Erastin/sorafenib induces cisplatin-resistant non-small cell lung cancer cell ferroptosis through inhibition of the Nrf2/xCT pathway

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Abstract. Non-small cell lung cancer (NSCLC) has long been one of the most lethal types of cancer due to its lack of typical clinical symptoms at early stages and high risk of tumour recurrence, even following complete surgical resection. Multicourse chemotherapy based on cisplatin (CDDP) is the standard adjuvant treatment for NSCLC; however, its benefits for the overall survival of patients are limited. In this study, NSCLC cells possessing CDDP-resistant characteristics (N5CP cells), obtained from surgical resection of clinical specimens of patients with NSCLC, were cultured and screened to generate research models. This study aimed to identify the mechanism underlying tumour cell resistance to CDDP and to identify a novel treatment for NSCLC following CDDP failure. CDDP-mediated NF-E2 related factor 2 (Nrf2)/light chain of System x_c^- (xCT) pathway activation was associated with the resistance of cells to CDDP. Therefore, erastin/sorafenib regulation of Nrf2 or xCT expression may alter the sensitivity of tumour cells to CDDP. The small molecules erastin and sorafenib effectively induced N5CP cell ferroptosis, which was mediated by the accumulation of intracellular lipid reactive oxygen species. Additionally, low doses of erastin or sorafenib could be used in association with CDDP to effectively trigger N5CP cell ferroptosis. Furthermore, it was indicated that erastin and sorafenib, alone or in combination with a low dose of CDDP, effectively inhibited the growth of N5CP cells *in vivo*. Therefore, ferroptosis inducers, including erastin and sorafenib, may be considered a novel treatment regimen for patients with NSCLC, particularly patients with CDDP failure.

Introduction

Lung cancer is one of the most common malignancies worldwide and is one of the most lethal types of cancer (1). Non-small cell lung cancer (NSCLC) accounts for ~85% of lung cancer cases (2). Early-stage lung cancer is characterised by an insufficiency of emblematic clinical manifestations, and for the majority of patients, tumours have advanced to later stages when diagnosed. Surgery is the first choice of treatment for patients with resectable NSCLC; however, even if pathological grade II or III tumours are completely removed, there remains a high risk of recurrence within 5 years, and their respective 5-year overall survival (OS) rates are only 56.9 and 23.6% (3). At present, prolongation of the OS of patients with advanced lung cancer mainly relies on multiple courses of chemotherapy. The chemotherapy regimen based on cisplatin (CDDP) has gradually become a standard adjuvant therapy for patients with advanced NSCLC. However, CDDP chemotherapy does not effectively improve OS due to apparent CDDP resistance (4). Therefore, improving the efficacy of CDDP chemotherapy and the subsequent treatment effects for CDDP-resistant patients are important to improving OS.

CDDP mainly destroys tumour cells by inducing apoptosis. However, multiple solid tumour cells, including NSCLC cells, exhibit an evident resistance to apoptosis (5). Cells can be induced to undergo various modes of programmed cell death, and apoptosis, which was identified early and is frequently reported on in research, is not the only mode. Ferroptosis is a widespread and promising mode of non-apoptotic programmed cell death. Ferroptosis is known for its distinct iron-dependence, which is induced by intracellular lipid reactive oxygen species (ROS) accumulation in the cytoplasm (6). A previous study indicated that ferroptosis is fundamentally different from apoptosis; therefore, the anti-apoptotic pathway of tumour cells cannot protect them from cell death mediated by ferroptosis; therefore, inducing ferroptosis may be an ideal solution for overcoming apoptosis resistance (7). Nevertheless, studies concerning ferroptosis in NSCLC are exceedingly scarce.

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The classical mechanism underlying CDDP-induced apoptosis is associated with formation of a CDDP-DNA complex, which can alter the structure of nucleotides and DNA, influencing DNA replication and homeostasis (8). However, CDDP can also induce cell death through increasing intracellular ROS levels, disrupting intracellular homeostasis and triggering oxidative stress (9). Since generated oxidative stress serves a crucial role in CDDP-mediated cytotoxicity, the activation of genes driven by transcription factor NF-E2 related factor 2 (Nrf2)/antioxidant response element (ARE) is considered a predominant reason for CDDP failure (10). In previous years, numerous Nrf2 downstream target genes that participate in ROS regulation, including NAD(P)H quinone dehydrogenase 1 (*NQO1*) (11), heme oxygenase 1 (*HO-1*) (12) and light chain of System x_c^- (*xCT*) (13), have been demonstrated to be involved in modulating the sensitivity of tumour cells to CDDP. Activation of the transmembrane transport protein xCT directly exerts its functions on the most important antioxidative stress molecule, glutathione (GSH), at the synthesis level (13). A number of studies have indicated that xCT significantly contributes to antioxidative stress of malignant tumour cells (13,14). However, how the Nrf2-xCT pathway and the activation of other downstream genes of Nrf2 regulate the CDDP sensitivity of NSCLC cells, and how to effectively eliminate CDDP-resistant NSCLC cells remains unknown. Notably, with further study of ferroptosis mechanisms, small molecular inducers of ferroptosis may be identified and these may promote intracellular lipid ROS, and eventually induce ferroptosis. Among these small molecular inducers, the classic inducers erastin and sorafenib, which are used to treat advanced renal and liver cancer, possess clinical development and application prospects. These two molecules exert their function by inhibiting the cystine import activity of xCT, resulting in a reduction of GSH synthetic materials and accumulation of lipid ROS, thus ultimately inducing ferroptosis (6,15). However, to the best of our knowledge, the effects of the aforementioned ferroptosis inducers on NSCLC, particularly NSCLC with CDDP treatment failure, have not been studied. Yet it is reasonable to hypothesize that the application of ferroptosis inducers is an ideal way of solving CDDP resistance in NSCLC.

In the present study, CDDP induced activation of the Nrf2/xCT pathway in different NSCLC cell lines, and its activation level was associated with the extent of CDDP resistance. Additionally, the expression levels of Nrf2/xCT in CDDP-resistant NSCLC cells were markedly increased, and the classical ferroptosis inducers erastin and sorafenib clearly induced ferroptosis in CDDP-resistant NSCLC cells. When a low dose of CDDP was used in combination with erastin/sorafenib, it effectively eliminated CDDP-resistant NSCLC cells. Finally, the present study demonstrated that erastin/sorafenib may inhibit CDDP-resistant NSCLC cell growth *in vivo*. This study indicated that ferroptosis induced by erastin/sorafenib may provide a novel perspective for the treatment of patients with NSCLC following failed CDDP treatment, and may improve the OS of patients.

Materials and methods

Cell lines and cell culture. The A549 and NCI-H1299 human NSCLC cell lines were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Cell Resource Center. N2 and N5 cells were surgically obtained from patients (N2: Male, 61 years old; N5: male, 58 years old) at the Shengjing Hospital of China Medical University from May to July of 2014, once written informed consent had been obtained. The study was approved by the Ethics Committee of China Medical University. Primary NSCLC cell isolation and expansion were performed as previously described (16). The N5 CDDP-resistant (N5CP) variant cell line was established by continuous culturing of N5 cells with increasing concentrations of CDDP (1-10 μg/ml; cat. no. C2210000; Sigma-Aldrich; Merck KGaA) for 6 months. The culture medium of N5CP cells contained 10 μg/ml CDDP to maintain drug resistance. All cells were cultured in RPMI-1640 medium (cat. no. 11875093; Gibco; Thermo Fisher Scientific, Inc.) containing 10% foetal bovine serum (cat. no. 10100147; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (cat. no. 15140122; Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere at 37˚C with 5% CO_2.

Cell survival rate analysis. Cell survival rate was assessed using the Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.), according to the manufacturer's protocol. Cells ($2x10^4$ cells/well) were seeded in 96-well plates and; after 24 h, the cells were subjected to with CDDP, erastin (E7781; Sigma-Aldrich; Merck KGaA) or sorafenib (284461-73-0; Sigma-Aldrich; Merck KGaA) with or without deferoxamine (DFO; 138-14-7, Sigma-Aldrich; Merck KGaA) or Vitamin E (Vit-E; 10191-41-0, Sigma-Aldrich; Merck KGaA) for an additional 48 h. After refreshing the culture media, 10 μl/well CCK-8 solution was added and the plates were incubated for an additional 45 min in a 37˚C incubator. The absorbance of cells at 450 nm was detected using a microplate reader (iMark™ Microplate Absorbance Reader; Bio-Rad Laboratories, Inc.).

Propidium iodide (PI) staining. The PI Flow Cytometry kit (cat. no. ab139418; Abcam) was used to determine cell death. Briefly, cells ($2x10^5$ cells/well) were seeded in 6-well plates and incubated for 24 h at 37˚C. Subsequently, the cells were treated with erastin (10 μM) or sorafenib (20 μM). After 48 h, the cells were harvested, washed twice with PBS and subsequently re-suspended in PI staining solution at room temperature for 15 min in the dark, according to the manufacturer's protocol. Samples were assessed by flow cytometry (Muse; Sigma-Aldrich; Merck KGaA) and the data were analysed using FlowJo software version 9 (FlowJo LLC).

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA of A549, N5 and N5CP cells was extracted using TRIzol® Reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.). Extracted RNA was quantified and RNA purity was confirmed by measuring 260/230 and 260/280 nm absorbance ratios (NanoDrop 2000 Spectrophotometer; NanoDrop Technologies; Thermo Fisher Scientific, Inc.). Subsequently, 1 μg total RNA was used for cDNA synthesis, using an iScript™ cDNA Synthesis kit (cat. no. 1708891; Bio-Rad Laboratories, Inc.), according to the manufacturer's protocols. SYBR Premix Ex Taq (cat. no. RR420A; Takara Bio, Inc., Otsu, Japan) was used to measure individual gene expression on the CFX96 Real-Time

PCR Detection system (Bio-Rad Laboratories, Inc.). The primer sequences used for RT-qPCR were as follows: Human *Nrf2*, forward, 5'-AGTCCTGGTCATCGGAAAAC-3', reverse 5'-ATGGAGAGCTTTTGCCCTAA-3'; human *xCT*, forward 5'-CCATGAACGGTGGTGTGTT-3', reverse 5'-GACCCT CTCGAGACGCAAC-3'); human *HO-1,* forward 5'-CTCTT GGCTGGCTTCCTTAC-3', reverse 5'-TCCTTCCTCCTT TCCAGAGA-3'); human *NQO1*, forward 5'-TGGCTCCAT GTACTCTCTGC-3', reverse 5'-CAGAAATGCAGAATG CCACT-3'); and human *GAPDH*, forward 5'-AGTCAGCCG CATCTTCTTTT-3' and reverse 5'-CAATACGACCAAATC CGTTG-3'. Gene expression was normalised to *GAPDH*. Each sample was analysed in triplicate. The PCR reaction was performed under the following conditions: One cycle at 95˚C for 2 min; followed by 45 cycles at 95˚C for 5 sec and extension at 62˚C for 30 sec; melting curves were recorded for 5 sec per temperature step during a temperature gradient from 65.0˚C to 95.0˚C with an increment of 0.5˚C (17). All expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (17).

Luciferase reporter assay. A549 and N5 cells were seeded in 12-well plates at a density of $1x10^5$ cells/well. The following day, cells were co-transfected with the ARE luciferase reporter vector and *Renilla* luciferase vector (ARE Reporter kit; cat. no. 60514; BPS Bioscience, Inc.) with Lipofectamine® LTX Reagent (cat. no. 15338100; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturers' protocols. *Renilla* luciferase activity was used as an internal control. A total of 24 h post-transfection, the culture media were changed and 20 µg/ml CDDP or DMSO (cat. no. M81802; Sigma-Aldrich, Merck KGaA) were added. After 12 h, the cells were collected and luciferase activity was detected using a Dual-Luciferase Reporter Assay system (cat. no. E1910; Promega Corporation). Mean values from triplicate analysis were presented.

Western blotting. The cells treated with CDDP, siRNA, overexpression plasmids, erastin or sorafenib were washed twice with ice-cold PBS at the end of the experiment. Whole cell protein lysates were prepared by dissolving the cell pellets in lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS and 10% glycerol]. Protein concentrations were measured with a Pierce™ Bicinchoninic Acid Protein Assay kit (cat. no. 23225; Thermo Fisher Scientific, Inc.). Total proteins (20 µg/lane) were separated by 8-10% SDS-PAGE. Subsequently, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (cat. no. IPVH09120; EMD Millipore) and the membranes were blocked with 1% skimmed milk for 1 h at room temperature. After three washes with Tris-buffered saline with 0.1% Tween-20 (TBST), the PVDF membranes were incubated with anti-human Nrf2 (1:1,000 dilution; cat. no. ab31163; Abcam), xCT (1:1,000 dilution; cat. no. ab175186; Abcam) and GAPDH (1:1,000 dilution; cat. no. ab8245; Abcam) antibodies diluted in TBST at room temperature for 1 h. After incubating with a goat anti-rabbit IgG H&L for detecting Nrf2 and xCT (1:10,000 dilution; cat. no. ab97051; Abcam) or a goat anti-mouse IgG H&L for detecting GAPDH (1:10,000 dilution; cat. no. ab6708; Abcam) at room temperature for 1 h, the membranes were visualised using Pierce™ Enhanced Chemiluminescence Western Blotting Substrate

(cat. no. 32106; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

ROS determination. ROS generation was determined using 6-carboxy-2',7'-dichlorofluorescein diacetate dye (H₂DCFDA; cat. no. D399; Thermo Fisher Scientific, Inc.). The medium was refreshed following treatment with CDDP, erastin, sorafenib or DMSO, and 20 µl/well H₂DCFDA was added to the medium 30 min prior to the end of the experiment at 37˚C. Subsequently, the cells were washed twice with ice-cold PBS and digested with trypsin. ROS production was analysed using a flow cytometer (Muse; Sigma-Aldrich; Merck KGaA) and FlowJo v.9 software.

Knockdown and overexpression experiment. For the knockdown experiment, A549 cells were seeded in 12-well plates at a density of $1.5x10^5$ cells/well. The following day, the cells were transfected with a final concentration of 20 nM anti-human Nrf2 small interfering RNA (siRNA; cat. no. 107966; Thermo Fisher Scientific, Inc.), anti-human xCT siRNA (cat. no. 108517; Thermo Fisher Scientific, Inc.) or scrambled siRNA (cat. no. AM4611; Thermo Fisher Scientific, Inc.) using Lipofectamine® RNAiMax reagent (cat. no. 13778150; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Subsequently, 24 h post-transfection, the medium was replaced with fresh medium containing 20 µg/ml CDDP and the cells were incubated for an additional 48 h. For the overexpression experiment, N5 cells were seeded as aforementioned and were then transfected with a final concentration of 0.5 ng/µl pcDNA3-human Nrf2, pcDNA3-human xCT or pcDNA3 vector using Lipofectamine® LTX Reagent. The plasmids of pcDNA3-hNrf2 and pcDNA3-hxCT were constructed as described previously (14). After 24 h, the medium was replaced with fresh medium containing 40 µg/ml CDDP. After 48 h, cell survival rate measurements were performed.

Xenograft assay. A total of 60 BALB/c-nu/nu nude mice (male; age, 4-6 weeks; weight, 16-22 g) were obtained from the Shanghai Laboratory Animal Co., Ltd. Mice were housed under pathogen-free conditions in barrier facilities under a 12-h dark/light cycle. The room temperature was maintained at 23˚C with a humidity of 50-60%; food and water were *ad libitum*. All animal research was performed in accordance with the approved animal protocols and the guidelines of the Institutional Animal Care and Use Committee of China Medical University. The present animal study was approved by the Institutional Animal Care and Use Committee of China Medical University. N5CP cells ($5x10^6$) were suspended in 200 µl DMEM and Matrigel (cat. no. 354234; BD Biosciences) mixture at a ratio of 1:1. Subsequently, the mixture was injected subcutaneously into the upper right flank of 20 nude mice. After 10 days, the mice were randomly divided into four groups and were treated with CDDP (5 mg/kg/2 days), erastin (10 mg/kg/2 days), sorafenib (10 mg/kg/2 days) or PBS by intraperitoneal injection. Two days after the third injection, the mice were sacrificed and tumours were carefully removed. For the combination experiment, CDDP (1 mg/kg) and erastin (5 mg/kg) or sorafenib (3 mg/kg) were also injected three times. Subsequently, tumour weight was measured. The mice were euthanised when a humane endpoint was reached (when

the mice were distressed by the tumour burden or tumour volume was >2,000 mm^3).

Statistical analysis. Statistical analysis was performed using SPSS v.20.0 software (IBM Corp.). All values are expressed as the mean ± standard error and are representative of at least three independent experiments. The data were analysed using one-way ANOVA. When the results were significant, post hoc testing of differences among groups was performed using a Bonferroni test. P<0.05 was considered to indicate a statistically significant difference.

Results

Sensitivity of NSCLC cells to CDDP is negatively associated with Nrf2 pathway activation. It has been demonstrated that Nrf2 expression is negatively associated with CDDP cytotoxicity; however, to the best of our knowledge, there are no detailed reports on either the key Nrf2 downstream target genes or the mechanisms of regulation (18). To further investigate the association between CDDP sensitivity and Nrf2, as well as to identify the downstream target genes, the cytotoxicity of CDDP in four NSCLC cell lines was compared. NCI-H1299 and A549 cells are commonly used in the laboratory, and N2 and N5 cells were primary cultured cells from patients with NSCLC obtained during surgical resection. As shown in Fig. 1A, CDDP exerted different cytotoxic effects on these cells. A549 cells were most resistant to CDDP, whereas N5 cell were most sensitive to CDDP. To investigate the differences in more detail, in a follow-up experiment, the two representative cell lines A549 and N5 were selected and the effects of CDDP on the Nrf2 pathway were compared. As shown in Fig. 1B and C, in A549 and N5 cell lines, CDDP markedly upregulated the expression of *Nrf2* and its downstream target gene *xCT* at the mRNA level, but no significant alterations in the expression of *HO-1* and *NQO1* were observed. In A549 cells, the expression of *Nrf2* was only slightly increased by CDDP, whereas *xCT* expression was increased 8.6-fold. By comparing the influence of CDDP on overall activation levels of the Nrf2 pathway in the two cell lines, the activation level of the CDDP-mediated Nrf2 pathway was revealed to be higher in A549 cells than in N5 cells (Fig. 1B and C). Western blot analysis demonstrated that Nrf2 and xCT protein expression were markedly increased in A549 cells in the presence of CDDP, which was consistent with the mRNA expression analysis results (Fig. 1D). In particular, Nrf2 protein was detected in unstimulated A549 cells, which may be caused by a Keap1 mutation, which hydrolyses Nrf2 by ubiquitination (19). Keap1 mutation leads to the sustained accumulation of Nrf2, as well as its downstream target genes. This may be why CDDP did not cause further significant upregulation of Nrf2 downstream target genes in A549 cells. The present study demonstrated that N5 cells with lower levels of Nrf2 activation were more sensitive to CDDP treatment than A549 cells with higher activation levels of Nrf2. To further explore the regulation of Nrf2/ARE-driven downstream gene transcription by CDDP, A549 and N5 cells were transiently transfected with a luciferase reporter plasmid containing ARE. CDDP increased the luciferase activity of reporter genes and had a clearer effect on Nrf2 transcription activity in A549 cells than in N5

cells (Fig. 1E). These results indicated that CDDP induced activation of the Nrf2/xCT pathway in NSCLC cells, and the activation level was negatively associated with the sensitivity of cells to CDDP.

Nrf2/xCT expression determines the sensitivity of cells to CDDP. The Nrf2/xCT pathway can resist oxidative stress and downregulate intracellular ROS levels by regulating glutathione synthesis (14). In the present study, the ROS levels of A549 and N5 cells following CDDP treatment were measured. As shown in Fig. 2A, CDDP significantly increased ROS accumulation in A549 and N5 cells, but the effect was greater in N5 cells (Fig. 2A). To further explain the contribution of Nrf2/xCT to CDDP sensitivity in NSCLC cells, A549 cells resistant to CDDP were transfected with siRNA to knockdown Nrf2 or xCT. The knockdown efficiency of individual siRNAs was confirmed through western blot analysis (Fig. 2C). As shown in Fig. 2B, when the expression of Nrf2 or xCT was decreased, the sensitivity of A549 cells to CDDP was markedly enhanced. Additionally, the effect of Nrf2 knockdown on CDDP cytotoxicity was stronger than that of xCT knockdown. Conversely, Nrf2 or xCT were transiently overexpressed in N5 cells that were relatively sensitive to CDDP (Fig. 2E). With increasing Nrf2 or xCT expression levels, CDDP cytotoxicity to N5 cells was significantly decreased (Fig. 2D). Compared with the results of Fig. 2A, overexpression or knockdown of xCT expression reversed the regulatory effect of CDDP on ROS levels in N5 and A549 cells (Fig. 2F).

Erastin and sorafenib effectively induce N5CP cell ferroptosis by modulating the Nrf2/xCT pathway. According to the aforementioned results, activation of the Nrf2/xCT pathway may be a major mechanism underlying NSCLC cell CDDP resistance, and inhibiting the Nrf2/xCT pathway effectively reversed resistance to CDDP in NSCLC cells. One induction mechanism of ferroptosis is to inhibit cystine import via xCT (6). Therefore, the induction of ferroptosis may be a feasible option to eliminate CDDP-resistant NSCLC cells. To obtain more clinically meaningful CDDP-resistant NSCLC cells, N5 cells that were relatively sensitive to CDDP were screened and cultured with gradually increasing concentrations of CDDP for 6 months, and the resulting cells were termed N5CP cells. Cell survival rate experiments demonstrated that N5CP cells exhibited marked CDDP resistance compared with N5 cells (Fig. 3A). The mRNA expression levels of *Nrf2* and *xCT* in N5 and N5CP cells were compared by RT-qPCR, and were revealed to be significantly higher in N5CP cells than in N5 cells (Fig. 3B). Erastin and sorafenib are traditional ferroptosis inducers that mainly inhibit the transport function of xCT. In the present study, erastin and sorafenib significantly reduced the survival rate of N5CP cells (Fig. 3C and D). Furthermore, erastin and sorafenib-induced cell death was markedly suppressed by the specific inhibitors of ferroptosis deferoxamine mesylate (DFO) and Vitamin E (Vit-E), thus verifying that the type of cell death induced was ferroptosis (Fig. 3C and D). Additionally, PI staining was employed to detect cell death induced by erastin and sorafenib, in order to validate the results of viability experiments previously conducted (20,21). Erastin and sorafenib markedly increased the number of PI-positive N5CP cells (Fig. 3E and F). In addition, the expression of Nrf2 and
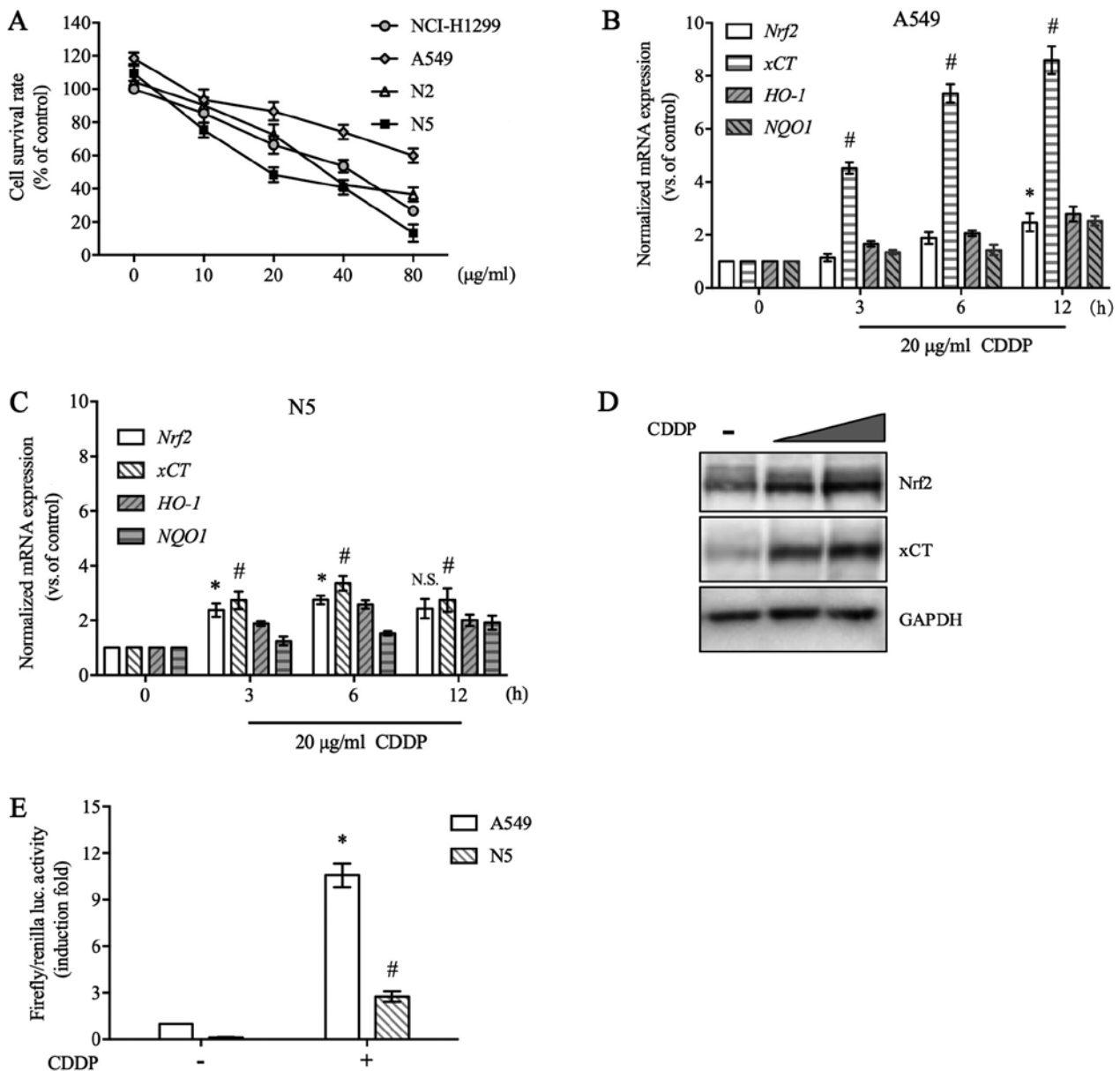
Figure 1. Nrf2 pathway is activated by CDDP and negatively regulates CDDP toxicity. (A) NCI-H1299, A549, N2 and N5 cells were treated with a series of CDDP doses (10-80 µg/ml) for 48 h, and the cell survival rate was analysed using a Cell Counting kit-8 assay. (B and C) A549 and N5 cells were exposed to 20 µg/ml CDDP for 3-12 h. The mRNA expression levels of *Nrf2*, *xCT*, *HO-1* and *NQO1* were evaluated by reverse transcription-quantitative PCR and normalised to *GAPDH*. (D) A549 cells were exposed to 20 or 40 µg/ml CDDP treatment for 12 h. Nrf2 and xCT protein expression was detected by western blotting. GAPDH was used as an internal control. (E) A549 and N5 cells were transfected with an antioxidant response element luciferase reporter vector and *Renilla* luciferase vector. After 24 h of transfection, the cells were incubated in the presence of 20 µg/ml CDDP for an additional 12 h and were then subjected to a luciferase assay. Luciferase activity was normalised to *Renilla* luciferase activity and then induction fold were compared with A549 cells without CDDP treatment. Data are presented as the mean ± standard error from at least three independent experiments. Differences among groups were assessed by one-way ANOVA with a Bonferroni post hoc test. *$P<0.05$ vs. Nrf2 in (B) and (C), vs. A549 in (E); #$P<0.05$ vs. xCT in (B) and (C), vs. N5 in (E). CDDP, cisplatin; HO-1, heme oxygenase 1; luc, luciferase; NQO1, NAD(P)H quinone dehydrogenase 1; Nrf2, NF-E2 related factor 2; N.S., not significant; xCT, light chain of System x_c^-.

xCT at the mRNA and protein levels were measured following erastin and sorafenib treatment. Erastin and sorafenib did not significantly alter Nrf2 expression at the mRNA level, but markedly reduced Nrf2 protein expression (Fig. 3G and H). This may be due to Nrf2 degradation being increased by erastin and sorafenib. As a target gene of Nrf2, xCT expression was significantly decreased at the mRNA and protein level. These findings indicated that erastin and sorafenib efficiently eliminated CDDP-resistant NSCLC cells by modulating the Nrf2/xCT pathway.

CDDP combined with erastin/sorafenib effectively induces N5CP cell ferroptosis. N5CP cells were treated with low doses of CDDP and/or erastin/sorafenib, as indicated, in order to explore the combined effect of low dose drugs, to allow for comparisons despite the inconsistent doses used across different experiments. Individual treatment with CDDP, erastin or sorafenib did not markedly affect cell survival rates. When cells were cultured in CDDP combined with erastin or sorafenib, the cell survival rate was significantly decreased (Fig. 4A), whereas ROS accumulation was increased at the
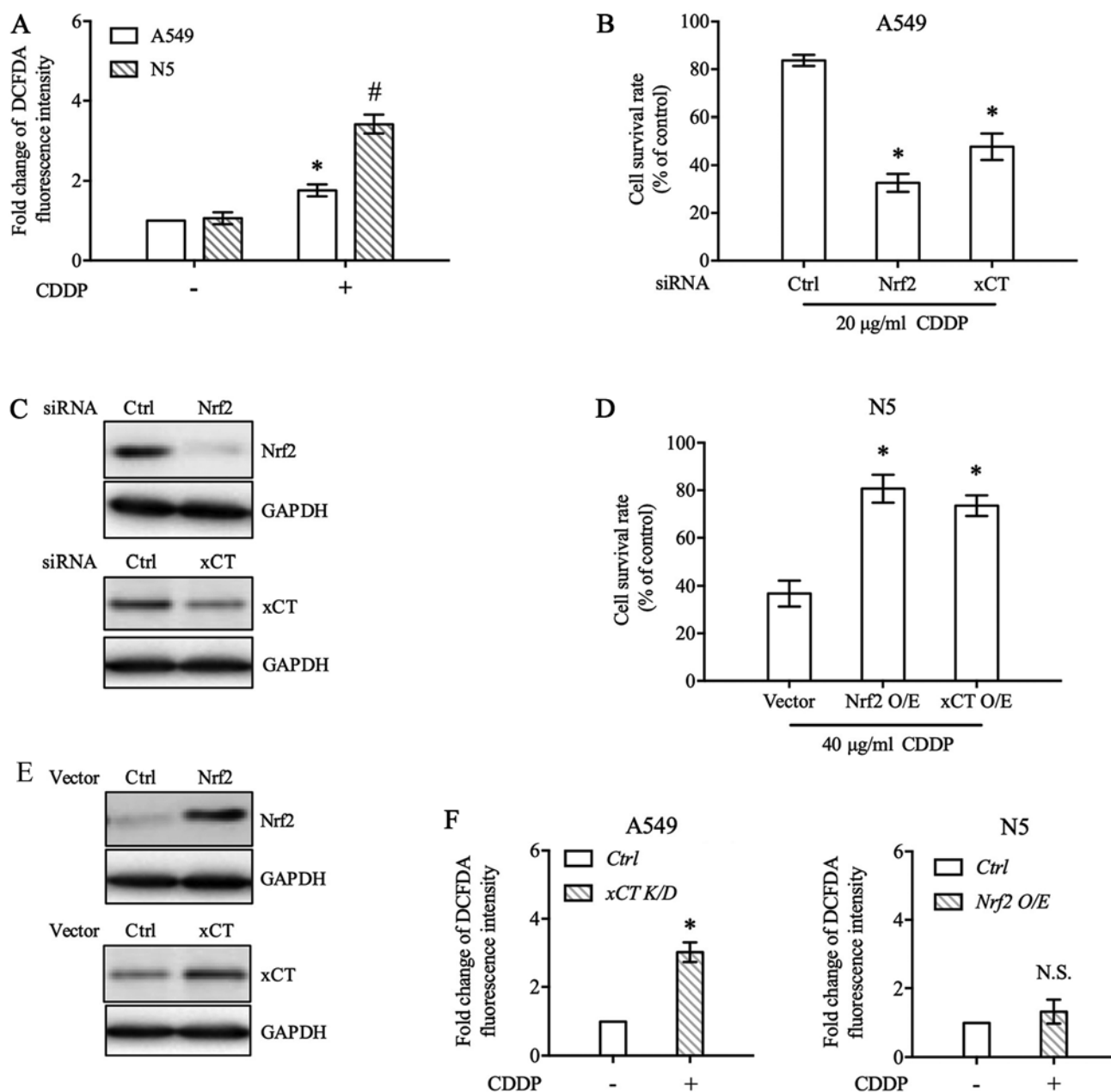
Figure 2. Nrf2/xCT expression determines CDDP sensitivity. (A) A549 and N5 cells were exposed to 20 μg/ml CDDP or DMSO for 6 h. DCFDA fluorescence, indicative of intracellular ROS level, was analysed by flow cytometry. Data were presented relative to the DMSO-treated control. (B) A549 cells were transfected with Nrf2 or xCT siRNA. After 24 h, the cells were treated with 20 μg/ml CDDP for 48 h, and cell survival rate was determined. (C) Nrf2 and xCT expression in A549 cells was inhibited by specific siRNAs. At 24 h post-transfection, whole cell lysate was extracted, and western blot analysis was performed to detect Nrf2 and xCT expression. GAPDH was used as a loading control. (D) N5 cells were transfected with Nrf2 or xCT expression vector. The pcDNA3 vector was used as a control. After 24 h of incubation, the cells were treated with 40 μg/ml CDDP for 48 h and subjected to cell survival rate analysis. (E) Western blot analysis was performed to detect the protein expression levels of Nrf2 and xCT following transfection with individual overexpression plasmids. (F) The expression of xCT was downregulated by siRNA in A549 cells and upregulated by overexpression plasmid in N5 cells. Cells were then treated with 20 μg/ml CDDP for 6 h, and ROS levels were detected by flow cytometry. Data are presented as the mean ± standard error from at least three independent experiments. Differences among groups were assessed by one-way ANOVA with a Bonferroni post hoc test. *P<0.05 vs. (A) A549, vs. (B) Ctrl, vs. (D) Vector. #P<0.05 vs. N5 (A). Ctrl, control; CDDP, cisplatin; DCFDA, 6-carboxy-2',7'-dichlorofluorescein diacetate dye; K/D, knockdown; N.S., not significant; Nrf2, NF-E2 related factor 2; O/E, overexpression; ROS, reactive oxygen species; siRNA, short interference RNA; xCT, light chain of System x_c^-.

same time (Fig. 4B). Furthermore, the cell death caused by cooperation of CDDP and erastin/sorafenib could be partially reversed by DFO, indicating that the type of cell death induced was ferroptosis (Fig. 4A).

Erastin/sorafenib restrains in vivo tumour growth in nude mice xenograft models. N5CP cells were seeded under nude

mice skin; ~10 days later, when the tumour volume reached ~600 mm³, the drugs CDDP (3 mg/kg), erastin (20 mg/kg) or sorafenib (10 mg/kg) were intraperitoneally injected into the nude mice every other day, and the tumour was resected and weighed after three injections (22). The results demonstrated that erastin/sorafenib inhibited tumour growth *in vivo* (Fig. 5A). Additionally, the present study demonstrated that
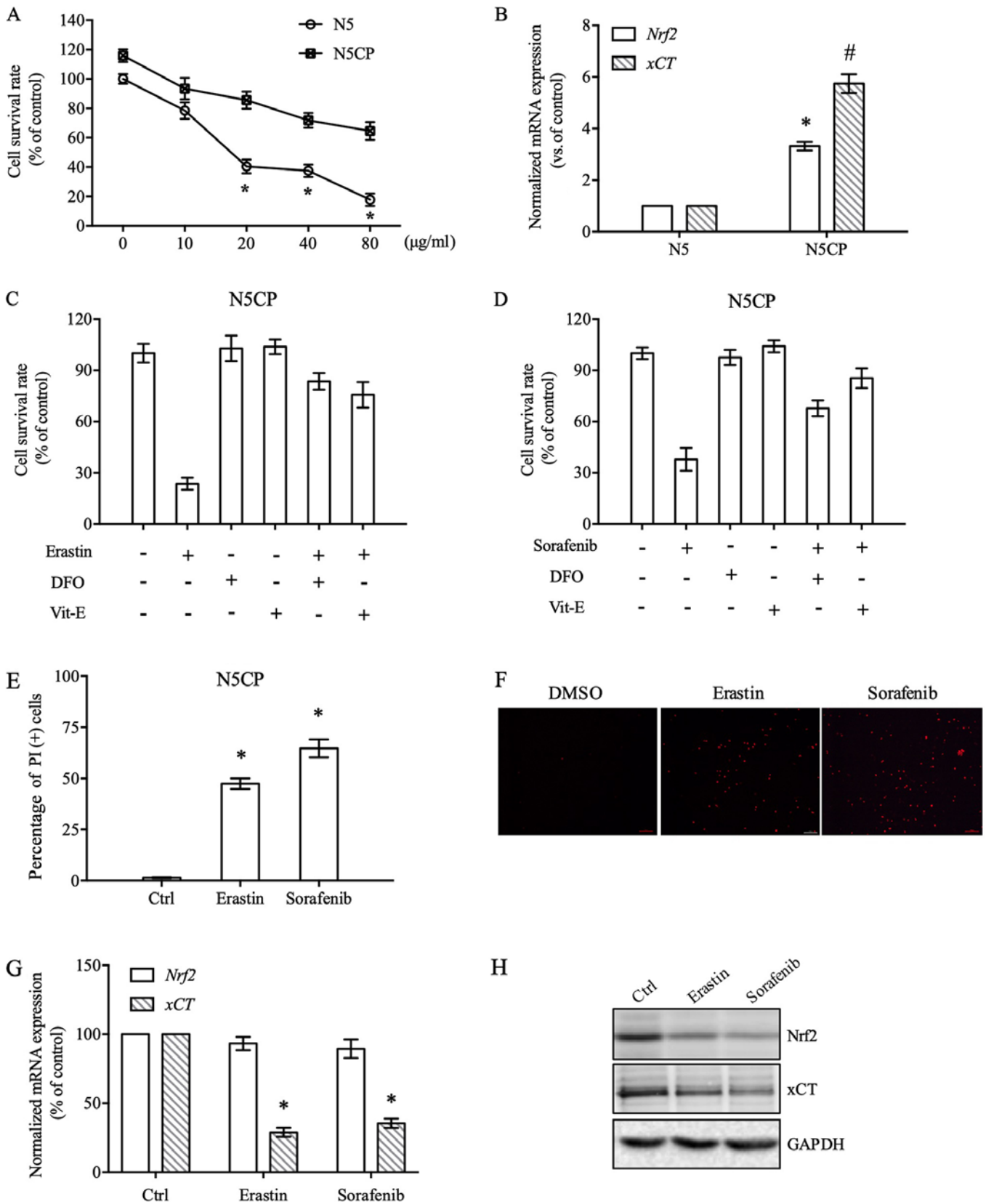
Figure 3. Erastin and sorafenib induce N5CP cell ferroptosis. (A) N5 and N5CP cells were exposed to different doses of CDDP, as indicated, for 48 h and were then subjected to cell survival rate determination. (B) The expression of *Nrf2* and *xCT* mRNA in N5 and N5CP cells was measured using RT-qPCR. The data were normalised to *GAPDH*. (C and D) N5CP cells were treated with erastin (10 μM), sorafenib (20 μM), DFO (50 μM) and Vit-E (100 μM) individually or in combination, as indicated, for 48 h, and then cell survival rate detection was performed. (E) N5CP cells were exposed to erastin (10 μM), sorafenib (20 μM) or DMSO for 48 h and subjected to PI staining analysis. Representative images are shown in (F). (G and H) N5CP cells were treated with erastin (10 μM), sorafenib (20 μM) or DMSO for 12 h, and the expression of Nrf2 and xCT was determined by RT-qPCR and western blotting. Data are presented as the mean ± standard error, and differences among groups were analysed by one-way ANOVA with a Bonferroni post hoc test. *P<0.05 vs. N5CP (A), vs. Nrf2 (B), vs. DMSO (C-E), vs. xCT (G); $^{#}$P<0.05 vs. xCT (B). CDDP, cisplatin; Ctrl, control; DFO, deferoxamine mesylate; Nrf2, NF-E2 related factor 2; PI, propidium iodide; RT-qPCR, reverse transcription-quantitative PCR; Vit-E, vitamin E; xCT, light chain of System x$_c^-$.
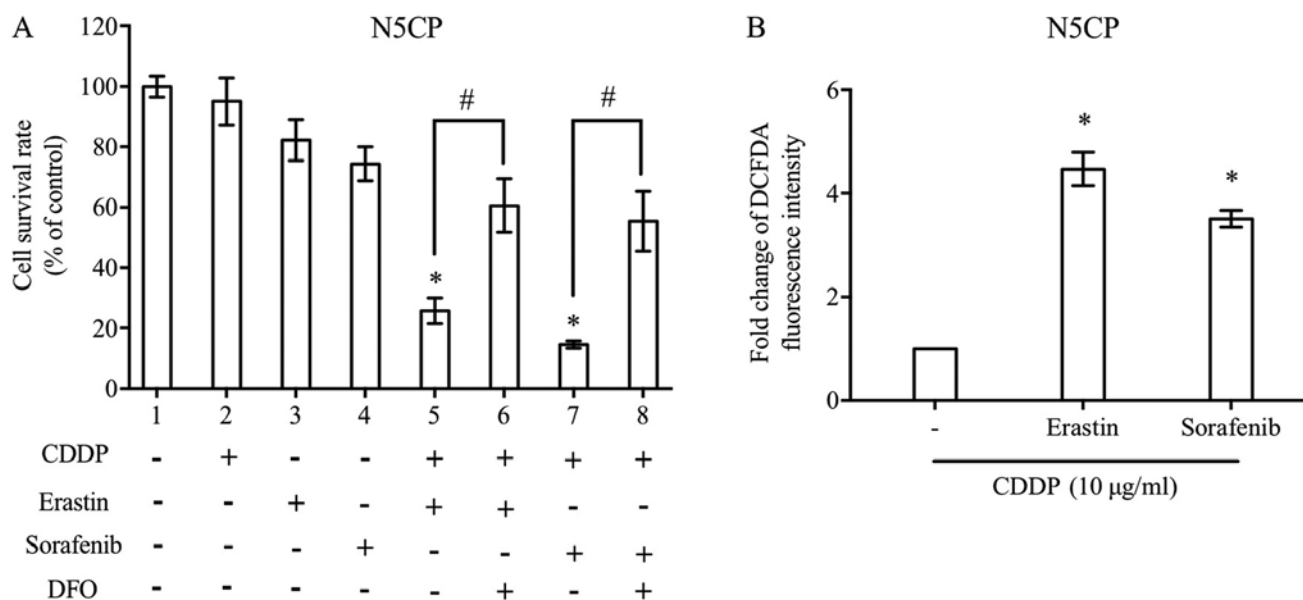
Figure 4. CDDP combined with erastin/sorafenib effectively induces N5CP cell ferroptosis. (A) N5CP cells were treated as indicated for 48 h. A Cell Counting kit-8 assay was used to detect the cell survival rate. The concentrations of CDDP, erastin, sorafenib and DFO were 10, 5, 10 and 50 μM, respectively. (B) N5CP cells were exposed to CDDP combined with erastin, sorafenib or control at the same concentrations for 12 h, and the reactive oxygen species level was detected using a DFCDA probe and flow cytometry. Data are presented as the mean ± standard error from at least three independent experiments. Differences among groups were assessed by one-way ANOVA with a Bonferroni post hoc test. *P<0.05 vs. (A) DMSO, vs. (B) CDDP only; #P<0.05. CDDP, cisplatin; DCFDA, 6-carboxy-2',7'-dichlorofluorescein diacetate dye; DFO, deferoxamine mesylate.

low doses of CDDP (1 mg/kg), erastin (5 mg/kg) and sorafenib (3 mg/kg) did not exhibit clear inhibitory effects on tumour growth effects when used alone; however, when used in combination, tumour growth was significantly inhibited (Fig. 5B). Based on the aforementioned results, it may be hypothesised that apparent reductions in tumour size due to treatment with erastin/sorafenib alone were possibly due to the dosages being high enough. It was evident that the effective concentrations of *in vivo* and *in vitro* experiments were inconsistent. It is a common phenomenon that the effects of drugs are not consistent. Evidence suggests that the effect of drugs is associated with numerous factors apart from concentration, including the route of administration and liposoluble activity (23).

Discussion

Resistance to drugs can usually be divided into two subtypes: Primary resistance, which is associated with chemoresistance prior to chemotherapy; and acquired resistance, which is mainly involved in drug resistance following chemotherapy (24). A variety of solid tumour cells, represented by NSCLC, exhibit inherent disorders of CDDP transport and metabolism, accompanied by high expression levels of multidrug resistance genes. Additionally, the anti-apoptotic pathway can be activated by CDDP; therefore, the coexistence of these two drug-resistance subtypes clearly restricts the curative effect and application of CDDP (25,26). In addition, although carboplatin (CBP) is widely used in NSCLC treatment as the second generation of platinum medicine and has excellent properties, including lower ototoxicity and nephrotoxicity, there is a cross-resistance to CBP and CDDP, such that CBP neither eliminates CDDP resistance nor markedly improves the prognosis of patients with NSCLC (27). In previous years, progress in NSCLC treatment

research has been made regarding targeted drugs in addition to the exploration of platinum. In particular, the introduction of drugs that specifically target ALK receptor tyrosine kinase, epidermal growth factor receptor and programmed cell death 1/programmed death-ligand 1 substantially improves the therapeutic outcomes of patients with certain gene mutations (28,29). However, the effect of these targeted drugs is unsatisfactory in patients without these mutations and gene expression characteristics. In the present study, a CDDP-resistant NSCLC cell model was used to reveal the CDDP sensitivity of tumour cells, which was reduced by the Nrf2/xCT pathway. Additionally, the results indicated that inducing ferroptosis could efficiently eliminate CDDP-resistant NSCLC cells.

Ferroptosis was fortuitously identified during the process of screening compounds that selectively produce lethal effects on RAS mutation-containing tumour cells. Two small molecules, erastin and Ras selective lethal (RSL) 3 compound (RSL3), of entirely different chemical structures are known as RSL compounds. The two can inactivate the intracellular GSH-dependent antioxidant defence and induce ferroptosis by different mechanisms (6). Erastin mainly inhibits the import of cystine through xCT, whereas RSL3 directly binds and restrains glutathione peroxidase 4 (Gpx4). Since Gpx4 is indispensable for the physiological function of cells, RSL3 exhibits cytotoxicity against normal cells, which limits the application of Gpx4 inhibitors (6,30). Similar to erastin, the small molecules sulfasalazine, glutamate, phosphatidylethanolamines (PE) and sorafenib mainly trigger ferroptosis by affecting xCT (31,32). Glutamate is rarely used clinically due to its neurotoxicity, and a study regarding the mechanism of ferroptosis induced by PE is currently in its infancy (33). The present study investigated whether erastin/sorafenib used individually or in combination with
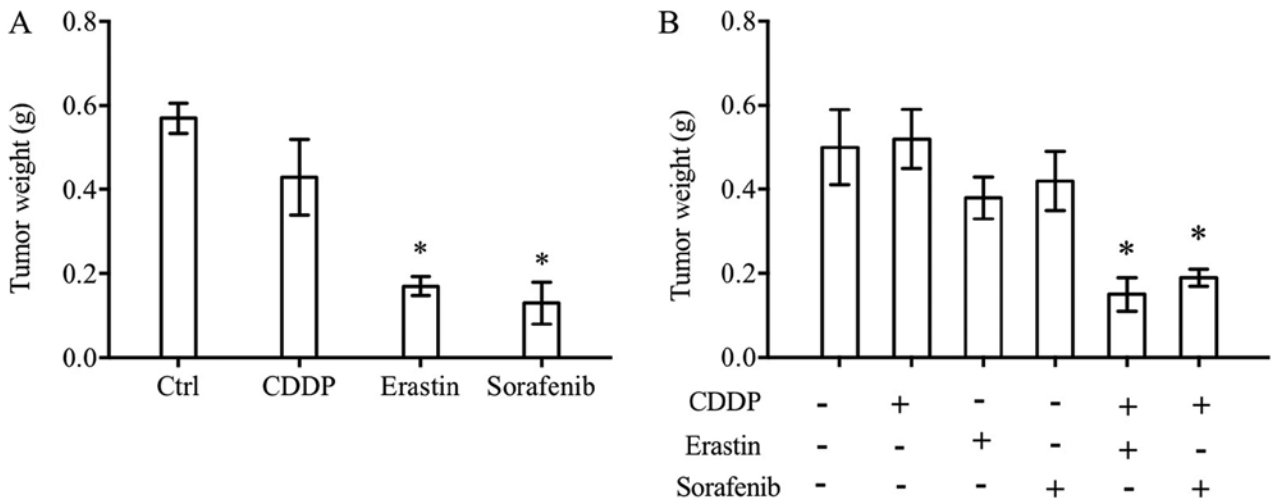
Figure 5. Erastin/sorafenib inhibits tumour growth *in vivo*. (A) Mice bearing N5CP cell subcutaneous xenografts were randomly divided into four groups and intraperitoneally injected with CDDP, erastin, sorafenib or PBS (Ctrl). After three injections, tumours were removed and weighed. (B) Low doses of CDDP, erastin and sorafenib were used in combination, and the tumour growth inhibitory effect was estimated by tumour weight. Data are presented as the mean ± standard error, and differences among groups were assessed by one-way ANOVA with a Bonferroni post hoc test. *$P<0.05$ vs. groups treated with PBS. CDDP, cisplatin; Ctrl, control.

a small dose of CDDP could effectively induce ferroptosis in CDDP-resistant NSCLC cells. Sulfasalazine is a clinical anti-inflammatory drug used to treat inflammatory bowel diseases and rheumatoid arthritis (34). Although a previous report claimed that sulfasalazine can increase the sensitivity of colon cancer cells to CDDP in a GSH-dependent manner (35), in Phase I clinical trials of patients with gastric cancer who were positive for splice variant isoforms of CD44, which can stabilize xCT, co-application of sulfasalazine and CDDP to CDDP-resistant advanced gastric cancer exhibited insufficient effectiveness (36). Consistent with the aforementioned studies, sulfasalazine did not exhibit clear cytotoxic effects with CDDP in the present CDDP-resistant NSCLC cell model (data not shown). These contradictory results may be explained by the specificity of tumour cells, xCT blocking the efficacy of the drug or another unknown reason. In the present study, only the role of erastin/sorafenib in one type of CDDP-resistant NSCLC cells was observed, so there are limitations to this analysis. In future studies, we will continue to explore the function of erastin/sorafenib in other CDDP-resistant NSCLC cell lines, as well as other pathological types of cells. Besides blocking xCT, sorafenib is also a multi-target tyrosine kinase inhibitor possessing anti-proliferative and anti-angiogenic effects (37). In the present study, the lethality of sorafenib, whether used individually or in combination with CDDP, on N5CP cells was clearer than that of erastin; however, it was inconsistent with the mediated intracellular lipid ROS accumulation levels. Additionally, DFO and Vit-E failed to completely inhibit the cytotoxicity of sorafenib. These results suggested that the cell death mechanism, induced by sorafenib alone or in combination with CDDP, may be mainly ferroptosis in combination with multiple other cell death mechanisms.

The transcription factor Nrf2 is the master regulator of cell antioxidants for oxidative or electrophilic stress. ROS can induce Nrf2 accumulation in the nucleus and then combine with basic leucine zipper proteins, including small MAF bZIP transcription factor, to eventually form a transactivation complex to bind to AREs, which regulate downstream gene transcription (38). The ARE sequence is mostly present in the promoter or enhancer regions of genes encoding phase II detoxification enzymes (39). These enzymes driven by Nrf2/ARE are principally involved in the regulation of intracellular ROS levels, and their expression level directly affects the occurrence of ferroptosis. HO-1 mediates ferroptosis induced by BAY11-7085 (40). NQO1, HO-1 and ferritin heavy chain 1 provide resistance to erastin- and sorafenib-induced hepatocellular carcinoma cell ferroptosis by regulating iron metabolism and lipid peroxidation (22). Metallothionein-1 has recently been revealed to be the downstream target gene of Nrf2, which is the biomarker of tumour cell ferroptosis induced by sorafenib (41). Therefore, Nrf2 influences ferroptosis through multiple downstream target genes and is considered the central regulatory factor of ferroptosis. The present study also revealed that the regulatory effect of knockdown or overexpression of Nrf2 on CDDP sensitivity was significantly higher than that of xCT, which may be associated with the simultaneous regulation of additional Nrf2 target genes. Therefore, the ferroptosis induction efficiency of Nrf2 inhibitors may be higher than that of downstream target gene inhibitors. Although a variety of small molecules have been reported to specifically inhibit Nrf2, their effects on ferroptosis induction have rarely been reported on (42).

In conclusion, the present study revealed that Nrf2 pathway activation is one of the predominant regulatory mechanisms underlying NSCLC cell resistance to CDDP. Erastin and sorafenib could efficiently induce CDDP-resistant NSCLC cell ferroptosis. In addition, a low concentration of CDDP in combination with erastin/sorafenib effectively induced N5CP cell ferroptosis; the potential mechanism by which sorafenib and erastin induced ferroptosis in CDDP-resistant NSCLC cells may be associated with inhibition of the expression of the Nrf2 downstream target gene xCT. In a nude mouse

xenograft model, erastin and sorafenib markedly restrained N5CP cell growth. Ferroptosis inducers, represented by erastin and sorafenib, may benefit the OS of patients with advanced NSCLC or even following CDDP treatment failure, which provides a novel perspective for the treatment of NSCLC.

Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YL and LZ designed the research and wrote the paper. YL, HYY, XMX and HBL implemented the experiments and data collection. CW performed the statistical analyses and inter-pretation. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of China Medical University, and written informed consent was obtained from the patients. The animal study was approved by the Institutional Animal Care and Use Committee of China Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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