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Review Article

Ferroptosis and Intrinsic Drug-induced Liver Injury by Acetaminophen and Other Drugs: A Critical Evaluation and Historical Perspective

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Abstract

Drug-induced hepatotoxicity is a significant clinical issue worldwide. Given the limited treatment options for these liver injuries, understanding the mechanisms and modes of cell death is crucial for identifying novel therapeutic targets. For the past 60 years, reactive oxygen species and iron-dependent lipid peroxidation (LPO) have been hypothesized to be involved in many models of acute drug-induced liver injury. However, this mechanism of toxicity was largely abandoned when apoptosis became the primary focus of cell death research. More recently, ferroptosis—a novel, non-apoptotic form of cell death—was identified in NRAS-mutant HT-1080 fibrosarcoma cells exposed to erastin and other NRLs. Ferroptosis is characterized by glutathione depletion and the impairment of glutathione peroxidase 4 activity, which hinders the detoxification of lipid hydroperoxides. These hydroperoxides then serve as substrates for iron-dependent LPO propagation. This cell death mechanism is now receiving widespread attention, extending well beyond its original identification in cancer research, including in the field of drug-induced liver injury. However, concerns arise when such mechanisms are applied across different cell types and disease states without sufficient validation. This review critically evaluated the historical evidence for iron-dependent LPO as a mechanism of drug-induced hepatotoxicity and explored how these earlier findings have led to the current concept of ferroptosis. Overall, the published data support the idea that multi-layered endogenous antioxidant defense mechanisms in the liver limit the occurrence of pathophysiologically relevant LPO under normal conditions. Only when these defense mechanisms are severely compromised does ferroptosis become a significant mode of drug-induced cell death.

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Introduction

Drug-induced liver injury (DILI) is a significant clinical problem worldwide. Fundamentally, DILI can be divided into two categories: intrinsic hepatotoxicity and idiosyncratic hepatotoxicity. The mechanism of cell death and liver injury caused by idiosyncratic drugs is unclear in most cases, aside from the involvement of the adaptive immune system. Consequently, the mode of cell death in idiosyncratic DILI is not well studied.[1,2] In contrast, the cell death mechanisms of intrinsic hepatotoxins, particularly acetaminophen (APAP), have been extensively investigated due to their clinical relevance.[3–5] Although safe and effective at therapeutic doses, an overdose of APAP can cause severe liver injury, acute liver failure, and even death.[4,5] A key advantage in the study of APAP toxicity is that its human pathophysiology can be effectively reproduced in a mouse model *in vivo* and in primary mouse hepatocytes.[6,7] Consequently, these models are frequently used to investigate cell death signaling mechanisms and to assess the therapeutic potential of new drug candidates.[8] The mechanisms of APAP-induced cell death, which apply to both animals and humans, include the formation of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) by cytochrome P450 2E1 (Cyp2E1), depletion of hepatic GSH, and protein adduct formation, primarily in mitochondria. This leads to the initiation of mitochondrial oxidative stress, which is amplified by the activation of c-Jun N-terminal kinase (JNK), resulting in mitochondrial dysfunction and opening of mitochondrial permeability transition pores. This process ultimately leads to the release of mitochondrial intermembrane proteins, such as endonuclease G, which translocate to the nucleus and trigger DNA fragmentation.[9,10] The culmination of these signaling events is oncotic necrosis[11] rather than apoptosis,[12] despite some overlap in mechanisms, such as the translocation of Bax and Bid to the mitochondria,[13–15] mitochondrial cytochrome c release,[13,14,16] and DNA fragmentation, as indicated by DNA ladder formation[17,18] and TUNEL staining.[19] However, over the last decade, the focus has shifted from comparing generic necrosis and apoptosis to exploring the signaling mechanisms of various necrotic cell death pathways.[20] As expected, it was suggested that APAP-induced liver injury might involve necroptosis,[21] pyroptosis,[22] or ferroptosis.[23,24] Although the importance of necroptosis, pyroptosis, and ferroptosis in APAP-induced liver injury has been questioned,[25,26] ferroptosis has emerged as a favored mode of cell death, with a dramatic increase in related pub-

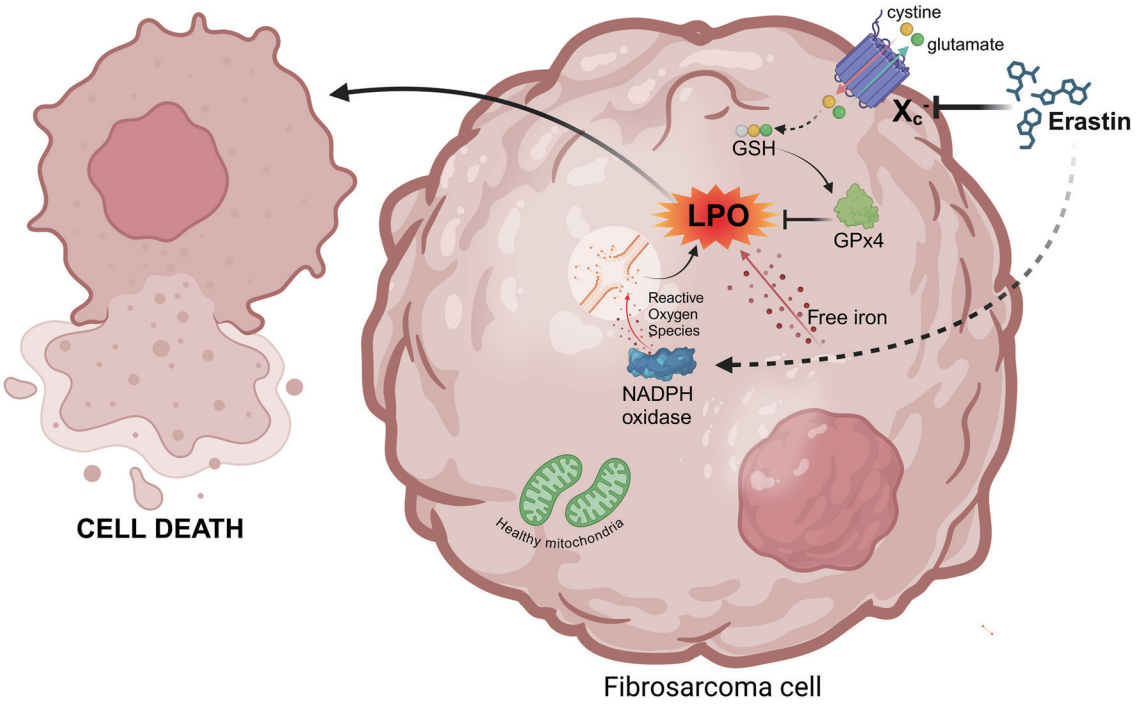

Fig. 1. The concept of ferroptosis in cancer cell death in response to specific stimuli. Ferroptosis, a cell death pathway distinct from apoptosis and necrosis, was described in fibrosarcoma cells exposed to erastin. Exposure to the drug induced a mitochondria-independent mode of cell death due to the induction of reactive oxygen species (ROS) through erastin-mediated activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Excessive ROS generation near lipid membranes, combined with free iron, facilitates lipid peroxidation (LPO). While enzymes such as glutathione peroxidase 4 (GPx4) typically utilize cellular glutathione (GSH) stores to scavenge lipid hydroperoxides and prevent LPO-induced damage, erastin-mediated inhibition of cysteine uptake through the Xc- transporter compromises this defense strategy, leading to cell death. (Created with biorender.com)

lications in recent years. Therefore, this review will critically assess the hypothesis of whether, and under what circumstances, ferroptosis is a relevant mode of cell death following APAP overdose and other intrinsically toxic drugs. Furthermore, it will explore whether, as proposed by an exponentially growing number of studies, ferroptosis signaling can serve as a therapeutic target for these patients.

Mechanisms of ferroptosis

The term ferroptosis was introduced by Stockwell and colleagues in 2012.[27] The background behind this discovery is that approximately 30% of all cancer cells exhibit mutations in the RAS family of GTPases.[28] Thus, identifying compounds that can selectively kill cells with RAS mutations (RAS-selective lethal compounds – RSLs) presents a potential anticancer strategy. Stockwell's group identified two chemicals, erastin and RSL3, which appear to selectively kill cancer cells with RAS mutations.[29,30] Interestingly, cell death induced by these RSLs did not involve classical features of apoptosis but seemed to depend on reactive oxygen species and iron.[29–31] Based on these findings, Dixon *et al*. explored the mechanism of cell death caused by erastin in an NRAS mutant HT-1080 fibrosarcoma cell line in culture.[27] The authors documented that erastin triggered mitochondria-independent oxidative stress causing cell death, which could be prevented by iron chelation.[27] Consistent with the lack of mitochondrial involvement, erastin did not cause ATP depletion, and morphologically, mitochondria appeared smaller and denser, but not swollen or ruptured as seen in classical necrotic cell death. Additionally, many other inhibitors of various features of necrotic, apoptotic, and autophagic cell death had no effect in this model. In contrast, the erastin-induced oxidative stress and cell death could be prevented in HT-1080 and other cancer cell lines by Trolox (a vitamin E analog), the iron chelator deferoxamine (DFO), the MEK inhibitor U0126, and the glutathione peroxidase mimetic ebselen.[27] These observations suggested that erastin-induced ferroptosis is clearly distinct from autophagy, apoptosis, and known forms of necrosis.[27] The authors also identified a small molecular inhibitor of ferroptosis, termed ferrostatin-1 (Fer-1), which appeared to selectively inhibit erastin-induced cell death but not apoptosis or other reactive oxygen species (ROS)-induced forms of cell death.[27] Because Fer-1 was more potent than lipophilic or soluble antioxidants, it was hypothesized that its aromatic amine structure "may confer a unique profile of radical reactivity upon Fer-1 that is better tuned to the RSL mechanism".[27] Interestingly, Fer-1 and an iron chelator were also effective in preventing glutamate-induced cell death in an organotypic hippocampal slice culture model, suggesting that neuronal cell death in this model resembles erastin-induced cell death in cancer cells.[27] However, when potential initiating events were investigated, the authors provided evidence that erastin inhibits system X_c^-, a Na^+-independent cystine/glutamate antiporter responsible for cystine uptake into the cell, thereby promoting glutathione synthesis.[27] The final experiment demonstrated the involvement of NADPH oxidase-dependent ROS formation following erastin exposure, leading to the emergence of ferroptosis as a unique cell death mechanism (Fig. 1). This mechanism involves a combination of oxidative stress and the formation of iron-dependent lipophilic ROS, e.g., lipid hydroperoxides. Although lipid hydroperoxides can be detoxified by GPx4, the inhibition of cystine uptake and limitation of GSH synthesis impair this detoxifi-

cation system, resulting in lipid peroxidation-dependent cell death (Fig. 1). However, several considerations must be taken into account. First, the detailed mechanisms of ferroptosis have been specifically investigated for cell death caused by erastin and other RSLs in NRAS mutant HT-1080 fibrosarcoma and related cancer cell lines, as well as for glutamate-induced cell death in brain slices. Both models are susceptible to ferroptosis due to inhibition of cystine uptake, elevated iron levels, and high concentrations of polyunsaturated fatty acids (PUFAs). Additionally, regular cell culture conditions using room air represent hyperoxic conditions for these cells, which are known to result in increased ROS formation.[32]

Thus, it is questionable whether this mechanism can be applied to other cell types, particularly hepatocytes with their complex, multi-layered antioxidant systems.[33] Nevertheless, ferroptosis has gained significant traction as a mode of cell death in various disease processes, including liver diseases and DILI, especially APAP-induced liver injury.[34]

Fenton reaction and lipid peroxidation in hepatotoxicity

According to a more recent review by Dixon and Stockwell,[35] there are "*three essential hallmarks that define ferroptosis: the loss of lipid peroxide repair capacity by the phospholipid hydroperoxidase GPx4, the availability of redox-active iron, and the oxidation of PUFA-containing phospholipids*." Although the term ferroptosis is new, the process of iron-dependent lipid peroxidation (LPO) as a mechanism of cell death in the liver has been recognized for 60 years. Carbon tetrachloride,[36,37] acetaminophen,[38] allyl alcohol,[39] ethanol,[40] and others have been suggested to involve LPO-induced cell death, with an essential role for iron. LPO is a free radical chain reaction initiated by ferrous iron, which catalyzes the formation of hydroxyl radicals from hydrogen peroxide (Fenton reaction),[41] attacking PUFAs and generating a self-perpetuating cycle that leads to their destruction.[42] This results in changes to membrane permeability, collapse of membrane potentials, and ion gradients, ultimately triggering cell and organelle swelling. For instance, the active exclusion of Ca^{2+} from the cytosol is impaired,[43] and the resulting increase in cytosolic Ca^{2+} induces its uptake by mitochondria, leading to mitochondrial dysfunction and the opening of the mitochondrial permeability transition pore (MPTP)as a cause of cell death.[44] Although cellular free iron concentrations are generally kept very low by binding to ferritin, iron can be mobilized from this binding protein by superoxide.[45] GPx4, an enzyme that specifically reduces lipid hydroperoxides, was recognized many years ago.[46] Most interventions involving LPO show hepatic GSH depletion, either from a reactive metabolite (acetaminophen – NAPQI; allyl alcohol – acrolein) that directly reacts with GSH[39,47] or that promotes GSH efflux (ethanol).[48,49] This GSH depletion impairs the capacity of GPx4 to reduce lipid hydroperoxides and to interrupt the propagation of LPO.

A prime example of this overall process is allyl alcohol hepatotoxicity. It has been shown that allyl alcohol causes massive GSH depletion through the formation of the reactive metabolite acrolein, followed by extensive LPO, as indicated by ethane and pentane exhalation and malondialdehyde (MDA) formation, resulting in severe liver injury.[39] LPO was also characterized by specific loss of PUFAs such as arachidonic acid (20:4) and docosahexaenoic acid (22:6) in the liver.[39] Treatment with either the iron chelator DFO or the lipid-soluble antioxidant vitamin E effectively protected against LPO and liver injury in the allyl alcohol model.[39] The cellular levels of free ferrous iron were increased by the excessive formation of NADH through the metabolism of allyl alcohol by alcohol dehydrogenase and acrolein by aldehyde dehydrogenase.[50] It was demonstrated that NADH, but not NADPH, can dose-dependently release iron from ferritin and also reduce ferric iron to ferrous iron.[50] The critical role of this metabolism for LPO and liver injury was shown by blocking toxicity with an alcohol dehydrogenase inhibitor and aggravating toxicity with an aldehyde dehydrogenase inhibitor.[39,50] These results, published more than 30 years ago, fulfill all the requirements of ferroptosis as outlined by Dixon and Stockwell.[35] However, what is often overlooked is the fact that the mice were fed a diet deficient in vitamin E and high in soybean oil, which substantially increased the hepatic contents of PUFAs (20:4; 22:6), making the animals highly susceptible to LPO.[39] In contrast, under normal conditions with a regular diet, allyl alcohol caused less LPO and less injury.[51] Importantly, chelation of iron with DFO reduced LPO but had no effect on the injury, suggesting a non-ferroptotic mode of cell death.[51]

These findings raise an important question: How much LPO is needed to kill hepatocytes? We have previously demonstrated extensive oxidative stress, mainly caused by immune cells, in hepatic ischemia-reperfusion injury.[52,53] However, despite the severe liver injury, highly specific LPO parameters such as hydroxy-eicosatetraenoic acids, products of GPx4 activity, and F2-isoprostanes were only increased two- to three-fold above baseline, suggesting minimal LPO.[54] In contrast, when animals were treated with tert-butylhydroperoxide to induce LPO-mediated liver injury, extensive LPO—indicated by a 12- to 30-fold increase in these LPO parameters—was necessary to even cause moderate liver injury.[54] Furthermore, in the allyl alcohol and APAP mouse models with vitamin E deficiency, there were up to 50-fold increases in ethane and pentane exhalation, which correlated with severe liver injury.[38,39] In contrast, under normal conditions, allyl alcohol- and APAP-induced liver injury involved only minimal LPO.[51,55] These data across various models consistently indicate that the final step of ferroptosis signaling, i.e., LPO, needs to be increased by 10- to 50-fold over constitutive levels to be biologically relevant.

Acetaminophen

Lipid peroxidation

The hypothesis that LPO is the main cause of APAP hepatotoxicity was introduced by Wendel and coworkers 35 years ago,[38,56–58] contrasting the previously established idea that protein binding of a reactive metabolite generated by cytochrome P450 is the key event in cell death.[47,59,60] This created controversies regarding the roles of reactive oxygen and LPO in the pathophysiology. First, the severe LPO reported in studies by Wendel and colleagues[38,56–58] could not be reproduced by others.[37] The main reason was that the mice used in Wendel's studies were kept on a vitamin E-deficient diet high in PUFAs, which made them highly susceptible to LPO. In fact, the massive LPO induced by an APAP overdose of 400 mg/kg decimated the liver and caused the death of the animal within 4–5 h.[38,56–58] In contrast, animals on a normal diet experienced significant centrilobular necrosis with only limited LPO and no death within 24 h (Fig. 2A).[37,55] Consistent with these observations, vitamin E effectively protected against APAP toxicity in susceptible mice[61] but not in animals on a normal diet, even if liver vitamin E levels were increased sevenfold.[55] Thus, it appears that relevant LPO sufficient to cause cell death is only observed under conditions that make the animals highly susceptible to LPO, but not under normal

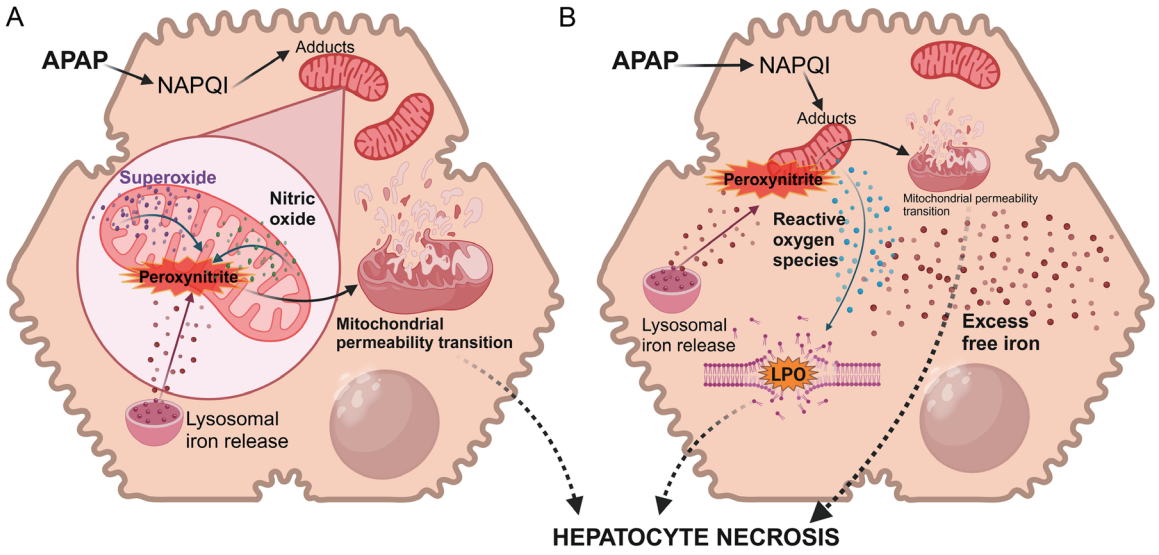

Fig. 2. Lipid peroxidation and acetaminophen-induced cell death. Lipid peroxidation is not a feature of acetaminophen (APAP)-induced hepatocyte necrosis without iron overload. A) Mitochondrial generation of superoxide is a characteristic feature of APAP-induced hepatocyte cell death, which reacts with nitric oxide to form the highly reactive oxidant and nitrating species peroxynitrite, causing extensive protein nitration in mitochondria. This process is facilitated by lysosomal iron release and its uptake into mitochondria, resulting in the induction of the mitochondrial permeability transition and ultimately hepatocyte necrosis. B) Under conditions of APAP overdose along with iron overload, excessive cellular free iron reacts with mitochondrial reactive oxygen species (ROS) to induce lipid peroxidation (LPO) of membranes. Iron-catalyzed lipid peroxidation, along with iron-catalyzed protein nitration, contributes to the induction of the mitochondrial permeability transition and cell necrosis. (Created with biorender.com). NAPQI, N-acetyl-p-benzoquinone imine.

conditions. This hypothesis was further supported when similar findings were obtained with ferrous iron treatment.[62] In these experiments, APAP caused severe liver injury with no significant LPO, but pretreatment with ferrous iron triggered extensive LPO and aggravated the injury (Fig. 2B).[62]

Oxidant stress

The second controversy surrounding APAP pathophysiology is whether APAP involves oxidant stress.[63] It was initially hypothesized that ROS are derived from the cytochrome P450 (Cyp) system during APAP metabolism, based on the observation that LPO can be inhibited by Cyp inhibitors and enhanced by Cyp inducers.[56] However, more direct assessments of intracellular oxidant stress through measurement of GSSG formation showed no evidence of oxidant stress during the metabolism phase in rats,[64] mice,[65] or mouse hepatocytes.[66] In contrast, GSSG accumulation was detected in mitochondria, suggesting mitochondrial oxidant stress.[67] APAP metabolism and protein adduct formation caused inhibition of mitochondrial respiration,[68,69] mainly at complex III,[70] leading to electron leaks from the electron transport chain. More recently, it was shown that initial electron leakage and superoxide formation from complex III occur mainly towards the mitochondrial intermembrane space, leading to a mild oxidant stress in the cytosol and activation of a MAP kinase cascade with phosphorylation of c-Jun N-terminal kinase (hereinafter referred to as pJNK).[71] pJNK translocates to mitochondria,[72] and through binding to the anchor protein Sab,[73] amplifies the oxidant stress.[74] The enhanced oxidant stress in the mitochondrial matrix is then mainly derived from complex I and, to a lesser degree, from complex III,[71] and the oxidant stress and injury correlate with protein adduct formation in mitochondria.[75,76] Although other sources of oxidants (H_2O_2) than the electron transport chain have been identified in relation to fatty acid metabolism within mitochondria,[77,78] the formation of peroxynitrite as the critical oxidant for mitochondrial dysfunction requires superoxide, which is generated by the electron transport chain. Together, these observations lead to the conclusion that the formation of NAPQI by Cyp2E1 and the formation of protein adducts, especially on mitochondria, are initiating events leading to mitochondrial dysfunction and mitochondrial oxidant stress (Fig. 2A).[8,9] Interestingly, Cyp2E1 expression in mitochondria may be critical for hepatotoxicity.[79–81] Thus, there is an overall consensus that an APAP overdose can trigger substantial oxidant stress in mitochondria, albeit with limited LPO.

Peroxynitrite

Although the idea that oxidant stress is critical for APAP-induced cell death was largely accepted by the late 1990s, the mechanisms of ROS-induced cell death remained unclear. During this time, peroxynitrite—a reaction product between the superoxide radical and nitric oxide—was discovered in biological systems.[82] Peroxynitrite is a potent oxidant and nitrating species.[83] Hinson and co-workers provided the first evidence for peroxynitrite formation in the liver during APAP hepatotoxicity by demonstrating nitrotyrosine protein adducts.[84] The initial hypothesis that inducible nitric oxide synthase (iNOS) in Kupffer cells is a significant source of NO formation[85] was quickly dismissed, as iNOS was not consistently induced in different models[86] and iNOS inhibitors did not provide protection.[74] Additionally, the fact that gp91phox knockout mice, which lack functional NADPH oxidase (the main producer of superoxide in phagocytes), neither showed reduced peroxynitrite formation nor protection against APAP toxicity suggested that phagocytes, including Kupffer cells, cannot be the source of superoxide.[87] In contrast, there is strong evidence for mitochondrial superoxide formation during APAP toxicity.[67,86] NO is more stable and can diffuse through membranes, while the superoxide anion has a shorter half-life and cannot cross membranes. Therefore, the observation that nitrotyrosine adducts are almost exclusively found inside mitochondria[18] suggests that superoxide is generated within the mitochondria and is not derived from other

cellular sources.

An important question remained: Is peroxynitrite a critical mediator of the injury process or merely an irrelevant side effect? To address this, we treated animals with GSH to restore hepatic GSH levels at different time points after APAP.[88] Intravenous GSH is not directly taken up by hepatocytes but is degraded in the kidney; the reabsorbed amino acids are then used to effectively resynthesize GSH in hepatocytes.[88] In fact, on a molar basis, GSH is superior to N-acetylcysteine,[89] the standard of care for APAP overdose. GSH not only detoxifies NAPQI but is also a potent scavenger of peroxynitrite.[88] In these experiments, we identified a window where drug metabolism and NAPQI formation were completed, but GSH could still effectively protect due to its scavenging of peroxynitrite, suggesting that this oxidant is critical for APAP-induced liver injury.[88] This conclusion was further supported by the demonstration of a dramatic increase in nitrotyrosine staining and liver injury in MnSOD (SOD2) partial knockout mice,[90] as well as the elimination of nitrotyrosine protein adducts and liver injury in animals treated with mitochondrial-targeted SOD mimetics such as Mito-TEMPO[91] and Mito-Q.[92] MnSOD is also a target of peroxynitrite, further impairing the intramitochondrial defense system.[93] Consistent with these data, JNK inhibitors were shown to prevent nitrotyrosine staining and effectively protect against APAP-induced liver injury.[74,94] Together, these observations provide strong evidence that peroxynitrite formation in mitochondria is a critical cytotoxic mediator in the pathophysiology of APAP hepatotoxicity.[95] MnSOD is important for limiting damage by removing superoxide, which is one of the reaction partners for peroxynitrite formation. Importantly, despite the enhanced formation of hydrogen peroxide in the dismutation reaction, LPO is very limited. This again suggests the critical role of peroxynitrite—rather than LPO—as the primary cause of oxidative damage to mitochondrial DNA[18] and protein nitration, which are central mechanisms of toxicity (Fig. 2A).

Iron

In 2008, Lemasters and colleagues recognized that an increase in chelatable iron in hepatocytes during severe oxidant stress induced by tert-butyl hydroperoxide originated from lysosomes.[96] The iron from lysosomes appears to be taken up into mitochondria, contributing alongside ROS to the opening of the MPTP and subsequent cell death.[96] Because APAP also causes lysosomal instability,[97] an APAP overdose can mobilize lysosomal ferrous iron, which, after uptake into mitochondria, triggers MPTP opening and cell necrosis.[98] The uptake of ferrous iron during APAP-induced cell death *in vitro* was mediated by the mitochondrial electrogenic Ca(2+), Fe(2+) uniporter (MCFU).[99] Interestingly, targeting the MCFU with the inhibitor minocycline was effective in protecting against APAP-induced liver injury *in vivo*.[62,100] Additionally, mitochondrial iron uptake, MPTP opening, and liver injury were significantly reduced in MCFU gene knockout mice exposed to APAP,[101] and selective chelation of iron in lysosomes with starch-desferal and the general iron chelator DFO also effectively prevented APAP-induced liver injury.[62,99,100] Together, these reports provide strong evidence that after an APAP overdose, *in vivo* and in isolated hepatocytes in culture, ferrous iron can be released from lysosomes and then taken up into mitochondria via the MCFU, where it contributes to MPTP opening and cell death (Fig. 2A).

If one assumes that the primary function of ferrous iron involves catalyzing the Fenton reaction to reductively cleave hydrogen peroxides and lipid hydroperoxides, initiating and propagating LPO, then the iron data clearly contradicts and is incompatible with the previous hypothesis that peroxynitrite is a critical mediator of APAP toxicity. However, more detailed mechanistic studies have shown that protein nitration by peroxynitrite requires transition metals, such as ferrous iron.[102] Based on this insight, we tested and confirmed the hypothesis that iron chelation prevents protein nitration in mitochondria and protects against APAP hepatotoxicity.[62] Thus, consistent with the very low levels of LPO,[55,62] lysosomal ferrous iron translocated into mitochondria promotes MPTP opening and APAP-induced cell death through facilitation of protein nitration, rather than generation of LPO under normal conditions.

The lack of relevant LPO and the documented critical role of ferrous iron in protein nitration suggest that the mode of cell death after an APAP overdose is unlikely to be ferroptosis under normal conditions. In contrast, as demonstrated by the massive LPO and aggravated liver injury in vitamin E-deficient mice,[38,56–58] there is potential for ferroptosis when antioxidant defenses are severely compromised. However, given the ubiquitous distribution of vitamin E, deficiency is difficult to achieve in animals and very rare in patients. Conversely, in suicide attempts where a patient indiscriminately takes drugs from a medicine cabinet, there is a possibility that an iron supplement is present.[103,104] When this scenario of acute iron overdose is mimicked in APAP-intoxicated animals, a severe aggravation of liver injury was observed, accompanied by enhanced protein nitration and a dramatic increase in LPO parameters.[62] An iron chelator effectively reduced both protein nitration and LPO, resulting in substantial protection.[62] These findings indicate that under conditions of acute iron overload, some of the excess iron triggers LPO and ferroptosis in addition to facilitating protein nitration (Fig. 2B).

Ferroptosis inhibitors

The protective effect of a ferroptosis inhibitor, such as ferrostatin-1, is considered evidence for ferroptosis.[105] Ferrostatin-1 is thought to be a lipophilic antioxidant that reacts with lipid free radicals more specifically than Trolox.[27] More recently, it has been suggested that ferrostatin-1 inhibits the propagation of LPO by preventing the formation of lipid alkoxyradicals.[106] Independent of the exact molecular mechanism, ferrostatin-1 has low bioavailability, a short half-life *in vivo*, and limited microsomal and plasma stability.[107] Therefore, analogs of ferrostatin-1, such as UAMC-3203, have been developed with improved stability and higher efficacy in inhibiting ferroptosis.[107] Although some studies showed beneficial effects of ferrostatin-1 in the APAP hepatotoxicity model despite very limited LPO,[23,24,108] we could not reproduce these findings.[109] Neither under normal conditions with no relevant LPO, nor under conditions of iron pretreatment with extensive LPO, did ferrostatin-1 at a dose identical to that used in previous studies show any beneficial effects.[109] In contrast, UAMC-3203 provided protection in the absence of relevant LPO. However, this is likely due to an off-target effect on downregulating Sab, the mitochondrial anchor protein for JNK.[109] It remains unclear whether the protective effect of UAMC-3203 in the iron/APAP model is due to reduced LPO alone or its combination with the effect on Sab.[109] Thus, a ferroptosis inhibitor alone cannot be used to support the conclusion of ferroptosis; the effect must be considered in the context of severe LPO (a 10- to 30-fold increase in LPO parameters).

Additional clinically relevant drugs and ferroptosis

APAP is an intrinsic hepatotoxin after an overdose. Besides APAP, only a limited number of clinically used drugs demon-

strate some capacity to cause hepatotoxicity in patients and animal models. The key issue is that the hepatotoxicity of these drugs occurs at therapeutic doses in a limited number of patients, placing them somewhere between intrinsic and idiosyncratic hepatotoxins. A few examples, derived from RUCAM-based clinical cases,[110] will be discussed in the following paragraphs. On the other hand, the cell death mechanisms of most idiosyncratic hepatotoxins are more difficult to assess due to the complexity of the disease.[26,110,111] It has been suggested that the injury mainly involves death-receptor-mediated apoptosis, followed by innate and adaptive immune responses.[26,111] Thus, the potential role of ferroptosis has not been investigated in these idiosyncratic hepatotoxic drugs.

Methotrexate

Methotrexate is a widely used drug to treat cancer and autoimmune diseases such as rheumatoid arthritis and psoriasis. Hepatotoxicity is a serious side effect of long-term methotrexate use. Many mechanisms of toxicity have been investigated, including oxidant stress, lipid peroxidation, mitochondrial dysfunction, and apoptosis through activation of the intrinsic pathway.[112] More recently, it was suggested that methotrexate causes ferroptosis through activation of autophagy, leading to increased degradation of ferritin and liberation of ferrous iron (ferritinophagy), which promotes LPO and liver injury.[113] The detrimental effect of autophagy induction was well demonstrated; however, LPO *in vitro* and *in vivo* never exceeded two-fold above baseline.[113] Nonetheless, the injury was mild, and ferrostatin-1 only partially protected against it.[113] Thus, future studies will need to address more directly the relevance of ferroptosis compared to other cell death mechanisms.

Amiodarone and valproic acid

Amiodarone is a drug used to treat arrhythmia, while valproic acid is an anticonvulsant. Both drugs can cause hepatotoxicity as a side effect. The toxic mechanisms of these drugs involve inhibition of mitochondrial beta-oxidation, leading to steatosis.[114–116] Both can also generate oxidant stress and LPO, which can be inhibited by lipophilic antioxidants such as vitamin E.[117–119] However, ferroptosis as a mode of cell death in this context has not been specifically investigated.

Rifampicin and isoniazid

The combination of rifampicin and isoniazid is used to treat tuberculosis. However, acute overdoses and long-term treatment can cause hepatotoxicity. Mechanisms have been extensively studied and include the formation of reactive metabolites, oxidative stress and lipid peroxidation, steatosis, apoptosis, cholestasis, and inflammation.[116,120] Ferroptosis as a mode of cell death was suggested more recently by Pan *et al*.[121] Treatment of mice with a combination of rifampicin and isoniazid caused ROS formation and LPO in hepatocytes, a reduction of GPx4, and an increase in acyl-CoA synthetase long-chain family member 4 (ACSL4).[121] ACSL4 is an enzyme that combines free long-chain fatty acids, especially arachidonic acid, with acyl-coenzyme A for incorporation into phospholipids.[122] The increase in PUFA levels in lipids enhances their susceptibility to LPO, and thus, ACSL4 deficiency can reduce ferroptosis.[123] The changes in these ferroptosis parameters, along with rifampicin and isoniazid-induced liver injury, were aggravated when ferrous succinate was added and significantly reduced with GSH treatment.[121] In addition to these mouse studies, liver biopsy samples from tuberculosis patients treated with rifampicin and isoniazid were analyzed. Similar to the mouse data, GPx4 and ACSL4 levels decreased and increased, respectively. A subset of patients receiving GSH treatment for liver protection showed less injury than untreated patients. Furthermore, a few patients who received iron supplements for iron deficiency experienced three times higher injury than untreated patients.[121] Together, these observations provide evidence for ferroptotic cell death in the livers of mice exposed to a single high-dose combination of rifampicin and isoniazid, as well as in patients treated with these drugs. However, more detailed mechanistic investigations are necessary to evaluate under which circumstances ferroptosis may be important.

Natural products

Although many natural products are protective in models of drug hepatotoxicity, including APAP-induced liver injury, some compounds can induce liver injury. Epimedium koreanum Nakai, an herbal supplement, is known to cause hepatotoxicity.[124] Treatment of rats with an ethanol extract of Epimedium koreanum Nakai (0.7 g/kg) for 28 days caused metabolic changes and mild liver injury. The authors observed a 50% increase in MDA, a reduction in SOD, GPx4, and Xc- protein expression, and a 60% increase in ACSL4 levels.[124] These changes are consistent with ferroptosis.[124] However, correlative changes in parameters that may be associated with but are not specific to ferroptosis do not prove that the mode of cell death is ferroptosis.

Another herbal compound, toosendanin (TSN), a triterpenoid derivative from Melia toosendan Sieb et Zucc,[125] has been identified as causing hepatotoxicity after both acute and chronic exposure.[126,127] Liang *et al*. exposed HepG2 cells to 5–20 μM TSN for up to 24 h and showed a 15% loss of cell viability that correlated with partial GSH depletion, a threefold increase in LPO, and reduced GPx4 expression.[128] A single treatment of Balb/c mice with 5–20 mg/kg TSN showed very mild liver injury (150 U/L ALT).[129] There was evidence of HNE staining, a 20% increase in MDA levels, and a 30% decline in GPx4 expression.[128] The authors concluded that TSN induced ferroptosis both in HepG2 cells and *in vivo*.[128] Again, the changes in these parameters were moderate, as was the injury, and the conclusions were based on correlations between injury and non-specific parameters.

Discussion

There is an exponential increase in publications suggesting that drug-induced liver injury, especially APAP hepatotoxicity, involves ferroptosis. These conclusions are generally based on moderate increases in LPO parameters, reduced hepatic GSH content, a decline in GPx4 protein levels, and an increase in ACSL4. However, none of these parameters are specific for ferroptosis. Additionally, while the changes in these parameters correlate with injury, causality is rarely established. Ferroptosis inhibitors are sometimes used to assess the role of ferroptosis in the pathophysiology; however, ferrostatin-1 has low bioavailability *in vivo*, and its effects are not reproducible.[107,109] Furthermore, it is questionable whether ferrostatin-1 can eliminate injury in the presence of significant damage but limited LPO.[24] More potent ferroptosis inhibitors may also protect due to off-target effects.[109] Thus, it is insufficient to simply measure non-specific parameters that could be somewhat associated with ferroptosis and correlate them with injury and protection. Among other considerations, quantitative aspects are important; substantial LPO (a 10-to-50-fold increase in LPO parameters) is necessary if there is severe liver injury caused by ferroptosis. Many "statistically" significant differences may be biologically irrelevant. An important tool to assess the pathophysiological relevance of a

mode of cell death or a specific mechanism such as LPO is to use a positive control, e.g., APAP + Fe^{2+} or APAP in vitamin E-deficient mice.[55,56,62] The increased LPO and injury under these conditions, along with the protection provided by relevant antioxidants such as vitamin E, can serve as strong evidence for the role of LPO and ferroptosis. However, potential pitfalls need to be considered when modulating an experimental model, as the mechanism of cell death may not be static. For example, when Wendel and coworkers sought to evaluate LPO as a mechanism of APAP-induced cell death, they intended to enhance susceptibility by feeding the animals a vitamin E-deficient diet high in PUFAs. This was meant to allow more reliable measurement of LPO parameters such as ethane and pentane exhalation.[38,56–58] However, instead of enhancing the injury mechanism as intended, this modulation changed the injury process altogether from a protein adduct-driven signaling process to one dominated by LPO.[95] More recently, when evaluating the role of ferrous iron, we showed that iron is involved in catalyzing peroxynitrite-mediated protein nitration and not LPO.[62] However, when the animals are pretreated with ferrous iron, not only is the nitration pathway enhanced, but LPO suddenly becomes a relevant part of the pathomechanism.[62,109] These examples demonstrate that LPO and ferroptosis may be biologically irrelevant for APAP hepatotoxicity under normal conditions because endogenous defense mechanisms (such as the lipid-soluble antioxidant vitamin E and the iron chelator ferritin) effectively prevent excessive LPO. However, when these defenses are compromised, LPO can become more prominent or even the dominant mechanism of cell death. Thus, the signaling mechanisms of drug-induced cell death involving ROS and peroxynitrite are flexible and can change depending on susceptibility (e.g., PUFA levels in membranes) and impairment of multi-layered antioxidant defense systems. This should be kept in mind when designing experiments and interpreting the results of studies on drug-induced cell death mechanisms.

Conclusions

Up to the 1980s, cell death caused by drugs, chemicals, and other pathophysiologies was primarily considered necrosis. This view radically changed with the discovery of apoptosis and its specific signaling pathways in the 1990s. By around 2000, apoptosis had become the prominent mode of cell death in liver diseases and other organ disorders. Despite this enthusiasm for apoptosis, there was limited concern that if this hypothesis were correct, potent pancaspase inhibitors could prevent all cell death and cure most diseases. As we know now, apoptosis is only relevant under specific circumstances, and we are still searching for therapies for most diseases. This may hint at what lies ahead as we navigate the (mostly erroneous) tendency to identify every cell death process in drug hepatotoxicity and other liver diseases as ferroptosis.

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Conflict of interest

The authors have been Editorial Board Members of the *Journal of Clinical and Translational Hepatology* since 2016 (HJ) and 2021 (AR).

Author contributions

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