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Review

Beyond ferrostatin-1: a comprehensive review of ferroptosis inhibitors

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Ferroptosis is an iron-catalysed form of regulated cell death, which is critically dependent on phospholipid peroxidation of cellular membranes. Ferrostatin 1 was one of the first synthetic radical-trapping antioxidants (RTAs) reported to block ferroptosis and it is widely used as reference compound. Ferroptosis has been linked to multiple diseases and the use of its inhibitors could have therapeutic potential. Although, novel biochemical pathways provide insights for different pharmacological targets, the use of lipophilic RTAs to block ferroptosis remains superior. In this Review, we provide a comprehensive overview of the different classes of ferroptosis inhibitors, focusing on endogenous and synthetic RTAs. A thorough analysis of their chemical, pharmacokinetic, and pharmacological properties and potential for *in vivo* use is provided.

Highlights

Ferroptosis is an iron-catalysed form of regulated cell death which is critically dependent on phospholipid peroxidation of the cell membrane.

Inhibiting ferroptosis has therapeutic potential in various diseases (ischemia–reperfusion injury, iron toxicity, neurological disorders, multiorgan disease, infarction, and stroke).

The exponential growth in research around ferroptosis during the past few years has unveiled different biochemical pathways and the potential of novel pharmacological targets.

Among the published ferroptosis inhibitors, lipophilic **RTAs** are currently recognized as the main strategy to block phospholipid peroxidation. Recently, RTAs with a right balance between potency and pharmacokinetic properties have been discovered, enabling successful *in vivo* proof of concept in different disease models.

Ferroptosis, a druggable form of regulated necrosis

In the past 20 years, new forms of regulated necrosis have been discovered alongside apoptosis [1]. The term ferroptosis was formally introduced in 2012 by the Stockwell Laboratory, defining it as an iron-dependent nonapoptotic type of cell death that is characterized by accumulation of phospholipid peroxides and regulated by multiple cellular metabolic pathways [2]. The benchmark ferroptosis inducing compounds, erastin and RAS-selective-lethal-3 (RSL3), were already described in the pre-ferroptosis era to induce an iron-dependent oxidative form of cell death [3,4]. In that period, Conrad and coworkers identified a previously unknown cell death pathway caused by **glutathione peroxidase (GPX)4** (see Glossary) inactivation. This pathway, which leads to neurodegeneration in mice, can be inhibited by α-tocopherol, an isoform of vitamin E [5].

The first synthetic ferroptosis inhibitor reported in literature was ferrostatin-1, which was extensively used as a reference compound in the past decade [2]. The ferroptosis field experienced an exponential growth in the past few years, with a significant increase in primary research. Although research mainly focused on ferroptosis induction for cancer therapy, ferroptosis inhibition remains a promising target for the prevention and management of diverse diseases such as ischemia–reperfusion injury or iron toxicity, neurological disorders, single or multiorgan injury, infarction, and stroke [6–8]. Therefore, several ferroptosis inhibitor classes have been developed; each with its own specific properties. While a substantial number of reviews on the different ferroptosis inducers and emerging regulatory mechanisms have been reported, less is described about ferroptosis inhibitors and their classification [6,9,10]. Following the review by Friedmann Angeli *et al.* in 2017, we summarize the most recent progress that has been published in the ferroptosis inhibitor field to date [11].

This Review provides a brief overview of the main regulatory mechanisms of ferroptosis involved in the phospholipid peroxide formation (Figure 1). It particularly highlights the development of ferroptosis inhibitors, placing significant emphasis on RTAs (Box 1), which are recognized as the pivotal strategy to tackle phospholipid peroxidation. We discuss the five established

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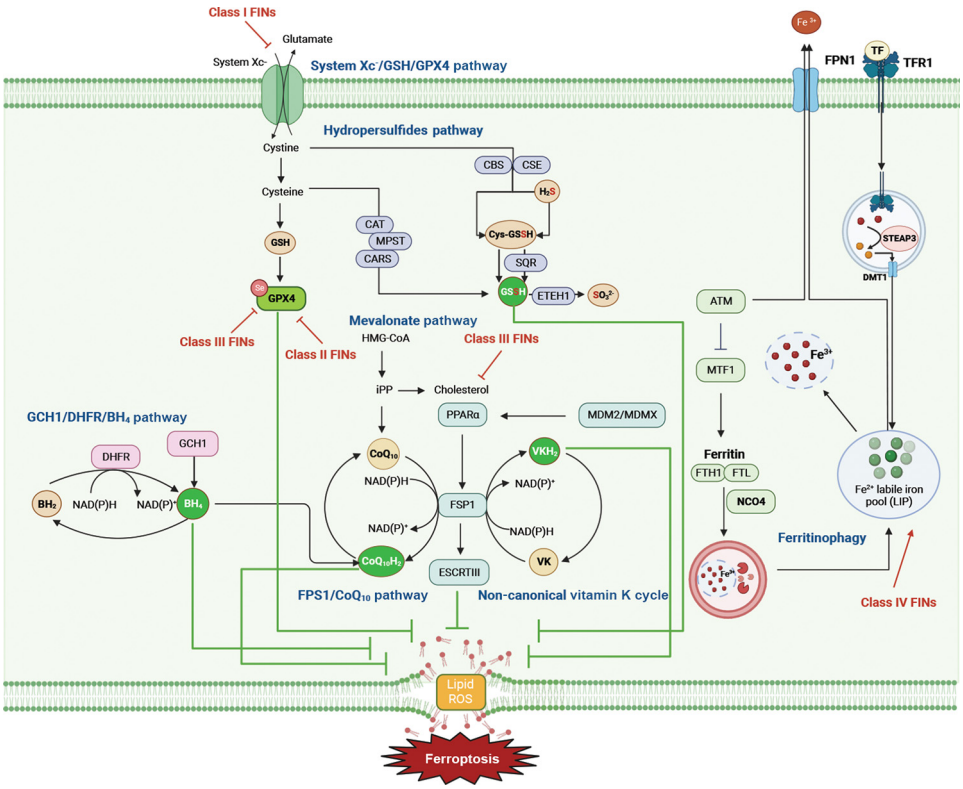

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Figure 1. Overview of the main endogenous regulatory pathways. System Xc-/GPX4 pathway: the system Xc- exchanges intracellular glutamate and extracellular cystine in a 1:1 ratio. From cystine, glutathione (GSH) is synthesized and available as a substrate for glutathione peroxidase 4 (GPX4), which is responsible for the antioxidant defense system limiting phospholipid peroxidation. Hydropersulfides pathway: glutathione hydropersulfides (GSSHs) are able to trap lipid reactive oxygen species (ROS) and can be obtained from CysSSH [generated through cystathionine β synthase (CBS) and cystathionine-γ-lyase (CSE)] or from cysteine [generated from sulfurtransferase (MPST), cysteine aminotransferase (CAT), and cysteinyl-tRNA synthetase (CARS)]. GSSH can be degraded by persulfide dioxygenase (ETHE1). GCH1/DHFR/BH_4 pathway: polyunsaturated fatty acid (PUFA) remodeling in the cell membrane is controlled by tetrahydrobiopterin (BH_4), which is obtained from dihydrobiopterin (BH_2) by reduction through dihydrofolate reductase (DHFR). GTP cyclohydrolase 1 (GCH1) is the rate-limiting enzyme for the synthesis of BH_4. BH_4 can also induce coenzyme Q_{10} (CoQ_{10}) synthesis as an additional mechanism to block ferroptosis. Mevalonate pathway: 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) is converted to the intermediate isopentenyl pyrophosphate (IPP), a common precursor of cholesterol and CoQ_{10}. FSP1/CoQ_{10} pathway: ferroptosis suppressor protein 1 (FSP1) can reduce CoQ_{10} to $CoQ_{10}H_2$, able to scavenge phospholipid peroxides. The antiferroptotic activity of FSP1 is also linked with the expression of endosomal sorting complex required for transport III (ESCRT III), which can promote membrane remodeling. Inhibition of murine double minute 2 and X (MDM2 and MDMX) (p53 suppressor proteins) can also increase FSP1 protein and CoQ_{10} levels. Noncanonical vitamin K cycle mediated by FSP1: FSP1 is responsible for the reduction of vitamin K (VK) to its hydroquinone (VKH_2), a potent radical-trapping antioxidant and inhibitor of (phospho)lipid peroxidation. Iron regulatory pathway: transferrin is responsible for the intake of Fe^{3+} which is reduced into a pool of Fe^{2+} (labile iron pool; LIP) by the metalloreductase six-transmembrane epithelial antigen of the prostate 3 (STEAP3). LIP can be exported through ferroportin-1 (FPN1) or stored in ferritin complexes under the Fe^{3+} form. The different classes of ferroptosis inducers (FINs) are reported in the glossary. The figure was created using BioRender.com.

endogenous RTAs [vitamin E, coenzyme Q_{10} (CoQ_{10}), tetrahydrobiopterin (BH_4), vitamin K (VK), and hydropersulfides (RSSHs)] and their biochemical pathways, and the main synthetic RTAs documented in the literature to date based on their chemical and pharmacological/pharmacokinetics aspects, mechanisms of action, and application in different disease models [12]. We present a critical evaluation of non-RTA inhibitors (perceived as more druggable due to their protein target) in comparison to RTAs.

Glossary

2,2-Diphenyl-1-picrylhydrazyl (DPPH): chemical compound widely used in the assay for the assessment of the scavenging capacity of anioxidants towards radicals.

Bond dissociation enthalpy (BDE): can be defined as the standard change in enthalpy when a bond is cleaved by homolytic fission and as such is a measure of the strength of that chemical bond [90] In ferroptosis and especially to compare the different synthetic and endogenous RTAs, this parameter is calculated for the specific N-H (or O-H bond as in the case of vit E) since the H-atom transfer is involved in the neutralization of lipid peroxyl radicals [27].

Ferroptosis inducers (FINs): molecules that are able to trigger ferroptosis and are divided in different classes by their mechanism of action [9]. Class I FINs act by depleting GSH, class II FINs directly target and inactivate GPX4, class III FINs deplete GPX4 and CoQ_{10}, and class IV FINs induce lipid peroxidation by increasing the LIP.

Fluorescence-enabled inhibited autoxidation (FENIX) assay: a spectrometric assay to quantify the reactivity of putative antioxidants with the phospholipid peroxyl radicals. It is performed in a liposome suspension to mimic lipid bilayers environment developed by Shah *et al.* in 2019 [29].

Glutathione peroxidase 4 (GPX4): a selenoprotein discovered in 1982 by Ursini F. *et al.*, which transforms phospholipid hydroperoxides to lipid alcohols in a glutathione (GSH)-dependent manner. GPX4 is the key antioxidant enzyme in ferroptosis which prevents phospholipid peroxide accumulation in the cell membrane [26].

Kinetic ISOTOPE effect (KIE): the chemical reaction rate where one atom has been substituted with one of its corresponding isotopes. For an enzymatic reaction, it represents the ratio of the reaction rate conducted with the original atom and the isotope [66].

Labile iron pool (LIP): represent a pool of chelatable and redox-active iron (Fe^{2+}) which is crucial for generation of oxygen radicals following the Fenton reaction. as well as catalyzing the phospholipid radical chain reaction [19].

Lipoxygenases (LOXs): iron-containing enzymes involved in arachidonic acid metabolism and synthesis of leukotrienes as inflammatory mediators [23].

Box 1. RTAs are superior ferroptosis inhibitors

The first class of ferroptosis inhibitors are RTAs. The autoxidation process of PUFAs is the driver of ferroptosis. The radical chain reaction can be stopped by RTAs leading to the formation of stable nonradical products and therefore preventing cell membrane disruption. Ideally, a good RTA inhibits ferroptosis by directly terminating the autoxidation chain reaction with high radical-trapping capacity. Among the specific properties, good RTAs should have labile H-atoms with a bond dissociation enthalpy (BDE) lower than ROO-H, and the derived radical from RTA must not propagate the radical chain reaction [90]. RTAs have been shown to be effective to reverse ferroptosis in cells and in *in vivo* models. However, since RTAs target the downstream lipid peroxides, once exhausted, they cannot suppress ferroptosis in the presence of insufficient GSH or G*px4* knockout.

Zilka *et al.* designed styrene autoxidation experiments based on the study of Ingold *et al*. to assess the RTA activity of Fer-1. When the experiment was conducted in chlorobenzene, the antioxidant capacity of Fer-1 was lower than the well-established lipophilic antioxidant vitamin E. This is explained by the predicted BDE of the O-H bond in vitamin E (77.7 kcal/mol) which is lower than the corresponding N-H bond BDE (83.3 kcal/mol) in Fer-1 [27]. In a liposome system mimicking the phospholipids bilayers of the cell membranes, the RTA activity of Fer-1 was significantly better than vitamin E. They hypothesized that the phenol-based antioxidants are generally less effective in lipid bilayers due to the stronger hydrogen bond formation between the phenolic proton and water at the lipid–water interface. Particularly, in Fer-1, the aromatic amine moieties are worse H-bond donors and thus more suited for scavenging radicals in a lipidic environment. In addition, Fer-1 can stabilize radicals since the newly formed radical can trap additional ROS. Similarly and as reported for Fer-1, Lip-1 acts as an RTA with higher potency in phospholipid bilayers than in organic solvent compared to phenolic RTAs [27]. Both Fer-1 and Lip-1 present a similar NH-moiety with almost identical BDE values (83.3 and 82.4 kcal/mol, respectively) able to trap radicals and therefore prevent the propagation of the lipid peroxidation in the phospholipid bilayer [23].

Currently, the FENIX assay developed in the Pratt group is the recognized method to determine the antioxidant activity of lipophilic RTAs [29,64].

Phospholipid peroxidation is the key mechanism of ferroptosis

Peroxidation of phospholipids plays a central role in ferroptotic cell death [12]. The presence and incorporation of **polyunsaturated fatty acids (PUFAs)** into phospholipids is essential for ferroptosis execution allowing **radical** phospholipid peroxidation [13]. Much progress has been made to understand how lipid metabolism regulates ferroptosis and how the radical chain reaction within the phospholipid bilayer leads to the accumulation of phospholipid hydroperoxides (PLOOH) (Figure 2) [14]. The discussion on the exact subcellular location of lipids susceptible to trigger ferroptosis is still ongoing: Stockwell's group identified the endoplasmic reticulum (ER), Jiang the mitochondria, and Rodriguez the lysosomes [15–17]. Pope and Dixon reported that independent from the ferroptosis inducing stimulus, phospholipid peroxides accumulate on the plasma membrane [12]. Probably there is not one unique location, but rather a dependence on the specific pathway that results in phospholipid peroxidation.

Nonenzymatic phospholipid peroxidation

The nonenzymatic generation of phospholipid peroxides is strictly dependent on intracellular iron availability [18]. Iron is abundantly present in our body in the form of reduced ferrous (Fe^{2+}) and oxidized ferric (Fe^{3+}) iron. Fe^{2+} from the **labile iron pool (LIP)** represents the redox-active pool of cellular iron, which can generate **reactive oxygen species (ROS)** directly via the Haber–Weiss reaction followed by the Fenton reaction [19]. These ROS can initiate phospholipid autoxidation and propagate the radical chain reaction through the phospholipid bilayer generating PLOOH. Excessive LIP in the cytosol or autophagic degradation of ferritin (ferritinophagy) are conditions that can start the lipid peroxidation process [20]. While phospholipid peroxidation has been widely studied in the context of ferroptosis, the precise contribution of iron and its sublocalization in ferroptosis still needs to be defined [14].

Enzymatic phospholipid peroxidation

The initiation of lipid peroxidation can also be mediated under certain context by **lipoxygenases (LOXs)**, NADPH oxidase (NOX) enzymes, and cytochrome P450 oxidoreductase (POR) [21].

Monounsaturated fatty acids (MUFAs): have only one unsaturated bond on the alkyl chain, making them inactive towards lipid peroxidation. They are the inactive substrate for ferroptosis and they can suppress lipid peroxidation [12].

Polyunsaturated fatty acids (PUFAs): represent the key substrates for autoxidation. They are characterized by the presence of a weak bis-allylic C-H bond, prone to autoxidation and ferroptosis initiation [25]. Free PUFAs are incorporated into phospholipids, especially phosphatidylethanolamine (PE) and phosphatidylcholine (PC), of the cell membrane by ACSL4 and LPCAT3 [69].

Radicals: atoms, ions, or molecules presenting at least one unpaired **valence electron**.

Radical-trapping antioxidants (RTAs): sometimes also referred as radical trapping agents. The term defined molecules able to trap (block) the reactive radicals preventing chain-carrying reactions.

Reactive oxygen species (ROS): defines molecular oxygen derivatives generated by reduction-oxidation (redox) reactions or electronic excitation. The term ROS includes two-electron no radical forms [H_2O_2, singlet molecular oxygen ($^1O_2$) and organic hydroperoxides (ROOHs)] and free radical forms [superoxide anion radical ($O2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), peroxyl radical ($ROO^{\bullet}$) and alkoxyl radical ($RO^{\bullet}$)] Excessive ROS formation can promote molecular or cellular damage, and stimulate different cell death pathways and inflammation [91].

System Xc-/GSH/GPX4 pathway: transmembrane cysteine/glutamate Na^+-dependent antiporter (system Xc-) imports cystine inside the cytosol followed by its reduction into cysteine, the precursor of GSH, which plays a protective role in cells against ROS [26]. GSH is the co-factor of GPX4.

Valence electron: represent a negatively charged particle in the outer shell of the atom which can be involved in bonds formation.

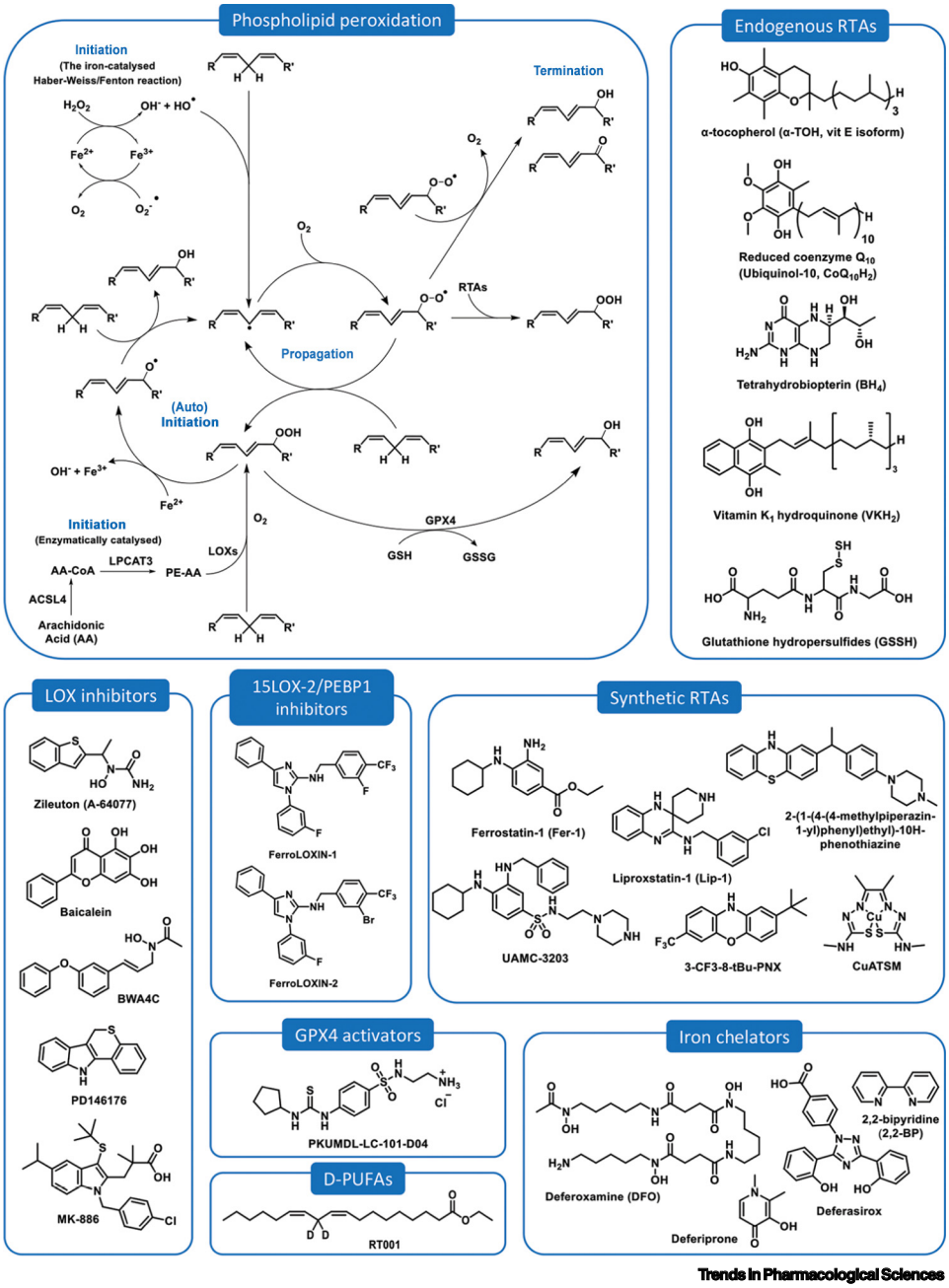

Figure 2. Mechanism of lipid peroxidation and the different classes of inhibitors. Overview of lipid peroxidation with its different steps: initiation, propagation, and termination and examples of the most active ferroptosis inhibitors. Initiation involves extracting the bis allylic hydrogen atom from polyunsaturated fatty acids (PUFAs) within the cell membrane phospholipid bilayer carried out by radicals such as an hydroxyl radical or a phospholipid alkoxyl radical. Consequently, the phospholipid radical (PL•) reacts with molecular oxygen generating phospholipid peroxyl radical (PLOO•), which reacts with another PUFA removing hydrogen and generating the corresponding lipid peroxide (PLOOH). The process can terminate when radical–radical interactions yield a nonradical product. In nature, besides glutathione peroxidase 4 (GPX4), several endogenous radical-trapping antioxidants (RTAs) can interfere with the lipid hydroperoxide process. In the past 10 years, many different synthetic RTAs have been developed by numerous groups working in the field. Additional strategies to block the lipid peroxidation chain process are iron chelators, lipoxygenase (LOX) inhibitors, GPX4 activators and deuterated (D)-PUFA.

How lipid peroxidation occurs on phospholipids through the action of LOXs during ferroptosis is still a mystery. Phosphatidylethanolamine binding protein (PEBP)1 was suggested to associate with 15LOX-2 to acquire specificity for the phosphatidylethanolamine (PE) phospholipids that are key to ferroptosis [22]. Although RTAs can rescue cells from ferroptosis by interfering with the autoxidation process, the inhibition of LOX cannot reverse ferroptotic cell death. Most of the reported LOX inhibitors can inhibit ferroptosis through an RTA off-target effect [23]. Recent studies have suggested that POR can initiate lipid peroxidation by donating electrons (accepted by NADPH) to the electron acceptor CYP450 and CYB5A as downstream effectors [24].

Considering the progress in the field, the discrimination between enzymatic and nonenzymatic phospholipid peroxidation in ferroptotic context starts to be outdated. It is clear now that the specific lipid composition, the presence of certain substrates and especially the balance between redox inactive **monounsaturated fatty acids (MUFAs)** versus PUFAs determine the initiation of phospholipid peroxidation and are relevant for the ferroptosis outcome [25].

Endogenous RTAs

Since oxygen is abundantly present in nature and plays a crucial role in life, it is no surprise that living organisms developed protective mechanisms against so called biological rust.

Besides the **system Xc-/GSH/GPX4 pathway** that inactivates toxic phospholipid hydroperoxides via a two-electron reduction, several endogenous RTAs dampen phospholipid peroxide propagation through trapping radicals using a one-electron reduction mechanism (Table 1) [26]. Lipophilic RTAs generally transfer an H atom to a lipid peroxyl radical, yielding a lipid hydroperoxide and a stable RTA-derived radical [27].

Vitamin E

α-Tocopherol (α-TOH), the most active form of vitamin E, and its analog pentamethyl chromanol (PMC) are well-recognized phenolic RTAs [28]. α-TOH demonstrated potential to inhibit ferroptosis *in vitro* in different cell lines [23]. Phenolic antioxidants are effective inhibitors of ferroptosis and lipid peroxidation, but their activity is limited by the strong H-bond formation between the phenolic -OH group and the polar phospholipids heads, making aminic RTAs superior in this respect [29]. Tocotrienols, a group of vitamin E isoforms, demonstrated to be more effective than α-TOH in inhibiting ferroptosis. [30]. The synthetic derivatives tetrahydronaphtyridinols (THNs), are even more effective based on an aza-phenol moiety with enhanced potency and stability resulting from the introduction of various alkyl chain substituents (C_{12}-THN was the best analog) [27].

Tetrahydrobiopterin (BH_4)

In 2018, Kraft *et al.* reported the GCH1/DHFR/BH_4 pathway where GTP cyclohydrolase (GCH)1 mediated the synthesis of BH_4 /BH_2, preventing the initiation of lipid peroxidation [31]. GCH1 is considered to be a rate-limiting enzyme for BH_4 synthesis. In parallel, Soula *et al.* described dihydrofolate reductase (DHFR) activity to convert dihydrobiopterin (BH_2) to BH_4 upon NAD(P)H consumption (Figure 1) [32]. GCH1 overexpression and high level of BH_4 promote reduced CoQ_{10} ($CoQ_{10}H_2$) decreasing ferroptosis sensitivity by depleting PUFAs-PL. Therefore, inhibition of GCH1 has been linked to the promotion of ferroptosis [33].

Coenzyme Q10 (CoQ_{10})

The FSP1/CoQ_{10} pathway, discovered by Bersuker, Doll and colleagues in 2019, involves ferroptosis suppressor protein (FSP)1, converting CoQ_{10} to its reduced form ($CoQ_{10}H_2$) and consuming NADPH (Figure 1) [34,35]. Inhibition of negative modulators of p53, homologous murine double minute 2 and X (MDM2 and MDMX, respectively), can increase CoQ_{10} and FSP1 levels, inhibiting ferroptosis in a

Table 1. Overview of the main classes of ferroptosis inhibitors[a]

Mechanism	Class	Structure	Refs
Endogenous RTA		α-TOH (vitamin E isoform)	[92]
		CoQ_{10} (ubiquinone 10)	[38]
		BH_4 (tetrahydrobiopterin)	[93]
		Vitamin K1 (phyllohydroquinone)	[40]
		GSSHs	[41]
Synthetic RTA	Ferrostatins	Fer-1	[2]
		UAMC-3203	[49]
		SRS11-92, SRS9-11, SRS16-86, UAMC-2418, CFI-4061, CFI-4082	[46,47]
	Liproxstatins	Lip-1	[54]
		Lip-2	[55]
	Tricyclic RTAs	Phenothiazine	[56]
		2-{1-[4-(4-methylpiperazin-1-yl)phenyl]ethyl}-10H-phenothiazine, 4-{4-[1-(10H-phenothiazin-2-yl)vinyl]phenyl}morpholine	[57,58]
		3-CF_3-8-tBu-PNX	[59]
	Other	CuATSM, CuATSP, SKI II, serdemetan, AZD3463, bazedoxifene	[45,60]
D-PUFA		RT-001	[94]
LOX inhibitors[b]		Zileuton (A-64077), baicalein, PD-146176, docebenone, MK-886, BWA4C	[23,54]
Inhibitors of 15LOX-2/PEBP1 complex[a]		FerroLOXIN-1, FerroLOXIN-2	[75]
GPX4 activators		PKUMDL-LC-101, PKUMDL-LC-101-D04	[78]
Iron chelator		Deferiprone, DFO, deferasirox, CPX, 2,2-BP, 1,10-phenanthroline, AKI-02	[2,80,95]
Others		See Table S1 in the supplemental information online	

[a]All the structures of the inhibitors discussed in the text are reported in the supplemental information (see Table S1 in the supplemental information online).
[b]Considering the discussion in the section on LOX inhibitors, these two groups of inhibitors could also be considered as RTAs.

p53-independent manner, as reported by Venkatesh *et al.* [36]. Dai *et al*. discovered that the antiferroptotic activity of FSP1 is connected with the recruitment of the endosomal sorting complex required for transport (ESCRT) III, and is responsible for membrane regeneration [37]. The discovery of ferroptosis inducer FIN56 revealed the link between lipid metabolism dysregulation and ferroptosis [38]. FIN56 binds SQS, a key enzyme in cholesterol synthesis, and suppresses CoQ_{10}.

Vitamin K (VK)

In 2022, Mishima *et al.* discovered that the long sought warfarin-resistant noncanonical reductase that reduces VK to VKH_2 is FSP1 (Figure 1) [39]. Both CoQ_{10}/$CoQ_{10}H_2$ and VK/VKH_2 belong to the 1,4-benzoquinone/hydroquinone antioxidant class. VKH_2 suppresses ferroptosis in a GPX4 deletion cell model by tackling lipid peroxides. Recently, VK1 was also reported by Kolbrink *et al*. as an effective endogenous antioxidant to prevent lipid peroxidation during acute kidney injury [40].

Hydropersulfides (RSSHs)

The latest reported endogenous regulatory mechanism that protects cells from lipid hydroperoxides is the RSSH/trans-sulfuration pathway. Dick and coworkers verified that the antioxidant potential of hydropersulfides (GSSHs) is strictly connected with the intracellular level of cysteine, but

independent from the GPX4 axis (Figure 1) [41]. In Pratt's group, the exceptional inhibitory capacity of RSSHs against phospholipid peroxidation was demonstrated *in vitro* in the **fluorescence-enabled inhibited autoxidation (FENIX) assay** [42]. Despite the lower antiferroptotic activity (micromolar range) of GSSHs in cells, they have the advantage of being endogenously produced through the transsulfuration pathway and are more effective than vitamin E due to their low H-bond acidity [43]. GSSHs function as early responders to ferroptosis induction, independent of dietary uptake (differently from vitamin E and VK). RSSHs not only act as effective RTAs but also serve as strong nucleophiles, suggesting their potential role in inhibiting lipid peroxide byproducts such as 4-hydroxynonenal (4-HNE). These hydropersulfides continuously regenerate through enzymatic activity, and the self-recombination of perthiyl radicals (GSS●).

Synthetic RTAs

Ferroptosis inhibitors are required when the physiological cellular antiferroptotic mechanisms are disrupted and the endogenous RTAs are not sufficient to tackle the formation of lipid peroxides (Table 1 and see Table S1 in the supplemental information online).

Ferrostatins

The first reported ferroptosis inhibitor was ferrostatin (Fer)-1 in 2012 [2]. Stockwell and coworkers identified Fer-1 through high-throughput screening of a small molecule library containing diverse drug-like soluble compounds. Fer-1 is an arylalkylamine that prevents lipid hydroperoxide accumulation in an erastin-mediated ferroptosis model in HT-1080 cells. Diarylamines and hindered dialkyl amines are commonly used as antioxidants in the food and material industries [44]. Therefore, a similar mechanism of action was hypothesized for Fer-1. While Stockwell's group demonstrated Fer-1 to be only a reductant, in 2017, Pratt's group shed light on the RTA mechanism of Fer-1. Structure–activity relationship (SAR) studies underlined that the presence of the *N*-cyclohexyl moiety, acting as a lipophilic anchor in biological membranes, is crucial [45].

To design novel ferroptosis inhibitors, the chemical structure of Fer-1 (EC_{50} = 95 nM) was investigated in an erastin-mediated ferroptosis model in HT-1080 cells. The maintenance of activity relies heavily on the amine group and the lipophilic anchor, as suggested by the reported SAR [46]. Further derivatization of the ethyl chain and introduction of a benzylic moiety on the aromatic amine improved potency (SRS11-92 EC_{50} = 6 nM). However they failed to improve the activity when an amide moiety was introduced to replace the ethyl ester with in SRS9-11 (EC_{50} = 950 nM). This result was contradicted by Hofmans *et al.* a few years later [47]. Plasma instability was attributed to the ethyl ester by Scouta *et al.*, leading to the synthesis of SRS16-86 (EC_{50} = 350 nM), featuring a *tert*-butyl ester, and an imine, showing improved stability but decreased activity [48]. These findings highlighted the importance of novel ferroptosis inhibitors that exhibit improved pharmacokinetic properties while retaining potency.

Hofmans *et al.* replaced the labile ester with either amide or sulfonamide moieties to improve stability, and a benzyl ring was introduced on the NH_2 to enhance stability and potency, resulting in sulfonamide analog UAMC-2418 [47]. The significant decrease in solubility drove the introduction of solubility-enhancing groups and the discovery of UAMC-3203 showing improved potency, stability, and solubility [49]. UAMC-3203 showed a lack of toxicity in a mouse model (Table 2), and currently is one of the most potent RTAs that can be used in *in vivo* disease models. Its use protected mice from multiorgan dysfunction and death induced by iron overload [50], improved post-resuscitation myocardial dysfunction in rats, and delayed relapse and ameliorated disease progression of relapsing–remitting multiple sclerosis in mice [51,52]. In our opinion, UAMC-3203 should replace Fer-1 as a reference compound in cellular and animal models due to its superior properties.

Table 2. Main pharmacokinetic properties and *in vivo* use of important RTAs[a]

Name	*In vivo* model / clinical trial	Additional information	Refs
Fer-1	$FeSO_4$ acute iron overload mouse model	5.2 mg/kg in 0.9% NaCl solution with 2% DMSO bol. i.p.	[50,53]
	Focal cerebral ischemia rat model	5 mg/kg in 2.5% (v/v) Tween-80, 2.5% (v/v) anhydrous ethanol and 95% (v/v) saline, i.v. bolus	[57]
	Relapsing–remitting experimental autoimmune encephalomyelitis (EAE) in mice	5.2 mg/kg in 0.9% NaCl solution with 2% DMSO i.p. bolus	[52]
Lip-1	Inducible $Gpx4^{-/-}$ acute renal failure and liver mouse model	10 mg/kg i.p., q.d.	[54]
	$FeSO_4$ acute iron overload mouse model	6.8 mg/kg in 0.9% NaCl solution with 2% DMSO i.p. bolus	[50]
	Relapsing–remitting EAE in mice	6.8 mg/kg in 0.9% NaCl solution with 2% DMSO i.p. bolus	[52]
UAMC-3203	Acute iron poisoning mouse model	10.16 mg/kg in 0.9% NaCl solution i.p. bolus	[49]
	$FeSO_4$ acute iron overload mouse model	12.35 mg/kg in 0.9% NaCl solution i.p. bolus	[50]
	Cardiac arrest and cardiopulmonary resuscitation rat model	5mg/kg i.p. bolus	[51]
	Relapsing–remitting EAE in mice	12.35 mg/kg in 0.9% NaCl solution with 2% DMSO i.p. bolus	[52]
Vitamin E	$FeSO_4$ acute iron overload mouse model	50 mg/kg i.p. bolus	[50]
(2-{1-[4-(4-methylpiperazin-1-yl)phenyl]ethyl}-10H-phenothiazine)	Focal cerebral ischemia rat model	5 mg/kg in 2.5% (v/v) Tween-80, 2.5% (v/v) anhydrous ethanol and 95% (v/v) saline, i.v. bolus	[57]
3-CF_3-8-tBu-PNX	Inducible $Gpx4^{-/-}$ acute renal failure mouse model	10 or 20 mg/kg q.d., i.p. (vehicle PEG400/5 % Solutol HS15/PBS; PEG E 400 and Solutol HS 15)	[59]
CuATSM	Clinical trial for early idiopathic Parkinson's disease (Phase 1)	30 participants p.o. 12 mg/day	NCT02870634
	Clinical trial for amyotrophic lateral sclerosis/motor neuron disease (ALC/MND) (Phases 2 and 3)	80 participants p.o. 72 mg/day	NCT04082832
	$SOD1^{G93A}$ mice with a C57BL/6 background	100 mg/kg/day p.o. b.i.d. (vehicle 0.9% w/v NaCl, 0.5% w/v Na-carboxym-ethylcellulose, 0.5% v/v benzyl alcohol, 0.4% v/v Tween-80)	[61]
RT-001	Clinical trial for infantile neuroaxonal dystrophy (Phases 2 and 3)	19 participants, p.o. 3.84 g/day	NCT03570931
	Clinical trial for Frederich's ataxia (Phases 1 and 2)	1.8 g (q.d.) or 9 g (b.i.d.), p.o.	NCT02445794
	$Aldh2^{-/-}$ mice with D-PUFA supplemented diet in AD model	1.2 g (1.2%) 11,11-D2 LA ethyl ester and 11,11,14,14-D4 α-ALA ethyl ester/100 g diet in a 1:1 ratio, 18 weeks	[96]
Deferiprone	Clinical trial for Parkinson's diseases (Phase 2)	22 participants, 20mg/kg/day or 30mg/kg/day, p.o. b.i.d.	NCT01539837
	Clinical trial for Frederich's ataxia (Phase 2)	36 participants, 20mg/kg/day or 40mg/kg/day, p.o. b.i.d.	NCT00897221
Zileuton	$NaIO_3$-induced acute retinal degeneration of murine model	20 mg/kg 10% DMSO in corn oil i.p. bis, at 24 h and 15 min before $NaIO_3$ treatment	[97]
Baicalein	Intracerebral hemorrhage mouse model	20 mg/kg in 0.5% carboxymethylcellulose sodium solution (0.5mg/ml) p.o. b.i.d.	[98]
	Myocardial ischemia/reperfusion injury rat model	100 mg/kg or 200 mg/kg p.o. gavage, q.d.	[99]
FerroLOXIN-1 FerroLOXIN-2	Total body irradiation mouse model	25 mg/kg i.p. bolus 24 h after radiation	[76]

[a]Structures of inhibitors reported in Table 2 are depicted in Figure 2.

To specifically target neurodegenerative diseases and the penetration through the blood–brain barrier (BBB) a novel series of Fer-1 analogs has been recently patented by Stockwell's group. In place of the ethyl ester/sulfonamide moiety, they introduced different heterocycles, including oxazole and oxadiazoles [53].

Liproxstatins

Similar to Fer-1, liproxstatin (Lip)-1 was selected from a small molecule screening in TAM-inducible gpx4$^{-/-}$ mouse embryonic fibroblasts (MEFs) [54]. With this study, a novel class containing a spiroquinoxalinamine scaffold with inhibitory potency against ferroptosis in the nanomolar range was introduced. The removal of the amine group (NH) from the quinoxaline was detrimental to the activity independently from the position of the -CH_3 or Cl on the benzyl ring. Considering the mechanism of Fer-1 as RTA, this should be explained by the H-bond donor function of NH to block phospholipid peroxidation. However, in the explanation provided by the study of Pratt and coworkers, where they confirmed the RTA activity of Lip-1, the quinaxoline ring was highlighted as a key blocker of peroxyl radicals [27]. Lip-1, together with UAMC-3203, was demonstrated to be a superior RTA in terms of activity, solubility, and especially stability in different mouse models [50]. Recently, Conrad and coworkers, reported a novel Lip-1 analog, Lip-2 (chemical structure not disclosed), with even improved pharmacokinetic properties, and effectiveness in lupus nephritis *in vitro* and *in vivo* [55]. So far, no other structural modifications of Lip-1 have been reported.

Tricyclic aromatic rings: phenoxazines and phenothiazines

Phenoxazines and phenothiazines are another class of aminic RTAs with excellent antiferroptotic potency [56]. in particular, phenothiazine and its analogs are known for their antipsychotic properties; therefore, their chemical structures are attractive for BBB permeability [56]. In the SAR reported by Yang *et al*., the introduction of a substituent presenting different alkyl/aryl moieties with different hydrophobicity in the C-10 position was detrimental to the activity of phenothiazines [57]. Instead, the derivatization at the C-2 position leads to a significant increase in antiferroptotic potency, with the most active compound 51 featuring a 2-phenyl-methyl piperazine moiety (EC_{50} = 0.5 nM in erastin-induced ferroptosis in HT-1080 cells). Another potent phenothiazine analog was reported by You *et al*. with a 2-vinyl-10H-substituent based on the previously reported SAR [58]. The compound showed an EC_{50} = 10 nM in erastin-induced ferroptosis in HT-1080 cell with no toxicity in an *in vivo* model (Table 2). Farmer *et al*. also investigated the potential of different substituents on phenothiazine and phenoxazines scaffolds [59]. They could not reproduce the result obtained by Yang *et al.* Since they tested their compounds in RSL3-induced ferroptosis in MEFs, the different IC_{50} values can be explained by the different assay conditions. In our opinion this demonstrates the importance of assay conditions on the variability of results: cell lines (HT-1080 vs Pfa-1 MEF), **ferroptosis inducers (FINs)** and their concentrations (erastin vs RSL3) and the specific cell density. All these parameters need to be carefully analyzed when discussing results and comparing inhibitors from different papers. It demonstrates the importance of taking control reference compounds along in each round of evaluating new inhibitors. The results obtained with the phenothiazines seem to confirm the study of Devisscher *et al*. that a piperazine ring is a favorable moiety for interaction with the phospholipid head group [49]. Similarly, phenoxazines, the most potent RTAs up to date, were investigated [59]. While the introduction on C2, C3, C7, and C8 of electron-withdrawing groups decreases activity, electron-donating groups increase the intrinsic activity. Lipophilicity correlates well with the potency and steric hindrance around the NH has a minimal impact on the RTAs activity. The presence of nonoxidizable substituents on C3 or C7 was however necessary to reduce metabolism in mouse liver microsomes. Compound 11 (EC_{50}= 3.6 nM in RSL3-induced ferroptosis in MEFs) was the most potent analog of the series 3-CF_3-8-tBu-PNX (CF_3 in C3 and *tert*-butyl in C8), with favorable pharmacokinetics properties.

Other RTAs

In an FDA-approved drugs screening, Dixon and coworkers identified SKI II, serdemetan, AZD3463, and bazedoxifene as potent RTAs able to suppress ferroptosis in erastin-2- and ML162-induced ferroptosis in HT-1080 cells, showing IC_{50} values <150 nM [60]. Bazedoxifene was more potent than Fer-1 and phenothiazine. The library was also screened in a **2,2-diphenyl-1-picrylhydrazyl (DPPH)** cell-free radical scavenging assay and ferrozine Fe^{2+} binding assay to verify their mechanism of action as RTAs or iron chelators.

Pratt and coworkers studied the RTA potential of the clinical candidate copper(II)-diacetyl-bis (*N*4-methyl thiosemicarbazone)(CuATSM) [45]. CuATSM slowed disease progression in amyotrophic lateral sclerosis (ALS) (Table 2) and inhibited RSL3-induced ferroptosis in rat mesencephalic (N27) cells [61,62]. Despite high **bond dissociation enthalpy (BDE)** values calculated for the N-H group of CuATSM (97.3 kcal/mol) compared to Fer-1 (83.3 kcal/mol) and Lip-1 (82.4 kcal/mol), CuATSM acted as a highly potent RTA (IC_{50} = 160 nM in RSL3 induced ferroptosis in Pfa1 cells) with a unique mechanism of action and BBB permeability. The structural analog CuATSP, which has two extra phenyl moieties on the two N-H of the bis(thiosemicarbazone), was 20-fold more active *in cellulo*. The increased lipophilicity of the molecule facilitates membrane permeability, making CuATSP one of the most potent ferroptosis inhibitors discovered to date (IC_{50} = 8.5 nM in RSL3 induced ferroptosis in Pfa1 cells). Next to its antioxidant properties the compound also works by improving mitochondrial respiration [63].

With the implementation of the FENIX-2 assay, the radical trapping potential of various ferroptosis inhibitors could be more precisely assessed [64]. Necrostatin-1, a well-known necroptosis inhibitor, was confirmed to act as an RTA in ferroptosis inhibition, as speculated previously [65]. Mallais *et al.* demonstrated that the thiohydantoin moiety reacts with hydroperoxides, but the sulfenic acid formed *in situ* effectively acts as an RTA [64].

Non-RTA ferroptosis inhibitors

Deuterated PUFAs

Cell treatment with PUFAs containing deuterium (D-PUFAs) at the peroxidation site can prevent ferroptosis by stopping the autoxidation process through the **kinetic isotope effect (KIE)** (Table 1) [66]. The protective effect of D-PUFAs was verified in erastin- and RSL3-induced ferroptosis models, with demonstrated efficacy in various disease models, particularly neurodegenerative disorders [67]. The company Retrotope started a clinical trials in 2018 with a candidate compound RT001 containing a deuterated form of linoleic acid (Table 2) [68].

LOX inhibitors and GPX4 activators

5-LOX, 12-LOX, and 15-LOX, are the three lipoxygenase isoforms responsible for enzymatic phospholipid peroxidation [69]. The most relevant inhibitors reported in the literature are: the 5-LOX inhibitors (zileuton, MK-886, and BWA4C) the 12/15-LOX inhibitor PD-146176; the 15-LOX inhibitor baicalein; and the 5/12-LOX inhibitor docebenone (AA-861) (Table 1) [70–72]. However, not all the LOX inhibitors have an antiferroptotic activity. This has been demonstrated by Shah *et al.* [23]. In their study, zileuton, PD-146176, baicalein, AA-861, and two additional 5-LOX inhibitors CJ-13610 and CAY-10649 were tested for their RTA potential in comparison with Fer-1 and Lip-1. Except for CJ-13610 and CAY-10649 which desensitize cell to ferroptosis induction, the antiferroptotic activity of LOX inhibitors was linked to their RTA mechanism. Zileuton displayed higher activity, likely due to the O-H bond in its hydroxamic acid moiety, while PD-146176 exhibited moderate activity due to the presence of an N-H bond in its arylamine moiety. Baicalein and AA-861 also demonstrated RTA activity attributed to the lipophilic pyrogallol in baicalein and the lipophilic quinone (similar to CoQ_{10}) in AA-861.

In the same study, the potential of RTAs such as Fer-1 to inhibit LOX was also investigated. They demonstrated comparable ferroptosis protection by the RTAs such as Fer-1 and Lip-1, and the LOX inhibitors zileuton and PD14676. However, the activity of Fer-1 and Lip-1 as 15-LOX inhibitors turned out to be poor compared to their RTA activity [27].

Recently, Anthonymuthu *et al.*, confirmed that Fer-1 does not inhibit 15-LOX directly, but they revealed that it can inhibit 15-hydroperoxy-eicosatetraenoyl phosphatidylethanolamine (15-HpETE-PE) production by the 15LOX-2/PEBP1 complex [73]. In a recent patent of 2022, a series of aryl-substituted imidazoles were reported as selective inhibitors of 15LOX-2/PEBP1 complex to treat necroinflammation associated with ferroptosis [74]. Recently, Kagan and coworkers reported FerroLOXIN-1 and 2 as inhibitors of the 15LOX-2/PEBP1 complex, suppressing lipid peroxidation and ferroptosis *in vitro* and *in vivo* [75]. However, considering the presence of the classical aromatic amine moieties typical of RTAs (Fer-1, Lip-1, and phenoxazines) it is likely that the antiferroptotic activity is at least in part due to an RTA-off target effect [76]. The inhibition of 15LOX-2/PEBP1 as an upstream mechanism of phospholipid peroxidation represents an emerging inhibition strategy that could complement the activity of RTAs [30].

Inducing GPX4 activation can be a promising approach to control the accumulation of lipid peroxides. However, designing a protein activator is challenging. In the past, ebselen was reported as a GPX4 (and GPX1) mimetic and included in several unsuccessful clinical trials [77]. More recently, with a computation-based approach, Li *et al.* managed to design and synthesize eight allosteric activators for GPX4 [78]. These compounds present a unique mechanism of action with no overlap with other strategies commonly used in ferroptosis inhibition. PKUMDL-LC-101 and its optimized analog PKUMDL-LC-101-D04 were among the most potent to increase GPX4 activity in a cellular model. However, the molecules showed IC_{50} > 100 μM (erastin-induced ferroptosis in HT-1080 cells), making them moderate ferroptosis inhibitors. This therapeutic strategy might be considered for a synergistic effect with RTAs.

Iron chelators

Iron chelators inhibit lipid peroxidation either by binding iron in the catalytic center of LOX or by chelating iron in the cytosolic LIP, thus inhibiting radical generation. The ones reported in the literature up to date are: deferoxamine (DFO); deferasirox, which is the first oral medication approved by the FDA for chronic iron overload; ciclopirox (CPX), an FDA-approved antifungal which acts as iron chelator and ALOX5/PTGS inhibitor; and 2,2′-bipyridine (2,2-BP), which can sequester Fe^{2+} from LIP similarly to 1,10-phenanthroline (Table 1) [2,19]. The LOX inhibitor baicalein also acts as an iron chelator due to the 5,6,7-hydroxyl groups that form complexes with iron in a stoichiometry of 1:1 [79]. Recently, a new hydroxypyridinone-based iron chelator, AKI-02, was reported as an effective iron-chelating agent in rhabdomyolysis (RM)-induced AKI [80].

The delicate equilibrium between redox active and inactive forms of iron can be altered and result in ferroptosis among the many possible outcomes. Considering the central role of iron in ferroptosis, iron chelators are effective inhibitors and an effective strategy to control ferroptosis [9]. However, the inhibitory potency of small RTAs seems to be superior. Nevertheless, we should question whether the mechanism of synthetic RTAs is only ascribable to their radical trapping capacity or perhaps an iron chelation mechanism is also involved.

Additional ferroptosis inhibitors

Bardoxolone methyl (BXM) is in Phase 3 clinical trials for the treatment of chronic diabetic kidney disease and it promotes the activation of Nrf2. In the p62/Keap1/Nrf2 pathway, Nrf2 protects from ferroptosis by binding with different antioxidant response elements [81]. ACSL4 inhibitors,

rosiglitazone and pioglitazone, activate Nrf2 through stimulation of peroxisome proliferator-activated receptor γ. The benzoxazole sepanisertib (INK128) and the pyridopyrimidine AZD8055 are orally bioavailable ATP-competitive mechanistic rapamycin complex (mTORC)1 inhibitors are in clinical trials (Phase 2 for pancreatic cancer and Phase 1 for glioma, respectively) and inhibited ferroptosis induced by class I FIN. The role of mTORC1 in ferroptosis seems to be context-dependent as described by Zhang *et al.* and Yi *et al*. [82,83]. Erastin-mediated ferroptosis in HL-60 cells can be inhibited by c-Jun N-terminal kinase inhibitor SP600125 and p38 inhibitor SB202190 at 10 μM in acute myeloid leukemia [84]. A769662 and AICAR are activators of AMP-activated protein kinase (AMPK) and responsible for decreased levels of PUFA-containing Pes [85]. 5-(tetradecyloxy)-2-furoic acid is a context-dependent allosteric acetyl CoA carboxylase 1 inhibitor; a downstream protein regulated by AMPK activation [86]. Go6983 and enzastaurin present a bisindolylmaleimide scaffold and act as protein kinase (PK)C inhibitors in an erastin and RLS3 ferroptosis cell model. Their activity is related to PKC inhibition, and involved in the execution of ferroptosis, as confirmed from their inactivity in the FENIX assay [87]. Vildagliptin, alogliptin, and linagliptin inhibit dipeptidyl-peptidase (DPP)4 and DPP4-mediated lipid peroxidation induced by ferroptosis inducers FIN I in p53 knockout cells [88] (see Table S1 in the supplemental information online for all the discussed structures). The rapid advances in ferroptosis have led to the necessity for an accessible tool to facilitate the classification and identification of novel ferroptosis inhibitors. FerrDb V2 is a collection of more than 1000 ferroptosis regulators now available and freely accessible (http://www.zhounan.org/ferrdb/current/), which also include 179 inhibitors [89].

Concluding remarks and future perspectives

The discovery of different metabolic pathways regulating ferroptosis sensitivity has improved the understanding of this type of cell death. Evidence for its implication in various diseases is increasingly convincing, and therefore the interest in novel ferroptosis inhibitors is high. The identification of specific *in vivo* biomarkers remains an important area to explore (see Outstanding questions). Another point of attention for the field is the crucial importance of various factors such as the specific class of FINs used, cell line, and cell density, when comparing the potency of ferroptosis inhibitors.

Endogenous RTAs are one of the main natural defense mechanisms and there is probably more to discover. In addition, library screening of drug-like/approved molecules led to the identification of many synthetic ferroptosis inhibitors showing RTA activity. This confirms the potential of lipophilic RTAs as a superior class of ferroptosis inhibitors. The recently discovered novel enzymatic targets open the door to new potential directions. However, initial results indicate that many of the inhibitors of these enzymes owe at least part of their ferroptosis inhibition to their potential to trap radicals.

Much has been done already to improve the initially discovered inhibitors Fer-1 and Lip-1, and this identified novel drug-like candidates to address different ferroptosis-driven diseases *in vivo* (e.g., UAMC-3203, Lip-2, and substituted phenothiazine/phenoxazine). Even if the lack of a protein target might remain a hurdle for further development, the data collected so far seem to indicate the blockade of phospholipid peroxidation through radical-trapping molecules as an unquestionable mechanism for ferroptosis inhibition. The assay recently developed in Pratt's group (FENIX-2) could facilitate the discrimination of RTAs versus non-RTAs during library screening and therefore accelerate the discovery of novel RTAs.

Outstanding questions

Currently lipophilic RTAs are the most effective ferroptosis inhibitors to tackle the formation of phospholipid peroxides. Are there any specific upstream pharmacological targets that can result in inhibitors with the same potency and efficiency in *in vivo* disease models?

What is the exact cellular or subcellular location of lipids that are most susceptible to peroxidation (if there is an unique one)?

Which are the most specific and sensitive biomarkers to detect ferroptosis *in vivo*?

How can these biomarkers help in translating the current ferroptosis inhibitors to the clinic?

Are there any additional endogenous RTAs to be considered in the complicated ferroptosis regulatory machinery?

How can we improve pharmacokinetic properties such as oral bioavailability, half-life and organ distribution (especially BBB permeability) to increase RTAs potential as therapeutic drugs?

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Declaration of interests

Professors Koen Augustyns and Tom Vanden Berghe are co-inventors of the following patents: 3,4-diaminobenzensulfonamide derivatives for inhibiting cell death(WO2016/075330Al) and 3-(benzylamino)-4(cyclohexylamino)-N-(2-piperazin-1-yl)ethyl) benzenesulfonamide derivatives and related ferrostatin-1 analogues as cell death inhibitors for treating for example stroke (WO2019/154795A1).

Supplemental information

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