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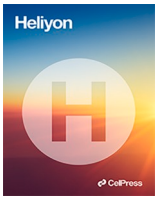

Research article

Naringenin enhances the efficacy of ferroptosis inducers by attenuating aerobic glycolysis by activating the AMPK-PGC1α signalling axis in liver cancer

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ABSTRACT

Liver cancer is a heterogeneous disease characterized by poor responses to standard therapies and therefore unfavourable clinical outcomes. Understanding the characteristics of liver cancer and developing novel therapeutic strategies are imperative. Ferroptosis, a type of programmed cell death induced by lipid peroxidation, has emerged as a potential target for treatment. Naringenin, a natural compound that modulates lipid metabolism by targeting AMPK, shows promise in enhancing the efficacy of ferroptosis inducers. In this study, we utilized liver cancer cell lines and xenograft mice to explore the synergistic effects of naringenin in combination with ferroptosis inducers, examining both phenotypic outcomes and molecular mechanisms. Our study results indicate that the use of naringenin at non-toxic doses to hepatocytes can significantly enhance the anticancer effects of ferroptosis inducers (erastin, RSL3, and sorafenib). The combination index method confirmed a synergistic effect between naringenin and ferroptosis inducers. In comparison to naringenin or ferroptosis inducers alone, the combined therapy caused more robust lipid peroxidation and hence more severe ferroptotic damage to cancer cells. The inhibition of aerobic glycolysis mediated by the AMPK-PGC1α signalling axis is the key to naringenin's effect on reducing ferroptosis resistance in liver cancer, and the synergistic cytotoxic effect of naringenin and ferroptosis inducers on cancer cells was reversed after pretreatment with an AMPK inhibitor or a PGC1α inhibitor. Taken together, these findings suggest that naringenin could boost cancer cell sensitivity to ferroptosis inducers, which has potential clinical translational value.

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1. Introduction

Liver cancer stands as one of the most widespread and deadliest malignancies worldwide, with its incidence rate steadily rising each year on a global scale [1]. Primary liver cancer is distinguished by its aggressiveness, rapid proliferation, and high recurrence rate [2]. Despite radical treatments like liver transplantation and surgical resection being potential treatment options for liver cancer, less than 30 % of patients are qualified to such treatment at the initial stage of diagnosis due to the insidious nature of the disease. Therefore, systemic anti-tumor therapies, including targeted drug therapy and immunotherapy, are crucial in treating mid-to late-stage liver cancer [3]. Most conventional anticancer medications achieve their therapeutic effects through the induction of apoptosis. Nonetheless, due to the diverse physical conditions of individual patients, there is frequently intrinsic or acquired resistance to apoptosis, resulting in limited drug efficacy [4]. As a result, novel treatment pathways based on mechanisms of alternative cell death are constantly being researched to increase overall therapeutic efficacy. Among the several possible pathways, targeting ferroptosis has promising clinical translation potential.

Ferroptosis, a type of programmed cell death reliant on iron and distinct from apoptosis, was initially discovered in 2012. This process is characterized by the buildup of free iron and cytotoxic lipid reactive oxygen species (ROS), leading to the degradation and perforation of lipid membranes. Key aspects of ferroptosis include increased ROS levels, lipid peroxidation, and the impairment of the antioxidant defense system involving glutathione peroxidase 4 (GPX4) and glutathione (GSH) [5]. Erastin and RSL3 are classical ferroptosis inducers [6,7] and the clinical first-line chemotherapy drug sorafenib can also induce ferroptosis [8,9]. Recent research has indicated that heightened extracellular lactate levels can confer resistance in liver cancer cells to ferroptosis induced by sorafenib and RSL3 [10]. Consequently, it is suggested that the responsiveness of liver cancer cells to ferroptosis-inducing agents like RSL3, sorafenib, and erastin could be enhanced by decreasing lactate levels, elevating ROS levels, and reducing GSH levels.

Cancer cells commonly exhibit a characteristic where their survival and proliferation are enhanced through the frequent conversion of pyruvate to lactate via the lactate pathway to produce energy, a phenomenon known as aerobic glycolysis, in contrast to the tricarboxylic acid cycle utilized by normal cells [11]. Hence, multiple enzymes are abnormally expressed in tumors compared to normal tissues during the process of glycolysis. For instance, pyruvate kinase [12] and lactate dehydrogenase [13] exhibit dysregulation in tumor cells. AMP-activated protein kinase (AMPK), which inhibits the process of glycolysis, is a crucial energy sensor and the regulatory center for metabolic homeostasis in mammalian cells [14]. It has been shown that activation of AMPK by genetic interference or pharmacological intervention might increase the toxicity of the FDA-approved anticancer medication sorafenib [15,16]. Therefore, we hypothesized that reducing lactate content by inhibiting glycolysis through activation of AMPK could enhance sensitivity to ferroptosis inducers.

The natural citrus flavonoid naringenin [17], which is abundant in medicinal plant, various fruits and vegetables, is an appropriate component for testing this hypothesis for two reasons. First, Research indicates that naringenin induces the activation of the AMPK system in various models and has been found to exhibit significant metabolic regulatory abilities by modulating several glycolysis-related enzymes, including LDHA and PKM2 [18–21]. Extensive and increasing preclinical evidence for its anticancer effect is accumulating [22,23]. Some of the pharmacologic properties of naringenin resemble those of ferroptosis inducers, including the promotion of ROS accumulation in cells [24], an accumulation in lipid peroxidation [25], and a decline in glutathione content [26]. This naringenin-mediated control of cellular energy metabolism and molecular mechanisms may also occur in liver cancer cells, suggesting that naringenin may interact synergistically with ferroptosis inducers to induce ferroptosis. Second, the findings of a recent clinical trial [27] on the safety and pharmacokinetics of naringenin were published and revealed no serious adverse effects in adults at doses of 150, 300, 600, and 900 mg, a lower likelihood of minor adverse effects in the treatment group than in the placebo group, and effective clearance within 24 h. With ongoing research and investigation of naringenin dosage forms and dosing strategies, naringenin is expected to have broad therapeutic application potential.

In this study, we studied the synergistic effect of naringenin with ferroptosis inducers in liver cancer, as well as the underlying processes. Our comprehensive molecular and functional analyses demonstrated that naringenin controls aerobic glycolysis in liver cancer through the AMPK-PGC1α axis and does so at concentrations nontoxic for normal cells. It also interacts synergistically with ferroptosis inducers to damage liver cancer cells at nontoxic concentrations. Therefore, combining naringenin with ferroptosis inducers may be a potential technique to enhance the effects of treatment for liver cancer.

2. Materials and methods

2.1. Reagents and antibodies

Naringenin (Nar), deferoxamine (DFO) mesylate and ferrostatin-1 (Fer-1) were obtained from Yuanye Bio-Technology (Shanghai, China); erastin and SR-18292 were obtained from Macklin (Shanghai, China); Sorafenib (Sora) and Compound C (Comp C) were obtained from MedChemExpress (Princeton, New Jersey, USA); RSL3 was obtained from Topscience (Shanghai, China); N-acetyl-L-cysteine (NAC), z-VAD-FMK (Z-VAD), Necrostatin-1 (Necro-1) and other chemicals were purchased from Beyotime (Shanghai, China).

Specific primary antibodies against GLUT4 (66846-1-Ig), Cyclin D1 (60186-1-Ig), Cyclin E1 (11554-1-AP) were purchased from Proteintech (Wuhan, China); LDHA (YN033) and GLUT1 (YT1928) were purchased from Immunoway (Hunan, China); PGC1α (#2178) was purchased from CST (Massachusetts, USA); β-actin (ab8227), PDK-1 (ab207450), p-AMPKα1/2 (ab133448), AMPKα1/2 (ab207442), CDK6 (ab124821), CDK2 (ab32147) were purchased from Abcam (United Kingdom).

2.2. Cell culture

The HepG2, Hep3B and SNU182 cell lines were procured from the ATCC (Manassas, USA). The Huh-7 cell line was purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The MIHA cell line was sourced from Fenghui Biotechnology (Hunan, China). STR authentication was performed on all cell lines. The cells were cultured in DMEM or RPMI-1640 media, supplemented with 10 % fetal bovine serum (Kibbutz Beit HaEmek, Israel), and incubated in a humidified atmosphere with 5 % CO2 at 37 °C.

2.3. Cell viability and proliferation assays

Cell viability was evaluated by CCK-8 assay (Dojindo, Kumamoto, Japan). The combination drug efficacy was evaluated using CompuSyn software through the calculation of Combination Index (CI) [28]. Two methods were employed to observe cell proliferation. In the first method, colony formation assay was conducted to evaluate the prolonged effects of the drug on cell proliferation. In brief, 1500 cells were seeded in 6-well plates, with medium replacement every 3 days. After two-week incubation, cells were fixed, stained with crystal violet, washed, and subsequently visualized. The second method involved EdU incorporation assay. Cells cultivated in 96-well plates received drug treatment for 24 h, followed by an 8-h EdU (Beyotime, China) staining. Subsequent procedures were conducted as per the manufacturer's guidelines. Cell nuclei stained with Hoechst 33342. Cell visualization was carried out using fluorescence microscopy (Olympus, Japan).

2.4. Glucose uptake and ROS assays

DCFH-DA (Beyotime, China) was utilized to detect ROS. Cellular glucose uptake was evaluated by using an assay kit provided by Dojindo Laboratories (Kumamoto, Japan). A total of 10000 cells were cultured with drugs for 12 or 24 h, after which glucose uptake and ROS were quantified by the protocol from the manufacturer. The fluorescence signals were analyzed by a microplate reader (BioTek).

2.5. GSH, MDA, ATP and lactate content assays

The GSH, MDA and ATP contents were measured following the protocols supplied by Beyotime Biotechnology (Shanghai, China) [29]. The lactate content was determined with the kit by measuring the colour reaction of WST to quantify the lactate content of the cell culture supernatant according to the instructions provided by Dojindo Laboratories (Kumamoto, Japan). All final results were obtained by the absorbance detection function of the microplate reader (BioTek).

2.6. Lipid peroxidation assay

Twenty-four hours before the treatment, 10^5 HepG2 cells were plated in six-well plates. The cells were subsequently exposed to the specified compounds for the specified period of time. Intracellular levels of lipid hydroperoxides were assessed by utilizing the Liperfluo probe. (Dojindo, Kumamoto, Japan) [30].

2.7. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen) and reverse transcribed with the cDNA Synthesis kit (TaKaRa, Otsu, Japan). qRT-PCR was conducted using SYBR PremixEx TaqTM (TaKaRa). The ACTB gene served as an internal housekeeping control. Primers used are listed in Table 1.

2.8. Western blot

Proteins in cell lysates were extracted. And proteins were transferred to PVDF membranes using SDS-PAGE method. Following blocking with nonfat milk in TBST, the membranes were sequentially exposed to the primary antibody and the secondary antibody (LI-COR, Lincoln, NE, USA). Subsequently, Data visualization and quantification were performed by the Odyssey system.

Table 1
Primer sequences information.

Gene	Species	Forward (5'→3')	Reverse (5'→3')
GLUT4	Human	TTTTGAGATTGGCCCTGGCCCCAT	CTCAGGTACTCTTAAGAAGGTGAAG
LDHA	Human	ATGGCAACTCTAAAGGATCA	GCAACTTGCAGTTCGGGC
PDK1	Human	CTGTGATACGGATCAGAAACCG	TCCACCAAACAATAAAGAGTGCT
β-ACTIN	Human	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA

2.9. Bioinformatic data

Liver hepatocellular carcinoma (LIHC) dataset from The Tumor Genome Atlas (TCGA) were analyzed using GEPIA2 (http://gepia2.cancer-pku.cn/#index) [30].

2.10. Molecular docking

The crystal structure of the human AMPK kinase protein (PDB code: 7M74, resolution: 3.93 Å) [31] was retrieved from the RCSB protein database (http://www.rcsb.org/). Subsequently, the protein polymorph was split using PyMOL software to obtain the AMPKα

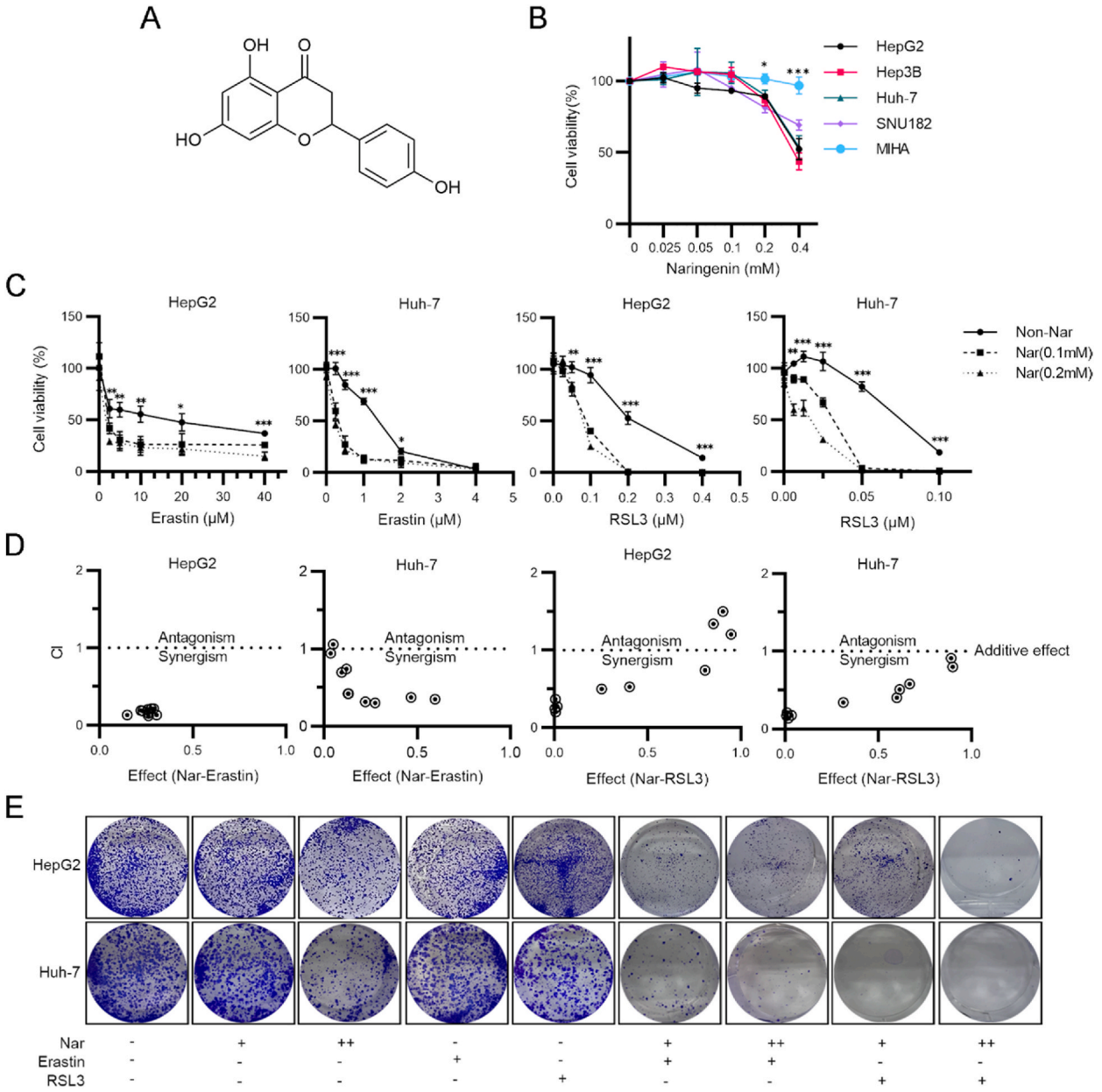

Fig. 1. The actions of naringenin and erastin/RSL3 are synergistic. (A) Chemical structure of naringenin. (B) The viability of cells post 24-h naringenin treatment was assessed using CCK-8 assay. (C) The impact of co-administration of naringenin and ferroptosis inducers on the viability of liver cancer cells was assessed by CCK-8 assay. (D) Combination index analysis of the induction of differentiation in liver cancer cells treated with combinations of Nar and erastin/RSL3. A combination index of 1.0 indicates additive effects, whereas combination indexes of greater than and less than 1.0 indicate antagonism and synergy, respectively. (E) The cell proliferation rate was assessed using clone formation assay. *p < 0.05, **p < 0.01, ***p < 0.001, n = 3 per group.

crystal structure for use as a molecular docking acceptor structure. The 3D structure of naringenin was sourced from PubChem (http://pubchem.ncbi.nlm.nih.gov/) and converted to MOL2 format through PyMOL software for utilization as the ligand structure in the molecular docking process. Molecular docking was performed using AutoDockTools software to obtain docking data. Finally, PyMOL software was employed to visualize the results.

2.11. Animal experiments

The mouse experiment was approved by the Guangxi Medical University Institutional Animal Care and Use Committees (approval no. 20210073) and conducted following the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. A total of 30 female BALB/c nude mice (Guangxi Medical University Laboratory Animal Center, Nanning, China) were maintained under standard conditions. HepG2 cells (approximately 5 million) were inoculated into the right flanks of the mice. Once the tumour volume reached 80–100 mm^3, the mice were randomly divided into 6 groups. They were given naringenin, RSL3, sorafenib, naringenin + RSL3, naringenin + sorafenib and an equal volume of PBS (control) by gavage every other day, respectively. The dosages of naringenin, RSL3, and sorafenib were 50 mg/kg, 5 mg/kg, and 20 mg/kg of mouse weight, respectively. The tumour nodules' size was assessed daily using digital caliper, and the volume was calculated employing the formula (length × width2)/2. After 14 days, the mice were anesthetized with 1.5 % isoflurane gas and then euthanized by cervical dislocation.

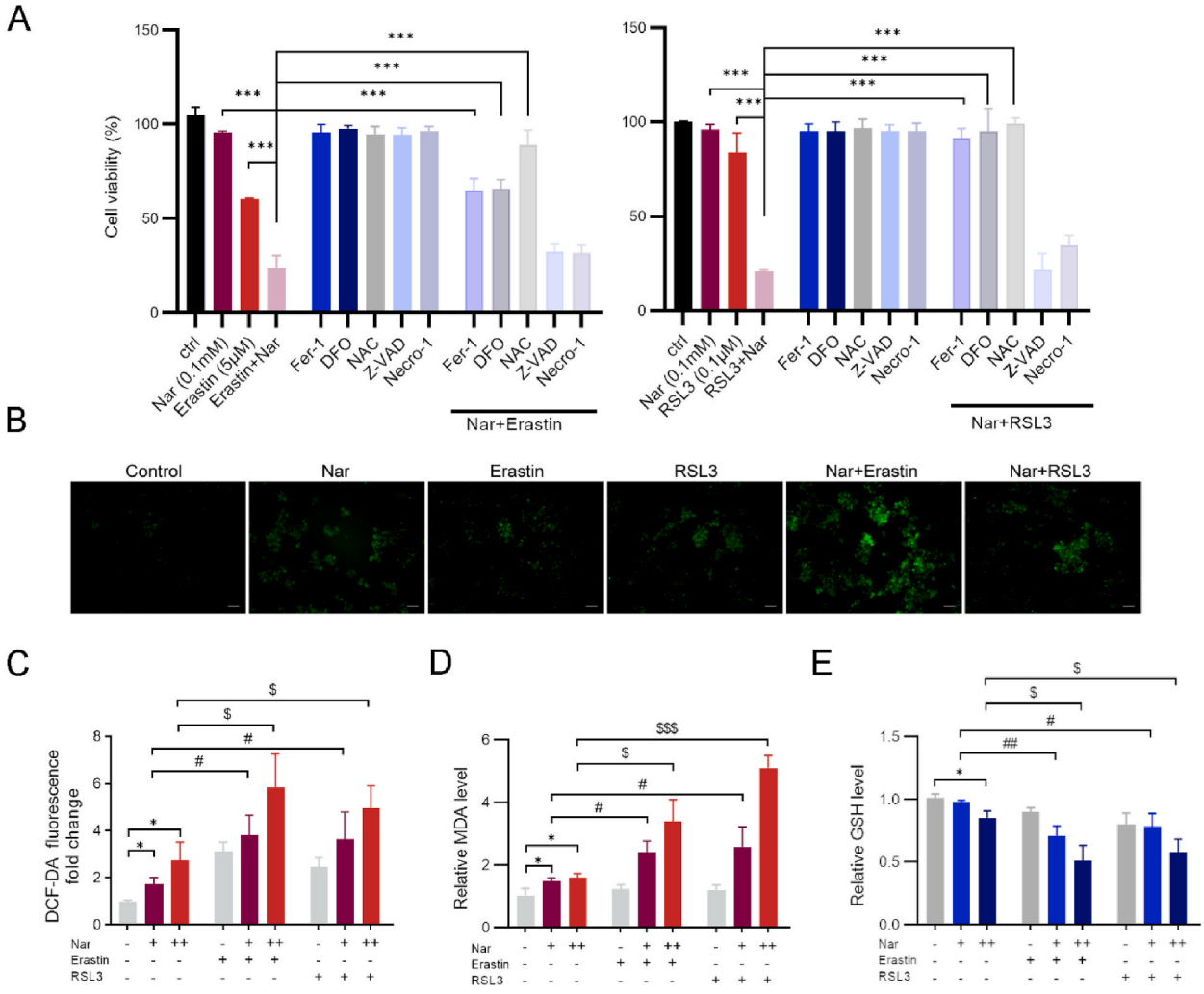

Fig. 2. Naringenin sensitizes liver cancer cells to erastin/RSL3-induced ferroptosis. (A) HepG2 cells were subjected to combined treatment with naringenin and a ferroptosis inducer, with or without pre-treatment with various programmed cell death inhibitors, and cell viability was assessed by CCK-8 assay. (B) Fluorescent imaging of lipid peroxides in HepG2 cells. The doses of Naringenin, erastin and RSL3 were fixed at 0.1 mM, 5 μM and 1 μM, respectively. Scale bar: 20 μm. (C–E) The combined treatment of naringenin and ferroptosis was examined for its impact on ROS levels (C), lipid peroxidation (D), and GSH levels (E) in HepG2 cells. The doses of naringenin, erastin, and RSL3 used were consistent with those indicated in Panel A. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 3$ per group.

2.12. Hematoxylin and eosin (H&E) staining

Briefly, tissue samples were first deparaffinized, followed by sectioning and staining with 0.5 % hematoxylin and eosin. The imaging was performed using an optical microscope (Olympus, Japan).

2.13. Statistical analysis

All statistical analyses were performed using GraphPad Prism 9 software. Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test, two-sided student's *t*-test and Pearson correlation coefficient as indicated. A P value < 0.05 was regarded as statistically significant.

3. Results

3.1. Naringenin sensitizes liver cancer cells to RSL3-and erastin-induced ferroptosis

Structure of naringenin is illustrated in Fig. 1A. We first examined toxic effects of naringenin on liver cancer cells and hepatic cells. Naringenin was found to be toxic to liver cancer cells at 0.2 mM, and 0.4 mM but not to hepatic cells. Concentrations below 0.2 mM were not toxic to either type of cell (Fig. 1B). When combined with escalating doses of erastin or RSL3, known ferroptosis inducers, naringenin further suppressed cell proliferation (Fig. 1C). The synergistic effect was obvious, even at nontoxic concentrations of erastin or RSL3 (Fig. 1E). The combination index method confirmed that the combined effect of ferroptosis inducers and naringenin was synergistic (Fig. 1D). Consequently, subsequent experiments utilized the following combinations and concentrations to investigate the molecular mechanisms underlying their synergy: less than 0.2 mM naringenin, 5 μM erastin, and 0.1 μM RSL3 for HepG2 cells, and 1 μM erastin and 0.05 μM RSL3 for Huh-7 cells.

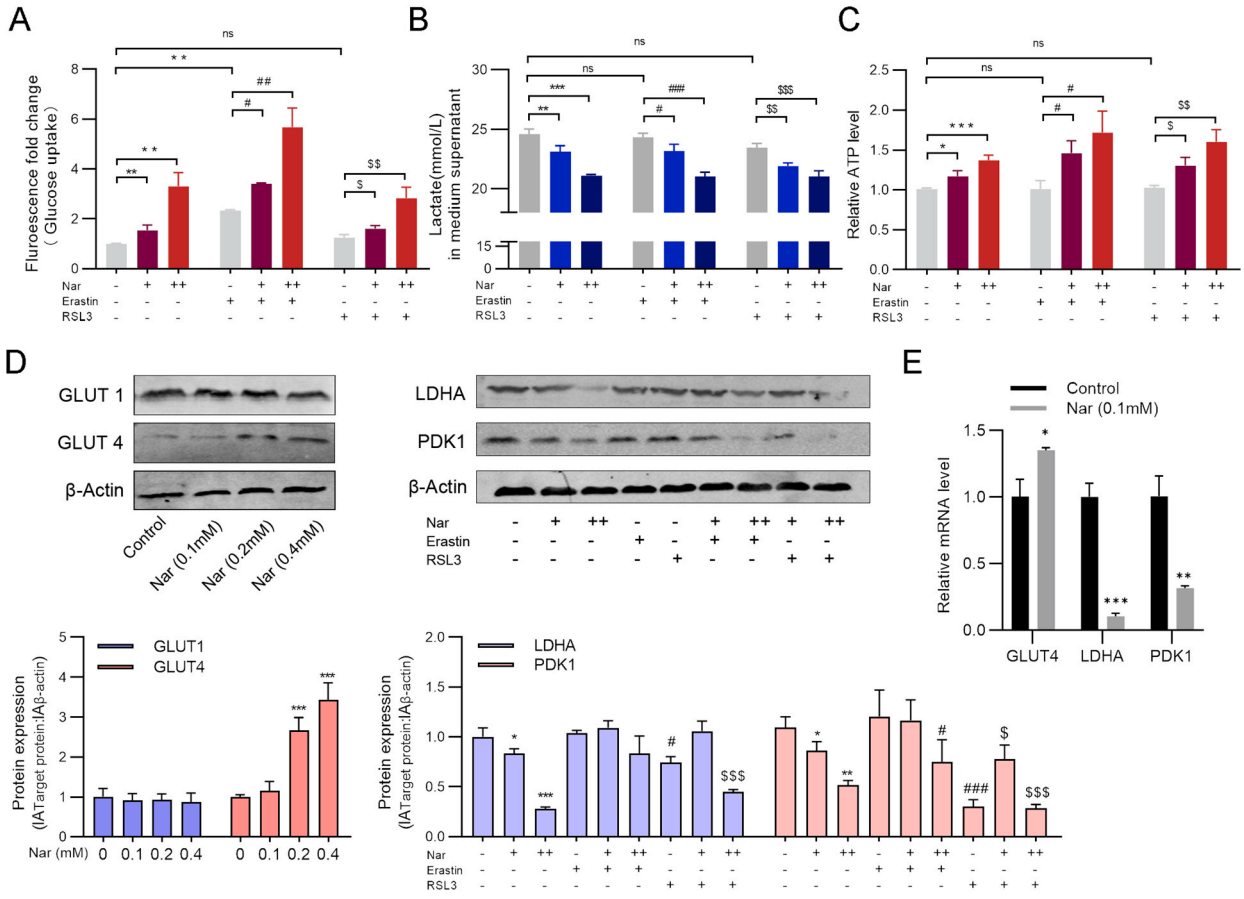

Fig. 3. Naringenin inhibits aerobic glycolysis in liver cancer cells. HepG2 cells were treated with Nar (0.1 mM/0.2 mM) and/or erastin (5 μM)/RSL3 (0.1 μM). (A) The glucose uptake levels of the cell. (B) Cellular lactate production. (C)the intracellular ATP level of the cells. (D) Western blot was utilized to determine the protein expression levels of GLUT1, GLUT4, LDHA, and PDK1 in HepG2 cells. (E) qRT-PCR was utilized to determine the mRNA expression levels of GLUT1, GLUT4, LDHA, and PDK1 in HepG2 cells. *P < 0.05, **P < 0.01, ***P < 0.001, n = 3 per group.

Cell survival analysis indicated a notable decrease in cell viability following the combined treatment. Importantly, pretreatment with DFO (an iron chelator), Fer-1 (a peroxidation inhibitor) and NAC (an ROS scavenger) effectively prevented this cell death, whereas inhibitors targeting distinct cell death pathways such as z-VAD (apoptosis inhibitor) and Necro-1 (necroptosis inhibitor) did not show the same protective effect (Fig. 2A, Fig. S1A). We thus speculated that the synergistic effect of naringenin with erastin and

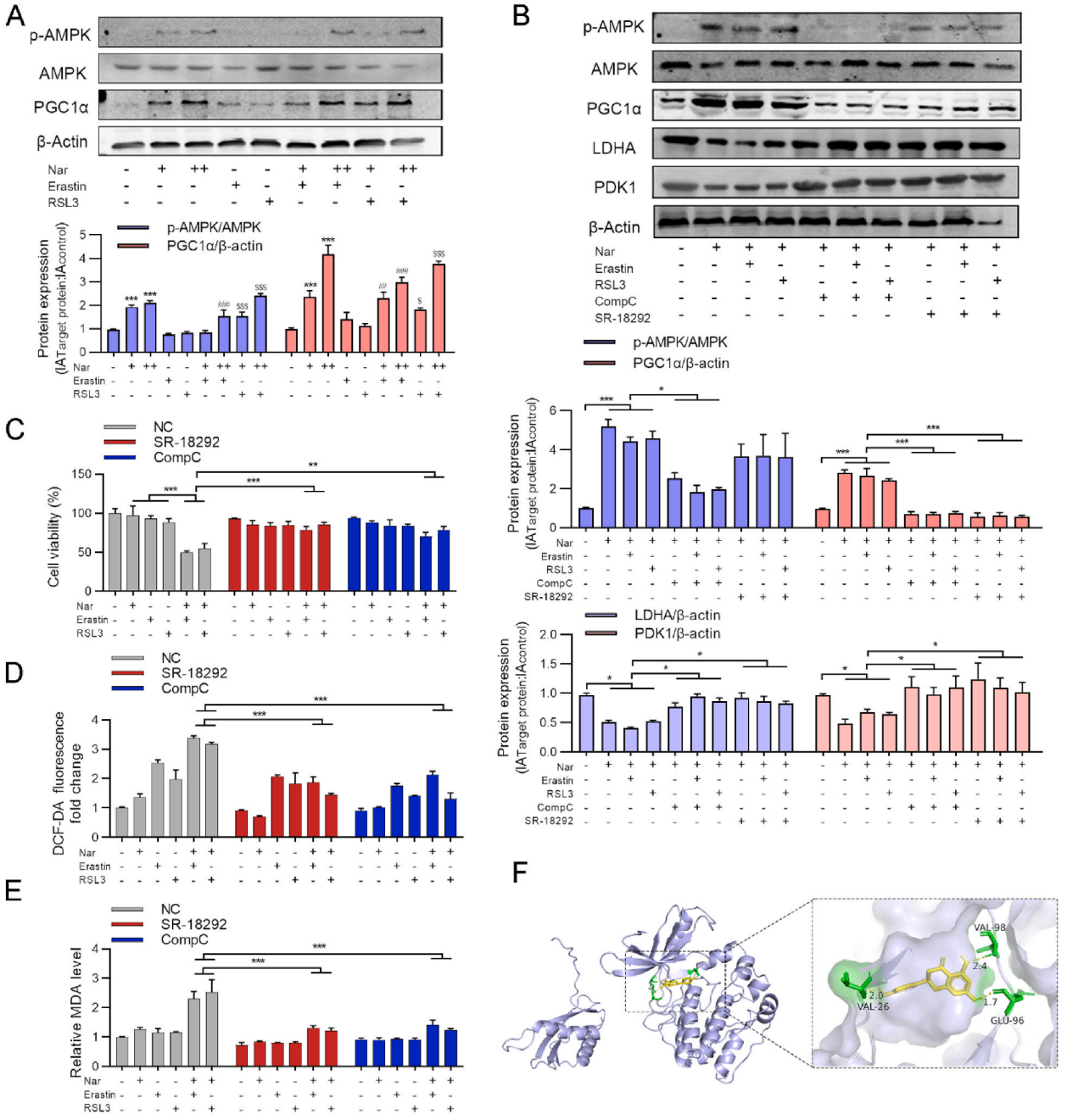

Fig. 4. By upregulating the AMPK-PGC1α signalling axis, naringenin inhibits aerobic glycolysis and thus reduces ferroptosis resistance in liver cancer cells. (A) The impact of the combined treatment of naringenin and ferroptosis on AMPK phosphorylation and PGC1α was assessed using Western blot. (B) HepG2 cells were treated with naringenin and/or ferroptosis inducers with or without pretreatment with different inhibitors for 24 h. These inhibitors included Compound C (a selective and ATP-competitive AMPK inhibitor, 4 μM) and SR-18292 (a PGC1α inhibitor, 25 μM). Western blot was utilized to determine the protein levels of p-AMPK, PGC1α, LDHA, and PDK1. (C–E) HepG2 cells were treated with a combination of naringenin and ferroptosis inducers, with or without pre-treatment of compound C or SR-18292. Subsequently, cellular viability (C), ROS levels (D), and lipid peroxidation levels (E) were assessed. (F) The molecular docking of naringenin with AMPKα. *P < 0.05, **P < 0.01, ***P < 0.001, n = 3 per group.

RSL3 induces ferroptosis in liver cancer.

To investigate this speculation, we examined the imapct of naringenin with ferroptosis inducers on ferroptotic biochemical processes, including the total ROS accumulation and lipid peroxidation and the reduction in intracellular glutathione (GSH) content [5]. Liver cancer cells were treated with naringenin and ferroptosis inducers, and relevant kits were used to detect related indicators. Unsurprisingly, naringenin increased ROS accumulation (Fig. 2C, Fig. S1B) as well as lipid peroxidation (Fig. 2B and. D, Fig. S1C) and reduced GSH production (Fig. 2E, Fig. S1D), and the above effects were similarly enhanced by combining it with a concentrations of ferroptosis inducers that were not effective alone in inducing the above effects.

The collective findings suggest that the synergistic impacts of naringenin in combination with erastin and RSL3 involve enhancing the susceptibility of liver cancer cells to ferroptosis induced by erastin and RSL3.

3.2. Naringenin inhibits liver cancer aerobic glycolysis

Next, we sought to investigate how naringenin increases the effect of the ferroptosis inducers. Recent research by Youbo Zhao et al. unveiled a lactate-mediated pathway that regulates ferroptosis, where lactate boosts resistance to ferroptosis via increasing the synthesis of unsaturated fatty acids [10]. Tumour cells, including liver cancer cells, take up glucose and metabolize it into pyruvate, which favours the replacement of the tricarboxylic acid cycle used in normal cells with the lactate pathway for energy production, a phenomenon called the Warburg effect [11]. This suggests that inhibition of tumour aerobic glycolysis and thus lactate production by tumour cells is a possible mechanism by which naringenin increases the toxic effect of ferroptosis inducers. To test this hypothesis, we measured glucose uptake, lactate levels, and ATP production in liver cancer cells treated with naringenin alone or in combination with erastin/RSL3. Our results demonstrated that naringenin dose-dependently increased cellular glucose uptake and ATP production while reducing lactate production in HepG2 (Fig. 3A–C) and Huh-7 cells (Figs. S2A–C). The modulation of glycolysis-associated proteins, such as GLUT4, LDHA, and PDK1, by naringenin further supported these findings (Fig. 3D–E). Interestingly, the addition of ferroptosis inducers did not significantly enhance the inhibitory effect on glycolysis, indicating that naringenin acts unilaterally in inhibiting glycolysis without synergizing with ferroptosis inducers.

In conclusion, naringenin decreases the resistance of liver cancer cells to ferroptosis by suppressing aerobic glycolysis.

3.3. By upregulating AMPK-PGC1α signalling axis, naringenin inhibits aerobic glycolysis and thus reduces ferroptosis resistance in liver cancer cells

To investigate the mechanism by which the inhibition naringenin inhibits glycolysis and reduces the tolerance of liver cancer cells to ferroptosis, we conducted a study to examine the impact of naringenin on PGC1α expression. Because Wenxin Qin's group recently reported [32] that overexpression of PGC1α could inhibit glycolysis by downregulating LDHA and PDK1 expression, which aligns with the observations detailed in the second section of our manuscript. Therefore, we posit that this provides a plausible explanation. Furthermore, naringenin was shown to activate the AMPK signalling pathway in a nonalcoholic fatty liver model, increasing energy expenditure, and PGC1α was proposed to be the downstream effector protein of this pathway [18]. As shown in Fig. 4A, treatment with naringenin alone and in combination with a ferroptosis inducer for 24 h effectively increased PGC1α expression at the protein levels, whereas the ferroptosis inducer alone did not have this effect. Consistent results were obtained from the evaluation of proteins related to the AMPK signalling pathway.

To better understand how naringenin regulates aerobic glycolysis via the AMPK-PGC1α signalling axis, we planned to use Compound C (a selective and ATP-competitive AMPK inhibitor) and SR-18292 (a PPAR gamma coactivator-1α (PGC1α) inhibitor) to pretreat the cells, create an AMPK inhibition model and a PGC1α inhibition model, treat the models with naringenin alone or in combination with a ferroptosis inducer, and measure the protein expression of LDHA and PDK1 in the models. After inhibition of the APMK-PGC1α regulatory axis, naringenin could no longer regulate LDHA and PDK1 activity, as shown in Fig. 4B. In the AMPK and PGC1α inhibition models, naringenin had no synergistic toxic effect with ferroptosis inducers (Fig. 4C, Fig. S3A), and the characteristics of ferroptosis were lost (Fig. 4D–E, Figs. S3B–C).

The AMPK pathway has been extensively researched as a signalling pathway that regulates bodily metabolism, but its direct relationship with ferroptosis has received little attention and thus piqued our interest. AMPK is a multiprotein complex that includes a1, a2, b1, b2, g1, g2 and g3 subunits and is the protein farthest upstream of the AMPK pathway. We investigated the relationship between the protein level of each AMPK complex subunit and those of the ferroptosis markers GPX4 [33], ACSL4 [8], and PTGS2 [33] by extracting gene expression data from TCGA-LIHC using bioinformatics methods and found that ferroptosis induction led to an upregulation of ACSL4 expression and a downregulation of GPX4 expression. Among all subunits, AMPKα1 expression shown a negative correlation with GPX4 expression and positive correlation with ASCL4 and PTGS2 expression (Fig. S3D, Table S1). The above results confirm that AMPKα1 expression is correlated with ferroptosis in liver cancer.

In addition, we obtained the protein structure of AMPKα1 from the PDB database, molecularly docked it with naringenin, and found that naringenin bound tightly to AMPKα1 in multiple modes (Table S2). The optimal binding conformation is shown in Fig. 4F, with a binding energy of −5.74 kJ/mol. This result supports the role of naringenin in regulating the AMPK pathway. Therefore, these findings have shown that naringenin's inhibitory impact on aerobic glycolysis led to a decrease in liver cancer resistance to ferroptosis by modulating the AMPK-PGC1α pathway.

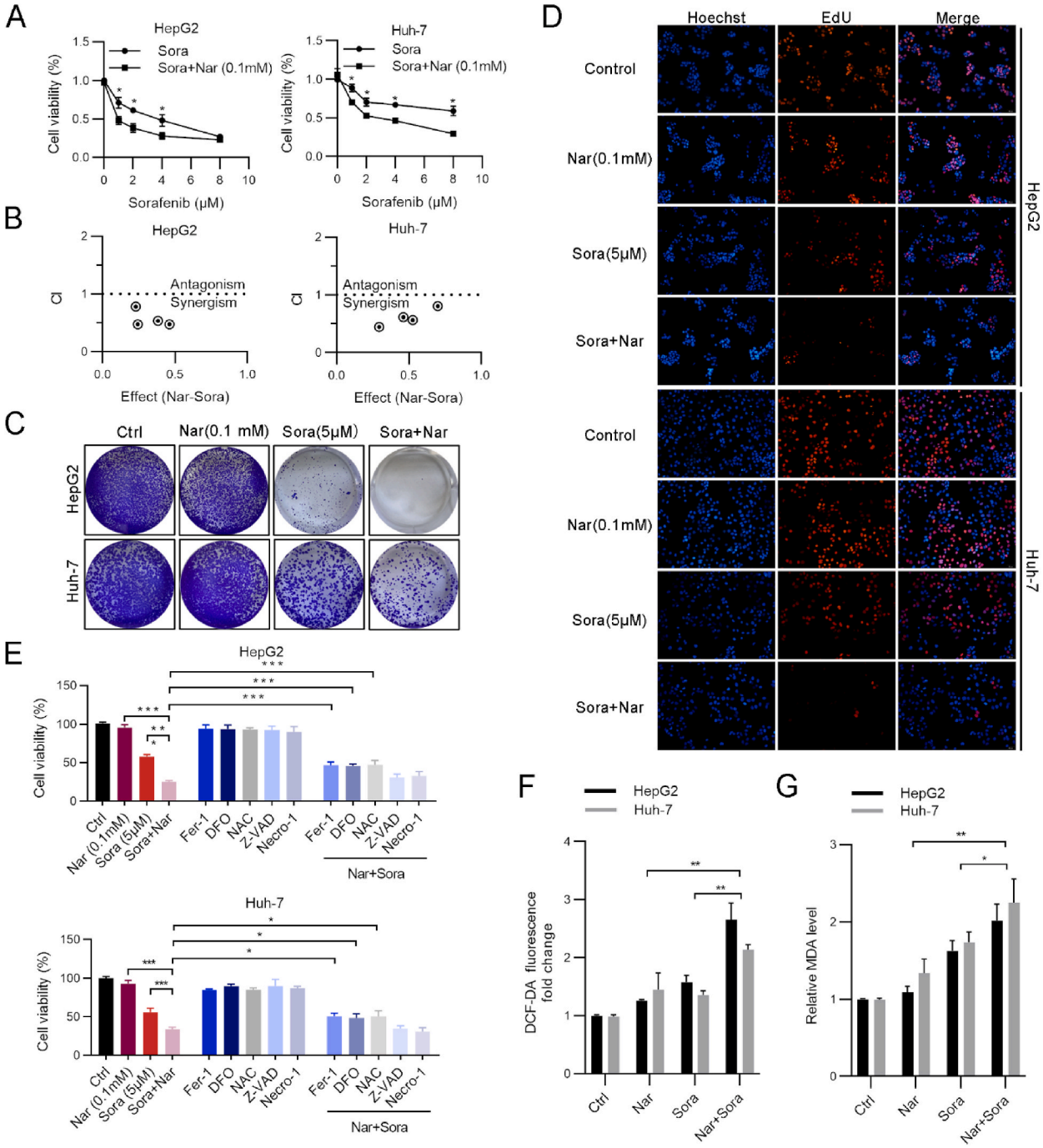

Fig. 5. Naringenin impairs the growth of liver cancer with sorafenib (Sora) in vitro. (A) The combined treatment of naringenin and sorafenib influences the cell viability of liver cancer cells. (B) Combination index analysis of the induction of liver cancer cells treated with combinations of naringenin and sorafenib. (C–D) The impact of combined treatment with naringenin and sorafenib on cell proliferation in liver cancer cells was assessed through clone formation (C) and EdU (D) assay. (Magnification, 400 ×) (E) The impact of various programmed cell death inhibitors on the potentiation of naringenin and sorafenib combination treatment in suppressing liver cancer cell viability. (F–G) The impact of the combined treatment of naringenin and sorafenib on the levels of ROS (F) and lipid peroxidation (G) in liver cancer cells. *P < 0.05, **P < 0.01, ***P < 0.001, n = 3 per group.

3.4. Naringenin impairs the growth of liver cancer cells treated with sorafenib

Sorafenib, a primary treatment in clinical practice for liver cancer, has been found to trigger ferroptosis [34]. Being among the extensively utilized targeted medications for liver cancer treatment over the past decade, the resistance of tumors to sorafenib has been a well-recognized challenge. Our previous study suggests that naringenin holds promise in addressing this issue.

Fig. 5A–D illustrates that sorafenib decreased the viability of HepG2 and Huh-7 cells in a dose-dependent fashion. We added a nontoxic concentration of naringenin to assess the possible synergistic impact of naringenin with sorafenib, and the findings revealed that the combination significantly decreased the activity of liver cancer cells compared to sorafenib alone. The combination index method validated the synergistic effect of sorafenib and naringenin (Fig. 5B). Correspondingly, this combined treatment led to a substantial rise in both ROS content and lipid peroxidation in comparison to individual treatments alone (Fig. 5F–G). In addition, pretreatment with ferroptosis, apoptosis, and necrosis inhibitors reduced the lethal effects of both sorafenib and naringenin, indicating that the synergistic impact of naringenin and sorafenib is attributable to ferroptosis in liver cancer (Fig. 5E).

3.5. Naringenin increases the efficacy of ferroptosis inducers in vivo

To expand on our in vitro findings, we examined whether naringenin has synergistic toxic effects with ferroptosis inducers on liver cancer in vivo. In clinical trials [27], doses of naringenin up to 600 mg/day were deemed safe for adults, which corresponds to a mouse equivalent dose of about 120 mg/kg according to Food and Drug Administration guidelines [35]. In addition, in many xenograft models, a naringenin concentration of 50 mg/kg has been demonstrated to be the anticancer starting concentration [36–38]. On this basis, we selected 50 mg/kg as the dose of naringenin to be utilized in the in vivo combination therapy with sorafenib (20 mg/kg) or RSL3 (5 mg/kg). As shown in Fig. 6A–C, naringenin enhanced the inhibitory effects of RSL3 and sorafenib on tumour growth compared to treatment with RSL3 or sorafenib alone. Histological examination of heart, liver, spleen, lung, and kidney tissues revealed no

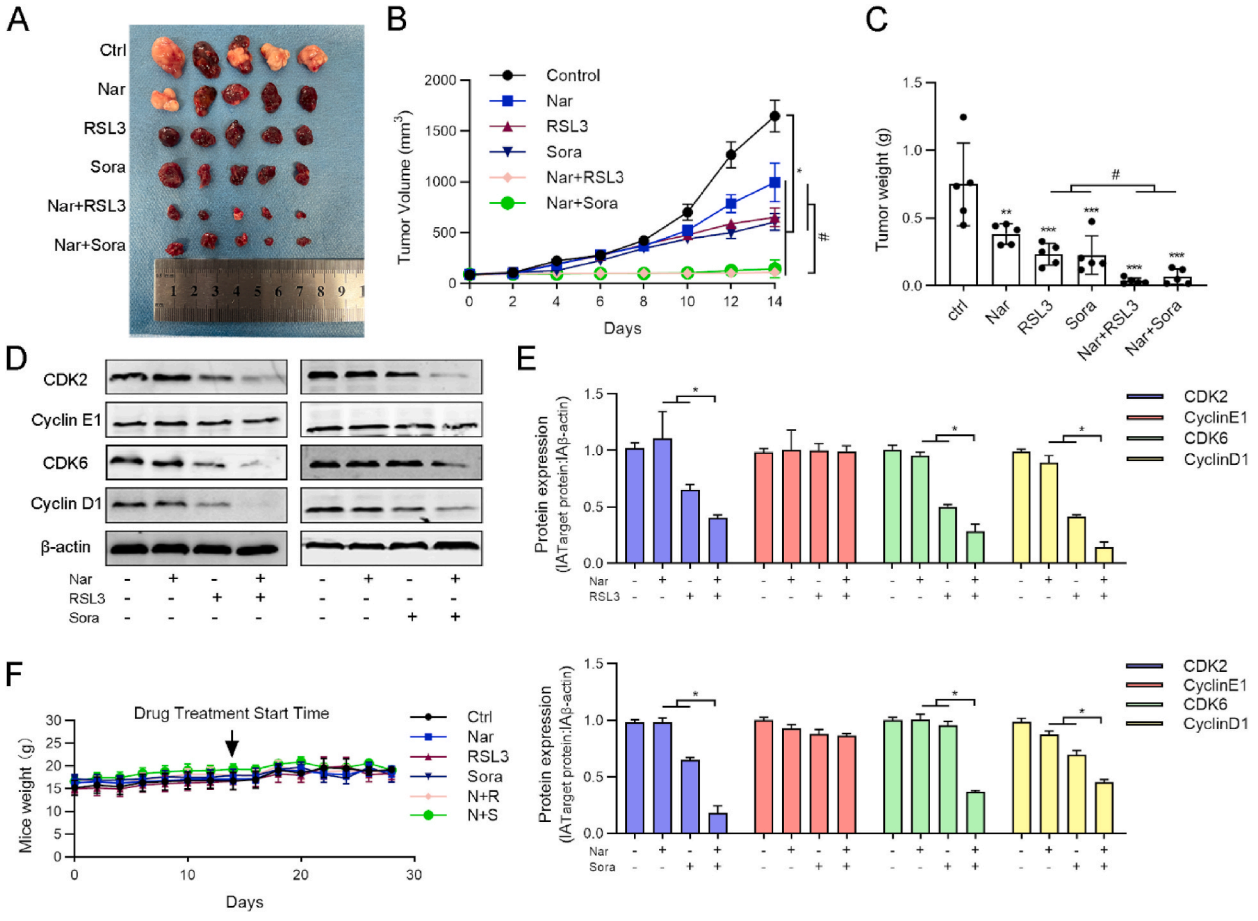

Fig. 6. Naringenin increases the efficacy of ferroptosis inducers in vivo. Naringenin (50 mg kg^{-1}, p. o., every other day); RSL3 (5 mg kg^{-1}, intratumorally injected, every other day); sorafenib (20 mg kg^{-1}, p. o., every other day); and naringenin plus RSL3/sorafenib treatment reduced the tumour volume (A–B) and tumour weight (C). (D–E) Western blot was utilized to determine the protein expression levels of CDK2, Cyclin E1, CDK6, and Cyclin D1 in tumour tissue. (F) The weights of the mice were monitored regularly. *P < 0.05, **P < 0.01, ***P < 0.001, n = 5 per group.

changes between the control and naringenin-treated groups, between the sorafenib-treated and the naringenin-treated groups, among the sorafenib combination groups, or between the RSL3-treated group and the naringenin/RSL3 combination-treated group (Fig. S4). Furthermore, there were essentially no variations in body weight changes across the groups of mice, suggesting that naringenin had no harmful effects on normal tissues (Fig. 6F), which is consistent with our in vitro findings. Western blot analysis showed that the combination treatment significantly decreased the expression levels of cell cycle proteins CDK2, CDK6, and cyclin D1 when compared to each individual treatment. This suggests that the combination treatment induced cell cycle arrest in the tumor cells, leading to a substantial reduction in cancer cell proliferation (Fig. 6D–E).

4. Discussion

Many patients with liver cancer are diagnosed in advanced stages, leading to an average survival time of only a few months. Chemotherapy remains one of the important means of comprehensive treatment of advanced liver cancer. Despite extensive research on apoptosis spanning over the last 50 years, its application in tumor therapy still encounters numerous challenges. Therefore, it is necessary to further study the mechanisms of liver cancer to improve the current treatment outcomes. Recent preclinical studies have increasingly showcased the efficacy of inducing ferroptosis as a promising therapeutic strategy for tumors [39]. The induction of ferroptosis offers an alternative strategy for impeding tumor growth. Naringenin has been shown to increase the ROS levels in human pancreatic cancer [42] and a human placental choriocarcinoma model [24], as well as decrease GSH content in a breast cancer model [26]. These changes in ROS and GSH levels are consistent with the biological characteristics of ferroptosis, suggesting that naringenin may heighten the vulnerability of liver cancer cells to ferroptosis. This study presents compelling evidence that non-toxic concentrations of naringenin enhance ferroptosis induced by erastin/RSL3 in liver cancer cells, focusing on a detailed exploration of how naringenin mechanisms heighten sensitivity to liver cancer ferroptosis.

In this study, we innovatively elucidated the impact and mechanism of naringenin on glycolysis, and further explored the changes in the tumour microenvironment (TME) after inhibiting glycolysis. Mechanistically, naringenin reduces LDHA and PDK1 expression while upregulating GLUT4 expression. Further experiments confirmed that naringenin reduces the lactate content and increased glucose uptake and the ATP content in liver cancer cells. These findings indicate that naringenin inhibits glycolysis and increases mitochondrial oxidative phosphorylation (OXPHOS) of glucose in liver cancer cells. Interestingly, numerous studies have shown that the suppression of aerobic glycolysis and enhanced mitochondrial OXPHOS cause an increase in the ROS level [40–43]. As previously described, lactate causes resistance to ferroptosis in liver cancer cells. Moreover, recent studies showed that inhibiting glycolysis leads to a significant reduction in the GSH content within TME, accompanied by a notable increase in ROS and lipid peroxidation levels [44]. These manifestations are all typical features of ferroptosis. Our study also demonstrated that naringenin can reduce the GSH content and elevate the level of lipid peroxidation and ROS. Therefore, it is reasonable to believe that naringenin heightens the sensitivity of liver cancer cells to ferroptosis by inhibiting glycolysis, leading to a decrease in lactate and GSH levels, as well as an elevation in the levels of lipid peroxidation and ROS.

AMPK has been identified as a negative regulator of glycolysis [14]. Previous studies have shown that the overexpression of PGC1α can inhibit glycolysis by downregulating PDK1 expression via WNT/β-catenin pathway, consequently inhibiting liver cancer progression [32]. Interestingly, recent studies have highlighted that the upregulation of PGC1α can potentiate the efficacy of sorafenib against liver cancer by elevating levels of ROS [45], but the exact mechanism is remains unclear. AMPK functions as an upstream activator of PGC1α [19]. However, the function of AMPK in regulating ferroptosis is remains debated. Some studies suggest that cells with AMPK activation display increased resistance to ferroptosis [46], while others indicate that AMPK activation promotes ferroptosis [47–50]. Our findings indicate that naringenin activates the AMPK-PGC1α signalling axis in liver cancer. Notably, upon application of AMPK or PGC1α inhibitors, the inhibitory effect of naringenin on LDH and PDK1, as well as its synergistic effect with ferroptosis inducers in liver cancer cells, were nullified. Thus, it is inferred that naringenin inhibits glycolysis by activating the AMPK-PGC1α signalling axis, ultimately enhancing the sensitivity of liver cancer cells to ferroptosis. Diminished AMPK activity in tumor patients is associated with an aggressive clinical phenotype and poor prognosis [51]. According to the meta-analysis conducted by Ji Cheng et al. cancer patients with high AMPK expression exhibit improved prognoses and disease-free survival. Additionally, we found a correlation between AMPKα1 and ferroptosis in liver cancer by bioinformatics analysis. Furthermore, molecular docking results revealed strong binding of naringenin to AMPK residues. These data from another perspective suggest that naringenin can enhance the sensitivity of liver cancer cells to ferroptosis by activating AMPK. In summary, this study creatively discovered the target of naringenin in inhibiting glycolysis and enhancing the sensitivity of liver cancer cells to ferroptosis, and innovatively explored the correlation between AMPK-PGC1α signalling pathway, glycolysis and ferroptosis.

Sorafenib is a primary chemotherapy agent used in the clinical setting for treating liver cancer. Recent studies have shown that sorafenib has the capability to trigger ferroptosis through the inhibition of System xc-activity [8,34]. However, the survival benefit from sorafenib treatment was moderate, and patients treated with sorafenib had an additional survival time of only 2–3 months [52,53]. Moreover, many liver cancer patients are intolerant to sorafenib, and some patients develop adaptive resistance [54]. Hence, it is imperative to develop an efficacious combination therapy that enhances the sensitivity to ferroptosis in liver cancer patients, thereby further enhancing their survival prognosis. In our study, we observed that a synergistic enhancement of cytotoxic effect on liver cancer cells when naringenin was combined with sorafenib. Additionally, naringenin in combination with sorafenib increased the level of ROS and lipid peroxidation. However, liver cancer cell viability was only increased by 15–20 % in the naringenin/sorafenib combination group after ferroptosis inhibitor pretreatment. Therefore, we speculated that sorafenib-mediated liver cancer killing involves both apoptosis and ferroptosis, and the mechanism by which naringenin synergistically enhances sorafenib-promoted apoptosis in liver cancer will be further elucidated in our subsequent study.

In addition, this study paves the way for further research of other flavonoids, glycolysis inhibitors, and AMPK agonists in combination with kinase inhibitors and ferroptosis inducers. In fact, studies have shown that many flavonoids are closely associated with ferroptosis. Our previous study revealed that quercetin induces lysosome activation mediated by EB and increases ferritin degradation, leading to ferroptosis and apoptosis involving bid [55]. This study posits that naringenin heightens the sensitivity of liver cancer to ferroptosis by activating AMPK to suppress glycolysis. However, naringenin is not a specific activator of AMPK or a traditional glycolysis inhibitor. Thus, future research could investigate the association of other AMPK activators or glycolysis inhibitors with tumor ferroptosis.

Our research findings suggest that naringenin can activate AMPK, inhibit aerobic glycolysis, and synergistically exhibit anti-tumor effects with ferroptosis inducers (Fig. 7). Nonetheless, further investigation is necessary to determine whether this mechanism still applies to actual individual in vivo environments. In fact, in addition to lactate, the tumor microenvironment also involves other metabolites and various types of immune cells, which play a critical role in the pathological development of diseases [56]. Therefore, it is imperative to conduct more research on these substances and immune cell populations to address the gaps in our study comprehensively. Furthermore, the limited water solubility and bioavailability of naringenin present obstacles to its clinical utility. Hence, investigating methods to enhance naringenin's bioavailability is crucial for optimizing its efficacy as a therapeutic agent. Researchers have extensively examined drug delivery systems for naringenin in cancer treatment [56]; however, further investigation is crucial to comprehensively ascertain the therapeutic advantages of naringenin in promoting human health.

5. Conclusion

In conclusion, the present study demonstrates that naringenin inhibits glycolysis through the activation of AMPK-PGC1α, which in turn increases the ROS level and reduces the GSH and lactate contents in the TME, causing intracellular lipid peroxidation and ultimately enhancing the sensitivity of liver cancer cells to ferroptosis. This study innovatively clarified the inhibitory effect of naringenin on glycolysis, exploring the association and mechanisms between glycolysis and ferroptosis, and innovatively revealed the correlation

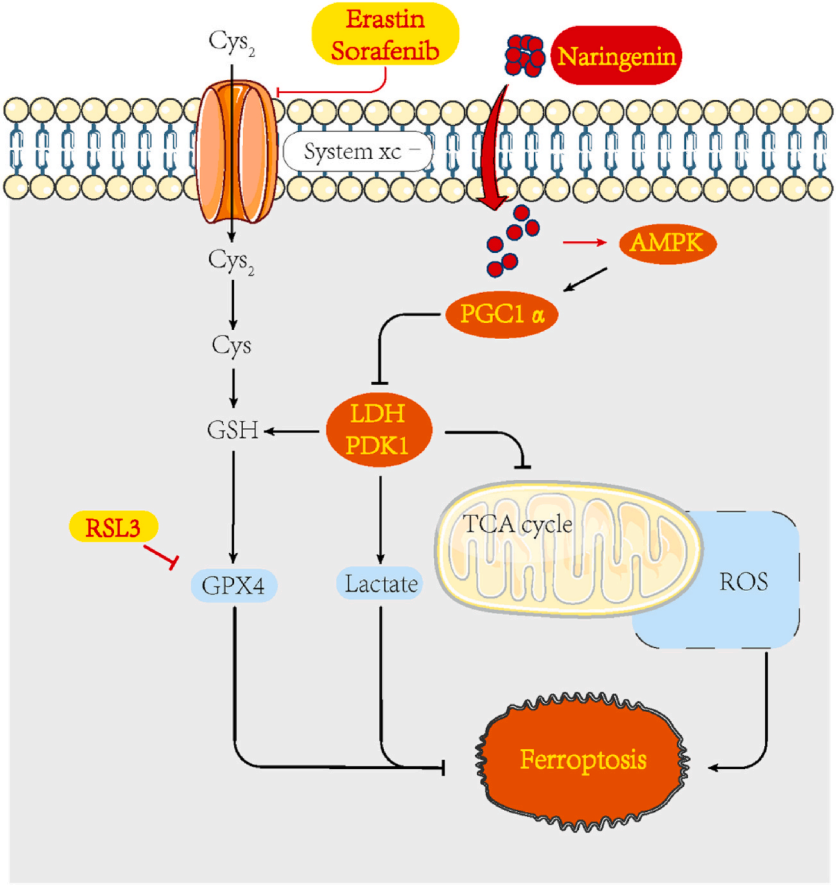

Fig. 7. Schematic representation. Erastin and sorafenib inhibit System xc-, whereas RSL3 is designed to inhibit GPX4 and disrupt the cell's antioxidant system in order to induce ferroptosis. Naringenin inhibits glycolysis in liver cancer cells by activating the AMPK-PGC1α signalling axis, which in turn increases ROS levels, decreases GSH and lactate levels in TME, causes intracellular lipid peroxidation, and ultimately enhances the sensitivity of liver cancer cells to ferroptosis.

and mechanism of AMPK and ferroptosis in a liver cancer model. Finally, it innovatively and systematically elucidated the effect and mechanism of naringenin in enhancing liver cancer sensitivity to ferroptosis by activating AMPK to inhibit glycolysis. The findings of this study will offer a fresh perspective on the examination of naringenin in different types of tumors, introduce novel concepts and a theoretical framework for the practical utilization of naringenin, both individually and in combination with sorafenib, and establish a solid theoretical foundation for enhancing the prognosis and survival rates of patients with liver cancer.

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Data availability statement

Data included in article/supp. Material/referenced in article.

Institutional review board statement

The animal study was reviewed and approved by the Institutional Animal Ethics Committee (IAEC, No. 20210073), Guangxi Medical University.

CRediT authorship contribution statement

Yong-Zhuo Li: Writing – original draft, Visualization, Investigation, Conceptualization. **Jing Deng:** Investigation, Formal analysis. **Li-Hui Yang:** Writing – review & editing, Supervision, Conceptualization. **Xiao-Dong Zhang:** Writing – review & editing, Investigation. **Dong-Yang Li:** Methodology, Investigation. **Li-Xi Su:** Methodology, Investigation. **Shan Li:** Methodology, Investigation. **Jian-Min Pan:** Formal analysis. **Lan Lu:** Formal analysis. **Jia-Qi Ya:** Formal analysis. **Nuo Yang:** Writing – review & editing, Resources. **Jing Zhou:** Writing – review & editing, Resources.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32288.

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