

Exploring the Potential Role of Manganese-Based Zeolitic Imidazolate Framework Nanoparticles in Cancer Therapy: *In vitro* Studies Using Lung Cancer Cells

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Purpose: Chemodynamic therapy (CDT) has emerged as a promising cancer treatment strategy leveraging tumor microenvironment conditions to generate reactive oxygen species (ROS) through Fenton-type reactions. This study reports the synthesis, in-depth characterization, and biological evaluation of novel manganese-based zeolitic imidazolate framework (ZIF) nanoparticles, ie, Mn-rods, as a carrier-free potential CDT platform with exceptionally high manganese loading.

Methods: Mn-rods were synthesized through coordination of Mn²⁺ ions with 2-methylimidazolate and characterized using transmission electron microscopy (TEM), Fourier-transform infrared spectroscopy (FTIR), and inductively coupled plasma optical emission spectroscopy (ICP-OES). Two human non-small cell lung cancer lines (A549 and Calu-3) were used to evaluate nanoparticle internalization and therapeutic response was assessed using cell viability assays, ROS generation measurements, and rescue experiments with pathway-specific inhibitors.

Results: The synthesized Mn-rods exhibited a rod-shaped morphology (226 ± 93 nm length x 26.5 ± 9.5 nm width) with an exceptional Mn²⁺ loading of 50 wt.%, surpassing existing manganese-based systems. Both A549 and Calu-3 cells internalized Mn-rods, however, only A549 cells exhibited marked dose-dependent cell viability reduction, highlighting the influence of cellular phenotype on therapeutic response. Mechanistic studies suggest that Mn-rods induce ferroptosis-like cell death in A549 cells through lipid peroxidation and redox imbalance, independent of apoptosis, necroptosis and iron-mediated pathways. Rescue experiments with ferroptosis inhibitors (ferrostatin-1 and liproxstatin-1) confirmed the lipid ROS-driven mechanism, further supported by increased intracellular ROS levels and progressive membrane damage.

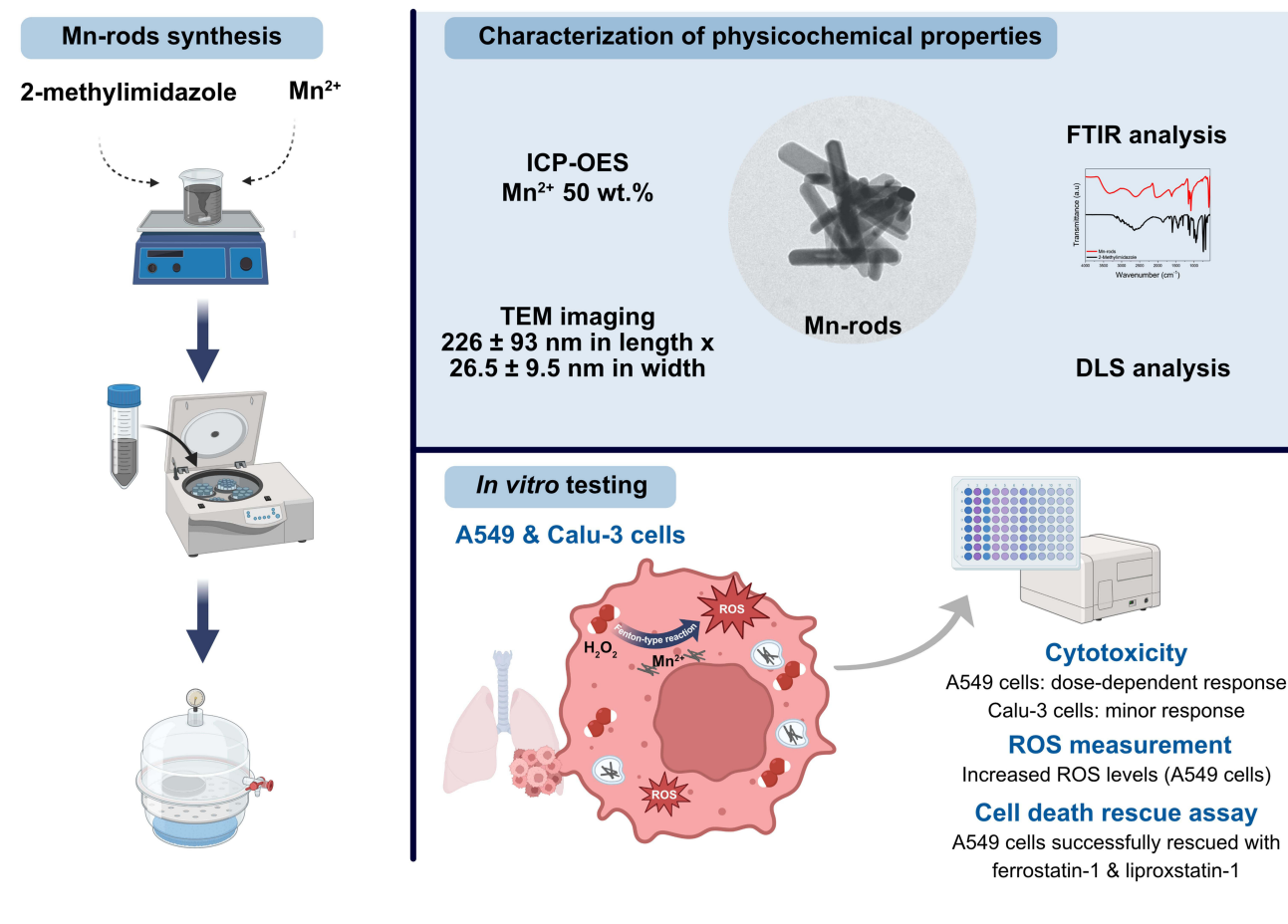
Conclusion: These findings establish Mn-rods as potent CDT agents whose efficacy is dictated by tumor cell oxidative vulnerability. Understanding such cell-specific responses is critical for optimizing nanoparticle design and tailoring therapeutic strategies in heterogeneous tumor environments. Future studies should extend these investigations across diverse cancer models to refine their translational potential.

Keywords: chemodynamic therapy, reactive oxygen species, tumor microenvironment, lipid peroxidation, ferroptosis, nanomedicine

Introduction

In the rapidly evolving field of nano-catalytic medicine, chemodynamic therapy (CDT) has emerged as a compelling alternative to conventional cancer treatments such as surgery, radiotherapy, and chemotherapy.^{1,2} CDT takes advantage of

Graphical Abstract



tumor microenvironment (TME) specific conditions, such as elevated hydrogen peroxide (H_2O_2) concentrations and acidic pH, to promote Fenton and Fenton-type reactions.^{3–8} These reactions produce highly toxic reactive oxygen species (ROS) such as hydroxyl radicals ($HO^\bullet$), which can damage proteins, lipids, and nucleic acids, leading to cancer cell death.³ This type of therapy is particularly attractive because it does not require external energy input and can selectively act within the TME.³ To date, most CDT platforms rely on iron-based nanoparticles due to the presence of ferric ions (Fe^{3+}) with a well-established role in the heterogeneous catalysis of the Fenton reaction, as described in the equations 1 and 2.⁹



Iron-containing platforms, including Fe_3O_4 , Fe-MOFs/ZIFs, and iron-doped mesoporous silica, have been extensively studied and often serve as benchmarks for CDT efficacy.^{10–15} However, these iron-based systems often exhibit limited activity at physiological pH.^{16,17} This challenge has led to increasing interest in manganese-based CDT agents, which can catalyze ROS generation across a broader pH range, target subcellular compartments such as mitochondria and the endoplasmic reticulum, and deplete cellular antioxidants such as glutathione.^{18–21} In the search for more effective catalytic agents, the selection of nanocarriers plays a pivotal role in shaping therapeutic outcomes, bioavailability, and tumor targeting.^{22–24} Metal-organic frameworks (MOFs), which are porous crystalline materials composed of metal ions and organic ligands, have emerged as highly versatile platforms for biomedical applications.^{25,26} Within this category, zeolitic imidazolate frameworks (ZIFs) are distinguished by their ease of

synthesis, chemical and thermal stability, and potential for functionalization to enhance biocompatibility and tumor specificity.^{27,28} ZIFs are constructed from metal nodes (commonly zinc – Zn^{2+} or cobalt – Co^{2+}) connected by imidazole linkers, forming robust structures that are suitable for cargo loading or intrinsic catalytic activity.^{14,29–31} Notably, the ability of imidazole to coordinate a variety of transition metals provides a unique opportunity to engineer ZIF-based nanoparticles with redox-active centers specifically optimized for Fenton-type chemistry. Although Zn^{2+} - and Co^{2+} -based ZIFs have been extensively explored for CDT, they are often designed as multi-component systems that incorporate additional metal ions, such as iron, copper, or manganese, to facilitate catalytic ROS generation.^{32–35} These platforms often integrate chemotherapeutic drugs or other therapeutic agents, leading to complex nanostructures that pose challenges in terms of synthetic reproducibility, batch-to-batch consistency, and regulatory approval pathways.^{14,36–38} In contrast, manganese-based ZIFs, wherein manganese serves dual roles as both structural nodes and catalytic center, remain relatively unexplored despite the beneficial redox properties of manganese ions and their wide pH reactivity range.^{39,40} Furthermore, ferroptosis, an iron-dependent form of regulated cell death characterized by lipid peroxidation, has emerged as an important mechanism relevant to CDT efficacy.⁴¹ Recent evidence suggests that manganese-based systems may induce ferroptosis-like pathways through ROS generation, independent of classical iron-mediated mechanisms, making ferroptosis investigation particularly relevant for evaluating manganese-based CDT platforms.⁴²

Having this in mind, the current study presents the synthesis, comprehensive characterization, and biological evaluation of novel manganese-based ZIF nanoparticles composed solely of Mn^{2+} and 2-methylimidazole. Compared to existing manganese-based platforms such as MnO_2 nanoparticles, Mn-doped MOFs, or multi-component hybrid systems, a carrier-free, single metal Mn-ZIF design offers distinct advantages, such as simplicity of synthesis, elimination of carrier-related toxicity concerns, and potential for achieving higher manganese content.^{18,43,44} Hence, these nanoparticles, referred to as Mn-rods due to their distinctive rod-like morphology, were rationally designed as carrier-free and intrinsically redox-active CDT platforms capable of inducing cancer cell death. To validate their biological efficacy, these nanoparticles were tested in two clinically relevant non-small cell lung cancer (NSCLC) cell lines: A549, representing alveolar epithelial type II cells,^{45,46} and Calu-3, modeling bronchial epithelium.^{47,48} NSCLC exhibits characteristics that make it particularly suitable for CDT evaluation, including oxidative stress vulnerability, redox imbalance, and heterogeneous metabolic profiles that influence therapeutic response.^{49–51} Significant emphasis was placed on investigating cellular uptake, redox activity, and the specific cell death pathways activated, with the aim of elucidating the therapeutic potential of Mn-rods as next-generation CDT platforms.

Methods

Preparation of Mn-Rods

The novel manganese-based ZIF nanoparticles, featuring Mn^{2+} as the central metal ion, were synthesized by adapting a previously established method.⁵² In brief, two separate aqueous solutions were prepared at room temperature (RT). Solution A was prepared by dissolving 1.47 g of manganese (II) chloride tetrahydrate ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$) in 4 mL of Milli-Q water. Solution B was prepared by dissolving 3.24 g of 2-methylimidazole in 40 mL of Milli-Q water. After complete dissolution, Solution A was slowly added dropwise to Solution B under continuous magnetic stirring. The mixture was stirred for 20 min at RT to allow the reaction to proceed. The resulting precipitate was collected by centrifugation at 4000 rpm for 10 min, washed thoroughly four times with ethanol, and dried under vacuum, see Figure 1 for a schematic illustration of the synthesis procedure. The resulting rod-shaped nanoparticles were designated as Mn-rods. The synthesized Mn-rods were stored as dry powder at RT, protected from direct light. For biological assays, Mn-rods were redispersed in endotoxin-free water and stored at 4 °C.

Physicochemical Characterization of Mn-Rods

The morphology and size of the Mn-rods were determined using transmission electron microscopy (TEM, using a Veleta digital camera (Olympus, Japan) on a FEI Tecnai Spirit transmission electron microscope (ThermoFischer, US) at 120 kV), and an open-source image processing program (ImageJ) was used for image analysis. Energy dispersive X-ray

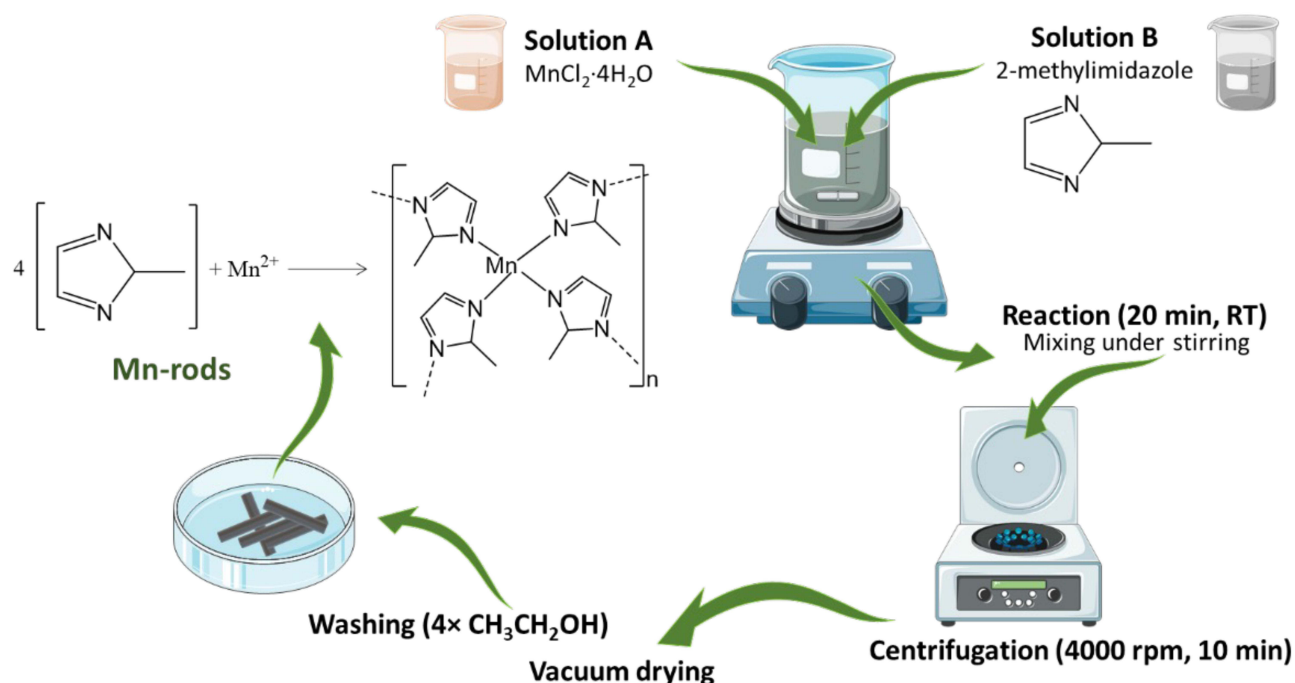


Figure 1 Schematic representation of the synthesis of Mn-rods procedure. Created with servier.com.

spectroscopy (EDS) was performed using an Xplore 30 mm² EDS detector, commercially available through Oxford Instruments (Abingdon, UK). Data was recorded at an output count rate of at least 15 k counts/second and a dead time of less than 20. The scan time was no less than 60 s. The analysis was performed using the Oxford Instruments AzTec Software (Version 5.0). The EDS detector was mounted on a Tescan Mira 3 scanning electron microscope (Brno, Czechia) equipped with a field emission gun. A 1.5 nm gold layer was deposited on the Mn-rod sample using a Cressington 205H sputter coater to ensure conductivity of the sample. The quantification of Mn^{2+} in Mn-rods was performed by inductively coupled plasma emission spectroscopy (ICP-OES), specifically ICPE-9000 Multitype ICP Emission Spectrometer (Shimadzu, Japan). A calibration curve was established for Mn within the range of 0.01 to 1 mg/L using a stock solution (Periodic Table Mix 1 for ICP, Merck) (Figure S1). A sample of Mn-rods was digested in 1 mL of HCl (37% v/v) for 48 h, diluted in Milli-Q water, and filtered using a sterile PES syringe filter (SFPE-24E.050) before analysis. Fourier transform infrared (FTIR) spectra of the Mn-rods were recorded in the range of 4000–550 cm⁻¹ using Spectrum 65 FTIR Spectrometer, with Spectrum 10 software (PerkinElmer Ltd., UK). The stability of Mn-rods, in the context of aggregation/agglomeration in complete cell culture media (10 µg/mL) was assessed through dynamic light scattering (DLS) at 0, 24, and 48 h at 37 °C and one scattering angle (175°) using the DLS Anton Paar Litesizer 500 particle analyzer (Anton Paar, Graz, Austria). For each sample, 15, 30-second-long repetitions were collected.

Endotoxin Content

The endotoxin content of the Mn-rods suspension (50 µg/mL) was assessed utilizing the end-point chromogenic Limulus Amebocyte Lysate (LAL) assay. The analysis was performed with the Pierce™ Chromogenic Endotoxin Quant Kit (Cat. No.: A39552, Thermo Fisher Scientific), following the manufacturer's protocol and employing the high-sensitivity detection method. Samples were collected in sterile, endotoxin-free tubes, and absorbance was measured to determine endotoxin levels. The measured endotoxin level in Mn-rods was 0.60 EU/mL, which is below the acceptable threshold (< 1 EU/mL). Additionally, all Mn-rods dispersions were prepared using endotoxin-free water.

Lung Cancer Cell Culture Conditions

Human alveolar epithelial type II cells (A549) and human bronchial epithelial cells (Calu-3) (passages 4–22) from the American Tissue Type Culture Collection (ATCC) were cultured at 37 °C in 5% CO₂ and 95% humidity. Subculturing was performed twice a week for A549 cells and once a week for Calu-3 cells to maintain optimal growth characteristics. Unless otherwise stated, all materials from cell culture were acquired from Gibco, Thermo Fisher Scientific (Switzerland). A549 cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 cell culture media supplemented with 10% fetal bovine serum (FBS), 1% L-Glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, further noted as cRPMI. Calu-3 cells were cultured in minimum essential medium (MEM) GlutaMAX supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 1% Non-Essential Amino Acids (NEAA) solution, referred as cMEMGlutaMAX. The absence of mycoplasma contamination in both cell cultures was confirmed by using the MycoAlert® Mycoplasma detection kit from Lonza Bioscience.

Cell Viability Assays

Cell viability was assessed by the Alamar Blue assay, which quantifies metabolic activity through the reduction resazurin (resazurin sodium salt (C₁₂H₆NNaO₄, Merck), a non-fluorescent indicator dye, to resorufin, its fluorescent counterpart, by metabolically active cells. A549 and Calu-3 cells were seeded in 96-well plates at a density of 5×10⁴ cells/mL (1×10⁴ cells/well) and allowed to adhere overnight. Subsequently, the cell culture medium was removed, and the cells were exposed to Mn-rods at concentrations ranging from 2.5 to 30 µg/mL for 24 and 48 h. Post-exposure, cell culture medium was removed, and the cells were washed with phosphate-buffered saline (PBS), after which 100 µL of Alamar Blue working solution (10% dilution of a 0.1 mg/mL stock solution in PBS, prepared in respective cell culture medium) was added to each well. Incubation proceeded for 3 h at 37 °C, after which fluorescence was quantified using a BioTek synergy H1 microplate reader (Excitation/Emission 560 nm/590 nm). Data were collected from at least three independent experiments. Cell viability was expressed as a percentage relative to the negative control (untreated cells), which was normalized to 100%.

Membrane integrity was assessed using Trypan Blue exclusion assay as a complementary viability measurement. A549 cells were seeded in a 24-well plate at an initial density of 6×10⁴ cells/well and allowed to adhere overnight. Cells were subsequently exposed to Mn-rods at concentrations of 10, 20 and 30 µg/mL for 24 and 48 h. At each time point, cells were harvested, stained with trypan blue solution (Sigma-Aldrich), 1:1 ratio (10 µL of cell suspension + 10 µL of trypan blue solution), and analyzed using an automated cell counter (EVE, NanoEnTek Inc.). The untreated cells served as the negative control (100% viability), with treated conditions expressed as relative percentages. Results represent data from at least three independent experiments performed in triplicate.

Analysis of Mn-Rods Cell Uptake by Transmission Electron Microscopy

To confirm the intracellular uptake of nanoparticles, A549 and Calu-3 were incubated with 10 µg/mL of Mn-rods in cell culture media, at 37 °C for 48 h, followed by fixation with 2% glutaraldehyde in 0.1 M Na-cacodylate with 0.1 M sucrose for 2 h at 20 °C, applied directly to the cells in the chamber slides. Samples were then rinsed three times in Milli-Q water and were then post-fixed in 1% OsO₄ in Milli-Q water for 1 h at 20 °C. The samples were then washed three times with Milli-Q water. Cells were dehydrated in a series of ethanol solutions in Milli-Q water (25% to 100% ethanol), each incubation with the duration of 15 min. The ethanol solution was then replaced with 100% acetone (anhydrous), incubated twice for 20 min. Infiltration and embedding were performed by applying a 50% epoxy resin mixture in acetone anhydrous (Epoxy Embedding Medium kit, Sigma-Aldrich, 45359) for 1 h at 20 °C. The solution was changed to 100% epoxy resin without the accelerator DMP-30 for 2 h at 20 °C. Finally, the solution was changed to fresh 100% epoxy resin with the accelerator DMP-30. The samples were transferred to a 60 °C incubator under a chemical hood and were left to polymerize for 72 h. After polymerization, the blocks were extracted from the chamber slide by successively warming up the slide and then immediately dipping it in liquid nitrogen to break the glass coverslip without damaging the sample. The sides of the blocks were then trimmed with a razor blade. Ultrathin sections (70-nm) were obtained by cutting the blocks on a Reichert-Jung Ultracut E ultramicrotome using an Ultra 45° diamond knife (Diatome). The

sections were transferred onto formvar/carbon coated copper slot grids (Plano EM, S162-5) for transmission electron microscopy. Samples were imaged on a 120-kV FEI Tecnai Spirit transmission electron microscope.

Determination of Manganese Uptake via ICP-OES

Intracellular Mn^{2+} content was quantified by ICP-OES following cellular exposure to Mn-rods. A549 cells (passage 4–18) were seeded in a 6 well plate at a density of 1.5×10^5 cell/ mL (3×10^5 cell/ well) and allowed to adhere. The following day, the cells were exposed to the Mn-rods ($10 \mu\text{g}/\text{mL}$). After 48 h, the cell culture medium was removed, the cells were washed three times with PBS. Subsequently, cells were trypsinized and centrifuged to obtain a pellet. Finally, the pellet was digested using 1 mL of HCl (37% v/v) for 48 h and diluted in Milli-Q water. The samples were then filtered (SFPE-24E-050) and analyzed. Results represent the total manganese content per well.

Cellular Reactive Oxygen Species (ROS) Assay

ROS formation in A549 cells was detected using a 2',7'-dichlorofluorescein-diacetate (DCFDA)/ H_2DCFDA -Cellular ROS Assay Kit (ab113851, Abcam, Cambridge, UK) according to the manufacturer's protocol. Briefly, A549 cells were seeded into black-walled, clear-bottom 96-well plates a density of 25000 cells per well and allowed to adhere overnight. The following day, the media was removed, and the cells were incubated with DCFDA solution for 45 min at 37°C in the dark to allow dye loading. Post-incubation, the DCFDA solution was removed, and the cells were treated with Mn-rods in phenol red-free RPMI medium ($10 \mu\text{g}/\text{mL}$) for 4 h. Finally, the fluorescence was measured using BioTek synergy H1 microplate reader (Excitation/Emission 485 nm/ 535nm). Tert-butyl hydroperoxide (TBHP), $250 \mu\text{M}$, was used as the positive control. The untreated cells provided baseline measurements, and ROS levels in treated groups were normalized relative to this baseline (set to 1).

Assessment of Cell Viability Following Inhibition of Signaling Pathways

Effective concentrations of cell death pathway inhibitors were determined through dose-response optimization assays. The following inhibitors were evaluated: the pan-caspase inhibitor zVAD-fmk, cathepsin B inhibitor CA-074, the receptor-interacting serine/threonine-protein kinase 1 (RIPK1) inhibitor necrostatin-1, the radical trapping antioxidant ferrostatin-1, the lipid reactive oxygen species scavenger liproxstatin-1 and the iron-chelating agent deferoxamine. All inhibitors were obtained from Sigma-Aldrich, St. Louis, USA, apart from zVAD-fmk that was acquired from Santa Cruz Biotechnology Inc., (Dallas, TX, USA). A549 cells were treated with various concentrations of each inhibitor, and the Alamar blue assay was used to identify the highest non-toxic concentration for subsequent mechanistic studies. For the positive control - RSL3 (Sigma-Aldrich, St. Louis, USA) - a potent ferroptosis inducer, the minimum effective concentration producing cytotoxicity comparable to Mn-rods was determined to validate inhibitor rescue efficacy. Following optimization A549 cells were pre-incubated for 1 h with either zVAD-fmk ($5 \mu\text{M}$), CA-074 ($10 \mu\text{M}$), necrostatin-1 ($20 \mu\text{M}$), ferrostatin-1 ($10 \mu\text{M}$), liproxstatin-1 ($10 \mu\text{M}$) or the deferoxamine ($3 \mu\text{M}$), prior to 48 h of exposure to Mn-rods ($10 \mu\text{g}/\text{mL}$). RSL3 ($2 \mu\text{M}$) was employed as ferroptosis-positive control. Cell viability was assessed using the Almar Blue assay to determine the protective efficacy of each inhibitor against Mn-rods-induced toxicity.

Results and Discussion

Synthesis and Characterization of Mn-Rods

The synthesis of manganese-based ZIFs nanoparticles was adapted from well-established protocols for ZIF-8 and ZIF-67, where Zn^{2+} and Co^{2+} -ions are coordinated with 2-methylimidazole to form highly crystalline structures.^{28,30,52} In this adaptation, Mn^{2+} ions were strategically employed as the metal nodes, leveraging their redox properties and relevance in the biomedical context.^{42,53} Water was selected as the reaction medium in preference to conventional organic solvents, such as methanol, due of the superior solubility of manganese-(II) salts in water.⁵⁴ This choice also aligns with the requirements of biomedical applications, as water-based synthesis minimizes toxic residues and enhances overall biocompatibility of the final product. The synthesized nanoparticles exhibited a characteristic grey color, typical of Mn^{2+} -containing compounds. The TEM images revealed a distinctive rod-shaped morphology (Figure 2A, Figure S1),

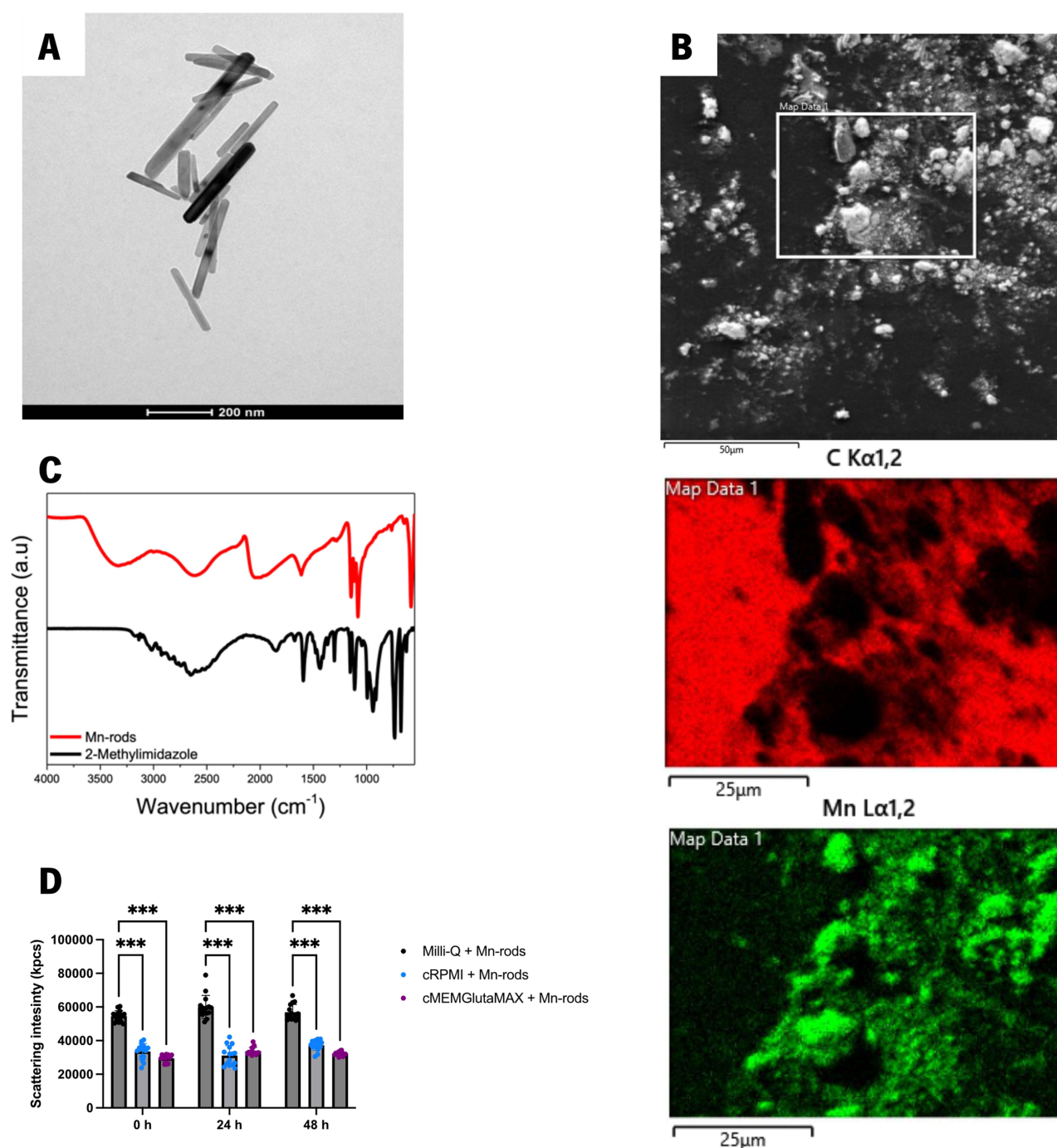


Figure 2 Comprehensive characterization of Mn-rods. **(A)** Transmission electron microscopy (TEM) image of Mn-rods; **(B)** Elemental mapping analysis of Map Data 1, showing carbon (red) and manganese (green); **(C)** FTIR spectra of Mn-rods and 2-methylimidazole; **(D)** Temporal colloidal stability assessment via DLS, monitoring static scattering intensity variation of Mn-rods (10 $\mu\text{g/mL}$) dispersed in Milli-Q water, cRPMI and cMEMGlutaMAX, at 0, 24 and 48 h ($T = 37^\circ\text{C}$, scattering angle 175° , $n=15$). The statistical analysis was conducted using two-way ANOVA, $p < 0.001$ (***) compared to Milli-Q + Mn-rods at corresponding time points.

prompting the designation of these nanoparticles as Mn-rods. Morphological analysis demonstrated average dimensions of 226 ± 93 nm in length and 26.5 ± 9.5 nm in width. The observed rod-shaped morphology significantly deviated from the traditional rhombic dodecahedron geometry characteristic of ZIF-8 and ZIF-67.⁵⁵ This difference is likely due to a synergistic interaction between the solvent choice (water) and the unique coordination characteristics of Mn^{2+} ions. The high polarity and hydrogen-bonding capability of water fundamentally alter crystal nucleation and growth dynamics, typically decelerating nucleation rates while promoting anisotropic growth patterns that favor less symmetric

morphologies and broader distribution of particle sizes.^{56,57} Additionally, the use of Mn^{2+} as the central ion, instead of Zn^{2+} or Co^{2+} , introduces further structural complexity, due to Mn^{2+} 's larger ionic radius and distinct coordination preferences, which collectively influence crystal packing arrangements and promote preferential growth along specific crystallographic axes.⁴⁴ Elemental mapping analysis using EDS provided confirmation of Mn^{2+} incorporation within Mn-rods structure. As illustrated in Figure 2B, the designated section indicated by the white box was selected for elemental mapping. The lower panels illustrate the distribution of carbon (C K α 1,2, represented in red) and manganese (Mn, L α 1,2, represented in green) within this area. The carbon signal was prevalent and associated with the carbon tape employed for mounting the sample. In contrast, manganese was concentrated in particle-rich zones, thereby confirming the presence of manganese in the sample. Quantitative analysis by ICP-OES determined the Mn^{2+} content in Mn-rods to be 50 wt.%, a value significantly exceeding typical Mn^{2+} concentrations reported in the literature.^{20,58–60} In single-metal Mn-ZIFs, the metal content is usually limited to 20–25 wt.% due to the substantial mass of the organic ligand.⁵⁸ In bimetallic systems such as Zn/Mn or Co/Mn-ZIFs, manganese is often incorporated as a dopant or co-metal in ratios up to 50 mol%; however, the overall manganese content in the final product is typically below 25 wt.%.^{59,61} Furthermore, in more complex systems designed to combined CDT and chemotherapeutics treatments, manganese is frequently co-loaded with cytotoxic drugs such as doxorubicin and 5-fluorouracil.^{60,62} These systems generally exhibit around 15–20 wt.% of manganese, as drug encapsulation and additional components like polymers or targeting ligands further reduce the metal amount.^{60,62} Hence, the significant amount of Mn^{2+} (50 wt.%) in the Mn-rods offers a distinct advantage by maximizing the availability of redox-active sites, making these nanoparticles promising candidates for effective CDT applications.

FTIR spectra analysis (4000–550 cm^{-1}) showed significant differences between Mn-rods and the precursor 2-methylimidazole (Figure 2C). In the region 3300–3500 cm^{-1} , a shift and weakening of the N-H stretching peak suggest the interaction between the manganese and the imidazole's nitrogen atom. In the fingerprint region (<1500 cm^{-1}), peaks shift, and broadening associated with imidazole ring deformations and C-H bending, confirm alterations in the ligand's electronic environment. Additionally, new peaks in the 700–550 cm^{-1} region are attributed to Mn-N bond stretching vibrations, characteristic of metal-ligand bonding.^{63,64} The stability of Mn-rods, in complete cell culture media for both cell lines used, ie. cRPMI and cMEMGlutaMAX, was characterized using DLS. Due to the heterogeneous nature and non-spherical morphology of Mn-rods, the evaluation focused on static scattering intensity measurements rather than conventional size distribution analysis, as particle aggregation/agglomeration would manifest as substantial increases in light scattering intensity.^{65,66} Temporal analysis over 48 h (0, 24, and 48 h) revealed no significant changes in static scattering intensity within individual media, including Milli-Q water, cRPMI, and cMEMGlutaMAX, indicating good temporal stability (Figure 2D). However, comparative analysis across different dispersion media revealed significant differences in scattering behavior. Mn-rods dispersed in Milli-Q water consistently exhibited higher scattering intensities than those in either cell culture medium, indicating increased particle aggregation/ agglomeration in the absence of stabilizing biomolecules.⁶⁷ This observation is consistent with established principles of nanoparticle colloidal chemistry, wherein cell culture media containing proteins, amino acids, and electrolytes facilitate the formation of biomolecular corona on nanoparticles surfaces, providing both steric and electrostatic stabilization mechanisms that effectively minimize aggregation/agglomeration.^{65,68} This behavior aligns with previous reports demonstrating that biological media can enhance nanoparticle colloidal stability by dynamic surface modification via protein corona formation, a phenomenon critical for maintain particle dispersion in physiological environments.⁶⁷

Effect of Mn-Rods on Cell Viability

CDT has gained significant interest as an emerging cancer treatment strategy, by leveraging the TME characteristics to promote the generation of ROS through the Fenton reaction and Fenton-type reactions.⁶⁹ Among various catalytic metals investigated for CDT applications, Mn^{2+} demonstrates exceptional versatility due to its capacity to mediate ROS-generating reactions across broad pH ranges (5.0–7.4).^{18,19} To investigate the therapeutic potential of synthesized Mn-rods as CDT platforms, a series of in vitro experiments were conducted using two clinically relevant human cell lines derived from NSCLC adenocarcinoma: A549 cells, which models alveolar epithelial type II cells,^{45,46} and Calu-3 cells, representing bronchial epithelium.^{47,48} The Alamar Blue metabolic assay was chosen for viability assessment based on its superior compatibility with manganese-based systems and methodological advantages over alternative approaches.⁷⁰ Unlike

tetrazolium-based viability assays (MTT, WST-1), that rely on intracellular reduction of tetrazolium salts to formazan products, Alamar Blue assay utilizes resazurin as a redox indicator that remains chemically inert toward Mn^{2+} under standard assay conditions.^{71,72} This distinction is critical, as Mn^{2+} can interfere with tetrazolium reduction, potentially generating misleading results.^{71,72} Similarly, lactate dehydrogenase (LDH) release assay may also produce unreliable results when coupled with tetrazolium-based detection systems, Mn^{2+} can interfere with the colorimetric readout.⁷² Previous studies have confirmed that resazurin is not chemically reduced by Mn^{2+} , and the fluorescence-based detection used in Alamar Blue assay avoids interference from nanoparticle light absorption or scattering.⁵⁹ These characteristics make Alamar Blue assay a reliable and validated method for accurately assessing Mn-rods-induced toxicity.

A549 and Calu-3 cells were exposed to increasing concentrations of Mn-rods (0, 2.5, 5, 10, 15, 20, 25, 30 $\mu\text{g/mL}$) over 24 and 48 h periods to establish dose-response relationships and temporal cytotoxicity profiles. The results indicated that Mn-rods induced a significant dose-dependent effect against A549 after 48 h exposure (Figure 3A). On the other hand, Calu-3 cells exhibited only minor reduction in viability after 48 h of exposure to the highest concentration used, 30 $\mu\text{g/mL}$, suggesting potential cell line-specific sensitivity differences (Figure 3B). The observed differential responses between A549 and Calu-3 cells can be attributed to fundamental differences in cellular morphology, metabolic profile, and redox homeostasis mechanisms. A549 cells display a fibroblast-like morphology, rapid proliferation rate (doubling time $\sim 22\text{--}24$ h) and form loosely organized monolayers lacking mature tight junctions complexes.^{45,46} Metabolically,

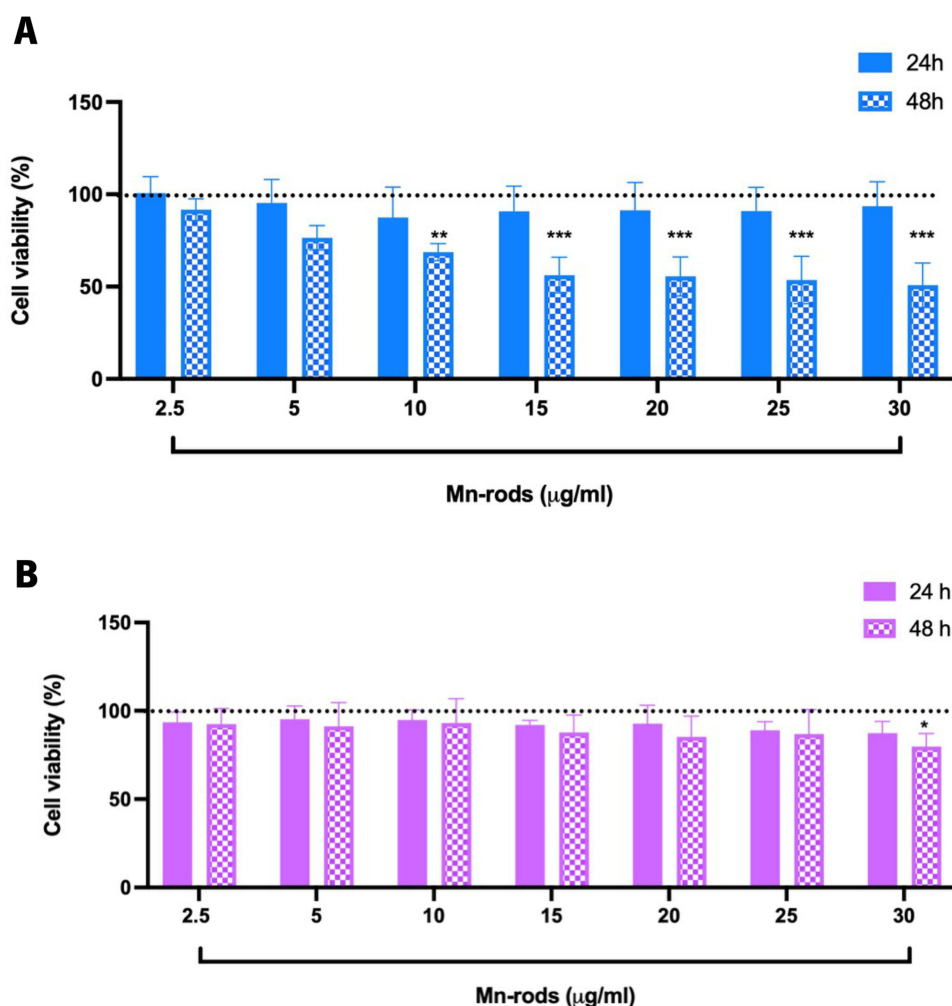


Figure 3 Dose-dependent effect of Mn-rods on the cell viability of (A) A549 and (B) Calu-3 cells after 24 and 48 h incubation at 37 °C. Cell viability was assessed using Alamar Blue metabolic assay and expressed as percentage relative to untreated cells (normalized to 100%). Data are shown as means $\pm$ SD. $n=3$. The statistical analysis was conducted using two-way ANOVA, $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***) compared to negative control (untreated cells) for each time point.

A549 cells are characterized by pronounced glycolytic activity, elevated glutamine metabolism, and increased basal ROS levels, reflecting their highly proliferative phenotype.^{46,73,74} These characteristics make A549 cells more vulnerable to oxidative stress. In contrast, Calu-3 cells exhibit a polygonal shape, form tight, polarized epithelial monolayers with robust transepithelial electrical resistance (TEER) and display lower proliferation rate (doubling time ~ 48–72 h).^{47,48} The well-developed tight junction architecture in Calu-3 cells likely restricts both paracellular transport and nanoparticle internalization pathways, potentially limiting Mn-rods uptake and subsequent intracellular accumulation. Whereas the less tightly monolayer structure of A549 cells may allow greater internalization and intracellular exposure.^{46–48} Metabolically, Calu-3 cells rely predominantly on oxidative phosphorylation, maintain lower baseline ROS levels, and possess more robust antioxidant defenses, such as glutathione-based detoxification mechanisms.^{47,48,75} Manganese-based nanoparticles can disrupt cellular redox homeostasis through multiple interconnected mechanisms.^{18,42,43,76} Following cellular internalization, the acidic pH within endosomal/lysosomal compartments can facilitate Mn^{2+} ions release from the ZIF framework, allowing subsequent diffusion to other cellular compartments including mitochondria. Once accumulated in mitochondria, Mn^{2+} ions can disrupt the electron transport chain, resulting in impaired ATP production, mitochondrial membrane depolarization, and excessive ROS generation.^{53,76,77} Furthermore, hydrogen peroxide-rich environments characteristic of cancer cells, Mn^{2+} ions can act as Fenton-type catalyst, promoting to the generation of $HO^{\bullet}$ that can further damage cellular components such as lipids, proteins, and nucleic acids.^{33–35} These mechanisms are likely amplified in A549 cells due to their elevated baseline ROS levels and inherent redox sensitivity.⁷⁸ Sustained elevation of intracellular levels of ROS not only contributes to generalized oxidative stress also but can also trigger lipid peroxidation cascades involving the oxidative degradation of polyunsaturated fatty acids (PUFAs) within plasma membrane phospholipids.^{41,79,80} This process compromises membrane structural integrity and, if not effectively countered by antioxidant defenses, can lead to ferroptosis, a regulated form of cell death characterized by lipid peroxidation.^{41,79–81} Though ferroptosis was originally defined as an Fe^{2+} -dependent process, emerging evidence indicates that Mn^{2+} can promote analogous redox reactions, suggesting the potential for ferroptosis-like cell death mechanisms.⁴⁰ The susceptibility to lipid peroxidation is significantly influenced by cellular membrane composition, particularly the relative abundance of PUFAs, which are more susceptible to oxidative damage.^{79,81} Although comparative data between A549 and Calu-3 cells are limited, previous studies suggest that A549 cells can undergo ferroptosis, suggesting a cellular environment favorable for lipid peroxidation.⁷⁸ In contrast, Calu-3 cells, which display a more differentiated epithelial phenotype and a lower proliferation rate, might have membranes with lower PUFA content, enhanced antioxidant systems and reduced nanoparticles uptake – factors that could confer resistance to ferroptosis-like damage.^{47,48,75} Hence, the enhanced sensitivity of A549 cells to Mn-rods might result from the convergence of multiple factors: elevated metabolic activity, compromised antioxidant capacity, and heightened susceptibility to ferroptosis and ROS-mediated cellular damage. These findings highlight the relevance of cellular phenotype in determining CDT efficacy and support the therapeutic potential of Mn-rods as CDT platform for redox-based cancer therapy.

Trypan Blue exclusion assay was employed to evaluate cell membrane integrity of A549 cells after exposure to Mn-rods, as a complementary method to assess cytotoxicity alongside the metabolic-based Alamar Blue assay. This method allows the differentiation between viable cells with intact membranes and non-viable cells that permit dye uptake, thereby offering a direct measurement of membrane disruption.⁸² Given the established role of manganese in promoting oxidative stress cascades and initiating lipid peroxidation processes, the evaluation of membrane integrity represents a particularly relevant endpoint for the mechanistic characterization.^{42,83} As previously discussed, lipid peroxidation – a key feature of ferroptosis and ferroptosis-like cell death mechanisms – involves the oxidative degradation of PUFAs, ultimately compromising membrane structural integrity, increasing permeability, and culminating in cell death.^{79,84,85} Therefore, Trypan Blue assay can provide mechanistic insights into whether Mn-rods induced decrease in A549 cells viability are accompanied by membrane destabilization events consistent with ferroptosis-like pathways. Based on the dose-response relationships established through Alamar Blue analysis, three representative concentrations were selected for membrane integrity assessment: 10 $\mu\text{g/mL}$, representing the lowest concentration with a significant effect; 20 $\mu\text{g/mL}$, as the intermediate concentration; and 30 $\mu\text{g/mL}$, as the highest concentration tested. The results demonstrated a significant dose-dependent decrease in the cell viability following Mn-rods treatment, corroborating previous findings from metabolic activity assessments (Figure 4A). Consistent with earlier observations, no significant effect was observed

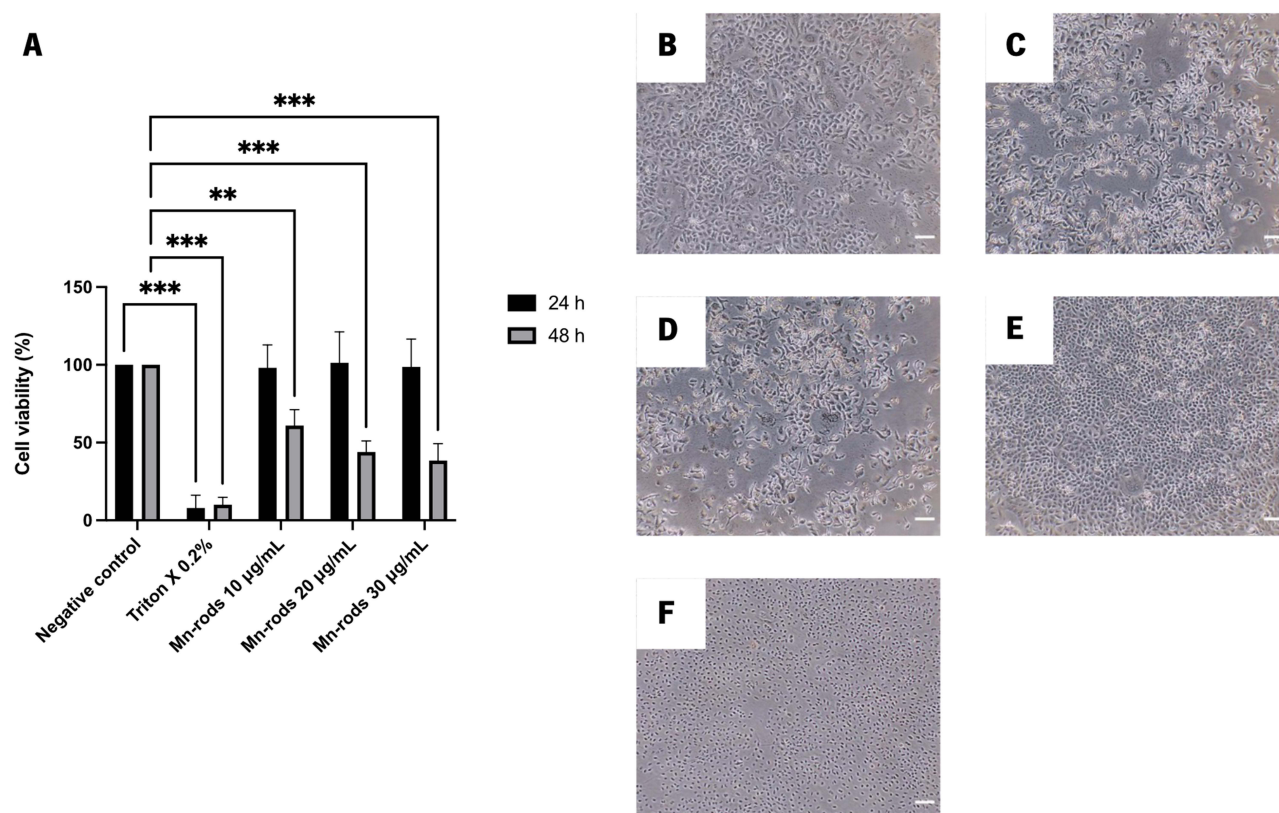


Figure 4 (A) Cell viability of A549 cells after 24 and 48 h of incubation with Mn-rods (at 10, 20 and 30 µg/mL), assessed by Trypan Blue exclusion assay. Triton X 0.2% used as positive control. Data are shown as means $\pm$ SD. $n=3$. The statistical analysis was conducted using two-way ANOVA, $p<0.01$ (**) and $p<0.001$ (***) compared to negative control (untreated A549 cells) for each time point. (B–F) correspond to phase-contrast microscopy images of A549 after 48 h of exposure to Mn-rods (B–D) – A549 cells treated with 10, 20 and 30 µg/mL of Mn-rods, respectively; (E) negative control (untreated A549 cells); (F) A549 cells incubated with Triton X 0.2%). The scale bar is 100 µm.

after 24 h of incubation; however, a pronounced decrease in cell viability became evident following 48 h exposure. Phase-contrast microscopy images captured at 48 h of incubation with Mn-rods revealed progressive morphological alterations with increasing concentrations of Mn-rods, including marked reductions in cell density, rounding, and shrinkage (Figure 4B–D), when compared to untreated cells (Figure 4E). As anticipated, cells exposed to Triton X (0.2%), a well-known positive control for membrane permeabilization, exhibited extensive cellular damage and membrane disruption (Figure 4F). The observed delayed onset of membrane integrity loss, accompanied by morphological changes are consistent with the temporal progression of intracellular ROS accumulation and subsequent lipid peroxidation, both recognized hallmarks of ferroptosis-like cellular damage.^{53,76} These outcomes support the proposed CDT mechanism of action, wherein Mn^{2+} -catalyzed ROS generation culminates in oxidative disruption of cellular membranes. Once again, the data reinforce the potential of Mn-rods as CDT platforms for cancer treatment applications.

Mn-Rods Uptake by Cells

TEM was performed to confirm the uptake of Mn-rods by A549 and Calu-3 cells after 24 and 48 h of exposure. As shown in Figure 5, Mn-rods were successfully internalized in both cell types and appeared clustered in membrane-enclosed compartments. The nanoparticles maintained the characteristic rod-shaped morphology even after internalization, indicating structural integrity during the uptake process. In A549 cells, multiple Mn-rods aggregates were observed at both 24 and 48 h post-exposure, commonly located in vesicular structures dispersed throughout the cytoplasm (Figure 5A and B). Notably, Mn-rods were consistently detected in nearly all A549 cells examined, suggesting widespread and efficient uptake in this highly proliferative cell line. Quantitative analysis via ICP-OES revealed that following 48h exposure to Mn-rods (10 µg), approximately 16.4% of the applied Mn^{2+} dose (equivalent to 1.64 µg from the total 10 µg) was recovered intracellularly in A549 cells, while Mn^{2+} remained undetectable in untreated

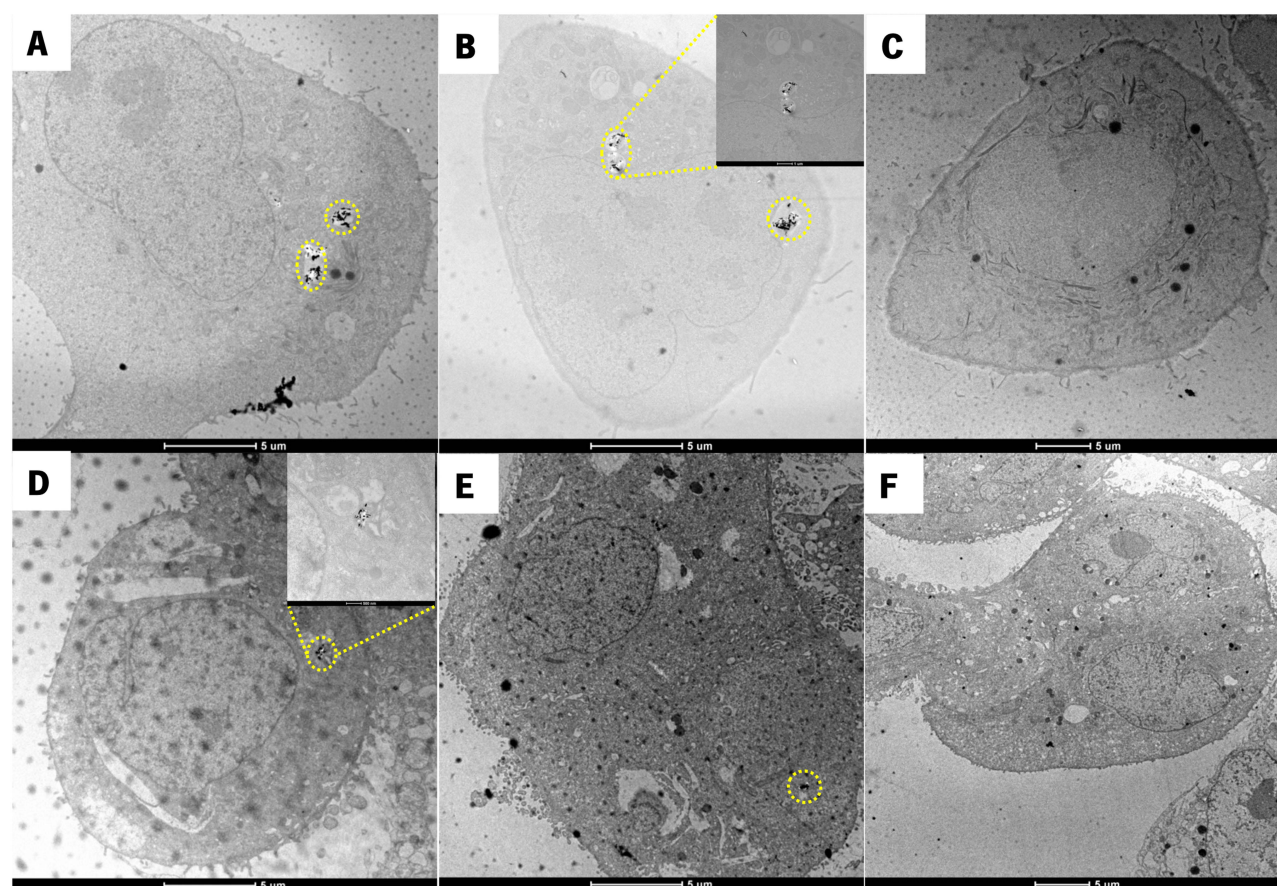


Figure 5 TEM images showing the cellular uptake of Mn-rods (10 $\mu\text{g/mL}$) by A549 and Calu-3 cells. Panels (A–C) correspond to A549 cells: (A and B) after 24 and 48 h of exposure to Mn-rods, respectively. (C) corresponds to untreated A549 cells. Panels (D–F) correspond to Calu-3 cells: (D and E) show Mn-rods internalization after 24 and 48 h of exposure, respectively. (F) corresponds to untreated Calu-3 cells. Yellow dashed circles highlight internalized Mn-rods, and yellow dashed lines mark the areas shown at higher magnification.

negative control cells (Figure 5C). Mn-rods were also internalized by Calu-3 cells, as confirmed by TEM imaging at both 24 and 48 h post-exposure (Figures 5D and E). No Mn-rods nanoparticles were observed in untreated negative control cells (Figure 5F). The internalization process of rod-shaped nanoparticles involves complex cellular mechanisms, as uptake efficacy depends on nanoparticle geometry, size, and spatial orientation. Successful internalization usually requires actin-dependent mechanisms such as micropinocytosis or clathrin-mediated endocytosis, along with substantial membrane remodeling.^{86–91} Additionally, nanoparticles orientation plays a critical role, as nanoparticles aligned tip-first to the cell membrane are more readily engulfed, whereas side-on interactions often result in particle wrapping or compromised endocytosis.⁸⁹ Previous DLS assay results showed that Mn-rods tend to form aggregates and agglomerates in aqueous environments. This behavior plays a dual role in modulating cellular uptake.⁹² On one hand, controlled aggregation and agglomeration can facilitate gravitational sedimentation, increase the local concentration of nanoparticles at the cell surface and thereby enhance the probability of cell-nanoparticle interactions and uptake.^{67,68} On the other hand, excessive aggregation/agglomeration may result in nanoparticle clusters that exceed the optimal size range for efficient nanoparticle internalization, impair nanoparticle dispersion and limit their bioavailability at the cellular surface.^{67,68} Consequently, while moderate aggregation/agglomeration can promote nanoparticle internalization, uncontrolled behavior can compromise the uptake. In the clinical context, controlled nanoparticle aggregation/agglomeration has emerged as a strategic approach for enhancing therapeutic efficacy. For instance, in cancer therapy, deliberate aggregation of drug-loaded nanoparticles can improve tumor accumulation through the enhanced permeability and retention effect.^{93,94} Similarly, in diagnostic imaging, aggregated contrast agents often demonstrate superior signal enhancement and prolonged circulation times compared to their dispersed counterparts.⁹⁴ However, uncontrolled

aggregation/agglomeration remains a significant obstacle in clinical translation, as it can lead to unpredictable pharmacokinetics, compromised biodistribution, and potential embolic complications.⁹⁵ Therefore, understating and controlling the aggregation/agglomeration behavior of nanotherapeutics is crucial for its successful clinical implementation.

Effect of Mn-Rods on the Levels of Cellular Reactive Oxygen Species (ROS)

Intracellular ROS levels in untreated and Mn-rod-treated A549 cells (10 $\mu\text{g/mL}$) were evaluated using the DCFDA/ H_2DCFDA fluorometric assay. This widely validated method relies on the oxidative conversion of a non-fluorescent probe - DCFDA into the highly fluorescent compound, DCF (2',7'-dichlorofluorescein) by intracellular ROS, offering a sensitive and quantitative measurement of overall cellular ROS production.⁹⁶ Given the importance of ROS production in CDT mechanisms, this assay provides a direct and biologically relevant evaluation of the oxidative response triggered by Mn-rods exposure in A549 cells. Fluorescence intensity measurements (Figure 6) indicated that A549 cells exposure to Mn-rods resulted in a significant elevation of intracellular ROS levels compared to untreated negative control (Figure 6). Although the oxidative response was lower than that induced by TBHP (positive control), the increase was statistically significant and biologically relevant, confirming that Mn-rods effectively promote intracellular ROS generation upon Mn-rods exposure. These findings align with the established mechanism of CDT, wherein redox-active metal ions such as Mn^{2+} catalyze the decomposition of endogenous H_2O_2 into ROS via Fenton-type reactions.⁹⁷ The DCFDA/ H_2DCFDA assay has been widely reported in the literature for evaluating nanoparticle-mediated ROS generation in the of CDT application, including studies focused on Mn^{2+} -containing therapeutic platforms.⁷⁷ The observed ROS elevation, when considered in conjunction with the demonstrated reductions in cell viability and progressive loss of membrane integrity, provides supports the conclusion that Mn-rods induce cell damage through oxidative stress pathways, reinforcing the role of Mn-rods as CDT platforms.

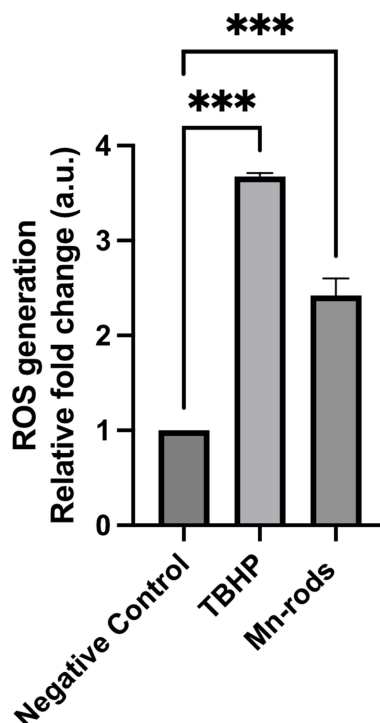


Figure 6 Relative intracellular ROS levels in A549 cells, measured by DCF fluorescence after 4 h of incubation with Mn-rods (10 $\mu\text{g/mL}$). Tert-butyl hydroperoxide (TBHP, 250 μM) was used as the positive control. Data are shown as means $\pm$ SD. $n=3$. The statistical analysis was conducted using one-way ANOVA, $p<0.001$ (***) compared to negative control (untreated A549 cells).

Effect on Cell Viability of Inhibitors of Distinct Signaling Pathways

Considering the significant effect on A549 cells' viability when exposed to Mn-rods, the aim of the following experiments was to elucidate the potential underlying cytotoxic mechanisms responsible for the cell viability results,

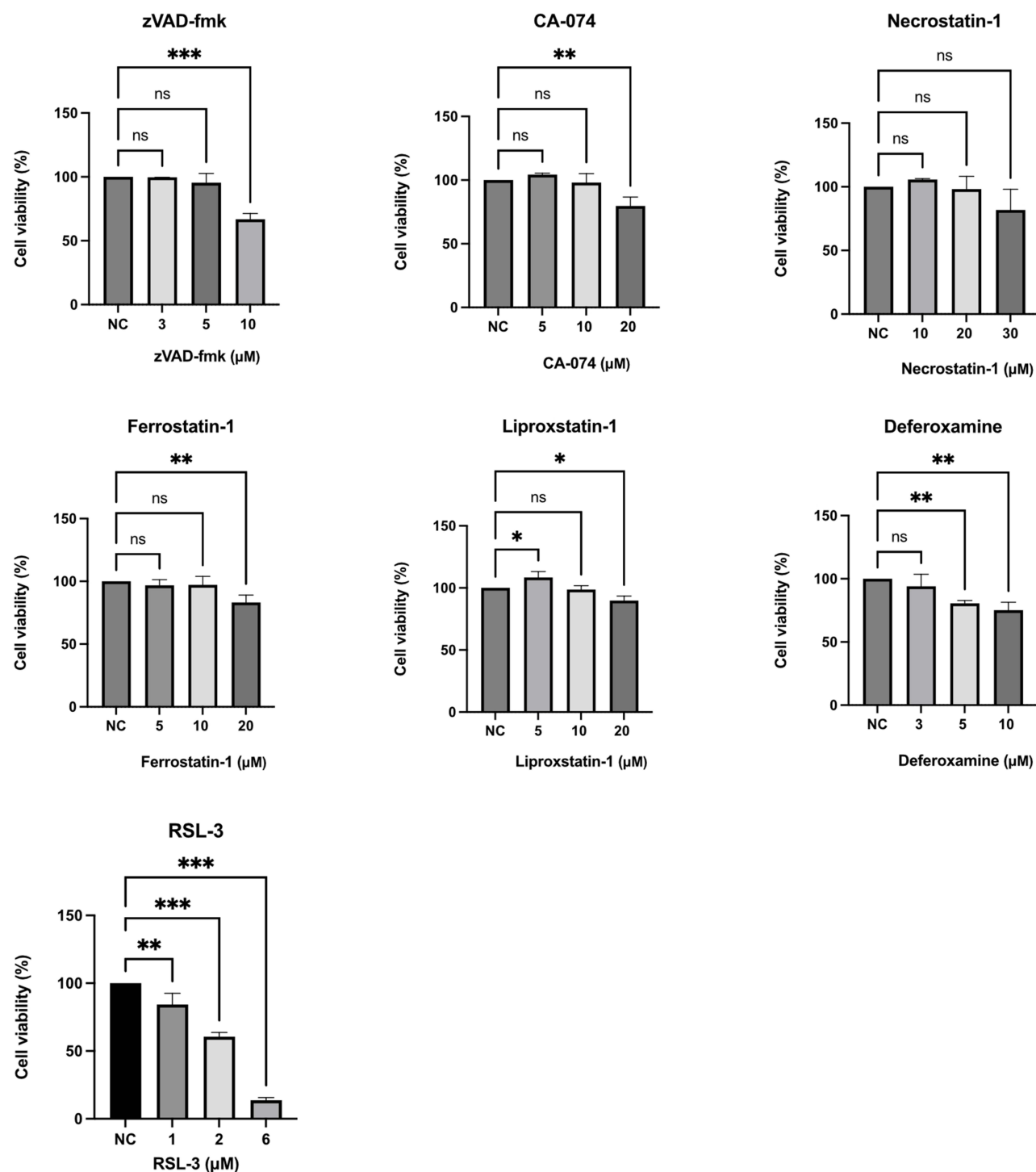


Figure 7 Identification of the highest non-toxic concentration of compounds targeting different signaling pathways in A549 cell line, using the Alamar Blue assay. Cells were incubated with the pan-caspase inhibitor zVAD-fmk, cathepsin B inhibitor CA-074, the receptor-interacting serine/threonine-protein kinase I (RIPK1) inhibitor necrostatin-1, the radical trapping antioxidant ferrostatin-1, the lipid reactive oxygen species scavenger liproxstatin-1, or the iron-chelating agent deferoxamine, and with the ferroptosis inducer, RSL-3, for 48 h at 37 °C. Data are shown as means $\pm$ SD. $n=3$. The statistical analysis was conducted using ordinary one-way ANOVA, ns – not significant, $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***). NC- negative control (untreated A549 cells).

since it is known that manganese is able to catalyze redox reactions and contribute to the generation of ROS.^{18,20} Thus, it was hypothesized that Mn-rods might initiate regulated cell death pathways, particularly those associated with oxidative stress and redox imbalance, such as apoptosis, necroptosis, and ferroptosis-like processes.⁹⁸ To evaluate this hypothesis, a panel of well-established inhibitors targeting distinct cell death pathways was used, including: the pan-caspase inhibitor zVAD-fmk that blocks caspase-dependent apoptotic cell death;^{99,100} the cathepsin B inhibitor, CA-074, to explore the involvement of lysosome-mediated pathways (autophagy);^{101,102} a specific inhibitor of receptor-interacting serine/threonine-protein kinase 1 (RIPK1), necrostatin-1, to assess the involvement of necroptosis;¹⁰³ and three ferroptosis-related inhibitors – ferrostatin-1^{104,105} and lipoxstatin-1,^{105,106} both acting as lipid ROS scavengers, and deferoxamine, an iron chelator that limits the availability of catalytic iron required for lipid peroxidation.^{105,107}

To confirm if ferroptosis contributes to Mn-rods-induced cytotoxicity, a well-established ferroptosis inducer, RSL-3, was used.^{108,109} Glutathione peroxidase (GPX4) is a crucial enzyme and a key regulator of ferroptosis, due to its ability to inhibit the accumulation of lipid peroxides, thereby preventing cells from undergoing this iron-dependent form of cell death.¹¹⁰ RSL-3 irreversibly inhibits GPX4, resulting in lipid peroxides accumulation and subsequent cell death.^{108,109} In this study, RSL-3 served as a positive control to validate the assay and allow direct comparison with Mn-rods-induced cytotoxicity. Initial dose-response studies were performed to optimize the concentration of each inhibitor and the positive control RSL-3. A549 cells were incubated for 48 h at 37 °C, with different concentrations of each compound, and the highest non-toxic concentration (corresponding to 90–100% viability) was selected for subsequent assays (Figure 7). For RSL-3, the lowest toxic concentration that induced cytotoxicity comparable to Mn-rods was selected to ensure the intended biological effect while preserving the potential for cellular rescue (Figure 7).

To confirm the ferroptotic mechanism of RSL-3-induced cytotoxicity, a rescue experiment was designed wherein A549 cells were exposed to RSL-3 (2 µM) for 48 h, both in the presence and absence of established ferroptosis inhibitors. Co-treatment with lipoxstatin-1 (10 µM), ferrostatin-1 (10 µM), and deferoxamine (3 µM) significantly restored cell viability (Figure 8). These results confirm that RSL-3-mediated cytotoxic effects are predominantly ferroptosis-dependent. The cellular rescue observed with lipoxstatin-1 and ferrostatin-1 highlights the central role of lipid peroxidation,¹¹¹ while the protective effect of deferoxamine supports the involvement of iron-catalyzed ROS formation.¹⁰⁵

Finally, A549 cells were exposed to Mn-rods (10 µg/mL) for 48 h in the presence or absence of various cell death pathways inhibitors (Figure 9). Treatment with zVAD-fmk, CA-074 and necrostatin-1 failed to rescue cells incubated with Mn-rods, suggesting that apoptosis, lysosomal-mediated cell death, and necroptosis are unlikely to represent the primary mechanisms responsible for the observed cytotoxicity (Figure 9). Notably, while ferrostatin-1 and lipoxstatin-1 effectively rescued A549 cells exposed to Mn-rods, deferoxamine showed no protective effect (Figure 9). These

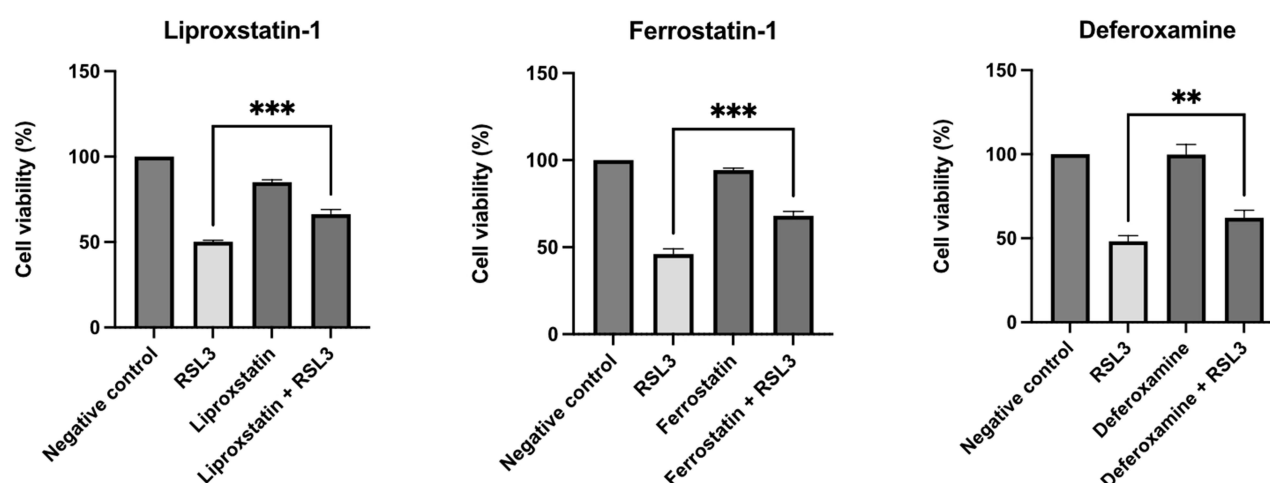


Figure 8 Effect of RSL-3 (2 µM) and different inhibitors (the radical trapping antioxidant ferrostatin-1, 10 µM, the lipid reactive oxygen species scavenger lipoxstatin-1, 10 µM, and the iron-chelating agent deferoxamine, 3 µM, on cell viability of A549 cells, following incubation for 48 h at 37 °C. Alamar Blue assay, data are shown as means ± SD. n=3. The statistical analysis was conducted using ordinary one-way ANOVA, $p < 0.01$ (**) and $p < 0.001$ (***). Negative control- negative control (untreated A549 cells).

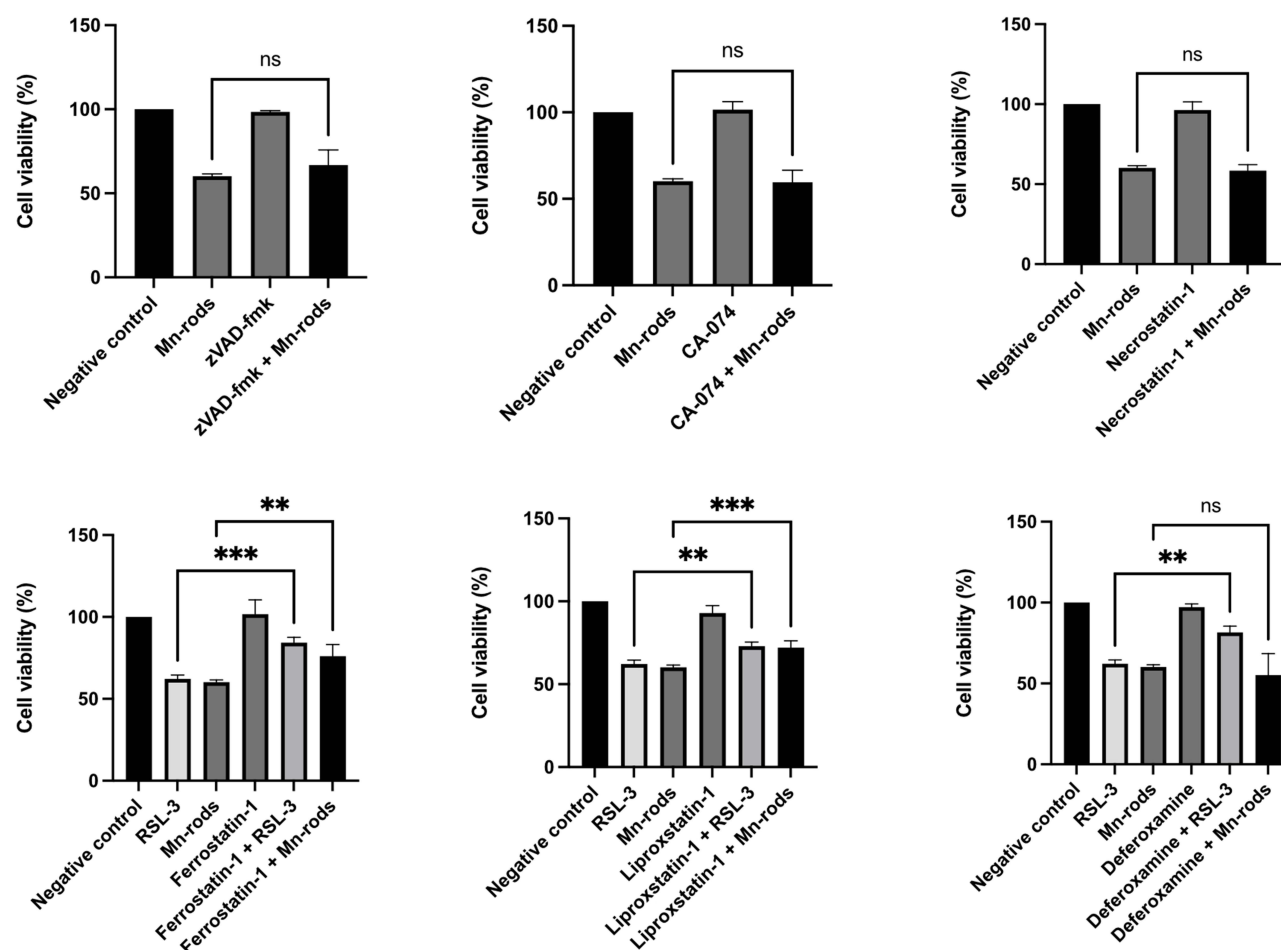


Figure 9 Study of the potential mechanisms underlying the effect on the metabolic activity of A549 cells after exposure for 48 h to Mn-rods in the presence or absence of the specified inhibitors (the pan-caspase inhibitor zVAD-fmk (5 μ M), cathepsin B inhibitor CA-074 (10 μ M), the receptor-interacting serine/threonine-protein kinase I (RIPK I) inhibitor necrostatin-1 (20 μ M), the radical trapping antioxidant ferrostatin-1 (10 μ M), the lipid reactive oxygen species scavenger lipoxstatin-1 (10 μ M), the iron-chelating agent deferoxamine (3 μ M), RSL-3 (2 μ M), ferroptosis inducer, was used as a positive control of ferroptosis. Data are shown as means $\pm$ SD, $n=3$. The statistical analysis was conducted using ordinary one-way ANOVA, ns – not significant, $p<0.01$ (**) and $p<0.001$ (***). Negative control- negative control (untreated A549 cells).

differences suggest that Mn-rods predominantly induce cell death through lipid peroxidation, a hallmark of ferroptosis,^{80,112} rather than through iron-mediated oxidative mechanisms. This finding is particularly significant as it reinforces the hypothesis that ferroptosis, or ferroptosis-like pathway induced by Mn^{2+} -triggered oxidative stress, may constitute the primary mechanism involved. Ferrostatin-1 and lipoxstatin-1 function as specific lipid ROS scavengers that inhibit ferroptosis by protecting membrane lipids from oxidative stress.¹¹¹ Their ability to promote cellular rescue highlights the central role of lipid peroxidation in Mn-rods-induced cytotoxicity. On the other hand, deferoxamine, which inhibits ferroptosis by chelating Fe^{2+} and limiting its availability for Fenton reactions,¹⁰⁵ had no effect in this context, suggesting that Mn^{2+} , rather than Fe^{2+} , may be the primary driver of lipid ROS formation and subsequent cytotoxicity. These findings support the hypothesis that Mn-rods induce a ferroptosis-like pathway, wherein lipid peroxidation plays a critical role in the observed effects, aligning with the principles of CDT.

Future work should aim to validate the CDT efficacy of Mn-rods in more intricate models, such as 3D tumor spheroids or co-culture systems that better mimic the complexities of the TME. Factors such as extracellular matrix composition, hypoxia, pH gradients, and cellular heterogeneity present in solid tumors may influence both nanoparticle internalization and catalytic activity. From a physicochemical perspective, comprehensive colloidal characterization including zeta potential measurements in aqueous and biological media would provide important insights into surface charge, electrostatic stabilization mechanisms, and protein corona effects that govern particle behavior in complex

biological environments. Additionally, while our pharmacological inhibitor-based approach provides strong evidence for ferroptosis-like cell death, the absence of direct molecular markers such as GPX4 depletion and specific lipid peroxidation assays represents a limitation.¹¹² Future studies incorporating these molecular validation techniques would further strengthen the mechanistic characterization of Mn-rods-induced cell death pathways. Surface functionalization of Mn-rods could enhance tumor-specific targeting capabilities, while combination approaches with chemotherapeutic agents may yield synergistic therapeutic outcomes. Furthermore, studies on the long-term behavior, biodegradation patterns, and sustained redox activity of Mn-rods in complex biological environments are essential to ensure both safety profiles and durable therapeutic efficacy.¹¹³ Finally, extending this approach to other cancer cell types, such as breast, melanoma, and colorectal, could broaden the therapeutic scope and enhance the translational potential of CDT-based treatments modalities. Such investigations would provide critical insights into the generalizability of Mn²⁺-mediated ferroptosis induction across diverse oncological contexts.

Conclusions

The development of Mn-rods, a novel manganese-based ZIF nanoparticle represents a significant advancement in CDT. The high Mn²⁺ loading (50 wt.% Mn²⁺) enhances ROS generation and therapeutic potential compared to existing systems. Mn-rods were internalized by both A549 and Calu-3 cells, but only A549 cells showed reduced viability, reflecting cell-type-specific susceptibility. This differential response reflects fundamental differences in cellular phenotype. A549 cells, characterized by high metabolic activity, rapid proliferation, and weak antioxidant defenses, proved highly vulnerable to oxidative stress and ferroptosis, whereas Calu-3 cells, with strong junctions, slower growth, and robust antioxidant systems, remained resistant to Mn-rods-induced cytotoxicity. Additionally, the observed differences between cell types may also reflect variations in nanoparticle internalization efficiency, which require further investigation. Mechanistic studies suggest that Mn-rods induced ferroptosis-like death in A549 cells via lipid peroxidation, independent of apoptosis or necroptosis pathways. Multiple lines of evidence support this conclusion: (i) both the Alamar Blue and Trypan Blue exclusion assays showed significant decrease in cell viability and membrane integrity, respectively, after exposure to Mn-rods; (ii) TEM imaging confirmed the successful internalization of Mn-rods by A549 cells, a crucial step for intracellular ROS generation; and (iii) intracellular ROS levels, measured by DCFDA/H₂DCFDA assay, were significantly elevated in Mn-rods-treated A549 cells. Together, these findings suggest that Mn-rods act as intracellular CDT platform that triggers oxidative stress-induced lipid peroxidation, culminating in ferroptosis-like cell death.

These results underscore the importance of incorporating cellular antioxidant capacity assessment into future CDT platforms development to guide therapeutic applications and improve treatment predictability across heterogeneous cancer cell populations. Future studies should aim to validate the CDT efficacy of Mn-rods in more intricate models, such as 3D tumor spheroids or co-culture systems, that better mimic the complexities of the tumor microenvironment.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no competing interests.

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