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Review

Ferroptosis: process and function

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Ferroptosis is a recently recognized form of regulated cell death. It is characterized morphologically by the presence of smaller than normal mitochondria with condensed mitochondrial membrane densities, reduction or vanishing of mitochondria crista, and outer mitochondrial membrane rupture. It can be induced by experimental compounds (e.g., erastin, Ras-selective lethal small molecule 3, and buthionine sulfoximine) or clinical drugs (e.g., sulfasalazine, sorafenib, and artesunate) in cancer cells and certain normal cells (e.g., kidney tubule cells, neurons, fibroblasts, and T cells). Activation of mitochondrial voltage-dependent anion channels and mitogen-activated protein kinases, upregulation of endoplasmic reticulum stress, and inhibition of cystine/glutamate antiporter is involved in the induction of ferroptosis. This process is characterized by the accumulation of lipid peroxidation products and lethal reactive oxygen species (ROS) derived from iron metabolism and can be pharmacologically inhibited by iron chelators (e.g., deferoxamine and desferrioxamine mesylate) and lipid peroxidation inhibitors (e.g., ferrostatin, liproxstatin, and zileuton). Glutathione peroxidase 4, heat shock protein beta-1, and nuclear factor erythroid 2-related factor 2 function as negative regulators of ferroptosis by limiting ROS production and reducing cellular iron uptake, respectively. In contrast, NADPH oxidase and p53 (especially acetylation-defective mutant p53) act as positive regulators of ferroptosis by promotion of ROS production and inhibition of expression of SLC7A11 (a specific light-chain subunit of the cystine/glutamate antiporter), respectively. Misregulated ferroptosis has been implicated in multiple physiological and pathological processes, including cancer cell death, neurotoxicity, neurodegenerative diseases, acute renal failure, drug-induced hepatotoxicity, hepatic and heart ischemia/reperfusion injury, and T-cell immunity. In this review, we summarize the regulation mechanisms and signaling pathways of ferroptosis and discuss the role of ferroptosis in disease.
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Facts

- Ferroptosis is an iron- and ROS-dependent form of regulated cell death (RCD).
- Ferroptosis is distinct from other forms of RCD at morphological, biochemical, and genetic levels.
- Several molecules (e.g., VDAC2/3, glutathione peroxidase (GPX4), heat shock protein beta-1 (HSPB1), nuclear factor E2-related factor 2 (NRF2), NADPH oxidase (NOX), p53, and SLC7A11) regulate ferroptosis by directly or indirectly targeting iron metabolism and lipid peroxidation.
- Misregulated ferroptosis has been implicated in multiple physiological and pathological processes such as cancer cell death, tissue injury, and T-cell immunity.

Open Questions

- How does the downstream signaling or executor of iron-dependent ROS metabolism identify and distinguish ferroptosis from other types of RCD?
- What controls the network of ferroptosis-signaling pathways?
- What explains the cross-regulation between ferroptosis and other types of RCD?
- How do mitochondrial dynamics and endoplasmic reticulum (ER) stressors affect ferroptosis?
- What is the specific role of ferroptosis in human disease?

Ferroptosis is the term for a form of RCD that was recently coined in 2012 by the lab of Dr. Brent R Stockwell.[1] According

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Abbreviations: AA, arachidonic acid; ACC1, acetyl-CoA carboxylase alpha; ACSF2, andacyl-CoA synthetase family member 2; ACSL4, acyl-CoA synthetase long-chain family member 4; AOA, aminooxyacetic acid; AKF, acute kidney failure; ATP5G3, ATP synthase F0 complex subunit C3; BSO, buthionine sulfoximine; BHT, butylated hydroxytoluene; CARS, cysteinyl-tRNA synthetase; CHAC1, cation transport regulator-like protein 1; COX-2, cyclooxygenase-2; CS, citrate synthase; CSSG, cysteine-glutathione disulfide; DAMP, damage-associated molecular pattern molecule; DMT1, divalent metal transporter 1; ER, endoplasmic reticulum; Fe3+, ferric iron; Fe2+, ferrous iron; FIN, ferroptosis-inducing agents; FLT-3, fms-like tyrosine kinase 3; FTH1, ferritin heavy chain 1; FTL, ferritin light chain; GPX4, glutathione peroxidase 4; GSH, glutathione; HMGB1, high mobility group box 1; HD, Huntington's disease; HETE, hydroxyeicosatetraenoic acid; HO-1, heme oxygenase-1; HPETE, hydroperoxyeicosatetraenoic acid; HSF-1, heat shock factor-1; HSPB1, heat shock protein beta-1; HCC, hepatocellular carcinoma; IREB2, iron-responsive element-binding protein 2; JNK, c-Jun NH$_2$-terminal kinase; Keap1, Kelch-like ECH-associated protein 1; LOX, lipoxygenases; LPCAT3, lysophosphatidylcholine acyltransferase 3; MAPK, mitogen-activated protein kinase; MDM2, murine double minute-2; MEFs, mouse embryonic fibroblasts; NADPH, nicotinamide adenine dinucleotide phosphate; NAPQI, *N*-acetyl-*p*-benzoquinone imine; NOX, NADPH oxidase; NRF2, nuclear factor erythroid 2-related factor 2; OLs, oligodendrocytes; PDAC, pancreatic ductal adenocarcinoma; PGSK, phen green SK; PKC, protein kinase C; PPP, pentose phosphate pathway; PTGS, prostaglandin-endoperoxide synthase; PUFAs, polyunsaturated fatty acids; PVL, periventricular leukomalacia; RCD, regulated cell death; ROS, reactive oxygen species; RPL8, ribosomal protein L8; RSLs, Ras-selective lethal small molecules; SCP2, sterol carrier protein 2; TFR1, transferrin receptor 1; TTC35, tetratricopeptide repeat domain 35; VDAC, mitochondrial voltage-dependent anion channel.

Table 1 The main morphological, biochemical, and immune features and core regulators of ferroptosis, apoptosis, necroptosis, and autophagy (modified from[45–47])

Type	Morphological features	Biochemical features	Immune features	Core regulators
Ferroptosis	Cell membrane: lack of rupture and blebbing of the plasma membrane; rounding-up of the cell Cytoplasm: small mitochondria with condensed mitochondrial membrane densities, reduction or vanishing of mitochondria crista, as well as outer mitochondrial membrane rupture Nucleus: normal nuclear size and lack of chromatin condensation	Iron and ROS accumulation Activation of MAPKs Inhibition of system X_c^- with decreased cystine uptake GSH depletion and increased NAPDH oxidation Release of arachidonic acid mediators (e.g., 11-HETE and 15-HETE) $\Delta\psi m$ dissipation	Pro-inflammatory due to release of DAMPs (e.g., HMGB1)	Positive • VDAC2/3 • Ras • NOX • TFR1 • p53 • CARS Negative • GPX4 • SLC7A11 • HSPB1 • NRF2
Apoptosis	Cell membrane: plasma membrane blebbing; rounding-up of the cell Cytoplasm: retraction of pseudopods; reduction of cellular volume Nucleus: reduction of nuclear volume; nuclear fragmentation; chromatin condensation	Activation of caspases Oligonucleosomal DNA fragmentation $\Delta\psi m$ dissipation PS exposure	Often anti-inflammatory and immune silent In some cases, eliciting an immune response due to exposure and release of DAMPs (e.g., histone, DNA and HMGB1)	Positive • p53 • Bax • Bak • Other pro-apoptotic Bcl-2 family proteins Negative • Bcl-2 • Bcl-XL • Other anti-apoptotic Bcl-2 family proteins
Necroptosis	Cell membrane: rupture of plasma membrane Cytoplasm: cytoplasmic swelling (oncosis); swelling of cytoplasmic organelles Nucleus: moderate chromatin condensation	Drop in ATP levels Activation of RIP1, RIP3, and MLKL Release of DAMPs (e.g., HMGB1) PARP1 hyperactivation	Most often pro-inflammatory due to release of DAMPs (e.g., HMGB1) In some cases, anti-inflammatory	Positive • RIP1 • RIP3 • MLKL
Autophagy	Cell membrane: lack of change Cytoplasm: accumulation of double-membraned autophagic vacuoles Nucleus: lack of chromatin condensation	LC3-I to LC3-II conversion Substrate (e.g., p62) degradation	Most often anti-inflammatory due to inhibition of inflammasome activation In some cases, pro-inflammatory due to mediation of non-classical secretion of cytokines	Positive • ATG5 • ATG7 • Beclin 1 • Other ATG family proteins

to their original study, ferroptosis is remarkably distinct from other types of RCD such as apoptosis, necroptosis, and autophagic cell death at morphological, biochemical, and genetic levels (Table 1). Multiple inducers and inhibitors of ferroptosis have been identified to affect accumulation of lipid peroxidation products and lethal reactive oxygen species (ROS) derived from iron metabolism (Table 2). Understanding the molecular mechanisms and signaling pathways (Figure 1) of ferroptosis may provide new diagnostic and therapeutic approaches to regulate cell survival and death in human disease.

Discovery

Ferroptosis inducers were discovered before the notion of ferroptosis was invented. First identified as a ferroptosis inducer in 2003, erastin was found to be synthetic lethal with expression of the engineered mutant Ras oncogene in human foreskin fibroblasts (BJeLR), but not their isogenic primary counterparts.[2] Ras-selective lethal small molecule (RSL)-3 and RSL5 were later identified in 2008 in another high-throughput small molecule-screening study that selectively killed BJeLR cells in a non-apoptotic manner.[3] Inhibition of apoptosis, necrosis, necroptosis, and autophagy by small molecule inhibitors (e.g., Z-VAD-FMK, BOC-D-FMK,

wortmannin, and necrostatin-1) cannot reverse RSL-induced cell death (Table 2).[1] In contrast, antioxidants (e.g., vitamin E) and iron chelators (e.g., deferoxamine mesylate) block RSLs-induced cell death (Table 2).[1] Thus, ferroptosis generally refers to an iron-dependent, non-apoptotic form of RCD.[1]

Morphology

Ferroptotic cancer cells are usually rounded up and detached in response to erastin.[1,2,4] Ferroptotic cells exhibit changed mitochondrial morphology and cristae structure. Smaller than normal mitochondria with increased mitochondrial membrane density and reduction/vanishing of mitochondria crista have been observed in ferroptosis following erastin treatment in BJeLR cells.[1,2,4] Induction of ferroptosis by genetic inactivation of GPX4 in immortalized fibroblasts and kidney tissue has been associated with outer mitochondrial membrane rupture, as observed using transmission electron microscopy.[5] In contrast, the structural integrity of the nucleus is retained following erastin treatment in cancer cells.[2] No nuclear condensation or chromatin margination are observed following erastin treatment in cancer cells.[2] These morphological features help us distinguish ferroptosis from apoptosis, necroptosis, and autophagy (Table 1).

Table 2 Small molecules and drugs that interfere with ferroptosis

Compound	Target	Effect	Molecular weight (MW)	Formula	Work concentration	Test cells	Test animals	Refs
Erastin	VDAC2/3 and system X_c^-	Induce ferroptosis	547.04	C30H31ClN4O4	9 µM 0.1–10 µM 2.5–20 µM 6.25 µM NA 0.01–100 µM 1 µM 0.1–10 µM 0.5–2 µM 5 µM	BJeLR HT1080 Calu-1 A-673 Hela 143B p^0 and p^+ cell Jurkat T cells Wild-type and Bax/Bak DKO MEFs U2OS DU-145		2,4 1,4 1 6 7
RSL3	GPX4	Induce ferroptosis	NA	NA	0.5 µg/ml 0.5–1.5 µg/ml 0.001–10 µg/ml	BJeLR HT1080 A549, Calu-1, HCT116, MIA PaCa-2 KBM7		3 21
DPI7 Buthioninesulfoximine	GPX4 GSH depletion	Induce ferroptosis Induce ferroptosis	477.40 222.305	C23H22Cl2N2O3S C8H18N2O3S	9 µM 100 µM 2.5 mM	KBM7 BJeLR HCT116/A549		21 6 7
Acetaminophen	GSH depletion	Induce ferroptosis	151.163	C8H9NO2	0~25 mM	HepG2/primary mouse hepatocytes		8
Sulfasalazine	System X_c^-	Induce ferroptosis	398.394	C18H14N4O5S	1 mM 500 µM $10^{-5.5}$–$10^{-2.5}$ µM	BJeLR/HT1080 HT1080/Calu-1 DU-145		1 7
Sorafenib	System X_c^-	Induce ferroptosis	464.825	C21H16ClF3N4O3	10 µM $10^{-5.5}$–$10^{-2.5}$ µM 2, 5, 10 µM 30 mg/kg 10 µM	HT1080/Calu-1 DU-145 Huh7 ACHN cells	Nude mice	7 14,15 14 16
Artesunate Lanperisone	N/A System X_c^-	Induce ferroptosis Induce ferroptosis	384.421 285.305	C19H28O8 C15H18F3NO	50 µM 1–40 µM 40 mg/kg	PDAC cell lines K-ras ^{G12D}-transfor med MEFs.	Nude mice	18 12
Piperazine erastin	VDACs and system X_c^-	Induce ferroptosis	645.19	C35H41ClN6O4	40 mg/kg NA	BJeLR	Nude mice	6
1 S,3 R-RSL3	GPX4	Induce ferroptosis	440.1139	C23H21ClN2O5	100 mg/kg 0.1–10 µM	BJeLR	Nude mice	6
Trolox	Lipophilic antioxidants	Inhibit ferroptosis	250.29	C14H18O4	50 µM 50 µM/200 µM 320 µM	HT1080/PUFA-oxidation-induced death model on *S. cerevisiae* Wild-type and Bax/Bak DKO MEFs HT1080, Calu-1, BJeLR		1,22
Cycloheximide	Protein synthesis	Inhibit ferroptosis	281.35	C15H23NO4	5 µM 10 µM	HT1080, Calu-1 BJeLR Wild-type and Bax/Bak DKO MEFs		1
Ebselen	Oxidative pathway (glutathione peroxidase mimetic)	Inhibit ferroptosis	274.17666	C13H9NOSe	5 µM 30 µM	HT1080, Calu-1 BJeLR GPX4-deficient T cells		1 26

Table 2 (Continued)

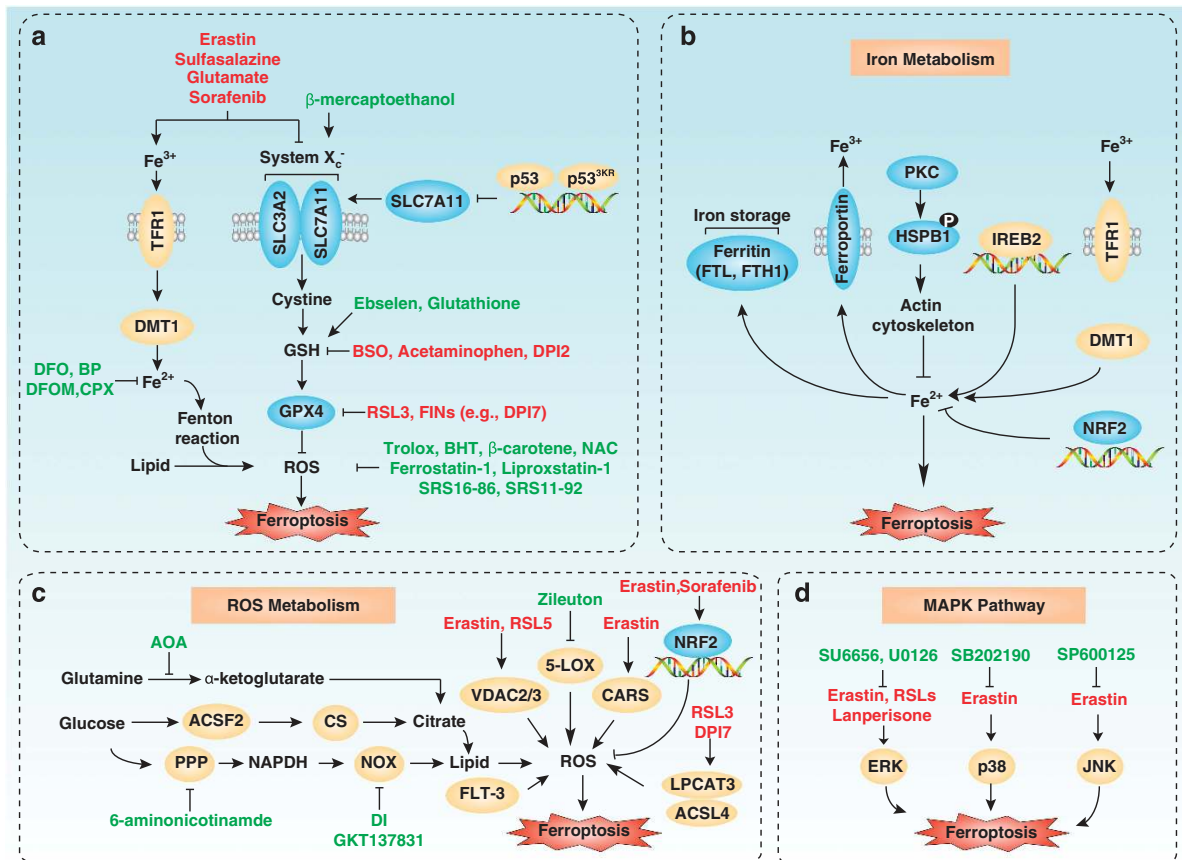| Compound	Effect	Target	Molecular weight (MW)	Formula	Work concentration	Test cells	Test animals	Refs
Aminooxyacetic acid	Inhibit ferroptosis	Fatty-acid synthesis	91.066	C2H5NO3	2 mM	HT1080, BJeLR		1
β-mercaptoethanol	Inhibit ferroptosis	Cystine uptake	78.133	C2H6SO	50 μM	HT1080		1
Ciclopirox olamine	Inhibit ferroptosis	Intracellular iron	207.269	C12H17NO2	5 μM / 10 μM	HT1080	OHSC	1 / 22
Diphenyleneiodonium	Inhibit ferroptosis	NOX	314.6	C12H8I	0.5 μM	HT1080/Calu-1		1
GKT137831	Inhibit ferroptosis	NOX1/4	394.86	C21H19ClN4O2	20 μM	HT1080/Calu-1		1
6-aminonicotinamde	Inhibit ferroptosis	NADPH-generating pentose phosphate pathway	137.14	C6H7N3O	200 μM	HT1080/Calu-1/BJeLR		1
Butylated hydroxyltoluene	Inhibit ferroptosis	Oxidative pathway	220.35	C15H24O	400 μM	HT1080/BReLR		1,4
α-tocopherol (vitamin E)	Inhibit ferroptosis	Oxidative pathway	430.71	C29H50O2	44 μM / 100 μM / 10 mg/kg ~ 500 mg/kg	BReLR	GPX4-deficient T-cell mice	4 / 3 / 26
β-carotene	Inhibit ferroptosis	Oxidative pathway	536.87	C40H56	0.2 μM	BJeLR		4
Glutathione	Inhibit ferroptosis	Oxidative pathway	307.32	C10H17N3O6S	5 mM	HT1080		6
N-acetylcysteine	Inhibit ferroptosis	Oxidative pathway	163.195	C5H9NO3S	1 mM	HT1080		1
2,2-bipyridyl	Inhibit ferroptosis	Intracellular iron	156.18	C10H8N2	100 μM	Wild-type and Bax/Bak DKO MEFs		1
Deferoxamine mesylate	Inhibit ferroptosis	Intracellular iron	656.8	C25H48N6O8•CH4O3S	150 μM	BJeLR		3
Deferoxamine	Inhibit ferroptosis	Fenton reaction	560.68	C25H48N6O8	100 μM/400 μM	Wild-type and Bax/Bak DKO MEFs, HT1080/Calu-1		1
SU6656	Inhibit ferroptosis	SRC kinase	371.45	C19H21N3O3S	50 μM	HT1080		3
U0126	Inhibit ferroptosis	MEK1/2	380.49	C18H16N6S2	5 μM/10 μM	Wild-type and Bax/Bak DKO MEFs/HT1080		1,3
Ferrostatin-1	Inhibit ferroptosis	ROS from lipid peroxidation	262.35	C15H22N2O2	0.5 μM/1 μM/5 μM / 2 μM / 1 nM-1 μM / 100 nM / 100 nM-2 μM / 10 μM	HT1080 / OHSC / Rat corticostriatal brain slice / PVL model / AKI model / Huh7		1,22 / 22 / 14
Liproxstatin-1	Inhibit ferroptosis	ROS from lipid peroxidation	340.85	C19H21ClN4	100 nM / 50nm / 10 mg/kg	HRPTEpiCs / GPX4−/− cells	GPX4−/− mice	5
SSRS11-92	Inhibit ferroptosis	ROS from lipid peroxidation	NA	NA	1 nM-1 μM / 100 nM / 1 μM	HD model / PVL model / HT1080		22
SRS 16-86	Inhibit ferroptosis	ROS from lipid peroxidation	432.2525	C16H24N2O2	1 μM / NA	HT1080/NIH 3T3	IRI mice model	28
Zileuton	Inhibit ferroptosis	5-LOX	236.291	C11H12N2O2S	1–100 μM	HT22		41
SB202190	Inhibit ferroptosis	p38	331.35	C20H14N3OF	10 μM	HL-60		23
SP600125	Inhibit ferroptosis	JNK	220.23	C14H8N2O	10 μM / 30 μM	HL-60 / HD model		23 / 22

Figure 1 Molecular mechanisms and signaling pathways of ferroptosis. (**a**) Core regulators of ferroptosis. (**b**–**d**) Roles of iron metabolism; (**b**), ROS metabolism (**c**), and the MAPK pathway (**d**) in ferroptosis

Inducer

Erastin. Cell death triggered by erastin is significantly inhibited by antioxidants (e.g., α-tocopherol, butylated hydroxytoluene, and β-carotene) and iron chelators (e.g., deferoxamine), suggesting that ROS- and iron-dependent signaling is required for erastin-induced ferroptosis (Figure 1a).[1,4] Six high-confidence genes regulating iron or mitochondrial fatty-acid metabolism are specifically required for erastin-induced ferroptosis.[1] These genes include ribosomal protein L8, iron-responsive element-binding protein 2 (IREB2), ATP synthase F_0 complex subunit C3, citrate synthase, tetratricopeptide repeat domain 35, and andacyl-CoA synthetase family member 2 (ACSF2).[1] Activation of RAF/MEK/ERK signaling seems to be important for erastin-induced ferroptosis in tumor cells bearing oncogenic Ras.[4] *In vivo*, piperazine erastin has been shown to have better stability and water solubility than erastin to inhibit cancer growth.[6]

One of the direct molecular targets of erastin is the mitochondrial voltage-dependent anion channel (VDAC) (Figure 1c).[4] Erastin can directly bind to VDAC2/3 in BJeLR cells. Knockdown of VDAC2 and VDAC3, but not VDAC1, leads to erastin resistance.[4] Furthermore, erastin has the ability to reduce glutathione (GSH) level by directly inhibiting cystine/glutamate antiporter system X_c^- activity (Figure 1a),[1]

with activation of the ER stress response.[7] This process could accelerate ROS accumulation during ferroptosis.

RSL3 and RSL5. Iron, ROS, and MEK are required for RSL3- and RSL5-induced ferroptosis in tumor cells bearing oncogenic Ras.[3] VDAC2/3 is required for RSL5-, but not RSL3-induced ferroptosis.[3] RSL3 is a direct inhibitor of GPX4, but not system X_c^-.[6] After binding to GPX4, RSL3 inactivates GPX4 to induce ROS production from lipid peroxidation (Figure 1a).[6] Thus, at least two types of RSLs exist. Type I RSLs such as erastin and RSL5 can target upstream regulators (e.g., VDAC and system X_c^-) to induce ferroptosis. Type II RSLs such as RSL3 can trigger ferroptosis by inhibiting downstream regulators (e.g., GPX4). The protein-synthesis inhibitor cycloheximide significantly inhibits RSL5-, but not RSL3-induced ferroptosis, indicating that protein synthesis is required for Type I RSL-induced ferroptosis.[3]

Buthioninesulfoximine. Buthioninesulfoximine (BSO) is an irreversible inhibitor of γ-glutamyl cysteine synthetase, the rate-limiting enzyme for GSH synthesis (Figure 1a). BSO inhibits GSH synthesis with decreased GPX activity and increased ROS levels, which results in ferroptosis in Ras-mutated cells.[6]

Acetaminophen. The reactive metabolite of acetaminophen has been identified as *N*-acetyl-*p*-benzoquinone imine, which causes GSH depletion and increases liver damage (Figure 1a). In addition to necrosis and apoptosis, acetaminophen induces ferroptosis in primary mouse hepatocytes, but not in HepG2 liver cancer cell lines.[8] The protective effect of ferrostatin-1 against acetaminophen-induced cell death does not occur from the reduced metabolism of acetaminophen or GSH depletion.[8]

FIN. In a larger screening to find ferroptosis-inducing compounds, a series of small molecule inducers, namely ferroptosis-inducing agents (FINs), were discovered.[9,10] Each of these additional FINs generates ROS, whereas BHT strongly suppresses this lethal ROS-induced cell death.[6] In the LC-MS-based GPX4 assay, cells treated with any of the seven DPI family members (DPI7, DPI10, DPI12, DPI13, DPI17, DPI18, and DPI19) are unable to detect reduced PC-OOH levels, which is a specific substrate of GPX4.[6] Like RSL3, these FIN compounds (class II FINs) directly inhibit GPX4 activity without GSH depletion (Figure 1a).[6] Class I FINs such as DPI2 enacts the same mechanism as erastin and BSO to inhibit GPX4 by GSH depletion.[6]

Lanperisone. Lanperisone is a modified form of tolperisone, which was developed as a muscle relaxant.[11] Lanperisone can selectively kill K-Ras-mutant mouse embryonic fibroblasts through the induction of ROS, which is mediated through iron and Ras/RAF/MEK/ERK signaling (Figure 1d). *In vivo*, lanperisone also showed efficacy against tumor growth in a K-Ras-driven mouse model of lung cancer.[12] The exact mechanism responsible for lanperisone-induced ROS generation is not known, but preliminary results suggest that it occurs through the perturbation of voltage-gated ion channels.[11]

Sulfasalazine. Sulfasalazine is broadly used to treat chronic inflammation in the gut, joints, and retina. In addition to inhibiting the NF-κB signaling pathway, sulfasalazine inhibits the system X_c^- transporter (Figure 1a).[13] Given that the disruption of system X_c^--mediated cystine uptake by erastin is sufficient to induce ferroptosis, treatment of cancer cells (e.g., BJeLR and HT1080) with sulfasalazine also triggers ferroptosis.[1,7]

Sorafenib. Sorafenib induces ferroptosis in certain cancer cells such as hepatocellular carcinoma (HCC) cells.[14,15] Sorafenib-induced ferroptosis occurs independent from the oncogenic status of Ras, RAF, PIK3CA, and p53.[16] However, the expression of Rb (the prototype tumor suppressor gene) and NRF2 could inhibit sorafenib-induced ferroptosis in HCC.[14,17] The mechanism and action of sorafenib in ferroptosis may depend on the inhibition of system X_c^- function, but not on GPX4 activity (Figure 1a).[7] This process is associated with upregulated ER stress.[7] Structure activity relationship analysis of 87 sorafenib analogs further indicates that sorafenib inhibits system X_c^- activity via a non-kinase target.[7]

Artesunate. Artesunate selectively induces ferroptosis in K-Ras-mutant pancreatic ductal adenocarcinoma (PDAC) cell lines, but not human pancreatic ductal epithelial cells or wild-type K-Ras PDAC cells.[18] This process can be blocked by the iron chelator deferoxamine, whereas it is enhanced by exogenous lysosomal form of iron.[18] In addition to iron chelator deferoxamine, the ROS inhibitors (e.g., trolox and ferrostatin-1), but not necrostatin-1, significantly inhibit artesunate-induced ferroptosis in PDAC cells.[18] Another study shows that 10 artemisinin derivatives, including artesunate, altered numerous iron-related gene mRNA levels, which contributes to cell death in cancer cells.[19]

Signaling pathway

Iron metabolism and lipid peroxidation signaling are increasingly recognized as central mediators of ferroptosis.[20] In addition, activation of the mitogen-activated protein kinase (MAPK) pathway contributes to ferroptotic cancer cell death.

Iron. Excessive iron contributes to ferroptosis through producing ROS by Fenton reaction (Figure 1a). Circulating iron exists in the form of ferric iron (Fe^{3+}) by binding to transferrin. Fe^{3+} is imported into cells through the membrane protein transferrin receptor 1 (TFR1) and then locates in the endosome. In the endosome, Fe^{3+} is reduced to ferrous iron (Fe^{2+}) by the ferrireductase activity of STEAP3. Finally, divalent metal transporter 1 (DMT1, also termed SLC11A2) mediates the release of Fe^{2+} from the endosome into a labile iron pool in the cytoplasm. Excess iron is stored in ferritin, an iron storage protein complex including ferritin light chain (FTL) and ferritin heavy chain 1 (FTH1). Iron export is mediated by the membrane protein ferroportin (an iron efflux pump, also termed SLC11A3), which can oxidize Fe^{2+} to Fe^{3+}.

Ferroptosis-sensitive cells with Ras mutation have increased TFR1 and decreased ferritin (FTL and FTH1) expression compared with ferroptosis-resistant cells.[3] This suggests that increased iron uptake and reduced iron storage may contribute to iron overload during ferroptosis. Indeed, decreased iron overload by iron chelators (e.g., deferoxamine, desferrioxamine mesylate, ciclopirox olamine) inhibits erastin-mediated ferroptosis, whereas supplying exogenous sources of iron (e.g., ferric ammonium citrate, ferric citrate, and iron chloridehexahydrate) enhances erastin-induced death.[1,3] More direct evidence for iron-dependent ferroptosis comes from the knockdown of IREB2, a master transcription factor of iron metabolism. Suppression of IREB2 expression by RNAi significantly increases iron metabolism-associated gene expression (e.g., F-box and leucine-rich repeat protein 5, iron-sulfur cluster assembly enzyme, FTH1, and FTL) and limits erastin-induced ferroptosis.[1,3] Thus, the cellular systems involved in the uptake and utilization of iron are required for the induction of ferroptosis.

ROS. The ROS origin of ferroptosis induction may involve multiple sources (Figure 1c). In addition to iron-mediated ROS production by Fenton reaction, nicotinamide adenine dinucleotide phosphate (NADPH)-dependent lipid peroxidation and GSH depletion are also important for the induction of ferroptosis.[1,6] Inactivation of GPX4 by GSH depletion triggers

ferroptosis by accumulation of ROS production from lipid peroxidation.[6] Mitochondrial fatty-acid metabolism provides the specific lipid precursor required for ferroptosis. In particular, ACSF2 and CS are required for mitochondrial fatty-acid metabolism in ferroptosis. Knockdown of ACSF2 and CS inhibits erastin-induced ferroptosis.[1] In addition to glucose metabolism, lipid production can generate from the conversion of glutamine to α-ketoglutarate (Figure 1c). This process can be blocked by the small molecule transaminase inhibitor aminooxyacetic acid.

ROS can react with the polyunsaturated fatty acids (PUFAs) of lipid membranes and induce lipid peroxidation. Two lipid metabolism-associated genes (lysophosphatidylcholine acyl-transferase 3 (LPCAT3) and acyl-CoA synthetase long-chain family member 4 (ACSL4)) have been identified to promote RSL3− and DPI7 (also known as ML162)−, but not erastin-induced ferroptosis by using haploid genetic screening in KBM7 cells.[21] The depletion of arachidonic acid (AA) and other PUFAs, following GPX4 inactivation, is required for the execution of ferroptosis.[5,22] ACSL4 acylates AA and LPCAT3 catalyzes the acylated AA into membrane phospholipids. Thus, suppression of ACSL4 and LPCAT3 may decrease the oxidization of a number of sensitive fatty acids in the membrane. In contrast, GPX4-deficient cells exhibit enriched oxidized membranes with AA, which contributes to ferroptosis.[5] The release of AA mediators, including 5-hydroxyeicosatetraenoic acid (HETE), 11-HETE, and 15-HETE, but not 12-HETE in the medium of GPX4$^{-/-}$ cells, has been specifically observed following ferroptosis stimuli (e.g., RSL3 and erastin), but not apoptosis stimuli (e.g., TNFα and staurosporine).[5] Treatment with 5-, 12-, and 15-hydroperoxyeicosatetraenoic acid (HPETE) accelerates the ferroptotic process.[5] Pharmacological inhibition of sterol carrier protein 2, a lipid transport protein for mitochondria, transiently rescues GPX4$^{-/-}$ cells from ferroptosis. The priming of lethal lipid signals from GPX4$^{-/-}$ cells appears to occur outside the mitochondrial matrix.[5] Thus, ferroptosis is triggered by extra-mitochondrial lipid peroxidation, which is associated with oxidized lipid mediator release.

Another study using erastin-resistant DU-145 cancer cells indicates that upregulation of AKR1C family genes, including AKR1C1-3, is associated with ferroptosis resistance.[7] AKR1C1-3 catalyzes the oxidation of 4-hydroxynonenal into various PUFAs. These erastin-resistant DU-145 cells also exhibit resistance to sulfasalazine, sorafenib, and RSL3, suggesting that the resistance is unlikely to be due to increased system X$_c^-$ activity.

Acetyl-CoA carboxylase alpha (encoding ACC1), the rate-limiting step in fatty-acid synthesis, seems to be not required for ferroptosis in certain cells.[21] Knockdown of ACC1 by RNAi or inhibition of ACC1 by 5-tetradecyloxy-2-furonic acid did not affect RSL3- and erastin-induced ferroptosis.[21] PUFAs such as AA and linolenic acid are substrates of lipoxygenases (LOX). LOX oxidizes PUFAs to their hydroperoxyl intermediates, including HPETE, in the case of AA. PUFAs decrease when lipid peroxidation increases. When AA is the substrate, different LOX isozymes can add a hydroperoxyl group at carbons 5, 12, or 15, and therefore are designated as 5-, 12- or 15-LOX. However, 12/15-LOX does not appear to be required for ferroptosis.[5]

MAPK. The mammalian family of MAPKs mainly includes ERK, p38, and c-Jun NH$_2$-terminal kinase (JNK). Blocking the Ras/Raf/MEK/ERK pathway inhibits erastin-induced ferroptosis in Ras-mutated cancer cells.[4] However, JNK and p38, but not ERK, appear important for erastin-induced cell death in leukemia cells.[23] SP600125 (an inhibitor of JNK phosphorylation) and SB202190 (an inhibitor of p38 activation) decrease the cytotoxicity induced by erastin in HL-60 cells.[23] These findings indicate that ferroptotic responses associated with a certain MAPK module can be cell type-specific (Figure 1d).

Molecular biology

Positive regulators of ferroptosis
Vdac2/3: VDAC2/3, but not VDAC1, has a positive role in ferroptosis because knockdown of VDAC2/3, but not VDAC1, inhibits erastin-induced ferroptosis in Ras-mutated cells (Figure 1c).[4] VDAC2/3 has been found to be a direct target of erastin via affinity purification assay.[4] Cells with more VDAC protein are more sensitive to erastin.[4] Erastin decreases the rate of NADH oxidation in isolated yeast mitochondria expressing a single mouse VDAC isoform[4] and increases the permeance of NADH into liposomes containing human VDAC2.[24] Erastin can enhance oxidative mitochondrial metabolism and limit aerobic glycolysis by disrupting the interaction between VDAC and tubulin in human liver cancer cells (e.g., HepG2), suggesting a potential role of energy metabolism and cytoskeleton in the modification of ferroptosis.[25]

Ras: Erastin exhibits gene-selective lethality in Ras-mutant cells, including H-Ras-mutant engineered cells, N-Ras-mutant HT1080 cells, and K-Ras-mutant Calu-1 cells.[4] However, induction of ferroptosis may exist in a both Ras-dependent and -independent manner. Artesunate induces pancreatic cancer death in a Ras-dependent manner,[18] whereas it induces leukemia cell death in a Ras-independent manner.[23] Indeed, several normal Ras wild-type cells such as kidney tubule cells, T cells, and fibroblasts are sensitive to erastin.[5,22,26–28] Even in some cases, overexpression of mutant Ras in rhabdomyosarcoma cells (e.g., RMS13 cells) promotes ferroptosis resistance to erastin and RLS3.[29]

TFR1: Compared with ferroptosis-resistant cells (e.g., BJ cells), the expression of TFR1 is upregulated in ferroptosis-sensitive cells (e.g., BJeLR cells).[3] In contrast, the expression of FTH1 and FTL is downregulated in ferroptosis-sensitive cells (e.g., BJeLR cells), indicating that iron storage also affects ferroptosis.[3] Knockdown of TFR1 by shRNA inhibits erastin-induced ferroptosis in BJeLR cells, confirming that inhibition of iron uptake prevents ferroptosis.[3] Transferrin and glutamine are two important components of full fetal bovine serum, which induces ferroptosis in fibroblasts upon amino acid starvation.[30]

NOX: The NOX protein family transfers electrons across biological membranes to reduce oxygen to superoxide. The canonical NOX inhibitor diphenyleneiodonium and the NOX1-/4-specific inhibitor GKT137831 partly inhibit erastin-induced ferroptosis in Calu-1 and HT1080 cells (Figure 1c).[1] The pentose phosphate pathway (PPP) is a metabolic pathway

parallel to glycolysis that generates NADPH and pentoses. Pharmacological inhibition of PPP by 6-aminonicotinamde (Figure 1c) or knockdown of two PPP enzymes (glucose-6-phosphate dehydrogenase and phosphoglycerate dehydrogenase) also partly prevents erastin-induced ferroptosis in Calu-1 cells.[1]

p53: Activation of p53 has been found to be required for ferroptosis in certain cancer cells.[27] This process depends on direct transcriptional inhibition of SLC7A11, a key component of system X_c^- (Figure 1a) (discussed later). Moreover, p53[3KR] (an acetylation-defective mutant) is responsible for the inhibition of SLC711A expression (Figure 1a), but not other known p53 target genes (e.g., p21 and BAX) involved in antiproliferative and pro-apoptotic activity.[27] p53[3KR] mice lacking cell-cycle arrest, apoptosis, or senescence still exhibit tumor suppression function.[31] This tumor suppressor function of p53[3KR] is dependent on the induction of ferroptosis.[27] Ferroptosis also mediates hyperactive p53 signaling in the promotion of embryonic lethality.[27] The E3 ubiquitin ligase murine double minute-2 (MDM2) regulates the proteasomal degradation of p53. Ferrostatin-1 alone cannot prevent knockdown of MDM2 by RNAi-induced cell death,[32] indicating that mixed cell death types are responsible for p53-induced death.

CARS: Cysteinyl-tRNA synthetase (CARS) is a positive regulator of ferroptosis upon cystine deprivation.[33] Knockdown of CARS inhibits erastin-induced ferroptosis, whereas overexpression of CARS enhances erastin sensitivity in several types of cancer cells (Figure 1c).[33] However, loss of CARS cannot prevent RSL3-, FIN56-, and BSO-induced cell death, suggesting that CARS regulates ferroptosis at the cysteine biosynthesis level.[33]

Negative regulators of ferroptosis

GPX4: GPX4 converts reduced GSH to oxidized glutathione (GSSG) while reducing lipid hydroperoxides to their corresponding alcohols or free hydrogen peroxide to water. Treatment with erastin or BSO can deplete GSH and GSSG and increase NADPH oxidation and lysophosphatidylcholines (an indicator of generation of ROS from lipid).[6] In contrast, GSH and *N*-acetylcysteine (a GSH biosynthetic precursor) prevent erastin lethality in U2OS cells (Figure 1a).[6] However, RSL3 can generate ROS in the absence of GSH depletion.[6] GPX4 is a direct target of RLS3. Knockdown of GPX4 induces ferroptosis in an iron-, MEK-, and ROS-dependent manner, whereas overexpression of GPX4 leads to resistance to RSL3.[6] Erastin is also able to cause GPX4 degradation in several types of cancer cells, suggesting that the protein degradation pathway is involved in ferroptosis.[23]

The function of ferroptosis *in vivo* is confirmed by using GPX4 conditional or inducible knockout mice. Inducible knockout of GPX4 in the kidney leads to acute renal failure, which can be rescued by ferrostatin-1 and necrostatin-1 (a necroptosis inhibitor).[5] Necrostatin-1 may have an off-target effect in the inhibition of ferroptosis in the kidney.[5] In the presence or absence of lymphocytic choriomeningitis virus infection, fewer CD8^{+} T cells have been observed in the spleens and lymph nodes of mice with conditional knockout of GPX4 in T cells by *Cd4-Cre*.[26] GPX4^{−/−} T cells rapidly accumulate lipid peroxides and die by ferroptosis, but not other types of RCD.[26] Inducible neuron-specific GPX4 knockout

mice have neuronal loss in the brain partly due to induction of ferroptosis.[34] In contrast, mice with deletion of GPX4 in hematopoietic cells develop anemia due to induction of RIP3-dependent necroptosis, but not apoptosis and ferroptosis.[35] These results indicate context-dependent functions of the GPX4 in cell death.

System Xc⁻: System X_c^- is responsible for maintaining redox homeostasis by importing cystine, where it is then reduced to cysteine and used to synthesize the major antioxidant GSH. Inhibiting system X_c^- with sulfasalazine can trigger ferroptosis, whereas increasing cystine uptake into cells by β-mercaptoethanol inhibits erastin-induced ferroptosis in HT1080 cells (Figure 1a).[1] System X_c^- structurally consists of SLC7A11 and SLC3A2. The upregulation of SLC7A11 by erastin is not dependent on iron and ROS.[1] Suppression of SLC7A11 expression by RNAi increases the anticancer activity of erastin, whereas overexpression of SLC7A11 by gene transfection diminishes erastin-induced ferroptosis.[1] In contrast, RSL3-mediated ferroptosis is system X_c^--independent.[1] Several ER stress markers such as phosphorylation of eIF2α and ATF4 protein upregulation has been observed in ferroptosis following treatment with erastin, sulfasalazine, and sorafenib.[7] As discussed above, p53 is a positive regulator of ferroptosis in certain cancer cells by inhibition of SLC7A11 expression, therefore inhibiting system X_c^- activity.[7]

HSPB1: HSPB1 expression is remarkably induced by the transcriptional factors heat shock factor-1 (HSF-1) after erastin treatment in several human cancer cells.[36] Inhibition of HSF-1-dependent HSPB1 expression increases, whereas overexpression of HSPB1 inhibits erastin-induced ferroptosis.[36] The phosphorylation of HSPB1 is important for its function in the regulation of actin dynamics and iron uptake. HSPB1 phosphorylation is increased by protein kinase C (PKC) following erastin treatment in HeLa cells, which facilitates ferroptotic resistance through blocking cytoskeleton-mediated iron uptake and subsequent ROS production (Figure 1b).[36]

NRF2: NRF2 has an anti-ferroptosis role in HCC cells.[17] p62 interacts with the NRF2-binding site of Kelch-like ECH-associated protein 1 (Keap1) and competitively inhibits Keap1-NRF2 interaction, which is responsible for NRF2 protein stability following treatment with FINs (e.g., erastin, sorafenib, and BSO).[17] Upregulated NRF2 protein promotes transcription of genes encoding antioxidant proteins (e.g., quinone oxidoreductase 1 and heme oxygenase-1 (HO-1)) and iron metabolism proteins (e.g., FTH1) in ferroptosis.[17] Knockdown of NRF2 and these NRF2-targeted genes accelerates erastin or sorafenib-induced ferroptosis in HCC cells.[17] In contrast, induction of HO-1 expression by erastin may promote cell death in HT1080 and fibroblasts, suggesting that HO-1 has a dual role in ferroptosis.[37]

Measuring methods

In vitro

Cell viability: Four methods of cell viability measurement have been reported in the evaluation of ferroptosis: calceinacetoxymethyl ester (Calcein AM) viability assay,[38] alamarBlue viability assay,[39] trypan blue assay,[40] and Cell Counting Kit-8 assay.[23]

Iron level: Phen Green SK (PGSK) is a cell membrane-permeable dye used to monitor the intracellular iron level in living cells via flow cytometry or confocal microscopy. It shows a diminished green autofluorescence of PGSK upon binding to sufficient cellular iron following erastin treatment.[3] The Iron Assay Kit provides a simple convenient means of measuring ferrous and/or ferric ions in samples.[17]

ROS level: Erastin-treated HT1080 cells are accompanied by a prolonged period of lipid peroxidation detection by C11-BODIPY probe and shift the fluorescence from red to green, whereas ferroptosis inhibitors restrict this shift. In contrast, MitoSOX (a mitochondrial ROS probe) is not changed following erastin treatment.[1] In addition, GSH depletion, glutamate release, and NADPH and cystine uptake assay can be used to monitor ferroptosis. GSH depletion and inhibition of glutamate release are observed in erastin- and sorafenib-induced ferroptosis.[6,7] Decreased cystine uptake and increased NADPH oxidation capability are associated with increased ferroptosis.[6]

In vivo. Prostaglandin-endoperoxide synthase (PTGS) is the key enzyme in prostaglandin biosynthesis. There are two isozymes of PTGS: a constitutive PTGS1 and an inducible PTGS2. PTGS2 encoding cyclooxygenase-2 (COX-2) is significantly upregulated after treatment with RSL3 and erastin in mice.[6] Upregulated PTGS2 only indicates ferroptosis onset and does not affect ferroptosis development.[6] Assessment of ferroptosis-associated genes/proteins in tissue and iron and GSH level in serum may be an alternative approach to monitor ferroptosis *in vivo*.

Inhibitor

Several ferroptosis inhibitors are listed in Table 2. Most of them are antioxidants or iron chelators. Here, we discuss three major compounds with specific anti-ferroptotic activity.

Ferrostatin. First-generation ferrostatin is termed ferrostatin-1 and acts to inhibit erastin- and RSL3-induced ferroptosis in HT1080 cells.[1] The activity of ferrostain-1 depends on the primary aromatic amine, which specifically inhibits accumulation of ROS from lipid oxidation.[1] Compared with ferrostatin-1, second- (termed SRS 11–92) and third-generation ferrostatins (termed SRS 16–86) have increased plasma and metabolic stability and significantly protect against tissue injury (e.g., acute kidney injury and ischemia-reperfusion injury) *in vivo* (Figure 1a).[22,28]

Liproxstatin-1. Liproxstatin-1 prevents ROS accumulation and cell death in GPX4$^{-/-}$ cells (Figure 1a).[5] Moreover, liproxstatin-1 inhibits FINs (e.g., erastin, RSL3, and BSO)-induced ferroptosis *in vitro*.[5] Intraperitoneal administration of liproxstatin-1 (10 mg/kg) in inducible GPX4$^{-/-}$ mice prolongs animal survival in response to kidney injury.[5] Liproxstatin-1 also protects against ischemia/reperfusion-induced liver injury in mice.[5]

Zileuton. Zileuton is an orally active specific inhibitor for 5-LOX, but not 12/15-LOX (Figure 1c). Zileuton provided significant protection from glutamate- and erastin- induced ferroptosis in HT22 cells (a mouse hippocampal cell line) by inhibition of cytosolic ROS production.[41]

Diseases

Cancer. NCI-60 is a panel of 60 diverse human cancer cell lines from eight diverse tissue types used by the US National Cancer Institute Developmental Therapeutics Program. Among them, kidney and leukemia cancer cells are more sensitive to erastin compared with cancer cells from the other six tissues (lung, colon, central nervous system, melanocytes, ovary, and breast).[6] Furthermore, the anticancer activity of erastin was tested in 117 cancer cells and no correlation between Ras mutation and erastin potency was identified.[6] Besides exerting single effects, erastin also enhances chemotherapy drugs such as temozolomide, cisplatin, cytarabine/ara-C, and doxorubicin/ adriamycin) in certain cancer cells.[23,42,43] Induction of ferroptosis by FDA-approved drugs such as sorafenib, sulfasalazine and artesunate holds great potential for cancer therapy. *In vivo*, erastin, piperazine erastin, and RSL3 prevented tumor growth in a xenograft model.[6,36] However, the role of ferroptosis in tumorigenesis still remains unclear.

Neurotoxicity. Inhibition of ferroptosis by ferrostatin-1 protects organisms from glutamate-induced neurotoxicity in a rat organotypic hippocampal slice culture model.[1] Fms-like tyrosine kinase 3 (FLT-3, also termed CD135) is a cytokine receptor, which is important for the normal development of hematopoietic stem cells and progenitor cells. Potent inhibitors for FLT-3 and its downstream signaling molecule phosphoinositide 3-kinase *α* (p110a) can suppress ROS production and lipid peroxidation to block ferroptotic cell death in neurons[44] (Figure 1c). Inhibition of ferroptosis by ferrostatins (ferrostatin-1 and SRS 11–92) also restored the number of healthy neurons in a Huntington's disease model.[22] Periventricular leukomalacia usually refers to the death of developing oligodendrocytes (OLs). Ferrostatins (e.g., ferrostatin-1 and SRS 11–92) remarkably protect OLs from cystine deprivation.[22]

AKF. Ferrostatins (e.g., ferrostatin-1 and SRS 11–92) had a protective role to prevent lethality of in a model of acute injury of freshly isolated renal tubules,[22] implicating ferroptosis-mediated cell death in acute kidney failure (AKF). Inhibition of ferroptosis by third-generation ferrostatins (SRS 16–86) limits acute ischemia-reperfusion injury and oxalate nephropathy-related AKF.[28] Similarity, inducible knockout of GPX4 in the kidney leads to ferroptosis, which contributes to AKF in mice.[5]

Liver injury. Acetaminophen overdoses are currently the most frequent cause of acute liver failure. Acetaminophen has been demonstrated to induce ferroptosis in primary liver cells and ferroptosis inhibitors such as ferrostain-1 can inhibit acetaminophen-induced death.[8] Ferroptosis is also implicated in hepatic ischemic damage in mice. Ischemia/reperfusion-induced liver injury can be ameliorated in mice by the ferroptosis inhibitor liproxstatin-1.[5]

Hear injury. Inhibiting glutaminolysis and ferroptosis by compound 968, DFO, or ferrastatin-1 limits ischemia/reperfusion-induced heart injury ex vivo.[30]

Conclusion & perspective

Several molecules have recently been identified to regulate ferroptosis by directly or indirectly targeting iron metabolism and lipid peroxidation. These so-called ferroptosis regulators are also implicated in other types of RCD. Thus, the most important objective in the study of ferroptosis is to identify the downstream signaling pathways or executors of iron-dependent ROS metabolism to distinguish ferroptosis from other types of RCD.

Induction of ferroptosis by drugs has been shown to inhibit cancer cell growth in both a Ras-dependent and -independent manner, suggesting that cancer cells display genetic hetero-geneity in the timing of the ferroptotic response. Further definition of the genotype-selective activity of ferroptosis in cancer and the mechanisms involved will be important to guide ferroptosis-based therapeutic intervention. Ferroptosis has an important role in sterile inflammatory conditions such as tissue acute injury, ischemic-reperfusion injury, and neurotoxicity. An improved understanding of the role of ferroptosis in cancer and injury-associated diseases will create a new opportunity for diagnosis and therapeutic intervention.

Conflict of Interest

The authors declare no conflict of interest.

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