

Original Article

Eupalinolide B exerts cytotoxic effects against KRAS-mutant NSCLC through Nrf2/HO-1-regulated ferroptosis

Wenjian Wang^{1,†}, Jinglu Yu^{2,†}, Tanxuan Huang¹, Kangdi Liu¹, and Lianxiang Luo^{1,*}

¹School of Ocean and Tropical Medicine, Guangdong Medical University, Zhanjiang 524023, China, and ²Pudong Traditional Chinese Medicine Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 201299, China

[†]These authors contributed equally to this work.

*Correspondence address. E-mail: luolianxiang321@gdmu.edu.cn

Received 15 April 2025 Accepted 15 July 2025 Published 4 December 2025

Abstract

Ferroptosis, an iron-dependent form of regulated cell death, is characterized by excessive reactive oxygen species (ROS) accumulation and lipid peroxidation of polyunsaturated fatty acids (PUFAs) in cellular membranes. Non-small cell lung cancer (NSCLC) harboring KRAS mutations often exhibits therapeutic resistance but may display high susceptibility to ferroptosis. Eupalinolide B (EB), a natural compound with documented anti-cancer activity, has not been thoroughly explored for its ferroptosis-inducing potential in KRAS-mutant NSCLC. In this study, we demonstrate that EB treatment significantly elevates ROS levels, intracellular iron accumulation, and lipid peroxidation in KRAS-mutant NSCLC cells, resulting in ferroptotic cell death. Molecular docking and cellular thermal shift assays reveal that EB directly binds to and activates heme oxygenase-1 (HO-1), a critical component of the Kelch-like ECH-associated protein 1 (Keap1)-Nrf2/HO-1 oxidative stress response pathway. Genetic or pharmacological inhibition of HO-1 attenuates EB-induced ferroptosis, underscoring the pivotal role of HO-1-mediated oxidative stress in this process. Furthermore, *in vivo* studies using KRAS-mutant H358 xenograft models confirm the potent anti-tumor effects of EB. Collectively, our findings establish that EB triggers ferroptosis in KRAS-mutant NSCLC by activating the Keap1-Nrf2/HO-1 pathway, suggesting a promising therapeutic strategy for this challenging malignancy.

Key words eupalinolide B, ferroptosis, KRAS-mutant NSCLC, Keap1-Nrf2/HO-1 pathway, oxidative stress

Introduction

Lung cancer ranks as the second most prevalent malignancy worldwide and remains the leading cause of cancer-related mortality [1,2]. Non-small cell lung cancer (NSCLC) constitutes approximately 85% of all lung cancer diagnoses [3] and has a poor overall prognosis despite advances in radiotherapy, chemotherapy, and surgical resection [4]. While these treatments provide temporary disease control, long-term outcomes for many patients remain suboptimal, particularly for those with mutations in KRAS, a common oncogenic driver in NSCLC [5]. Although targeted therapies have shown success in treating other genetic subtypes of NSCLC, KRAS-mutant NSCLC remains resistant to conventional treatments, highlighting the necessity for innovative therapeutic approaches.

Inducing tumor cell death is a promising strategy in cancer therapy. Various well-characterized forms of programmed cell death, such as apoptosis [6], necroptosis [7], and autophagy [8],

have been extensively studied for their roles in cancer treatment. However, recent research has revealed the therapeutic potential of ferroptosis, a distinct form of iron-dependent cell death characterized by lipid peroxidation and iron accumulation [9,10]. Unlike other forms of cell death, ferroptosis is driven by oxidative damage to cell membranes through the peroxidation of polyunsaturated fatty acids (PUFAs) and is further amplified by reactive oxygen species (ROS) when the cell's antioxidant defenses, such as glutathione (GSH) and glutathione peroxidase 4 (GPX4), are compromised [11].

Importantly, increasing evidence suggests that ferroptosis inducers have the potential to sensitize resistant cancer cells to chemotherapy by exploiting vulnerabilities in their redox balance [12]. This phenomenon is especially important for KRAS-mutant NSCLC, a subtype known for its high levels of ROS production caused by metabolic imbalances. KRAS mutations disrupt various pathways related to oxidative stress and redox balance, resulting in

elevated ROS levels that both support tumor survival and increase the susceptibility of cancer cells to ferroptosis. Therefore, ferroptosis inducers present a unique opportunity to target KRAS-mutant cancer cells by increasing ROS levels and promoting iron-dependent lipid peroxidation, thus overcoming resistance mechanisms.

Natural products have the potential to induce tumor cell death by regulating ferroptosis [13]. Eupalinolide B (EB) is a bioactive sesquiterpenoid discovered from *Eupatorium lindleyanum* DC [14]. Research has shown that EB has effective therapeutic effects on a variety of diseases, including neurodegenerative disease, acute lung injury (ALI), and hepatic carcinoma [15–17]. However, its effects on NSCLC, particularly KRAS-mutant NSCLC, remain largely unexplored. Preliminary findings suggest that EB may induce ferroptosis by activating the Kelch-like ECH-associated protein (Keap1)-Nrf2/HO-1 pathway, a critical regulator of oxidative stress responses in cancer cells [17]. Activating this pathway could increase the accumulation of ROS and iron inside cancer cells, leading to lipid peroxidation and cell death via ferroptosis.

In this study, we explored the ability of EB to induce ferroptosis in KRAS-mutant NSCLC by activating the Keap1-Nrf2/HO-1 signaling pathway both *in vitro* and *in vivo*. Our results reveal that EB promotes ferroptosis by modulating oxidative stress, highlighting its potential as a therapeutic strategy for overcoming drug resistance in KRAS-mutant NSCLC. These findings offer a promising avenue for improving treatment outcomes in this challenging subtype of lung cancer.

Materials and Methods

Cell culture

Human non-small cell lung cancer cell lines (H358, H1975, H2122, A549, H1299, H1650, and 16HBE) were obtained from American Type Culture Collection (ATCC, Manassas, USA) and were cultured in RPMI-1640 medium (Pricella, Wuhan, China) supplemented with 10% fetal bovine serum (FBS; Pricella) and 1% penicillin-streptomycin (PS; Solarbio, Beijing, China). All the cells were cultured and passaged in an incubator containing 5% CO₂ at 37°C.

Cell viability and colony formation assay

Cell viability was assessed by CCK-8 assay. Cultured cells were plated in 96-well plates at a seeding density of 5×10^3 cells/well and exposed to varying concentrations of the drug for 24 h. Subsequently, a CCK-8 solution (Beyotime Biotechnology, Shanghai, China) was introduced and allowed to incubate within a controlled environment for a period of 2 h. Subsequent to this step, the absorbance was measured at a wavelength of 450 nm, enabling the determination of the relative viability of the cells in comparison with that of the control group. For the colony formation assay, cells (7×10^3 cells/well) were plated in 6-well plates and cultured for 24 h. The medium was changed every 3 days. After 7 days, the cells were fixed and stained with a crystal violet solution (Beyotime Biotechnology) for 10 min, and images of the colonies were captured and the number of colonies were counted.

Identification of potential targets of EB for the treatment of NSCLC

The PubChem database was utilized to retrieve detailed information on eupalinolide B, including its SMILES notation and CAS number. Potential target proteins of eupalinolide B were identified by inputting the relevant data into databases such as SwissTargetPre-

diction, STITCH, and BATMAN-TCM. In the STITCH database and BATMAN-TCM database, all potential target proteins of eupalinolide B can be found. With the keyword “non-small cell lung cancer” as the key word, a total of 16,270 disease-related genes were collected from the GeneCards database (<https://www.genecards.org/>) and CTD database (<https://www.ctdbase.org/>). Moreover, we obtained 564 ferroptosis-related genes from FerrDb V2 (<https://ferddb.org/>). With the help of the R package “Venny”, we mapped the targets of NSCLC, ferroptosis and eupalinolide B and constructed Venn diagrams to identify the potential targets of eupalinolide B for the treatment of NSCLC via ferroptosis.

Network analysis and enrichment analysis of the intersection genes

To explore the linkages between intersection genes, protein-protein interaction (PPI) data for target proteins were exported from the STRING database in TSV format. With the help of Cytoscape 3.7.2, we used the NetworkAnalyzer tool in cytoHubba to analyze the PPI network to obtain information such as the degree and MCC of protein nodes, and finally, we constructed a graph of the Hub target PPI network. To gain deeper insights into the primary processes associated with the interaction targets, the identified target protein genes were submitted to the Metascape platform (<https://metascape.org/>) for Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment.

Antibodies and reagents

All primary antibodies used for subsequent experiments were anti-GPX4 (ABclonal, Wuhan, China), anti-ACSL4 (ABclonal), anti-GAPDH (ABclonal), anti-SLC7A11 (CST, Shanghai, China), anti-FTH1 (ABclonal), anti-cyclin D1 (CST), anti-p21 (Wanleibio, Wuhan, China), anti-p27 (Wanleibio), anti-Nrf2 (CST), anti-Keap1 (CST), anti-HO-1 (CST), anti- β -actin (CST), and HRP-linked secondary antibodies (1:5000; CST). The inhibitors mentioned in the experiments were ferrostatin-1 (Fer-1), deferoxamine (DFO), Z-VAD-FMK, necrostatin-1 (Nec-1), and wortmannin (Wort), which were purchased from MedChemexpress (New Jersey, USA).

Cell cycle assay

Drug-treated cells were collected, centrifuged, and washed three times with phosphate-buffered saline (PBS) without Ca²⁺ or Mg²⁺ to perform a cell cycle analysis. After that, the cells were fixed overnight in 75% precooled ethanol. After being washed twice with PBS devoid of Ca²⁺ and Mg²⁺ to eliminate any remaining ethanol, the cells were left in the dark for 15 min in a complex with propidium iodide/RNase staining solution. The cells were then filtered through a 200-mesh filter. Finally, flow cytometry was used to examine the distribution of cells in different stages of the cell cycle.

Metabolomics analysis

The metabolic data underwent logarithmic (LOG) transformation and mean centering via SIMCA software (V16.0.2) for normalization. Principal component analysis (PCA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA) were subsequently conducted. Differentially abundant metabolites were identified on the basis of the criteria of a *P* value less than 0.05 and a variable importance in projection (VIP) score exceeding 1. R software (V4.1.3) was used to construct a visual volcano map and heatmap. MetaboAnalyst (<https://www.metaboanalyst.ca/>) was

subsequently used to conduct KEGG and the Small Molecule Pathway Database (SMPDB) enrichment analyses of the differentially abundant metabolites.

Molecular docking

The protein structure of heme oxygenase-1 (HO-1) was acquired from the Protein Data Bank (PDB: 6EHA), whereas the 3D SDF-format structure of EB was obtained from the PubChem repository (<https://pubchem.ncbi.nlm.nih.gov/>) as the ligand. Protein–ligand docking was performed via the Molecule Operating Environment molecular simulation tool (Chemical Computing Group ULC, Montreal, Canada). The triangle matching method was employed to place the ligand within the defined activity threshold of the receptor protein. The London dG function was subsequently used to calculate the total energy of the ligand at different positions to identify its lowest-energy binding conformation. Finally, Discovery Studio software (BIOVIA, Paris, France) was used for analysis of the molecular interactions.

Western blot analysis

The cells in the plate were subjected to digestion and subsequent collection, after which the cells were separated via centrifugation at 11,000 *g* for 4 min. Subsequently, the cells were subjected to lysis via a radioimmunoprecipitation assay (RIPA; Solarbio) supplemented with PMSF (Beyotime Biotechnology) on ice for 10 min, followed by centrifugation at 80,000 *g* for 10 min, leading to the collection of the supernatant. The concentration of proteins in the lysates was determined via a bicinchoninic acid (BCA) assay kit (Sangon Biotech, Shanghai, China). The proteins were subsequently separated by 10% SDS-PAGE and transferred to NC membranes. To prevent nonspecific binding, a 5% bovine serum albumin (BSA) solution was applied to block the membranes for 1 h, after which the membranes were exposed to primary antibodies overnight at 4°C. The next day, after being washed three times with TBST, the membranes were subjected to incubation with HRP-linked secondary antibodies (1:5000) for 1 h at ambient temperature. The membranes were subsequently washed three times with TBST. The process of color development was ultimately carried out using the BeyoECL Moon kit (Beyotime Biotechnology).

Lipid peroxidation assay

Following a 24-h EB (Herbest, Baoji, China) treatment, H358 cells were treated with C11-BODIPY 581/591 (ABclonal) and incubated for 1 h at 37°C in a 5% CO₂ incubator. The cells were then washed twice with PBS and subjected to flow cytometry analysis. Flow cytometry was performed using a flow cytometry instrument (Cytek Biosciences, Shanghai, China), with excitation wavelengths set at 581 nm for detection of the reduced form and 488 nm for the oxidized form of the dye.

Iron assay

After 24 h of exposure to EB, the cells were incubated with FerroOrange (1 μM; Dojindo, Kumamoto, Japan) in a 5% CO₂ incubator at 37°C for 30 min. Subsequently, the cells were examined via a fluorescence confocal microscope (FV3000; Olympus, Tokyo, Japan) using an excitation wavelength of 640 nm and appropriate emission filters to detect the fluorescence signal.

Assessment of GSH and MDA

Protein samples were obtained from H358 cells in accordance with the guidelines provided by the manufacturer for the assessment of GSH and MDA concentrations. The GSH concentrations were assessed via GSH and GSSG assay kits (Beyotime Biotechnology). MDA levels were determined via a lipid peroxidation MDA assay kit (Beyotime Biotechnology).

siRNA transfection

The si-HO-1 construct was introduced into H358 cells, which were subsequently allowed to incubate for a period of 48 h prior to being stimulated with EB. The si-HO-1 reagent sourced from Sangon Biotech was utilized to suppress the expression of the *HO-1* gene. The si-HO-1 sequence used in this study was as follows: 5'-CAGUUGCUGUAGGGCUUUATT-3'. The si-NC sequence used in this study was as follows: 5'-UUCUCCGAACGU-GUCACGUTT-3'. For transfection, the cells were seeded into 12-well plates at a density of 8 × 10⁴ cells/well. Additionally, cells were transfected at a final dose of 20 nM with si-HO-1/si-NC (negative control) via siRNA transfection reagent (Polyplus, Ilkirre, France). Finally, 48 h after transfection, cells were used to examine the target protein expression level. In the subsequent tests, cells that had undergone successful transfection were employed.

Mouse models

The animal research conducted in this study was overseen by the Animal Ethics Committee at Guangdong Medical University, with ethical approval number GDY2502726. Two groups of 6-week-old female BALB/c mice were procured from SPF Biotechnology (Beijing, China) and were randomized into 2 groups: the vehicle group and the experimental group. The mice were given free access to food and kept in separate ventilated cages. H358 cells (5 × 10⁶) were resuspended in FBS-free medium and injected subcutaneously into the right dorsum of each mouse in a volume of 100 μL. The experimental group was treated daily by intraperitoneal injection of EB (20 mg/kg; Herbest, Baoji, China) for 18 days. Body weight was recorded, and toxic effects were monitored every day. The tumor volume was measured every three days with Vernier calipers and calculated via the following formula: tumor volume (mm³) = {length (mm) × [width (mm)]²}/2. After 18 days, the mice were sacrificed via cervical dislocation, and tumor tissues were obtained.

Histopathology (H&E) and immunohistochemistry (IHC)

The tumor samples were fixed in 4% paraformaldehyde (PFA) and then embedded in paraffin for further analysis. The subsequent steps involved dewaxing the sections with environmentally friendly transparent dewaxing liquid (Solarbio) and dehydrating them through the addition of alcohol solutions. The application of H&E staining followed established protocols. For IHC analysis, sections of tissue embedded in paraffin were subjected to treatment with citric acid buffer solution and subsequently placed in a microwave oven for 10 min, allowing the temperature to equilibrate to room temperature. Following this step, the tissues were incubated for 10 min with 3% hydrogen peroxide. Then, the sections were blocked with 5% goat serum for 30 min at room temperature. Finally, antibodies (such as those against ACSL4, SLC7A11, GPX4, FTH1, and HO-1) were added, and the samples were incubated overnight at 4°C. The next day, the sections were treated with an HRP-conjugated secondary antibody for 30 min at ambient tem-

perature. Next, the samples were exposed to DAB and counterstained with hematoxylin. The sections were subsequently dehydrated, clarified via environmentally friendly transparent dewaxing liquid, and finally sealed with neutral resin and examined under a microscope.

CETSA-western blotting

The soluble protein lysate derived from H358 cells was dispensed into tubes and exposed to EB (20 μ M) or DMSO for 1.5 h at ambient temperature to perform a CETSA heat pulse. The solutions were heated at temperatures within the range of 40 to 80°C for 10 min. Following a 3-min equilibration at room temperature, the solution was analyzed via western blotting.

DARTS assay

M-PER lysis buffer was used to lyse untreated H358 cells on ice for 10 min, and the mixture was then centrifuged at 80,000 *g* for 10 min, after which the supernatant was collected. Equal amounts of DMSO and different concentrations of EB were added to the lysis mixture, and the mixture was incubated at room temperature for 1 h. The samples were incubated for 30 min at a ratio of pronase to total protein of 1:300. The lysate samples were mixed with 5 $\times$ loading buffer and boiled for 5 min at 100°C, after which the samples were subjected to western blot analysis.

Statistical analysis

The data are presented as the mean $\pm$ standard deviation (SD). GraphPad Prism 9 software was used to conduct the statistical analysis. The significance of the variations was evaluated through either one-way ANOVA or two-way ANOVA. Statistical significance was set when $P < 0.05$.

Results

EB suppresses the growth of lung cancer harboring KRAS mutations

We used the CCK-8 assay to evaluate the ability of EB to affect the metabolic status of various NSCLC cells and normal lung cells. Compared with other cancer cell lines, KRAS (G12C)-mutant H358 and H2122 cells are more sensitive to EB (Figure 1A). Its effect on H358 cell viability was greater than that of cisplatin (Figure 1B). To determine the antiproliferative effect of EB, we conducted an experiment involving colony formation. The results demonstrated that EB treatment dramatically reduced the ability of H358 cells to form colonies (Figure 1C). We used flow cytometry and discovered that EB induces cell cycle arrest at the G2/M phase, hence reducing cell viability. The H358 cell cycle was stopped by EB in the G2/M phase (Figure 1D). Moreover, EB increased p21 expression while simultaneously reducing the expression levels of the cell cycle-associated proteins cyclin D1 and p27 (Figure 1E). In summary, EB treatment effectively decreased the viability of H358 cells and induced cell cycle arrest at the G2/M phase.

Screening and analysis of potential targets of EB in NSCLC

The 2D and 3D structures and related information of EBs were obtained from the PubChem database (Figure 2A). A total of 26 target genes of EB were obtained from three databases (SwissTargetPrediction database, STITCH database and BATMAN-TCM database). We mapped the targets of EB to the potential targets of

NSCLC via a Venn diagram (Figure 2B). Notably, by searching the ferroptosis database, we identified potential targets of EB for the treatment of NSCLC via ferroptosis (Figure 2B), which revealed that EB acts on a protein-protein interaction (PPI) network of nine potential ferroptosis targets in NSCLC. After irrelevant genes were filtered from the STRING database and the remaining genes were visualized with Cytoscape software, a PPI network was constructed. As illustrated on the left side of Figure 2C, the “EB target-NSCLC target-ferroptosis target” network consists of eight solid-colored nodes in the PPI network interconnected by 15 edges. On the right side of Figure 2C, a refined network was generated by applying parameters (MCC > 6 and degree > 6), resulting in five intersecting genes (*PTGS2*, *KEAP1*, *RELA*, *HMOX1* and *GPX4*) that are likely to play pivotal roles.

Enrichment analysis of potential ferroptosis targets of EB for the treatment of NSCLC

We imported 9 genes encoding potential ferroptosis targets of EB for the treatment of NSCLC into the metscape database for GO and KEGG enrichment analysis. As depicted in Figure 2D,E, GO analysis of the identified targets was conducted across three categories: biological process (BP), cellular component (CC), and molecular function (MF). The BP analysis suggested involvement in processes such as “cellular response to oxidative stress”, “cellular response to hydrogen peroxide”, “cellular response to iron ions”, and “positive regulation of lipid metabolic processes”. CC analysis highlighted associations with the “membrane region”, “peroxisomal matrix”, and “nuclear matrix”. MF analysis revealed activities such as “peroxidase activity”, “glutathione peroxidase activity”, “NADP binding”, and “phospholipase activity”. Additionally, KEGG pathway enrichment analysis revealed potential roles in “pathways in cancer”, “ferroptosis”, “HIF-1 signaling pathway”, and “micro-RNAs in cancer” (Figure 2F). The findings from the analyses indicate that EB may exert therapeutic effects on NSCLC by modulating these identified processes and pathways. These results suggest that the biological mechanisms underlying the effects of EB in the treatment of NSCLC are closely associated with ferroptosis.

EB induces ferroptosis in KRAS-mutant NSCLC

According to earlier research, EB might cause H358 and H2122 cell death. To determine the mechanism of H358 and H2122 cell death, we subjected H358 and H2122 cells to treatments consisting of EB, a ferroptosis inhibitor, an iron chelator, a necrosis inhibitor, an apoptosis inhibitor, and an autophagy inhibitor, either alone or in combination. CCK-8 assay revealed that EB induced cell death, and Fer-1 and DFO reversed this effect (Figure 3A), indicating that EB may induce H358 and H2122 cell death via ferroptosis. To ascertain the efficacy of EB in triggering ferroptosis in H358 cells and H2122 cells, some assessments were conducted to examine the influence of EB on the initiation of ferroptosis in H358 cells and H2122 cells. As shown in Figure 3, the levels of lipid peroxidation, MDA, and intracellular ferrous iron increased in a concentration-dependent manner after EB treatment (Figure 3B,C,F). Moreover, in H358 cells, western blot analysis revealed increased expression level of the ferroptosis-associated protein ACSL4, whereas notable decreases in the expression levels of SLC7A11, GPX4 and FTH1 were detected. In H2122 cells, the expression level of ACSL4 increased, and the expression levels of SLC7A11, GPX4, and FTH1 decreased (Figure 3D). As an important intracellular antioxidant molecule, the GSH

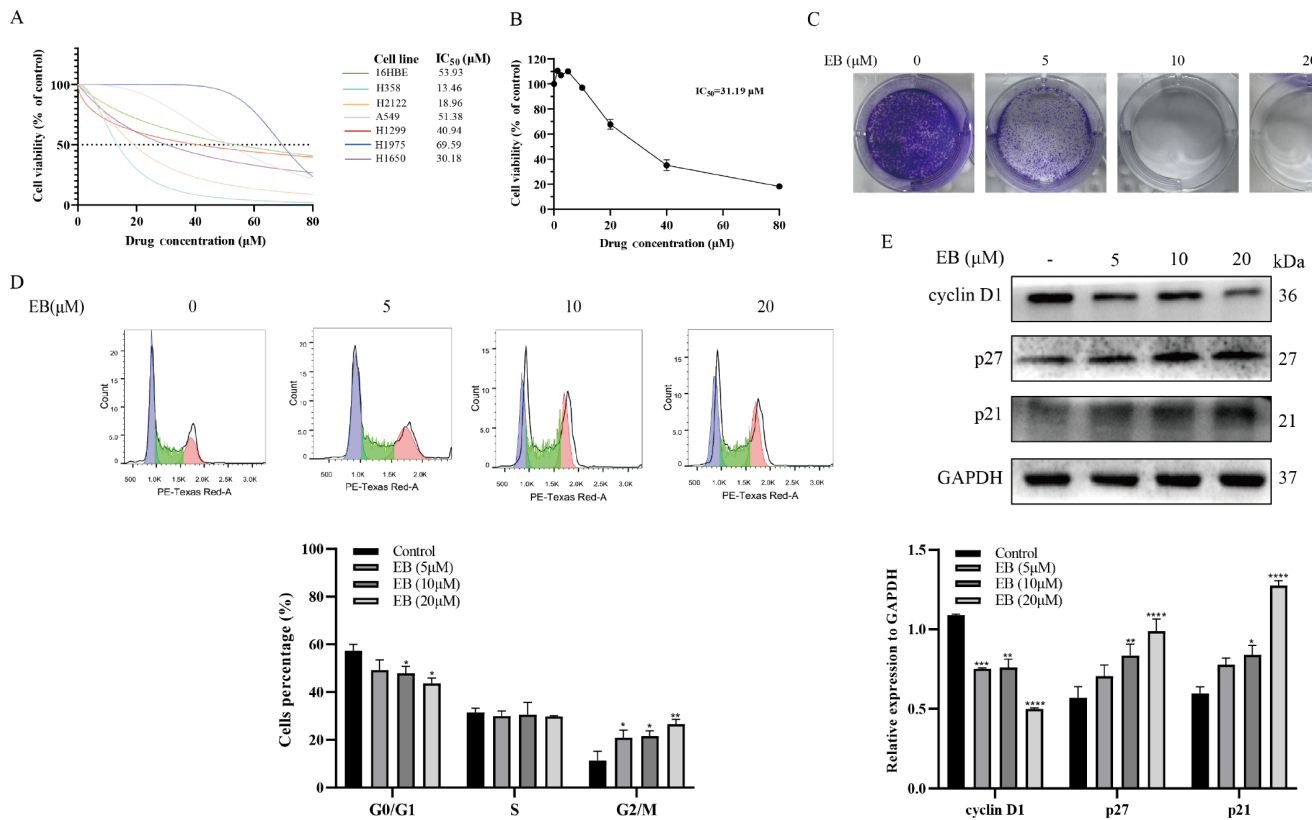


Figure 1. EB arrests the cell cycle of H358 cells by regulating cycle-related proteins (A) Various NSCLC cells and normal lung cells were treated with different concentrations of EB for 24 h. Cell viability is determined by CCK-8 assay. (B) Effect of cisplatin on H358 cells. Cell viability was determined via CCK-8 assay. (C) Representative images of H358 cell colony formation after treatment with EB. (D) Representative results of the cell cycle and quantitative analysis. (E) western blot analysis of cyclin D1, p27 and p21 protein expression in H358 cells treated with different concentrations of EB (0, 5, 10 and 20 μM) for 24 h. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

level continued to decrease as the concentration of EB increased (Figure 3E).

EB affects lipid metabolism

In an effort to uncover the mechanisms responsible for the alterations in the metabolic profile of H358 cells following EB treatment, a differential expression analysis was performed. The resulting volcano plot indicated a marked increase in 41 metabolites and a marked decrease in 57 metabolites (Figure 4A). A comparative analysis between the EB and control groups revealed 42 key metabolites that exhibited significant variation ($VIP > 1$, $P < 0.05$) (Figure 4B). Through KEGG enrichment analysis, we observed that EB influences lipid metabolism, including pathways such as ferroptosis and the HIF-1 signaling pathway, and is connected with pyruvate metabolism (Figure 4C,D). Further validation of these results was provided by SMPDB enrichment analysis, which additionally revealed a relationship between EB and both amino sugar metabolism and glutathione metabolism (Figure 4E). By ranking the differentially expressed metabolites by their abundance, the top 20 metabolites with the highest absolute values were selected (Figure 4F). This analysis led to the identification of several metabolites that showed substantial changes post-administration, which may have contributed to the partial suppression of the production of reactive oxygen species (ROS), including cysteinyl-alanine, L-homocysteic acid, and linoleic acid (Figure 4G).

Consequently, our findings suggest a significant link between EB and the processes of lipid peroxidation and ferroptosis in KRAS-mutated NSCLC.

EB targets HO-1 through the Keap1-Nrf2/HO-1 pathway

An evaluation of the docking energy score of small molecules and proteins revealed that a docking score lower than -5.0 can indicate a good binding effect between the ligand and the receptor. The optimal conformation score of the small molecule EB for docking with the HO-1 protein was -5.9671, indicating that at the energy level, the binding effect of EB and the receptor protein was relatively ideal. However, the number of interactions formed between molecules and proteins was relatively limited. As shown in Figure 5A, EB only formed hydrogen bonds with the ARG-136 residue of the protein. From the perspective of the number of interactions, the HIS-25 residue and PHE207 residue of the receptor play a key role in the ligand-receptor binding. They both form Pi-donor hydrogen bonds and alkyl interactions with the molecule. In particular, the hydrogen ring on the ten-membered ring of the molecule formed a water-hydrogen bond with the crystal water molecules of chain A (Figure 5B). DARTS assay revealed that EB inhibited the proteolysis of HO-1 by pronase (Figure 5C). In summary, EB was predicted to have better HO-1-binding properties in molecular docking studies. To further verify the interaction of EB with HO-1, we performed a CETSA in H358 cells. The addition of EB (20 μM) stabilized HO-1

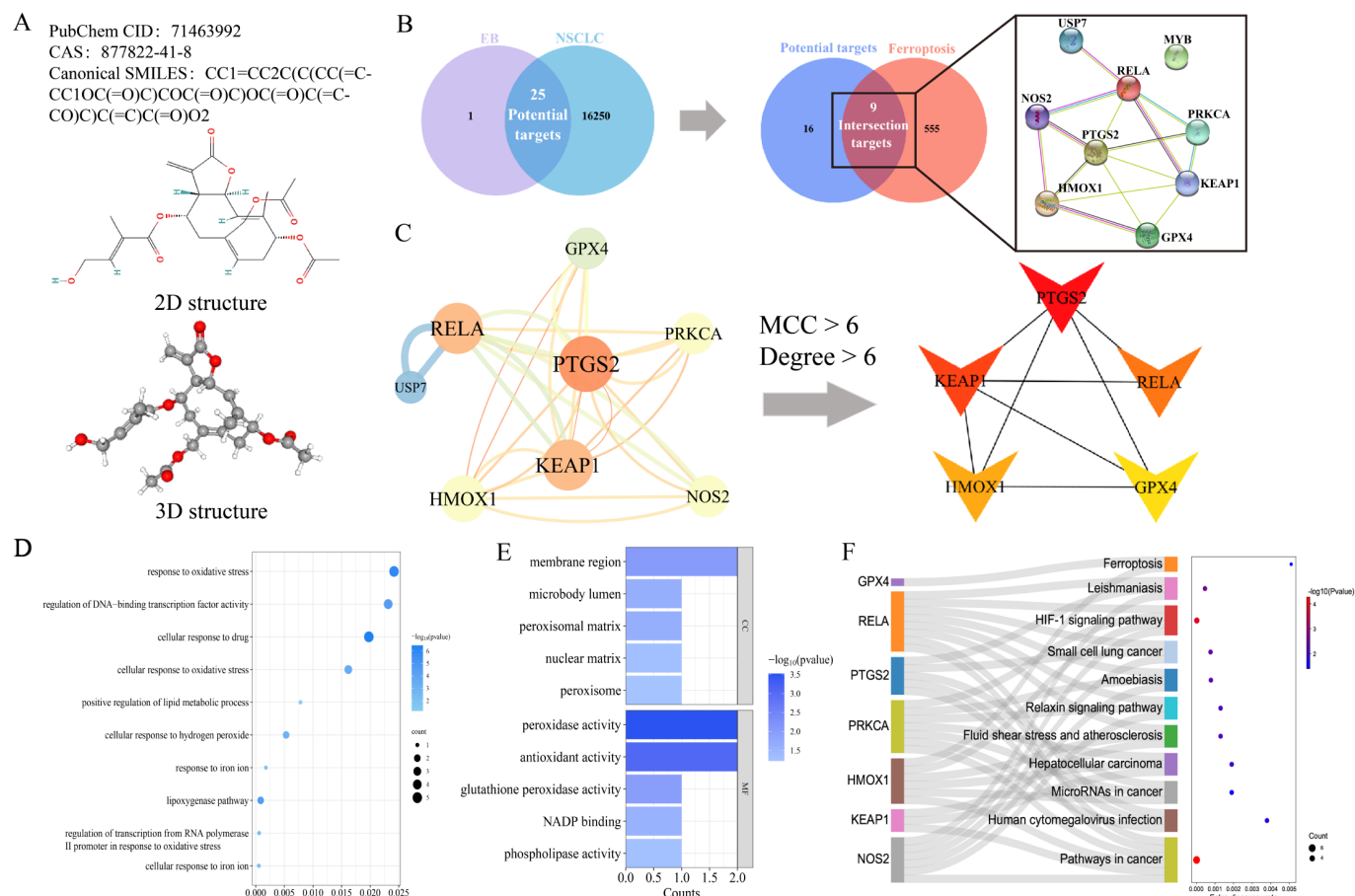


Figure 2. Screening and analysis of potential ferroptosis targets of EB in NSCLC (A) 2D and 3D structures of EB and related information. (B) Venn diagrams depicting the number of EB targets, NSCLC targets and ferroptosis-related targets. (C) The PPI network illustrates interactions among potential ferroptosis-related targets of EB in NSCLC, with a network diagram highlighting five intersecting genes. In this diagram, nodes represent target proteins, and lines denote interactions between them. Targets are ranked by their degree value; larger, more deeply colored nodes signify higher degree values. (D) The KEGG signaling pathways are depicted in Sankey bubble plots, demonstrating pathway enrichment. (E) The BP enrichment analysis results are visualized via bubble plots, revealing the biological processes involved. (F) The CC and MF enrichment outcomes are presented in bar charts, highlighting the cellular components and molecular functions associated with these targets.

(Figure 5D). After treatment with EB, the thermal stability of HO-1 increased, indicating that EB interacts with HO-1. Collectively, these results indicate that EB can bind directly to HO-1. Both reactive oxygen species elimination and cellular tolerance to oxidative stress are significantly influenced by the Keap1-Nrf2/HO-1 pathway [18]. Currently, the Keap1-Nrf2/HO-1 pathway is considered a crucial signaling mechanism that controls ferroptosis [19]. Thus, we hypothesized that the induction of ferroptosis by EB may involve the Keap1-Nrf2/HO-1 pathway. The experimental results demonstrated that exposure to EB resulted in a significant reduction in the level of Keap1 protein expression, along with a notable increase in the expression levels of Nrf2 and HO-1 (Figure 5E). These findings revealed that the Keap1-Nrf2/HO-1 pathway is involved in how EB targets HO-1 and causes ferroptosis in H358 cells.

Knockdown of HO-1 inhibits ferroptosis

To verify that the HO-1 protein is the key protein through which EB induces ferroptosis to exert its anti-tumor function, si-RNA targeting this protein was designed and synthesized. H358 cells were transfected with si-HO-1 for 48 h and subsequently analyzed via flow cytometry, western blot analysis, and ferrous ion level

measurement. First, the knockdown efficiency of si-HO-1 was assessed (Figure 6A). The findings indicated that downregulation of HO-1 resulted in reduced levels of ROS following treatment with EB (Figure 6B), decreased expression of ACSL4 protein, increased expression levels of GPX4 and FTH1 proteins (Figure 6C), and decreased accumulation of ferrous ions (Fe^{2+}) in the cells (Figure 6D). These findings suggest that HO-1 is critical for EB-induced ferroptosis in H358 cells.

ZnPP inhibits ferroptosis in H358 cells by inhibiting the expression of HO-1

Zinc protoporphyrin (ZnPP) is an HO-1 inhibitor [20]. In this study, H358 cells were pretreated with ZnPP (1 μM) for 1 h, after which EB was added. A series of experiments were conducted to verify whether ZnPP could rescue EB-induced ferroptosis. The results showed that ZnPP reduced the accumulation of lipid peroxidation caused by EB after inhibiting the expression of HO-1 (Figure 7A). The western blot analysis results revealed that the expression levels of ACSL4 and HO-1 were reduced, the expression of GPX4 was increased (Figure 7B), and the increase in ferrous iron levels in the cells was inhibited (Figure 7C). In conclusion, ZnPP can alleviate

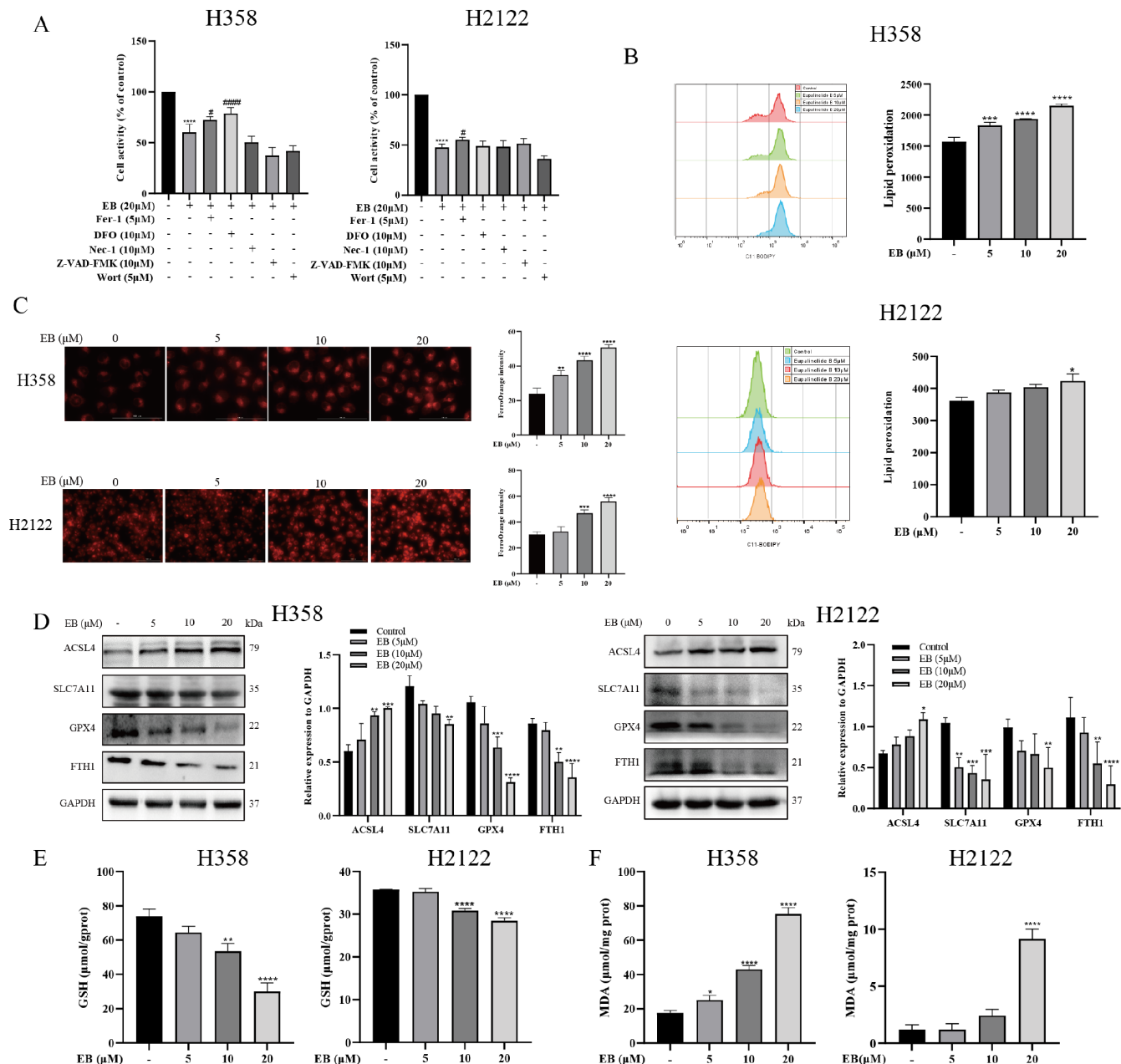


Figure 3. EB induces ferroptotic death in H358 and H2122 cells (A) H358 and H2122 cells were treated with EB (20 μM) and the cell death inhibitors Fer-1 (5 μM), DFO (10 μM), Nec-1 (10 μM), Z-VAD-FMK (10 μM), or Wort (5 μM) for 24 h. (B) The level of lipid peroxidation was measured via flow cytometry. (C) Detection of intracellular ferrous iron levels. (D) ACSL4, SLC7A11, GPX4 and FTH1 expression levels were detected via western blotting, and the relative intensities were quantified via ImageJ software. (E,F) Detection of GSH and MDA levels. * $P < 0.05$; ** $P < 0.01$; vs control. **** $P < 0.0001$ vs Fer-1.

EB-induced ferroptosis by inhibiting the expression of HO-1.

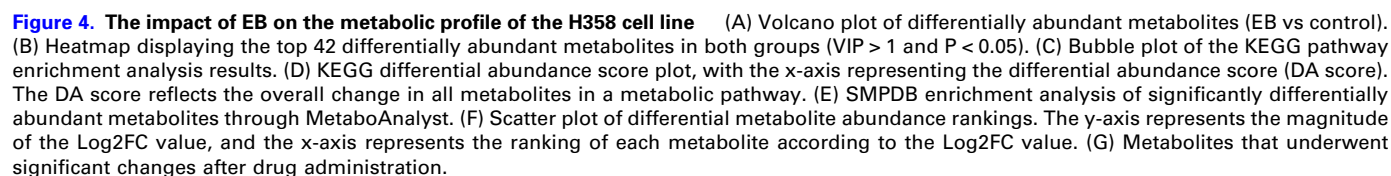
ML385 inhibits ferroptosis in H358 cells by inhibiting the expression of Nrf2

ML385, an Nrf2 inhibitor, was used to pretreat H358 cells for 1 h, after which EB was added. A series of experiments were conducted to verify whether ML385 could rescue EB-induced ferroptosis. The results showed that ML385 reduced the accumulation of lipid peroxidation caused by EB after the expression of Nrf2 was inhibited (Figure 8A). The western blot analysis results revealed

that the expression of ACSL4 was reduced, the expression levels of SLC7A11 and GPX4 were increased (Figure 8B), and the increase in ferrous iron levels in the cells was inhibited (Figure 8C). In conclusion, ML385 can alleviate EB-induced ferroptosis by inhibiting the expression of Nrf2.

EB induces ferroptosis *in vivo*

On the basis of *in vitro* experiments, a human lung carcinoma murine xenograft model was established to examine the *in vivo* anti-tumor effect of EB in BALB/c mice. H358 tumor-bearing mice



tumor cell damage (Figure 9E). Additionally, as shown in Figure 9F, EB influences the variations in the expressions of ferroptosis marker proteins in tumor tissues compared with those in the vehicle group (including the downregulation of FTH1 and GPX4 expression and the upregulation of ACSL4 and HO-1 expression). These findings suggest that EB can cause ferroptosis *in vivo* in malignancies.

Over the past two decades, molecular targeted therapy and

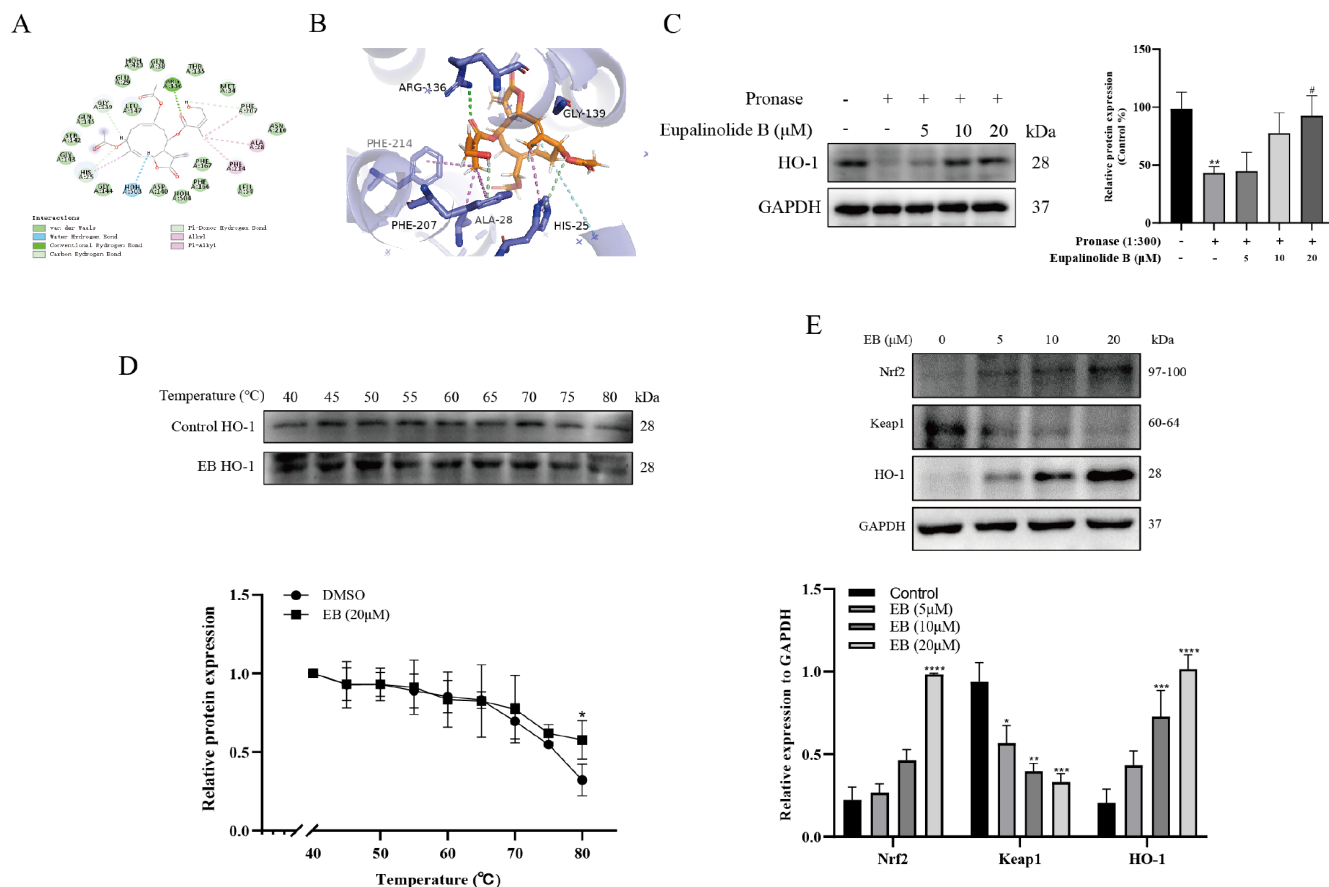


Figure 5. EB targets HO-1 through the Keap1-Nrf2/HO-1 pathway (A) Plan view of the interaction between EB and the HO-1 protein. (B) 3D schematic diagram of EB and the receptor protein HO-1. (C) H358 cell lysates were incubated with DMSO or different concentrations of EB in the presence or absence of pronase (1:300), after which HO-1 was detected by western blot analysis. (D) The effects of EB on the thermal stability of HO-1 were detected by CETSA and western blot analysis. The thermal stability of HO-1 in the range of 40°C to 80°C was quantified. (E) Nrf2, Keap1 and HO-1 expression levels were detected via western blotting, and the relative intensities were quantified via ImageJ software. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ vs control; # $P < 0.05$ vs [in the presence of pronase (1:300) and absence of EB].

immunotherapy for NSCLC have significantly enhanced patient outcomes [21]. However, for the subset of NSCLC patients harboring KRAS mutations, therapeutic progress has been limited. KRAS has long been considered an undruggable target because of the complexity of its downstream signaling pathways and the lack of effective inhibitors [22]. Therefore, the identification of novel therapeutic approaches to overcome the resistance of KRAS-mutant NSCLC remains a high priority in cancer research.

Because of their distinctive and innovative architectures, drugs derived from plants are frequently employed in the research of protein function and cell death pathways [13,23]. EB, a derivative extracted from *Eupatorium lindleyanum* DC, has been identified as a potential anti-tumor drug due to its significant anti-proliferative activity [14]. However, its anti-tumor mechanism has not yet been fully elucidated. In addition, we collected data and reported that the therapeutic effect of EB on KRAS-mutant NSCLC has not yet been reported. In this study, we provide new evidence that EB arrests H358 cells in the G2/M phase and induces ferroptosis by regulating the Keap1-Nrf2/HO-1 pathway. In addition, we also examined the *in vivo* anti-tumor effect of EB in mice.

Ferroptosis is different from apoptosis, necrosis, and autophagy. It is a new form of programmed cell death that is characterized

mainly by lipid peroxidation and the accumulation of ferrous ions in cells. An increasing number of studies have shown that ferroptosis plays an important role in inflammatory responses in multiple organs and in targeted cancer therapy [24,25]. To date, a vast number of recognized commercial medications can target ferroptosis, and ferroptosis therapy is becoming more diverse [2]. Unlike other cell death mechanisms, ferroptosis is driven by iron-dependent lipid peroxidation and is amplified by reactive oxygen species (ROS) accumulation. Importantly, many tumors, including KRAS-mutant NSCLC, exhibit elevated basal ROS levels due to metabolic reprogramming, which sensitizes them to ferroptosis when antioxidant defenses are overwhelmed [26].

In this study, we found that EB had a significant cytotoxic effect on a variety of tumor cell lines *in vitro*, especially H358 cells. At the same time, flow cytometry and FerroOrange probe imaging revealed that the levels of reactive oxygen species and ferrous ions in cells continued to increase with EB treatment. We also found that the level of GSH, an important antioxidant that protects H358 and H2122 cells from oxidative stress, continued to decrease with increasing EB concentration after treatment for 24 h. This finding is similar to the findings of GSH depletion and further induction of ferroptosis in cells after erastin treatment [27,28]. As the final

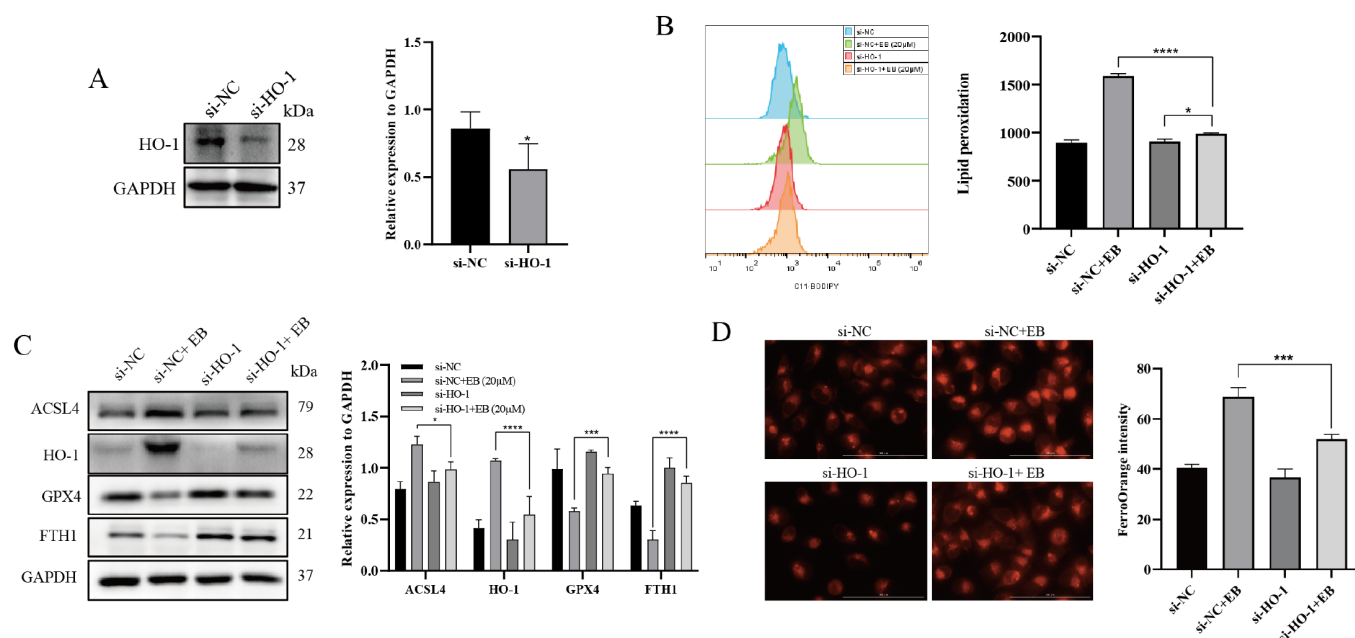


Figure 6. Inhibition of ROS and ferroptosis by downregulation of the HO-1 protein (A) Western blot analysis confirmed the successful knockdown of the *HO-1* gene via siRNA in H358 cells. (B) The level of lipid peroxidation in H358 cells after treatment with si-HO-1 or the negative control si-NC. (C) ACSL4, HO-1, GPX4 and FTH1 expression levels were detected via western blotting, and the relative intensity was quantified via ImageJ software; the treatments were similar to those in Figure 6B. (D) Detection of intracellular ferrous iron levels in response to treatments similar to those in Figure 6B. * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$.

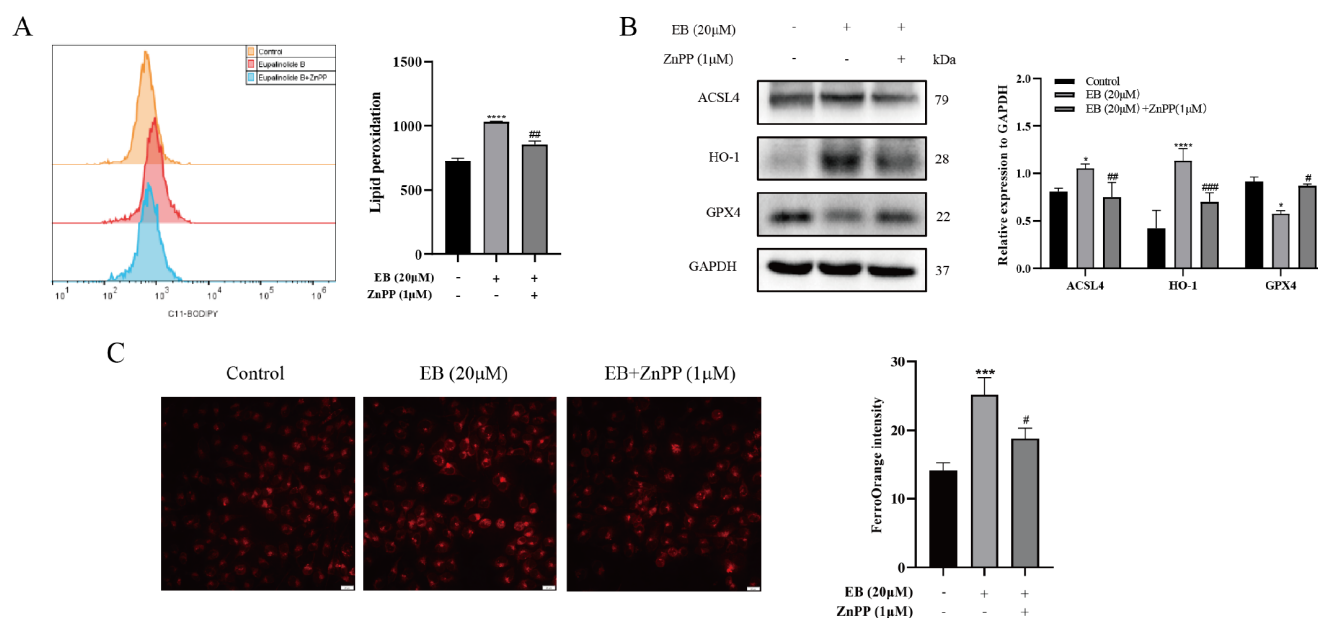


Figure 7. ZnPP inhibits ferroptosis in H358 cells (A) Levels of lipid peroxidation in H358 cells after treatment with Znpp (1 μM). (B) ACSL4, HO-1, and GPX4 expression levels were detected via western blotting, and the relative intensities were quantified via ImageJ software; the treatments were similar to those in (A). (C) Detection of intracellular ferrous iron levels in response to treatments similar to those in (A). * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$ vs control; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ vs EB (20 μM).

product of lipid peroxidation, the MDA content gradually increases in H358 and H2122 cells [29], indicating that EB induced lipid peroxidation in these cells. In addition, ferroptosis inhibitors such as Fer-1 and DFO can inhibit EB-induced cytotoxicity. Taken together, these results provide new evidence that EB-induced

cytotoxicity in H358 and H2122 cells is iron-dependent.

The targeted treatment of diseases has always been a research hotspot, and previous studies have shown that EB induces ferroptosis in liver cancer cells by regulating the ROS-ER-JNK signaling pathway [30]. In addition, EB also targets the TAK1

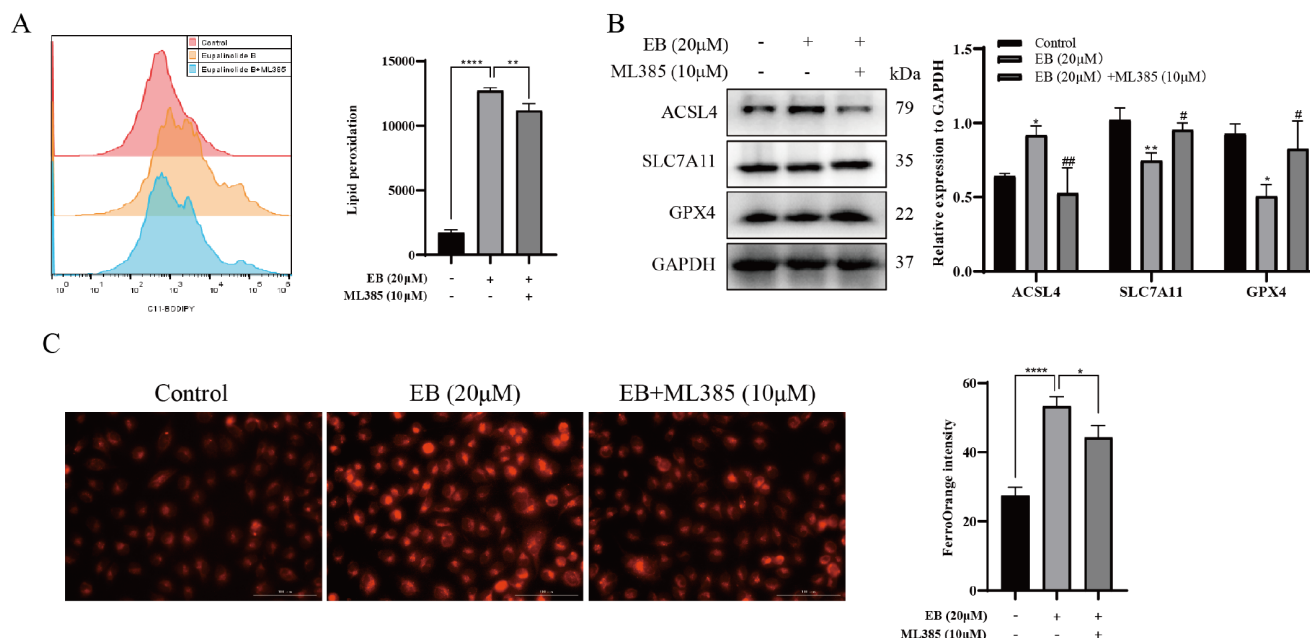


Figure 8. ML385 inhibits ferroptosis in H358 cells (A) The level of lipid peroxidation in H358 cells after treatment with ML385 (10 μM). (B) ACSL4, SLC7A11, and GPX4 expression levels were detected via western blotting, and the relative intensities were quantified via ImageJ software; the treatments were similar to those in (A). (C) Detection of intracellular ferrous iron levels in response to treatments similar to those in (A). * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$ vs control; # $P < 0.05$; ## $P < 0.01$ vs EB (20 μM).

protein to inhibit ALI by inhibiting NF-κB and MAPKs signaling [16]. We found through network pharmacology and validation analysis that the HO-1 protein is a target protein of EB and that downregulating HO-1 protein expression in H358 cells reversed EB-induced ferroptosis. HO-1 is not only the primary target of Nrf2 but also a crucial target for the development of ferroptosis. It performs dual roles in ferroptosis depending on different cell types [31]. In some cases, low expression of HO-1 protects cells from ferroptosis [32]. In other cases, high expression of HO-1 can lead to lipid peroxidation, causing the accumulation of ROS and inducing ferroptosis in cells [33,34]. The Keap1-Nrf2/HO-1 pathway is one of the key antioxidant pathways and is closely related to a variety of inflammatory diseases [35,36]. Nrf2 plays a crucial role in regulating antioxidant activity in cells and is involved in the regulation of ferroptosis and lipid peroxidation [37]. Keap1 is an inhibitory protein of Nrf2 that generally negatively regulates Nrf2 to maintain the expression of Nrf2 at a low level [38,39]. However, when cells are damaged and oxidative stress occurs, Keap1 dissociates and releases Nrf2, which then enters the nucleus and subsequently activates various genes, including the downstream molecule HO-1 [40]. Numerous studies have demonstrated the inhibitory effects of various pharmaceutical compounds on ferroptosis through the modulation of the Nrf2/HO-1 signaling pathway. For instance, the compound urolithin A mitigates ferroptosis induced by LPS through the activation of the Keap1-Nrf2/HO-1 pathway [41]; tagitinin C induces ferroptosis in colorectal cancer cells by activating the endoplasmic reticulum stress-mediated PERK-Nrf2-HO-1 signaling pathway [42]; and astaxanthin activates the Nrf2/HO-1 signaling pathway to inhibit ferroptosis and improve acetaminophen-induced liver injury [43]. In this study, the results revealed that EB induces ferroptosis in KRAS-mutant NSCLC cells through the Keap1-Nrf2/HO-1 pathway and that EB may be a

candidate target for the treatment of KRAS-mutant NSCLC.

In summary, EB has the potential to induce ferroptosis in KRAS-mutant NSCLC cells via the Keap1-Nrf2/HO-1 pathway. Moreover, the results of this study revealed that EB promotes the accumulation of ferrous iron and GSH depletion, resulting in the production of excess lipid peroxides. EB also downregulates the expression of the GPX4 protein, inducing ferroptosis in H358 cells. Furthermore, we determined that EB has the same tumor suppressive effects both *in vivo* and *in vitro*. These findings suggest that EB could be developed as a targeted therapy to overcome the resistance associated with KRAS mutations in NSCLC. The ability of EB to modulate key redox pathways and induce ferroptosis makes it a promising candidate for future therapeutic development. Further studies are warranted to explore the broader clinical implications of EB and its potential combination with other therapies to maximize its anti-tumor effects in KRAS-mutant cancers.

Acknowledgement

We thank the members of the Public Service Platform of the South China Sea for R&D Marine Biomedicine Resources for their support in providing marine biomedicine resources and technical services.

Funding

This work was supported by the grant from the Science and Technology Program of Guangdong Province (No. 2023A1515010850).

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A,

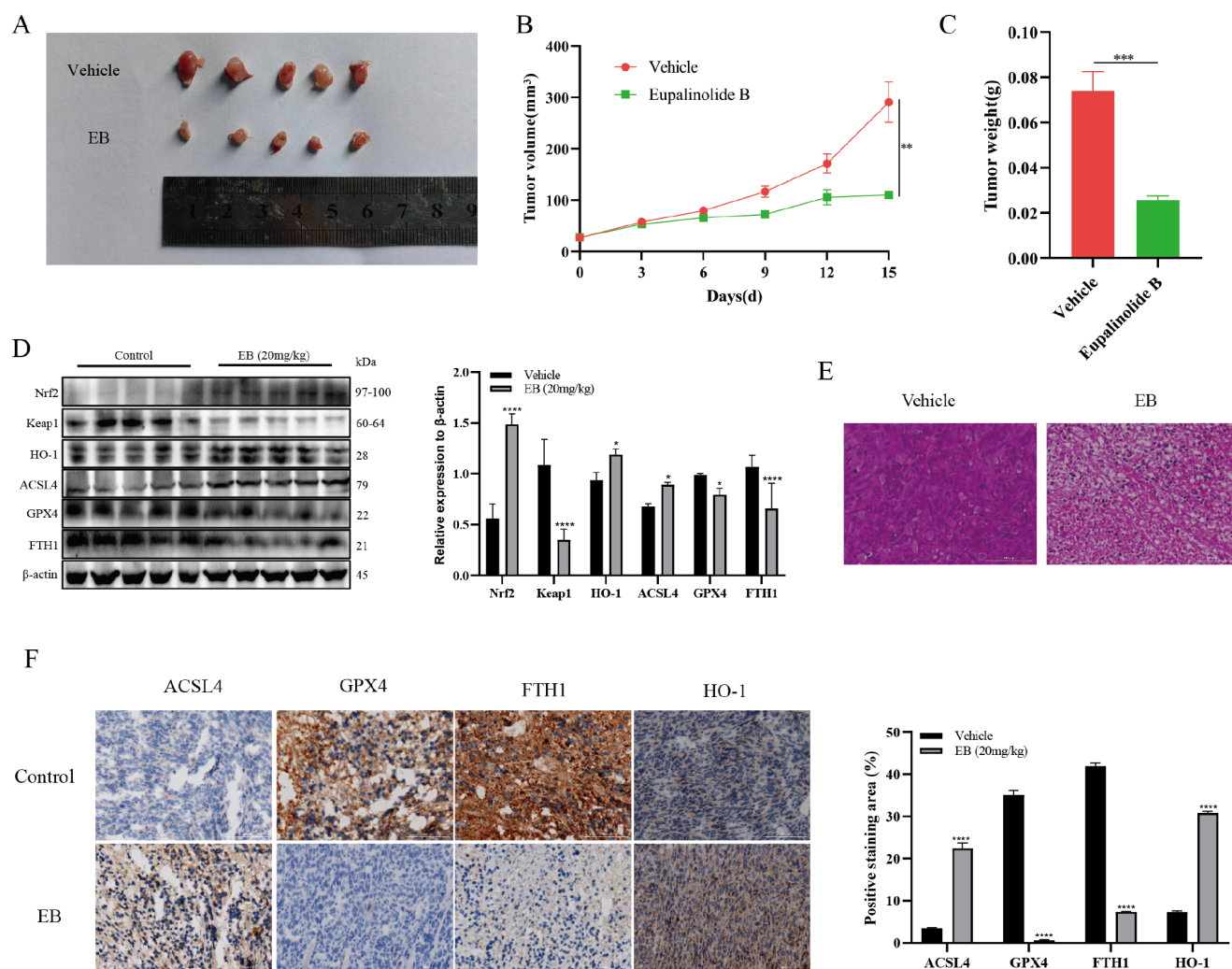


Figure 9. EB induces ferroptosis *in vivo* (A) Images of excised tumor bulks from each group. (B,C) Tumor volume and weight changes in each group. (D) Nrf2, Keap1, HO-1, ACSL4, GPX4 and FTH1 expression levels in tumor tissues were detected via Western blotting, and the relative intensities were quantified via ImageJ software. (E) H&E staining of tumor tissues under 200× magnification. (F) Images of ACSL4, GPX4, FTH1 and HO-1 immunohistochemical staining of tumor tissues under 200× magnification; the relative positive staining area was quantified via ImageJ software. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

- Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021, 71: 209–249
- Xu G, Wang H, Li X, Huang R, Luo L. Recent progress on targeting ferroptosis for cancer therapy. *Biochem Pharmacol* 2021, 190: 114584
- Miller M, Hanna N. Advances in systemic therapy for non-small cell lung cancer. *BMJ* 2021, 375: n2363
- Schabath MB, Cote ML. Cancer progress and priorities: lung cancer. *Cancer Epidemiol Biomarkers Prev* 2019, 28: 1563–1579
- Reck M, Carbone DP, Garassino M, Barlesi F. Targeting KRAS in non-small-cell lung cancer: recent progress and new approaches. *Ann Oncol* 2021, 32: 1101–1110
- Wong RS. Apoptosis in cancer: from pathogenesis to treatment. *J Exp Clin Cancer Res* 2011, 30: 87
- Yan J, Wan P, Choksi S, Liu ZG. Necroptosis and tumor progression. *Trends Cancer* 2022, 8: 21–27
- Patel NH, Bloukh S, Alwohosh E, Alhesa A, Saleh T, Gewirtz DA. Autophagy and senescence in cancer therapy. *Adv Cancer Res* 2021, 150: 1–74
- Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, Patel DN, *et al.* Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* 2012, 149: 1060–1072
- Jiang X, Stockwell BR, Conrad M. Ferroptosis: mechanisms, biology and role in disease. *Nat Rev Mol Cell Biol* 2021, 22: 266–282
- Ursini F, Maiorino M. Lipid peroxidation and ferroptosis: the role of GSH and GPX4. *Free Radical Biol Med* 2020, 152: 175–185
- Xie Y, Hou W, Song X, Yu Y, Huang J, Sun X, Kang R, *et al.* Ferroptosis: process and function. *Cell Death Differ* 2016, 23: 369–379
- Deng L, Tian W, Luo L. Application of natural products in regulating ferroptosis in human diseases. *Phytomedicine* 2024, 128: 155384
- Wang X, Ma S, Lai F, Wang Y, Lou C. Traditional applications, phytochemistry, and pharmacological activities of eupatorium lindleyanum DC: a comprehensive review. *Front Pharmacol* 2020, 8: 577124
- Zhang XW, Feng N, Liu YC, Guo Q, Wang JK, Bai YZ, Ye XM, *et al.* Neuroinflammation inhibition by small-molecule targeting USP7 non-catalytic domain for neurodegenerative disease therapy. *Sci Adv* 2022, 8:

- eabo0789
16. Yang L, Chen H, Hu Q, Liu L, Yuan Y, Zhang C, Tang J, *et al.* Eupalinolide B attenuates lipopolysaccharide-induced acute lung injury through inhibition of NF- κ B and MAPKs signaling by targeting TAK1 protein. *Int Immunopharmacol* 2022, 111: 109148
 17. Zhang Y, Zhang H, Mu J, Han M, Cao Z, Dong F, Wang T, *et al.* Eupalinolide B inhibits hepatic carcinoma by inducing ferroptosis and ROS-ER-JNK pathway. *Acta Biochim Biophys Sin* 2022, 54: 974–986
 18. Nguyen T, Nioi P, Pickett CB. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J Biol Chem* 2009, 284: 13291–13295
 19. Li J, Lu K, Sun F, Tan S, Zhang X, Sheng W, Hao W, *et al.* Panaxydol attenuates ferroptosis against LPS-induced acute lung injury in mice by Keap1-Nrf2/HO-1 pathway. *J Transl Med* 2021, 19: 96
 20. Tang Z, Ju Y, Dai X, Ni N, Liu Y, Zhang D, Gao H, *et al.* HO-1-mediated ferroptosis as a target for protection against retinal pigment epithelium degeneration. *Redox Biol* 2021, 43: 101971
 21. Wang M, Herbst RS, Boshoff C. Toward personalized treatment approaches for non-small-cell lung cancer. *Nat Med* 2021, 27: 1345–1356
 22. Lim TKH, Skoulidis F, Kerr KM, Ahn MJ, Kapp JR, Soares FA, Yatabe Y. KRAS G12C in advanced NSCLC: prevalence, co-mutations, and testing. *Lung Cancer* 2023, 184: 107293
 23. Huang S, Cao B, Zhang J, Feng Y, Wang L, Chen X, Su H, *et al.* Induction of ferroptosis in human nasopharyngeal cancer cells by cucurbitacin B: molecular mechanism and therapeutic potential. *Cell Death Dis* 2021, 12: 237
 24. Lei G, Zhuang L, Gan B. Targeting ferroptosis as a vulnerability in cancer. *Nat Rev Cancer* 2022, 22: 381–396
 25. Chen Y, Fang ZM, Yi X, Wei X, Jiang DS. The interaction between ferroptosis and inflammatory signaling pathways. *Cell Death Dis* 2023, 14: 205
 26. Zou Y, Schreiber SL. Progress in understanding ferroptosis and challenges in its targeting for therapeutic benefit. *Cell Chem Biol* 2020, 27: 463–471
 27. Zhao Y, Li Y, Zhang R, Wang F, Wang T, Jiao Y. The role of erastin in ferroptosis and its prospects in cancer therapy. *Onco Targets Ther* 2020, Volume 13: 5429–5441
 28. Kazan K, Kalaipandian S. Ferroptosis: yet another way to die. *Trends Plant Sci* 2019, 24: 479–481
 29. Zhang Z, Lu M, Chen C, Tong X, Li Y, Yang K, Lv H, *et al.* Holo-lactoferrin: the link between ferroptosis and radiotherapy in triple-negative breast cancer. *Theranostics* 2021, 11: 3167–3182
 30. Zhou S, Li D, Xiao D, Wu T, Hu X, Zhang Y, Deng J, *et al.* Inhibition of PKM2 enhances sensitivity of olaparib to ovarian cancer cells and induces DNA damage. *Int J Biol Sci* 2022, 18: 1555–1568
 31. Guo S, Zhong A, Zhang D, Gao J, Ni Y, Zhao R, Ma W. ATP2B3 inhibition alleviates erastin-induced ferroptosis in HT-22 cells through the P62KEAP1NRF2HO-1 pathway. *Int J Mol Sci* 2023, 24: 9199
 32. Luo L, Huang F, Zhong S, Ding R, Su J, Li X. Astaxanthin attenuates ferroptosis via Keap1-Nrf2/HO-1 signaling pathways in LPS-induced acute lung injury. *Life Sci* 2022, 311: 121091
 33. Chang LC, Chiang SK, Chen SE, Yu YL, Chou RH, Chang WC. Heme oxygenase-1 mediates BAY 11-7085 induced ferroptosis. *Cancer Lett* 2018, 416: 124–137
 34. Luo P, Liu D, Zhang Q, Yang F, Wong YK, Xia F, Zhang J, *et al.* Celastrol induces ferroptosis in activated HSCs to ameliorate hepatic fibrosis via targeting peroxiredoxins and HO-1. *Acta Pharm Sin B* 2022, 12: 2300–2314
 35. Lu MC, Zhao J, Liu YT, Liu T, Tao MM, You QD, Jiang ZY. CPUY192018, a potent inhibitor of the Keap1-Nrf2 protein-protein interaction, alleviates renal inflammation in mice by restricting oxidative stress and NF- κ B activation. *Redox Biol* 2019, 26: 101266
 36. Luo RR, Yang J, Sun YL, Zhou BY, Zhou SX, Zhang GX, Yang AX. Dexmedetomidine attenuates ferroptosis by Keap1-Nrf2/HO-1 pathway in LPS-induced acute kidney injury. *Naunyn Schmiedebergs Arch Pharmacol* 2024, 397: 7785–7796
 37. Dodson M, Castro-Portuguez R, Zhang DD. NRF2 plays a critical role in mitigating lipid peroxidation and ferroptosis. *Redox Biol* 2019, 23: 101107
 38. Sihvola V, Levonen AL. Keap1 as the redox sensor of the antioxidant response. *Arch Biochem Biophys* 2017, 617: 94–100
 39. Dinkova-Kostova AT, Kostov RV, Canning P. Keap1, the cysteine-based mammalian intracellular sensor for electrophiles and oxidants. *Arch Biochem Biophys* 2017, 617: 84–93
 40. Baird L, Swift S, Llères D, Dinkova-Kostova AT. Monitoring Keap1-Nrf2 interactions in single live cells. *Biotechnol Adv* 2014, 32: 1133–1144
 41. Lou L, Wang M, He J, Yang S, Meng F, Wang S, Jin X, *et al.* Urolithin A (UA) attenuates ferroptosis in LPS-induced acute lung injury in mice by upregulating Keap1-Nrf2/HO-1 signaling pathway. *Front Pharmacol* 2023, 14: 1067402
 42. Wei R, Zhao Y, Wang J, Yang X, Li S, Wang Y, Yang X, *et al.* Tagitinin C induces ferroptosis through PERK-Nrf2-HO-1 signaling pathway in colorectal cancer cells. *Int J Biol Sci* 2021, 17: 2703–2717
 43. Cai X, Hua S, Deng J, Du Z, Zhang D, Liu Z, Khan NU, *et al.* Astaxanthin activated the Nrf2/HO-1 pathway to enhance autophagy and inhibit ferroptosis, ameliorating acetaminophen-induced liver injury. *ACS Appl Mater Interfaces* 2022, 14: 42887–42903