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Lattice Distortion-Enhanced Cuproptosis Nanoplatforrm for Synergistic Catalytic-Autophagy Tumor Therapy

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ABSTRACT

Cuproptosis holds promise for tumor therapy, yet its efficacy is constrained by insufficient copper bioactivity and metabolic adaptation of tumor cells. To address this challenge, we developed a lattice-distorted Dy₄-CuBi/GDY nanocomposite via a lattice engineering strategy, sequentially introducing bismuth (Bi) and tetranuclear dysprosium (Dy) clusters onto copper (Cu) quantum dots anchored on ultrathin graphdiyne (GDY). Within this system, Bi induces Cu lattice expansion, while Dy triggers lattice distortion and electronic reconstruction, significantly enhancing peroxidase (POD)- and glutathione oxidase (GSHOx)-like catalytic activities alongside lysosomal targeting capability. In vitro and in vivo evaluations demonstrate that Dy₄-CuBi/GDY selectively accumulates within tumor lysosomes and sustainably releases copper ions. This precise intervention disrupts lysosomal integrity and blocks autophagosome-lysosome fusion by impairing the Rab7-HOPS-SNARE axis. The resulting autophagic flux blockade sensitizes osteosarcoma cells to copper, cooperatively pairing with elevated oxidative stress to trigger robust cuproptosis for efficient tumor eradication with biosafety. Overall, this lattice engineering strategy establishes a powerful paradigm for designing highly efficient cuproptosis amplifiers for solid tumor therapy.

KEY WORDS: Cuproptosis, Nanocomposites, Enzyme catalytic activity, Cancer treatment, Lysosomal damage

Introduction

As a vital physiological regulator, copper (Cu) homeostasis disruption can trigger a unique regulated cell death known as cuproptosis[1]. Consequently, tumor-specific amplification of cuproptosis signaling has emerged as a promising oncological strategy[2]. Current approaches primarily utilize copper ionophores (e.g., DSF and ES) or copper-based nanomaterials (e.g., alloys and MOF) to elevate intratumoral Cu levels, often combined with modalities like photothermal therapy (PTT), chemodynamic therapy (CDT), and immunotherapy for synergistic efficacy[3–6]. However, these combination strategies face critical bottlenecks: conventional small-molecule ionophores suffer from poor targeting and low bioavailability, while elevated glutathione (GSH) in the tumor microenvironment neutralizes oxidative damage. Furthermore, PTT is constrained by limited tissue penetration, immunotherapy often induces systemic toxicity, and tumor cells adaptation to Cu-induced metabolic stress further compromises sensitivity[7]. These compounding limitations lead to suboptimal therapeutic outcomes and hinder clinical translation. Therefore, developing a multifunctional cuproptosis amplifier that simultaneously enhances Cu bioactivity, sensitizes tumor cells, intensifies oxidative stress, and enables active tumor targeting is highly desirable for safe, efficient, and precise cancer therapy.

Cuproptosis initiation and progression are executionally governed by cellular redox homeostasis and autophagy[8,9]. Intracellular reactive oxygen species (ROS) accumulation drives cuproptosis by promoting Cu^{2+} reduction to Cu^+ , accelerating lipoylated protein aggregation, and disrupting mitochondrial membranes[10]. However, overexpressed GSH in the tumor microenvironment (TME) counteracts this process by chelating free Cu^+ and scavenging ROS, thereby establishing a major therapeutic barrier[11–13]. Consequently, concurrent ROS generation and GSH depletion hold great promise for amplifying cuproptosis. Concurrently, under copper or oxidative stress, tumor cells upregulate lysosomal autophagy to clear damaged mitochondria and aberrant proteins, maintaining cell survival[14]. Disrupting this self-protective mechanism by inhibiting key autophagic proteins (e.g., ATG4B) or impairing lysosomal function can impede the clearance of cuproptosis-associated toxic aggregates, thereby lowering the death threshold[15,16]. Therefore, orchestrating TME-responsive ROS generation, GSH depletion, and autophagic flux blockade represents a highly effective cascade strategy for enhanced tumor therapy[17–19].

Multienzyme-mimetic nanomaterials with peroxidase (POD) and glutathione oxidase (GSHOx) activities can amplify oxidative stress and block lysosomal autophagy[20,21]. However, conventional copper

nanomaterials suffer from low atom utilization, insufficient catalytic sites, and weak substrate adsorption[22,23]. To overcome these bottlenecks, graphdiyne (GDY), featuring an sp/sp^2 carbon skeleton and electron-rich acetylenic bonds ($-C\equiv C-$)[24], establishes a strong metal-support interaction (SMSI) with copper. This SMSI suppresses nanoparticle aggregation and accelerates interfacial electron transfer[25], promoting asymmetric active sites and driving valence reconstruction toward toxic Cu^+ [26]. To address bioavailability and autophagy blockade limitations, 8-hydroxyquinoline (8-HQ) polynuclear complexes are introduced[27,28]. These hydrophobic and weakly basic complexes exhibit high membrane permeability and lysosomal enrichment, acting as ionophores to increase copper accumulation while coordinating with metals for synergistic lysosomal damage and tumor eradication[29,30]. Consequently, integrating copper nanomaterials with 8-HQ complexes leverages multinuclear electron transfer to boost catalytic efficiency while capitalizing on 8-HQ-mediated autophagy blockade, establishing a precise paradigm for cuproptosis-driven oncological therapy.

Herein, via a lattice engineering strategy, we developed a Dy_4-CuBi/GDY nanocomposite to mediate Fenton-like reactions and space-specifically disrupt lysosomal autophagy, amplifying cuproptosis for efficient osteosarcoma therapy. This platform utilizes defect-rich, ultrathin GDY to stabilize copper quantum dots via SMSI, while incorporating Bi to form ultrafine Cu-Bi alloys for accelerated charge transfer and optimized active site distribution. Crucially, doping with a designed tetranuclear dysprosium cluster (Dy_4L_2) induces pronounced lattice distortion and defects within the CuBi alloy. This structural modulation shifts the Cu d -band center toward the Fermi level, tuning intermediate adsorption energies into the optimal Sabatier regime to enhance POD-like and GSHOx-like catalytic activities. *In vitro* and *in vivo* evaluations revealed that Dy_4-CuBi/GDY selectively accumulates within lysosomes to sustainably release copper ions, directly damaging lysosomes and blocking autophagosome-lysosome fusion by impairing the Rab7-HOPS-SNARE axis. The resulting autophagic flux obstruction sensitizes osteosarcoma cells to copper, cooperatively pairing with exacerbated oxidative stress to trigger cuproptosis (Fig. 1). Overall, this work delivers a lattice-engineering strategy to construct functional cuproptosis amplifiers for clinical translation.

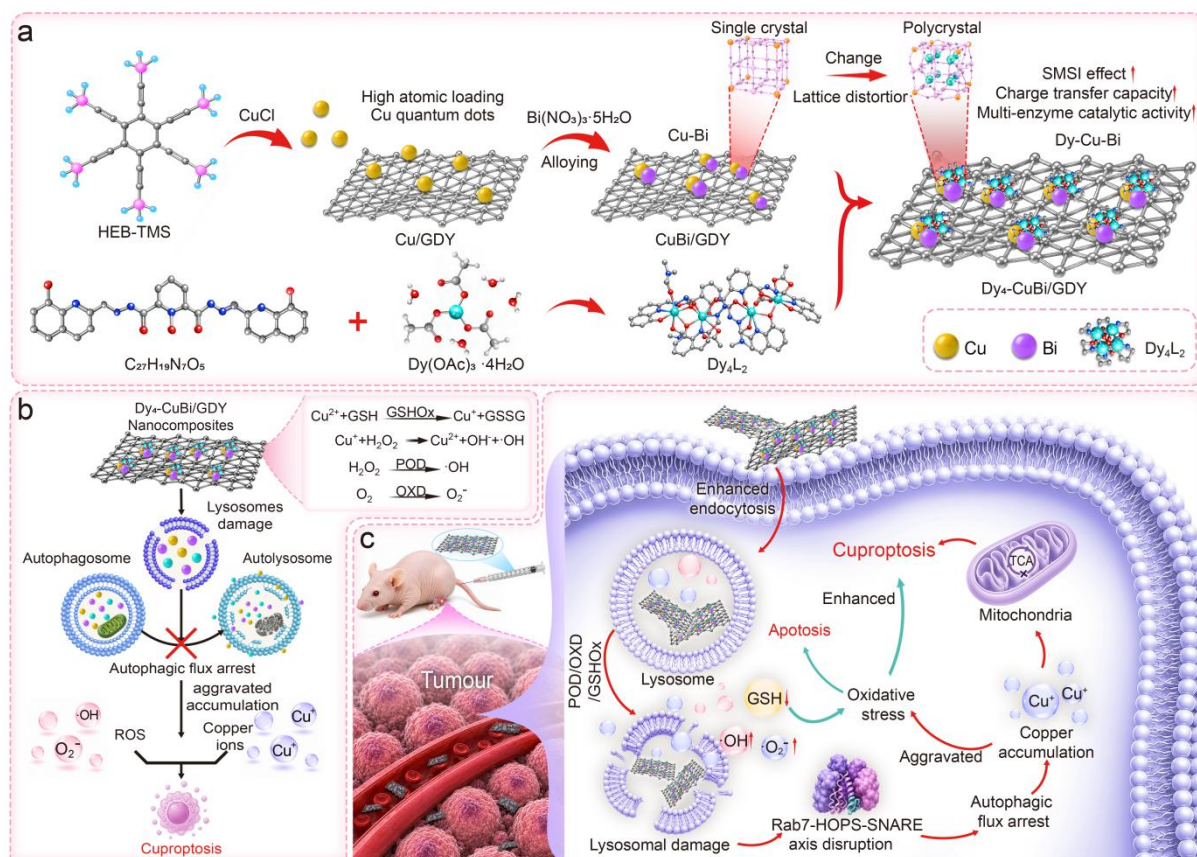


Fig. 1. Schematic illustration of the synthesis of Dy₄-CuBi/GDY nanocomposites and their antitumor mechanism via inducing cuproptosis. (a) Preparation of Dy₄-CuBi/GDY nanocomposites. (b) Dy₄-CuBi/GDY nanocomposites damage lysosomes through their enzyme-like activities, leading to blockage of autophagic flux, which exacerbates the intracellular accumulation of ROS and copper ions, collectively triggering cuproptosis in tumor cells. (c) In vivo antitumor mechanism of Dy₄-CuBi/GDY nanocomposites.

Results and Discussion

Characterization of Dy₄-CuBi/GDY Nanocomposites

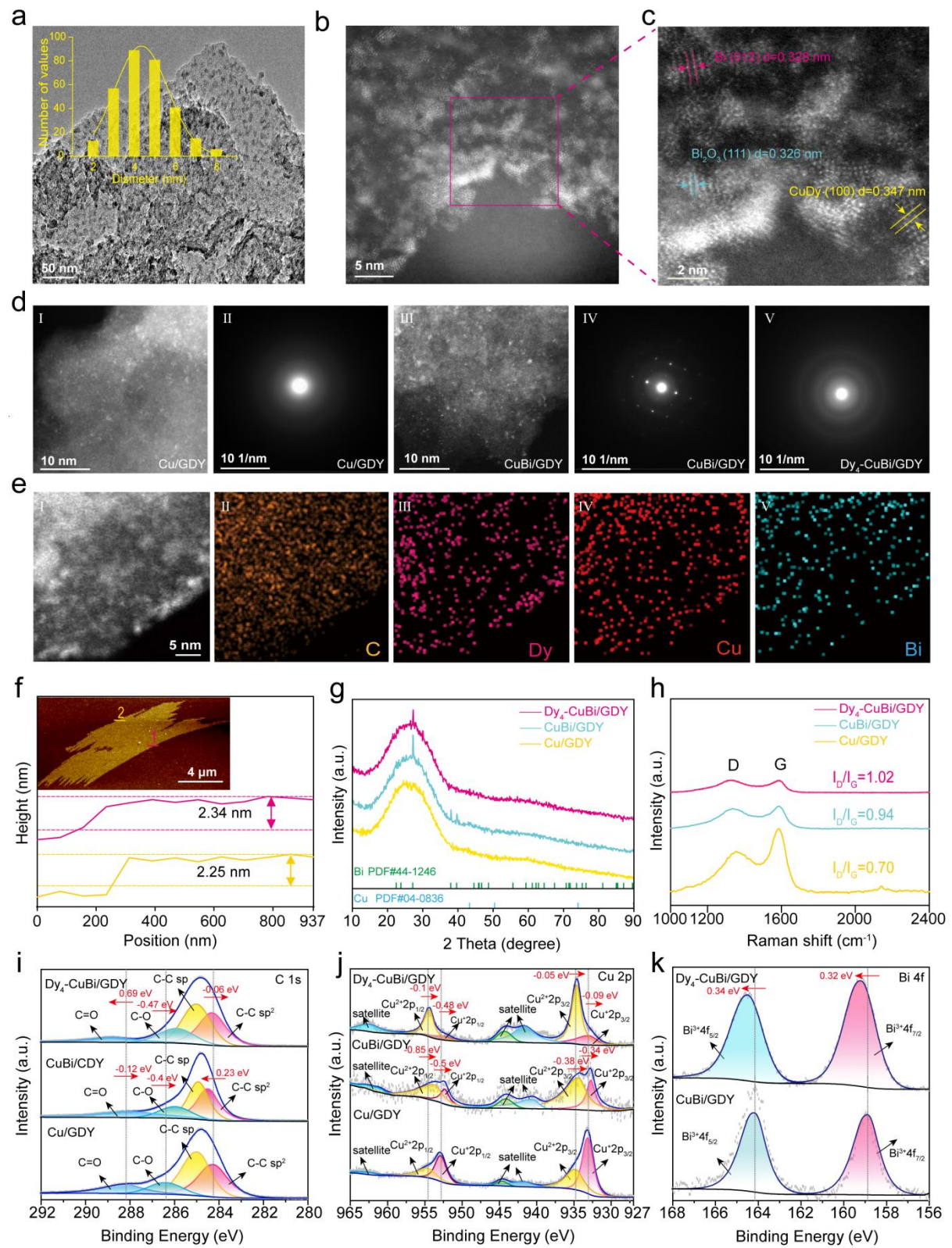


Fig. 2. Characterization of the Dy₄-CuBi/GDY nanocomposites. (a) Transmission electron microscopy

(TEM) image and particle size distribution of Dy₄-CuBi/GDY. (b) and (c) High-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) images of Dy₄-CuBi/GDY. (d) HAADF-STEM images and selected area electron diffraction (SAED) patterns of different samples. I: HAADF-STEM image of Cu/GDY. II: SAED pattern of Cu/GDY. III: HAADF-STEM image of CuBi/GDY. IV: SAED pattern of CuBi/GDY. V: SAED pattern of Dy₄-CuBi/GDY. (e) HAADF-STEM image and energy-dispersive X-ray spectroscopy (EDS) elemental mapping images of Dy₄-CuBi/GDY. (f) Atomic force microscopy (AFM) image of Dy₄-CuBi/GDY. (g) X-ray diffraction (XRD) patterns of Dy₄-CuBi/GDY, CuBi/GDY and Cu/GDY. (h) Raman spectra of Dy₄-CuBi/GDY, CuBi/GDY and Cu/GDY. (i-k) X-ray photoelectron spectroscopy (XPS) analyses of Dy₄-CuBi/GDY, CuBi/GDY and Cu/GDY.

To develop a nanocomposite combining multi-enzyme activity with lysosomal targeting capability, a strategy integrating bimetallic synergistic assembly with in situ coordination modification was employed. Cu-Bi bimetallic composites were first prepared using graphdiyne (GDY) as the carrier, followed by the introduction of tetranuclear dysprosium clusters to ultimately yield the Dy₄-CuBi/GDY nanocomposites. The 8-hydroxyquinoline-2-carbaldehyde condensed *N*-oxopyridine bishydrazone ligand (L) was synthesized via a multi-step liquid-phase organic synthesis method (Fig. S1). High-resolution mass spectrometry (HRMS) data confirmed the successful synthesis of ligand L (C₂₇H₁₉N₇O₅), exhibiting a molecular mass of 522.1530 (Fig. S2) and an isotopic pattern in high agreement with the theoretical value (Fig. S3). Ligand L was coordinated with Dy(OAc)₃·4H₂O to synthesize the Dy₄L₂. Therein, the framework of Dy₄L₂ comprises four Dy(III) centers bridged by one μ₃-OH⁻ and one μ₂-OH⁻ group, coordinated with three DMF molecules, four CH₃COO⁻, and two protonated L ligands, yielding a molecular formula of C₇₁H₆₃Dy₄N₁₇O₂₁ (Fig. S4,S5). The refined crystallographic data, bond lengths, and bond angles are summarized in Tables S1-S3. Powder X-ray diffraction (PXRD) results indicated that the experimental diffraction peaks of Dy₄L₂ in the low-angle region (2θ<10°) aligned well with the theoretical simulation, showing no detectable impurity phases (Fig. S6). Fourier transform infrared (FTIR) spectra (Fig. S7) confirmed the presence of its core structural components including hydrazone bonds, benzene rings, and N–O bonds, thereby verifying the successful coordination between Dy(III) and ligand L[31].

Transmission electron microscopy (TEM) images reveal that Dy₄-CuBi/GDY retains the characteristic flake-like structure of GDY[32], with immobilized metal nanoparticles exhibiting an average size of approximately 4.8 nm (Fig. 2a) and a zeta potential of -11.77 mV (Fig. S8). Aberration-corrected TEM (AC-

TEM) imaging further resolves the Bi (012), Bi₂O₃ (111) and CuDy (100) lattice planes of the metal nanoclusters[33,34] (Fig. 2b, c). The selected area electron diffraction (SAED) pattern reveals a predominantly amorphous structure for Cu/GDY, whereas CuBi/GDY displays distinct diffraction spots indicative of partially ordered domains. Notably, these diffraction features disappear upon Dy doping, confirming that the incorporation of Dy into the Cu-Bi alloy network drives the amorphization process (Fig. 2d). Energy-dispersive X-ray spectroscopy (EDS) analysis and elemental mapping confirm the uniform distribution of C, Dy, Cu, and Bi elements (Fig. S9, Fig. 2e). The atomic force microscopy (AFM) topography reveals that the Dy₄-CuBi/GDY nanosheets possess an average thickness of approximately 2.3 nm (Fig. 2f). X-ray diffraction (XRD) patterns (Fig. 2g) show no metallic Cu or copper oxide signals in Cu/GDY, indicating highly dispersed Cu species. For CuBi/GDY, the Bi peaks are accompanied by a new reflection at $2\theta = 28.89^\circ$. Positioned between the signature peaks of pure Cu and Bi, this peak confirms Cu-Bi metallic bond formation according to Vegard's law[35]. Additionally, Dy doping suppresses alloy crystallization, driving an amorphous transition that aligns with the SAED results. Raman spectra confirm successful GDY synthesis, featuring the D band (1350 cm⁻¹), G band (1580 cm⁻¹), and an alkyne peak (~2100 cm⁻¹). The I_D/I_G ratio increases markedly with Bi and Dy introduction (Fig. 2h), indicating that Bi induces structural distortions and defects on GDY, an effect further enhanced by Dy doping[36,37]. This defect-rich carbon environment stabilizes multimetallic or amorphous structures and enhances metal-support electronic coupling.

X-ray photoelectron spectroscopy (XPS) full-spectrum analysis further confirmed the successful integration of all elements, supporting the effective synthesis of the nanocomposites (Fig. S10). The formation of Cu-Bi alloy alters the local electronic distribution of GDY, as evidenced by the positive shift of C=C and negative shifts of C–O, C=O peaks[38] (Fig. 2i). Subsequent Dy doping induces distinct peak adjustments, proving an intricate charge redistribution between the GDY framework and metals. The effective loading of Dy³⁺ is confirmed by the Dy 3d spectrum (Fig. S11). Compared to Cu/GDY, alloying with Bi causes a negative shift in Cu 2p peaks, which is suppressed upon Dy introduction, suggesting further electronic rearrangement at active centers (Fig. 2j). Concurrently, the Bi 4f peaks in Dy₄-CuBi/GDY shift toward higher binding energies (Fig. 2k), confirming that electrons transfer from Bi to Dy and Cu[35]. Collectively, these findings demonstrate that the introduction of Bi and Dy successfully modulates the charge

distribution of Cu/GDY, establishing an optimal geometric and electronic foundation for the outstanding multienzyme-like catalytic performance of Dy₄-CuBi/GDY in tumor therapy.

Evaluation of Enzyme-like Activities and DFT Calculations of Dy₄-CuBi/GDY Nanocomposites

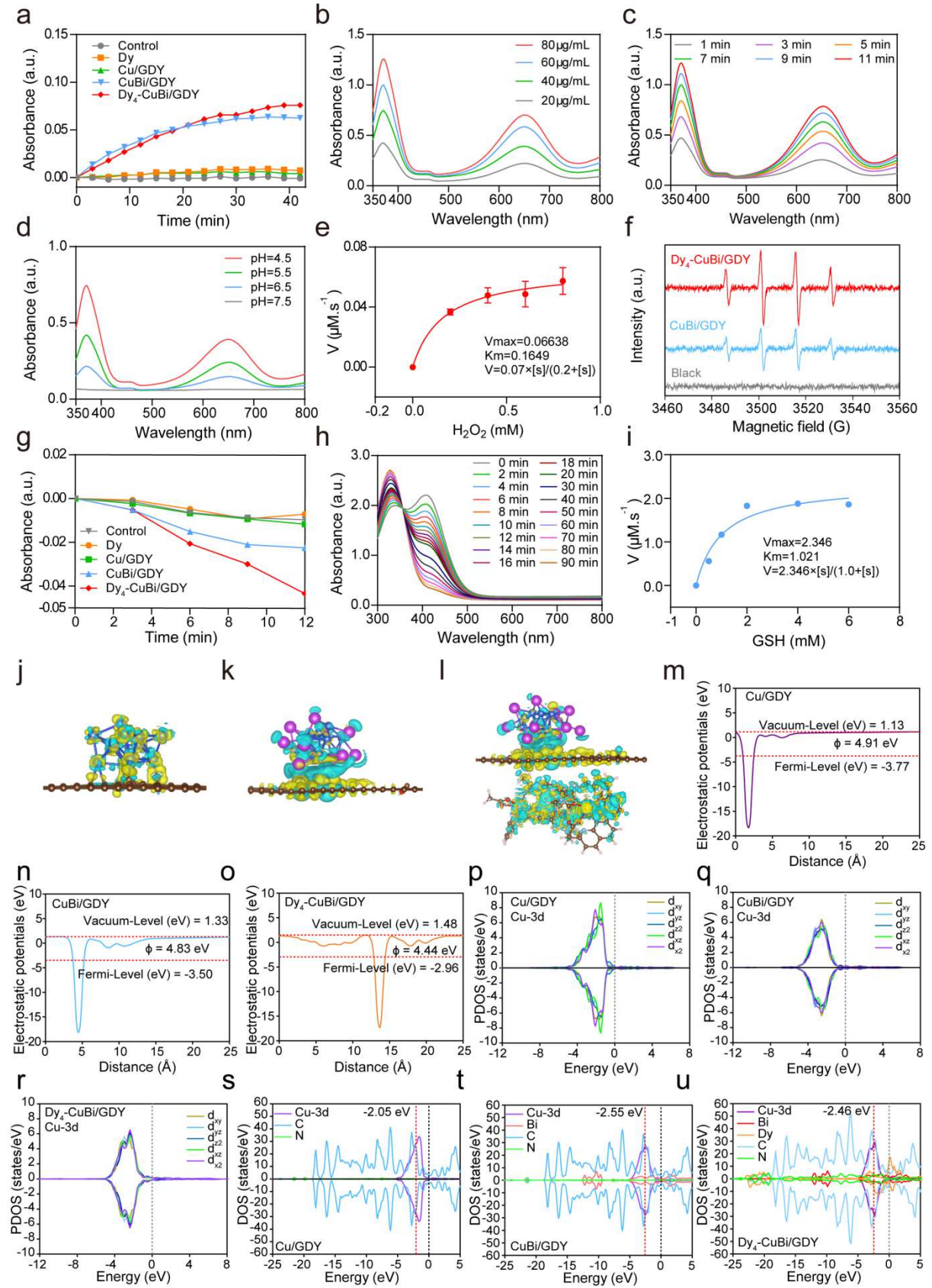


Fig. 3. POD-like and GSHOx-like catalytic activities of the Dy₄-CuBi/GDY nanocomposites with DFT calculations. (a) Reaction-time curves of the TMB colorimetric reaction catalyzed by Dy₄-CuBi/GDY, CuBi/GDY, Cu/GDY or Dy at 652 nm. (b) UV–vis absorption spectra of TMB oxidation catalyzed by varying concentrations of Dy₄-CuBi/GDY. (c) Time-dependent UV–vis absorption spectra of the TMB reaction catalyzed by Dy₄-CuBi/GDY. (d) UV–vis absorption spectra of the TMB reaction catalyzed by Dy₄-CuBi/GDY under different pH conditions. (e) Kinetic curves of the peroxidase-like (POD-like) activity of Dy₄-CuBi/GDY. (f) Electron spin resonance (ESR) spectra of Dy₄-CuBi/GDY and CuBi/GDY in H₂O₂ solution. (g) Reaction-time curves of the DTNB colorimetric reaction catalyzed by Dy₄-CuBi/GDY, CuBi/GDY, Cu/GDY or Dy at 412 nm. (h) Time-dependent UV–vis absorption spectra of glutathione (GSH) consumption catalyzed by Dy₄-CuBi/GDY. (i) Kinetic curves of the glutathione peroxidase-like (GSHOx-like) activity of Dy₄-CuBi/GDY. (j) Differential charge density distribution of Cu/GDY, (k) CuBi/GDY and (l) Dy₄-CuBi/GDY. (m) Calculated work functions of the Cu/GDY, (n) CuBi/GDY and (o) Dy₄-CuBi/GDY surfaces. (p) Projected density of states (PDOS) calculations for the Cu/GDY, (q) CuBi/GDY and (r) Dy₄-CuBi/GDY surfaces. (s) The calculated density of states (DOS) with Cu d-band center for the Cu/GDY, (t) CuBi/GDY and (u) Dy₄-CuBi/GDY surfaces .

The tumor microenvironment (TME) is characterized by hypoxia, mild acidity, elevated levels of ROS, and high GSH concentrations. These abnormal metabolic features create a favorable environment for the deployment of nanocomposites. By mimicking the enzymatic activities of POD, oxidase (OXD), and GSHOx, these nanocomposites respond to the TME to catalyze the conversion of H₂O₂ into highly cytotoxic hydroxyl radicals $\cdot\text{OH}$ for tumor cells eradication. Concurrently, they oxidize GSH to deplete intracellular antioxidants, thereby amplifying oxidative stress damage[39–41]. Therefore, we evaluated the POD-like activity of Dy₄-CuBi/GDY. The results showed that the absorbance of Dy₄-CuBi/GDY and CuBi/GDY at 652 nm continued to increase over time, and the Dy₄-CuBi/GDY group exhibited a stronger ability to decompose H₂O₂ as time progressed (Fig. 3a). The POD activity of the Dy₄-CuBi/GDY gradually increased with increasing concentration, prolonged reaction time and decreasing pH values (Fig. 3b-d). Steady-state kinetic evaluations yielded a maximum reaction velocity V_{max} of 0.06638 $\mu\text{M s}^{-1}$ and a Michaelis constant K_m of 0.1649 μM (Fig. S12a, Fig. 3e). Electron spin resonance (ESR) spectroscopy corroborated the generation of $\cdot\text{OH}$ radicals, with Dy₄-CuBi/GDY producing stronger characteristic signals than CuBi/GDY, which confirms that Dy loading boosts the POD-like activity (Fig. 3f). Concurrently, Dy₄-CuBi/GDY possessed OXD-like activity,

enabling the generation of $\cdot\text{O}_2^-$ within the simulated TME (Fig. S12b). GSH, an endogenous antioxidant that maintains intratumoral redox homeostasis and promotes tumor growth, presents a critical target; thus, depleting GSH levels is essential for enhancing the efficacy of cancer therapies[42]. The GSH scavenging capacity of the nanocomposites was investigated employing the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) assay. Under identical reaction times and concentrations, Dy₄-CuBi/GDY exhibited a time-dependent GSH depletion capacity that was notably superior to those of Cu/GDY and CuBi/GDY (Fig. 3g,h, Fig. S13a,b), confirming that the loading of Dy successfully boosts the GSHOx-like activity of the nanocomposites. Steady-state kinetic analysis further revealed that this biomimetic GSHOx activity escalated alongside the substrate concentration, yielding a maximum reaction velocity V_{max} of 2.2346 $\mu\text{M s}^{-1}$ and a Michaelis constant K_m of 1.021 μM (Fig. S13c, Fig. 3i). Collectively, these findings underscore that the Dy₄-CuBi/GDY nanocomposite possesses excellent concurrent POD- and GSHOx-like catalytic properties, which can effectively elevate intracellular ROS levels to pave the way for enhanced tumor therapy.

Density functional theory (DFT) calculations were performed to elucidate the biomimetic catalytic mechanism (Fig. S14). Differential charge density analysis (Fig. 3j–l) reveals that the sequential introduction of Bi and Dy efficiently enhances interfacial electronic coupling and charge transfer, with Dy₄-CuBi/GDY exhibiting the most pronounced electron delocalization and interfacial polarization. This local electronic restructuring substantially lowers the calculated work function from 4.905 eV (Cu/GDY) and 4.834 eV (CuBi/GDY) to 4.443 eV (Dy₄-CuBi/GDY) (Fig. 3m–o), reducing the electron escape barrier to accelerate transport kinetics and redox reactions. Furthermore, PDOS analysis (Fig. 3p–u) demonstrates that Bi and Dy doping modulates the Cu-3d orbitals and shifts the d-band center, optimizing the adsorption/desorption equilibrium of radical intermediates. Concurrently, enhanced orbital overlap near the Fermi level highlights strong hybridization among the Cu-3d, Bi-p, Dy-f, and C/N-p orbitals, boosting interfacial electronic transport and multienzyme-like catalytic activity. Gibbs free energy profiles (Fig. S15a, b) reveal that CuBi/GDY possesses significantly lower reaction barriers across both POD- and GSHOx-like pathways compared to Cu/GDY. Specifically, Cu–Bi alloy sites effectively stabilize the transition state during critical O–O bond cleavage, facilitating electron transfer for sustained $\cdot\text{OH}$ generation. Concurrently, in the GSH-to-GSSG conversion pathway, CuBi/GDY efficiently stabilizes sulfur radical intermediates to diminish the overall energetic barrier, showcasing superior GSHOx-like activity through synergistic electronic regulation. Consequently, the tailored electronic structure of Dy₄-CuBi/GDY endows it with robust multienzyme-like

performance to trigger ROS accumulation, which disrupted intratumoral redox homeostasis for potent antitumor outcomes.

In vitro antitumor activity of Dy₄-CuBi/GDY nanocomposites

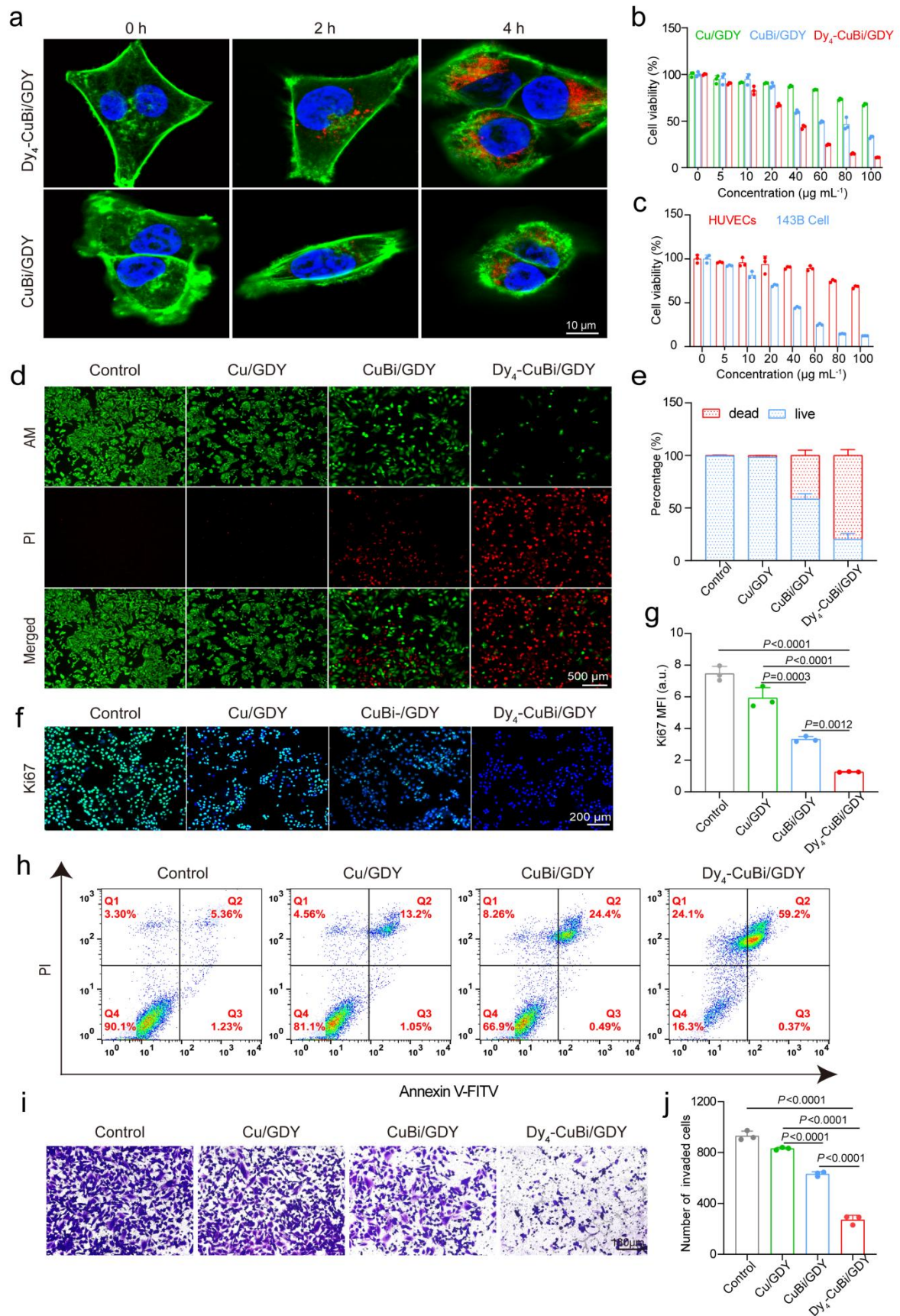


Fig. 4. In vitro antitumor activity of Dy₄-CuBi/GDY nanocomposites against osteosarcoma cells. (a) Confocal Laser Scanning Microscopy (CLSM) images showing the cellular uptake of Dy₄-CuBi/GDY and CuBi/GDY in 143B cells. (b) Cell viability of 143B cells after different treatments (n = 3). (c) Cell viability of HUVECs and 143B cells after treatment with Dy₄-CuBi/GDY (n = 3). (d) Live/dead staining of 143B cells after different treatments (green: Calcein-AM; red: PI) and quantitative analysis (e) (n = 3). (f) Representative immunofluorescence images of Ki67 expression in 143B cells after different treatments and quantitative analysis (g) (n = 3). (h) Flow cytometric analysis of apoptosis in 143B cells after different treatments. (i) Invasion assay of 143B cells after different treatments and quantitative analysis (j) (n = 3). *p* values are determined by one-wayANOVA. Data are presented as mean ± SD.

After elucidating the enzyme-like catalytic mechanism of the nanocomposites, we further evaluated the cellular effects of Dy₄-CuBi/GDY and its intracellular ROS-generating capability. Confocal laser scanning microscopy (CLSM) imaging confirmed internalization of both CuBi/GDY and Dy₄-CuBi/GDY, with Dy complexation further boosting cellular uptake (Fig. 4a). This efficient internalization translated into pronounced cytotoxicity; cell viability assays showed that Dy₄-CuBi/GDY reduced cell survival to 24.6% in a concentration-dependent manner, significantly outperforming Cu/GDY (83.6%) and CuBi/GDY (49.1%) (Fig. 4b). While Cu delivery alone was insufficient to eradicate most tumor cells, the Cu-Bi alloying triggered ROS evolution to enhance cytotoxicity, an effect drastically amplified by Dy incorporation. Crucially, Dy₄-CuBi/GDY maintained good biocompatibility toward normal cells up to 60 µg mL⁻¹ (Fig. 4c). This tumor-specific killing capability was further corroborated by live/dead staining and flow cytometric apoptosis analyses (Fig. 4d,e,h). Moreover, Dy₄-CuBi/GDY exhibited superior efficacy over its Cu/GDY and CuBi/GDY counterparts in suppressing 143B cell proliferation (Fig. 4f,g), invasion (Fig. 4i,j), and migration (Fig. S16a,b).

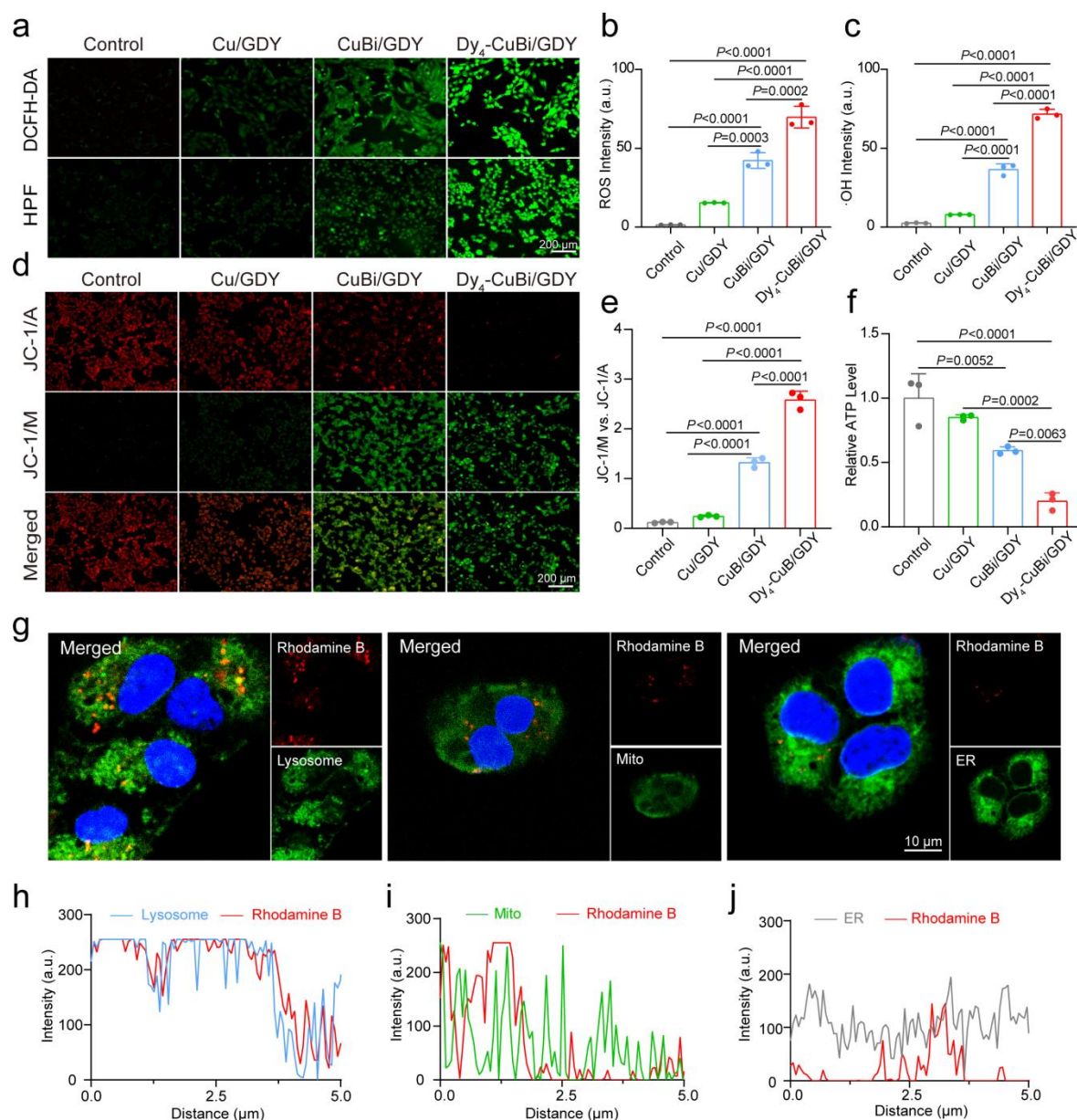


Fig. 5. Intracellular ROS generation and organelle colocalization of Dy₄-CuBi/GDY nanocomposites.

(a) Intracellular total ROS and ·OH levels in 143B cells after different treatments and corresponding quantitative analyses (b,c) (n = 3). (d) Mitochondrial membrane potential (MMP) changes in 143B cells after different treatments (red: JC-1 aggregates; green: JC-1 monomers) and quantitative analysis (e) (n = 3). (f) Relative intracellular ATP levels in 143B cells after different treatments (n = 3). (g) CLSM images showing the intracellular distribution of Dy₄-CuBi/GDY nanocomposites in 143B cells. (h) Colocalization analysis of Dy₄-CuBi/GDY nanocomposites with lysosomes, (i) mitochondria, and (j) endoplasmic reticula, respectively. *p* values are determined by one-way ANOVA. Data are presented as mean ± SD.

Dy₄-CuBi/GDY exhibits POD-, OXD-, and GSHOx-like activities, mediating its antitumor effects via

ROS generation and GSH depletion to amplify oxidative stress. Compared with Cu/GDY and CuBi/GDY, Dy₄-CuBi/GDY induces higher intracellular ROS levels, including hydroxyl radicals $\cdot\text{OH}$ (Fig. 5a–c). This elevated ROS generation is accompanied by a decrease in mitochondrial membrane potential and adenosine triphosphate (ATP) production (Fig. 5d–f), along with abnormal Ca^{2+} accumulation (Fig. S17), collectively indicating mitochondrial dysfunction. These findings demonstrate that Dy₄-CuBi/GDY-generated ROS suppress mitochondrial oxidative phosphorylation and impair respiratory chain function, resulting in energy insufficiency. Concurrently, mitochondrial damage disrupts Ca^{2+} homeostasis, and the subsequent intracellular Ca^{2+} accumulation further exacerbates mitochondrial injury. Consequently, Dy₄-CuBi/GDY inhibits tumor cells growth primarily through ROS-mediated mitochondrial dysfunction.

Dy₄-CuBi/GDY Nanocomposites Induce Lysosomal Dysfunction and Trigger Cuproptosis in Tumor Cells

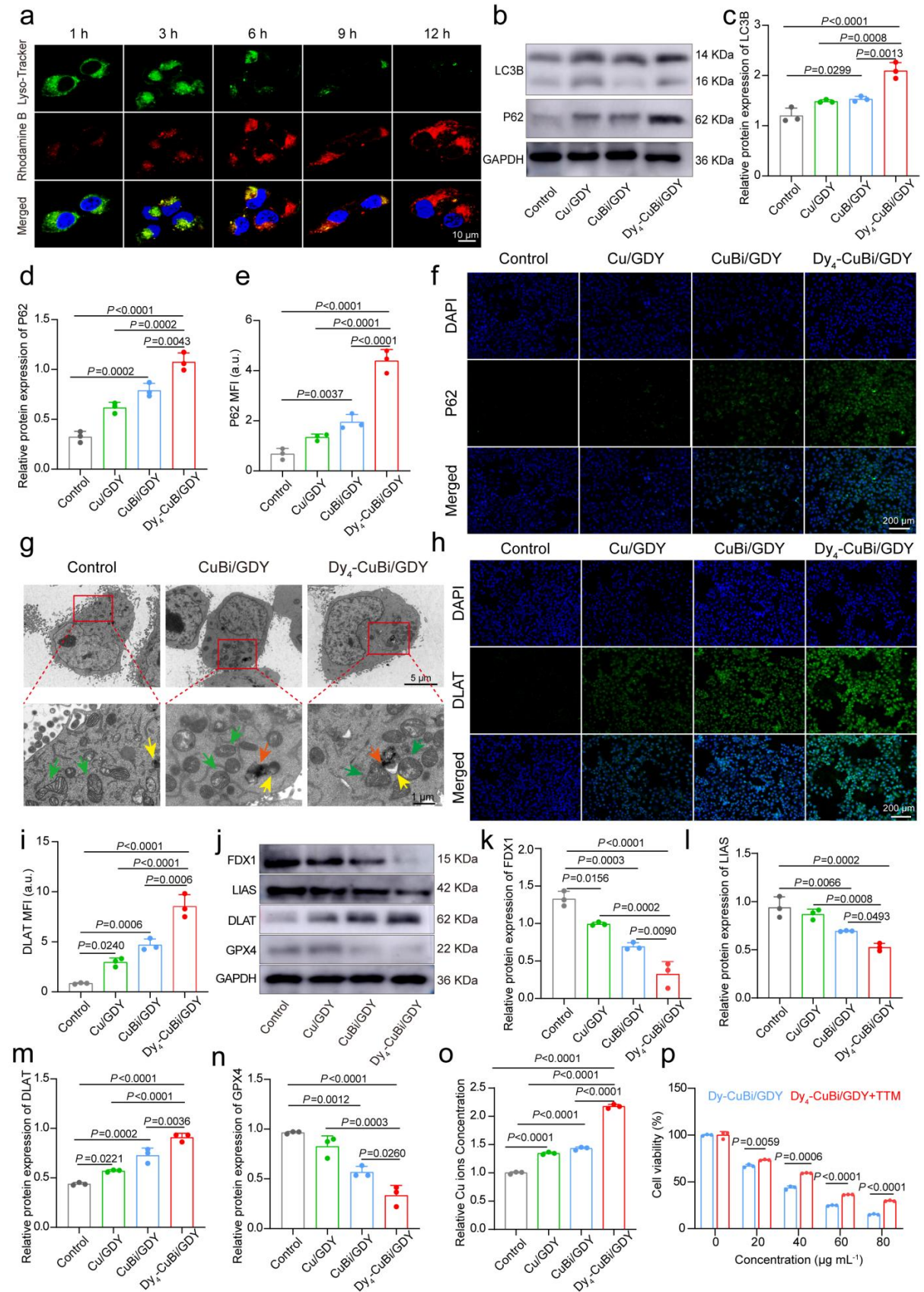


Fig. 6. Dy₄-CuBi/GDY nanocomposites induce lysosomal damage, autophagic dysfunction, and subsequent cuproptosis. (a) CLSM images showing the lysosomal targeting ability of Dy₄-CuBi/GDY nanocomposites and its effect on lysosomal integrity in 143B cells. (b) Western blot analysis of LC3B and P62 expression in 143B cells after different treatments and quantitative analyses (c,d) (n = 3). (f) Immunofluorescence images of P62 expression in 143B cells after different treatments and quantitative analysis (e) (n = 3). (g) TEM images of 143B cells after treatment with CuBi/GDY and Dy₄-CuBi/GDY nanocomposites (Green arrows represent mitochondria, red arrows represent nanomaterials, and yellow arrows represent lysosomes.). (h) Immunofluorescence images of DLAT expression in 143B cells after different treatments and quantitative analysis (i) (n = 3). (j) Western blot analysis of FDX1, LIAS, DLAT, and GPX4 expression in 143B cells after different treatments and quantitative analyses (k-n) (n = 3). (o) Relative intracellular Cu ions levels in 143B cells after different treatments (n = 3). (p) Rescue experiments of Dy₄-CuBi/GDY induced cell death in 143B cells using the copper chelator tetrathiomolybdate (TTM) (n = 3, *p* values are determined by unpaired two-tailed t-test). *p* values are determined by one-way ANOVA. Data are presented as mean ± SD.

Having demonstrated the efficacy of the nanocomposites in eradicating tumor cells, we further investigated the underlying intracellular mechanism of Dy₄-CuBi/GDY. Quinoline derivatives typically accumulate in lysosomes owing to their weakly basic and hydrophobic properties[43]. To verify the lysosome-targeting capability of Dy₄-CuBi/GDY, colocalization assays with lysosomes, mitochondria, and the endoplasmic reticulum (ER) were performed using CLSM. Following the incubation of 143B cells with rhodamine B-labeled Dy₄-CuBi/GDY for 30 min, the lysosomes, ER, and mitochondria were labeled with Lyso-Tracker Green, ER-Tracker Green, and Mito-Tracker Green, respectively. Confocal imaging revealed that the red fluorescence of Dy₄-CuBi/GDY-rhodamine B substantially overlapped with the green fluorescence of Lyso-Tracker Green (Fig. 5g-j). The corresponding colocalization coefficient for lysosomes was higher than those for the other organelles, confirming the preferential lysosomal accumulation of Dy₄-CuBi/GDY.

The impact of Dy₄-CuBi/GDY on lysosomal integrity was subsequently evaluated. Following a 3-h incubation, the fluorescence signals of Dy₄-CuBi/GDY and lysosomes remained colocalized, confirming efficient cellular internalization and lysosomal trafficking. By 6 h, the fluorescence intensity of Dy₄-CuBi/GDY sustained an increase, whereas the lysosomal fluorescence progressively diminished and nearly

disappeared, indicating Dy₄-CuBi/GDY-induced lysosomal impairment (Fig. 6a). Because lysosomes function as the primary degradation compartment of autophagy, lysosomal dysfunction typically disrupts autophagic flux. Accordingly, the expression levels of the key autophagy markers microtubule-associated protein 1 light chain 3B (LC3B) and sequestosome 1 (SQSTM1/p62) were analyzed. Compared with the CuBi/GDY cohort, Dy₄-CuBi/GDY treatment elevated both the LC3B-II/I ratio and p62 expression (Fig. 6b-f). These findings indicate that incorporation of the Dy₄L₂ exacerbates lysosomal damage, thereby impeding autophagosome–lysosome fusion and inducing autophagosome accumulation; this blockage promotes LC3B-I to LC3B-II conversion while inhibiting p62 degradation. TEM observations further revealed swollen lysosomes with disrupted membranes in Dy₄-CuBi/GDY-treated cells (Fig. 6g), substantiating the occurrence of lysosomal structural damage and defective autophagic degradation.

Lysosomal dysfunction concurrently impairs the efflux of Dy₄-CuBi/GDY, leading to intracellular Cu accumulation. This elevated Cu burden disrupts mitochondrial respiration and the tricarboxylic acid (TCA) cycle, thereby triggering cuproptosis. Ferredoxin 1 (FDX1), lipoic acid synthase (LIAS), and dihydrolipoamide S-acetyltransferase (DLAT) serve as critical regulators of this cuproptosis pathway. Compared with the CuBi/GDY group, Dy₄-CuBi/GDY treatment downregulated FDX1 and LIAS expression while significantly upregulating DLAT expression (Fig. 6h-m). These alterations promote DLAT protein lipoylation and subsequent toxic oligomerization, which exacerbates mitochondrial metabolic dysfunction. Concurrently, the reduction in LIAS expression compromises cellular copper detoxification, further sustaining intracellular Cu accumulation (Fig. 6o). Furthermore, GPX4 expression was downregulated in the Dy₄-CuBi/GDY group (Fig. 6n), a phenomenon attributed to the GSHOx-like activity of the nanocomposites and the subsequent depletion of intracellular GSH (Fig. S18). To validate the involvement of cuproptosis, a cell viability rescue assay was performed using the copper chelator ammonium tetrathiomolybdate (TTM). Compared with Dy₄-CuBi/GDY monotherapy, cotreatment with TTM significantly restored cell viability (Fig. 6p), confirming that Dy₄-CuBi/GDY-induced cell death is closely linked to copper homeostasis disruption and cuproptosis activation. Collectively, Dy₄-CuBi/GDY induces lysosomal impairment via ROS generation and simultaneously depletes intracellular GSH. This cascade compromises the cellular antioxidant defense system and amplifies oxidative damage to both lysosomes and mitochondria, ultimately accelerating cuproptosis execution.

Mechanistic Insight into the Antitumor Activity of Dy₄-CuBi/GDY nanocomposites

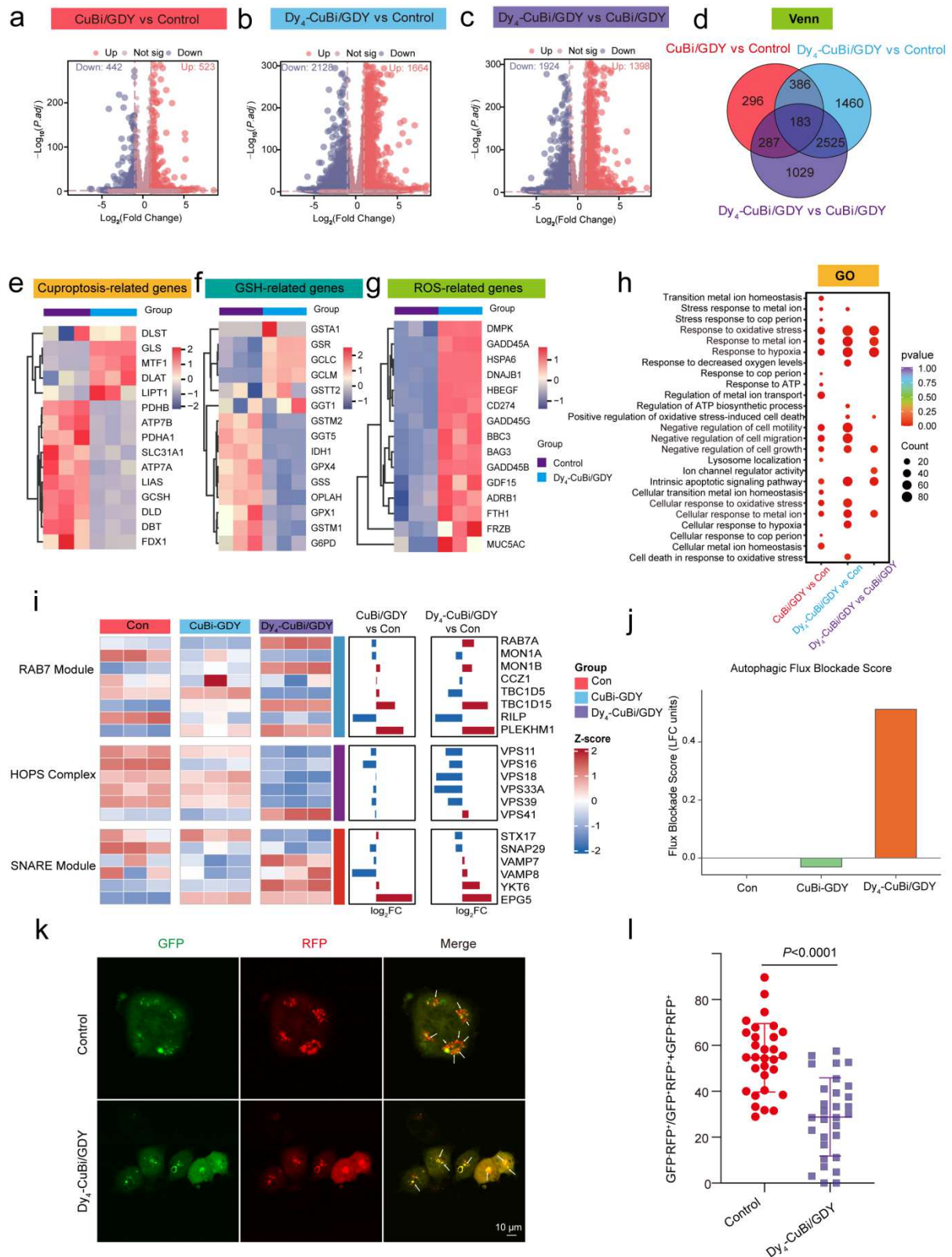


Fig. 7. Dy₄-CuBi/GDY induces transcriptomic remodelling and perturbs the autophagosome-lysosome

fusion axis. (a-c) Volcano plots showing differentially expressed genes in CuBi/GDY versus Control, Dy₄-CuBi/GDY versus Control, and Dy₄-CuBi/GDY versus CuBi/GDY comparisons. Red and purple dots indicate upregulated and downregulated genes, respectively. (d) Venn analysis of differentially expressed genes across the three comparisons. (e-g) Hierarchical clustering heatmaps of cuproptosis-related, GSH-related and ROS-related genes. (h) GO enrichment analyses of differentially expressed genes. (i) Expression heatmap and log₂FC changes of key genes in the RAB7 module, HOPS complex and SNARE module. (j) Autophagic flux blockade score calculated from expression changes in fusion-axis-related genes. (k) CLSM images of 143B cells transfected with RFP-GFP-LC3, followed by Dy₄-CuBi/GDY treatment for 24 h. (l) The number of GFP⁺RFP⁺ and GFP⁻RFP⁺ puncta in each cell was counted. Data are represented as the percentage (%) of GFP⁻RFP⁺ over the total of RFP⁺ (GFP⁺RFP⁺ and GFP⁻RFP⁺) puncta (n > 30). *p* values are determined by unpaired two-tailed t-test. Data are presented as mean ± SD.

To further elucidate the molecular basis underlying Dy₄-CuBi/GDY-induced lysosomal damage, autophagic substrate accumulation, and cuproptosis amplification, transcriptomic analysis was performed in 143B osteosarcoma cells. PCA showed clear separation among the Control, CuBi/GDY, and Dy₄-CuBi/GDY groups, with PC1 and PC2 explaining 40.13% and 21.07% of the variance, respectively (Fig. S19a). Boxplot, correlation, and hierarchical clustering analyses further confirmed good sample consistency and reproducibility (Fig. S19b–d). Enrichment analysis of differentially expressed genes (DEGs) indicated significant association with osteosarcoma-related diseases (Fig. S20). Compared with the Control group, CuBi/GDY treatment resulted in 523 upregulated and 442 downregulated genes (Fig. 7a), whereas Dy₄-CuBi/GDY induced a broader transcriptional response, with 1664 upregulated and 2128 downregulated genes (Fig. 7b). Direct comparison between Dy₄-CuBi/GDY and CuBi/GDY identified 1398 upregulated and 1924 downregulated genes (Fig. 7c). Venn analysis revealed 183 shared DEGs among the three comparisons (Fig. 7d), indicating that Dy₄-CuBi/GDY elicited both CuBi/GDY-associated responses and Dy₄-specific regulatory effects. Hierarchical clustering further showed substantial remodeling of cuproptosis-, GSH metabolism-, and ROS response-related genes following Dy₄-CuBi/GDY treatment (Fig. 7e–g), consistent with the enhanced oxidative and copper-ion stress observed at the cellular level. GO enrichment analysis indicated that DEGs were mainly associated with oxidative stress, metal ion/copper ion response, hypoxia response, lysosomal localization, and negative regulation of cell migration and proliferation (Fig. 7h). KEGG analysis further suggested alterations in cancer-related pathways, apoptosis, and autophagy (Fig. S21).

Consistently, GSEA demonstrated positive enrichment of the cuproptosis-related gene set, response to oxidative stress, and cellular response to metal ion, whereas the tricarboxylic acid cycle and oxidative phosphorylation gene sets were negatively enriched in the Dy₄-CuBi/GDY group (Fig. S22), suggesting enhanced cuproptosis, oxidative stress, and metal-ion responses accompanied by suppression of mitochondrial metabolic programs.

Notably, completion of autophagic flux requires not only autophagosome formation but also autophagosome transport, tethering, and fusion with lysosomes. Analysis of genes involved in the RAB7-HOPS-SNARE fusion axis revealed marked transcriptional alterations following Dy₄-CuBi/GDY treatment, among which HOPS complex-associated genes exhibited the most pronounced downregulation (Fig. 7i). Changes were also observed in RAB7- and SNARE-related fusion factors, suggesting coordinated disruption of autophagosome transport, recognition, and membrane fusion. Consistently, the autophagic flux blockade score calculated from fusion-axis genes was significantly elevated in the Dy₄-CuBi/GDY group (Fig. 7j). Functional validation further supported these transcriptomic findings. The mRFP-GFP-LC3 assay showed marked accumulation of yellow LC3 puncta without a corresponding increase in red-only autolysosome signals (Fig. 7k,l), indicating impaired autophagic flux. Moreover, LC3/LAMP1 colocalization analysis supported defective autophagosome–lysosome fusion (Fig. S23). Together, these results suggest that Dy₄-CuBi/GDY does not simply inhibit autophagy initiation but rather blocks the late-stage fusion/maturation process between autophagosomes and lysosomes after autophagy induction, thereby causing autophagic flux arrest and further amplifying ROS accumulation, metal-ion stress, and cuproptosis-related damage.

In vivo antitumor activity of Dy₄-CuBi/GDY nanocomposites

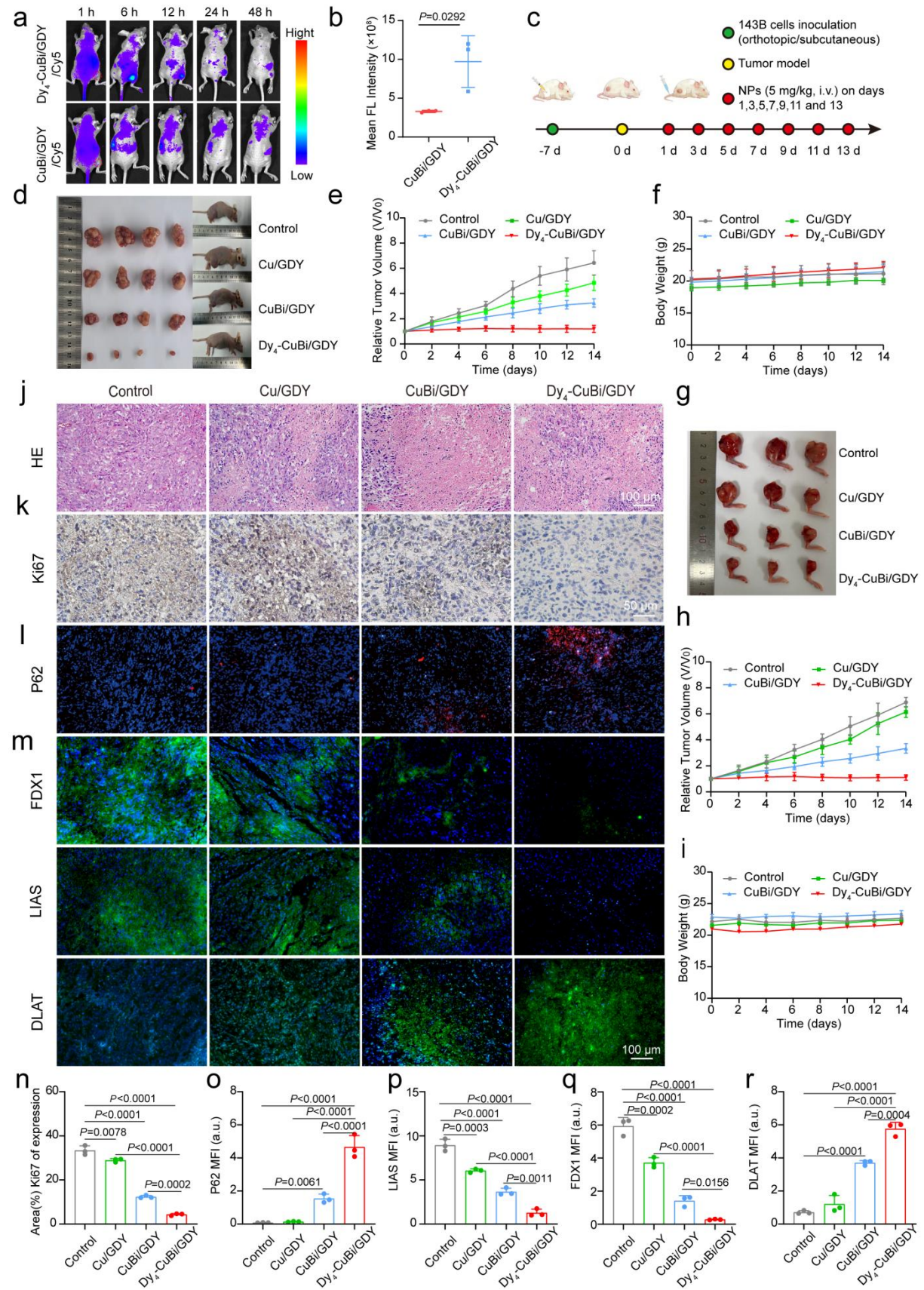


Fig. 8. In vivo antitumor therapeutic efficacy of Dy₄-CuBi/GDY nanocomposites. (a) In vivo

fluorescence images of tumor-bearing nude mice after intravenous injection of CuBi/GDY and Dy₄-CuBi/GDY. (b) Quantitative analysis of fluorescence signals at 6 h post intravenous injection of CuBi/GDY and Dy₄-CuBi/GDY. (n = 3 mice per group, *p* values are determined by unpaired two-tailed t-test). (c) Schematic illustration of the treatment protocol for orthotopic and subcutaneous 143B tumor-bearing mouse models. (d) Representative photographs of subcutaneous 143B tumors after 14 days of different treatments. (n = 4 mice per group). (e) Relative tumor volume growth curves (n = 4 mice per group). (f) Body weight changes during treatment (n = 4 mice per group). (g) Representative photographs of orthotopic 143B tumors after 14 days of different treatments (n = 3 mice per group). (h) Relative tumor volume growth curves of orthotopic 143B tumor-bearing mice under different treatments (n = 3 mice per group). (i) Body weight changes of orthotopic tumor-bearing mice during treatment (n = 3 mice per group). (j) Hematoxylin and eosin (H&E) staining of tumor tissue sections collected after different treatments. (k) Representative immunohistochemistry images of Ki67 expression in tumor sections after different treatments and quantitative analysis (n) (n = 3). (l) Representative immunofluorescence images of P62 expression in tumor sections after different treatments and quantitative analysis (o) (n = 3). (m) Representative immunofluorescence images of FDX1, LIAS, and DLAT expression in tumor sections after different treatments and quantitative analyses (p-r) (n = 3). *p* values are determined by one-wayANOVA. Data are presented as mean ± SD.

Encouraged by the in vitro therapeutic efficacy of the Dy₄-CuBi/GDY nanocomposite mediated by autophagic flux blockage, oxidative stress amplification, and cuproptosis induction, we subsequently evaluated its in vivo antitumor performance. First, cyanine 5 (Cy5)-labeled Dy₄-CuBi/GDY (Dy₄-CuBi/GDY/Cy5) and CuBi/GDY (CuBi/GDY/Cy5) nanocomposites were intravenously administered into mice bearing subcutaneous osteosarcoma tumors, followed by real-time in vivo fluorescence imaging at predefined intervals (1, 6, 12, 24, and 48 h). The results revealed that Dy₄-CuBi/GDY efficiently accumulated at the tumor site by 6 h, exhibiting superior targeting capability compared with CuBi/GDY/Cy5 (Fig. 8a,b). Ex vivo fluorescence imaging of major organs further indicated that the Dy₄-CuBi/GDY nanocomposite was primarily cleared via hepatic and renal pathways (Fig. S24). Next, both subcutaneous and orthotopic osteosarcoma models were established to systematically validate the therapeutic efficacy of the nanocomposites (Fig. 8c). Compared with the saline, Cu/GDY, and CuBi/GDY control groups, the Dy₄-CuBi/GDY treatment group exhibited pronounced suppression of tumor volume progression, while the body

weights of the mice remained stable throughout the therapeutic period (Fig. 8d-f, 8g-i). These findings demonstrate the potent *in vivo* antitumor therapeutic performance of the Dy₄-CuBi/GDY nanocomposites.

Furthermore, histopathological analysis revealed that the Dy₄-CuBi/GDY treatment group exhibited the most extensive tumor tissue necrosis compared with the saline, Cu/GDY, and CuBi/GDY groups, accompanied by downregulated Ki67 expression and diminished tumor proliferation capability (Fig. 8j-k, n). Notably, relative to the other cohorts, the Dy₄-CuBi/GDY group displayed elevated p62 expression, downregulated FDX1 and LIAS expression, and upregulated DLAT expression within the tumor tissues; these alterations confirm that the therapy suppressed autophagic degradation and promoted intratumoral cuproptosis (Fig. 8l-m, o-r).

Concurrently, the biosafety profile of Dy₄-CuBi/GDY was systematically evaluated. Post-treatment, no appreciable histological damage was detected in the major organs, including the heart, liver, spleen, lungs, and kidneys (Fig. S25). Moreover, all serum biochemical and routine hematological parameters remained within physiological ranges (Fig. S26). Inductively coupled plasma mass spectrometry (ICP-MS) quantitative analysis indicated no substantial accumulation of constituent metals (Cu, Bi, and Dy) in the major organs (Fig. S27). Taken together, these results demonstrate that the Dy₄-CuBi/GDY nanocomposite possesses favorable biocompatibility and achieves robust osteosarcoma eradication by inducing autophagic dysfunction to amplify intratumoral cuproptosis, thereby offering promising translational potential for clinical research.

Conclusions

In summary, leveraging a lattice engineering strategy, we developed a lattice-distorted Dy₄-CuBi/GDY nanocomposite to amplify cuproptosis for efficient osteosarcoma therapy. Within this system, ultrathin GDY anchors ultrafine CuBi catalytic sites via SMSI. Bi incorporation and novel tetranuclear dysprosium cluster doping cooperatively trigger lattice distortion, shifting the Cu d-band center toward the Fermi level to optimize intermediate adsorption energies within the Sabatier regime. Consequently, Dy₄-CuBi/GDY exhibits enhanced POD- and GSHOx-like cascade catalytic activities alongside lysosomal targeting capability. *In vitro* and *in vivo* evaluations demonstrated that Dy₄-CuBi/GDY selectively accumulates within tumor lysosomes and continuously releases copper ions. This precise intervention disrupts lysosomal integrity and blocks autophagosome-lysosome fusion by impairing the Rab7-HOPS-SNARE axis, obstructing autophagic flux to lower the copper tolerance threshold. Concurrently, cascade-enhanced

oxidative stress synergizes with accumulated copper ions to trigger cuproptosis, achieving specific osteosarcoma eradication with high biosafety. Overall, this study offers a potent strategy for cuproptosis-based cancer therapy and establishes a design paradigm for cuproptosis-sensitizing nanomedicines governed by autophagic flux regulation.

Experimental Section

Materials

hexa[(trimethylsilyl)ethynyl]benzene (HEB-TMS) was obtained from XFNANO (Nanjing, China). CuCl and triethylamine were obtained from Macklin (Shanghai, China). Bi(NO₃)₃·5H₂O and N,N-dimethylformamide (DMF) were obtained from Aladdin (Shanghai, China). Dimethyl sulfoxide (DMSO) was obtained from Jinshan Chemical Reagent (Chengdu, China). Ethanol, methanol (CH₃OH) and concentrated sulfuric acid were obtained from HU SHI (Shanghai, China). Hydrazine hydrate (N₂H₄·H₂O) was obtained from Merck (Shanghai, China).

Synthesis of 8-hydroxyquinoline-2-carbaldehyde-condensed N-oxopyridine bis-hydrazone tetranuclear dysprosium complex (Dy₄L₂).

N-O Pyridine dicarboxylic acid (1 mmol) was placed in a round-bottom flask with 100 mL of CH₃OH and 10 mL of conc. H₂SO₄. The mixture then underwent heating in an oil bath at 90 °C for 12 h. Upon cooling, white needle-like crystals precipitated. These were identified as dimethyl N-oxopyridinedicarboxylate. Refluxing this intermediate (1 mmol) with 50 mL of CH₃OH and 10 mL of N₂H₄·H₂O at 80 °C for 12 h followed. After cooling, the mixture was filtered. The precipitate was collected, washed with small portions of CH₃OH, and air-dried. A white flocculent solid thus formed, namely N-oxopyridinedicarboxylic dihydrazide. Dissolving this dihydrazide (1 mmol) in 50 mL of CH₃OH and heating to reflux at 80°C came next. 8-Hydroxyquinoline-2-carbaldehyde (2 mmol) was then added in two portions. The reaction continued for 12 h. Cooling and filtration of the mixture followed. The precipitate was washed with CH₃OH and air-dried, yielding a pale yellow powder. This powder corresponded to the Schiff-base ligand H₄L (8-hydroxyquinoline-2-carbaldehyde condensed N-oxopyridine bis-hydrazone). H₄L (0.01 mmol), Dy(OAc)₃·4H₂O (0.05 mmol), CH₃OH (2 mL), DMF (0.5 mL), and triethylamine (10 μL) were added to a 15 mL Pyrex tube. The tube was evacuated and sealed under vacuum. Heating at 90°C in an oven for three days followed. Slow cooling to room temperature gave

orange-yellow crystals. These crystals were collected, washed with CH_3OH , and dried to afford the tetranuclear dysprosium cluster complex, designated as Dy_4L_2

Synthesis of CuBi/GDY nanocomposites

HEB-TMS, CuCl and dimethylformamide were mixed in a hydrothermal reactor with a polytetrafluoroethylene lining, with the molar ratio of CuCl to HEB-TMS being 1:2. The flask was sealed and then placed in an oven at $100\text{ }^\circ\text{C}$ for 12 h. After the reaction, the product was successively washed with dimethylformamide, ethanol and ultrapure water and dried in a vacuum oven to get a dark brown powder, which was named as Cu/GDY. Subsequently, Cu/GDY (70 mg) powder and $\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ (24 mg) were dissolved in 40 mL of dimethylsulfoxide. The solution was stirred magnetically until it was evenly dispersed and then poured into an oil bath and heated at $60\text{ }^\circ\text{C}$ for 4 h. Then, the reaction system was heated to $180\text{ }^\circ\text{C}$ in a fume hood to eliminate all the dimethylsulfoxide. The obtained sample was dried in a vacuum oven, packed into a ceramic crucible and calcined in a tube furnace at $800\text{ }^\circ\text{C}$ for 3 h under a nitrogen atmosphere, resulting in CuBi/GDY. Finally, the product was ground into fine particles and stored in darkness after drying.

Synthesis of $\text{Dy}_4\text{-CuBi/GDY}$ nanocomposites

CuBi/GDY (2 mg) and Dy_4L_2 (1 mg) were put into a glass vial and dissolved in the freshly prepared ultra-pure water. The mixture was stirred at room temperature for 12 h to guarantee adequate interaction between the components. When the reaction was finished, the product was separated and washed with methanol to remove the unbound substances. The obtained residue was then dried in a vacuum oven to obtain the final product. Afterwards, the dried solid was crushed into a fine powder by using a mortar and a pestle and stored in a brown glass vial under light-protected condition.

Characterization of Cu/GDY, CuBi/GDY and $\text{Dy}_4\text{-CuBi/GDY}$ nanocomposites

Transmission electron microscopy (TEM) imaging and elemental analysis were performed using a JEM-1230 field-emission electron microscope (JEOL, Japan) equipped with an energy-dispersive X-ray spectroscopy (EDS) detector (Oxford Instruments, UK). The hydrodynamic size and zeta potential were measured by dynamic light scattering (DLS) using a Zetasizer Pro instrument (Malvern Panalytical, UK). X-ray diffraction (XRD) patterns were recorded on a Rigaku Ultima IV diffractometer (Rigaku, Japan). X-ray photoelectron spectroscopy (XPS) analysis was conducted using a Thermo Fisher ESCALAB 250Xi

system (Thermo Fisher Scientific, USA). The morphology and microstructure of the samples were characterized by atomic force microscopy (AFM, MFP-3D system, Asylum Research, USA). Raman spectra were obtained using a LabRAM HR Evolution micro-Raman spectrometer (HORIBA Jobin Yvon, France) with an excitation wavelength of 532 nm and a laser spot diameter of approximately 600 nm.

Determination of POD-like activity

The peroxidase (POD)-like activity of Cu/GDY, CuBi/GDY and Dy₄-CuBi/GDY nanocomposites was determined using 3,3',5,5'-tetramethylbenzidine (TMB) as the substrate in the presence of H₂O₂. H₂O₂ (1 mM) and TMB (0.02 mM) were employed as substrates in sodium acetate buffer (pH 4.5). Different nanosystems (PBS, Dy, Cu/GDY, CuBi/GDY and Dy₄-CuBi/GDY) with a concentration of 10 µg mL⁻¹ were added to start the reaction. Then, the reaction mixtures were transferred into a 96-well plate and the absorbance at 652 nm was recorded continuously for 40 min by a microplate reader. To study the enzyme kinetics of Dy₄-CuBi/GDY, different concentrations of H₂O₂ (0, 0.2, 0.4, 0.6 and 0.8 mM) with a constant TMB concentration (0.02 mM) were used under the same buffer conditions (pH 4.5). The absorbance at 652 nm was monitored continuously for 15 min after adding Dy₄-CuBi/GDY (10 µg mL⁻¹) into the reaction system. The POD-like activity of Dy₄-CuBi/GDY was quantitatively evaluated by plotting the Michaelis–Menten saturation curve to determine the kinetic parameters according to the equation $v = (V_{\max} \times [S]) / (K_m + [S])$.

To evaluate the effects of concentration and pH on the POD-like activity, Dy₄-CuBi/GDY suspensions at varying concentrations (20, 40, 60, and 80 µg mL⁻¹) were added to TMB solution (0.02 mM, pH 4.5) containing H₂O₂ (1 mM), and the absorbance at 652 nm was recorded. Separately, the pH effect was assessed by adding Dy₄-CuBi/GDY (60 µg mL⁻¹) to the same TMB/H₂O₂ system at pH 4.5, 5.5, 6.5, and 7.4, with absorbance measured at 652 nm.

Determination of OXD-like activity

The oxidase (OXD)-like activity of Dy₄-CuBi/GDY was evaluated using TMB as the substrate. Dy₄-CuBi/GDY (60 µg mL⁻¹) suspension was added to TMB solution (0.02 mM, pH 4.5) in the absence of H₂O₂. The absorbance at 652 nm.

ESR measurement of •OH generation by Dy₄-CuBi/GDY

Hydroxyl radicals ($\bullet\text{OH}$) were generated by irradiating an H_2O_2 solution with a full-spectrum xenon lamp for 5 min. The generated $\bullet\text{OH}$ radicals were captured using 5-methyl-1-pyrroline N-oxide (BMPO, 10 mM). Upon the formation of hydroxyl radicals, four characteristic peaks appeared in the electron spin resonance (ESR) spectra. The scavenging ability of Cu/GDY, CuBi/GDY, and $\text{Dy}_4\text{-CuBi/GDY}$ nanocomposites at $60\ \mu\text{g mL}^{-1}$ toward $\bullet\text{OH}$ was evaluated by monitoring the changes in the peak intensities.

Determination of GSHOx-like activity

To evaluate glutathione (GSH) depletion using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as a probe, several nanosystems were tested: PBS, Dy, Cu/GDY, CuBi/GDY, and $\text{Dy}_4\text{-CuBi/GDY}$. Each nanosystem at $10\ \mu\text{g mL}^{-1}$ reacted with GSH (10 mM) and DTNB (10 mM). After the reaction, the mixtures were transferred to a 96-well plate, and a microplate reader recorded the absorbance at 420 nm continuously for 12 min. For kinetic studies, $\text{Dy}_4\text{-CuBi/GDY}$ ($10\ \mu\text{g mL}^{-1}$), DTNB (10 mM), and GSH at varying concentrations (0, 0.15, 0.3, 0.45, 0.6, and 0.75 mM) were added to 2 mL centrifuge tubes. After mixing, the solutions were transferred to a 96-well plate, and the absorbance at 420 nm was monitored for 15 min. The Michaelis–Menten curve was plotted from these data, and kinetic parameters were obtained by fitting to the equation: $v = (V_{\text{max}} \times [\text{S}]) / (K_{\text{m}} + [\text{S}])$.

In order to observe how the spectra changed over time, we prepared three suspensions (Cu/GDY, CuBi/GDY, and $\text{Dy}_4\text{-CuBi/GDY}$) at $60\ \mu\text{g mL}^{-1}$. These were mixed with DTNB solution (10 mM) and GSH (10 mM). Absorption spectra were collected at various time points.

Density Functional Theory (DFT) Calculations

DFT calculations in this work were performed with the Vienna ab initio simulation package (VASP). Exchange-correlation effects were treated via the Perdew-Burke-Ernzerhof (PBE) functional. For structural relaxations, we adopted a plane-wave basis set with a kinetic energy cutoff of 400 eV, together with convergence thresholds of 10^{-4} eV for total energy and $0.05\ \text{eV \AA}^{-1}$ for atomic forces, and a $(1 \times 1 \times 1)$ Monkhorst-Pack grid for k-point sampling. A vacuum layer larger than $10\ \text{\AA}$ was introduced to avoid spurious interactions between periodic replicas. Isolated molecules (GSH, GSSG, H_2O , and H_2O_2) were placed in cubic boxes of $25\ \text{\AA} \times 25\ \text{\AA} \times 25\ \text{\AA}$, employing only the gamma point for Brillouin-zone integration. Finally, Bader charge analysis was employed to evaluate atomic charges and the extent of charge transfer.

Cell lines and culture

143B osteosarcoma cells (CL-1031) were obtained from Wuhan Procell (Wuhan, China), and HUVECs (iCell-h110) were obtained from iCell Bioscience (Shanghai, China). 143B cells and HUVECs were grown in DMEM/F12 (Biosharp, Beijing, China) and DMEM basic media (Gibco, Shanghai, China), respectively. Both supplemented with 10% FBS (Gibco, Shanghai, China) and 1% penicillin/streptomycin (Biosharp, Beijing, China), and maintained at 37 °C under 5% CO₂ in a humidified incubator.

Cellular Uptake and Organelle Colocalization

The Dy₄-CuBi/GDY or CuBi/GDY nanoparticles were labeled with Rhodamine B (5 mg mL⁻¹), (Beyotime, Shanghai, China) and then centrifuged, washed and dried after 12 h of stirring. 143B cells were seeded in confocal dishes. Subsequently, different time treatments of Rhodamine B-labeled nanoparticles (10 µg mL⁻¹) were performed, namely 0, 2 and 4 h. The actin filaments were stained with F-actin probe and the cell nuclei were stained with DAPI (Beyotime, Shanghai, China). The intracellular uptake images were obtained using CLSM (Leica TCS SP8 confocal microscope, Leica Microsystems, Wetzlar, Germany).

For organelle colocalization studies, cells were incubated with Dy₄-CuBi/GDY-Rhodamine B (10 µg mL⁻¹) for 30 min, then stained with Lyso-Tracker Green, ER-Tracker Green, or Mito-Tracker Green (Beyotime, Shanghai, China) for 30 min at 37°C. CLSM was used to monitor colocalization within lysosomes, endoplasmic reticulum, and mitochondria.

Treatment in vitro

Except for the Control group (complete medium without nanomaterials), Cells were treated with Cu/GDY, CuBi/GDY and Dy₄-CuBi/GDY nanocomposites at 60 µg mL⁻¹ for another 24 h.

Cell viability assay

143B cells (1 × 10⁴ cells/well) were seeded in 96-well plates and incubated for 24 h. After washing with PBS, cells were treated with different concentrations (0, 5, 10, 20, 40, 60, 80, 100 µg mL⁻¹) of Dy₄-CuBi/GDY, CuBi/GDY, or Cu/GDY for 24 h. Subsequently, 100 µL of CCK-8 (Biosharp, Beijing, China) working solution was added per well and incubated at 37°C for 2 h, followed by measurement of absorbance at 450 nm.

Live/Dead Cell Staining

143B cells were seeded in 6-well plates for 24 h. After PBS washing, the assigned treatments were applied as described in Section 1.13. Cells were stained with Calcein-AM and PI (Beyotime, Shanghai, China) for 30 min. Live/dead cell images were captured using a fluorescence microscope

Intracellular ROS Generation Assay

143B cells were seeded in 6-well plates for 24 h. After PBS washing, the assigned treatments were applied as described in Section 1.13. Then incubated with DCFH-DA (Beyotime, Shanghai, China) or HPF (Maokang, Shanghai, China) in the dark at 37 °C with 5% CO₂ for 20–30 min. Fluorescence images were acquired via fluorescence microscope.

Flow Cytometric Analysis of Cell Apoptosis

143B cells were seeded in 6-well plates for 24 h. After PBS washing, the assigned treatments were applied as described in Section 1.13. Subsequent steps were performed according to the manufacturer's instructions of the Annexin V-FITC kit (Multi Sciences, Hangzhou, China).

Immunofluorescence

143B cells were seeded in 6-well plates for 24 h. After PBS washing, the assigned treatments were applied as described in Section 1.13. Cells were fixed with 4% paraformaldehyde. Primary antibodies against Ki67 (27309-1-AP), P62 (18420-1-AP), DLAT (13426-1-AP), FDX1 (12592-1-AP) and LIAS (11577-1-AP) (Proteintech, Wuhan, China) were incubated overnight at 4°C. Then, incubate the corresponding fluorescent secondary antibody, and stain the cell nuclei with DAPI. Images were acquired by fluorescence microscopy.

Mitochondrial Membrane Potential Assay

143B cells were seeded in 6-well plates for 24 h. After PBS washing, the assigned treatments were applied as described in Section 1.13. Cells were incubated with JC-1 working solution (Beyotime, Shanghai, China) at 37 °C for 20 min in the dark. After cleaning, images were captured using a fluorescence microscope.

Intracellular Ca²⁺ Concentration Detection

143B cells were seeded in 6-well plates for 24 h. After PBS washing, the assigned treatments were

applied as described in Section 1.13. HBSS served as the washing buffer. Cells were incubated with 0.5–5 μM Fluo-4 AM working solution (Solarbio, Beijing, China) at 37 °C for 20 min. Removal of the staining solution and rinsing with HBSS followed. A final wash was conducted after 40 min. The intracellular Ca^{2+} signal was recorded by a fluorescence microplate reader.

Intracellular ATP Level Detection

143B cells were seeded in 6-well plates for 24 h. After PBS washing, the assigned treatments were applied as described in Section 1.13. The processed cells were lysed on ice, centrifuged at 4 °C for 5–10 min, and the supernatant was collected. The ATP level was measured using the Enhanced ATP Assay Kit (Beyotime, Shanghai, China). After removing the background ATP in the system, 20–50 μL of the supernatant was added to the detection well and the luminescence signal was detected using a fluorescence/chemiluminescence microplate reader.

Lysosomal Membrane Permeabilization Assay

143B cells in confocal dishes were treated with Rhodamine B-loaded $\text{Dy}_4\text{-CuBi/GDY}$ (60 $\mu\text{g mL}^{-1}$) for 1, 3, 6, 9, and 12 h, then stained with 50 nM Lyso-Tracker Green for 30 min. After fixation with 4% paraformaldehyde and DAPI nuclear staining, fluorescent signals were observed by CLSM.

Intracellular Cu ions Detection

143B cells were seeded in 6-well plates for 24 h. After PBS washing, the assigned treatments were applied as described in Section 1.13. Treated cells were digested, centrifuged, and ultrasonically disrupted (ice bath, 200 W, 3 s sonication/7 s interval, 3 min), then centrifuged at 10000 $\times g$ for 10 min. The supernatant was assayed using the Cell Copper Content Assay Kit (Solarbio, Beijing, China). The absorbance was read at 580 nm using a microplate reader.

Cell Invasion Assay

1×10^4 143B cells were seeded into Matrigel-coated Transwell upper chambers (8.0 μm pore size), with lower chambers containing control medium or nanocomposites-containing medium. After 24 h incubation, non-invading cells were removed, and invaded cells were fixed, stained with crystal violet, and imaged by microscope.

Intracellular GSH Detection

143B cells were seeded in 6-well plates for 24 h. After PBS washing, the assigned treatments were applied as described in Section 1.13. Treated cells were lysed by ultrasonication (300 W, 30 min), and the supernatant was collected. Subsequently, 0.5 mL of the supernatant was transferred to a 96-well plate and incubated with GSH detection reagent (Solarbio, Beijing, China), followed by absorbance measurement at 412 nm.

Western Blotting

143B cells were seeded in 6-well plates for 24 h. After PBS washing, the assigned treatments were applied as described in Section 1.13. Lysed in RIPA buffer containing PMSF (Beyotime, Shanghai, China), and protein concentration was determined by BCA assay (Beyotime, Shanghai, China). Proteins were separated on 10% SDS-PAGE gels (Epizyme, Shanghai, China) and transferred to PVDF membranes (LABGIC, Beijing, China). After blocking with Blocking Buffer (Beyotime, Shanghai, China), membranes were incubated with primary antibodies (P62 (18420-1-AP), LC3 (14600-1-AP), FDX1 (12592-1-AP), DLAT (13426-1-AP), LIAS (11577-1-AP), GPX4 (30388-1-AP), GAPDH (10494-1-AP)) (Proteintech, Wuhan, China) overnight at 4°C, followed by HRP-conjugated secondary antibodies (Beyotime, Shanghai, China) for 60 min at room temperature. Signals were developed using ECL substrate (Beyotime, Shanghai, China).

Rescue Assays

143B cells were seeded in 96-well plates for 24 h, then pretreated with 20 μ M tetrathiomolybdate (TTM) for 12 h, followed by incubation with Dy₄-CuBi/GDY at concentrations of 0, 20, 40, 60, and 80 μ g mL⁻¹ for another 12 h. Cell viability was determined using the CCK-8 assay.

RNA sequencing and data processing

143B osteosarcoma cells were assigned to the Control, CuBi/GDY and Dy₄-CuBi/GDY groups, with three biological replicates per group. Total RNA was extracted using the TRIzol method. RNA concentration and fragment integrity were assessed using a Qubit fluorometer and a Qsep400 high-throughput biofragment analyzer, after which poly(A) mRNA was enriched and used for cDNA library construction. Qualified libraries were sequenced by Metware Biotechnology on an Illumina platform to generate 150 bp paired-end reads. Raw reads were processed with fastp (v0.23.2) to remove adaptor contamination, reads with excessive N bases and low-quality reads. Clean reads were aligned to the

Ensembl human reference genome (Homo_sapiens.GRCh38.109) using HISAT2 (v2.2.1), and gene-level read counts were obtained using featureCounts (v2.0.3). FPKM values were used for expression visualization, whereas raw read counts were used for differential expression analysis.

Differential expression and functional enrichment analysis

Differential expression analysis was performed using the featureCounts-derived count matrix. Duplicate gene symbols were merged, lowly expressed genes were removed, and DESeq2 (v1.38.3) was applied with condition as the design factor. Pairwise comparisons were performed for CuBi/GDY versus Control, Dy₄-CuBi/GDY versus Control and Dy₄-CuBi/GDY versus CuBi/GDY. *P* values were adjusted using the Benjamini-Hochberg method, and genes with an adjusted *P* value ≤ 0.05 and $|\log_2 \text{fold change}| \geq 1$ were defined as differentially expressed genes. Volcano plots and Venn diagrams were generated from the differential expression results. GO and KEGG enrichment analyses were then performed for differentially expressed genes. Gene set enrichment analysis (GSEA) was conducted using ranked whole-transcriptome gene lists, with a focus on mechanism-related gene sets including cuproptosis, oxidative stress response, cellular response to metal ions, the tricarboxylic acid cycle and oxidative phosphorylation.

Autophagy fusion-axis analysis

To further investigate the transcriptomic basis of copper/oxidative stress injury and autophagic flux blockade after material treatment, curated gene sets related to cuproptosis, GSH metabolism, ROS response and the autophagosome-lysosome fusion axis were analyzed. Expression patterns of cuproptosis-, GSH- and ROS-related genes were visualized using heatmaps based on normalized expression matrices. The autophagosome-lysosome fusion-axis analysis included the RAB7 module, the HOPS tethering complex and SNARE-associated fusion factors, including STX17/SNAP29/VAMP7/VAMP8/YKT6/EPG5. Normalized expression values and log₂ fold changes for these genes were extracted and visualized. The autophagic flux blockade score was calculated as the difference between the mean log₂ fold change of autophagosome-formation-related genes and that of fusion/lysosomal degradation completion-related genes, and was used to summarize the transcriptomic tendency toward increased autophagosome formation with impaired late-stage fusion/maturation. Unless otherwise stated, statistical analysis and visualization were performed in R and Python, and adjusted *P* value < 0.05 was considered statistically significant.

Tandem RFP-GFP-LC3 in 143B cell

143B cells were transduced with RFP-GFP-hLC3 lentivirus (Genechem, Shanghai, China). Transfected cells were seeded into confocal dishes. The cells were imaged using a CLSM, after treatment with Dy₄-CuBi/GDY for 24 h. The number of GFP and RFP puncta was manually counted. The quantities of GFP⁺RFP⁺ and GFP-RFP⁺ puncta were calculated accordingly.

Cellular colocalization of LAMP1 and LC3 in 143B cells

143B cells were seeded into confocal dishes, and then treated with Dy₄-CuBi/GDY for 24 h. Subsequently, the cells were labeled with anti-LAMP1 antibody (21997-1-AP, Proteintech, Wuhan, China), anti-LC3B antibody (81004-1-RR, Proteintech, Wuhan, China), and DAPI. After incubation with the fluorescent secondary antibody, take pictures using a CLSM.

Animals and tumour models

All studies complied with relevant ethical regulations, and animal experiments were approved by the Animal Ethics Committee of Guangxi Medical University (approval No.: 202310039). Six-week-old male BALB/c nude mice (22 g) were obtained from the Animal Research Center of Guangxi Medical University, housed 5 per cage under SPF conditions (22 ± 1 °C, 40–70% relative humidity, 12 h light-dark cycle) with free access to food and water.

Tumor volume was calculated as $V = (L \times W^2)/2$. Tumor burden was limited to ≤5% of normal body weight in general experiments and ≤10% in therapeutic experiments (RB/T 173-2018). Mice were humanely euthanized with isoflurane at the end of experiments. For in vivo osteosarcoma models, 143B cells were diluted 1:1 with Matrigel. Twenty male nude mice were randomly divided into four groups. 1×10^6 143B cells were subcutaneously injected above the patella to establish subcutaneous models. In another batch, 1×10^5 143B cells were injected into the tibial cavity of the knee joint for orthotopic models. In vivo studies started when tumor volume reached ~100 mm³.

In Vivo Studies

Mice were randomly assigned to Control, Cu/GDY, CuBi/GDY, or Dy₄-CuBi/GDY groups (5 mg/kg, tail vein injection every other day for 14 days). Tumor volume was calculated as $V = (L \times W^2)/2$. At the endpoint, tumors and organs were harvested for H&E staining, immunohistochemistry, immunofluorescence and histological analysis.

Hematoxylin and Eosin (H&E) Staining

Tissue sections were deparaffinized in xylene I and II (20 min each) and rehydrated through a graded ethanol series (100%, 95%, 85%, and 75% for 5 min each) into distilled water. Sections were then stained with hematoxylin for 5 min, rinsed with running tap water, and differentiated in 1% acid alcohol for up to 30 s. After stopping the differentiation with a 3-min tap water wash, sections were immersed in a bluing reagent for 30 s and rinsed again for 3 min. Subsequently, sections were counterstained with eosin for 15 s, rapidly dehydrated through 75% and 85% ethanol (20 s each), followed by 95% (1 min) and 100% ethanol (2 min). Finally, sections were cleared in xylene I and II (2 min each) and mounted with neutral balsam under a coverslip, ensuring no air bubbles. Representative images were acquired using an optical microscope.

Immunohistochemistry (IHC)

Tissue sections were deparaffinized in xylene I and II (20 min each) and rehydrated through a graded ethanol series (100%, 95%, 85%, and 75% for 5 min each). Antigen retrieval was performed using EDTA buffer, followed by permeabilization with 0.1% Triton X-100 at room temperature for 10-15 min. After blocking with normal goat serum, sections were incubated with primary anti-Ki67 antibody overnight at 4 °C. The next day, sections were washed with PBS (2-3 times) and incubated with HRP-conjugated secondary antibody (1:100) at 37 °C for 30-60 min in the dark. After washing with PBS, standard 3,3'-diaminobenzidine (DAB) development was applied for 5-10 min and terminated with tap water. Sections were then counterstained with hematoxylin, differentiated, dehydrated, cleared, and mounted with neutral balsam. Representative images were captured using an optical microscope.

Immunofluorescence (IF) Staining

Tissue sections were deparaffinized in xylene I and II (20 min each) and rehydrated through a graded ethanol series (100%, 95%, 85%, and 75% for 5 min each). After blocking with normal goat serum at room temperature for 30 min, sections were incubated with the designated primary antibody overnight at 4°C in the dark. The following day, sections were washed with PBS (2-3 times) and incubated with a FITC-conjugated goat anti-rabbit IgG secondary antibody (1:100) at 37°C for 30-60 min in the dark. After subsequent PBS washes, nuclei were counterstained with DAPI. Finally, sections were mounted using an anti-fade mounting medium. Representative images were acquired using a laser scanning confocal

microscope.

ICP-MS Analysis

Tumor and organ samples were fixed in 4% paraformaldehyde for 24 h. Metal contents were quantified by inductively coupled plasma mass spectrometry (ICP-MS) following standard protocols.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8, R and Python software. Data are expressed as mean $\pm$ standard deviation (S.D.).

Ethics approval and consent to participate

All studies complied with relevant ethical regulations, and animal experiments were approved by the Animal Ethics Committee of Guangxi Medical University (approval No.: 202310039).

Declaration of competing interest

All authors declare that there are no competing interests.

Author contributions

Chuwen Ma: Writing - original draft, Data curation, Investigation, Validation. Hanjie Wang: Conceptualization, Data curation, Investigation. Zhengtian Li: Methodology, Software, Visualization. Biying Tan: Writing - review & editing, Supervision. Yuting Ye: Supervision. Tianjie Li: Investigation. Jiahao Li: Investigation. Haijuan Liu: Investigation. Li Zheng: Funding acquisition, Resources, Supervision. Ting Meng: Supervision, Writing - review & editing. Jingping Zhong: Conceptualization, Funding acquisition, Supervision, Writing - review & editing. Jianwei Liu: Funding acquisition, Supervision.

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Data availability

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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