

Review Article

Ferroptosis, cuproptosis, and metal ion-modulated cytotoxicity in osteosarcoma: regulatory mechanisms and emerging therapeutic strategies

Xuehai Liu¹, Linzhu Li¹, Yufei Yang², Binkui Jia¹, Zhaoyu Li³, Xiaoyan Long⁴, Haolong Zhang¹, Zhijing Song^{1,5}

¹Clinical College of Traditional Chinese Medicine, Gansu University of Chinese Medicine, No. 35 Dingxi East Road, Lanzhou 730000, Gansu, China; ²School of Public Health, Gansu University of Chinese Medicine, No. 35 Dingxi East Road, Lanzhou 730000, Gansu, China; ³College of Acupuncture-Moxibustion and Tuina, Gansu University of Chinese Medicine, No. 35 Dingxi East Road, Lanzhou 730000, Gansu, China; ⁴The First School of Clinical Medicine, Yunnan University of Chinese Medicine, No. 88 Baita Road, Panlong District, Kunming 650011, Yunnan, China; ⁵Dunhuang Key Laboratory of Medicine and Transformation, Ministry of Education, Lanzhou 730000, Gansu, China

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Abstract: Osteosarcoma is characterized by early metastatic potential, frequent postoperative recurrence, and resistance to conventional chemotherapy, and therapeutic progress remains limited. Osteosarcoma may have exploitable vulnerabilities due to metal homeostasis and redox control dysregulation. However, metal-associated cytotoxic processes do not constitute a single class of regulated cell death. This review examines ferroptosis and cuproptosis as mechanistically defined metal-dependent regulated cell-death modalities and distinguishes them from broader metal ion-modulated cytotoxic processes. Ferroptosis is due to iron-dependent phospholipid peroxidation and the collapse of antioxidant defenses, while cuproptosis is induced by the interaction of copper with lipoylated mitochondrial proteins, which induces protein aggregation, loss of iron-sulfur cluster proteins, and proteotoxic stress. In addition, perturbations in metal homeostasis are likely to affect chemosensitivity, immune responses and the osteolytic microenvironment; these effects may be either beneficial or adverse depending on the cellular compartment. Preclinical agents, including small molecules, nanomedicine-based delivery systems, metal-active scaffolds and immunotherapy combinations, have demonstrated remarkable potential but varying degrees of mechanistic validation and translational maturity. Osteosarcoma-specific mechanistic evidence is currently strongest for ferroptosis, whereas evidence for canonical cuproptosis remains limited and is concentrated mainly in material-based experimental systems with incomplete mechanistic validation. Studies in the future should combine molecular subtype with intraosseous microenvironmental context, cell-type-specific toxicity and therapeutic response to classify osteosarcoma subgroups for prospective evaluation of metal-targeted treatments.

Keywords: Osteosarcoma, ferroptosis, cuproptosis, metal ion-modulated cytotoxicity, metal homeostasis, therapeutic translation

Introduction

Despite contemporary multimodal treatment, the long-term outcome of patients with metastatic, recurrent, or chemotherapy-refractory osteosarcoma remains poor. The current standard of care is surgical resection with multi-agent chemotherapy. Clinical outcomes are much better for patients with localized disease than for those presenting with metastases at diagnosis, postoperative relapse, or a poor histological response to neoadjuvant chemo-

therapy. Intrinsic resistance and acquired resistance restrict the activity of conventional cytotoxic therapy, suggesting that further intensifying treatment alone is not likely to produce significant therapeutic progress. These limitations have spurred interest in therapeutically exploitable metabolic dependencies, redox dysregulation, and vulnerabilities within regulated cell-death pathways [1].

This is supported by the heterogeneous molecular profile of osteosarcoma, which creates

opportunities to exploit metabolic and redox vulnerabilities. Multi-omics analyses have proposed different molecular subtypes, such as the immune-activated subtype, immunosuppressive subtype, homologous-recombination-deficiency-dominant subtype, and MYC-driven subtype. The MYC-driven subtype has a more robust signature of oxidative phosphorylation and is linked to resistance to neoadjuvant chemotherapy [2]. Taken together, these observations suggest that the metabolic state and immune context may determine therapeutic response. However, no molecular subtype of osteosarcoma has been prospectively validated as a biomarker to predict sensitivity to ferroptosis or cuproptosis.

Reprogramming of iron and copper homeostasis may alter the sensitivity of tumor cells to oxidative and mitochondrial stress. Ferroptosis is an iron-dependent form of regulated cell death driven by fatal phospholipid peroxidation and antagonized mainly by the SLC7A11-glutathione (GSH)-glutathione peroxidase 4 (GPX4) axis in concert with complementary antioxidant defense systems [3]. Cuproptosis is conceptually and mechanistically distinct from other canonically defined forms of regulated cell death and entails copper binding to lipoylated proteins of the mitochondrial tricarboxylic acid cycle, aggregation of lipoylated proteins, loss of iron-sulfur cluster proteins, and mitochondrial proteotoxicity [4]. Iron and copper are hence important metabolic cofactors but can also become foes of cell survival when the intracellular levels, trafficking, or redox activity of these elements exceed the buffering capacity of the cell.

For clarity of conceptualization, the levels of evidence covered here are divided into three categories. Mechanistically defined metal-dependent regulated cell-death modalities are classified as Level I: ferroptosis and cuproptosis [5]. Level II encompasses metal ion-modulated cytotoxicity or sensitization to established cell-death pathways, including Zn²⁺-associated mitochondrial injury, Mn²⁺-mediated sensitization to ferroptosis, Ca²⁺-overload-associated injury, and selenium-associated oxidative stress. Therefore, in the current review, these processes are regarded as metal-coupled injury or regulation of established death pathways rather than as mechanistically distinct regulated cell-death modalities within the

classical mechanistic nomenclature [6]. Level III comprises metal-containing nanomaterials, ion-release systems, hydrogels, scaffolds, and implants that mediate antitumor effects through multiple overlapping cytotoxic mechanisms. This review used this hierarchical framework in the review to differentiate true regulated cell-death modalities from more general metal toxicity and material-related therapeutic benefits. This hierarchy is utilized in **Table 1**, which ranks each cited mechanism according to disease specificity, model type, canonical marker assessment, and functional rescue or dependency testing.

Based on the above mechanisms, osteosarcoma resistance to ferroptosis and other metal-associated cytotoxic stresses has been attributed to augmented antioxidant defenses, microenvironmental signalling, and dysregulated metal homeostasis. MLX, driven by superenhancers, regulates SLC7A11, which enhances cystine uptake and maintains redox homeostasis, providing direct evidence for the MLX-SLC7A11 axis acting as an anti-ferroptotic defense mechanism in osteosarcoma [7]. ZEB1 has been reported to be targeted by exosomal miR-144-3p, thus inhibiting osteosarcoma cell proliferation, migration, and invasion along with ferroptosis-related molecular changes [8]. It is concluded that the results indicate a role for extracellular-vesicle-mediated communication in modulating ferroptosis susceptibility.

Tirapazamine, a hypoxia-activated prodrug, could also induce ferroptosis-associated injury in osteosarcoma cells due to disruption of the SLC7A11-GPX4 pathway [9]. In contrast, the evidence for an additional ferroptotic contribution is only indirect, based on inhibitor-rescue and SLC7A11-related experiments, while direct C11-BODIPY analysis and thorough validation involving iron chelation remain limited. These observations suggest that hypoxia could serve as a double-edged sword: an instigator of therapeutic resistance and, if appropriately exploited, a context to therapeutically undermine antioxidant defenses.

Although antitumor activity in osteosarcoma models has recently been shown by copper-delivery platforms, direct evidence that this response is mediated through canonical cuproptosis remains limited. In other studies, metal-loaded nano-heterojunctions exacerbated the local copper burden and oxidative stress and

Metal-associated cytotoxicity in osteosarcoma

Table 1. Evidence hierarchy for metal-dependent regulated cell death and metal ion-modulated cytotoxicity relevant to osteosarcoma

Mechanism or regulatory module	Evidence source	Model type	Proposed classification	Canonical markers assessed	Functional rescue or dependency test	Main finding	Major limitations	References
Super-enhancer-driven MLX-SLC7A11 axis	Direct OS evidence	OS cells; xenografts	Ferroptosis-defense module	SLC7A11; cystine uptake; GSH; ROS	MLX knockdown; SLC7A11 rescue	MLX maintains SLC7A11-dependent redox defense	No patient-derived or metastatic validation	[7]
Exosomal miR-144-3p-ZEB1 axis	Direct OS evidence	Patient samples; OS cells; xenografts	Ferroptosis-associated regulation	Fe ²⁺ ; ROS; MDA; GSH; GPX4	miR-144-3p/ZEB1 gain- and loss-of-function	miR-144-3p targets ZEB1 and enhances ferroptosis-associated injury	Canonical inhibitor and chelator validation incomplete	[8]
Tirapazamine-SLC7A11-GPX4 axis	Direct OS evidence	Hypoxic HOS, 143B and U2OS cells	Ferroptosis sensitization	SLC7A11; GPX4; ROS; intracellular iron	SLC7A11 restoration; ferroptosis-inhibitor rescue	Tirapazamine weakens antioxidant defense under hypoxia	Limited iron-chelation and in vivo validation	[9]
EF24-HMOX1-GPX4 axis	Direct OS evidence	U2OS and Saos-2 cells	Ferroptotic component	Iron; ROS; MDA; HMOX1; GPX4	Fer-1 rescue; HMOX1 perturbation	EF24 promotes HMOX1-dependent oxidative injury	No direct C11-BODIPY or chelator rescue	[83]
Casticin-HMOX1-LC3-NCOA4 module	Direct OS evidence	Human OS cell lines	Ferritinophagy-linked ferroptosis-associated cytotoxicity	Fe ²⁺ ; ROS; HMOX1; LC3; NCOA4	HMOX1/LC3-NCOA4 perturbation; canonical rescue NR	Casticin promotes iron overload and ferritinophagy-associated injury	Apoptosis coexists; rescue and direct lipid-peroxidation evidence incomplete	[15]
p62-mediated SLC7A11 degradation by sulforaphane	Direct OS evidence	OS cells; subcutaneous and intratibial models	Mechanistically supported ferroptosis	p62; SLC7A11; GSH; lipid ROS	Fer-1; Lip-1; DFO; p62 silencing	Sulforaphane promotes autolysosomal SLC7A11 degradation	Clinical pharmacology and bone toxicity unknown	[16]
Fe(hino) ₃ -induced iron overload	Direct OS evidence	OS cells; xenograft and immunocompetent models	Dose-dependent ferroptosis- and pyroptosis-associated cell death	Lipid ROS; GPX4; NLRC4; caspase-1; GSDMD	Ferroptosis- and pyroptosis-pathway inhibition	Lower exposure favors ferroptosis; higher exposure activates pyroptosis	Mixed death pathways; recent preclinical evidence	[36]
FDX1-dependent protein lipoylation	Non-OS mechanistic evidence	Cancer cell lines; CRISPR screens	Bona fide cuproptosis mechanism (foundational non-osteosarcoma evidence)	FDX1; DLAT aggregation; Fe-S protein loss	Copper chelation; FDX1 knockout; respiration perturbation	Copper targets lipoylated TCA-cycle proteins	Foundational mechanism not validated in OS	[4]
Copper-vorinostat injectable hydrogel	OS material-based evidence	OS cells; local rat model	Copper-associated material cytotoxicity with a possible cuproptotic component	Copper burden; FDX1; lipoylated proteins; ROS	Component comparison; FDX1/protein-lipoylation dependency and copper-chelator rescue not reported	Local copper delivery enhances photothermal tumor suppression	PTT confounding; cuproptosis dependency and skeletal safety incomplete	[23]
Cuproptosis-related molecular classification	OS bioinformatic evidence	Public transcriptomic datasets	Bioinformatic association; not cell-death evidence	Cuproptosis-related gene signature	Not applicable	Associated with prognosis and immune infiltration	Does not demonstrate cuproptotic cell death	[43]
DSF/Cu-ROS-JNK axis	Direct OS evidence	OS cells; xenografts	Copper-associated apoptosis/autophagy	ROS; JNK; apoptosis; autophagy	ROS scavenging; JNK inhibition	DSF/Cu induces ROS-dependent apoptosis and autophagy	No canonical cuproptosis assessment	[92]
TRPML1-mediated lysosomal Zn ²⁺ release	Non-OS mechanistic evidence	Metastatic melanoma cells; in vivo model	Zn ²⁺ -mediated necrotic cytotoxicity	Lysosomal Zn ²⁺ ; ATP loss; mitochondrial injury	TRPML1 inhibition or silencing	Lysosomal Zn ²⁺ release causes rapid necrosis-like injury	Not validated in OS; not a consensus RCD modality	[25]
Mn ²⁺ -cGAS-STING-IFN-DHODH axis	Non-OS mechanistic evidence	Tumor cells; tumor-bearing mice	Mn ²⁺ -mediated ferroptosis sensitization	cGAS-STING; IFN-I; DHODH; mitochondrial lipid ROS	STING/IFNAR inhibition; DHODH restoration	Mn ²⁺ suppresses DHODH and lowers the ferroptosis threshold	Not validated in OS; incomplete iron-dependency assessment	[26]
ZnO-HIF-1α-BNIP3-LC3B-β-catenin axis	Direct OS material evidence	OS cells; in vivo metastasis model	ZnO-associated mitophagy and antimetastatic activity	HIF-1α; BNIP3; LC3B; β-catenin	Mitophagy/autophagy perturbation	ZnO promotes mitophagic β-catenin degradation	Not an independent RCD; nanoparticle and Zn ²⁺ effects overlap	[29]
PTBP1-SLC31A1/CTR1 module	Direct OS evidence	Patient tissues; resistant OS cells; xenografts	Platinum-transport regulation	PTBP1; SLC31A1; cisplatin uptake	PTBP1 knockdown; SLC31A1 dependency	PTBP1 reduces CTR1-mediated cisplatin uptake	Platinum pharmacology, not cuproptosis	[37]

Table notes: OS, osteosarcoma; RCD, regulated cell death; NR, not reported in the primary study; Fer-1, ferrostatin-1; Lip-1, liprostatin-1; DFO, deferoxamine; Fe-S, iron-sulfur; PTT, photothermal therapy. "Direct OS evidence" indicates experimental evidence from osteosarcoma cells, tissues or animal models and does not imply clinical validation. Ferroptosis evidence was considered stronger when functional inhibitor rescue, direct lipid-peroxidation assessment and iron dependency were evaluated. Cuproptosis evidence was considered stronger when copper dependency, FDX1/protein-lipoylation dependency, lipoylated-protein aggregation and Fe-S protein loss were assessed.

induced cuproptosis-associated molecular changes in orthotopic osteosarcoma models [10]. These results provide evidence that local copper homeostasis can be perturbed by materials but do not necessarily validate canonical cuproptosis unless Ferredoxin 1 (FDX1) dependency, lipoylated-protein aggregation, loss of iron-sulfur cluster proteins, and rescue by copper chelation are also demonstrated.

This article reviews some emerging insights into the basic biological processes of ferroptosis and cuproptosis, metal ion-modulated cytotoxic effects, dysregulation of metal metabolism in osteosarcoma, microenvironmental regulation, pathway crosstalk, and drug resistance mechanisms. The current review subsequently classifies small-molecule agents, nanomedicine-based delivery systems, local metal-active materials, and combinations with immunotherapy based on osteosarcoma specificity and the extent of mechanistic validation. We have sought to differentiate direct evidence from osteosarcoma from comparative sarcoma evidence, non-osteosarcoma evidence, bioinformatic associations, and material-based proof-of-concept studies.

Instead of considering ferroptosis and cuproptosis as segregated biological events, the current review integrates these regulated cell-death modes within the overall context of metabolic reprogramming, adaptation to hypoxia, the osteolytic microenvironment, and the impact of these factors on osteosarcoma chemotherapy resistance. Currently, ferroptosis is a comparatively well-validated therapeutic vulnerability associated with osteosarcoma based on the evidence accrued in this review, whereas cuproptosis is a more novel candidate mechanism that requires further formal validation using well-established canonical approaches. Hence, instead of being regarded as equivalent regulated cell-death modalities, other metal-associated processes should be regarded as modulators of death susceptibility or as material-mediated therapeutic platforms.

Mechanistic hierarchy of metal-dependent regulated cell death and metal ion-modulated cytotoxicity

In this section we touch upon ferroptosis and cuproptosis as mechanistically defined metal-

dependent regulated cell-death modalities, while other metal-associated processes are classified as cytotoxic modulators or material-mediated effects.

Ferroptosis

Ferroptosis is the result of iron-dependent peroxidation of membrane phospholipids exceeding cellular lipid-peroxide repair capacity. Together, expansion of the labile iron pool, increased availability of oxidizable polyunsaturated phospholipids and impairment of antioxidant defences boost the accumulation of phospholipid hydroperoxides. Phospholipid hydroperoxides accumulate because they can no longer be reduced efficiently when GPX4 activity is inhibited, when GSH is depleted, or when coenzyme Q10 (CoQH₂) cannot be regenerated. This initiates a cascade of radical reactions that propagate membrane lipid peroxidation, cause loss of membrane integrity, and then end in ferroptotic cell death [11].

In this review, aberrations of ROS, Malondialdehyde (MDA), intracellular iron, SLC7A11, or GPX4 per se are interpreted as ferroptosis-related alterations but not as conclusive evidence for ferroptotic cell death. Stronger identification should rely on several convergent observations, such as functional rescue of ferroptotic cell death by ferrostatin-1 or liprostatin-1 and suppression by the iron chelator deferoxamine (DFO), alongside direct measurement of membrane lipid peroxidation, if possible by C11-BODIPY 581/591 oxidation or oxidized-phospholipid quantification. Furthermore, measurements of labile iron or Fe²⁺, the GSH/GSSG ratio, and SLC7A11 and GPX4 protein amounts or activity support mechanistic involvement. The term “ferroptosis-associated cell death” is used instead of “ferroptosis” when pharmacological rescue and direct evidence of membrane lipid peroxidation are absent [12].

Dihydroorotate dehydrogenase (DHODH), which is localized to the inner mitochondrial membrane, provides a parallel mitochondrial defense against lipid peroxidation in cases where GPX4-based protection is insufficient. Mitochondrial DHODH maintains the CoQ/CoQH₂ redox cycle and works in concert with mitochondrial GPX4 to repress lipid peroxidation within mitochondrial membranes. DHODH inhi-

bition also sensitizes tumor cells with low GPX4 expression or impaired GPX4 function, which were found to be markers of higher sensitivity, and furthermore, combined inhibition of both enzymes strongly enhances ferroptotic cell death [13]. Thus, mitochondria serve as energetic hubs but also as critical loci for ferroptosis protection. Since these results were mostly established in non-osteosarcoma models, they point to the possibility that osteosarcoma cells primarily reliant on oxidative phosphorylation may thus depend on the DHODH-CoQH₂-GPX4 defense route. However, this hypothesis definitely needs direct validation in osteosarcoma models.

Besides the well-defined system xc⁻-GSH-GPX4 axis, cysteine metabolism can inhibit ferroptosis via noncanonical antioxidant routes. Cysteine serves as a rate-limiting substrate for the synthesis of GSH and is also a precursor for sulfane sulfur biosynthesis, therefore contributing to hydropersulfide production. Hydropersulfides effectively scavenge free radicals, attenuate lipid peroxidation, and inhibit ferroptosis [14]. Existing cellular defenses such as sulfane sulfur metabolism, the Ferroptosis suppressor protein 1 (FSP1)-CoQH₂ system, and even the GTP cyclohydrolase 1 (GCH1)-Tetrahydrobiopterin (BH4) pathway allow tumor cells to still reduce membrane lipid peroxides after inhibition of SLC7A11 or GPX4. While these additional antioxidant systems are well characterized in the broader field of ferroptosis biology, their contributions to ferroptosis resistance in osteosarcoma remain insufficiently defined. These functions therefore need to be examined in drug-resistant cell lines, organoid models, and intraosseous orthotopic models.

The ferroptosis-targeting activities of casticin and sulforaphane were also studied in osteosarcoma models; however, only some studies provide mechanistic evidence. Casticin dose-dependently elevated intracellular Fe²⁺ and ROS levels, disrupted the GSH/GSSG balance, and induced HMOX1- and nuclear receptor coactivator 4 (NCOA4)-mediated ferritinophagy while inhibiting osteosarcoma cell proliferation/metastatic phenotypes [15]. These results are consistent with ferroptosis-related cytotoxicity, but direct lipid-peroxidation measurements and broader pharmacological rescue or iron-dependency experiments are lack-

ing. On the other hand, sulforaphane enhanced p62-dependent autolysosomal degradation of SLC7A11, which was associated with increased lipid ROS and was rescued by ferrostatin-1, liproxstatin-1, and deferoxamine, concomitantly with p62 perturbation [16]. Thus, the sulforaphane study offers mechanistically supported evidence for ferroptosis (rather than simply ferroptosis-related injury).

Most of the direct evidence for ferroptosis in osteosarcoma has come from cell-line and xenograft models. The current review calls for additional studies in orthotopic tumors, pulmonary-metastasis models, drug-resistant models, and patient-derived systems to dissect ferroptosis sensitivity as a function of molecular subtype, chemotherapy response, intraosseous hypoxia, and the osteolytic microenvironment.

In the late period of ferroptosis, massive peroxidation of membrane phospholipids causes increased membrane tension and ion permeability, which activate Piezo1 and TRP channels to promote Na⁺ and Ca²⁺ influx and K⁺ efflux, leading to terminal membrane rupture [17]. These biophysical changes translate lipid peroxidation into terminal membrane injury. Such ion-flux events must be viewed as downstream consequences or amplifiers of membrane injury rather than initial mechanisms like iron accumulation or GPX4 failure. It is unclear whether this terminal phase of ferroptosis in osteosarcoma is altered by the mechanically stressed bone microenvironment.

Cuproptosis

Excess intracellular copper targets lipoylated proteins of the mitochondrial tricarboxylic acid (TCA) cycle, which initiates the cuproptosis event. Copper binding induces aggregation of lipoylated proteins, depletion of iron-sulfur cluster proteins, and mitochondrial proteotoxic stress. Of particular significance, the cytotoxic activity of the copper ionophore elesclomol is dependent on copper transport and is influenced by the metabolic state of mitochondria. Cuproptosis sensitivity is directly correlated with mitochondrial respiratory activity: in cells extensively dependent on oxidative phosphorylation, copper overload increases susceptibility to cell death, while glycolytic cells demonstrate much higher resistance [18]. Thus, cu-

apoptosis should be defined not as nonspecific metal toxicity but as a regulated form of cell death dependent on mitochondrial function.

These findings, alongside evidence that osteosarcoma appears to have a hyperactivated oxidative phosphorylation program [19], lead to the hypothesis that those models with high oxidative phosphorylation activity may be selectively sensitive to treatment with copper ionophores or copper-delivery systems. To current knowledge, however, this hypothesis has not been directly tested in patient-derived osteosarcoma models or prospectively studied in clinical cohorts. Additionally, cuproptosis should not automatically be assigned to copper-associated cytotoxicity. Robust evidence for cuproptosis should consist of the copper dependence of cell death and rescue by an appropriate copper chelator; functional dependence on FDX1 or the mitochondrial protein-lipoylation machinery; aggregation of lipoylated proteins, specifically Dihydrolipoamide S-acetyltransferase (DLAT); depletion of iron-sulfur cluster proteins; and dependence on mitochondrial respiration. The accumulation of copper, generation of reactive oxygen species (ROS), mitochondrial depolarization, or apoptosis alone is not sufficient to provide evidence for cuproptosis [4].

Expression of FDX1, TCA-cycle activity, mitochondrial respiration, and the abundance of lipoylated proteins are important determinants of sensitivity to cuproptosis. Copper stress was also found to trigger METTL16 lactylation and stabilize FDX1 mRNA through m6A-related regulation in non-osteosarcoma models, ultimately controlling FDX1-dependent cuproptosis [20]. This regulatory axis connects lactate-driven metabolism with RNA epigenetic modification and cuproptosis, offering a mechanistic rationale to explain the differential sensitivity to cuproptosis under conditions of hypoxia and metabolic reprogramming. While lactate accumulation and hypoxia are frequently observed in osteosarcoma tissues, direct evidence that enhanced METTL16 lactylation regulates FDX1 expression to modulate the cuproptosis threshold is still unavailable.

In non-osteosarcoma models, PPP1R15A also has a role in regulating translational stress and G2/M-phase arrest during cuproptosis [21]. Such results indicate that cuproptosis

sensitivity can be altered by translational stress and cell-cycle status. Nonetheless, it is still unknown whether the METTL16-FDX1 axis or PPP1R15A-related mechanisms operate in osteosarcoma cells with varying proliferative and metabolic states.

Nanomaterials containing copper have been studied as locoregional therapeutic platforms in osteosarcoma models, showing effects on both tumor growth and osteolytic damage. It has been observed that multifunctional copper-containing nanomaterials were capable of repressing osteosarcoma growth while augmenting the local copper burden, oxidative stress, and cuproptosis-associated molecular changes [22]. These observations should be interpreted as indicative of copper-related cytotoxicity with a potential cuproptotic element until demonstrated through copper-chelator rescue, FDX1 dependence, lipoylated-protein aggregation, and loss or depletion of iron-sulfur cluster proteins. Besides their direct actions on tumor cells, copper-based interventions with a potential cuproptotic component may also affect the remodeling of the tumor-bone interface.

A hyaluronic acid-L-cysteine hydrogel co-delivering copper ions and vorinostat enhanced photothermal therapy in an osteosarcoma model. Changes in lipoylated TCA-cycle proteins were detected following treatment with this platform, while vorinostat enhanced FDX1 expression, therefore promoting increased sensitivity to copper-related cytotoxicity, likely with a cuproptotic component [23]. This therapeutic platform encompasses concepts of local delivery, epigenetic regulation, and copper-associated cytotoxicity. However, causal dependence on the FDX1-protein-lipoylation pathway, intraosseous copper-release kinetics, and long-term skeletal safety remain unproven.

A copper-delivery platform improved the response to both radiotherapy and PD-1 blockade in osteosarcoma models, accompanied by cuproptosis-related and immunogenic cell-death signals [24]. Nonetheless, the specific contributions of canonical cuproptosis, non-specific oxidative injury, radiotherapy, and immune activation still need to be clarified. However, regarding the functional link between cuproptosis and the tumor immune microenvi-

ronment, mechanistic studies are required to understand how these effects may relate to antigen release, exposure of damage-associated molecular patterns (DAMPs), and immune-cell recruitment. They also allow us to test whether immunosuppressive or radioresistant osteosarcoma models are specifically sensitive to combinatorial copper delivery, radiotherapy, and immune modulation, though such preferential sensitivity has not yet been established.

Cuproptosis lays the groundwork for mechanistic exploration of mitochondrial metabolic vulnerabilities, copper-delivery systems, and immunotherapy combinations in osteosarcoma. Despite this, osteosarcoma-specific evidence is still in relative preclinical infancy and based largely on studies of copper-containing materials, transcriptomic associations, and assays utilizing incomplete panels of canonical markers. Therefore, copper-associated tumor suppression should not be categorized as verified cuproptosis until dependence on the FDX1-protein-lipoylation pathway and other canonical features is demonstrated.

Potential candidate variables to investigate in the future include copper uptake capacity, FDX1 expression, TCA-cycle activity, abundance of lipoylated proteins, and markers of immunogenic cell death (ICD). Subsequent studies should integrate these indicators with molecular subtype, hypoxic status, the degree of bone destruction, and response to chemotherapy to form testable hypotheses regarding differential sensitivity to copper-targeted therapies.

Other metal ion-modulated cytotoxic processes

Indeed, zinc, manganese, calcium, and selenium can modulate tumor-cell death by perturbing organelle homeostasis, redox balance, calcium signaling, and/or immunogenic signaling. In contrast to ferroptosis and cuproptosis, however, these processes lack a specific execution mechanism that has been independently validated in studies. Thus, they are not categorized here as independent regulated cell-death modalities, but rather discussed in the context of metal ion-modulated cytotoxicity or as sensitizers of established cell-death pathways.

Activation of TRPML1 in metastatic melanoma models promotes lysosomal Zn^{2+} release, mi-

tochondrial dysfunction, ATP depletion, and necrosis-like cell death [25]. Zinc homeostasis under physiological conditions maintains enzymatic activity and signal transduction, while disruption of lysosomal zinc compartmentalization leads to mitochondrial dysfunction and bioenergetic stress. As this mechanism has not been shown in osteosarcoma, it is presented as mechanistic data from non-osteosarcoma models and is not claimed to represent a unique zinc-dependent regulated cell-death modality.

$MnCl_2$ has been shown to inhibit mitochondrial DHODH, enhance mitochondrial ROS production and lipid peroxidation, and lower the threshold for ferroptosis in non-osteosarcoma tumor models through a type I interferon-dependent mechanism [26]. With this background, Mn^{2+} is a mitochondrial redox modulator and ferroptosis sensitizer rather than an initiator of an independent cell-death modality. In a similar approach, prolonged Ca^{2+} release in non-osteosarcoma models caused endoplasmic-reticulum stress, mitochondrial Ca^{2+} overload, and loss of mitochondrial membrane potential, accompanied by apoptosis- or necrosis-like cell death [27].

Hence, Ca^{2+} overload should be considered an amplifier of cell death rather than a distinct regulated cell-death modality or pathway. Zinc complexes induced mitochondrial Ca^{2+} overload, endoplasmic-reticulum stress, and ROS generation in non-osteosarcoma tumor models. These effects could facilitate the release or exposure of damage-associated signals characteristic of immunogenic cell death [28]. Further, in osteosarcoma models, it was shown that ZnO nanoparticles released Zn^{2+} and inhibited metastatic phenotypes via Hypoxia-inducible factor-1 (HIF-1 α)/BNIP3/LC3B-mediated mitophagy and inhibition of β -catenin signaling [29]. This study provides direct osteosarcoma evidence of zinc-associated mitochondrial and signaling perturbations, but does not demonstrate a specific zinc-dependent regulated cell-death modality.

Manganese-containing hybrid nanozymes have also been reported to produce hydroxyl radicals through Fenton-like reactions and inhibit osteosarcoma growth in experimental models [30].

Metal-associated cytotoxicity in osteosarcoma

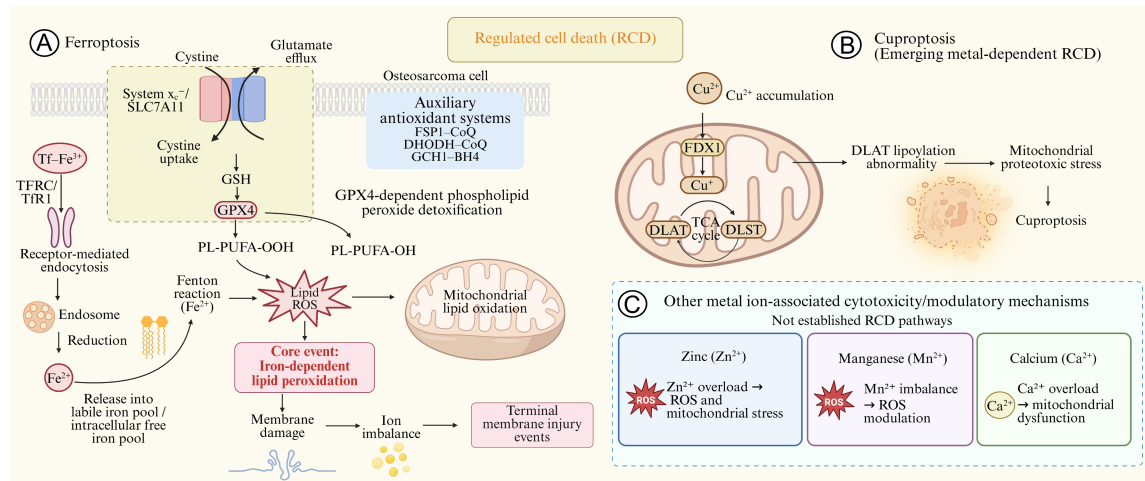


Figure 1. Mechanistic hierarchy of ferroptosis, cuproptosis, and other metal ion-associated cytotoxicity in osteosarcoma. A. Ferroptosis is centered on iron-dependent lipid peroxidation and is regulated mainly by the SLC7A11-GSH-GPX4 axis together with auxiliary antioxidant systems. Progressive lipid damage leads to membrane dysfunction, ion imbalance, and terminal membrane injury. B. Cuproptosis involves intracellular copper accumulation, FDX1-associated reduction of Cu²⁺ to Cu⁺, interaction with lipoylated TCA-cycle proteins, and mitochondrial proteotoxic stress. C. Zn²⁺, Mn²⁺, and Ca²⁺-associated processes represent metal ion-modulated cytotoxic or regulatory mechanisms and are not established as independent regulated cell-death pathways. Abbreviations: RCD, regulated cell death; GSH, glutathione; GPX4, glutathione peroxidase 4; FSP1, ferroptosis suppressor protein 1; DHODH, dihydroorotate dehydrogenase; BH4, tetrahydrobiopterin; TCA, tricarboxylic acid; ROS, reactive oxygen species.

In the absence of evidence showing functional reliance on a given regulated cell-death pathway, such effects should be considered material-mediated oxidative cytotoxicity.

In summary, metals other than iron and copper primarily modulate ROS generation (oxidative stress), organelle injury such as mitochondrial and endoplasmic-reticulum injury, calcium homeostasis disruption, and immunogenic signal dysregulation. Whether these ions induce lysosomal, mitochondrial, or endoplasmic-reticulum injury directly or serve as sensitizers of ferroptosis, cuproptosis, apoptosis, and immunogenic cell death should be determined in future studies in osteosarcoma. Those studies should include pathway-specific rescue experiments, kinetics of ion release, subcellular localization, cell-type selectivity testing, and validation in the hypoxic and osteolytic intraosseous environment.

Thus, such processes should not be considered independent metal-dependent regulated cell-death modalities but rather types of metal ion-modulated cytotoxicity. The mechanistic hierarchy distinguishing ferroptosis and cuproptosis from broader metal ion-associated cyto-

toxic or modulatory mechanisms is summarized in **Figure 1**.

Aberrant metal metabolism and cell-death sensitivity in osteosarcoma

Iron metabolism and osteosarcoma

Dysregulated iron homeostasis in osteosarcoma involves more than excess iron accumulation but rather reflects an orchestrated restructuring of iron uptake, storage, release, export, and redox utilization. Important elements of this network are transferrin receptor 1 (TFRC), divalent metal transporter 1 (DMT1/SLC11A2), ferroportin (FPN/SLC40A1), ferritin-mediated iron storage, ferritinophagy, and the use of redox-active iron. Perhaps, osteosarcoma cells control the labile iron pool to maintain proliferation, mitochondrial metabolism, and redox homeostasis [31]. Dysregulated iron metabolism not only provides the substrate foundation for ferroptosis but also acts as a regulatory framework for tumor-cell proliferation, invasion, and therapeutic response. Notably, iron dependence for the proliferation of tumor cells does not necessarily coincide with iron dependence for ferroptotic cell death.

In osteosarcoma, one indicated feature of iron-metabolic remodelling is increased TFRC-mediated iron uptake. Mechanistically, upregulated TFRC enhances cellular iron availability and the expression or activity of RRM2 to promote osteosarcoma cell proliferation, migration, and invasion. TFRC silencing causes an arrest in cell proliferation, which is partially restored by iron supplementation or RRM2 overexpression [32]. These findings are compatible with an iron-dependent proliferative phenotype linked to nucleotide synthesis. They do not, per se, establish increased sensitivity to ferroptosis.

Some iron chelators, such as deferoxamine and deferasirox, interfere with intracellular iron homeostasis to inhibit osteosarcoma cell proliferation and may promote apoptosis through ROS-MAPK signaling [33]. As iron chelation decreases the availability of iron required for lipid peroxidation, such cytotoxicity may not be referred to as ferroptosis unless independently validated by functional evidence.

The ratio of ferritin-directed iron storage to ferritinophagic iron release regulates the labile iron pool and consequently modulates sensitivity to ferroptosis. Comparative evidence from dogs with osteoblastic osteosarcoma lends support to this hypothesis through identification of altered FTH1-NCOA4-associated ferritin turnover [34]. These data represent comparative canine osteosarcoma evidence rather than direct evidence from human osteosarcoma. In human osteosarcoma models, high expression of TFRC/TfR1 and DMT1 is associated with NCOA4-mediated ferritinophagy followed by iron release, ROS accumulation, and lipid peroxidation [35]. Ferritin should therefore not be regarded as an inert iron-storage structure, since its autophagic degradation can enlarge the pool of readily accessible iron and alter the threshold of oxidative-stress tolerance in osteosarcoma cells.

Intervention strategies aimed at modulating iron metabolism may also switch the predominant mode of cell death. Literature has demonstrated that $\text{Fe}(\text{hino})_3$ triggers ferroptosis-associated alterations in osteosarcoma cells and, at high concentrations, activates the NLRC4/caspase-1/GSDMD pyroptotic pathway [36]. These results suggest that different cell-death programs are engaged in a dose-dependent

manner. As a result, ferroptosis and pyroptosis must be independently validated using pathway-specific rescue experiments rather than being inferred solely from shared markers of oxidative stress or membrane injury. Therefore, the response to iron overload may depend on dose, activating different death pathways rather than producing a uniform ferroptotic response.

Future studies should encompass TFRC/RRM2-mediated iron uptake, FTH1-NCOA4-mediated ferritinophagy, and FPN-mediated iron efflux in a coherent analytical context. This framework should subsequently be linked with chemotherapy response, hypoxic status, and the extent of osteolytic destruction to delineate subgroups in osteosarcoma that are dependent on iron-metabolic remodeling.

Copper metabolism and osteosarcoma

Copper metabolism in osteosarcoma may involve abnormal copper acquisition, altered trafficking into and out of tumor cells, increased efflux, and modified mitochondrial utilization. While both copper and cisplatin transport are important to osteosarcoma biology, neither should be considered evidence of cuproptosis. Cisplatin enters tumor cells via CTR1, encoded by SLC31A1, which mediates cellular copper uptake. PTBP1 can inhibit the expression of SLC31A1 and decrease cisplatin uptake to promote cisplatin tolerance in osteosarcoma cells [37]. Note that this mechanism relates specifically to platinum transport and chemoresistance and should not be understood as indicating evasion of cuproptosis in cisplatin-resistant cells. Although downregulation of CTR1 may help to explain inadequate intracellular levels of platinum agents, clinical drug resistance is generally multifactorial and involves additional mechanisms such as increased drug efflux, enhanced DNA-damage repair, and evasion of cell death.

Also, p53 has been reported to limit CTR1-mediated cisplatin uptake by inhibiting SP1 nuclear translocation [38]. Based on the results, TP53 status might influence CTR1-dependent cisplatin transport through SP1. Therefore, this association should not be interpreted as direct regulation of cuproptosis, but rather through the lens of platinum pharmacology.

Chaperone-mediated trafficking governs the distribution of intracellular copper and is not an unrestricted diffusion process. Inhibition of ATOX1 and CCS sensitized human and canine osteosarcoma cells to treatment with platinum-based therapies [39], while increased ATOX1 expression and altered copper content have been detected in canine osteosarcoma specimens [40]. These results are consistent with a role for copper trafficking in platinum pharmacology, but do not prove cuproptosis. Canine osteosarcoma evidence should be classified as comparative canine osteosarcoma evidence and requires validation in larger human cohorts, ideally with paired pre- and post-treatment specimens, before ATOX1 can serve as a predictive biomarker of platinum sensitivity.

Dysregulated copper metabolism might also play a role in remodeling the immune state and the prognostic heterogeneity of osteosarcoma. Public transcriptomic analyses have reported associations between expression levels of copper metabolism-related genes, patient prognosis, and immune-cell infiltration in osteosarcoma datasets [41, 42]. Other cuproptosis-related gene-based classifications have also been linked with prognosis, immune infiltration, and predicted drug sensitivity [43]. PLCD3-based models have also been correlated with immune-cell infiltration and predicted therapeutic response [44]. These results may enable the generation of new hypotheses and tools for biomarker development, but do not prove intracellular copper overload, FDX1 dependency, DLAT aggregation, aberrant lipoylation of mitochondrial proteins, cuproptotic cell death, or a causal role for copper homeostasis in tumor progression or therapeutic resistance.

Next steps will involve the simultaneous measurement of Copper transporter 1 (CTR1)/SLC31A1, ATOX1, ATPase copper transporting alpha/beta (ATP7A/ATP7B, FDX1), and DLAT in patient cohorts, together with assessment of copper content, transporter localization, mitochondrial protein lipoylation, and response to platinum-based therapy. It is important that such analyses also consider hypoxic status, the intraosseous microenvironment, and drug-resistant phenotypes to stratify candidate osteosarcoma subgroups for prospective evaluation of copper-targeted or cuproptosis-oriented therapeutics.

Other metal ions and osteosarcoma

In addition to iron and copper, other metal ions such as zinc, manganese, calcium, and magnesium and metalloids such as selenium may play roles in modulating oxidative stress, Ca^{2+} homeostasis, mitochondrial function, and remodeling at the tumor-bone interface in osteosarcoma. In contrast to ferroptosis and cuproptosis, these processes are not classified under a common execution mechanism and can be more accurately characterized as modulators of oxidative injury, organelle function, cell-death susceptibility, or remodeling of the bone microenvironment. Research on zinc and manganese metabolism has been mainly based on pan-cancer models, focusing predominantly on changes in metalloenzyme activity, antioxidant defenses, and immune-cell function. These studies yield valuable mechanistic reference points to shed light on how disrupted zinc or manganese homeostasis could modulate ROS thresholds and immune responses in osteosarcoma [45].

Rotenone activated ROS/ Ca^{2+} /AMPK signaling and changed the expression and subcellular localization of ZO-2 in osteosarcoma cells, thereby inhibiting migratory and invasive phenotypes [46]. The present study supports a role for redox and calcium signalling in regulating osteosarcoma behaviour but does not establish Ca^{2+} overload as an independent cell-death modality. Rather, elevated ROS production, changes in intracellular Ca^{2+} , and energetic stress may change the junctional architecture and consequently affect the migration of osteosarcoma cells.

Selenium-containing mesoporous silica nanoparticles induced oxidative stress and cell death in osteosarcoma cells, but were less cytotoxic to the normal-cell controls analysed [47]. Thereafter, mesoporous silica nanoparticles co-loaded with doxorubicin and selenium were designed to achieve both chemotherapeutic cytotoxicity and selenium-mediated oxidative injury [48]. These studies are proof of principle for postoperative local treatment with materials rather than evidence for selenium-dependent regulated cell death and should be regarded as material-mediated cytotoxicity. However, there are still many unknowns regarding the redox activity of selenium and its potential for biomaterial functionalization, specifically in terms of selenium-release kinetics, local tissue distribution, and long-term safety.

As postoperative management of osteosarcoma might demand simultaneous eradication of residual tumor cells and repair of bone defects, multi-element biomaterials may offer relevant platforms for local therapy. Selenium-doped TiO₂ nanotube arrays may endow orthopedic implants with enhanced function through suppression of residual osteosarcoma cells while maintaining acceptable biocompatibility [49]. In a similar context, selenium nanoparticle/MgFe-layered double hydroxide composite nanosheets could address these challenges by integrating antitumor, antibacterial, and osteogenic functions to reduce residual tumor burden, infection risk, and impaired bone repair during local treatment [50].

Other examples include the utilization of magnesium-calcium mineralized collagen materials to inhibit osteosarcoma cell invasion and metastasis-associated phenotypes. Moreover, besides supporting bone repair, Mg²⁺ and Ca²⁺ could also modulate tumor-cell migration by locally altering the ionic microenvironment, extracellular-matrix properties, and the osteoblast-osteoclast balance at the tumor-bone interface [51].

Osteosarcoma-specific and material-based evidence to date suggests that metals and metal-oids other than iron and copper may influence tumor behavior and therapeutic response via ROS accumulation, Ca²⁺ dysregulation, mitochondrial injury, immunogenic signaling, and modification of the bone-repair microenvironment. Mechanistic studies suggest that these interventions could directly kill tumor cells, sensitize them to established cell-death pathways, or indirectly enhance therapeutic efficacy by remodeling the local bone microenvironment.

Thus, future studies should complement tumor growth inhibition measurements with a systematic characterization of ion-release kinetics, tissue distribution, subcellular localization, the main responsive cell populations, and the final mode of cell death. Since osteosarcoma involves malignant-cell proliferation and osteolytic destruction, evaluative frameworks should also include the assessment of newly formed bone architecture, biomechanical properties, long-term safety, and the risk of local recurrence.

Cell-type-specific therapeutic window of metal-associated cell death in the osteosarcoma microenvironment

Importantly, the therapeutic effects of ferroptosis- or cuproptosis-based therapies critically depend on the cellular compartment in which metal-loaded and redox stress is activated. While this may trigger antitumor effects through the oxidative or copper-associated injury of osteosarcoma cells, similar stress in CD8⁺ T cells, myeloid cells, osteoblasts, osteocytes, and/or osteoclasts may inhibit antitumor immunity and compromise bone homeostasis. Thus, the question is not only whether the lethal effects of metals associated with cell death could be induced but also whether these effects could be selectively confined to malignancies in ways that preserve immune-cell function and bone-resident cell populations.

Osteosarcoma cells as the intended therapeutic target

Ferroptosis- and cuproptosis-oriented agents target osteosarcoma cells. Direct osteosarcoma evidence suggests that reducing tumor-cell availability of SLC7A11-dependent cystine uptake, GSH accessibility, GPX4-mediated lipid-peroxide repair or iron handling can diminish tumor-cell survival and augment ferroptosis-associated cellular injury [7, 35, 36]. However, the ability to classify ferroptosis can vary between studies because direct assays for membrane lipid peroxidation, iron-chelation rescue and canonical inhibitor rescue have not always been evaluated concurrently.

Additionally, copper-loaded materials and copper-delivery systems have inhibited the growth of osteosarcoma cells whilst also eliciting cuproptosis-associated molecular alterations [22-24]. These findings support copper-associated tumor-cell cytotoxicity but do not establish canonical cuproptosis without FDX1 or protein-lipoylation dependency, aggregation of lipoylated proteins, loss of iron-sulfur cluster proteins, mitochondrial respiratory dependence, and rescue by copper chelation. Thus, the expected benefit within the tumor-cell compartment is direct malignant-cell killing, whereas the principal uncertainty is whether this effect can be achieved without comparable injury to immune and bone-resident cells.

Cell-type-specific effects on immune cells

Dendritic cells and immune priming: The tumor microenvironment (TME) in osteosarcoma is largely considered to be immunosuppressive. Single-cell atlases have demonstrated an abundance of myeloid cells, T cells, and dendritic cells in the osteosarcoma TME, including a population of mature regulatory dendritic cells (mregDCs) defined by the CCR7⁺LAMP3⁺CD83⁺ phenotype. These mregDCs are correlated with the recruitment of Tregs and can also demonstrate an immunoregulatory phenotype characterized by upregulated expression of immune regulatory or checkpoint molecules. This accumulation is not necessarily indicative of intrinsic deficiencies in antigen presentation, but can lead to suboptimal T-cell priming via immunoregulation and interactions with Tregs. Concurrently, downregulated major histocompatibility complex class I (MHC-I) expression by tumor cells may diminish proper antigen recognition by cytotoxic T cells, while upregulation of CD24-mediated antiphagocytic signaling can decrease macrophage-dependent tumor-cell clearance [52].

Therefore, DAMPs released from ferroptotic or copper-stressed osteosarcoma cells might not result in significant antitumor immunity. Phagocytic clearance, antigen uptake and processing, dendritic-cell maturation and migration, the regulatory state of dendritic cells, and downstream T-cell priming within the osteosarcoma microenvironment all dictate their immunological consequences.

CD8⁺ T-cell ferroptosis and impaired antitumor immunity: In non-osteosarcoma tumor models, oxidized lipids were shown to be taken up by CD8⁺ T cells via CD36, thereby facilitating lipid peroxidation, resulting in diminished cytokine production and cytolytic activity [53, 54]. If these ferroptosis-inducing strategies allow lipid peroxidation to extend into CD8⁺ T cells, they may hinder T-cell-mediated antitumor immunity. In models without osteosarcoma, PD-1 signaling has similarly been associated with increased ferroptosis in CD8⁺ T cells via PLPP1-dependent regulation. It remains to be seen whether this mechanism is operative in osteosarcoma.

These observations suggest a potential safety concern: global promotion of lipid peroxidation

in the osteosarcoma microenvironment could eliminate osteosarcoma cells but impair T-cell antitumor function. Accordingly, the best approach will ideally induce ferroptosis-related damage only in osteosarcoma cells while preserving cytokine production and cytotoxic activity as well as antioxidant defense in CD8⁺ T cells. At present, this cell-type-specific therapeutic window is hypothetical due to a lack of direct cell-resolved evidence in osteosarcoma.

Myeloid cells, TAMs, and MDSCs: Ferroptosis of intratumoral PMN-Myeloid-derived suppressor cells (MDSCs) releases oxidized lipids that induce local immunosuppressive effects. This process can decrease the abundance of Polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs); however, the oxidized lipids then released may inhibit T-cell effector function, leading to a local environment of continued immunosuppression [55]. In other words, if MDSCs or neutrophil-like cells in the osteosarcoma microenvironment are sensitive to ferroptosis induction and oxidized lipids inhibit T-cell function, this could partially offset the therapeutic benefit. Direct validation of this possibility in osteosarcoma models is now warranted.

Tumor-associated macrophages (TAMs) could also regulate the susceptibility of tumor cells to ferroptosis. M2-polarized macrophage-derived exosomal APOC1 induces ACSF2 degradation and strengthens the resistance of osteosarcoma cells to ferroptosis [56]. This observation indicates that TAMs may shield tumor cells from the lethal effects of lipid peroxidation, even when the macrophages themselves are not undergoing ferroptosis. Consequently, the effects of metal-targeted interventions on both TAM polarization and exosome-mediated signaling, in conjunction with the susceptibility of tumor cells to ferroptosis, should be evaluated as independent yet interacting processes.

Hypoxia-dependent modulation of tumor-cell sensitivity

Hypoxia is proposed here as a permissive factor in osteosarcoma cell sensitivity, not an independent mechanism of cell death. Intraosseous osteosarcoma lesions are often defined by impaired perfusion, diffusion-limited oxygen delivery, and metabolic acidification that drive chronic hypoxia-inducible factor (HIF) signaling.

HIF signaling reshapes other essential metabolic processes including glycolysis, mitochondrial metabolism, and redox defenses and modulates iron and copper homeostasis. Its effects on metal-dependent cell death are not unidirectional and are contingent on tumor-cell metabolic state, patterns of iron and copper utilization, and antioxidant capacity.

HIF-2 α activity has been linked to increased intracellular iron levels and oxidative vulnerability in colorectal cancer models [57]. However, this finding, which is based on non-osteosarcoma models, gives a mechanistic rationale for testing whether hypoxia alters iron uptake and storage as well as labile iron availability in osteosarcoma.

Hydroxysafflor yellow A suppresses glycolytic metabolism and promotes ferroptosis by inhibiting the HIF-1 α /HK2/SLC7A11 pathway in human osteosarcoma cells [58]. This conclusion would be further bolstered by direct measurements of membrane lipid peroxidation and ferroptosis-specific rescue. These observations imply that HIF-1 α -dependent glycolytic adaptation sustains SLC7A11-mediated cystine uptake and GSH synthesis to maintain a higher barrier against ferroptosis.

Additionally, shikonin has been shown to induce osteosarcoma cell ferroptosis-associated oxidative injury by promoting mitochondrial ROS generation and regulating the HIF-1 α /HO-1-related pathway [59]. Prolonged generation of mitochondrial ROS along with HO-1-associated iron release could exceed antioxidant-buffering capacity and drive cell death associated with lipid peroxidation. However, thorough proof of membrane lipid peroxidation and functional rescue by standard ferroptosis inhibitors is needed for definitive classification as ferroptosis.

Hypoxic osteosarcoma is frequently a problem for photodynamic therapy because ROS production is restricted by limited oxygen availability and oxygen consumption during treatment. Capsaicin and photodynamic therapy together offered more potent tumor suppression with activation of TRPV1/Ca²⁺ signaling, down-regulation of HIF-1 α expression, and promotion of ferroptosis-associated oxidative injury in osteosarcoma models [60]. Nevertheless, pathway-specific validation should be conducted

to dissect the respective contributions of photodynamic cytotoxicity and ferroptosis.

HIF-1 α signaling has been implicated in non-osteosarcoma models via PDK1/3-mediated repression of DLAT and increased metallothionein-dependent copper buffering [61]. This raises the hypothesis that regions of osteosarcoma afflicted by hypoxia may acquire cuproptosis resistance through reduced mitochondrial protein lipoylation and increased copper detoxification. But direct evidence in osteosarcoma is lacking. The hypothesized role of DLAT in cuproptosis resistance needs to be tested directly, while SLC7A11 upregulation associated with hypoxia may enhance ferroptosis resistance.

Future studies utilizing intraosseous orthotopic models and patient specimens should assess HIF-1 α , HK2, SLC7A11, HO-1, DLAT, PDK1/3, and metallothioneins in relation to measurements of oxygen tension, iron and copper content, lipid peroxidation, and mitochondrial protein lipoylation. It is concluded that HIF inhibition, hypoxia-activated prodrugs, metal-delivery strategies, and photodynamic therapy should be studied as context-dependent combinations rather than broadly effective treatments.

Effects on bone-resident cells and the osteolytic microenvironment

Osteosarcoma arises within a mineralized bone matrix and harbors tumor and immune cells, as well as osteoclasts, osteoblasts, osteocytes, components of the mineralized matrix, and local ionic gradients. Osteosarcoma cells interfere with homeostatic bone remodeling at the tumor-bone interface, resulting in increased osteoclast-mediated bone resorption and inhibited new bone formation. Thus, the relevance of metal-associated cell death goes beyond direct tumor-cell killing and is also implicated in bone resorption, osteogenic repair potential, and reconstruction of postoperative bone defects.

Osteoclasts and osteolytic remodeling: The osteolytic context of osteosarcoma is defined by the following osteoclast-related mechanisms, which themselves should not be construed as evidence of ferroptosis or cuproptosis. Osteosarcoma-derived small extracellular

vesicles (sEVs) mediate transfer of miR-19a-3p into osteoclast precursors, thereby promoting migration and differentiation as well as bone-resorptive activity [62]. In contrast, sEVs harboring miR-146a-5p inhibit NF- κ B signaling activity and osteoclast maturation as well as increase angiogenesis and distant metastasis [63]. Single-cell studies have also identified an MKI67⁺ osteoclast subpopulation, with PPARG implicated in osteoclast activation and bone-matrix degradation [64]. This highlights a need to assess osteoclast differentiation, resorptive activity and structural characteristics at the tumor-bone interface when evaluating metal-associated cell-death interventions.

Osteocytes: Research in osteoporosis mouse models supports a role for ferroptosis of osteocytes in promoting RANKL-dependent osteoclastogenesis and contributing to bone loss [65]. Although such a mechanism has not been shown in osteosarcoma, this raises a safety concern that nonselective ferroptosis-inducing strategies could cause damage to normal osteocytes, increase osteoclast activation, and exacerbate osteolytic destruction. Thus, in intraosseous osteosarcoma models, monitoring of osteocyte survival and RANKL-related signaling needs to be considered separately from tumor-cell killing.

Osteoblast-lineage cells: Bone-forming, post-operative regenerative, and reconstructive-material integration activities involve osteoblast-lineage cells. Direct evidence for ferroptosis or cuproptosis in osteosarcoma-associated osteoblasts has, to date, been limited, but it remains plausible that even nonselective metal accumulation or oxidative stress could negatively affect osteoblast viability, differentiation and mineralization. In light of these considerations, antitumor efficacy should be assessed together with osteoblast survival, osteogenic differentiation, new-bone mineralization, and the biomechanical quality of regenerated bone.

Therapeutic window and cellular selectivity

Most importantly, the therapeutic window of metal-associated cell-death strategies relies on selectively killing tumor cells while sparing antitumor immune function and normal bone-resident cells. Studies in other types of tumor models and osteoporosis models have shown that ferroptotic stress impairs the function of

tumor-infiltrating CD8⁺ T cells, that immunosuppressive oxidized lipids are released from ferroptotic PMN-MDSCs, and that ferroptosis within the osteogenic lineage can lead to bone loss [54, 55, 66]. Consequently, in future studies of osteosarcoma, ferroptosis- and cuproptosis-related changes should be examined separately in tumor cells, CD8⁺ T cells, TAMs, MDSCs, osteoblasts, osteocytes, and osteoclasts. Measurements of ROS, lipid peroxidation, or metal content in whole tumors cannot reveal the cellular compartments in which these changes happen. Only cell-type-resolved analyses will allow differentiation between true antitumor efficacy and treatment-induced immune dysfunction or aggravation of osteolytic destruction. **Figure 2** summarizes how the osteosarcoma-specific microenvironment shapes the therapeutic window of metal-dependent cell death by influencing tumor-cell sensitivity, immune-cell safety, and bone-cell homeostasis.

Pathway crosstalk between ferroptosis and cuproptosis and mechanisms of therapeutic resistance

Molecular nodes linking ferroptosis and cuproptosis

Ferroptosis and cuproptosis are characterized by different terminal mechanisms. Ferroptosis is induced by iron-dependent phospholipid peroxidation and the consequent loss of membrane integrity, while cuproptosis involves copper binding to lipoylated tricarboxylic acid (TCA)-cycle proteins, aggregation of lipoylated proteins, loss of iron-sulfur cluster proteins, and mitochondrial proteotoxic stress. A common terminal execution mechanism has still not been established [4, 11]. However, the two processes may converge through metal handling, GSH metabolism, mitochondrial oxidative metabolism and protein lipoylation. In pre-clinical liver cancer models, for instance, ferroptosis inducers such as sorafenib and erastin have been shown to enhance copper-iono-phore-induced cuproptosis through the stabilization of FDX1, increases in protein lipoylation and copper-dependent aggregation of lipoylated proteins, and inhibition of GSH synthesis. As this experimental evidence was obtained in non-osteosarcoma model systems, the molecular links that are mentioned in this section should be regarded as mechanistically relevant

Metal-associated cytotoxicity in osteosarcoma

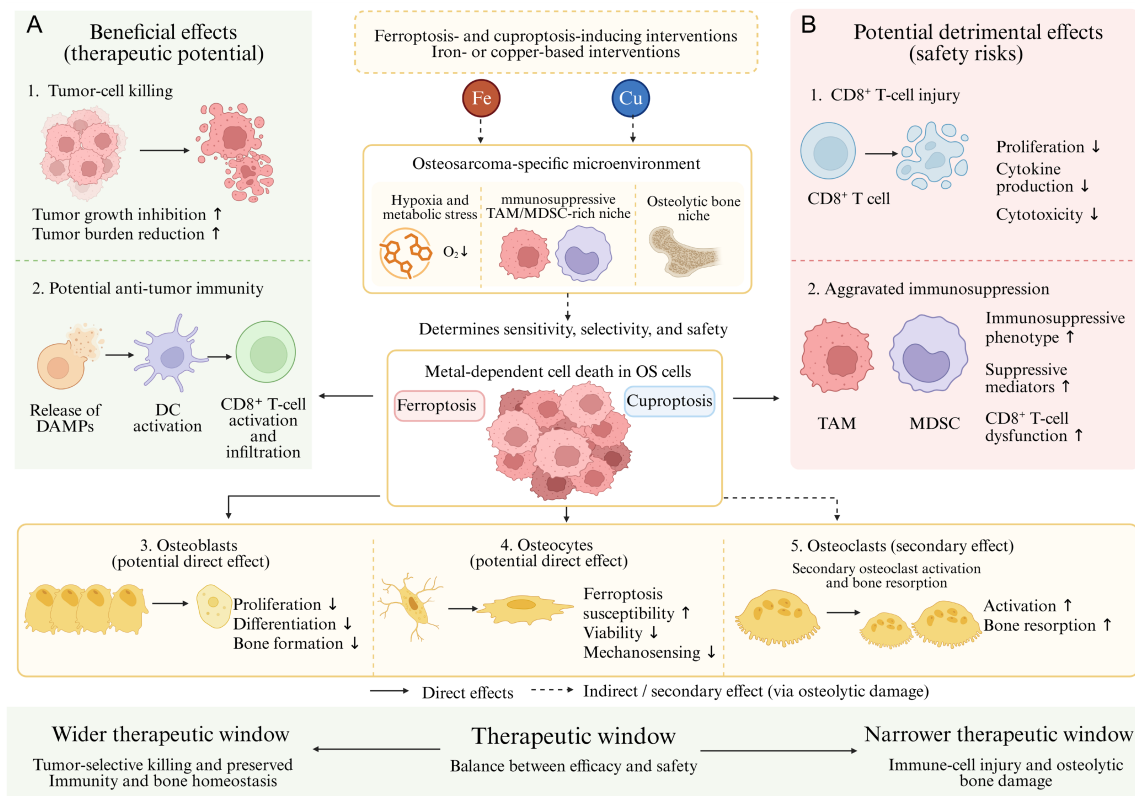


Figure 2. The osteosarcoma microenvironment determines the therapeutic window of metal-dependent cell death. Ferroptosis- or cuproptosis-inducing, and iron- or copper-based interventions could have promising antitumor efficacy through direct osteosarcoma cell killing and by possibly enhancing antitumor immunity via damage-associated molecular pattern (DAMP) release, dendritic-cell activation, and CD8⁺ T-cell recruitment. Yet, these interventions may inflict adverse effects as well by impairing CD8⁺ T cells, aggravating immunosuppression orchestrated by TAMs and MDSCs, compromising osteoblast and osteocyte function, and secondarily promoting the activation of osteoclasts leading to bone resorption. Ultimately, the balance between efficacy and off-target toxicity within the hypoxic, immunosuppressive and osteolytic osteosarcoma microenvironment determines whether the therapeutic window is wider or narrower. Abbreviations: OS, osteosarcoma; TAM, tumor-associated macrophage; MDSC, myeloid-derived suppressor cell; DAMPs, damage-associated molecular patterns; DC, dendritic cell.

hypotheses and not as established osteosarcoma-specific crosstalk mechanisms [66].

One possibility for the crosstalk is that excess copper impacts GPX4 stability. Copper also binds cysteine residues in GPX4 and leads to GPX4 aggregation and ubiquitination, in turn leading to TAX1BP1-mediated autophagic degradation of GPX4 with a reduction in lipid-peroxide clearance capacity, thereby highlighting the role of copper as an enhancer of ferroptosis [67]. Thus, ferroptosis and copper-associated cell-death pathways are not fully independent. Rather, perturbations in iron and copper homeostasis may converge on antioxidative regulatory hubs like GPX4 that subsequently influence cellular susceptibility to oxidative damage. Importantly, this mechanism was es-

tablished in contexts other than osteosarcoma and has not been functionally validated in osteosarcoma models.

Data from non-osteosarcoma models also indicate that intracellular copper overload due to impaired copper efflux may compromise the SLC7A11-GSH-GPX4 antioxidant axis. Elesclomol and its copper complex induce the degradation of ATP7A to promote mitochondrial copper retention and ROS generation in colorectal cancer models, while also promoting SLC7A11 degradation and ferroptosis [68]. Thus, impaired copper efflux may influence not only copper-associated cytotoxicity but also sensitivity to ferroptosis through disruption of the SLC7A11-GSH-GPX4 axis. An analogous mechanism of dysregulated copper transport may similarly

alter ferroptosis susceptibility in osteosarcoma; however, the causal relationships among ATP7A, SLC7A11, and lipid peroxidation remain to be validated directly in osteosarcoma cells and orthotopic models.

GPX4-dependent detoxification of phospholipid hydroperoxides may be compromised by GSH depletion, and the intracellular copper-buffering capacity may also be weakened. The availability of GSH in cells allows it to bind intracellular copper ions and thus limits their interaction with lipoylated proteins. The ferroptosis inducers sorafenib and erastin, which reduce cystine uptake, deplete GSH and stabilize FDX1 in hepatocellular carcinoma models, may enhance copper-ionophore-induced cuproptosis and increase protein lipoylation [66]. Together, these findings suggest that the SLC7A11-GSH axis may influence both lipid-peroxide detoxification and intracellular copper buffering. It is not clear if such a shared metabolic interface works in osteosarcoma.

Similarly, GSH utilization during disulfiram/copper treatment may also promote lipid peroxidation and copper-associated cytotoxicity. In non-osteosarcoma models, disulfiram/copper depleted GSH and induced ferroptosis- and cuproptosis-associated changes, whereas compensatory upregulation of xCT restored cystine uptake. With inhibition of xCT, intracellular GSH replenishment was blocked, while lipid peroxidation and copper-associated cytotoxicity were further aggravated [69]. These results suggest that osteosarcoma cells with high SLC7A11 expression, or those undergoing therapy-induced antioxidant compensation, may require simultaneous disruption of cystine uptake and copper buffering in order to achieve sustained cytotoxicity. In this regard, combined inhibition of xCT or GSH synthesis may simultaneously disrupt both lipid-peroxide detoxification and copper buffering, providing a tractable rationale for co-targeting ferroptosis- and cuproptosis-related susceptibilities.

FDX1 function has been implicated in TCA-cycle protein lipoylation, mitochondrial respiration and ROS production. Structural information on FDX2 further revealed the functional distinction between these two mitochondrial proteins, with FDX1 supporting protein lipoylation and FDX2 primarily supporting iron-sulfur cluster biosynthesis [70]. The direct interac-

tion of FDX1 with LIAS promotes the lipoylation of TCA-cycle-associated proteins and fosters mitochondrial respiration and cell viability under glucose-deprived conditions [71]. FDX1 may influence responsiveness to cuproptosis and ferroptosis through its effects on protein lipoylation, which is directly relevant to cuproptosis, and mitochondrial ROS generation and oxidative metabolism, which may influence ferroptosis susceptibility. Nonetheless, such an inference in osteosarcoma is devoid of direct evidence. FDX1 is therefore not a common effector of ferroptosis and cuproptosis. Its known function is in mitochondrial protein lipoylation and cuproptosis sensitivity, but its effects on ferroptosis are likely indirect.

Moreover, activation of NRF2 increases the bioavailability of GSH and supports iron homeostasis, as well as mitochondrial antioxidant capacity. NFE2L2 and SLC25A39 modulate mitochondrial antioxidant defences via GSH metabolism in pancreatic ductal adenocarcinoma and confer cuproptosis resistance [72]. NRF2 also controls the expression of genes involved in antioxidant activity, iron homeostasis and GSH metabolism, thus protecting cells against ferroptosis. In osteosarcoma cells, elevated SLC7A11 expression, activation of NRF2 and elevated oxidative phosphorylation activity may work together to create a composite state of tolerance with continuous GSH support, efficient clearance of lipid peroxides and enhanced mitochondrial antioxidant defense.

Therefore, future studies should assess the SLC7A11/GSH/GPX4 axis, FDX1/LIAS/DLAT pathway components, iron-sulfur cluster proteins and NRF2 target genes in parallel within the same osteosarcoma models. Genetic and pharmacological perturbations in combination will need to be employed to establish the hierarchy of dependence among these regulatory nodes as well as a mechanistic rationale for co-targeting strategies.

Intrinsic and microenvironment-mediated ferroptosis resistance

Compelling evidence in osteosarcoma favors inherent and microenvironment-mediated ferroptosis resistance more strongly than cuproptosis resistance. Direct evidence from osteosarcoma shows that the MLX-SLC7A11 axis is crucial for maintaining cystine uptake and

redox defense [7], whereas M2-macrophage-derived exosomal APOC1 promotes ACSF2 degradation, resulting in reduced tumor-cell vulnerability to ferroptosis [56]. One should differentiate these mechanisms from those that underlie escape from ferroptosis induced by chemotherapy, which is described separately below.

MLX positively regulates SLC7A11 through a super-enhancer and maintains cystine uptake, GSH availability, and redox homeostasis in osteosarcoma cells [7]. These data give direct evidence that transcriptional maintenance of SLC7A11 contributes to anti-ferroptotic defense. Further studies will need to establish whether MLX dependence is enriched in particular molecular subtypes, drug-resistant models or pulmonary metastases.

Exosomal APOC1 from M2-polarized macrophages inhibits USP40-mediated deubiquitination of ACSF2, induces ACSF2 degradation, and promotes ferroptosis resistance in osteosarcoma cells [56]. Thus, not only tumor-cell antioxidant machinery but also myeloid-cell-derived signaling augments ferroptosis tolerance. As ACSF2 is involved in fatty-acid metabolism, subsequent studies should determine whether the APOC1-USP40-ACSF2 axis decreases the availability of polyunsaturated fatty-acid substrates and hence hinders lipid peroxidation.

However, comparative evidence from Ewing sarcoma points to other potential hypotheses rather than direct support in osteosarcoma. SETD8 also has been shown to modulate the YBX1/RAC3 pathway, which is related to resistance to apoptosis and ferroptosis [73], whereas inhibition of carbonic anhydrase promotes ferroptosis-associated cell death through AKT-FTH1 pathway suppression [74]. These mechanisms need to be directly validated before being extrapolated to osteosarcoma.

Altogether, cystine uptake regulated by SLC7A11, lipid-metabolic regulation, and myeloid-cell-derived exosomal signaling are candidate mechanisms of ferroptosis resistance in osteosarcoma. Data derived from comparative sarcoma findings may help guide experimental design, but should not be presented as established mechanisms in osteosarcoma.

Chemotherapy-associated ferroptosis escape

The evidence supporting ferroptosis escape in osteosarcoma is substantially stronger than

that for cuproptosis resistance following chemotherapy. Putative mechanisms of resistance to lipid-peroxidation-associated death based on available studies implicate SLC7A11-mediated cystine uptake, GPX4 stabilization, NRF2 and AKT signaling, OTULIN-dependent protein regulation, noncoding RNAs and exosome-mediated transfer of resistance signals.

SNHG14 and SLC7A11 are upregulated in Nutlin-3a-resistant osteosarcoma cells and linked to decreased lipid peroxidation. Further, SNHG14 promotes SLC7A11 expression through the miR-206/SLC7A11 axis, whereas SNHG14 downregulation decreases Nutlin-3a resistance, while this effect can be partly reversed by ferroptosis inhibitors [75]. These results indicate a role for the SNHG14-miR-206-SLC7A11 axis in Nutlin-3a resistance and ferroptosis escape.

Cisplatin-resistant osteosarcoma cells can activate the DNA-PKcs/AKT pathway and NRF2-dependent GPX4 expression, while eicosapentaenoic acid increases cisplatin sensitivity by inhibiting this antioxidant program [76]. These findings show a stronger association with chemotherapy-associated ferroptosis escape than with cuproptosis resistance. Functional dissection of the specific contributions from DNA repair, AKT activation, NRF2-GPX4-dependent antioxidant defense, and immune evasion is still a prerequisite.

Cisplatin-tolerant osteosarcoma cells were sensitized to cisplatin with formononetin through inhibition of the MAZ-GPX4 axis and induction of lipid peroxidation [77]. Nevertheless, this observation indicates that some platinum-resistant cells maintain a dependency on GPX4-mediated lipid-peroxide repair that can be functionally exploited. Yet, the restoration of cisplatin sensitivity cannot be ascribed solely to ferroptosis unless other cell-death pathways are ruled out.

The combination of eriodictyol with cisplatin raised ROS levels and lipid peroxidation-associated changes while improving the sensitivity to cisplatin in osteosarcoma cells [78]. Since the platform combines chemotherapy with oxidative injury, its contribution to ferroptosis should be verified using direct membrane lipid-peroxidation assays, iron-dependency testing and canonical inhibitor rescue.

OTULIN has been shown to stabilize GPX4 and lessen mitochondrial apoptosis, and OTULIN is upregulated in cisplatin-resistant osteosarcoma cells [79]. OTULIN may therefore play a role in both apoptosis resistance and ferroptosis escape. Studies in the future will need to couple pathway-specific rescue and genetic perturbation to separate its effects on GPX4 protein homeostasis from those on mitochondrial apoptosis.

In doxorubicin-resistant osteosarcoma tissues and cells, the expression levels of GPX4 and hsa_circ_0078767 are increased, while those of hsa-miR-188-3p are decreased. The hsa_circ_0078767/miR-188-3p/GPX4 axis contributes to doxorubicin resistance by inhibiting ferroptosis-related responses [80]. This result substantiates a mechanism of chemotherapy-associated ferroptosis escape through non-coding-RNA-mediated GPX4 regulation.

MiR-20a-5p is enriched in exosomes released from cisplatin-resistant osteosarcoma cells. After exosomal uptake by sensitive cells, miR-20a-5p targets RUNX3, inhibits ferroptosis-associated responses and enhances cisplatin resistance [81]. This finding directly demonstrates that resistant osteosarcoma cells can transfer a ferroptosis-resistant phenotype via extracellular vesicles.

Actively targeted doxorubicin nanoformulations have been shown to enhance intracellular drug accumulation and ferroptosis-associated oxidative damage in multidrug-resistant osteosarcoma cells [82]. But sensitization with nanodelivery does not establish ferroptosis as a leading death mode. Each of these parameters, including intracellular doxorubicin exposure, direct membrane lipid peroxidation, rescue by ferroptosis-specific inhibitors and activation of alternative death pathways, needs to be assessed in parallel.

Together, available evidence supports GPX4 stabilization, SLC7A11-mediated cystine uptake, NRF2 and AKT signaling, OTULIN-dependent regulation, noncoding RNAs, and transfer of resistance signals through exosomes as mechanisms of chemotherapy-associated ferroptosis escape. These mechanisms should be studied in the context of matched parental and resistant cell lines, orthotopic tumors, pulmonary-metastasis models, and paired pre- and post-treatment specimens.

Cuproptosis resistance remains hypothetical

None of the studies addressing osteosarcoma-based mechanisms have demonstrated that chemotherapy resistance comes about due to escape from canonical cuproptosis. Altered CTR1/SLC31A1, ATOX1, ATP7A, or ATP7B may also contribute to copper or platinum handling by the cell, but changes in these factors cannot be considered cuproptosis resistance without corresponding parental and drug-resistant models defined by differences in mitochondrial copper burden, FDX1 or protein-lipoylation dependency, DLAT aggregation, loss of iron-sulfur cluster proteins, mitochondrial respiratory dependence, and rescue by copper chelation [4].

Hence, CTR1/SLC31A1, ATP7A/ATP7B, FDX1, DLAT lipoylation and aggregation, mitochondrial copper content, and iron-sulfur cluster proteins should be measured in the same chemotherapy-resistant osteosarcoma models in future studies. In the absence of such evidence, chemotherapy-associated cuproptosis resistance should be considered a testable hypothesis rather than an established mechanism.

Therapeutic strategies stratified by evidence maturity and translational distance

The following summarizes potential therapeutic strategies with varying degrees of mechanistic validation and translational maturity. For exposition, we categorized them into ferroptosis-oriented approaches tested in osteosarcoma systems, copper-based interventions whose validation of canonical cuproptosis is not yet fully complete, local delivery systems or biomaterials that integrate therapeutics with bone repair, and immunotherapy-based combinations that exist largely at the proof-of-concept stage. Each strategy is evaluated on the basis of route of administration, intraosseous biodistribution, potential activity against pulmonary metastases, toxicity to normal osteoblast-lineage cells, effects on bone-marrow immunity, long-term metal retention, and compatibility with standard methotrexate, doxorubicin, and cisplatin (MAP) chemotherapy. **Table 2** compares and summarizes the osteosarcoma-specific evidence, mechanistic validation, delivery context, local and metastatic relevance, and main translational limitations of these pre-clinical therapeutic strategies.

Metal-associated cytotoxicity in osteosarcoma

Table 2. Preclinical therapeutic strategies stratified by mechanistic validation and translational maturity in osteosarcoma

Strategy category	Representative intervention	Delivery route and model	Direct osteosarcoma evidence	Proposed mechanism	Canonical validation	Local and metastatic relevance	Major translational limitations	References
Small-molecule ferroptosis strategy	EF24	Free compound; U2OS and Saos-2 cells	Yes; in vitro	HMOX1 upregulation, GPX4 suppression and iron-associated oxidative injury	Partial: Fer-1 rescue and HMOX1 perturbation; no C11-BODIPY or DFO rescue	No orthotopic or lung-metastasis assessment	No in vivo efficacy, pharmacokinetics, bone-cell toxicity or MAP-combination data	[83]
Small-molecule ferroptosis strategy	Baicalin	Free compound; OS cells and xenograft model	Yes; in vitro and in vivo	NRF2 degradation and suppression of SLC7A11-GPX4 defense	Partial: Fer-1 rescue; Fe, ROS, MDA and GSH/GSSG assessed; no C11-BODIPY	Primary xenograft only; lung metastasis not assessed	Bioavailability, iron-chelation rescue, normal osteoblast toxicity and MAP compatibility unknown	[84]
Small-molecule ferroptosis strategy	Curcumin	Free compound; MG-63 and MNNG/HOS cells; xenograft	Yes; in vitro and in vivo	KEAP1-NRF2-SLC7A11-GPX4 disruption with ferroptosis and apoptosis	Partial: Lip-1 and NRF2-activator rescue; ROS, MDA, GSH and GPX4 assessed; no iron-chelation test	Primary xenograft only; metastatic efficacy not tested	Ferroptosis-apoptosis overlap, poor bioavailability and no bone-specific distribution data	[85]
Small-molecule ferroptosis strategy	Naringenin	Free compound; OS cells and subcutaneous xenograft	Yes; in vitro and in vivo	STAT3 inhibition, MGST2 suppression and iron-dependent oxidative injury	Partial: DFO rescue and STAT3/MGST2 perturbation; Fe ²⁺ , ROS and MDA assessed; no Fer-1 or C11-BODIPY	Primary subcutaneous tumor only; lung metastasis not assessed	No canonical lipid-peroxidation assay, limited pharmacokinetics and no MAP-combination data	[86]
PDT nanomedicine with ferroptosis-associated readouts	HOS membrane-coated PLGA-IR780 nanoparticles	Nanoparticle plus NIR irradiation; HOS cells and xenografts	Yes; in vitro and in vivo	Homologous targeting and ROS-mediated photodynamic cytotoxicity	Insufficient for ferroptosis: ROS assessed; no iron dependency or ferroptosis-specific rescue	Tumor accumulation demonstrated; lung metastasis not evaluated	PDT mechanism is not ferroptosis-specific; light penetration and nanoparticle manufacturing remain limiting	[87]
Ferroptosis-oriented nanomedicine	mFeP@si carrying siGPX4 plus sonodynamic therapy	Intravenous nanoparticle plus ultrasound; 143B/chemoresistant cells and xenograft	Yes; in vitro and in vivo	GPX4 silencing, GSH depletion, PL-OOH accumulation and ICD	Relatively strong: GPX4 dependency, GSH and PL-OOH assessed; inhibitor/DFO rescue not clearly reported	Primary tumor and resistant-cell relevance; lung metastasis not tested	Complex manufacture, ultrasound parameters, systemic biodistribution and long-term immune safety	[89]
Ferroptosis-oriented nanomedicine	PDA-Fe-MOF-erastin with OS-cell membrane coating	Intravenous nanoparticle plus NIR; 143B cells and tibial tumor model	Yes; in vitro and intratibial in vivo model	Iron delivery, SLC7A11 inhibition, lipid peroxidation and photothermal enhancement	Partial: intracellular Fe, LPO and ferroptosis-related proteins assessed; canonical rescue not reported	Relevant to intraosseous tumor and osteolysis; lung metastasis not assessed	PTT and ferroptosis effects are difficult to separate; complex manufacture and long-term iron burden	[90]
Copper-related agent with unconfirmed cuproptosis	Disulfiram/copper complex	Free-drug combination; OS cells and xenograft model	Yes; in vitro and in vivo	ROS-JNK activation with apoptosis and autophagy	No canonical cuproptosis validation: ROS/JNK dependency assessed; no FDX1, DLAT aggregation or Fe-S loss	Primary xenograft only; lung metastasis not assessed	Represents copper-associated apoptosis, not validated cuproptosis; copper pharmacokinetics and bone toxicity unknown	[92]
Copper-related agent with unconfirmed cuproptosis	DDTC-Cu(I)	Free complex; U2OS, SaOS2 and MG-63 cells; SaOS2 xenograft	Yes; in vitro and in vivo	MET-PI3K-AKT inhibition and apoptosis	No cuproptosis validation: MET overexpression rescue; no FDX1/DLAT or chelator-dependency test	Primary xenograft only; metastatic efficacy not assessed	Very early evidence; mechanism is survival-signaling inhibition rather than cuproptosis	[93]
Local delivery and bone-repair platform	Copper-vorinostat hyaluronate-L-cysteine hydrogel	Local injectable hydrogel plus NIR; OS cells and local tumor model	Yes; material-based in vitro and in vivo evidence	Local copper release, FDX1-related sensitization and photothermal cytotoxicity	Partial cuproptosis validation: FDX1/lipoylation-related changes; no clear chelator rescue or FDX1 loss-of-function	Strong local postoperative relevance; unlikely to treat occult lung metastases alone	PTT confounding, copper-release kinetics, degradation, skeletal toxicity and systemic coverage unknown	[23]

Metal-associated cytotoxicity in osteosarcoma

Local delivery and bone-repair platform	Single-atom Cu nanozyme-loaded PLLA/bioactive-glass scaffold	Local scaffold implantation plus mild PTT; OS and bone-regeneration models	Yes; material-based in vitro and in vivo evidence	Peroxidase/GSH-oxidase-like activity, ROS/LPO elevation and ferroptosis-associated injury	Partial: ROS, LPO and GPX4 activity assessed; Fer-1/DFO rescue not clearly reported	Designed for local residual tumor control and bone regeneration; no metastatic coverage	Long-term Cu release, scaffold degradation, mechanical reliability and canonical ferroptosis rescue unresolved	[94]
Local delivery and bone-repair platform	TME-responsive Cu-containing hydrogel combined with a 3D-printed scaffold	Postoperative local implantation; residual-tumor and bone-defect model	Yes; material-based in vitro and in vivo evidence	Chemodynamic ROS generation, copper-associated cytotoxicity with cuproptosis-related readouts, and bone regeneration	Partial: cuproptosis-related markers reported; chelator rescue, FDX1 dependency and Fe-S loss incompletely established	High local recurrence/ bone-defect relevance; no expected systemic lung-metastasis coverage	Complex fabrication, local copper kinetics, long-term skeletal safety and cuproptosis attribution	[95]
Local implant strategy	Nano-Se-doped TiO ₂ nanotube array on titanium	Implant-surface exposure; Saos-2, MC3T3-E1 and L929 cells in vitro	Yes; direct OS-cell evidence, no animal OS model	ROS-mediated intrinsic and extrinsic caspase-dependent apoptosis	Non-ferroptotic/non-cuproptotic: ROS, BAX, cytochrome c and caspases assessed	Intended for postoperative local implant surfaces; metastatic relevance absent	In-vitro only, concentration-dependent effects, no recurrence model and uncertain long-term Se release	[49]
Immunotherapy combination/ proof of concept	Mitochondria-targeted MXene@ MnO ₂ -TPP plus sonodynamic therapy	Systemic nanoparticle plus ultrasound; K7M2/HOS cells and mouse tumor model	Yes; in vitro and in vivo	Mitochondria-targeted oxidative injury, lipid peroxidation, mtDNA release, cGAS-STING activation, and immune remodeling	Relatively strong ferroptosis panel: C11-BODIPY, GPX4, SLC7A11, ACSL4, GSH and MCU dependency; DFO/Fer-1 rescue not clearly reported	Systemic delivery and immune activation shown; direct lung-metastasis control not established	Complex heterojunction manufacture, ultrasound delivery, biodistribution and long-term immune/material safety	[96]
Immunotherapy combination/ proof of concept	Copper nanoparticles plus radiotherapy and anti-PD-1	Copper nanoplateform combined with RT and αPD-1; OS cells and mouse tumor model	Yes; in vitro and in vivo proof of concept	Copper-associated cell death, ICD and immune-checkpoint sensitization	Partial cuproptosis validation: cuproptosis-related changes were reported; complete copper-chelator rescue and FDX1/DLAT/Fe-S dependency testing was not reported	Relevant to primary tumor and immune microenvironment; metastatic efficacy not clearly established	Difficult separation of copper toxicity, cuproptosis, RT and immune effects; bone-marrow toxicity and MAP compatibility unknown	[24]

Table notes: OS, osteosarcoma; Fer-1, ferrostatin-1; Lip-1, liprostatin-1; DFO, deferoxamine; LPO, lipid peroxidation; PL-OOH, phospholipid hydroperoxides; PTT, photothermal therapy; PDT, photodynamic therapy; SDT, sonodynamic therapy; ICD, immunogenic cell death; RT, radiotherapy; MAP, methotrexate, doxorubicin and cisplatin. "Direct osteosarcoma evidence" refers to experiments performed using osteosarcoma cells or osteosarcoma animal models and does not indicate clinical validation. "Canonical validation" was graded conservatively: ROS, MDA, Fe²⁺ or GPX4 changes alone were not treated as definitive ferroptosis evidence; copper accumulation or ROS alone was not treated as definitive cuproptosis evidence.

Ferroptosis-oriented strategies evaluated in osteosarcoma models

Ferroptosis-oriented small molecules: Representative small-molecule interventions assessed in osteosarcoma models with ferroptosis validation of varying comprehensiveness include EF24, baicalin, curcumin and naringenin. EF24 raised the intracellular levels of MDA, ROS and iron in osteosarcoma cells; however, ferrostatin-1 partially reversed the cytotoxicity. EF24 also upregulated HMOX1 expression while downregulating GPX4 levels, associating HMOX1-associated iron release with ferroptosis-related lipid oxidative injury [83]. Ferrostatin-1 partially ameliorated the cytotoxicity, supporting a role for ferroptosis in EF24 action. However, direct assessment of membrane lipid peroxidation and protection by iron chelation would consolidate its mechanistic categorization. The ultimate translational potential of EF24 for intraosseous osteosarcoma will need to be gauged by its pharmacokinetics, effects on normal osteoblast-lineage cells and bone repair, and overall therapeutic index.

Effects of baicalin on the NRF2-SLC7A11-GPX4 antioxidant defense system in osteosarcoma cells. It promotes increased NRF2 ubiquitination and degradation while inhibiting SLC7A11 and GPX4 expression, yet is rescued by Fer-1, indicating a ferroptotic contribution to the observed cytotoxicity [84]. Whether this means that osteosarcoma cells with high SLC7A11 or GPX4 expression are more dependent on cystine uptake and lipid-peroxide reduction needs to be explored in detail. Thus, this new study should validate in vivo exposure at optimal pharmacologically effective concentrations and the therapeutic window in combination with cisplatin or doxorubicin using greater diversity of preclinical models beyond those available for viability assays. Future studies will complement the existing Fer-1 rescue evidence with direct measurements of membrane lipid peroxidation and, if feasible, further iron-dependency or chelation experiments to strengthen the mechanistic classification of baicalin-induced ferroptosis.

In osteosarcoma cells and xenograft models, curcumin augmented ROS levels and lipid peroxidation-related products while modulating

the KEAP1-NRF2 pathway. These effects were partially blunted by liproxstatin-1 or by manipulation of KEAP1-NRF2 signaling [85]. Thus, curcumin is better described as a redox homeostasis disruptor with a ferroptotic component than as a pathway-selective ferroptosis inducer. Due to low solubility and limited bioavailability, curcumin may be better suited to nanodelivery or local sustained-release platforms than to delivery as a free drug.

Naringenin inhibits the STAT3-MGST2 axis and impairs lipid-redox defenses in osteosarcoma cells. It increased ROS, intracellular iron and MDA levels, downregulated STAT3-MGST2 signaling, and inhibited tumor growth in a xenograft model [86]. Therefore, MGST2 is involved in glutathione-related redox metabolism, and its downregulation may reduce the capacity of cells to shield membrane lipids against oxidative damage. These results imply that lipid-redox regulation mediated by STAT3 and MGST2 may influence susceptibility to ferroptosis beyond the canonical GPX4-centered defense circuitry. Future studies should investigate changes in polyunsaturated fatty acid (PUFA)-containing phospholipids as well as in specific lipid-peroxidation products to ascertain whether naringenin-induced oxidative injury fulfills the criteria for ferroptotic cell death.

In summary, EF24, baicalin, curcumin and naringenin have been evaluated directly in osteosarcoma models and reported to interfere with iron handling, antioxidant defenses or lipid-redox homeostasis. However, direct evaluation in osteosarcoma does not provide full mechanistic validation of ferroptosis. The quality of the mechanistic evidence is variable between studies, and future investigations should include pharmacokinetic evaluation, intraosseous distribution, toxicity towards normal osteoblast-lineage cells, effects on bone repair and compatibility with standard methotrexate, doxorubicin and cisplatin chemotherapy.

Ferroptosis-oriented nanodelivery strategies: Nanoplateforms have been designed mainly to amplify ROS generation, ferritinophagy, GSH depletion or GPX4 inhibition in different osteosarcoma models to combine chemotherapy, photodynamic therapy (PDT), photothermal therapy (PTT), or sonodynamic therapy (SDT) with ferroptosis-associated oxidative injury. For

instance, HOS osteosarcoma cell membrane-coated poly(lactic-co-glycolic acid) (PLGA) nanoparticles loaded with IR780 can increase the uptake and retention of the photosensitizer by homotypic adhesion. NIR irradiation can enhance intracellular ROS levels, alter ferritinophagy-related markers, and reduce GPX4 expression, allowing PDT-induced oxidative stress to coalesce with ferroptosis-associated injury [87]. Bone-targeted exosome-based nanoparticles, which have been primarily designed to enhance intraosseous delivery, have also been shown to modulate the Keap1/Nrf2/GPX4 axis, increase ROS and Fe²⁺ levels and lipid peroxidation-associated products, and promote ferroptosis-associated cell death in osteosarcoma cells [88]. Since PDT produces ROS and therefore has the potential to activate multiple cell-death pathways, direct assessment of membrane lipid peroxidation, rescue with iron chelation, and rescue with canonical ferroptosis inhibitors are necessary in order to assess the contribution of ferroptosis specifically.

Delivery of siGPX4 via metal-organic frameworks (MOFs), combined with sonodynamic therapy (SDT), in osteosarcoma models suppresses GPX4-dependent antioxidant defenses and boosts the production of ROS through ultrasound activation, inducing changes associated with ferroptosis and immunogenic cell death [89]. SDT generally provides greater tissue penetration depth in comparison to PDT, potentially rendering it a more suitable therapy option for deep-seated osteosarcoma lesions. However, the relative contributions of ferroptosis, sonodynamic cytotoxicity and immunogenic cell death should be separated by pathway-specific validation.

When antioxidant protective mechanisms are compromised, nanotherapeutic-induced ROS are more easily converted into membrane lipid-peroxidation injury. An erastin-loaded polydopamine (PDA)-based biomimetic nanoplateform can combine PTT with inhibition of system Xc⁻, thus decreasing cystine uptake and inducing lipid peroxidation to promote ferroptosis-associated cell death [90]. These nanomedicines may not only further impair GPX4-mediated reduction of phospholipid hydroperoxides by depleting GSH in the TME, but also enhance oxidative stress generated by photo-

dynamic therapy (PDT) or photothermal therapy (PTT). Cumulatively, this may overwhelm the antioxidant-buffering ability of osteosarcoma cells and sensitise them to ferroptosis [91].

These systems should not be evaluated solely with cell-viability assays. Future studies should evaluate GSH depletion, GPX4 activity, MDA and 4-hydroxynonenal (4-HNE) levels, C11-BODIPY oxidation, rescue by ferroptosis inhibitors or iron chelators and toxicity toward normal osteoblast-lineage cells.

Copper-related agents with incomplete cuproptosis validation

Copper agents show anti-osteosarcoma activity, but most of the evidence available today reflects copper-associated oxidative stress, apoptosis, autophagy or inhibition of prosurvival signaling rather than convincingly validated canonical cuproptosis. The interventions are therefore considered copper-related therapies unless they demonstrate dependence on copper and FDX1, aggregation of lipoylated proteins, loss of iron-sulfur cluster proteins, dependence on mitochondrial respiration, and rescue by copper chelation.

Disulfiram/copper (DSF/Cu) produced increased levels of ROS, led in turn to activation of the JNK signaling pathway, cell-cycle arrest, induction of autophagy and apoptosis, and inhibited osteosarcoma growth in xenograft models [92]. These data indicate copper-associated cytotoxicity, but do not demonstrate FDX1-DLAT-dependent cuproptosis. Rescue by copper chelation, functional perturbation of FDX1, examination of lipoylated DLAT aggregation and iron-sulfur cluster protein loss, and dependence on mitochondrial respiration should be considered in studies attempting to classify cell death induced by DSF/Cu as cuproptosis.

Copper(I) diethyldithiocarbamate complex (DDTC-Cu(I)) inhibited the MET/PI3K/AKT signaling pathway and reduced proliferation, migration, and invasion of osteosarcoma cells [93]. The mechanism now reported is more consistent with copper-associated inhibition of prosurvival signaling than with validated cuproptosis. In vivo efficacy, skeletal toxicity, copper distribution and canonical markers of cuproptosis need to be tested in future studies.

In general, copper-associated agents are a reasonable approach to targeting aberrant copper handling and mitochondrial vulnerability in osteosarcoma; however, direct evidence of canonical cuproptosis remains scarce. It follows that future studies should examine CTR1/SLC31A1-dependent copper uptake, ATP7A/ATP7B-dependent copper efflux, mitochondrial copper burden, FDX1 dependence, DLAT lipoylation and aggregation, loss of iron-sulfur cluster proteins, dependence on mitochondrial respiration, and rescue by copper chelation within the same osteosarcoma models.

Local delivery and bone-repair-integrated materials

Because an irregular bone defect may coexist with microscopic residual tumor cells following tumor resection, local delivery systems as well as bone-repair-integrated materials are especially relevant to osteosarcoma. By embedding metal-active moieties, like copper or iron, into bone scaffolds, it may be possible to allow concurrent defect repair and suppression of residual tumor cells through local ion release, amplification of ROS, and ferroptosis- or cuproptosis-associated cytotoxicity.

In vivo, single-atom copper-loaded bone scaffolds exhibited the ability to inhibit osteosarcoma growth through ferroptosis-associated oxidative injury complemented by mild photothermal therapy, in conjunction with their capacity to promote bone regeneration [94]. Pathway-specific rescue experiments should then be used to define the relative contributions of ferroptosis, copper-associated oxidative stress, and photothermal injury.

The scaffolds involved TME-responsive hydrogels combined with three-dimensional (3D)-printed structures that inhibited postoperative residual tumors via a chemodynamic effect and copper-associated stress with cuproptosis-related readouts while still supporting osteoblast adhesion, migration, and new-bone formation [95]. The combination of local tumor control and structural support for bone regeneration is provided by these platforms. However, osteoblast compatibility in material-based models may not preclude potential delayed toxicity due to sustained copper release or material degradation.

A hyaluronic acid-L-cysteine hydrogel co-delivery system of copper and vorinostat combines local copper delivery, epigenetic regulation and photothermal therapy. For vorinostat, FDX1 expression was elevated in the reported osteosarcoma models, and for the copper-containing hydrogel, alterations in DLAT and other mitochondrial cuproptosis-associated readouts indicate a potential cuproptotic element. Nonetheless, neither causal dependence on FDX1 nor dependence on the protein-lipoylation machinery was established through genetic or functional rescue experiments, and copper-release kinetics under intraosseous conditions, spatial distribution within bone and long-term safety in the skeleton have yet to be demonstrated [23].

Selenium-doped TiO₂ nanotube implants, selenium nanoparticle/MgFe-layered double-hydroxide-functionalized bioactive-glass scaffolds, and magnesium-calcium alloy/mineralized-collagen composites are additional platforms for integrated bone repair. These systems not only provide local osteosarcoma suppression but also offer implant biocompatibility, antibacterial and osteogenic activity, bone reconstruction or structural support. Because their reported antitumor effects involve ROS-related apoptosis, GSH depletion and Fenton-like oxidative chemistry or more global suppression of tumor-cell proliferation, migration and invasion, they are better categorized as material-mediated cytotoxicity or microenvironmental modulation rather than as independent metal-dependent regulated cell-death modalities [49-51].

Despite their potential advantages, local material-based strategies need systematic assessment of implantation procedures, ion-release kinetics, intraosseous distribution, material degradation, toxicity toward osteoblasts and osteocytes, responses from osteoclasts, quality of osteogenesis, effects on bone-marrow immunity and long-term metal accumulation. Local control of a postoperative bone defect should not be interpreted as efficacy against occult or established pulmonary metastases. Thus, future studies should evaluate local recurrence, lung metastatic burden, continuity and biomechanical strength of newly formed bone and compatibility with systemic methotrexate-, doxorubicin- and cisplatin-based chemotherapy.

Immunotherapy combinations as proof-of-concept strategies

Preclinical and mostly proof-of-concept data are available to support combinations of ferroptosis- or cuproptosis-based therapies with immune-checkpoint blockade in osteosarcoma. Any data on osteosarcoma itself should be clearly separated from studies of non-osteosarcoma models that are only utilized to develop mechanistic hypotheses. Moreover, since lipid peroxidation may promote tumor-cell killing while also impairing CD8⁺ T-cell function or increasing immunosuppressive oxidized-lipid signaling from myeloid cells [54, 55], potential benefit is also dependent on cellular selectivity.

In osteosarcoma models, a Cu₂xSe(R837)-iRGD copper-delivery nanoformulation elicited cuproptosis-associated and immunogenic cell-death signals, remodeled the tumor immune microenvironment, and enhanced radiotherapy combined with PD-1 blockade [24]. This study presents direct preclinical proof of concept for the simultaneous use of copper-associated cytotoxicity, radiotherapy, and immune-checkpoint inhibition. Nevertheless, the exact relative roles of canonical cuproptosis, nonspecific oxidative injury, radiation-induced cell death, R837-mediated immune stimulation and downstream immune activation are less well-defined.

Triphenylphosphonium-modified MXene@MnO₂ nano-heterojunctions accumulated in mitochondria and, after ultrasound activation, augmented mitochondrial ROS, induced alterations associated with lipid peroxidation, facilitated cytosolic release of mitochondrial DNA, and activated cGAS-STING signaling in osteosarcoma models [96]. This platform is an osteosarcoma-specific proof of concept for the coupling of mitochondria-targeted oxidative injury with innate immune activation. However, scalable preparation, biodistribution, effects on bone-marrow immunity and long-term material safety still need to be characterized.

Additional proof of principle that ferroptosis-associated injury to tumor cells can be combined with immune-checkpoint blockade or innate immune activation has been provided in non-osteosarcoma models [97-100]. Collectively, these studies suggest that inhibition of

NRF2, iron-based nanomaterials, IFN-γ-dependent membrane-lipid remodeling or combined apoptosis-ferroptosis signaling may enhance antigen release and T-cell responses. None of these studies establishes efficacy or cellular selectivity in osteosarcoma, and all approaches require validation in intraosseous, pulmonary-metastasis, and immunocompetent osteosarcoma models.

Therefore, the therapeutic utility of these combinations will rely on selectively inducing tumor-cell damage that does not compromise CD8⁺ T-cell viability and function. Future studies must differentiate between alterations consistent with ferroptosis and cuproptosis across tumor cells, CD8⁺ T cells, TAMs, MDSCs and dendritic cells while also evaluating parameters including bone-marrow immunity, pulmonary metastatic burden, systemic toxicity and MAP chemotherapy compatibility. **Figure 3** integrates the major crosstalk and resistance mechanisms underlying metal-dependent cell death in osteosarcoma and stratifies these new approaches by level of evidence and mechanistic validation. Non-osteosarcoma evidence or inferred mechanisms are shown with dashed boxes.

Discussion

This review clearly differentiates mechanistically defined metal-dependent regulated cell-death modalities, ferroptosis and cuproptosis, from the broader cytotoxic processes involved in osteosarcoma that are modulated by metal ions. SLC7A11, GPX4 or FDX1 alone are unlikely to define sensitivity to metal-dependent regulated cell death in osteosarcoma. Rather, it likely represents the cumulative effect of GSH availability, lipid-peroxide detoxification capacity, mitochondrial metabolic state, membrane phospholipid composition and the immunological microenvironment. GPX4 is known as a recurrent anti-ferroptotic node on which multiple prosurvival signals may converge, and the PVT1-STAT3-GPX4 axis has been reported to suppress lipid peroxidation and promote osteosarcoma progression [101]. In a similar fashion, the POU3F3-IGF2BP2-NRF2-GPX4 axis links noncoding RNAs, RNA-binding proteins, antioxidant transcriptional programs and ferroptosis tolerance [102]. The SLC7A11-GSH-GPX4 axis is the most studied ferroptosis-defense mechanism in osteosarcoma to date.

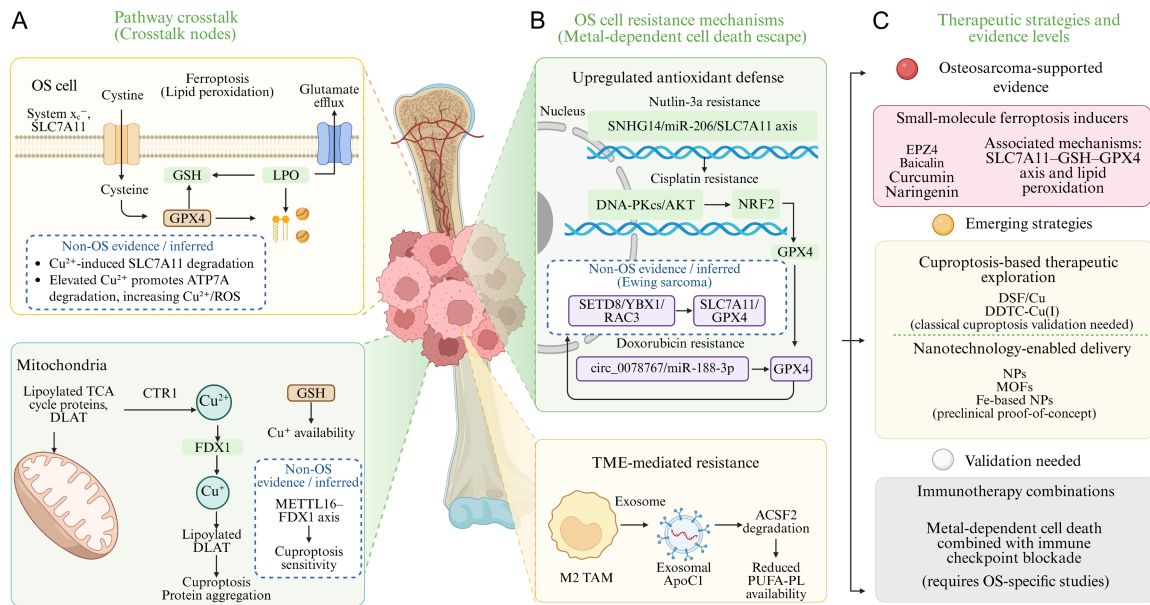


Figure 3. Crosstalk, resistance mechanisms, and evidence-stratified therapeutic strategies associated with metal-dependent cell death in osteosarcoma. **A.** Potential ferroptosis-cuproptosis crosstalk involves the SLC7A11-GSH-GPX4 antioxidant axis, copper handling, FDX1-dependent mitochondrial protein lipoylation, and related metabolic regulatory nodes; dashed boxes indicate non-osteosarcoma evidence or inferred mechanisms. **B.** Osteosarcoma-cell-intrinsic and tumor-microenvironment-mediated resistance involves antioxidant signaling, noncoding RNA-associated regulation, GPX4-related defense, and TAM-derived exosomal signaling, whereas dashed boxes denote non-osteosarcoma or inferred mechanisms. **C.** Therapeutic strategies are stratified according to osteosarcoma evidence and mechanistic maturity, including osteosarcoma-supported ferroptosis-oriented approaches, copper-related strategies with incomplete canonical cuproptosis validation, nanotechnology-enabled approaches, and immunotherapy combinations requiring further validation. Abbreviations: OS, osteosarcoma; GSH, glutathione; GPX4, glutathione peroxidase 4; LPO, lipid peroxidation; TCA, tricarboxylic acid; FDX1, ferredoxin 1; CTR1, copper transporter 1; NRF2, nuclear factor erythroid 2-related factor 2; TAM, tumor-associated macrophage; TME, tumor microenvironment; PUFA-PL, polyunsaturated fatty acid-containing phospholipid; NP, nanoparticle; MOF, metal-organic framework.

Nevertheless, most of the supporting data have come from cell lines and subcutaneous xenograft models that poorly mimic the interplay between intraosseous hypoxia, molecular subtype, chemotherapy pressure, and the tumor-bone interface.

So far, current research on ferroptosis in OS has been based on the detection of some classical endpoints, such as Fe^{2+} , MDA, and GPX4. Subsequent studies will need to go beyond these endpoints to define membrane lipid substrates, organelle-specific localization and thresholds for lethal oxidative injury. Mechanistic studies in non-osteosarcoma models demonstrate that the endoplasmic-reticulum membrane is one of the main locations for lipid-peroxidation amplification [103]. Work outside of osteosarcoma shows that MBOAT1/2-mediated phospholipid remodeling can confer a ferroptosis-resistant state independently of GPX4 and FSP1 [104]. The PUFA-containing

phospholipids that are preferentially oxidized in osteosarcoma are unknown, as are how membrane lipid composition impacts tolerance to oxidative stress and whether chemotherapy or hypoxia remodels the lipid profile. In doing so, future studies may harness spatial lipidomics and PUFA-phospholipid profiling in combination with patient-derived organoids and intraosseous orthotopic models to uncover biomarker-defined subgroups for which susceptibility to ferroptosis differs. This approach would enable the field to go from asking whether ferroptosis can be induced toward identifying in which metabolic contexts it is most likely to occur.

Copper-based materials are a newly emerging platform for local tumor control, postoperative residual disease treatment, and bone defect reconstruction. However, the degree to which their antitumor efficacy relies on canonical cuproptosis remains uncertain. Biomimetic

copper-based nanocomposites have been described to alleviate hypoxia, promote copper-associated stress responses, and inhibit tumor growth in orthotopic osteosarcoma and pulmonary-metastasis models [105]. Whether these effects are mediated by canonical cuproptosis remains to be confirmed by copper-chelator rescue, FDX1 perturbation, assessment of lipoylated-protein aggregation, and analysis of iron-sulfur cluster proteins. Injectable TFP-Cu gels similarly combine TME-responsive copper release, cuproptosis-associated cytotoxicity, immune modulation, and osteogenic support [106]. Yet, their translational potential is limited by the controllability of copper release, dependence on the FDX1-DLAT axis, tolerance of normal bone-resident cells, and the long-term safety of copper accumulation. In a recent osteosarcoma study, GSH depletion by ferroptosis inducers enhanced copper-associated cell death and antitumor immunity [107]. Independent validation of whether this interaction meets canonical criteria for cuproptosis and retains specificity for tumor cells is needed.

Hence, studies moving forward should ideally evaluate iron and copper fluxes, oxidation of PUFA-containing phospholipids, the FDX1-DLAT axis, GSH availability, and immune-effector-cell function together in a common set of experimental systems. These analyses should be performed in conjunction with intraosseous orthotopic models, patient-derived organoids and paired pre- and post-treatment specimens. This strategy might reveal biomarker-defined candidate subgroups for future testing, for example, neoplasms characterized by high expression of SLC7A11/GPX4, strong dependence on GSH availability, preserved FDX1-DLAT signaling or elevated oxidative phosphorylation activity. These profiles should be seen as testable hypotheses rather than established predictors of therapeutic benefit. This change will be crucial in advancing research on metal-dependent cell death in osteosarcoma from descriptive mechanistic studies to rationally stratified therapeutic design.

Tumor-cell susceptibility coupled with preservation of antitumor immune function should be integrated into patient stratification. Candidate variables include responsiveness to IFN- γ , LPCAT3-mediated membrane-lipid remodel-

ing, SLC7A11/GPX4-dependent antioxidant status, FDX1/DLAT-associated cuproptosis vulnerability, myeloid-cell infiltration and Programmed cell death protein 1/programmed death-ligand 1 (PD-1/PD-L1) expression. However, these variables remain testable hypotheses that need to be evaluated in cell-type-resolved osteosarcoma models. Durable benefit will require selective elimination of osteosarcoma cells while sparing CD8⁺ T-cell function and limiting myeloid-cell-mediated immunosuppression.

Conclusions

Ferroptosis and cuproptosis are two mechanistically distinct forms of metal-dependent regulated cell death. In contrast, the Zn²⁺, Mn²⁺, Ca²⁺, Mg²⁺, and selenium-associated processes discussed in this review better reflect metal ion-modulated cytotoxicity, sensitization to established cell-death pathways or material-mediated therapeutic effects. Of the mechanisms discussed here, osteosarcoma-specific mechanistic evidence is most developed with regard to ferroptosis, particularly for pathways involving iron availability and phospholipid peroxidation as well as the SLC7A11-GSH-GPX4 antioxidant axis [108].

However, direct evidence for canonical cuproptosis in osteosarcoma is still lacking. Most of the published literature focuses on therapeutic effects associated with copper-containing materials and local-delivery systems, as well as transcriptomic associations with cuproptosis-related genes, molecular subtypes, immune infiltration, prognosis or drug response predictions [109]. Copper-associated suppression of tumors should not be confused with cuproptosis unless documented copper dependence and copper-chelation rescue, functional dependence on FDX1 or the mitochondrial protein-lipoylation machinery, aggregation of lipoylated proteins such as DLAT, loss of iron-sulfur cluster proteins and dependence on mitochondrial respiration are each demonstrated [4].

Future therapeutic approaches need to prioritize osteosarcoma-specific mechanistic validation, cell-type selectivity, evaluation in intraosseous orthotopic and pulmonary-metastasis models, safety for normal bone-resident cells and the bone-marrow immune compartment,

assessment of long-term metal accumulation, and compatibility with MAP chemotherapy. These criteria will be critical for converting metal-associated vulnerabilities into clinically relevant, selective and mechanistically validated therapies for osteosarcoma.

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Disclosure of conflict of interest

None.

Abbreviations

ACSL4, Acyl-CoA synthetase long-chain family member 4; ATP7A/ATP7B, ATPase copper transporting alpha/beta; BH4, Tetrahydrobiopterin; CTR1/SLC31A1, Copper transporter 1; DAMPs, Damage-associated molecular patterns; DFO, Deferoxamine; DHODH, Dihydroorotate dehydrogenase; DLAT, Dihydrolipoamide S-acetyltransferase; FDX1, Ferredoxin 1; FSP1, Ferroptosis suppressor protein 1; GCH1, GTP cyclohydrolase 1; GPX4, Glutathione peroxidase 4; GSH, Glutathione; HIF-1 α , Hypoxia-inducible factor-1; ICD, Immunogenic cell death; MAP, Methotrexate, doxorubicin, and cisplatin; MDA, Malondialdehyde; MDSCs, Myeloid-derived suppressor cells; MOF, Metal-organic framework; NCOA4, Nuclear receptor coactivator 4; OS, Osteosarcoma; PD-1/PD-L1, Programmed cell death protein 1/programmed death-ligand 1; PMN-MDSCs, Polymorphonuclear myeloid-derived suppressor cells; PUFA, Polyunsaturated fatty acid; ROS, Reactive oxygen species; TAMs, Tumor-associated macrophages; Tregs, Regulatory T cells; TCA, Tricarboxylic acid cycle; TFRC, Transferrin receptor 1; TME, Tumor microenvironment.

Address correspondence to: Haolong Zhang and Zhijing Song, Clinical College of Traditional Chinese Medicine, Gansu University of Chinese Medicine,

No. 35 Dingxi East Road, Lanzhou 730000, Gansu, China. E-mail: zhanghaolong_ac@139.com (HLZ); songzhijing2020@163.com (ZJS)

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