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Ferrous Iron–Dependent Pharmacology

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Abstract

The recent emergence of oxidation state selective probes of cellular iron has produced a more nuanced understanding of how cells utilize this crucial nutrient to empower enzyme function, and also how ‘labile’ ferrous iron contributes to iron-dependent cell death (ferroptosis) and other disease pathologies including cancer, bacterial infections, and neurodegeneration. These findings, viewed in light of the Fenton chemistry promoted by ferrous iron, suggest a new category of therapeutics exhibiting ferrous iron–dependent pharmacology. While still in its infancy, this nascent field draws inspiration from the remarkable activity and tremendous clinical impact of the antimalarial artemisinin. Here we review recent insights into the role of labile ferrous iron in biology and disease, and describe new therapeutic approaches designed to exploit this divalent transition metal.

Keywords

Iron homeostasis; ferroptosis; ferritinophagy; targeted prodrugs; reactivity-based probes; activity-based probes

Iron Acquisition and Homeostasis

Around a third of all proteins are metalloproteins, with zinc, copper, and iron most prominent among the divalent metal ions that drive co-factor dependent enzyme function [1]. These activities include DNA & RNA synthesis and repair, epigenetic regulation, and cellular respiration, among many others. The ability to redox cycle between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states enables iron-dependent cofactors to fulfil their catalytic roles

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Resources

in a wide range of biochemical processes. Homeostatic control of cellular iron is carried out via dynamic mechanisms that regulate its absorption, transport, storage and mobilization [2]. Because the ferric ion is essentially water-insoluble, it must be bound to protein chaperones such as transferrin for transport and ferritin for storage. While ferric iron storage and transport has been well understood for decades, the much less well characterized cellular pool of ferrous iron has historically been referred to, somewhat unhelpfully, as the ‘chelateable’ or ‘labile’ iron pool (LIP) – the pool of iron subject to binding by exogenous reagents. Unlike enzyme-regulated iron co-factors or ferric iron stored in cellular ferritin, iron in the LIP undergoes spontaneous redox cycling, promoting Fenton-type reaction with oxygen and cellular hydroperoxides [2,3], producing oxidative stress and causing damage to cellular macromolecules and lipids [4]. Accordingly, the LIP and iron homeostasis generally must be carefully regulated, both at the level of the individual cell and the whole organism.

In mammals, total systemic iron is regulated through dietary uptake, with ferric iron absorbed at the apical membrane of the enterocyte through coordinated iron reduction by duodenal cytochrome B (DCYTB, encoded by the *CYBRD1* gene) and imported via the divalent metal transporter (*DMT1*) [5]. At the basolateral membrane, an analogous process of export (via ferroportin) followed by oxidation (by hephaestin) enables loading of ferric iron ions onto transferrin for entry into systemic circulation. The distribution of iron between tissues and organs in mammals is primarily mediated by the cellular iron exporter ferroportin and its negative regulator hepcidin, a peptide hormone produced in the liver (or in autocrine fashion in some tissues) that binds to ferroportin and leads to its internalization and degradation [3]. Major sites of ferric iron storage include the liver and within plasma ferritin, while systemic iron is recycled from damaged and aged erythrocytes by splenic macrophages [6]. Thus, ferrous iron is produced transiently, as needed and “just in time”, while ferric iron in storage or transit comprises the major iron stores of the organism.

Iron handling at the cellular level involves analogous modes of iron uptake, recycling, storage, and export (Figure 1). Transferrin mediated iron uptake involves binding of holo-transferrin to the transferrin receptor, followed by endocytosis and endosome-lysosome acidification to release the otherwise insoluble ferric ion; reduction of the liberated Fe^{3+} to Fe^{2+} by STEAP3 is then followed by export via endolysosomal DMT1. The lysosome is also involved in ferritin iron recycling through the process of ferritinophagy [7]. A recent CRISPR functional screen found that maintenance of adequate cellular iron was an indispensable function of lysosome acidity [8], consistent with an essential role for the lysosome in iron uptake and ferritin recycling. As the Fe^{2+} ion exits the lysosome it enters the cytosolic labile iron pool (LIP), where it can be utilized directly by essential enzymes like ribonucleotide reductase that utilize a mononuclear iron cofactor, oxidized and incorporated into ferritin for storage, or incorporated into heme or Fe/S prosthetic groups in the mitochondria. Until very recently, it was unknown whether iron in the LIP was truly ‘labile’ (i.e., unbound) or whether cellular chaperones were involved in its trafficking. It has been proposed more recently that poly(rC)-binding protein (PCBP1) is an Fe^{2+} binding protein involved in the delivery of cytosolic iron to ferritin [9], and also to proteins involved in heme iron biosynthesis [10] and Fe/S cluster assembly [11]. Iron bound to PCBP1 is further ligated by a glutathione molecule, consistent with earlier work [12] suggesting glutathione-ligated Fe^{2+} as a component of the LIP. For the discussion that follows, it is

important to appreciate that the LIP comprises a small percentage of total cellular iron, the vast majority of iron being safely stored in ferritin, or actively utilized in metalloproteins to perform specific tasks.

With this understanding of iron metabolism, two principles for iron(II)-dependent pharmacology can be enunciated, 1) that redox-active ferrous iron is present at pharmacologically relevant concentrations within cells, but generally not in plasma or other extracellular compartments, and 2) that disease pathology is more likely to result from elevated ferrous iron levels (e.g., in the LIP, in mitochondria, in hypoxic cells) than from changes to total iron levels, which will be predominantly Fe^{3+} in storage. Unfortunately, traditional methods to measure total cellular iron, such as ICP-MS, are unable to distinguish ferrous from ferric states. Similarly, any approach that breaks the cell membrane prior to analysis will result in rapid oxidation of labile ferrous iron, unless special precautions are taken. PhenGreen SK, until recently the most widely utilized probe of 'labile' iron, is a chelation-based probe unable to reliably distinguish iron oxidation state and is furthermore susceptible to interference from other metal ions. Fortunately, the past five years have witnessed a revolution in the detection of the ferrous ion specifically, and this in turn has motivated new approaches and interest in iron(II)-dependent pharmacology.

First Selective Probes of Labile Ferrous Iron

The study of cellular ferrous iron has historically been hampered by a lack of chemical probes that could distinguish Fe^{2+} and Fe^{3+} ions [13]. Being relatively electron-poor, chelation of the ferric ion is characterized by sigma donation from electron-rich oxygen atoms of catechol or hydroxamate ligands. The more electron-rich ferrous iron can additionally engage in back-donation from metal *d*-orbitals to ligands capable of accepting such bonds – typically aromatic nitrogen heterocycles. This is why canonical (ferric) iron chelators like desferoxamine (DFO) employ hydroxamate ligands, while divalent metal ion probes like PhenGreen SK utilize a heteroaromatic 1,10-phenanthroline ligand. While some measure of selectivity can be achieved in this way, the key breakthrough in the detection of Fe^{2+} was a move from chelation-based to reactivity-based detection, as has been recently reviewed [14]. Thus, in 2014 Hirayama and Nagasawa described RhoNox-1 [15], the N-oxide form of fluorescein that is selectively reduced by Fe^{2+} , producing a turn-on response (Figure 2). Further improvements to the RhoNox scaffold in terms of sensitivity and organelle-selective staining were subsequently achieved, yielding probes such as ER-SiRhoNox-1 [16], Mem-RhoNox [17], and Lyso-RhoNox [18], among others. Contemporaneously, our laboratory described 1,2,4-trioxolane (TRX) based scaffolds for Fe^{2+} detection [19,20], including a caged form of puromycin (TRX-PURO, Figure 2) able to detect Fe^{2+} in malaria parasites [21] and cancer cell lines [22] by immunofluorescence imaging with high specificity. Analogous to the reduction of the N–O bond in RhoNox probes, it is iron(II)-promoted reduction of a hindered O–O bond in TRX-based probes that promotes their activation. The TRX scaffold was subsequently utilized by us and others to produce FRET-based probes such as TRX-FRET [22] and FIP-1 [23] and the *in vivo* probes ICL-1 [24] and HNG [25], the later showing particular sensitivity to labile iron(II)heme. In 2019, Evans and Renslo described ^{18}F -TRX [26], a Fe^{2+} probe for positron emission tomography (PET) that revealed an avidity for Fe^{2+} in several tumor types.

The availability of these new tools has enabled new insights into the role of ferrous iron in biology and disease pathology. Thus, RhoNox-class probes have confirmed iron reduction at the plasma and endolysosomal membranes [15,17] during cellular iron uptake, and revealed an elevation of lysosomal and ER-associated ferrous iron during ferroptotic cell death [18]. RhoNox probes have also linked hypoxia with elevated intracellular Fe²⁺ in tumor spheroids [16], and in cellular models of the blood-brain barrier [27] and the neural vascular barrier [28]. The trioxolane-based probes TRX-PURO and FIP-1 respectively revealed an elevation of the LIP in cancer cell lines [22] and in cells undergoing ferroptosis induced with the cystine/glutamate antiporter inhibitor erastin [23]. These probes have also revealed more unexpected roles for Fe²⁺ in the cell, such as a link between GPCR signaling and epigenetic regulation mediated by the LIP [29,30], and a possibly causative link between elevated Fe²⁺ and hepatic steatosis [31]. Among *in vivo* active TRX-type probes, ICL-1 has revealed elevated Fe²⁺ in mice infected with the bacterium *Acinetobacter baumannii* [24], while HNG detected heme(II)iron in the livers of mice following an acute hemolytic insult with phenylhydrazine [25]. Greater appreciation for the role of ferrous iron in disease will only increase as these new probes become more widely available and supplant the use of earlier, less selective probes.

Ferrous Iron-Dependent Pharmacology

The dysregulation of iron homeostasis has been associated with a wide range of disease states, from iron storage disorders [32], to cancer [33–35], neurodegenerative [36–38] and metabolic disease [39,40], and in infection by bacterial [41–43], viral [44], and eukaryotic [45–47] pathogens. Much as iron detection has historically relied on iron chelation, so have therapeutic strategies targeting iron been largely based on (ferric) iron chelation therapy [35,48,49] or on active drug uptake via conjugation to transferrin or bacterial iron siderophores [50–52]. While effective in certain disease contexts, chelation therapy can lead to anemia, and has been disappointing in the clinic as an approach to anti-infective or cancer therapy. However, the combination of natural chemical diversity and empiric traditional medicine has revealed an orthogonal approach to iron-based therapy exemplified today by standard-of-care antimalarial therapy with artemisinin-based agents. These endoperoxide-bearing sesquiterpenes are activated by ferrous iron heme liberated during parasite catabolism of hemoglobin in the erythrocyte [46]. Clinical success of this agent has driven a search for new chemotypes with analogous pharmacology, leading most notably to the 1,2,4-trioxolanes arterolane [53] and artefenomel [54,55] reported by Vennerstrom and co-workers. These wholly synthetic agents, like the artemisinins, exhibit highly iron(II)-selective reactivity (Box 1). Informed by careful mechanistic studies [56–59] detailing their iron(II)-dependent fragmentation, we and others have been inspired to expand the concept of ferrous iron-dependent pharmacology beyond its origins in antimalarial chemotherapy.

Ferrous Iron-Dependent Pharmacology in Bacterial Infection

The innate immune response to bacterial infection in mammals, known as nutritional immunity, comprises a diverse array of iron limiting and withholding strategies carried out via the ferroportin/hepcidin axis [43,60], the iron-binding protein lactoferrin [41], and the iron siderophore-scavenging protein lipocalin-2 [61]. In response, pathogenic bacteria

deploy diverse adaptive responses to overcome iron withholding, including uptake and degradation of host heme iron, and assimilation of iron from transferrin, ferritin and lactoferrin [41]. The latter is achieved through the expression of small molecule siderophores with exceptionally high binding affinity for the Fe^{3+} ion [42]. The reliance of bacteria on siderophore mediated iron uptake has motivated an impressive volume of work on antibiotic-siderophore conjugates as notional ‘Trojan Horse’ therapies [52,62–64], the agent cefiderocol [65] being the first to receive marketing approval from the FDA, in 2019.

While siderophore-antibiotic conjugates necessarily exploit binding and uptake of Fe^{3+} , several recent studies provide evidence for Fe^{2+} utilization by bacterial pathogens, which in turn suggests the potential to develop reactivity-based, ferrous iron-targeted therapy. Thus, Nolan and co-workers recently showed that the mammalian protein calprotectin sequesters the Fe^{2+} ion in response to bacterial infection and induces an iron starvation response in the pathogen *Pseudomonas aeruginosa* [66,67]. Evolution of a host response around Fe^{2+} (analogous to Fe^{3+} binding by lactoferrin) suggests that bacterial pathogens are able to exploit Fe^{2+} during infection. Indeed, organisms that evolved on the anoxic earth (prior to the ‘great oxidation’ that accompanied early photosynthesis) would require the means to exploit forms iron that predominate under such conditions. The most widely distributed of these bacterial Fe^{2+} transporters are members of the Feo system [68,69] and are known to be exploited by pathogens such as *P. aeruginosa* that often colonize hypoxic niches, paradoxically including the lungs of cystic fibrosis (CF) patients [70]. Most provocatively, a recent study [71] of live *Escherichia coli* using Mössbauer and EPR spectroscopy paints a dramatically different picture of bacterial iron homeostasis in which labile Fe^{2+} is in fact the predominant iron species present during exponential stages of bacterial growth.

The *in vivo* probe ICL-1 was recently applied [24] to study mice infected with the Gram-negative pathogen *Acinetobacter baumannii* (Figure 3A). Comprising the TRX-caged form of D-aminoluciferin, ICL-1 was shown in a series of cell free and cell-based experiments to luminesce (with luciferin and ATP), strictly contingent on its initial uncaging via reaction with Fe^{2+} . Other metal ions (including Fe^{3+}) and cellular reductants or oxidants were unable to uncage ICL-1, thereby confirming its utility as a selective probe of Fe^{2+} . In luciferase-expressing mice infected with *A. baumannii*, or mock-infected as a control, ICL-1 revealed a substantial increases in bioluminescent signal across a range of tissues that were subsequently shown by *ex vivo* analysis to also be major sites of infection. By contrast, mock infected controls showed only minimal bioluminescent signal that was focused around the site of ICL-1 injection in the peritoneum (Figure 3A). Further *ex vivo* analysis of organs/tissues from both infected and mock-infected mice revealed that total iron in these tissues was not significantly elevated as a result of infection (the exception being in the liver, where ferritin iron accumulation in response to infection is to be expected). These findings with ICL-1 suggest that ferrous iron concentrations are elevated at sites of infection in a live animal, a perhaps surprising result given that nutritional immunity predicts iron limitation during infection. Further studies will be required to determine whether the observed effects derive from changes in host iron disposition, bacterial utilization or acquisition of iron, an emergent property of the infection microenvironment, or some combination of these factors. Whatever the case, the iron avidity of infected tissues revealed by ICL-1 clearly suggests the

possibility of targeting antibiotics to infected tissues with analogous TRX-based drug conjugates.

The bacterial deacetylase LpxC performs the first committed step in lipopolysaccharide biosynthesis and this, combined with an absence of the pathway in mammals, has made this metalloenzyme a promising antibacterial drug target. Inhibition of LpxC has most often been approached with hydroxamate-based ligation of the enzyme's zinc center. While hydroxamates can be highly potent inhibitors, the susceptibility of this functional group to proteolytic or metabolic degradation, including the formation of genotoxic intermediates [72], has hampered their clinical development. Our laboratory explored the use of a known LpxC inhibitor (PF-5081090) as the hydroxamate-linked conjugate TRX-PF508, which we surmised would be inactive toward LpxC and other metalloenzyme off-targets in the absence of Fe^{2+} . Activation by Fe^{2+} at infection sites would liberate the LpxC inhibitor in its parent form (Figure 3B), and the desired iron(II)-dependent reactivity was confirmed in control experiments [73]. The antimicrobial activity of the conjugate in Minimum Inhibitory Concentration (MIC) assays was modest however, which suggested inefficient activation and/or limited access to Fe^{2+} in the bacterial cytoplasm in this *in vitro* setting. In contrast, when administered to mice in an acute *P. aeruginosa* lung infection model, TRX-PF508 produced significant reductions in bacterial load in the lung, and did so more robustly than in mice treated with the parent LpxC inhibitor at an equimolar dose. This implied a drug concentrating effect at sites of infection and was consistent with the compound's predicted iron(II)-dependent pharmacology. Moreover, the observation with this compound of robust *in vivo* efficacy despite poor MICs, suggested that *P. aeruginosa* can utilize pools of Fe^{2+} *in vivo* that are not present in *in vitro* MIC assays. In fact, a microenvironment rich in Fe^{2+} has been demonstrated clinically in the case of chronic lung infection by *P. aeruginosa* in cystic fibrosis patients [74].

Ferrous Iron-Dependent Pharmacology in Cancer

It has been long been recognized that cancers exhibit an avidity for iron that seems to drive proliferation and metastasis [33,34]. Upregulation of the transferrin receptor is the canonical mechanism associated with this archetype but recent years have seen a striking array of novel mechanisms come to light. The appetite of the cancer cell for iron has been dubbed 'iron addiction' [75] and indeed, appears to follow in lockstep with dependence upon ongoing signaling from commonly mutated oncogenes. Thus, the seminal finding [76] that c-Myc regulates iron storage (ferritin) and regulatory (IRP2) genes was further corroborated when the c-Myc driven cell line PC3 was found, using TRX-PURO, to be among the most avid for Fe^{2+} across a panel of lines from different tumor types [77]. In two important studies from 2008, the lipid hydroperoxidase glutathione peroxidase 4 (GPX4) was found to promote survival of neurons [78] and mutant Ras driven cancer cells [79], seminal work that would help position GPX4 at the center of a pro-survival pathway that protects cells from an iron-dependent, nonapoptotic form of cell death subsequently dubbed ferroptosis [80]. Drug-tolerant 'persister' cells were similarly found to be highly dependent on GPX4 function for survival and therefore highly susceptible to GPX4 inhibition [81,82]. The hepcidin/ferroportin axis (Figure 1) can also contribute to 'ferroaddiction' via systemic liver hepcidin, or via hepcidin produced by the tumor itself in autocrine fashion, retaining tumoral iron and

leading to more aggressive disease and poorer prognosis in breast [83], prostate [84], and pancreatic [85] cancers. In the case of gliomas, high-grade tumors of a mesenchymal subtype have been shown to possess significantly higher expression of transferrin receptor and lysosomal ferrireductase (STEAP3) as compared to low grade tumors [86]. How avidity for iron, and ferrous iron specifically, supports and promotes these aggressive and resistant cancer phenotypes remains largely unknown. One can speculate however, that ready access to soluble and metabolically active Fe^{2+} ion would be of significant benefit to rapidly proliferating cells, insofar as this would support the various iron-dependent enzyme functions required for cell growth and division. It appears a Faustian bargain is struck in which the benefits of elevated Fe^{2+} are exploited by managing (e.g., through greater reliance on GPX4) the dangerous levels of oxidative stress that result.

Given the avidity of cancer cells for iron, it is unsurprising that artemisinins and other antimalarial peroxides have been evaluated for their anticancer effects. In the most comprehensive of these studies, artesunate, dihydroartemisinin, arterolane, and artefenomel were profiled across 91 human cancer cell lines [87]. While these compounds showed measurable cytotoxicity in nearly all cell lines examined, their potencies landed squarely in the micromolar range, which compares unfavorably with the low-nanomolar effect of these same compounds in malaria parasites. Likely the difference stems from the presence in parasites of pathogenic free iron(II)heme, and a more robust cellular response to oxidative stress in cancer cells, many of which are likely pre-adapted to survive in a ferroaddicted state. Although these antimalarial compounds would appear to have limited clinical potential as single agents, they might yet find utility as adjuvant therapies, for example in combination with a GPX4 inhibitor as has been recently proposed [88]. Testing such hypotheses will however require the development of GPX4 inhibitors with suitable *in vivo* properties. It is also worth noting that the ferroptosis-inducing small molecule FINO2 [89,90] which bears an endoperoxide function, can be regarded as a starting point for the development of anticancer endoperoxides that exploit the ferroptosis pathway for therapeutic ends.

While arterolane itself has modest effects on cancer cells, we judged that TRX-based ferrous iron-targeting could be employed to direct much more potent therapeutics selectively to ferroaddicted cancer cells. To explore this notion, we employed a highly potent cyclobenzindoline (CBI) class DNA-alkylator and studied the resulting conjugate TRX-CBI in xenograft models of predicted high iron avidity [77]. Consistent with expectations, TRX-CBI conferred a potent low-nM insult on cell lines like PC3 that are ferroaddicted as judged with TRX-PURO. Importantly, TRX controls lacking the CBI payload, or non-peroxidic conjugates unable to release the CBI payload were at least 1000-fold less potent than TRX-CBI, confirming that the potent cell-killing effects of TRX-CBI derive from release of the free CBI payload. The effective caging of CBI toxicity by the TRX moiety allowed us to safely achieve >20-fold higher dose and *in vivo* exposure levels for TRX-CBI in mouse xenografts, which in turn produced dramatically superior cytotoxic effect response when compared to CBI administered at its MTD. Moreover, the tumor-shrinking effects of TRX-CBI were achieved while largely avoiding CBI-associated hepatotoxicity in the same mice. These encouraging findings suggest that selective targeting of cancer cells through ferrous iron-dependent pharmacology represents a promising new way to deliver cancer chemotherapeutics.

In a recent preprint [bioRxiv 2020.05.12.088971] the Collisson and Renslo laboratories explored ferrous iron-dependent drug delivery in mutant KRAS-driven cancer cells and xenograft models. Several FDA-approved inhibitors of the KRAS effector MEK are currently available, but these agents uniformly exhibit dose-limiting toxicity resulting from chronic MEK1/2 inhibition in healthy tissues [91] at and below MTDs established in phase I trials. After establishing with both TRX-PURO and SiRhoNox [16] an elevated pool of Fe²⁺ in KRAS-driven pancreatic ductal adenocarcinoma (PDA) cells, we synthesized TRX-COBI, the ferrous iron-activated form of the approved MEK inhibitor cobimetinib. As hoped, TRX-COBI behaved as a bona fide MEK inhibitor in PDA cells, and ablated MAPK signaling in tumors of patient derived xenograft (PDX) and syngeneic PDA mouse models, as judged by pERK staining. Most importantly, TRX-COBI achieved effective MAPK blockade in PDA models as well as in autochothanous *Kras*^{LSL-G12D}-driven lung adenocarcinoma, while sparing MAPK signaling in normal, tumor-free tissues from the same animals. Moreover, thinning of the epidermal layer in mouse tail skin (a model of clinically relevant MEK-related toxicity) was wholly avoided in TRX-COBI treated animals, while clearly evident in cobimetinib and binimetinib treated animals. Finally, we observed superior efficacy (Figure 4) with reduced weigh loss when employing TRX-COBI (in place of cobimetinib) in SHP-2 inhibitor (SHP2i) combination therapy of a KRAS^{G12C}-driven lung adenocarcinoma PDX model, consistent with improved systemic tolerability of TRX-COBI as compared to cobimetinib. Thus the significance of ferrous-iron dependent pharmacology in the context of mutant *KRAS*-driven malignancies could lie in the ability to achieve potent yet tolerable parallel and/or vertical pathway blockade, thus avoiding the inter- and intra-pathway signaling loops that enable tumor escape from single-agent blockade [92].

Concluding Remarks and Future Perspectives

Here we have detailed recent progress in the detection of iron with oxidation-state specificity and described some of the new biological findings and therapeutic approaches that have resulted from these advances. In science and in drug discovery, inquiry is often biased toward what is ‘easy’ to study (or to drug), rather than what is most important or relevant to study (or drug). The study of iron homeostasis has thus relied on (ferric) iron sequestration or supplementation experiments, and quantified iron load using experimental approaches that are blind to what is almost certainly the more relevant analyte – labile ferrous iron. Mirroring this state of affairs, iron-focused drug therapy is overwhelmingly focused on ferric iron chelation (e.g., in iron overload diseases), when it is labile ferrous iron that is associated with diseases of much greater burden to humankind – malaria most notably, but apparently also many of the most aggressive and untreatable cancers and, more tenuously, also in neurodegenerative disease.

Much remains to be learned and understood about the role of ferrous iron under conditions of normal and aberrant homeostasis, and its relative distribution in normal and diseased tissues (see Outstanding Questions). Will these conditions be best treated with drugs designed to restore a normal homeostatic state, or through reactivity-based approaches that exploit elevated ferrous iron levels to achieve tissue selectivity, as detailed in some recent work highlighted herein. The unprecedented clinical impact of artemisinin therapy in malaria would seem to argue for the latter approach, and yet decades after its introduction,

artemisinin resides in a lonely niche of the pharmacopeia. This fact can be attributed in part to a reticence of drug discovery scientists to pursue reactivity-based pharmacology. However, a significant proportion of currently used drugs have been found – often well after their discovery and approval – to act unexpectedly via covalent, reactive mechanisms [93]. Increasing appreciation for this fact has seen a resurgence of work on drugs designed from the outset to exhibit reactivity-based pharmacology, with cysteine-reactive drugs and drug candidates becoming commonplace. Next-generation trioxolane antimalarials like artefenomel combine exquisitely selective iron(II)-dependent reactivity with excellent pharmacokinetic and safety profiles in human subjects [94]. The utility of this chemotype as applied to reactivity-based probes and in ferrous-iron dependent drug delivery, as reviewed here, suggests a bright future for the study of iron homeostasis and metabolism, and in the treatment of any disease state in which ferrous iron levels are dysregulated.

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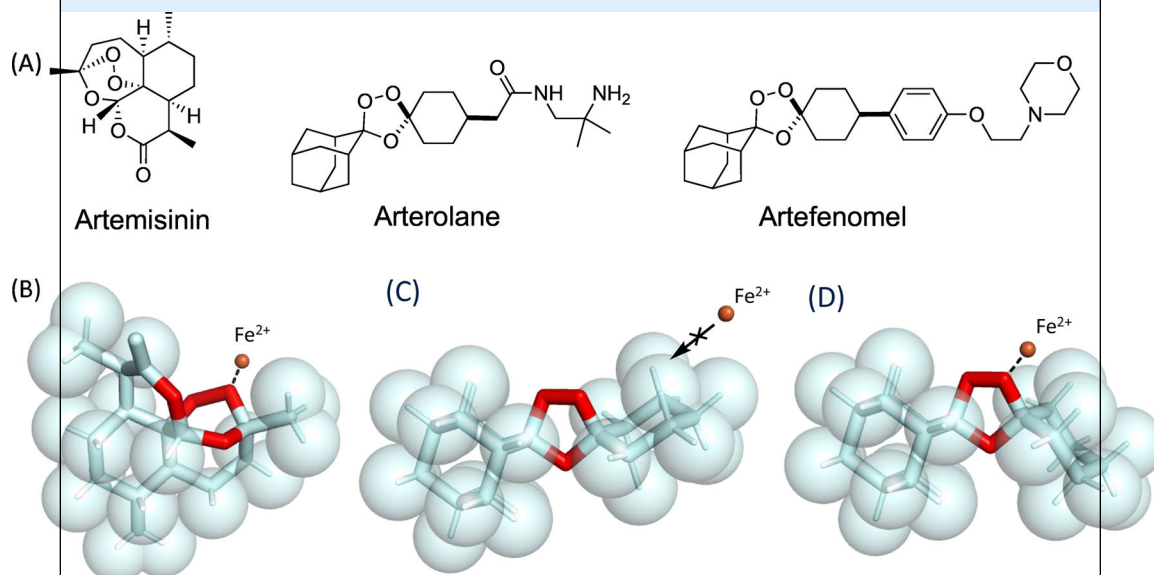
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Box 1.**Antimalarial Endoperoxides**

The sesquiterpene artemisinin (qinghaosu) was first isolated in China in the 1970s as part of an effort to identify the active components in traditional Chinese herbal remedies [95]. While there was initial skepticism about the drug on account of the peroxide bond, clinical development of artemisinin-derived agents was ultimately undertaken and their clinical utility confirmed [96]. This success bred keen interest in the molecular pharmacology of these agents. Seminal mechanistic work by Posner in the 1990s involving synthetic artemisinin analogs, and later work by Vennerstrom involving synthetic 1,2,4-trioxolanes, established the crucial role of the peroxide bond in mediating the Fe^{2+} reactivity and the antimalarial effects of the artemisinin, arterolane, and artefenomel [46,59]. Space-filling models (Box 1, Figure I) of these agents reveal a hindered steric environment surround the peroxide bond. Fenton-type reaction with iron requires close inner-sphere coordination by Fe^{2+} prior to single-electron transfer that leads to O–O bond scission. Thus, the various C–H bonds surrounding the peroxide likely preclude interaction with co-factor Fe^{2+} bound to metalloproteins, while the major ferritin iron stores of the cell are both physically inaccessible and in a mineralized ferric and redox-stable state. Hence, it is the loosely chelated Fe^{2+} of the LIP and labile, unbound iron(II)heme that are in the correct redox state, and also able to physically approach the peroxide bond for reaction. These features are retained and exploited in the TRX-based probes and drug conjugates described herein.

**Box 1, Figure I.**

Structure-reactivity relationships of antimalarial peroxides. **A.** Structures of antimalarials artemisinin, arterolane, and artefenomel. **B.** Spacefilling representation of artemisinin showing approach (dashed line) of Fe^{2+} ion (orange sphere) to the more exposed of two peroxidic oxygen atoms. **C.** Spacefilling representation of arterolane pharmacophore in its major, unreactive conformer, with approach of the Fe^{2+} ion sterically blocked by

proximal axial C–H bonds of the adamantane and cyclohexane rings. **D.** Spacefilling representation of the corresponding iron-reactive, peroxide equatorial conformer showing approach of the Fe^{2+} ion (orange sphere) to the exposed peroxidic oxygen atom. Artemisinin structure retrieved from the Cambridge Crystallographic Data Centreⁱ and visualized using PyMol 2.4.0 (pymol.org). Arterolane pharmacophore structure minimized using MarvinSketch 6.3.0 (chemaxon.com) and visualized using PyMol 2.4.0 (pymol.org).

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Outstanding Questions

- How precisely do elevated labile ferrous iron concentrations promote cell growth and proliferation in cancer?
- Is the labile iron pool necessarily elevated in cells that are sensitive to, or actively undergoing ferroptosis?
- What is the source and nature of the free ferrous iron that appears to be mobilized during bacterial infection?
- Which normal tissues and cells harbor elevated ferrous iron concentrations under conditions of normal iron homeostasis?
- Is there a link between ferroptotic cell death, aberrant accumulation of ferrous iron, and neurodegenerative disease?
- Which iron regulatory proteins might be targeted to limit the labile iron pool and what effect would such intervention have in proliferative or neurodegenerative disease?

Highlights

- Iron cycles between the ferric and ferrous oxidation states to empower enzyme function but also during its uptake, export, and storage, with the ferrous state being generated transiently and in limited concentrations to prevent oxidative damage to the cell.
- Until very recently, it has not been possible to study the ferrous ion specifically, largely due to its much poorer ligation by small molecule or protein siderophores, as compared to the ferric ion.
- The emerging role of ferrous iron in a form of iron-dependent cell death (ferroptosis), and in supporting the proliferation of some of the most aggressive cancers has given new urgency to the study of cellular ferrous iron in biology and disease.
- The clinical success of the antimalarial artemisinin provides inspiration as well as a chemical and conceptual framework for the development of a new class of therapeutic agents exhibiting ferrous iron dependent pharmacology.

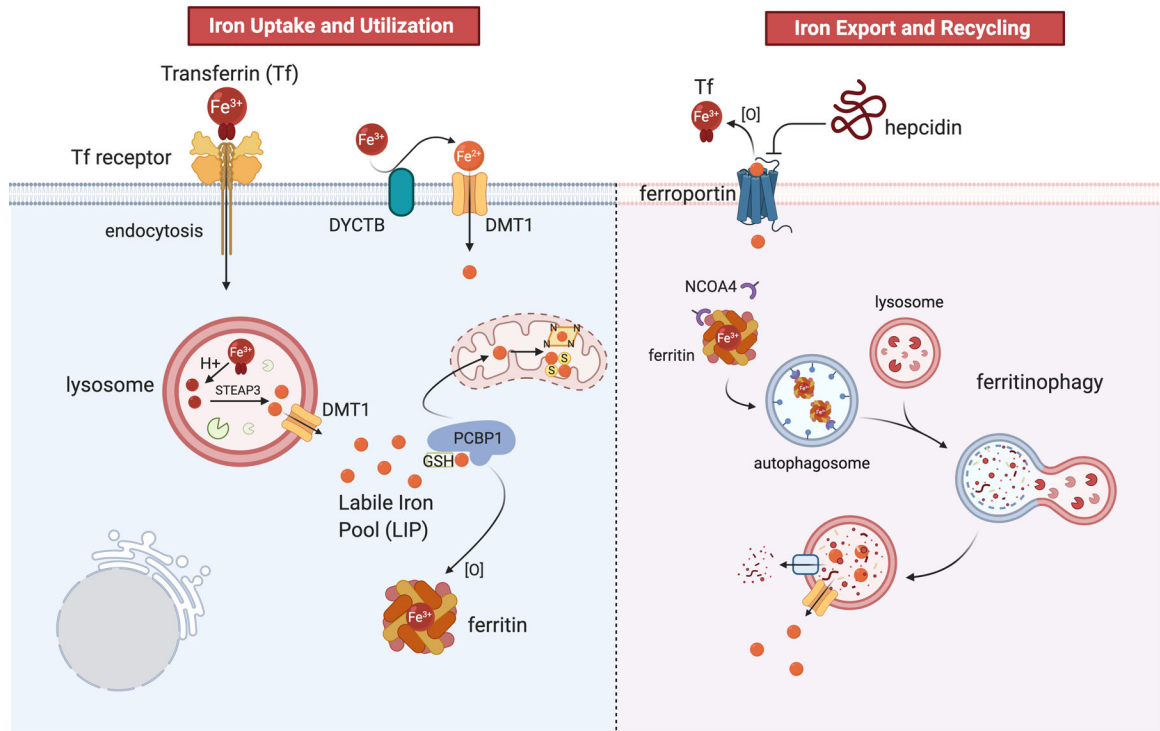


Figure 1. Summary of iron homeostasis in mammalian cells with a focus on ferrous iron (orange spheres). Figure created in BioRender ([biorender.com](https://www.biorender.com))

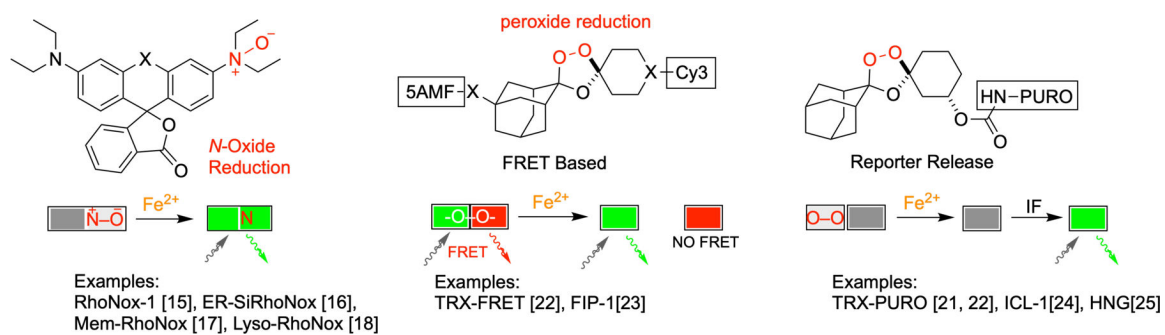


Figure 2.

The first oxidation-state selective probes of ferrous iron rely on reactivity-based sensing. Three important classes of reactivity-based probes are shown, along with schematic representations of their modes of activation. RhoNox-based probes undergo iron(II)-promoted reduction of an *N*-oxide to produce a fluorescent signal. TRX-based probes include FRET-based turn-on and ratiometric probes, and caged reporter probes.

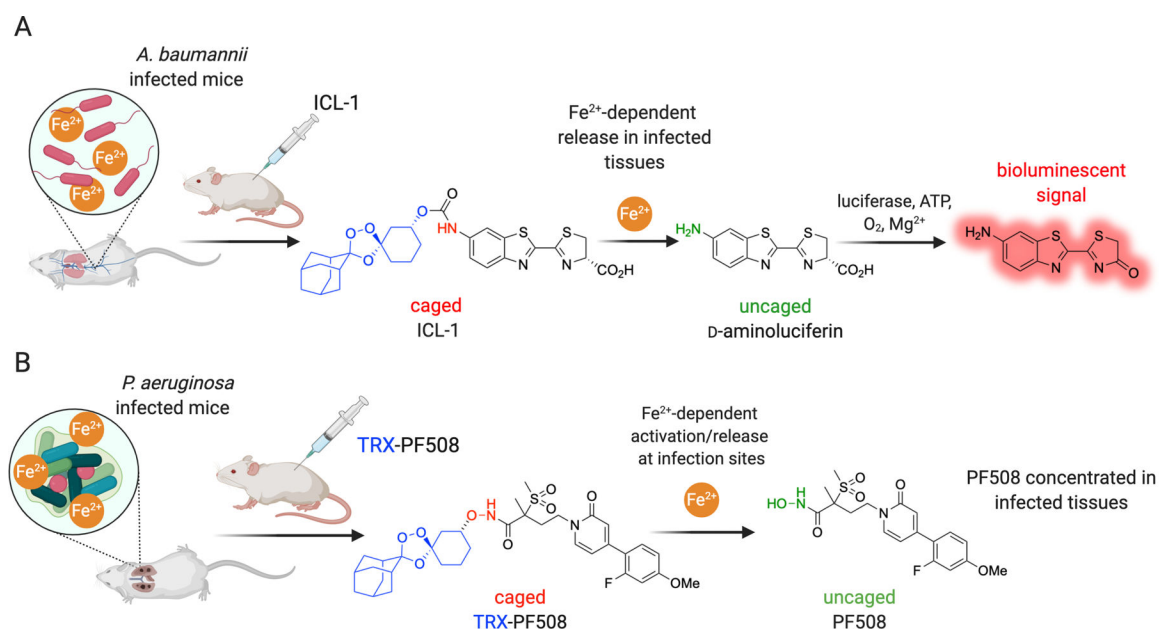
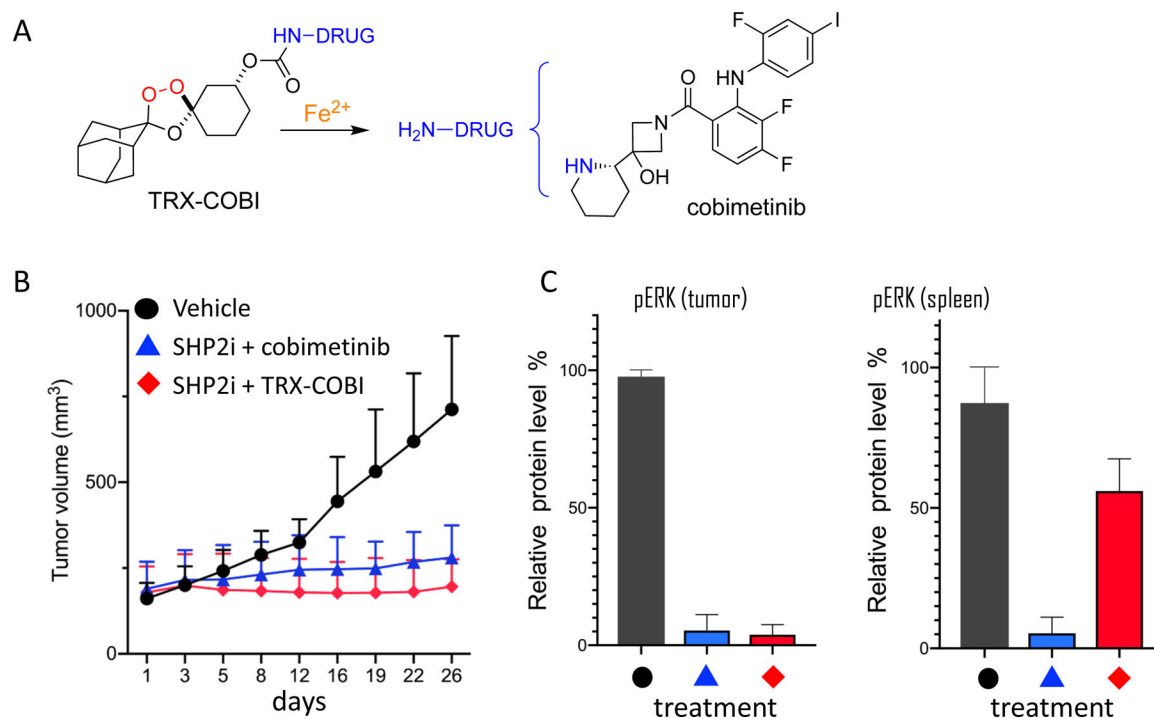


Figure 3. Iron(II)-based detection and treatment of mice infected with Gram-negative pathogens. **A.** Scheme illustrating bioluminescence imaging of Fe²⁺ at sites of infection using ICL-1 in FVB-Luc⁺ mice infected with *A. baumannii*. **B.** Scheme illustrating Fe²⁺ targeted therapy using the caged LpxC inhibitor TRX-PF508 in BALB/c mice bearing an acute lung infection with the Gram-negative pathogen *P. aeruginosa*.

**Figure 4.**

Efficacy of iron(II)-targeted forms of FDA-approved MEK inhibitor cobimetinib. **A.** Structure of TRX-DRUG conjugates such as TRX-COBI, which releases free cobimetinib upon reaction with Fe^{2+} . **B.** Tumor volume over time in mice bearing KRAS lung adenocarcinoma and treated with vehicle, SHP2 inhibitor + cobimetinib, or SHP2i + TRX-COBI at equivalent, equimolar doses. **C.** Phospho-ERK staining of tumor in spleen from animals studied in panel B, indicating selective activation of TRX-COBI in tumor. Unpublished data reproduced from bioRxiv 2020.05.12.088971.