereby ameliorating CDDP-induced renal injury.

Nrf2 is a core transcription factor that regulates cellular antioxidant balance and metabolic homeostasis. It protects cells by activating the expression of downstream antioxidant enzymes that scavenge ROS. Notably, Nrf2 plays a pivotal role in regulating ferroptosis: during ferroptosis, Nrf2 is activated to enhance antioxidant defense and reduce ROS levels, thereby inhibiting the progression of ferroptosis. A substantial body of evidence indicates that Nrf2 inhibits ferroptosis by promoting the transcription and expression of GPX4⁴⁵⁻⁴⁸. In this study, we observed that the expression of Nrf2 and GPX4 was significantly downregulated in the renal tissues of CI-AKI mice and Erastin-treated HK-2 cells, while TLB intervention effectively reversed this phenomenon, suggesting that TLB may inhibit ferroptosis in CI-AKI by activating the Nrf2/GPX4 axis. Network pharmacology analysis further identified Nrf2 as a key target. Functional loss experiments confirmed that in ML385-inhibited Nrf2-deficient HK-2 cells, the protective effect of TLB against Erastin-induced ferroptosis and lipid peroxidation was significantly attenuated, strongly supporting the central role of the Nrf2/GPX4 axis in the anti-ferroptosis mechanism of TLB. In addition to renal tubular cell death, inflammation is another important feature of CI-AKI⁴⁹. Dead cells release inflammatory factors, exacerbating tissue damage. Studies have shown that ferroptosis is immunogenic and can significantly promote the

development of inflammation. Our research confirmed that TLB can alleviate inflammatory damage in CI-AKI and Erastin-induced HK-2 cells.

As a natural product characterized by high solubility, low toxicity, and ease of isolation, TLB has shown therapeutic potential across various disease models, indicating its viability as an intervention strategy for patients with CI-AKI. Nevertheless, this inference necessitates further experimental validation. This study acknowledges several limitations: first, there is a lack of ultrastructural observations of renal tissues and ferroptosis renal tubular epithelial cells, particularly regarding changes in mitochondrial morphology; second, the assessment of iron metabolism-related indicators, such as ferritin and transferrin receptor, is insufficient; finally, although network pharmacology has identified multiple potential targets, the specific pathway network through which TLB regulates ferroptosis in CI-AKI, as well as the molecular mechanisms by which ferroptosis triggers inflammation, require further investigation.

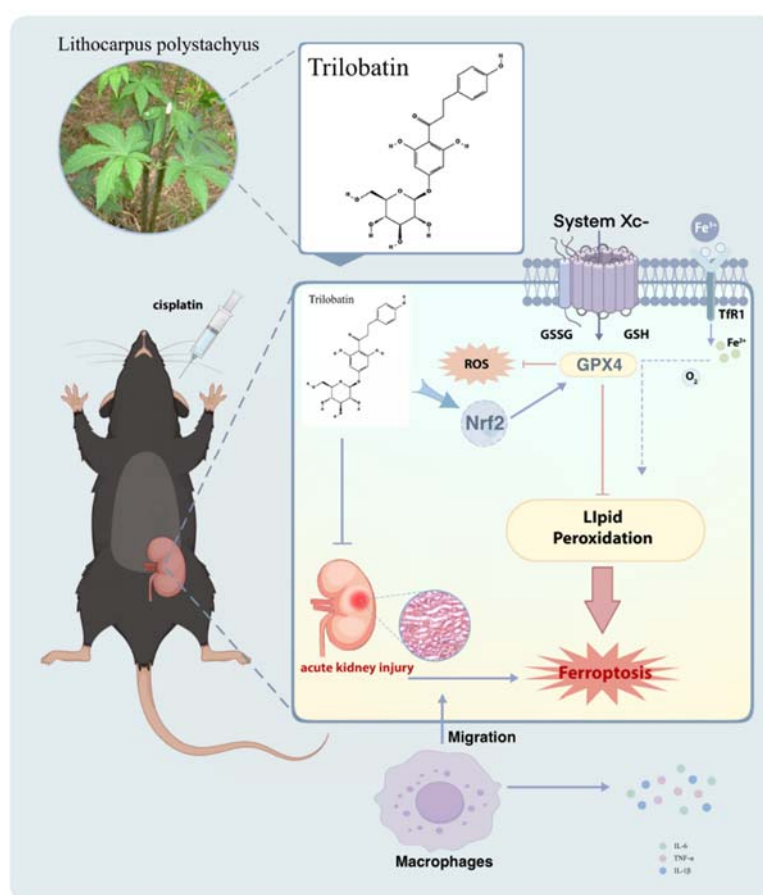


Fig.9 The main pathway of TLB against CI-AKI. It illustrates the role of TLB in inhibiting ferroptosis, regulating oxidative stress, and suppressing inflammatory responses, thereby protecting renal function in mice.

5. Conclusion

This study is the first to elucidate the protective effects and underlying mechanism of TLB against CI-AKI and Erastin-induced ferroptosis in HK-2 cells. The findings suggest that TLB is a promising therapeutic agent, as it effectively alleviates nephrotoxicity associated with CDDP-based anticancer therapies. Key mechanistic studies have demonstrated that TLB confers renal protective effects by activating the Nrf2 signaling pathway. Future clinical trials are essential to assess the safety and efficacy of

TLB in the treatment of CI-AKI and to clarify its clinical application value in CDDP-containing anticancer regimens.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflict of Interest

The authors declare that they have no Conflict of Interest. We agree to publish in Food Science and Human wellness.

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Ethics approval and consent to participate

Our experiments receive ethical approval and review and consent to the participation of animal experiments

Consent for publication

We promise that both materials and data are available.

Data availability statement

We promise that all raw data is available.

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