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Expert recommendation

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Recommendations for robust and reproducible research on ferroptosis

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Abstract

Ferroptosis is a necrotic, non-apoptotic cell death modality triggered by unrestrained iron-dependent lipid peroxidation. By unveiling the regulatory mechanisms of ferroptosis and its relevance to various diseases, research over the past decade has positioned ferroptosis as a promising therapeutic target. The rapid growth of this research field presents challenges, associated with potentially inadequate experimental approaches that may lead to misinterpretations in the assessment of ferroptosis. Typical examples include assessing whether an observed phenotype is indeed linked to ferroptosis, and selecting appropriate animal models and small-molecule modulators of ferroptotic cell death. This Expert Recommendation outlines state-of-the-art methods and tools to reliably study ferroptosis and increase the reproducibility and robustness of experimental results. We present highly validated compounds and animal models, and discuss their advantages and limitations. Furthermore, we provide an overview of the regulatory mechanisms and the best-studied players in ferroptosis regulation, such as GPX4, FSP1, SLC7A11 and ACSL4, discussing frequent pitfalls in experimental design and relevant guidance. These recommendations are intended for researchers at all levels, including those entering the expanding and exciting field of ferroptosis research.

Sections

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Introduction

Ferroptosis is a non-apoptotic cell death modality characterized by iron-dependent unrestrained phospholipid peroxidation[1,2], which is caused by the disruption of the antioxidant defence system and/or imbalanced cellular metabolism. Over the past decade, research has revealed the major regulatory nodes of ferroptosis and established its central role in various human diseases, including neurodegeneration and organ injury, as well as in tumour suppression[3–17]. This has led to increasing interest in modulating ferroptosis therapeutically[18]. For example, the suppression of ferroptosis is expected to alleviate acute organ damage, such as ischaemia-reperfusion injury (IRI)[7,17]. Conversely, therapy-resistant mesenchymal cancers acquire a vulnerability to ferroptosis[8,9], making the induction of ferroptosis in cancer cells a promising therapeutic approach[19,20]. However, several open questions remain before the modulation of ferroptosis can be fully exploited to treat disease. Notably, reliable molecular biomarkers are lacking to faithfully detect ferroptosis in human disease. Against this backdrop, the rapid surge in ferroptosis research worldwide carries a risk of erroneous identification and assessment of this cell death modality. This highlights the need for careful assessment of ferroptosis to avoid experimental misinterpretation. In this Expert Recommendation, we first provide an overview of our current understanding of ferroptosis regulatory mechanisms and the key factors involved. To improve the robustness and reproducibility of experimental results, we then discuss appropriate and validated methods for evaluating ferroptosis, including recommended compounds and animal models for ferroptosis research.

Major challenges in ferroptosis research

The study of ferroptosis presents several challenges. One hurdle is the variability and complexity of factors that determine the susceptibility to ferroptosis in different cellular contexts and organisms. The regulatory mechanisms of ferroptosis are governed by interactions of three main processes: redox signalling, lipid metabolism and iron homeostasis, all of which affect phospholipid peroxidation. Therefore, understanding all the relevant cues, such as the expression and function of the key regulatory factors involved, is essential for accurately assessing ferroptosis. Second, the lack of specific biomarkers to detect ferroptosis, especially in vivo, poses a challenge in monitoring the execution of ferroptosis and evaluating its association with disease. Furthermore, recognizing differences between in vitro and in vivo redox environments is crucial when studying the regulatory mechanisms of ferroptosis. This complexity is further compounded by the influence of various experimental factors on the sensitivity of cells to ferroptosis, which immediately affects the interpretation of results, as discussed in the following sections.

Ferroptosis regulation

Ferroptosis results from unrestrained phospholipid peroxidation, eventually leading to plasma membrane rupture[21]. It was termed 'ferroptosis' (from *ferrum*, Latin for iron) to reflect the requirement of iron in this process[1], and it is now accepted that excessive phospholipid peroxidation, driven by iron, is its defining characteristic[22]. The pleiotropic roles of ferroptosis in pathology have been extensively reviewed elsewhere[18]. We focus here on the main cellular pathways regulating ferroptosis (Fig. 1).

Ferroptosis surveillance systems

Cells have various systems to suppress excessive lipid peroxidation and prevent ferroptosis. The prime anti-ferroptotic pathway is the cysteine–glutathione (GSH)–glutathione peroxidase 4 (GPX4) axis[7,10,23]. GPX4, a member of the family of selenoproteins, converts potentially detrimental lipid hydroperoxides, into their comparatively non-toxic alcohols at the expense of GSH[4,24]. As GSH is required for full GPX4 activity, GSH biosynthesis is critical to ferroptosis suppression. Cysteine derived from cystine (the dimeric, oxidized form of cysteine) taken up via the cystine-glutamate antiporter system x_c^- (ref. 25) is used for the biosynthesis of GSH. Accordingly, the pharmacological or genetic disruption of the cysteine–GSH–GPX4 axis can induce ferroptosis. Inhibitors of GPX4 and system x_c^- are the most common agents used to induce ferroptosis in experimental studies.

Ferroptosis suppressor protein 1 (FSP1, encoded by *AIFM2*) prevents ferroptosis independently of GPX4 (refs. 26,27). FSP1 consumes NAD(P)H to reduce extramitochondrial ubiquinone (also known as coenzyme Q_{10} or CoQ_{10}) or vitamin K to either ubiquinol (the reduced form of CoQ_{10}) or vitamin K hydroquinone, respectively[28]. These reduced forms act as radical-trapping antioxidants (RTAs), effectively trapping the lipid peroxyl radicals that propagate lipid peroxidation, thereby preventing ferroptosis. The GTP cyclohydrolase 1 (GCH1)–tetrahydrobiopterin (BH_4) pathway also suppresses ferroptosis[29,30]. GCH1 catalyses the rate-limiting step in the synthesis of BH_4, which functions as an endogenous RTA and is recycled by dihydrofolate reductase. In addition, vitamin E protects against lipid peroxidation and ferroptosis as a potent natural lipophilic RTA[31]. The cholesterol biosynthesis pathway (that is, the mevalonate pathway) and its metabolites also contribute to ferroptosis suppression. Intermediate metabolites of the cholesterol biosynthesis pathway, such as squalene and 7-dehydrocholesterol, suppress ferroptosis by sparing phospholipid autoxidation[32–34]. Moreover, metabolites produced in the mevalonate pathway are essential for CoQ_{10} synthesis and for the stabilization of selenocysteinyl-tRNA[35], which are crucial for FSP1 function and GPX4 expression, respectively.

PUFAs and iron

Peroxidized polyunsaturated fatty acids (PUFAs) and, in particular, the lipid radicals derived from them are the primary triggers of ferroptosis[36–38]. The cells undergoing ferroptosis have elevated levels of oxidized phospholipids, including phosphatidylcholines and phosphatidylethanolamines containing PUFA chains[22,39,40]. As PUFAs are inherently more prone to undergo lipid peroxidation than monounsaturated fatty acids (MUFAs) and saturated fatty acids[41], the balance of PUFAs and MUFAs in cell membranes is the major determinant of the sensitivity of cells to ferroptosis[37,42] (Fig. 1). Increased PUFAs in the plasma membrane enhance ferroptosis susceptibility[39,40,43], whereas increased MUFAs promote ferroptosis resistance[44]. Mechanistically, increased expression of ACSL4 and LPCAT3 promotes the incorporation of PUFAs into phospholipids[39,40,45,46]. ACSL1 facilitates the incorporation of conjugated PUFAs, such as α-eleostearic acid, which is highly susceptible to peroxidation[46]. By contrast, high expression of SCD1, ACSL3, MBOAT1 and MBOAT2 increases MUFA content and confers resistance to ferroptosis[13,47–49].

Iron also has a crucial role in ferroptosis[1], although its exact contribution to the execution of ferroptosis is still debated, at least in an in vivo context. A small fraction of redox-active iron in the labile iron pool triggers the Fenton reaction, initiating lipid peroxidation chain reactions by forming lipid alkoxyl and hydroxyl radicals[50]. Consequently, cellular contexts with increased labile iron make cells more prone to ferroptosis. Thus, iron homeostasis, the mechanisms of which are discussed elsewhere[51], should be tightly regulated. It is important to note that inducing ferroptosis through system x_c^- inhibition can increase intracellular labile iron[52,53], but this does not occur in all ferroptotic cells.

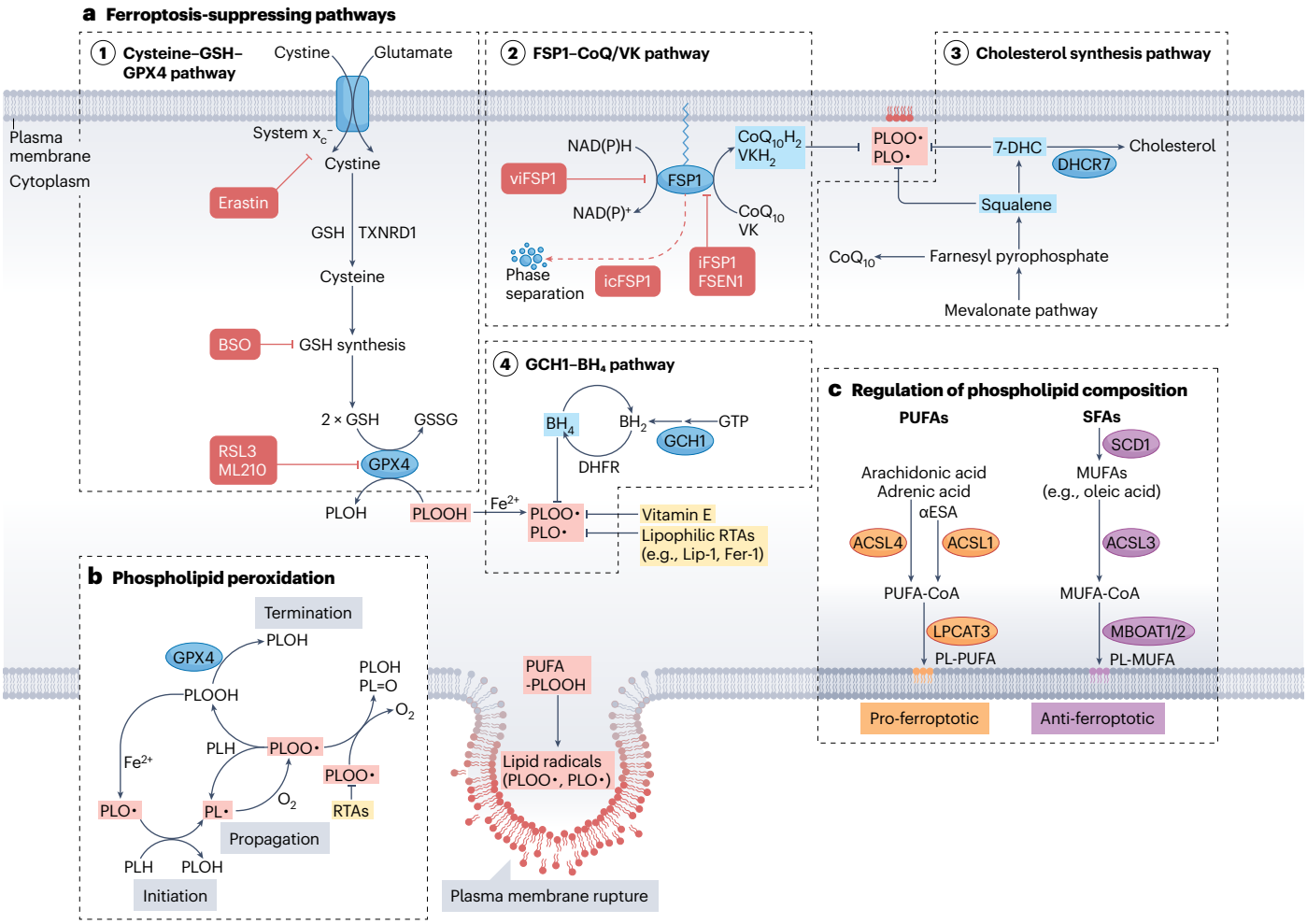
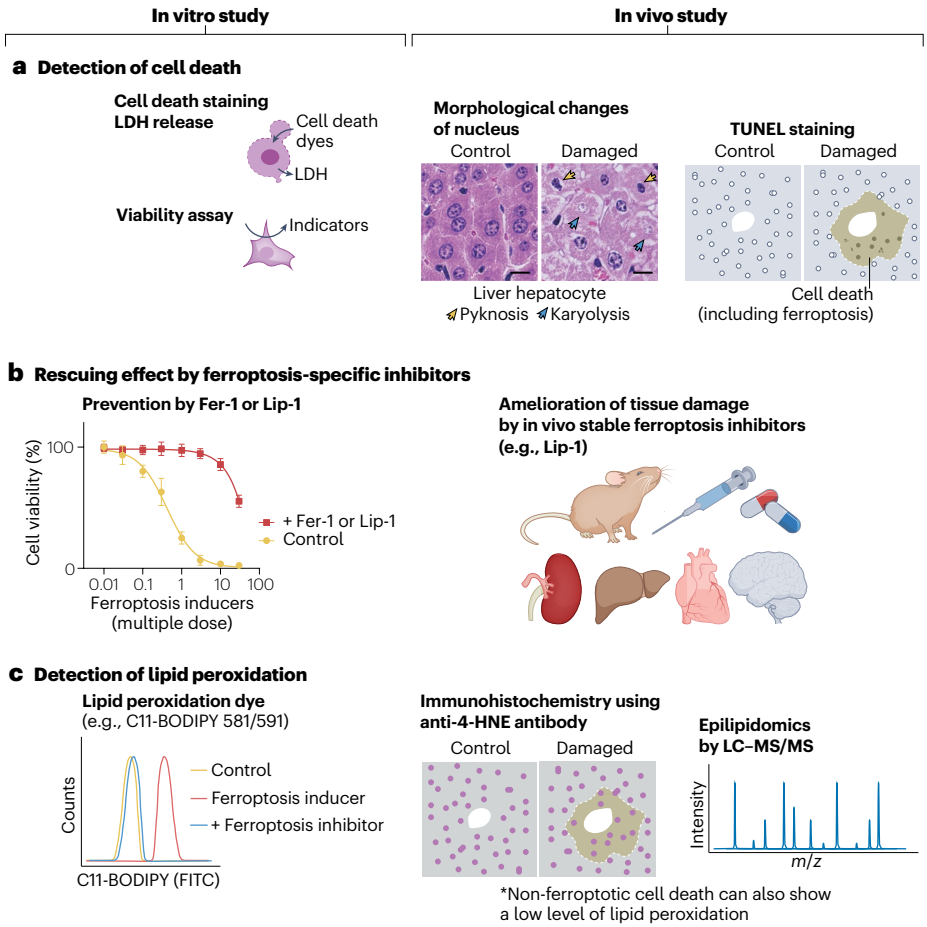


Fig. 1 | Overview of intracellular ferroptosis regulatory pathways. **a**, Ferroptosis-suppressing pathways. (1) The cysteine–glutathione–glutathione peroxidase 4 pathway (cysteine–GSH–GPX4) pathway. Cystine, taken up via the cystine-glutamate antiporter system x_c^-, is converted to its reduced form cysteine by GSH or thioredoxin reductase 1 (TXNRD1). Cysteine is used for GSH biosynthesis. GPX4 reduces phospholipid hydroperoxides (PLOOH) to the corresponding alcohols (PLOH) at the expense of GSH. GPX4 inhibitors (for example RSL3 and ML210), system x_c^- inhibitors (for example erastin) and GSH biosynthesis inhibitors (for example L-buthionine sulfoximine, BSO) induce ferroptosis by disrupting the cysteine–GSH–GPX4 pathway. (2) The system involving ferroptosis suppressor protein 1 and ubiquinone or vitamin K (FSP1–CoQ_{10}/VK) suppresses ferroptosis in parallel to the GPX4 pathway. FSP1 prevents lipid peroxidation by reducing ubiquinone (also known as coenzyme Q_{10}, CoQ_{10}) and VK, whose reduced forms (that is, ubiquinol ($CoQH_2$) and VK hydroquinone (VKH_2)) act as radical-trapping antioxidants (RTAs). (3) The metabolites of the mevalonate pathway, such as squalene and 7-dehydrocholesterol (7-DHC), act as ferroptosis suppressors. (4) The GTP cyclohydrolase 1 (GCH1)–tetrahydrobiopterin (BH_4) pathway, including dihydrofolate reductase (DHFR), protects against lipid peroxidation. Vitamin E and lipophilic RTAs, such as ferrostatin-1 (Fer-1) and liproxstatin-1 (Lip-1), also suppress ferroptosis by acting as RTAs. **b**, Mechanisms of phospholipid peroxidation. Lipid peroxidation, the hallmark of ferroptosis, is a free radical chain reaction, which comprises initiation (Fenton-type chemistry), propagation (the reaction between lipids and lipid peroxyl radicals and oxygenation of the lipid-derived radicals) and termination (radical–radical coupling to give non-radical products) reactions. GPX4 prevents initiation by reducing (P)LOOH that can undergo Fenton-type reactions, whereas RTAs prevent propagation by trapping lipid peroxyl radicals. **c**, The phospholipid composition dictates ferroptosis sensitivity. Esterification of phospholipids with polyunsaturated fatty acids (PUFAs), such as arachidonic acid, adrenic acid and α-eleostearic acid (α-ESA), renders cells susceptible to ferroptosis. By contrast, the esterification of phospholipids with saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs, such as oleic acid) renders cells resistant to ferroptosis. ACSL1, acyl-CoA synthetase long-chain family member 1; DHCR7, 7-dehydrocholesterol reductase; LPCAT3, lysophosphatidylcholine acyltransferase 3; MBOAT1, membrane-bound *O*-acyltransferase domain-containing 1; PLOO·, phospholipid peroxyl radical; PLO·, phospholipid alkoxyl radical; PL, phospholipid; PL=O, lipid-derived carbonyl compound; SCD1, stearoyl-CoA desaturase.

Ferroptosis detection

A fundamental problem in ferroptosis research is the absence of established specific markers for directly detecting ferroptosis. Therefore, researchers use complementary approaches to assert the contribution of ferroptosis to tissue damage and cancer regression. The following four approaches are currently used to conclude whether an observed phenotype is indeed linked to ferroptosis: (1) detection of cell death, (2) rescue of cell death by ferroptosis-specific inhibitors, (3) detection



d Exclusion of other cell death modalities

	Inhibitors	Markers
Apoptosis	zVAD-FMK (caspase inhibitor)	Cleaved caspase-3
Necroptosis	Nec-1s (RIPK1 inhibitor)** GSK'872 (RIPK3 inhibitor)	p-MLKL, p-RIPK3
Pyroptosis	MCC950 (NLRP3 inhibitor)***	Cleaved GSDMD
Parthanatos	Olaparib (PARP inhibitor)	Nuclear translocation of AIF
CypD-dependent regulated cell death	Cyclosporin A (CypD inhibitor)	

of lipid peroxidation, and (4) exclusion of other cell death modalities (Fig. 2 and Table 1).

Detection of cell death

In vitro, cell death can be evaluated by conventional cell death stains, measuring lactate dehydrogenase release, or cell viability assays[54]. Morphologically, in vitro, ferroptosis manifests as 'balloon-bursting' cell death (Supplementary Video 1). As these assays cannot be applied to in vivo experiments, evaluating morphological changes in nuclei may help to assess cell death in vivo. Histologically, cells undergoing ferroptosis typically exhibit pyknosis or karyolysis, features associated with necrotic cell death, whereas nuclear fragmentation indicates apoptosis[55–57]. Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) staining, which detects double-strand DNA breaks, provides substantial evidence for the occurrence of cell death in tissues. Although TUNEL staining was classically thought to be specific for apoptosis, it is also positively associated with other regulated cell death modalities, including ferroptosis[28,58].

Rescue of cell death by ferroptosis-specific inhibitors

Evaluating whether ferroptosis-specific inhibitors can entirely prevent the detected cell death is crucial to validate whether ferroptosis is occurring. For this purpose, ferrostatin-1 (Fer-1)[1] or liproxstatin-1 (Lip-1)[7] are commonly used. Although iron chelators, such as deferoxamine, can also rescue ferroptosis[1], their effective dose range is relatively narrow and unsuitable for long-term treatment (over 48 h) owing to substantial cell toxicity in vitro. It is essential to note that iron chelators also prevent non-ferroptotic cell death forms, such as hydrogen-peroxide-induced cell death[1]. Moreover, the possibility that RTAs, such as Lip-1, might inhibit other, yet-undefined, radical-dependent cell death

Fig. 2 | Experimental procedures for determining ferroptosis. a–**d**, Observed phenotypes linked to ferroptosis: detection of cell death (**a**), rescue of cell death by ferroptosis-specific inhibitors **b**), detection of lipid peroxidation (**c**) and exclusion of other cell death modalities (**d**). There are currently no specific markers that alone can conclusively characterize ferroptosis; therefore, it is recommended to use a combination of **a**–**d** to determine whether an observed phenotype is linked to ferroptosis. Relying on the evidence from one assay alone as an indicator of ferroptosis is insufficient. The evaluation methods that can be used in vivo and in vitro may differ and the advantages and limitations of each method should be carefully considered. For **a**, the cell death occurring in vitro can be evaluated by cell death staining, measuring lactate dehydrogenase (LDH) release, or cell viability assays. Evaluating morphological changes in nuclei may help to assess cell death in vivo. Images of hematoxylin and eosin-stained sections from control (wild-type mice) and damaged liver (hepatocyte-specific *Gpx4*-knockout mice fed a diet low in vitamin E[28]) illustrate nuclei exhibiting pyknosis (yellow arrowheads) and karyolysis (blue arrowheads). Scale bar, 10 μm. These unpublished images were obtained from the tissue sections prepared in the previous study[28]. Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) staining, which detects double-strand DNA breaks, provides substantial evidence for the occurrence of cell death in tissues. For **b**, in vitro, ferroptosis-specific inhibitors, such as ferrostatin-1 (Fer-1) or liproxstatin-1 (Lip-1), are commonly used to determine whether the observed cell death is ferroptosis by assessing their protective effects. In animal experiments, in vivo-stable ferroptosis inhibitors such as Lip-1 or UAMC-3203 are recommended to evaluate their ameliorating effects on tissue damage in target organs of animal models, such as conditional *Gpx4*-knockout mice. For **c**, in cell culture, fluorescent probes such as C11-BODIPY 581/591 are often used to detect lipid peroxidation. To evaluate lipid peroxidation in vivo, the detection of lipid-peroxide-derived breakdown products, such as by immunohistochemistry using anti-4-hydroxy-2-nonenal (4-HNE) antibody, can serve as an indicator of lipid oxidation. Epilipidomic analysis using liquid chromatography–tandem mass spectrometry (LC–MS/MS) enables a comprehensive assessment of individual oxidized lipids. Of note, regions affected by oxidative stress and non-ferroptotic cell death can also show 4-HNE positivity and a low level of increased lipid peroxidation. In **b** and **c**, hypothetical graphs were shown as examples of the result. In **d**, ferroptotic cells do not exhibit the characteristic hallmarks of other cell death modes (for example cleaved caspase-3 as an apoptosis marker), and inhibitors of other types of cell death (for example the apoptosis inhibitor zVAD-FMK) should not prevent ferroptosis. As many other cell death modes exist in addition to those listed in the table, attempts should be made to rule out other forms of cell death. However, in in vivo models, testing the rescue effects of each cell death inhibitor can be highly challenging as these inhibitors often do not sufficiently block the targeted cell death in animal models, owing to limited in vivo efficacy. **Necrostatin-1s (Nec-1s) also inhibits RIPK1-dependent apoptosis; ***MCC950 does not inhibit NLRP3-independent pyroptosis. FITC, fluorescein isothiocyanate.

pathways, and signalling events sensitive to lipid peroxides cannot be excluded at this time.

To assess the rescuing effect of ferroptosis inhibitors in animal experiments, in vivo-stable ferroptosis inhibitors such as Lip-1 or UAMC-3203 (ref. 59) (a Fer-1 analogue with better stability) are recommended. Treatment with these ferroptosis inhibitors mitigates the tissue damage and prolongs survival in animal models with ferroptosis-associated pathophysiology, such as conditional *Gpx4*-knockout mice[7,60]. However, these ferroptosis inhibitors cannot entirely prevent tissue damage in the animal models[7], unlike the complete prevention of ferroptosis observed in cell culture. This suggests that lipid peroxidation cannot be entirely inhibited in vivo by ferroptosis inhibitors, owing to factors such as drug pharmacokinetics, tissue distribution and the downstream pathological consequences following widespread cell death and/or necroinflammatory processes[61].

Detection of lipid peroxidation

Lipid peroxidation is the hallmark of ferroptosis, and it is therefore essential to evaluate its occurrence to confirm that cells are dying via ferroptosis. In cell culture, fluorescent probes are often used to detect lipid radical formation or lipid peroxidation. C11-BODIPY 581/591 is a fatty-acid-based dye with conjugated double bonds that are susceptible to peroxyl radical addition[62], and it competitively reacts with propagating lipid peroxyl radicals, resulting in a shift in fluorescence emission from red to green. STY-BODIPY is a slower-reacting analogue[63] and is therefore more suitable for live-cell imaging[64,65]. However, these lipid peroxidation probes are unsuitable for tissue staining or in vivo use, unless isolated cells collected from fresh tissue samples are used[13].

To evaluate lipid peroxidation in vivo, the detection of lipid-peroxide-derived breakdown products, such as 4-hydroxy-2-nonenal (4-HNE), malondialdehyde and 4-hydroxy-2-hexenal, can serve as an indicator of lipid oxidation[57,66]. Immunohistochemical detection using anti-4-HNE antibodies is feasible in formalin-fixed, paraffin-embedded sections[55,57]. However, all of these lipid oxidation products are also formed by oxidation of lipids other than phospholipids, such as free fatty acids.

Epilipidomic analysis using liquid chromatography–tandem mass spectrometry (LC–MS/MS) enables a comprehensive assessment of individual oxidized lipids[67]. Despite its effectiveness, this method is not widely implemented because of its technical challenges and the necessity for specialized equipment and bioinformatic analysis[68,69]. In addition, several important aspects must be considered to ensure the accuracy of the obtained results (Box 1).

Epilipidomic studies have identified a wide variety of PUFA-containing phospholipids, not limited to phosphatidylcholines and phosphatidylethanolamines with arachidonoyl (20:4) and adrenoyl (22:4) chains, as substrates for lipid peroxidation during ferroptosis[33,39,40]. Other phospholipids, including phosphatidylserine, phosphatidylinositol and cardiolipin, are also prone to peroxidation when they contain PUFA acyl chains[22,70]. Ether phospholipids, specifically plasmalogens containing PUFA chains, are preferential lipid peroxidation substrates and oxidized during ferroptosis, but whether this process prevents or promotes ferroptosis remains under debate[71–73]. A key challenge in evaluating lipid peroxidation as an indicator of ferroptosis is the lack of a clear definition regarding which specific phospholipid species or the extent of their increase definitively indicates ferroptosis. In addition, the signature of lipid peroxidation during ferroptosis may vary depending on the lipid composition of different cell types and the induction method.

It is important to note that increased oxidized lipids can only be detected just before and after the onset of ferroptosis. Therefore, it is necessary to optimize the timing of the measurements; otherwise, the increase in oxidized lipids might be missed if the timing of sample processing is too early or too late after exposure to a ferroptosis-inducing stimulus. Moreover, these indicators of lipid peroxidation are not direct markers of ferroptosis. Non-ferroptotic cell death can also show a low level of increased lipid peroxidation[4,22]. Similarly, 4-HNE can also be positive in non-ferroptotic cells, such as regions affected by oxidative stress[74]. Therefore, relying solely on evidence of these lipid

Table 1 | Advantages and limitations of each method to detect ferroptosis

Category	In vitro use suitability	In vivo use suitability	Considerations and limitations
Detection of cell death			
Cell viability assay (for example WST-8, resazurin and ATP content)	✓	–	Cell proliferation rates, ATP production rates and general cell metabolism may affect the results
Lactate dehydrogenase release	✓	–	A general and reliable indicator of necrotic cell death
Dead cell staining dyes (for example propidium iodide and SYTOX Green)	✓	–	When using Trypan blue for cell death assessment, it should be noted that Trypan blue begins to be toxic to cells after a short exposure, and even viable cells will eventually be stained
TUNEL staining	✓	✓	Other types of cell death involving double-strand DNA breaks also yield positive results
Morphological changes in the nucleus	–	✓	Ferroptosis can show morphological changes in the nucleus, such as pyknosis and karyolysis, as findings of necrotic cell death
Detection of lipid peroxidation			
Lipid peroxidation fluorescent probes (for example C11-BODIPY 581/591 and STY-BODIPY)	✓	–	Can only be used in live cells and is not specific to oxidation of phospholipids
Immunodetection of 4-HNE protein adducts	✓	✓	Suitable for fixed cells/tissues or immunoblotting[55,57] and can also be increased in cells and tissues exposed to oxidative stress without cell death
LC–MS/MS-based epilipidomics	✓	✓	Special equipment and appropriate sample preparation are required and is used for unfixed cells and tissues
Rescue effect by ferroptosis inhibitors			
Radical-trapping antioxidants	✓	✓	In vitro study, Fer-1 and Lip-1 are recommended. For in vivo studies, Lip-1 and UAMC-3203 are recommended. Fer-1 is unsuitable for in vivo use, owing to poor metabolic stability in plasma. Radical-trapping antioxidants may influence other forms of oxidative cell death and may affect inflammatory processes
Iron chelators	✓	–	Not suitable for long-term treatment (>48 h) owing to general cellular toxicity in vitro, has a narrow dose range for anti-ferroptotic effect and also prevents hydrogen peroxide-induced cell death. Poor, if any, rescuing activity in *Gpx4* conditional knockout-induced ferroptosis mouse models
Exclusion of other type of cell death			
Absence of characteristic signs for each type of cell death (for example cleaved caspase-3 in apoptotic cells)	✓	✓	To include positive control samples is mandatory (for example cleaved caspase-3 in spontaneous apoptosis in the spleen or testis when analysing tissue samples)
Absence of rescuing effects by specific inhibitors for each type of cell death	✓	–	Some inhibitors also suppress other forms of cell death (for example necrostatin-1 inhibits ferroptosis in addition to necroptosis). Many inhibitors are unsuitable for in vivo use
Changes in signature genes or proteins			
Transferrin receptor mobilization	(✓)[a]	(✓)[a]	Does not always occur during ferroptosis and is context dependent
PTGS2 expression	(✓)[a]	(✓)[a]	Expression levels are also upregulated during inflammatory conditions
CHAC1 expression	(✓)[a]	–	Expression is upregulated by system x_c^- inhibition but not by GPX4 inhibition
PRDX3 overoxidation	(✓)[a]	(✓)[a]	Hyperoxidation can be induced by other forms of oxidative stress, such as isothiocyanate and H_2O_2 (ref. 84)

[a]These changes are not specific to ferroptosis.

oxidation products or fluorescent probes as indicators of ferroptosis is insufficient.

Exclusion of other forms of cell death

Finally, when analysing a potential ferroptotic phenotype, assessing the potential involvement of other known cell death modalities can be important and useful. Ferroptotic cells do not exhibit the characteristic hallmarks observed in different modes of cell death. In addition, inhibitors of other types of cell death should not prevent ferroptosis. For example, to exclude apoptosis, the absence of the rescuing effect by apoptosis inhibitors and the absence of cleaved caspase-3 can be assessed (Fig. 2). Similarly, the impact of necroptosis inhibitors can

be tested to exclude necroptosis. Of note, the necroptosis inhibitor necrostatin-1 (Nec-1) also inhibits ferroptosis at higher concentrations by being converted in situ into an RTA[7,75]. Hence, the use of Nec-1s, an analogue of Nec-1 that does not impede ferroptosis, is recommended to distinguish between necroptosis and ferroptosis.

As our knowledge of cell death expands, the number of potential alternative cell death pathways to be excluded continues to grow. This presents a practical challenge in determining how thoroughly to verify the exclusion of other cell death pathways. Especially, in in vivo models, testing the rescue effects of each cell death inhibitor can be highly challenging or even impossible, as these inhibitors often do not sufficiently block the targeted cell death in in vivo models, owing to limited in vivo efficacy. Therefore, from a practical standpoint, ferroptosis should be considered in an in vivo model when there is clear evidence of cell death and lipid peroxidation, along with suppression of the cell death by ferroptosis inhibitors, and the absence of markers indicating other major forms of cell death.

Limitation of signature genes and proteins suggesting ferroptosis

Certain transcriptional or translational changes have been proposed as signatures of ferroptosis[76]. Although these changes may correlate with ferroptosis, it is important to note that these signatures can also occur under non-ferroptotic conditions, emphasizing the need for careful consideration of their specificity. For example, increased anti-transferrin receptor 1 (TFR1) staining intensity has been reported as a potential marker for cells undergoing ferroptosis[77]. However, the localization of TFR1 in the plasma membrane is not always specific to

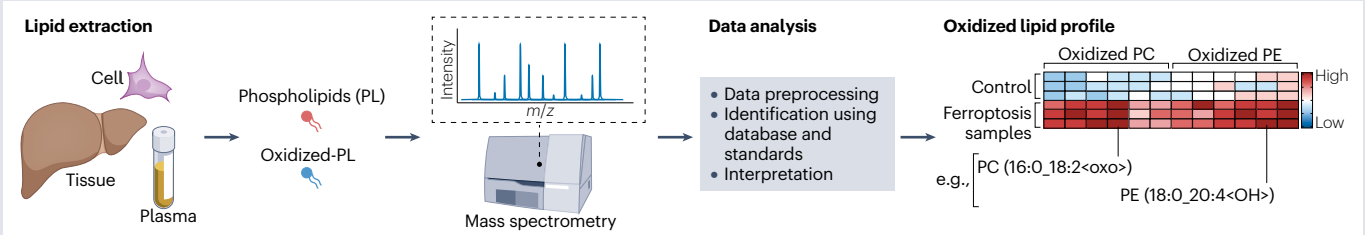
Box 1 | Critical recommendations for epilipidomic surveys

When planning liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis of oxidized lipids from any biological material (for example cells, tissues and biofluids; see the figure), it is crucial to prevent lipid autoxidation during sample collection, storage and lipid extraction. Lipids readily undergo autoxidation when exposed to air and ambient temperature[175]. Artificially formed products can mask the endogenous signature of ferroptosis. It is recommended that the solutions used to collect cells or perfuse animal tissues be spiked with radical-trapping antioxidants (RTAs) such as butylated hydroxytoluene (BHT). Once collected, the samples must be immediately frozen and stored at −80 °C or lower until use. The lipid extraction should be performed on ice using precooled solvents spiked with BHT.

Liquid chromatography–mass spectrometry (LC–MS) analysis of oxidized lipids is challenging because of their low endogenous concentrations (relative to the concentrations of bulk, unoxidized cellular lipids) and the high diversity of possible structures formed during ferroptosis. When the ferroptosis signature of oxidized lipids (epilipidome) is known, targeted LC–MS/MS approaches such as multiple or parallel reaction monitoring, characterized by high selectivity and sensitivity, are strongly recommended. However, when no prior knowledge of the oxidized lipid species in the studied samples is available, initial identification is necessary. Initially formed lipid peroxides, when not efficiently reduced by glutathione peroxidase 4 (GPX4), undergo a variety of secondary reactions, forming a diverse set of products including hydroxy-modified, keto-modified and epoxy-modified polyunsaturated fatty acids (PUFA acyl chains), as well as truncated keto and carboxylic acid derivatives. Given the wide variety of natural phospholipids carrying PUFA acyl chains, a complex and sample-specific mixture of oxidized lipids is formed during ferroptosis execution[28,33].

To target the diversity of oxidized lipids in ferroptotic conditions, a workflow based on the in silico prediction of oxidized lipidome followed by semi-targeted LC–MS/MS analysis was developed and automated with the help of LPPtiger2 open-source software[67]. Tandem mass spectra (MS/MS) of oxidized lipids can be used for structural annotation of detected lipid species. Typically, informative MS/MS spectra allow for the assignment of not only the class of oxidized lipid but also the type and even the modification position. To assist such structural annotation, fragmentation patterns of oxidized lipids were characterized and made publicly available[67]. To avoid false positive identification, attention should be paid to the annotation of isomeric oxidized lipids (for example, hydroperoxy-modified versus dihydroxy-modified lipids will have the same mass and similar fragmentation patterns but can be resolved by reverse-phase chromatography) and artefacts derived from in-source fragmentation of lipid ions (for example, lipid hydroperoxide can undergo in-source fragmentation with formation of the corresponding truncated carbonyl derivative)[67,176].

Retention time mapping (for example using Kendrick mass defects plots) is usually beneficial in accounting for possible misannotations. Although current LC–MS/MS workflows allow for the detection and identification of oxidized lipids even in complex biological matrices, absolute quantification of oxidized lipid species remains challenging owing to the lack of commercially available isotopically labelled standards capable of accounting for diverse lipid peroxidation products. Thus, so far, only relative quantification (such as fold change increase in ferroptotic versus non-ferroptotic conditions) of oxidized species is possible. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid.



ferroptosis, as it is present on the cell surface even under normal conditions. *PTGS2* and *CHAC1* can be upregulated during ferroptosis[10,78], yet the changes in expression of these genes are also observed under non-ferroptotic conditions. *PTGS2* expression is also upregulated in inflammatory conditions[79]. Although the expression of *CHAC1* is upregulated by system x_c^- inhibition via ATF4-dependent endoplasmic reticulum stress response[78,80,81], it is not upregulated by GPX4 inhibition. Several stress-responsive, post-translational protein modifications, such as hyperoxidation of peroxiredoxin 3 (PRDX3)[82] and phosphorylation of apoptosis signal-regulating kinase 1 (ASK1)[83], have also been reported as indirect markers of ferroptosis. However, these findings can be caused by ferroptosis-independent oxidative stress events[84,85].

Similar limitations apply to the analysis of gene signatures using omics data. Many genes involved in the ferroptosis pathway overlap with those of the oxidative stress response. Indeed, NFE2-like bZIP transcription factor 2 (NRF2), a master transcription factor of the antioxidant response, transcriptionally regulates the gene expression involved in ferroptosis regulation, contributing to GSH biosynthesis (for example, *SLC7A11* and *GCLC*), iron metabolism (*FTH1* and *HMOX1*) and CoQ reduction (*AIFM2*)[86,87]. Therefore, even if transcriptional profiling indicates correlative changes in several ferroptosis signature genes, this finding alone cannot conclude the actual occurrence of ferroptosis and may only reflect an adaptive response against oxidative stress in general.

Analysing the regulators of ferroptosis

GPX4, SLC7A11, FSP1 and ACSL4 are the best-studied regulators of ferroptosis, but improper investigation of their function or assessment of their expression levels may lead to misinterpretations. Here we focus on key aspects to consider when evaluating these molecules in ferroptosis studies (Supplementary Figs. 1 and 2). Moreover, we summarize the available antibodies against these proteins and the single-guide RNA sequences for generating knockout cells using the CRISPR–Cas9 system to yield reliable experimental results (Supplementary Tables 1 and 2).

GPX4

Isoforms and functions of GPX4. GPX4 exists in three isoforms with varying functions: the short form, the mitochondrial matrix form and the nuclear form. Each isoform is derived from its distinct transcription initiation start site and has distinct physiological roles (Supplementary Fig. 1). Notably, only the short form (also referred to as cytosolic or somatic form) is essential to prevent ferroptosis and for embryonic development[88–90]. The mitochondrial matrix form, which has a cognate mitochondrial targeting signal at its N-terminus, has an important role in sperm maturation and the development of photoreceptor cells[91–93] but does not contribute to ferroptosis regulation, owing to its restricted expression, mainly in spermatocytes[93]. The longest isoform is the nuclear form, which has an N terminus encoded by an alternative exon and contains a nuclear localization sequence, along with arginine-rich and lysine-rich domains that facilitate binding to sperm DNA. Nuclear GPX4 is not involved in ferroptosis prevention as it is exclusively present in late spermatids and contributes to sperm chromatin condensation[94,95].

Recommendations for studying GPX4. Despite GPX4 being the most studied gene in ferroptosis research, its expression levels and activity are, at times, not adequately evaluated. To properly assess the expression and function of the GPX4, it is necessary to consider that GPX4 is a selenoprotein. Mammalian cells require a unique machinery to incorporate selenocysteine into selenoproteins[96]. Although selenocysteine is encoded by the stop codon UGA, this machinery allows the successful decoding of UGA as selenocysteine. Given that the selenocysteine incorporation during translation is the rate-limiting step determining the protein expression level of GPX4 (ref. 97), *GPX4* mRNA levels do not necessarily correlate with the protein level. Indeed, selenium supplementation in the culture medium promotes GPX4 translation[98]. Therefore, to investigate the possible contribution of GPX4 to ferroptosis regulation in a given context, GPX4 protein expression, rather than mRNA expression, should be evaluated. To directly assess the degree of selenium incorporation into selenoproteins, including GPX4, a ^{75}Se-radioisotope labelling assay can be used[4]. In overexpression studies, the GPX4 expression construct must contain the 3′-UTR selenocysteine insertion sequence element and the protein-coding sequence to allow the expression of proper selenocysteine-containing GPX4 (ref. 99). GPX4 enzymatic activity can be measured by assessing the GSH-dependent reduction of phospholipid hydroperoxides or cholesterol hydroperoxides by mass spectrometry[99,100] or indirectly monitored by measuring NADPH consumption[99,101]. The latter is achieved through GSH-reductase-coupled reduction, in which NADPH is consumed as GSH is regenerated during the reduction of substrate hydroperoxides by GPX4 (Supplementary Fig. 2). To measure GPX4-specific activity, lysates of whole cells or tissues are not suitable samples, owing to the presence of other oxidoreductases. Therefore, purified GPX4 from mammalian cells or tissues is recommended to assess native GPX4-specific activity accurately[99,101].

In most cell lines, the deletion of *GPX4* triggers ferroptosis, necessitating the supplementation of a ferroptosis inhibitor in the culture medium to maintain *GPX4*-knockout cells. Notably, some cancer cell lines exhibit high resistance to GPX4 inhibitors and can even proliferate after *GPX4* deletion[27]. In these cells, alternative anti-ferroptosis pathways are responsible for survival. Similarly, the lethality of *GPX4*-deficient cells can be prevented by overexpressing other protective proteins such as FSP1, GCH1, MBOAT1 and MBOAT2 (refs. 26,29,49) or deleting *ACSL4* (ref. 39).

System x_c^-

Function of system x_c^-. System x_c^- is a heterodimer composed of two solute carriers, SLC7A11 (also known as xCT) and SLC3A2 (ref. 102). To evaluate the role of system x_c^-, it is crucial to recognize the substantial differences in redox conditions between cell culture and in vivo settings. In cell culture, virtually all cysteine in the medium is oxidized to cystine, the oxidized (dimeric) form of cysteine. Thus, *SLC7A11*-knockout is lethal in many cell lines, owing to depletion of cellular cysteine and GSH. By contrast, *Slc7a11*-deficient mice are fully viable[103]. This is because cysteine exists in its reduced form in vivo, such as in plasma and extracellular body fluids, and is transported into cells by neutral amino acid transporters independently of system x_c^- (ref. 103). Moreover, the catabolism of cysteine-rich extracellular proteins, such as albumin, may provide an alternative source of cysteine in the body[104], thereby reducing the dependence of the cells on system x_c^-. Therefore, it is essential to emphasize that findings on the anti-ferroptotic role of system x_c^- obtained from in vitro studies may not directly translate to in vivo settings.

Under in vitro conditions, the maintenance of *SLC7A11*-knockout cells requires the supplementation of β-mercaptoethanol or *N*-acetyl L-cysteine, promoting the supply of cysteine to the cells in

a system x_c^--independent manner[105]. Although ferroptosis inhibitors such as Lip-1 can also prevent cell death in *SLC7A11*-knockout cells, they fail to rescue the phenotype of decreased cell growth caused by cellular cysteine starvation, as cysteine is also needed to synthesize proteins and other metabolites[106]. There are some exceptions, as *Slc7a11*-deficient hepatocytes and macrophages can survive without β-mercaptoethanol, indicating the presence of system x_c^--independent cysteine sources[107,108].

Recommendations for studying system x_c^-. An experimentally crucial point is that the band size of xCT detected by immunoblotting differs from the predicted molecular mass. Although the predicted molecular mass is 55 kDa, the actual band of xCT migrates at approximately 35–40 kDa (refs. 105,109). Several commercially available xCT antibodies detect a nonspecific band at ~55 kDa, requiring essential controls such as *SLC7A11*-knockout cells for validation. It is also important to note that the samples should not be boiled before immunoblotting but rather heated at 50 °C; otherwise, the actual band corresponding to xCT cannot be detected.

FSP1

Isoforms and function of FSP1. Although only one FSP1 isoform is known in humans, two isoforms with distinct C termini are present in rodents (isoforms 1 and 2) (Supplementary Fig. 1). The anti-ferroptosis function has been demonstrated for isoform 1 (ref. 26), but the functional role of isoform 2 remains unclear. Beyond its anti-ferroptotic effects, FSP1 plays a vital role in thermogenesis in brown adipose tissues[110] and the vitamin K cycle, which is essential for blood coagulation[28,111]. Whereas overexpression of FSP1 confers ferroptosis resistance to cells, *AIFM2*-knockout cells are more sensitive to ferroptosis[26,27]. However, unlike *GPX4* and *SLC7A11*-knockout cells, *AIFM2*-knockout cell lines are fully viable as long as GPX4 is functional. *Aifm2*-knockout mice do not show any overt phenotypes, at least under steady-state conditions[28], albeit they show more severe renal damage by IRI[112].

Recommendations for studying FSP1. In addition to CoQ_{10}, several quinone compounds can also serve as reducing substrates for FSP1 (refs. 26,28,113). For a functional assay of FSP1, recombinant FSP1 is incubated with the cofactor NAD(P)H and a substrate, such as resazurin, menadione, CoQ_0 or CoQ_1. FSP1 activity is assessed by monitoring the fluorescence of reduced resazurin or by kinetic measurement of NAD(P)H consumption[26,28,114,115]. When using CoQ_{10} as a substrate, liposomes-based assays are required because of its low solubility in aqueous solutions. As an experimental note, when tagged-FSP1 expression constructs are used, the tag sequence should be placed at the C terminus, because the N-terminal myristoylation site of FSP1 is crucial for membrane localization and ferroptosis prevention[26]. To avoid confusion, it should be noted that another protein, fibroblast-specific protein 1 (also known as S100A4) is also referred to as FSP1.

ACSL4

Isoforms and function of ACSL4. ACSL4 exists in the short and long isoforms (Supplementary Fig. 1), each with a context-dependent pro-ferroptotic function[39,116]. Functionally, both isoforms are nearly identical, but expression profiles may vary. Whereas the human ACSL4 short form (NP_001305439.1) is more abundant, the long form (NP_001305438.1) is restricted to the brain[117–119]. *ACSL4*-knockout cells contain lower levels of PUFAs esterified in phospholipids and are accordingly more resistant to ferroptosis induced by GPX4 inhibitors[39], although these effects can be less pronounced when using system x_c^- inhibitors[116]. Thus, to measure ACSL4 activity, assessing phospholipid profiles, especially the PUFA/MUFA ratios[39,40], is required.

Recommendations for studying ACSL4. Given that the expression profiles of ACSL family members vary across cell types, and their substrate preferences can overlap[120,121], relying solely on expression levels to assess their role in ferroptosis regulation is insufficient. Therefore, analysing downstream lipid composition is crucial to confirm the specific contribution of each ACSL. Thiazolidinedione compounds, such as rosiglitazone (excluding troglitazone owing to its antioxidant function), inhibit ACSL4, decreasing PUFA levels[39]. To assess the impact of these agents on lipid composition, cells should be pretreated with the agents for at least 24 h before inducing ferroptosis, allowing sufficient time for phospholipid remodelling. As some commercial antibodies against ACSL4 also recognize ACSL3 (ref. 48) (Supplementary Table 1), knockout samples are necessary to confirm which band corresponds to the target protein.

Compounds inducing or inhibiting ferroptosis

Pharmacological approaches have been crucial to ascertaining the importance of ferroptosis in physiological and pathophysiological contexts. Although many compounds targeting ferroptosis are available, optimal concentrations, potential off-target effects, in vivo availability and species specificity must be considered. We summarize the commonly used compounds in ferroptosis studies and discuss their limitations and advantages (Table 2).

Ferroptosis suppressors

Radical-trapping antioxidants. Fer-1 and Lip-1 are the most frequently used ferroptosis inhibitors, acting as RTAs[31,122,123]. Whereas Fer-1 is unsuitable for in vivo studies, especially those involving long-term administration, owing to its poor microsomal stability and rapid clearance[59], Lip-1 is suitable for in vivo use[7]. When identifying novel compounds as ferroptosis inhibitors, the potential for 'hidden' RTA activities must be carefully evaluated, as off-target RTA effects frequently occur[50,124,125]. For instance, several lipoxygenase inhibitors, including PD146176 and zileuton, and the MEK inhibitor U0126 suppress ferroptosis by acting as RTAs, independent of their on-target actions[50,63,126]. Although several commonly used assays, such as the DPPH assay, are available to evaluate the antioxidant potential of compounds, they are not ideal for assessing the lipid-centric RTA activity associated with anti-ferroptotic activity. In light of this, the fluorescence-enabled inhibited autoxidation (FENIX) assay was specifically designed to enable the direct quantification of the reactivity of a compound to propagate radicals in a phospholipid bilayer, thereby predicting its anti-ferroptotic potency[63] (Box 2).

Ferroptosis induction

To induce ferroptosis in vitro, it is recommended to first genetically target *GPX4* or use GPX4 inhibitors and/or xCT inhibitors. Findings from one approach should be corroborated by those obtained using different methods to induce ferroptosis. This is because ferroptosis triggered by different mechanisms may result in distinct phenotypes, potentially due to off-target effects of inducers or epiphenomena occurring alongside ferroptosis, such as endoplasmic reticulum stress induced by xCT inhibition or cysteine deprivation[80,127].

Table 2 | Inhibitors and inducers commonly used for ferroptosis studies

Compounds	In vivo use	Optimal dose	Timing of triggering cell death in vitro	Considerations and limitations (optimal concentrations and assay timing can vary depending on the cell type and conditions)
Ferroptosis inhibitors				
Fer-1	Not recommended	≤10 μM	n.a.	Not recommended for in vivo studies, especially those involving long-term administration, owing to low microsomal stability
Lip-1	Yes	≤1 μM (in vitro), 10–20 mg kg^{-1} (in vivo, i.p.)	n.a.	In vivo applicable and bioavailability >50%, high doses show unspecific toxicity
Ferroptosis inducers				
GPX4 inhibitors				
(1*S*,3*R*)-RSL3	No	≤3 μM	6–24 h	RSL3 also targets other selenoproteins besides GPX4. A high concentration of RSL3 (>5 μM) shows cell toxicity that cannot be prevented by ferroptosis inhibitors; unsuitable for systemic in vivo use
ML210	No	≤30 μM	6–24 h	Less off-target effect than RSL3; prodrug; unsuitable for systemic in vivo use
Compound **28** (GPX4-IN-2)	Yes	≤1 μM, 60 mg kg^{-1} (in vivo, p.o.)	6–24 h	Orally bioavailable GPX4 inhibitor; covalent binding via its alkyne
System x_c^- inhibitor				
Erastin	No	≤30 μM	12–48 h	Not suitable for in vivo use; an irreversible system x_c^- inhibitor that also induces endoplasmic reticulum stress due to cysteine deprivation
Imidazol ketone erastin	Yes	≤30 μM	12–48 h	Improved metabolic stability and solubility as compared with erastin
GSH depletion				
BSO	Yes	≤1 mM, 20 mM in drinking water (in vivo, p.o.)	60–72 h	γ-GCS inhibitor. BSO alone hardly triggers cell death in many cancer cell lines. Resistance mechanisms frequently occur in vivo during prolonged treatment
FSP1 inhibitors				
iFSP1	No	≤10 μM	6–24 h with GPX4 inhibition	Inhibits only human FSP1, and a high concentration of iFSP1 (>10 μM) shows off-target toxicity; not suitable for in vivo use
FSEN1	Not determined	≤10 μM	6–24 h with GPX4 inhibition	Inhibits only human FSP1 and may be applicable for in vivo use, but appropriate dose levels to be determined
viFSP1	No	≤30 μM	6–24 h with GPX4 inhibition	Inhibits FSP1 of different organisms, including human and mouse
icFSP1	Yes	≤30 μM, 50 mg kg^{-1} (in vivo, i.p.)	6–24 h with GPX4 inhibition	Inhibits only human FSP1 by inducing phase separation of myristoylated FSP1 and is in vivo applicable by intraperitoneal injection
Others				
FINO2	No	≤30 μM	6–24 h	Endoperoxide-containing 1,2-dioxolane and directly oxidizes ferrous iron and indirectly inhibits GPX4 activity
FIN56	No	≤30 μM	6–24 h	Degradation of GPX4 and suppression of CoQ_{10} synthesis

i.p., intraperitoneal administration; n.a., not available; p.o., oral administration.

GPX4 knockout and GPX4 inhibitors. Genetic *GPX4* deletion is a reliable method for studying ferroptosis, which can exclude possible off-target effects of pharmacological inducers. This can be accomplished, for instance, by using tamoxifen-inducible *Gpx4*-knockout cells (such as Pfa1 cells)[23] with the caveat that tamoxifen can alter lipid/sterol biosynthesis and membrane lipid composition. The removal of ferroptosis inhibitors from *GPX4*-knockout cells maintained in a medium containing the inhibitor can also be used for ferroptosis induction[128].

Most GPX4 inhibitors, including (1*S*,3*R*)-RSL3 (RSL3), covalently react with the selenocysteine residue of GPX4 owing to their electrophilic warhead, irreversibly inactivating the enzyme. However, these covalent GPX4 inhibitors also react with selenocysteine in other selenoproteins, affecting their functions[129,130]. In addition, attention should be paid to whether GPX4 inhibitors are used in the appropriate concentration range where ferroptosis inhibitors can rescue the induced cell death. High concentrations of RSL3 (more than 5 μM) can cause nonspecific cell toxicity insensitive to ferroptosis inhibitors. ML210 and JKE-1674 are GPX4 inhibitors designed with increased selectivity for GPX4 and are recommended for in vitro experiments[131]. Owing to its poor metabolic stability, RSL3 cannot be used for systemic administration in animal models[10]. Thus, in vivo available GPX4 inhibitors have recently been developed. Compound **18** and compound **28** inhibit GPX4 through intraperitoneal and oral administration, respectively, in tumour animal models and showed synergistic tumour-suppressing effects when combined with anticancer drugs[48,132]. PACMA31 has also been reported as a GPX4 inhibitor with potential

application in vivo[133,134]. N6F11 and dGPX4@401-TK-12 degrade GPX4 in tumour models in a proteasome-dependent manner[135,136]. Further investigation is needed to determine whether these inhibitors can be reliably applied in different models and whether their antitumour effects are indeed due to GPX4 inhibition.

To evaluate the reactivity of a covalent GPX4 inhibitor towards GPX4 in specific tissues, mass spectrometry analysis or immunoblotting analysis can be used. After treatment with the inhibitors, the band corresponding to GPX4 should shift slightly upward in western blotting[48,99] (Supplementary Fig. 2). GPX4-inhibitor adducts can also be detected by mass spectrometry analysis[131]. While RSL3 inhibits the enzymatic activity of affinity-purified human GPX4 collected from mammalian cells[99], a recent study reported that it unexpectedly failed to inhibit the enzymatic activity of recombinant selenocysteine-containing GPX4 protein expressed and purified from bacteria[137]. This finding suggests that yet-unrecognized cofactors or post-translational modifications present in the native condition might be required for RSL3 and other GPX4 inhibitors to effectively inhibit GPX4 (ref. 101).

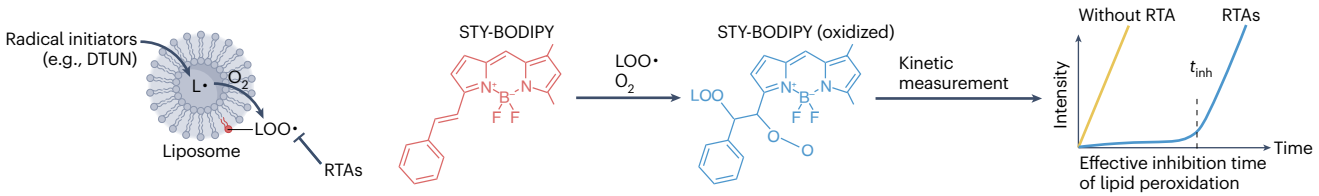
xCT inhibitors and cysteine starvation. Erastin and its derivatives are widely used as xCT inhibitors, causing ferroptosis[1]. Similarly, cysteine starvation by culturing cells in a cysteine-free medium triggers ferroptosis[138]. When using erastin, it is essential to consider that some cells, such as mature neurons, exhibit low xCT expression levels[139]. Moreover, x_c^- inhibition and cysteine starvation deplete not only GSH but also other cysteine-containing metabolites. These effects cause an integrated stress response and mitochondrial stress via glutaminolysis independently of ferroptosis[78,140]. As erastin is unsuitable for in vivo use, imidazole ketone erastin has been developed to improve its metabolic stability and solubility[141]. Imidazole ketone erastin can reduce tumour growth in a xenograft mouse model[141]; however, further experimental evidence is needed to determine whether the antitumour effect is solely due to ferroptosis or a non-ferroptotic mechanism related to cysteine deprivation. Sulfasalazine, a clinically approved drug for treating rheumatoid arthritis, is known to inhibit system x_c^- (ref. 142). However, the therapeutic window for its ferroptosis-inducing effect is small, and the exact mechanism of the antitumour effect of sulfasalazine, either by induction of ferroptosis or by suppression of inflammatory responses, remains uncertain. Thus, it is not recommended for in vivo use as a ferroptosis inducer. Earlier findings suggested that sorafenib, a multikinase inhibitor used in cancer treatment, can induce ferroptosis by inhibiting system x_c^- (ref. 78). However, a recent study showed that the inhibitory activity on system x_c^- of sorafenib is weak and insufficient to induce ferroptosis in most cell lines[105].

GSH depletion. L-buthionine sulfoximine (BSO) is a long-known inhibitor of γ-glutamylcysteine-synthetase, the rate-limiting step in GSH biosynthesis[143]. BSO blocks the biosynthesis of GSH, triggering ferroptosis, at least in certain cultured cells. Nevertheless, BSO treatment alone is often insufficient to induce ferroptosis in many cancer cell lines[144]. This is probably because GPX4 can utilize other thiol-containing molecules, such as cysteine, instead of GSH[145]. Although BSO is bioavailable in animal models[146,147], treatment with BSO alone is insufficient to induce ferroptosis in vivo and may lead to rapid drug resistance[147]. APR-246 (eprenetapopt), an anticancer drug in clinical trials, induces ferroptosis by depleting GSH, at least in vitro[148]; however, the putative in vivo anticancer effect due to ferroptosis induction requires further investigation.

FSP1 inhibitors. FSP1 inhibitors show synergistic effects with GPX4 inhibition in various cancer cells[26,114,149]. By contrast, FSP1 inhibitors alone are insufficient to induce ferroptosis in cultured cells as long as GPX4 remains functional, except in conditions where the cells are highly prone to ferroptosis, such as ferroptosis-sensitive cells seeded at very low cell densities[149]. When using FSP1 inhibitors, attention should be paid to species specificity and their suitability for in vivo use. The first reported FSP1 inhibitor, iFSP1, only inhibits human FSP1 and is unsuitable for in vivo studies owing to limited metabolic stability[26,114,115]. FSEN1 is a human FSP1 inhibitor with reasonable pharmacokinetic properties; however, its in vivo activity and efficacious dose levels have yet to be verified[150]. viFSP1 is a multi-species FSP1 inhibitor, inhibiting both human and mouse FSP1, but it is unstable under in vivo conditions[114]. icFSP1 is the first in vivo-applicable human-specific FSP1 inhibitor with

Box 2 | Assessment of RTAs using FENIX assay

In the context of ferroptosis inhibition, radical-trapping antioxidants (RTA) activity depends on the kinetic competition between an inhibitor and an oxidizable lipid for a chain-propagating phospholipid peroxyl radical. Thus, an assay based on this competition is essential to the characterization of a ferroptosis inhibitor as an RTA — or not. The fluorescence-enabled inhibited autoxidation (FENIX) assay was designed with this in mind[63]. As this assay directly quantitates a compound's reactivity to propagating radicals in a phospholipid bilayer, it can predict anti-ferroptotic potency. In the FENIX assay (see the figure), a lipid-soluble hyponitrite (DTUN) is added into liposomes of polyunsaturated phospholipids to initiate lipid peroxidation at a well-defined rate, and lipid peroxidation is monitored indirectly by the increase in fluorescence of oxidized STY-BODIPY (λ_{ex} = 488 nm, λ_{em} = 518 nm). Added RTAs slow STY-BODIPY oxidation by trapping propagating phospholipid-derived radicals, and the radical-trapping kinetics can be determined directly from the inhibited reaction rate using standard formulae[63]. In the figure, t_{inh} denotes the inhibited period, defined by the intersection of the lines of best fit to the inhibited and uninhibited phases.



a unique mechanism of action, reversibly inactivating FSP1 by triggering phase separation and detaching FSP1 from the plasma membrane[149]. Interestingly, some inhibitors of dihydroorotate dehydrogenase, such as brequinar, also inhibit FSP1 at relatively high concentrations[88]. These unexpected findings result from careful observation of potential off-target effects of pharmacological inhibitors.

Experimental factors influencing susceptibility to ferroptosis in vitro. It is important to note that various experimental factors influence the susceptibility of cells to ferroptosis in cell culture experiments (Fig. 3). For example, high cell density can render cells more resistant to ferroptosis[12,88,151]. Differences between fetal bovine serum batches can influence ferroptosis susceptibility owing to different amounts of selenium (in the form of selenoprotein P)[98,152], vitamin E, other antioxidants and lipid constituents[153]. Growth media nutrients, such as glucose, pyruvate and glutamine, can also influence susceptibility to ferroptosis by affecting metabolic pathways and cell proliferation rates[154–157]. Notably, in the medium, glutamine and several other amino acids are essential for triggering ferroptosis induced by erastin or cysteine deprivation[125,140,154]. The absence of phenol red, commonly contained in culture media as a pH indicator, reduces ferroptosis sensitivity by influencing the expression of ferroptosis regulator genes[158]. Therefore, these experimental factors are critical to interpreting and reproducing results.

Some cell lines are highly resistant to ferroptosis inducers. In such cases, a combination of ferroptosis inducers with different mechanisms of action or supplementation of culture media with PUFAs such as arachidonic acid may increase the ferroptosis sensitivity of the cells[42,159]. Lastly, when assessing the impact of an inhibitor or inducer of ferroptosis in cellular experiments, examining the effects across multiple concentrations spanning a broad range is imperative. This approach is essential to avoid underestimating or overestimating effects by focusing on only a single compound concentration.

Ferroptosis animal models

Redox conditions differ substantially between cell or organoid cultures and in vivo settings. Thus, it is crucial to translate in vitro findings to the in vivo context using animal models to validate the mechanisms involved in ferroptosis.

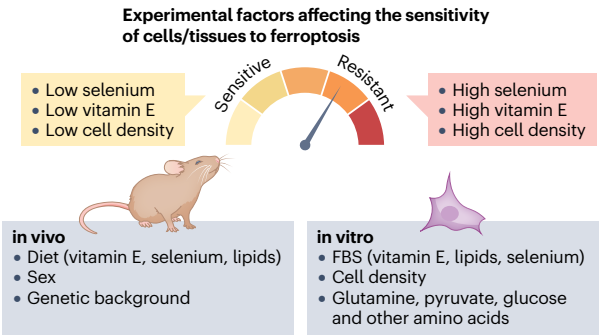


Fig. 3 | Experimental factors affecting the sensitivity of cells to undergo ferroptosis. In vivo, diet (for example the amount of vitamin E, selenium and lipids contained in chow), sex and genetic background of mouse strains influence the vulnerability of tissues to ferroptosis. In vitro, the composition of fetal bovine serum (FBS) (for example the amount of vitamin E, lipids and selenium), cell density and other medium components (for example glucose, glutamine, other amino acids, pyruvate and phenol red) can affect the susceptibility of cultured cells to ferroptosis.

Genetically engineered models

Conditional *Gpx4*-knockout mice are considered bona fide ferroptosis models and are valuable for studying ferroptosis. Whereas whole-body knockout of *Gpx4* is embryonic lethal[23,89,160], tissue-specific conditional deletion of *Gpx4* causes cell death and dysfunction in the target tissues[161] (Supplementary Table 3). Conditional whole-body deletion of *Gpx4* (except brain) results in acute kidney injury and associated mortality[7]. Conditional knockout of *Gpx4* in neurons results in neurodegeneration and behavioural dysfunction[162,163]. As ferroptosis inhibitors mitigate tissue damage inflicted by conditional *Gpx4* deletion[7], *Gpx4*-deficient mice are available for evaluating the in vivo effects of anti-ferroptotic compounds. When assessing the phenotype of conditional *Gpx4*-knockout mice, an important point to consider is the amount of dietary vitamin E. For instance, high vitamin E intake rescues ferroptotic tissue damage induced by deletion of *Gpx4* in mouse hepatocytes, endothelial or T cells[28,164–166]. Therefore, these models require the use of a vitamin-E-deficient diet for ferroptosis induction. Besides, mice engineered with other ferroptosis-related genes, such as *Slc7a11*, *Aifm2* and *Acsl4*, provide valuable information on their physiological importance (see Supplementary Table 3 for a summary).

Other models of tissue damage

Animal models of tissue damage induced by surgical procedures such as ischaemia induction or chemical interventions with toxic substances have also been used in ferroptosis research. Compelling evidence has demonstrated that ferroptosis plays a crucial role in IRI[7,17,167]. Ferroptosis inhibitors mitigate organ damage in IRI models[7,17]. However, other cell death modalities and necroinflammatory processes may be involved in the pathogenesis of IRI, making it challenging to identify the exact contribution of ferroptosis to IRI. Numerous other animal models of organ damage have also been used in ferroptosis studies. Representative models include acetaminophen-induced liver injury[168], folic acid-induced nephropathy[169] and experimental autoimmune encephalomyelitis[170]. However, it can be challenging to ascertain how much ferroptosis contributes to pathogenesis in each model. Thus, the implication of ferroptosis in each condition must be carefully evaluated as highlighted above, in the section on ferroptosis detection.

Tumour animal models

Tumour animal models, such as xenografts, syngenic models and chemically induced and genetically engineered mouse models, have been used to assess the antitumour effects of ferroptosis-inducing agents[135,149]. In these studies, it is crucial to discriminate whether the antitumour effect of a compound is mediated by its ferroptosis-inducing property or by alternative off-target effects or nonspecific toxicity. To ascertain whether the antitumour efficacy of a compound is indeed attributable to ferroptosis induction, experiments have been conducted to show whether the antitumour effect can be reversed by coadministration of a ferroptosis inhibitor. However, it must be acknowledged that the observation of diminished antitumour efficacy by ferroptosis inhibitor treatment does not conclusively confirm ferroptosis induction in the tumour because ferroptosis inhibitor effects might be confounded by enhancing tumour growth through their RTA activity. Therefore, excluding this possibility by incorporating a control group treated with the ferroptosis inhibitor alone can be important.

Experimental factors affecting ferroptosis in animal studies

In animal experiments, various factors such as diet, sex and genetic background can affect the ferroptotic phenotype, such as tissue

Glossary

3′-UTR selenocysteine insertion sequence element
A stem loop-like secondary structure located in the 3′-untranslated region (UTR) of selenoprotein mRNAs, which affords decoding of UGA as a selenocysteine during translation.

4-hydroxy-2-nonenal
(4-HNE). An α,β-unsaturated hydroxyalkenal produced downstream of lipid peroxidation, which can react with specific amino acid residues (that is, histidine, cysteine and lysine) in proteins to generate Michael adducts in cells and tissues.

C57BL/6J×129S6/SvEv mixed background
F_1 mice with mixed background strains derived from two inbred strains of C57BL/6J and 129S6/SvEv mice.

Epilipidomic studies
A method to analyse a subset of natural lipidome formed by lipid modifications (for example oxidation) required to regulate complex biological functions.

Fenton reaction
A reaction in which iron or other transition metals catalyse the disproportionation of hydrogen peroxide into highly reactive hydroxyl radical and a hydroperoxide ion.

***Gpx4* U46C mutant mice**
Transgenic mice carrying a targeted mutation of the catalytically active site selenocysteine (U46) to Cys of *Gpx4*.

Ischaemia-reperfusion injury
(IRI). Transient ischaemia, followed by reperfusion, generates oxygen-centred radicals that trigger extensive cell death and inflammatory responses in the affected organs, leading to acute tissue damage.

Karyolysis
Complete dissolution of nuclear components in a dying cell.

Lipid hydroperoxides
The primary products of lipid peroxidation, resulting from propagation by H-atom transfer, can be further reduced or oxidized to yield radicals that initiate additional lipid peroxidation and/or produce secondary reactive lipid aldehydes.

Lipid peroxidation
Generally refers to the autoxidation of lipids, a free radical chain reaction in which oxygen is incorporated into hydrocarbons to form peroxides, resulting in the production of lipid hydroperoxides when the chain reaction is propagated by H-atom transfer from a lipid or lipid peroxides when propagated by addition to a lipid.

Necroptosis
A regulated, necrotic cell death modality mediated by receptor-interacting protein kinase 3 (RIPK3) activity and ensuing pore formation by mixed-lineage kinase domain-like pseudokinase.

Parvalbumin-expressing GABAergic interneurons
The principal inhibitory interneurons in the brain cortex.

Phospholipids
Amphiphilic molecules with a hydrophilic head containing a phosphate group (for example phosphocholine and phosphoethanolamine) and two hydrophobic fatty acid tails esterified to the glycerol moiety, which are key component of cell membranes.

Plasmalogens
A unique class of phospholipids containing a vinyl ether bond at the sn-1 position, with its synthesis initiated in peroxisomes and endoplasmic reticulum.

Pyknosis
Condensation of the nucleus and chromatin, often observed in cells undergoing cell death.

Radical-trapping antioxidants
(RTAs). Compounds that react with radical chain-propagating radicals to form non-propagating radicals[31,123].

Regulated necrosis
A type of programmed cell death involving plasma membrane rupture and including various modalities, such as ferroptosis and necroptosis.

Selenocysteinyl-tRNA
A specific tRNA responsible for incorporating selenocysteine into selenoproteins during translation.

Selenoproteins
An exclusive group of selenium-containing proteins in which selenocysteine, the 21st proteinogenic amino acid, is cotranslationally incorporated into the protein and is usually present at the catalytically active site.

Tetrahydrobiopterin
(BH_4). A redox-active cofactor for several biosynthetic enzymes, also functioning as an RTA by reacting with peroxyl radicals, yielding oxidation products such as dihydrobiopterin (BH_2), which can be reduced back to BH_4 by the enzyme dihydrofolate reductase.

Thiol-containing molecules
Organic molecules that contain a sulfhydryl group.

damage and tumour regression (Fig. 3). In addition to the amount of dietary vitamin E, a low selenium diet increases susceptibility to ferroptosis in tumour experiments[98]. Female mice are less sensitive to ferroptotic stress in the kidney than male mice[171]. Sex hormone signalling also influences ferroptosis-related pathologies and variations in drug effects by regulating the expression of the phospholipid-modifying enzymes MBOAT1 and MBOAT2 (ref. 49). The genetic background also affects ferroptosis. Whereas homozygous *Gpx4* U46C mutant mice mice present with profound seizures due to the loss of parvalbumin-expressing GABAergic interneurons in a mixed genetic background of C57BL/6J and 129S6/SvEv, the same mice on a congenic C57BL/6J background die in utero[4]. As C57BL/6J strains are known to be deficient in nucleotide nicotinamide transhydrogenase, an enzyme that regulates mitochondrial redox balance[172], it is crucial to consider that any phenotype observed in a C57BL/6J strain occurs under nucleotide nicotinamide transhydrogenase-deficient conditions. These experimental factors should be noted when reporting the results of the animal experiments targeting ferroptosis. Moreover, conclusions from different mouse strains, experimental conditions and laboratories will yield more robust results.

Conclusions and perspectives

We compiled the recommendations presented above to promote and enable the rapidly growing field of ferroptosis research. Several important challenges remain in the study of ferroptosis. These include (1) identifying a molecular biomarker capable of precisely and reliably detecting ferroptotic cells; (2) determining the principal molecular contributors to plasma membrane rupture during ferroptosis; (3) determining the precise impact of ferroptosis on inflammation and immunomodulation in neighbouring cells and tissues; and lastly, (4) exploring potential physiological roles of ferroptotic cell death[173,174] beyond its pathophysiological involvement. Clarifying the complexity of ferroptosis and exploiting its therapeutic potential will require appropriate experimental and systematic approaches.

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E.M., T.N., A.W. and M.C. wrote the article. S.D., B.P., M.F., D.A.P., J.P.F.A. and S.J.D. contributed substantially to the discussion of the content. All authors reviewed and edited the manuscript before submission.

Competing interests

M.C. and B.P. are cofounders and shareholders of ROSCUE Therapeutics GmbH. D.A.P. is a cofounder and shareholder of Prothegen Inc. S.J.D. holds patents related to ferroptosis. M.C., B.P. and T.N. have filed patents for some of the compounds described herein.

Additional information

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