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SUPPLEMENTARY MATERIAL TO

4-Aminoquinolines promote redox-dependent ferroptosis sensitization in pancreatic ductal adenocarcinoma cells

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CONTENT

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Table S1. LC-MS/MS detection parameters for the analyzed metabolites.

Analyte	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)	Detection mode
GSH	305.91	143.04	21	MRM
GSSG	610.94	305.75	26	MRM
Cystine	238.92	74.30	20	MRM
MDA	71.19	53.37	19	MRM
Cysteine	120.01	–	–	Molecular ion
Tartaric acid	149.0	–	–	Internal standard

MS source parameters: spray voltage, 3500 V; vaporizer temperature, 300 °C; capillary temperature, 300 °C; sheath gas pressure, 20 arbitrary units; auxiliary gas pressure, 10 arbitrary units; capillary offset, 35; tube lens offset, 44.

Determination of Active Caspase-3/7 by FAM FLICA Assay

Caspase-3/7 activity was assessed using a FAM-FLICA assay (ICT094, Bio-Rad, California, USA), which employs a cell-permeable fluorescent probe that binds to active caspase-3/7. Cells were stained according to the manufacturer's protocol and analyzed by flow cytometry.

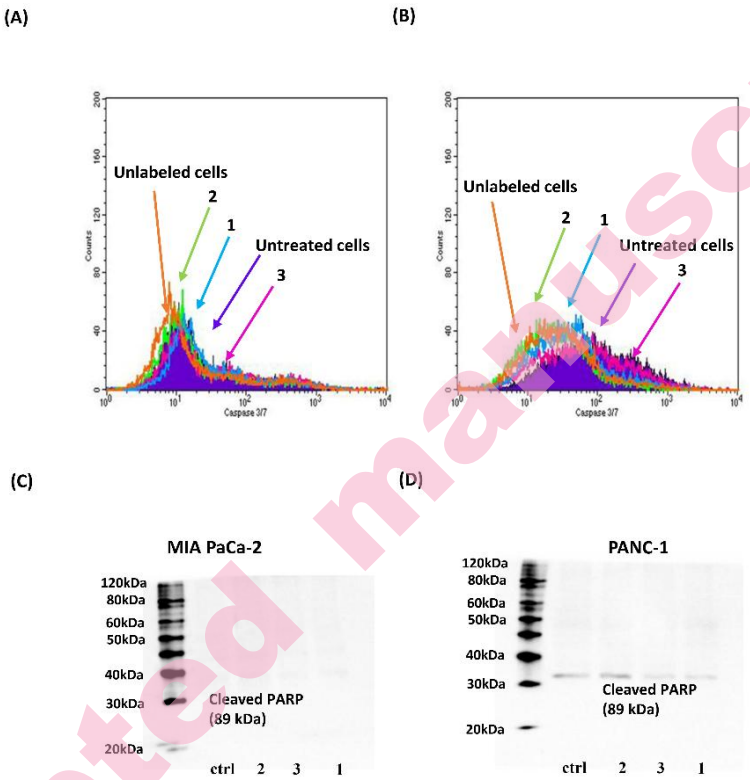

Fig. S1. Effects of 4-aminoquinoline derivatives on caspase-dependent apoptotic markers in PDAC cells. Representative flow cytometry histograms showing caspase-3/7 activity in MIA PaCa-2 (A) and PANC-1 (B) cells following 24 h treatment with compounds **1**–**3** (2 µM and 4 µM, respectively). Caspase-3/7 activity was assessed using the FAM-FLICA assay. Representative Western blots showing the absence of cleaved PARP (89 kDa) in MIA PaCa-2 (C) and PANC-1 (D) cells following treatment with compounds **1**–**3**. No detectable increase in caspase-3/7 activity or PARP cleavage was observed under the investigated conditions. Flow cytometry data are representative of three independent experiments.

Intracellular staining for phosphorylated p38

Cells stained for FACS analysis were treated as described above. Briefly, cells (5×10^5 cells/flask 25 cm^2) were allowed to adhere for 24 hours in standard conditions and then treated as described in the results section. After the stimulation period, cells were fixed immediately by adding pre-warmed Cytofix Buffer for 10–12 minutes at 37 °C and washed twice with PBS containing 1% BSA. After permeabilizing the cells using Perm Buffer for 20 minutes at room temperature and washing, cells were incubated with mouse anti-human phospho-p38 MAPK (Thr180/Tyr182) antibody, clone 28B10 (1:800, Cell Signaling, Danvers, MA, USA) at room temperature for 60 minutes protected from light. After the incubation, cells were washed three

times with PBS containing 1% BSA and incubated with the FITC-coupled secondary antibody (dilution 1:100, BD Pharmingen, San Diego, CA, USA). Samples were analyzed by flow cytometer.

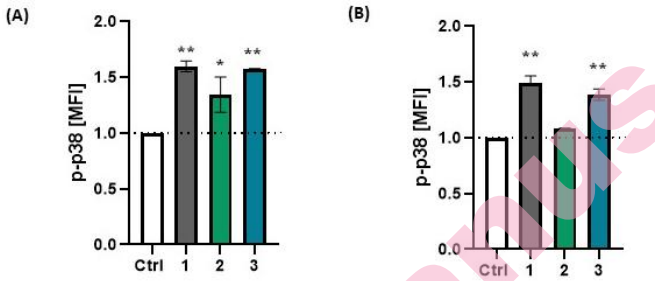

Fig. S2. Flow cytometric analysis of phosphorylated p38 levels in PDAC cells following treatment with 4-aminoquinoline derivatives. After 24 hours of treatment of MIA PaCa-2 (A) and PANC-1 (B) cells with investigated compounds (2 μM for MIA PaCa-2 and 4 μM for PANC-1) phosphorylated p38 protein levels were analyzed by flow cytometry. Data are presented as mean fluorescence intensity (MFI). Bar graph showing results presented as mean ± SD from three independent experiments. Asterisks denote statistical significance compared to control cells * $p < 0.05$, ** $p < 0.01$ (one-way ANOVA).

Changes in mitochondrial transmembrane potential

Mitochondrial transmembrane potential (MMP) was assessed using a cationic dye JC-1 (5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide, 65-0851, Thermo Fischer Scientific, Waltham, MA, USA). Following treatment cells were stained with JC-1 dye at final concentration of 2 μM, according to the manufacturer's protocol, followed by flow cytometric analysis.

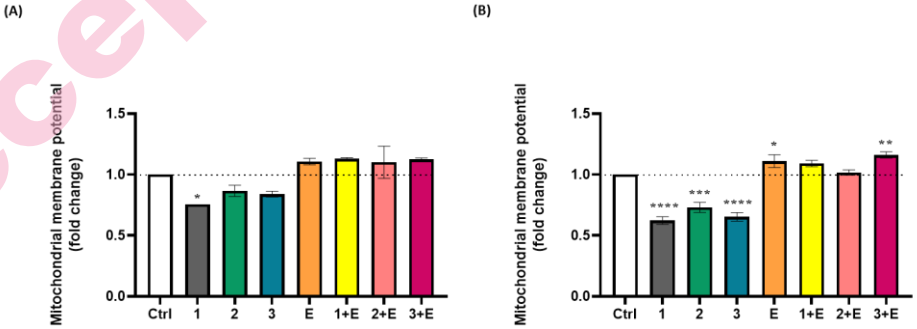

Fig. S3. Effects of the investigated compounds combined with erastin (E) on mitochondrial membrane potential of PDAC cells. Mitochondrial membrane potential of MIA PaCa-2 (A) and PANC-1 (B) cells after treatment with compounds **1**–**3** (2 μM for MIA PaCa-2 and 4 μM for PANC-1) alone and combined with erastin (10 μM) for 24 hours was assessed by flow cytometry using JC-1 dye. Bar graph showing results presented as mean ± SD from three independent experiments. Asterisks denote statistical significance compared to control cells *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (one-way ANOVA).

Gene and protein expression analysis

Transcriptomic expression data of ferroptosis regulators (GPX4, SLC7A11, NFE2L2, KEAP1, and SQSTM1) in pancreatic ductal adenocarcinoma (PDAC) were analyzed using the GEPIA2 web platform (http://gepia2.cancer-pku.cn) accessed on June 21, 2025. Boxplots comparing gene expression levels between tumor and normal pancreatic tissues were generated from TCGA-PAAD and GTEx data, with values expressed in transcripts per million (TPM). Data were downloaded and annotated together according to the GEPIA2 web platform's legends. mRNA expression in PDAC cell lines was derived from Human Protein Atlas (HPA) transcriptomic profiles, and protein expression data were sourced from DepMap proteomics (mass spec and RPPA) accessed on July 20, 2025.

Survival Analysis

Survival analysis was performed using GEPIA2. Patients were stratified into high- and low-expression groups based on the median expression level of each gene. Kaplan-Meier survival curves were generated, and differences between the groups were evaluated using the log-rank test. Hazard ratios (HRs) with 95% confidence intervals were reported.

Genomic alteration analysis (OncoPrint)

Experimental details. Genetic alterations in selected ferroptosis regulators were examined using cBioPortal for Cancer Genomics (https://www.cbioportal.org/), focusing on the TCGA-PAAD dataset (accessed on June 21, 2025). OncoPrint plots were generated to visualize the frequency and types of genomic alterations, including missense mutations, truncating mutations, amplifications, deep deletions, and changes in mRNA/protein expression. Data were downloaded and annotated according to the cBioPortal legends.

Expression and survival relevance of ferroptosis-associated genes in PDAC

The expression of key ferroptosis regulators was analyzed in the TCGA-PAAD cohort using the GEPIA2 platform. NFE2L2, KEAP1, SQSTM1, and GPX4 were significantly upregulated in PDAC compared with normal pancreatic tissues, indicating alterations in antioxidant and ferroptosis-associated pathways in human PDAC (Fig. S4A). In contrast, SLC7A11 expression was not significantly altered. Survival analysis did not reveal significant associations between the expression of these genes and overall survival, although higher SLC7A11 expression showed a trend toward poorer outcome (Fig. S5).[1]

To further characterize ferroptosis-related pathways in the PDAC cell models used in this study, transcriptomic (HPA) and proteomic (DepMap) datasets were analyzed for MIA PaCa-2 and PANC-1 cells (Fig. S4B-D). Transcriptomic analysis revealed high GPX4 expression in both cell lines, with higher NFE2L2 and SQSTM1 expression in PANC-1 cells, whereas TP53 expression was higher in MIA PaCa-2 cells (Fig. S4B).

Proteomic data showed only partial correspondence with transcript levels (Fig. S4C). Protein expression data for NFE2L2 in MIA PaCa-2 cells and SQSTM1 in PANC-1 cells were not available in the database. SLC7A11 protein expression was higher in PANC-1 than in MIA PaCa-2 cells, suggesting

differences in cystine metabolism and redox regulation between the two cell lines. TP53 protein levels were markedly higher in PANC-1 cells. Comparison of mRNA and protein expression further highlighted discrepancies between transcript abundance and protein levels, particularly for GPX4 and SLC7A11 (Fig. S4D).

OncoPrint analysis of TCGA-PAAD data revealed frequent alterations in TP53 (63%) and alterations in components of the KEAP1-Nrf2-p62 axis (Fig. S4E). Moderate alterations were observed in NFE2L2 (9%), SQSTM1 (8%), and KEAP1 (6%), including mRNA upregulation and occasional amplifications. SLC7A11 and GPX4 exhibited lower alteration frequencies (4% and 2.7%, respectively), predominantly involving increased mRNA expression. Collectively, these findings indicate dysregulation of ferroptosis- and redox-associated pathways in PDAC.

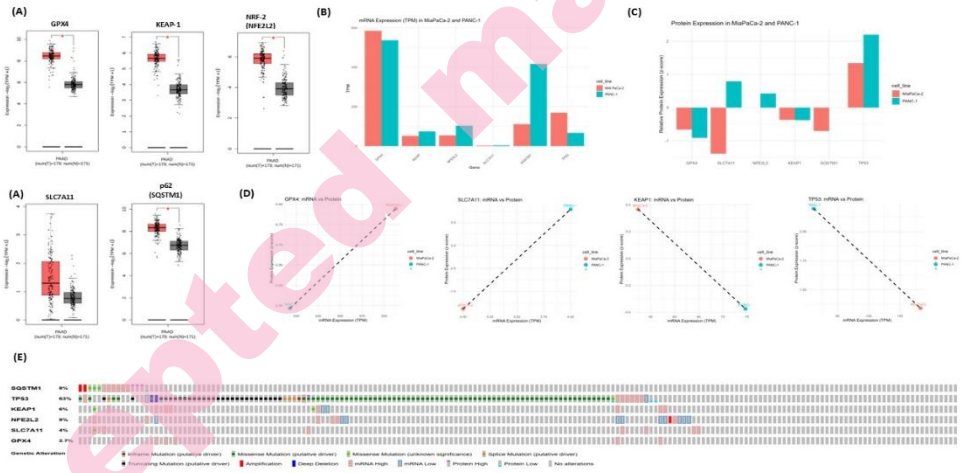

Fig. S4. Bioinformatic analysis of ferroptosis- and redox-related genes in pancreatic ductal adenocarcinoma. (A) mRNA expression (TPM) of NFE2L2, KEAP1, SQSTM1, SLC7A11, and GPX4 in PDAC (PAAD in GEPIA2) and normal pancreatic tissues analyzed using GEPIA2. (B) Comparative mRNA expression of ferroptosis- and redox-related genes in MIA PaCa-2 and PANC-1 cells based on HPA transcriptomic data. (C) Corresponding protein expression based on DepMap proteomic data. (D) Comparison of mRNA and protein expression levels in MIA PaCa-2 and PANC-1 cells. (E) OncoPrint representation of genomic and molecular alterations in SQSTM1, TP53, KEAP1, NFE2L2, SLC7A11, and GPX4 in the TCGA-PAAD cohort.

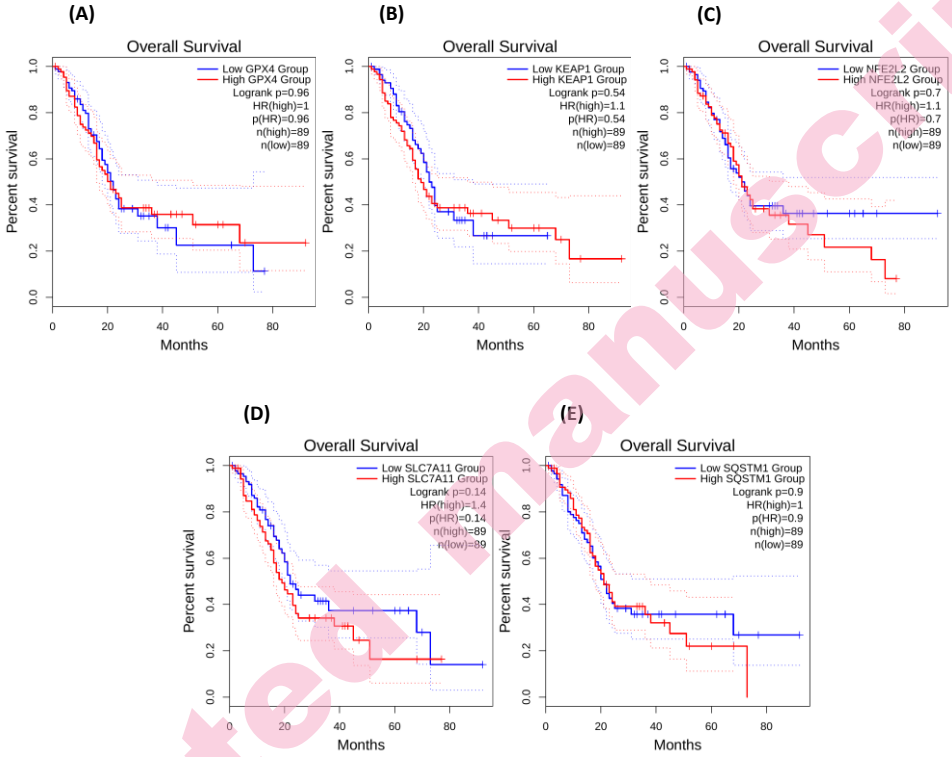

Fig. S5. Overall survival according to the expression of ferroptosis- and redox-related genes in PDAC. Kaplan-Meier overall survival curves for PDAC patients stratified according to the expression of NFE2L2, KEAP1, SQSTM1, SLC7A11, and GPX4 using GEPIA2. Patients were stratified into high- and low-expression groups based on median gene expression. Statistical significance was assessed using the log-rank test.

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