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Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. **00(0)** 1-18 (2026)
JSCS–14049
Original scientific paper
Published DD MM, 2026

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

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(Received 6 July; revised 6 August; accepted 21 September 2026)

Abstract: Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive cancer marked by oxidative stress, metabolic rewiring, and resistance to cell death. Ferroptosis, a regulated cell death driven by iron-dependent lipid peroxidation, represents a promising therapeutic vulnerability in PDAC; however, intrinsic resistance to ferroptosis limits its therapeutic exploitation. This study shows that 4-aminoquinoline derivatives sensitize PDAC cell lines (MIA PaCa-2 and PANC-1) to erastin-induced ferroptotic cell death. Ferroptosis sensitization occurred independently of caspase-3/7 activation and was associated with increased ROS production, mitochondrial oxidative stress, lipid peroxidation, and redox imbalance. Importantly, sensitivity to ferroptosis was accompanied by decreased GSH levels, reduced intracellular cystine levels, and differential regulation of the KEAP1-Nrf2-p62 axis. While PANC-1 cells exhibited enhanced oxidative stress and KEAP1 upregulation, MIA PaCa-2 cells maintained relative redox homeostasis unless exposed to combined treatment conditions. To our knowledge, this study provides the first evidence that 4-aminoquinoline derivatives promote sensitization to ferroptosis in PDAC and highlights the therapeutic potential of targeting redox metabolism and stress-adaptive signaling to overcome resistance to therapy in pancreatic cancer.

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https://doi.org/10.2298/JSC260706057Z

Keywords: redox homeostasis; KEAP1-Nrf2-p62 axis; oxidative stress; cysteine metabolism; erastin.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive cancer, with a 5-year survival rate below 10%, and is extremely difficult to treat due to resistance to conventional treatments, including chemotherapy, radiotherapy, and immunotherapy.[1] Consequently, there is an urgent need to identify alternative therapeutic vulnerabilities that can overcome treatment resistance in PDAC. Ferroptosis, an iron-dependent form of regulated cell death driven by lipid peroxidation, has emerged as a promising therapeutic vulnerability in PDAC.[2,3] Ferroptosis is characterized by iron-dependent accumulation of lipid peroxides and failure of cellular antioxidant defenses, particularly those involving glutathione peroxidase 4 (GPX4), a key enzyme that protects cellular membranes from peroxidative damage.[2,3]

Redox imbalance is a common feature of cancer cells, which often exhibit persistently elevated reactive oxygen species (ROS) levels due to oncogenic signaling, metabolic activity, mitochondrial dysfunction, and the inflammatory tumor microenvironment.[4,5] One consequence of mutant-KRAS signaling, which occurs in approximately 90% of PDAC cases, is increased ROS production, which can damage cellular structures. In response, PDAC cells activate compensatory metabolic pathways that rely on cysteine-derived metabolites such as reduced glutathione (GSH).[6]

Among the key cellular defense systems is the KEAP1-Nrf2 pathway, which regulates the antioxidative response. When the cell is in redox homeostasis, KEAP1 binds one Nrf2 molecule and promotes its degradation. In contrast, oxidative stress disrupts this interaction, allowing Nrf2 to translocate to the nucleus and activate transcription of antioxidant genes, including NQO1, GCLM, and GPX. In addition to canonical activation through oxidative modification of KEAP1,[7–13] Nrf2 can also be activated via a noncanonical mechanism involving p62-mediated KEAP1 degradation through autophagy.[14–18] While Nrf2 exerts tumor-suppressive functions during early tumorigenesis,[19] sustained activation of the KEAP1-Nrf2 axis in PDAC promotes redox adaptation, therapy resistance, and tumor progression.

Small-molecule compounds like erastin or RAS-selective-lethal-3 small molecule (RSL3) act as pharmacological ferroptosis inducers by targeting the cystine/glutamate antiporter system Xc^- and GPX4, respectively.[3,20] GPX4 is a key suppressor of ferroptosis that protects cells from lipid peroxidation and oxidative membrane damage. Manipulating ferroptosis, either through induction or inhibition, has emerged as a promising therapeutic strategy in cancer and other oxidative stress-related diseases.[21,22]

Ferroptosis has emerged as a promising therapeutic vulnerability in PDAC due to the tumor's distinct redox and metabolic dependencies.[23–26] PDAC cells display variable sensitivity to ferroptosis based on differences in antioxidant capacity, lipid composition, and iron metabolism.[27,28] In particular, the cystine transporter SLC7A11 and glutathione metabolism play critical roles in maintaining redox balance and ferroptosis resistance in PDAC cells.[29,30]

Although ferroptosis plays a role in various metabolic processes, its specific role in PDAC metabolism remains unclear. Our previous work demonstrated that 4-aminoquinoline derivatives inhibit autophagy and induce ROS accumulation in PDAC cells, which can be partially reversed by antioxidants such as *N*-acetylcysteine (NAC) or GSH.[31] Given their structural similarity to chloroquine, which has been shown to reduce cystine uptake[29] and the ability of other antimalarial agents to enhance ferroptosis through iron-mediated ROS production,[32] we hypothesized that our compounds might similarly modulate ferroptotic susceptibility. Therefore, this study investigated whether 4-aminoquinoline derivatives promote a ferroptosis-sensitizing phenotype in PDAC and explored the underlying redox-dependent mechanisms in MIA PaCa-2 and PANC-1 cells.

EXPERIMENTAL

Pharmacological/biological assays

Reagents and antibodies

Erastin (5449, Tocris Bioscience, MN, USA) was used as a chemical inducer of ferroptosis. 4-aminoquinoline derivatives **1**–**3** (Fig. 1A) have been previously reported by Konstantinović *et al.*[33] The primary antibodies used in this study anti-human p62 (5114, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-human xCT/SLC7A11 (12691, Cell Signaling Technology, Danvers, MA, USA), mouse anti-human KEAP1 (MA5-17106, Invitrogen, Thermo Fisher Scientific, Waltham, MA USA), rabbit anti-human NRF2 (PA5-27882, Invitrogen, Thermo Fisher Scientific, Waltham, MA USA) mouse anti-human p53 (2524, Cell Signaling Technology, Danvers, MA, USA), mouse anti-human phospho-p38 MAPK (Thr180/Tyr182) antibody, clone 28B10 (1:800, Cell Signaling, Danvers, MA, USA), and rabbit anti-human cleaved PARP (5625, Cell Signaling Technology, Danvers, MA, USA).

Cell culture

Cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Human pancreatic cancer cell lines MIA PaCa-2 and PANC-1 were maintained as monolayer cultures in Dulbecco's Modified Eagle's Medium (DMEM). The nutrient medium DMEM was prepared in sterile deionized water and supplemented with penicillin (192 U/mL), streptomycin (200 mg/mL), and 10% heat-inactivated FCS. The cells were grown at 37 °C in 5% CO_2 and a humidified air atmosphere.

General flow cytometry

All flow cytometric analyses were performed using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, USA), and data acquisition and analysis were conducted using CellQuest software (BD Biosciences, San Diego, CA, USA).

Assessment of cell death by propidium iodide (PI) staining and flow cytometry

Cell death was evaluated by propidium iodide staining, which detects loss of membrane integrity. Following treatment, cells were stained with PI at a final concentration of 2.5 µg/mL according to the manufacturer's protocol and analyzed by flow cytometry.

Total intracellular reactive oxygen species evaluation

Measurement of total intracellular reactive oxygen species ROS was performed using the oxidative stress-sensitive fluorescent probe, 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) (D3999, Thermo Fischer Scientific, Waltham, MA, USA). Cellular esterases deacetylate the probe to the non-fluorescent compound DCFH, which ROS can oxidize into 2',7'–dichlorofluorescein (DCF), thereby increasing green fluorescence intensity. Briefly, the treated cells were resuspended in 50 µM H2DCF-DA, according to the manufacturer's protocol. The levels of intracellular ROS were measured with flow cytometry. The excitation wavelength was 485 nm, and the fluorescence was measured at 530 nm.

Measurement of mitochondrial reactive oxygen species

The generation of mitochondrial reactive oxygen species (mROS) was evaluated using a ROS-sensitive probe MitoSOX Red (M36008, Thermo Fisher, Waltham, MA USA). This positively charged probe rapidly accumulates in mitochondria and is oxidized by superoxide but not by other ROS and reactive nitrogen species (RNS). Briefly, the treated cells were resuspended in 5 µM MitoSOX Red, according to the manufacturer's instructions. The levels of mROS were measured with flow cytometry. The excitation wavelength was 396 nm, and the fluorescence was measured at 610 nm.

Liquid chromatography with tandem mass spectrometry analysis of lipid peroxidation and redox status

Cells (approximately 500,000 cells per sample) treated with the tested compounds were collected and lysed in 100 µL of RIPA buffer and stored at -80 °C until analysis. After thawing, 10 µL of internal standard solution and 200 µL of cold acetonitrile were added to the samples. The obtained solution was vortex-mixed for 30 seconds, then centrifuged at 14,000 rpm for 10 minutes at 4 °C. The supernatants were filtered through a membrane and transferred to autosampler vials. Tartaric acid (100 ng/mL of acetonitrile) was used as an internal standard. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was performed using an ACELLA liquid chromatograph coupled to a TSQ Quantum Access MAX triple-quadrupole mass spectrometer (Thermo Fisher Scientific Inc., Madison, WI, USA). Chromatographic separation was performed on an Acclaim™ HILIC-10 column (3 µm, 120 Å, 2.1 × 150 mm) maintained at 30 °C, with the autosampler temperature set at 10 °C. The mobile phase consisted of acetonitrile and 0.1% formic acid using the following gradient: 0–3 min, 70% acetonitrile; 3–3.1 min, 70–20%; 3.1–8 min, 20%; 8–8.1 min, 20–70%; and 8.1–13 min, 70%. The flow rate was 500 µL/min and the injection volume was 20 µL. MS/MS analysis was performed in negative electrospray ionization mode. Detailed MRM transitions and mass spectrometer parameters are provided in Table S1.

Western blot analysis

Following four hours of treatment with the tested compounds, protein samples extracted from PDAC cells were used for a Western blot assay. Cells were washed twice with ice-cold PBS and lysed in RIPA lysis and extraction buffer (89901, Thermo Fischer Scientific, Waltham, MA, USA). Total protein concentration in cell lysates was determined by the BCA assay. Proteins were separated by sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis

(PAGE), transferred to nitrocellulose membranes and membranes were blocked with TBST containing 5% non-fat milk. Following overnight incubation of membranes with primary antibodies at 4 °C, membranes were washed with TBST containing 0.1% Tween 20 and incubated with goat anti-mouse peroxidase-conjugated secondary antibodies for 60 min at room temperature. Protein expression was normalized to loading controls, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Blots were probed with an enhanced chemiluminescence (ECL) substrate (32132, Thermo Fischer Scientific, Waltham, MA, USA) and visualized using an Azure Biosystems 600 Imaging System.

Statistical analysis

Experimental results are presented as mean ± SD. Differences between untreated and treated groups were evaluated by one-way ANOVA followed by Dunnett's multiple comparisons test. Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

4-Aminoquinoline derivatives sensitize PDAC cells to ferroptosis

Since our previous findings demonstrated that 4-aminoquinoline derivatives induce ROS accumulation, mitochondrial stress, and redox-dependent cytotoxicity in PDAC cells,[31] we next investigated whether these stress-associated alterations might also contribute to ferroptosis sensitization, a process closely linked to oxidative stress and the disruption of redox homeostasis. The IC_{50} values of compounds **1**–**3**, previously determined by the MTT assay (after 48h hours), were approximately 2 µM for MIA PaCa-2 and 4 µM for PANC-1 cells; therefore, these concentrations were used throughout the present study to ensure biological comparability between the two cell lines.[31] Importantly, under experimental conditions used in this study (24 h treatment), compounds **1**–**3** predominantly induced early apoptotic changes, without substantial accumulation of PI-positive late-apoptotic or necrotic cells.[31] This experimental setting allowed evaluation of ferroptosis-associated redox alterations with minimal interference from secondary cell death processes. To investigate whether compounds **1**–**3** modulate ferroptosis sensitivity, PDAC cell lines with distinct sensitivity to cysteine deprivation and erastin treatment were selected.[30] PANC-1 cells are characterized by higher SLC7A11 expression and are more dependent on cystine uptake and thus more susceptible to erastin-induced ferroptosis, whereas MIA PaCa-2 cells exhibit relative resistance to ferroptosis due to lower dependence on this pathway.[30]

Cells were treated with compounds **1**–**3** (Fig. 1A) in the presence or absence of erastin (10 µM) for 24 h (Fig. 1B–1D). Subsequently, cell death was determined by propidium iodide (PI) uptake and flow cytometry. Results presented in Fig. 1B–1D indicate that erastin alone induced cell death in PANC-1 cells (12.5%, $p = 0.005$) but not in MIA PaCa-2 cells, which is consistent with previous findings.[30] In contrast, combination treatment significantly increases cell death in both MIA PaCa-2 and PANC-1 cells ($p < 0.01$ for all combinations, Fig. 1B–1D). The synergistic effect was more pronounced in MIA PaCa-2 cells, indicating

sensitization of otherwise resistant cells to ferroptosis (Fig. 1B and 1C). Although the enhanced cell death observed under combined treatment conditions functionally supports ferroptosis sensitization, contributions of other cell death pathways cannot be fully excluded.

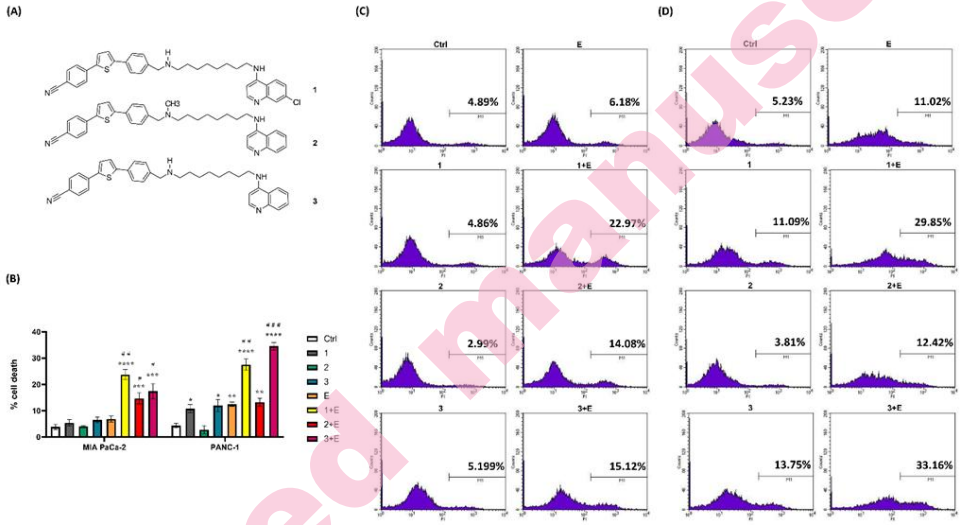

Fig. 1. 4-aminoquinolines enhance the sensitivity of PDAC cells to erastin-induced cell death. Quinoline derivatives **1**–**3** investigated for ferroptotic activities (A). The ability of quinoline derivatives to induce cell death (B) was determined after 24 hours of treatment of MIA PaCa-2 (C) and PANC-1 (D) cells with investigated compounds (2 μM for MIA PaCa-2 and 4 μM for PANC-1), and erastin (10 μM) alone or in combination. Bar graph of flow cytometric quantification of PI-positive cells presented as the mean ± SD of three independent experiments (B). Asterisks denote statistical significance compared to control cells (*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$) (B). Representative histograms of the cells stained with PI from three independent experiments after 24 hours of treatment are shown (C and D).

Moreover, treatment with compounds **1**–**3** did not significantly increase caspase-3/7 activity (Fig. S1A and S1B) or induce detectable PARP cleavage (Fig. S1C and S1D), indicating no substantial activation of the canonical caspase-dependent apoptotic pathway under these conditions. Together, these findings suggest that 4-aminoquinoline derivatives enhance sensitivity to erastin-induced ferroptosis, particularly in cells intrinsically resistant to erastin. Based on these observations, we further investigated the molecular basis underlying ferroptosis sensitization by 4-aminoquinoline derivatives.

The effects of 4-aminoquinolines and erastin on ROS accumulation in PDAC cells

Next, due to iron-dependent ROS generation being a key driver of ferroptotic cell death,[3] both mitochondrial ROS (mROS) and total ROS (tROS) levels in MIA PaCa-2 and PANC-1 cells following treatment were assessed.

Fig. 2 shows a pronounced increase in both mROS and tROS levels in PANC-1 cells upon treatment with erastin alone (3.8-fold for mROS and 8.9-fold for tROS, with $p < 0.0001$ for mROS and tROS) or in combination with compounds **1**–**3** (4-fold for mROS and approximately 6- to 11-fold for tROS, with $p < 0.0001$ for mROS and tROS), indicating that elevated oxidative stress may contribute to their ferroptosis induction sensitivity.

In contrast, MIA PaCa-2 cells did not exhibit increased ROS levels following erastin treatment alone, and showed a moderate mROS levels increase when combined with compound **2** (2-fold, $p = 0.0012$) and a more pronounced mROS levels increase in combination with compounds **1** and **3** (approximately 3- to 4-fold, $p < 0.0001$) (Fig. 2A and 2B), suggesting a more robust redox response in MIA PaCa-2 cells. These findings indicate that ferroptosis sensitization of PDAC cells seems to be closely associated with mitochondrial oxidative stress and an altered cellular redox balance.[34] Compounds **1** and **3** also increased p38 MAPK phosphorylation after 24 hours treatment in both MIA PaCa-2 and PANC-1 cells, consistent with activation of cellular stress-responsive signaling accompanying ROS accumulation (Fig. S2).

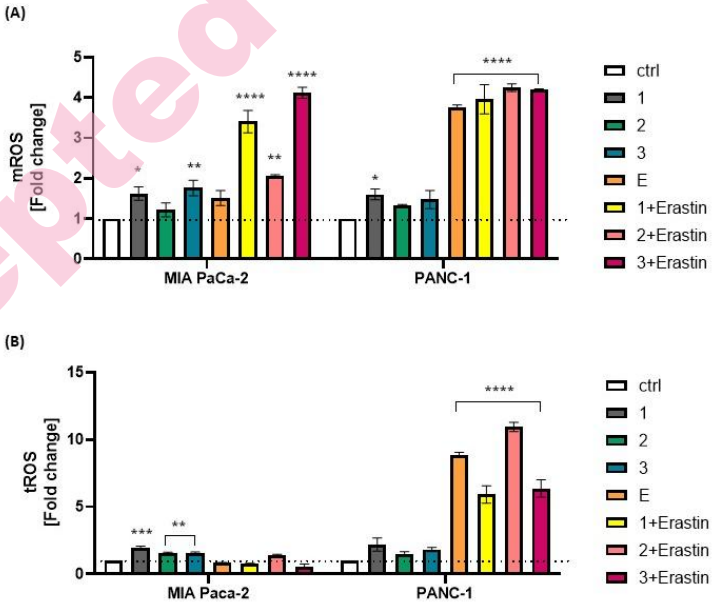

Fig. 2. Accumulation of intracellular ROS level in pancreatic adenocarcinoma cells after 24 h treatment with a combination of quinoline derivatives and erastin. After 24 hours of treatment of MIA PaCa-2 and PANC-1 cells with investigated compounds (2 μM for MIA PaCa-2 and 4 μM for PANC-1), mitochondrial (A) and total ROS (B) levels in PDAC cells were measured. Bar graph showing results presented as mean ± SD from three independent experiments. Asterisks denote statistical significance compared to control cells *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

In parallel, mitochondrial membrane potential analysis revealed distinct responses to erastin and the 4-aminoquinoline derivatives. Erastin induced mitochondrial hyperpolarization, whereas compounds **1**–**3** caused a reduction in mitochondrial membrane potential (MMP), indicating mitochondrial depolarization (Fig. S3). These findings further demonstrate that the compounds and erastin differentially affect mitochondrial function under the investigated conditions.

The effects of 4-aminoquinoline derivatives on PDAC cells lipid peroxidation and redox status

Since the combined treatments demonstrated ferroptosis sensitization, subsequent analyses focused on the effects of 4-aminoquinoline derivatives alone to identify the redox-related molecular alterations that may underlie this sensitizing effect. In this context, we investigated whether the compounds induce changes in glutathione metabolism, cystine utilization, and ferroptosis-associated signaling pathways that could create a ferroptosis-permissive cellular environment. Therefore, to better understand and elucidate the mechanisms underlying ferroptosis sensitization by the 4-aminoquinoline derivatives (**1**–**3**), key biochemical markers of oxidative stress and redox homeostasis, including lipid peroxidation by MDA determination, glutathione status (GSH/GSSG), and the cysteine/cystine ratio in PDAC cell lines MIA PaCa-2 and PANC-1, were determined.

MDA, as one of the final secondary byproducts of lipid peroxidation, and in ferroptosis, serves as a primary biochemical marker for measuring cellular damage.[35] Fig. 3A shows that 4-hour exposure to compound **1** significantly increased MDA level in MIA PaCa-2 cells ($p < 0.05$), while compounds **1** and **3** induced a significant increase in PANC-1 cells ($p < 0.05$ for both **1** and **3**, Fig. 3B). This effect was rapid and transient, as no changes in MDA levels were detected after 24 hours of treatment (Fig. 3A and 3B). These findings suggest that PANC-1 cells exhibit increased susceptibility to lipid peroxidation, consistent with elevated tROS levels,[30] whereas MIA PaCa-2 cells exhibited a more limited lipid damage response.

Next, given the central role of GSH in redox homeostasis and ferroptosis regulation, we examined whether compounds **1**–**3** affect GSH and GSSG levels in PDAC cells. As shown in Fig. 4A, MIA PaCa-2 cells showed no significant changes in GSH levels following treatment with **1**–**3**; however, GSSG levels decreased slightly after treatment with compound **1**, resulting in a minor increase in the GSH/GSSG ratio. Meanwhile, all compounds significantly reduced GSH levels after 4 hours of treatment on PANC-1 cells ($p < 0.05$ for all compounds) without affecting GSSG levels (Fig. 4B). This resulted in a decreased GSH/GSSG ratio, indicating impaired antioxidative capacity and increased oxidative stress.

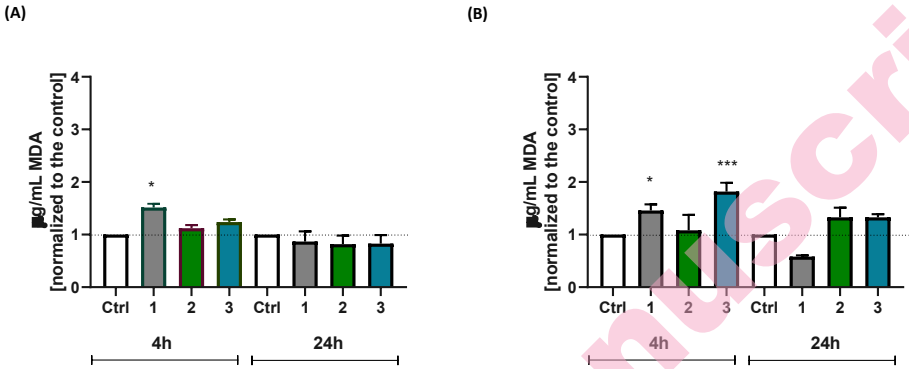

Fig. 3. Quantification of malondialdehyde by mass spectrometry. MDA levels were quantified by LC-MS/MS. After 4 hours and 24 hours of treatment of MIA PaCa-2 (A) and PANC-1 (B) cells with investigated compounds (2 µM for MIA PaCa-2 and 4 µM for PANC-1), cell lysates were prepared and separated by liquid chromatography and detected using tandem mass spectrometry in multiple reaction monitoring (MRM) mode. The MDA concentration was calculated based on an external calibration curve and normalized to protein content. Bar graph showing results presented as mean ± SD from two independent experiments. Asterisks denote statistical significance compared to control cells *$p < 0.05$, ***$p < 0.001$.

After 24 hours of treatment, no significant changes in GSH levels were detected in either cell line (Fig. 4A and 4B). Only minor changes in GSSG levels were observed in MIA PaCa-2 cells after treatment with compound **2** (Fig. 4A). However, no significant differences in the GSH/GSSG ratio were observed across all tested compounds (Fig. 4A). Whereas GSSG levels increased in PANC-1 cells following treatment with compounds **2** and **3** ($p < 0.05$ for **2** and **3**), this did not translate into a significant change in the GSH/GSSG ratio (Fig. 4B).

The alterations in MDA and GSSG indicate that 4-aminoquinoline derivatives induce an early, transient disruption of redox homeostasis, particularly in PANC-1 cells, characterized by increased lipid peroxidation and GSH depletion. This redox imbalance likely contributes to enhanced sensitivity to ferroptosis, whereas the relatively stable redox state in MIA PaCa-2 cells may underlie their resistance. Although these changes, induced by compounds **1**–**3**, are strongly associated with ferroptosis susceptibility, they do not independently cause ferroptotic cell death; rather, they may indicate a disruption of key redox buffering systems in PDAC cells.

Cysteine is a key determinant of redox homeostasis and ferroptosis sensitivity[36,37] as PDAC cells depend on cystine uptake and its reduction to cysteine. To assess whether 4-aminoquinoline derivatives affect this pathway, we measured intracellular cystine and cysteine levels over time.

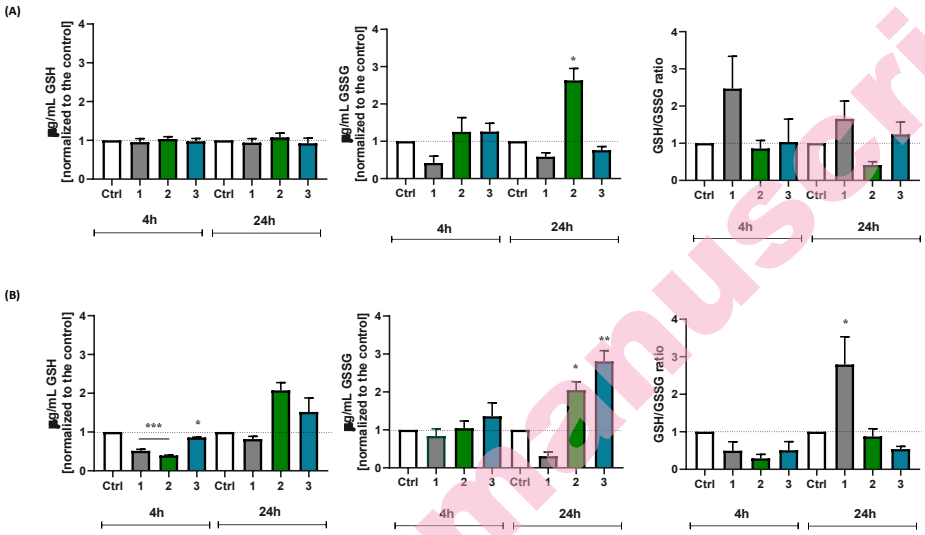

Fig. 4. Quantification of Reduced (GSH) and Oxidized (GSSG) Glutathione by LC-MS/MS. After 4 hours of treatment of MIA PaCa-2 (A) and PANC-1 (B) cells with investigated compounds (2 μM for MIA PaCa-2 and 4 μM for PANC-1), cells were lysed in ice-cold buffer. Samples were centrifuged and analyzed by LC-MS/MS in multiple reaction monitoring (MRM) mode. Quantification was performed using standard calibration curves, and values were normalized to total protein content. Bar graph showing results presented as mean ± SD from two independent experiments. Asterisks denote statistical significance compared to control cells *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$.

As shown in Fig. 5A, cystine levels were slightly reduced after 4 hours of treatment with **1**–**3** in both cell lines, although this decrease did not reach statistical significance. In PANC-1 cells, this trend became significant at 24 hours ($p < 0.05$ for all tested compounds, Fig. 5B), whereas cystine levels in MIA PaCa-2 cells remained largely unchanged (Fig. 5A). In parallel, cysteine levels increased in MIA PaCa-2 cells, while a slight decrease in PANC-1 cells after 4 hours of treatment (Fig. 5A and 5B). Consequently, the cysteine/cystine ratio was elevated in MIA PaCa-2 cells (Fig. 5A), indicating preserved intracellular cysteine availability. These findings suggest that MIA PaCa-2 cells maintain cysteine homeostasis despite treatment, which may support sustained glutathione synthesis and contribute to their resistance to ferroptosis. In contrast, reduced cystine availability in PANC-1 cells is consistent with impaired redox buffering and increased susceptibility to ferroptotic stress. These differences between the two cancer cell types further support the existence of a ferroptosis-permissive redox environment rather than representing a direct marker of ferroptotic cell death.

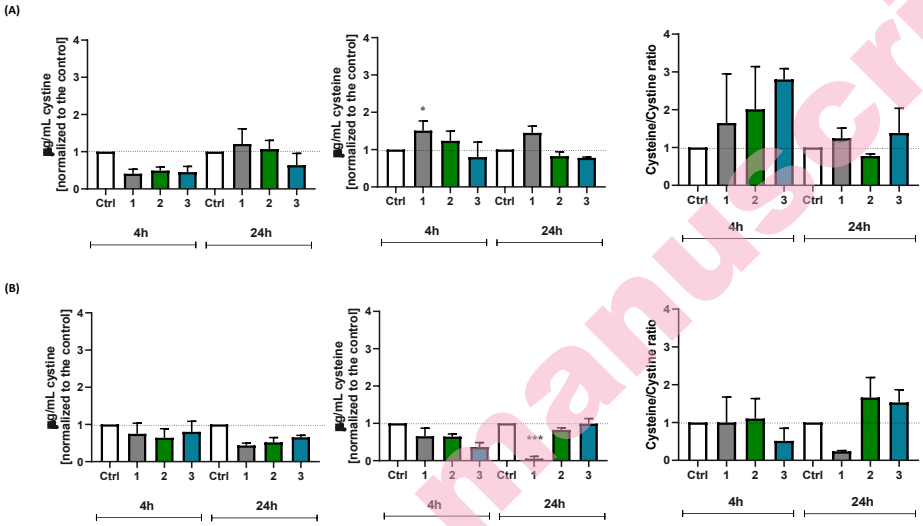

Fig. 5. Quantification of Cystine and Cysteine by LC-MS/MS. After 4 hours of treatment of MIA PaCa-2 (A) and PANC-1 (B) cells with investigated compounds (2 µM for MIA PaCa-2 and 4 µM for PANC-1), cells were lysed in ice-cold methanol. Samples were centrifuged and the supernatants analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) in multiple reaction monitoring (MRM) mode. Quantification was performed using external standards, and values were normalized to protein content. Bar graph showing results presented as the mean ± SD of two independent experiments. Asterisks denote statistical significance compared to control cells *p < 0.05, **p < 0.005, ***p < 0.001.

To place the experimentally observed redox alterations and ferroptosis sensitization in a clinically relevant PDAC context, we examined the expression, genomic alterations, and prognostic associations of key ferroptosis- and redox-regulatory genes using publicly available datasets (Fig. S4 and S5). The analysis supported the relevance of the SLC7A11/GPX4 antioxidant defense and the KEAP1-Nrf2-p62 regulatory axis in PDAC, although the associations varied among individual genes (Fig. S4). Based on these observations and their established roles in cellular redox homeostasis and ferroptosis regulation, we next examined the effects of compounds **1**–**3** on the corresponding proteins by Western blot analysis.

The impact of 4-aminoquinoline derivatives on KEAP-1/Nrf2 and p62 axis

Cells have evolved various antioxidant defense mechanisms to maintain redox homeostasis and to prevent excessive ROS accumulation. Among them, Nrf2 acts as a master regulator of antioxidant response by controlling the expression of genes involved in redox balance.[38] Therefore, we further investigated the effects of compounds **1**–**3** on Nrf2 and related ferroptosis-associated proteins. A 4-hour time point was selected to capture early redox-sensitive signaling events preceding

transcriptional feedback and cell death. This decision was supported by LC-MS/MS data showing that key redox perturbations, including GSH depletion, altered cysteine/cystine ratios and increased lipid peroxidation, were most pronounced at this early stage. In addition, previous studies have shown that modulation of ferroptosis-related proteins such as Nrf2, KEAP, and SLC7A11 occurs within the first hours of exposure to ferroptotic stimuli.[39–41] Prolonged exposure (24 hours) with 4-aminoquinoline derivatives was avoided, as apoptotic features were already detectable under these conditions.[31]

Western blot analysis revealed two Nrf2 forms with distinct electrophoretic mobility, corresponding to the nuclear Nrf2 (n-Nrf2) and the cytosolic Nrf2 (c-Nrf2) fraction (Fig. 6A–6D). The basal level of active n-Nrf2 was higher in untreated PANC-1 cells, indicating an intrinsically elevated antioxidant defense. This observation is consistent with previous reports demonstrating that mutant p53 (R273H) present in PANC-1 cells can enhance Nrf2 transcriptional activity and promote redox adaptation.[42] Upon treatment with 4-aminoquinoline derivatives (Fig. 6A–6D), n-Nrf2 levels increased in both MIA PaCa-2 and PANC-1 cells. In contrast, c-Nrf2 levels were higher in MIA PaCa-2 at baseline and remained largely unchanged under treatment, whereas in PANC-1 cells, c-Nrf2 expression decreased slightly (Fig. 8A–8D). This shift suggested enhanced nuclear translocation of Nrf2 and activation of stress-responsive antioxidant signaling. Previous studies have shown that ferroptosis-inducing agents, such as erastin and sorafenib, increase Nrf2 protein stability and prolong its half-life, supporting its role in adaptive stress responses.[43]

Recent studies have shown that the substrate adaptor protein p62 upregulates Nrf2 expression levels by directly interacting with KEAP-1 and inactivating it under various stress conditions.[44] Therefore, the effects of 4-aminoquinoline derivatives on the p62 and KEAP-1 expression were examined. A slight increase in p62 level was observed in MIA PaCa-2 cells following treatment with all compounds (Fig. 6A and 6F), whereas in PANC-1 cells this effect was evident primarily for compounds **1** and **3** (Fig. 6B and 6G). KEAP-1 could not be detected in either untreated or treated MIA PaCa-2 cells (Fig. 6A), which explains the accumulation of cNrf2 in this cell line. In contrast, KEAP-1 levels were significantly increased in PANC-1 cells upon treatment with compounds **1** and **3** (Fig. 6B and 6E). Collectively, these findings indicate an activation of the Nrf2-driven antioxidant response in PDAC cells following treatment. While Nrf2 upregulation is generally associated with protection against ferroptosis by maintaining cellular antioxidant defenses and redox homeostasis,[43] the observed redox imbalance and lipid peroxidation suggest that this response may be insufficient to fully counteract ferroptotic stress under the tested conditions.

System Xc^-, composed of solute carrier family 3 member 2 (SLC3A2) and solute carrier family 7 member 11 (SLC7A11), mediates cystine uptake and plays

a role in maintaining intracellular redox balance. Imported cystine is reduced to cysteine, a key precursor for GSH synthesis, supporting GPX4-dependent detoxification of lipid peroxides.[20,45] Accordingly, the SLC7A11-GSH-GPX4 axis represents a major defense system against ferroptosis. Therefore, we examined whether compounds **1**–**3** modulate SLC7A11 expression. As shown in Fig. 6A and 6H, treatment with compounds **1**–**3** did not significantly alter SLC7A11 expression in MIA PaCa-2 cells. In contrast, compounds **1** and **3** decreased SLC7A11 expression in PANC-1 cells. Notably, this decrease coincided with increased p62 expression (Fig. 6B and 6I), suggesting a potential link between stress signaling and modulation of cystine metabolism.

Given that both PDAC cell lines harbor mutant p53[46] (R248W in MIA PaCa-2 and R273H in PANC-1), and considering the reported involvement of p53 status in p62-mediated ferroptosis,[47] we further examined p53 expression. Basal p53 level was relatively higher in MIA PaCa-2 cells, while treatment with compounds **1**–**3** did not alter p53 expression (Fig. 6A and 6J). In contrast, a reduction in total p53 levels was observed in PANC-1 cells following treatment (Fig. 6B and 6K). These findings suggest that p53 status may contribute to the differential regulation of ferroptosis-related pathways, including SLC7A11 expression.[47] It should be noted that the antibody used detects total p53, and certain mutations may affect epitope recognition or protein stability, potentially influencing the observed expression levels.

Overall, these results support a model in which differential regulation of the Nrf2–p62–KEAP1 axis, together with modulation of SLC7A11 and p53, contributes to variability in ferroptosis sensitivity among PDAC cell lines, with PANC-1 cells exhibiting greater susceptibility due to impaired cystine-dependent antioxidant defenses.[30,48,49]

Recent clinical advances in RAS pathway inhibition in PDAC have renewed interest in therapeutic strategies that target vulnerabilities emerging during targeted therapy.[50] Importantly, resistance to KRAS inhibition has been linked to suppression of ferroptosis, as shown in KRAS G12D inhibitor-resistant PDAC models, in which MGST1-mediated inhibition of lipid peroxidation reduced ferroptotic sensitivity.[51] In this context, agents capable of disrupting redox buffering systems and promoting a ferroptosis-permissive state, such as the 4-aminoquinoline derivatives investigated here, may represent promising candidates for future combination strategies to potentially overcome or delay resistance to RAS-targeted therapies. Interestingly, quinoline-based compounds have previously been reported to interfere with stress-associated signaling pathways and mitochondrial redox homeostasis,[31,52] suggesting that the observed ferroptosis sensitization may reflect broader adaptive stress-response mechanisms that are increasingly recognized as contributors to targeted therapy resistance.[53]

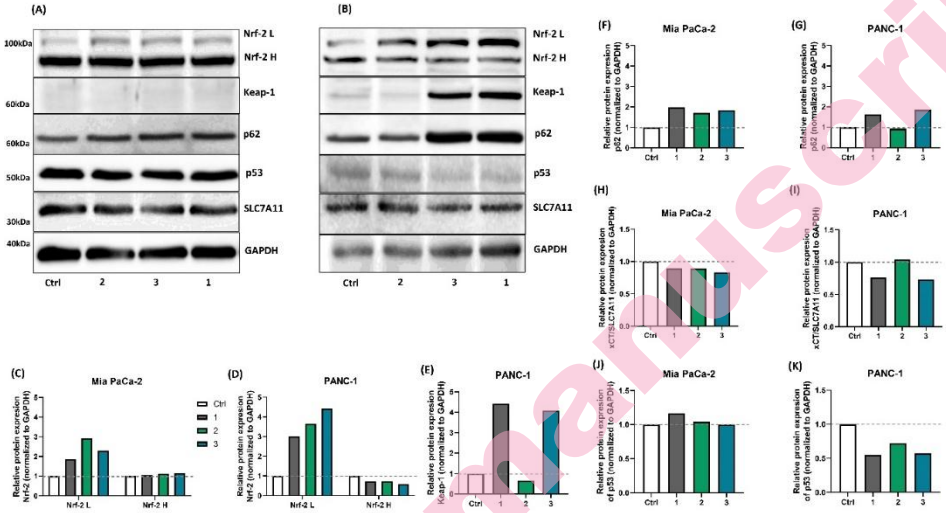

Fig. 6. Detection of Nrf-2, Keap-1, SLC7A11, p62, and p53 proteins associated with the regulation of ferroptosis by Western blotting. MIA PaCa-2 cells (A) and PANC-1 cells (B) were treated with the investigated compounds (2 μM for MIA PaCa-2 and 4 μM for PANC-1) for 4 hours. Densitometry of protein bands for MIA PaCa-2 cells (C, F, H, J) and PANC-1 cells (D, G, I, K) was performed using ImageJ software.

CONCLUSION

In conclusion, this study demontrates that 4-aminoquinoline derivatives sensitize PDAC cells to ferroptosis by disrupting redox homeostasis and modulating the KEAP1–Nrf2–p62 signaling axis. Ferroptosis sensitization was associated with mitochondrial ROS accumulation, altered glutathione metabolism, and altered cystine metabolism, highlighting the importance of the redox buffering system in regulating ferroptosis susceptibility. The distinct responses observed between PANC-1 and MIA PaCa-2 cells further emphasize the role of intrinsic antioxidant capacity and metabolic context in determining ferroptosis sensitivity. While PANC-1 cells exhibit pronounced redox disruption and ferroptotic vulnerability, MIA PaCa-2 cells maintain redox stability but can be sensitized by combined treatment. Together, our results identify redox regulation and cysteine metabolism as potential therapeutic vulnerabilities in PDAC and support further investigation of 4-aminoquinoline derivatives as ferroptosis-sensitizing agents in pancreatic cancer.

SUPPLEMENTARY MATERIAL

Additional data are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/14049, or from the corresponding author on request.

Acknowledgements: This research was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia, Contract numbers 451-03-33/2026-03/200288, 451-03-33/2026-03/200168, 451-03-33/2026-03/200161, 451-03-33/2026-03/200015, 451-03-33/2026-03/200043 and is in accordance with The 2030 Agenda for Sustainable Development of the UN (Goal 3: Good health and well-being and Goal 17: Partnership for the goals). This work has also received funding from the Serbian Academy of Sciences and Arts under the strategic projects program (grant agreement No 01-2019-F65).

И З В О Д

4-АМИНОХИНОЛИНИ СЕНЗИТИВИШУ ЋЕЛИЈЕ ДУКТАЛНОГ АДЕНОКАРЦИНОМА ПАНРЕАСА НА РЕДОКС-ЗАВИСНУ ФЕРОПТОЗУ

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Дуктални аденокарцином панкреаса је изузетно агресивно малигно обољење, које карактеришу повишен ниво оксидативног стреса, метаболичка адаптација и изражена отпорност на терапију. Фероптоза је облик програмиране ћелијске смрти покренут гвожђе-зависном пероксидацијом липида и представља обећавајућу терапијску мету код аденокарцинома панкреаса. Међутим, њена клиничка примена ограничена је развојем резистенције. Ово истраживање показује да деривати 4-аминохинолина повећавају осетљивост ћелија аденокарцинома панкреаса MIA PaCa-2 и PANC-1 на фероптозу изазвану еластином. Повећана осетљивост на фероптозу одвија се независно од активације каспаза 3 и 7 и укључује повишен ниво реактивних кисеоничних врста, митохондријски оксидативни стрес, пероксидацију липида и поремећај редокс равнотеже. Важно, осетљивост на фероптозу праћена је снижењем нивоа глутатиона, смањењем унутарћелијског цистина и диференцијалном регулацијом сигналне осе KEAP1–Nrf2–p62. Ћелијска линија PANC-1 показала је израженији оксидативни стрес и повећану експресију KEAP1, док су ћелије MIA PaCa-2 одржале релативну редокс хомеостазу, осим у условима комбинованог третмана. Према доступним сазнањима, ово истраживање, по први пут, показује да деривати 4-аминохинолина повећавају осетљивост ћелија аденокарцинома панкреаса на фероптозу, указујући на терапијски потенцијал циљања редокс метаболизма и сигналних путева адаптације на стрес у превазилажењу терапијске резистенције код карцинома панкреаса.

(Примљено 6. јула; ревидирано 6. августа; прихваћено 21. септембра 2026.)

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