Ferroptosis inhibition, mechanisms and applications

Jose Pedro Friedmann Angeli [1*], Ron Shah[2], Derek A. Pratt[2], Marcus Conrad[1]

1 Helmholtz Zentrum München, Institute of Developmental Genetics, 85764 Neuherberg, Germany

2 Department of Chemistry and Biomolecular Sciences University of Ottawa, Ottawa, Ontario K1N 6N5, Canada

ABSTRACT

The last decade has yielded tremendous insights into our perception of how cells die. This has come in part by our understanding that several distinct forms of cell death are encompassed under the umbrella term necrosis. Among these distinct forms of regulated necrotic cell death, ferroptosis has attracted considerable attention due to its putative involvement in diverse patho-physiological processes. A key feature of the ferroptosis process is the requirement of phospholipid peroxidation, a process that has been associated with several human pathologies. Now with a direct link between lipid peroxidation and a distinctive cell death pathway, the search for new small molecules able to suppress lipid peroxidation has gained momentum and may yield novel cytoprotective strategies. We review here advances in our understanding of the ferroptotic process and summarize the development of lipid peroxidation inhibitors with the ultimate goal of suppressing ferroptosis-relevant cell death and related pathologies.

Correspondence should be addressed to:

Dr. José Pedro F. Angeli, Helmholtz Zentrum München, German Research Center for Environmental Health, Institute of Developmental Genetics, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany
Tel: 0049-89-31872197; E-Mail: pedro.angeli@helmholtz-muenchen.de

Dr. Marcus Conrad, Helmholtz Zentrum München, German Research Center for Environmental Health, Institute of Developmental Genetics, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany
Tel: 0049-89-31874608; E-Mail: marcus.conrad@helmholtz-muenchen.de

Keywords: Regulated necrosis, cell death, lipid peroxidation, small molecules, glutathione peroxidase, cysteine metabolism

Ferroptosis

Understanding of how cells die presents an intrinsic and enormous translational potential as it offers unique opportunities to interfere with processes associated with cell death and survival. Approaches trying to halt cell death have been strongly biased towards the study of apoptosis because it has long been considered the only form of cell death thought to be amenable to pharmacological and genetic intervention. However, during the past decade, several landmark studies have identified additional regulatory mechanisms and signaling pathways that govern previously unrecognized necrotic cell death routines [1]. Among these novel regulated forms of necrosis, ferroptosis has attracted considerable attention due to its implication in several patho-physiological contexts, such as tumor suppression [2, 3], antiviral immunity [4], neurodegeneration [5] and ischemia/reperfusion injury (IRI) [6]. Based on morphological, biochemical and genetic criteria, ferroptosis was shown to be distinct from other described cell death modalities [7]. Morphological changes during apoptosis include cell shrinking and blebbing, whereas ferroptosis is characterized by cell swelling, a process known as oncosis. At the ultrastructural level, ferroptotic cells show altered mitochondrial morphology including electron-dense mitochondria and outer membrane rupture [6]. Importantly, mitochondria permeabilization seen in ferroptosis is independent of known apoptosis players, such as BAX/BAK or BCL2 [7]. Ferroptosis presents some unique features; for instance, lipid peroxidation is essential for ferroptosis and involves a preferential oxidation of phosphatidylethanolamine (PE) [8], whereas the intrinsic apoptosis pathway requires cardiolipin oxidation [9]. The generation of these oxidized phospholipids (oxPL) species is key to ferroptosis execution as it was reported that suppression of their formation halts the cell death process [8]. Execution mechanisms that ultimately lead to cell death caused by accumulation of oxPL are not clear and are still a matter of intense studies. Current hypotheses include physical disruption with subsequent loss of membrane integrity, or alternatively, the formation of electrophilic products able to inactive critical proteins required for cell viability. The inability of the cells to repair oxPL can be a consequence of dysfunction in cysteine metabolism. Dysfunction in cysteine metabolism ultimately leads to depletion of intracellular glutathione (GSH) levels as cysteine is the limiting factor for the biosynthesis of the tripeptide, which serves as a reducing co-substrate for the sole enzyme responsible for the repair of oxPL, glutathione peroxidase 4 (GPX4). As a consequence of GPX4

inactivation, oxPL accumulate and ultimately lead to cell death (**Figure 1**). Therefore, approaches aiming to inhibit ferroptosis have mainly focused on suppressing the formation of these oxPL species, thus providing novel cytoprotective strategies that can translate into improved therapeutics in pathological relevant scenarios. In the present review, we will thus present the reader with the basic aspects of lipid peroxidation and the potential contribution to disease. We emphasize what is presently known about ferroptosis and discuss the potential pharmacological approaches aiming to subvert lipid peroxidation and suppress ferroptosis.

(Patho)Physiological Relevance of Ferroptosis

The association of lipid peroxidation with human pathological conditions has long been appreciated; however, whether lipid peroxidation plays a causal role in disease onset or progression remains to be directly demonstrated. The recent characterization of a specific cell death pathway triggered by the accumulation of oxPL may finally link lipid peroxidation and disease development. Thus, disruption of the regulatory framework that keeps ferroptosis in check may contribute to the pathogenesis of degenerative diseases in which oxPL accumulation has been implicated.

Our current understanding has been largely built on the identification of tissues that are prone to undergo ferroptosis upon disruption of *Gpx4*, the most downstream regulator of ferroptosis [6, 10] (**Figure 2**). Due to early embryonic lethality of systemic *Gpx4* knockout mice [11], determination of the tissues sensitive to ferroptosis only became possible with the generation of the first conditional knockout model for *Gpx4* [12]. Since then, various conditional knockout models for *Gpx4* have been cross-bred with tissue-specific Cre expressing strains, and studies therewith revealed the significance of ferroptosis in many tissues and cell types including specific neuronal subpopulations [12-15], photoreceptor cells [16], kidney tubular epithelial cells [6], hepatocytes [17], T-cells [4], and endothelial cells [18]. Most of these studies were carried out before the term ferroptosis was actually coined, and therefore, no attempts were made to inhibit this form of cell death to preserve tissue function in these models. The only exception was the work carried out using a whole body conditional *Gpx4* KO model [6], whereby using an *in vivo* efficacious ferroptosis inhibitor, Liproxstatin-1 (Lip-1), it was shown that acute renal failure induced by *Gpx4* loss could be mitigated.

This study demonstrated that small-molecules can be used to inhibit ferroptosis in pathological settings. Indeed two groups working independently demonstrated that ferroptosis inhibitors could mitigate tissue damage in a model of ischemia/reperfusion injury (IRI) in both kidney [19] and liver [6]. Hence, this underscores ferroptosis as a key contributor to tissue demise in an IRI setting in two different organs.

In addition to the important role of ferroptosis for tissue IRI, ferroptosis has also been implicated in neurodegeneration [20]. This is not surprising as the nervous system contains the highest content of polyunsaturated fatty acids (PUFAs) [21], and aberrant GSH metabolism and lipid peroxidation are associated with several pathologies of the central nervous system (CNS), such as Alzheimer disease [22], Parkinson's disease [23], Friedrich Ataxia [24] and Huntington's disease (HD) [25]. Of particular relevance is HD, as recent studies have suggested that ferroptosis inhibitors could inhibit cell death in neurons expressing a mutant form of huntingtin (*HTT*), the causative agent of the disease [26]. This is further supported by data showing that glutathione peroxidase activity is essential in suppressing mutant HTT toxicity in yeast models of the disease [27]. Nevertheless, it is important to note that definitive evidence for the contribution of ferroptosis to disease onset/development is still missing, which can be partially ascribed to the lack of tools and biomarkers to probe the contribution of ferroptosis to CNS diseases. Therefore, ferroptosis inhibitors capable of efficiently crossing the blood-brain barrier are urgently needed to unravel the contribution of ferroptosis to neuronal demise and to provide new therapeutic opportunities for these still poorly treatable diseases.

Lipid Peroxidation

Lipid-derived peroxides are formed via autoxidation, a free radical chain reaction, as well as by enzyme-catalyzed processes (**Figure 1**). The enzymatic oxidation of lipids is primarily mediated by lipoxygenases, but also cyclooxygenases. To date, only the LOX family of enzymes has been associated with ferroptotic cell death [12, 28, 29]. Lipoxygenases are a family of enzymes consisting of six isoforms in humans (ALOXe3, *ALOX*5, *ALOX*12, *ALOX*12B, *ALOX*15, *ALOX*15b), and seven in mice (*Aloxe*3, *Alox*5, *Alox*12, *Alox*12b, *Alox*12e *Alox*15, *Alox*15b) [30, 31]). The first step in lipoxygenase catalysis is the abstraction of one of the labile hydrogen atoms from a bis-allylic position on a polyunsaturated fatty acid (PUFA). These sites are at positions 7, 10 and

13 of arachidonic acid, the primary substrate of lipoxygenases, giving rise to delocalized pentadienyl radicals that span C5-C9, C8-C12 and C11-C15, respectively. Molecular oxygen adds to the intermediate carbon-centered radical to yield a peroxyl radical, which is then reduced by the enzyme to yield the hydroperoxide product [30]. The regioselectivity and stereoselectivity of O_2 addition and subsequent trapping is controlled by the enzyme, leading to exquisite positional selectivity of hydroperoxide formation from the different isozymes of lipoxygenases [32].

Lipid peroxidation by autoxidation is triggered by any species that is sufficiently reactive to abstract a hydrogen atom the bis-allylic position of a PUFA, which include hydroxyl and hydroperoxyl radicals. This step initiates the free radical chain reaction as the pentadienyl radical reacts with molecular oxygen to generate a chain carrying peroxyl radical capable of propagating the chain reaction by abstracting a hydrogen atom from an adjacent lipid [33]. The autoxidation of lipids can be an autocatalytic process since the hydroperoxides which result can undergo O-O bond fragmentation to yield radicals capable of initiating a new radical chain. The fragmentation of peroxidic O-O bonds can be induced by heat, UV light and low-valent transition metals, such as Fe^{2+}, in what is generally referred to as the Fenton reaction.

Inhibition of Lipid Peroxidation

With the increasing recognition of the essential role of lipid peroxidation in ferroptosis execution, and the possible contribution of ferroptosis to degenerative disease, strategies aiming at the inhibition of lipid peroxidation are re-emerging as attractive cytoprotective strategies. Given that lipid peroxidation can be ignited through enzymatic and non-enzymatic pathways, strategies aiming to halt this process can be divided into two major groups: lipid autoxidation inhibitors (e.g. radical-trapping antioxidants) and lipoxygenase inhibitors (**Figure 3**).

Lipoxygenase Inhibitors

Lipoxygenase inhibitors can be divided into five major groups based on their mechanism of action: i) redox inhibitors that maintain the active site iron atom in its (inactive) reduced form; ii) iron chelators which inactivate the enzyme by removal of

the active site iron; iii) competitive inhibitors that can occupy the active site, precluding binding of the substrate; iv) suicide substrates which irreversibly inactivate the enzyme; and v) allosteric inhibitors which affect enzyme activity by binding to a site remote the substrate binding pocket [30]. Since there are six different lipoxygenase isoforms in humans, a major challenge in the field of lipoxygenase inhibition is isoform specificity. This has been compounded by the lack of three-dimensional structural information of the human lipoxygenases beyond ALOX5 [34]. Nevertheless, researchers have gone to great lengths to develop isoform-specific inhibitors.

No single lipoxygenase isoform has been convincingly implicated in ferroptosis to date. For instance, genetic studies showing that deficiency in *Alox15*, the only lipoxygenase able to oxidize esterified FA and presumed to be responsible for the cell death, could not rescue loss of *Gpx4* [6, 35]. Nevertheless, loss of *Alox15* could rescue male subfertility in heterozygous *Gpx4* mice, thus suggesting a complex tissue specific interplay between lipoxygenase and *Gpx4* [35, 36]. Interestingly a few reports have indicated that multiple lipoxygenase inhibition presents a stronger protective effect when comparing with single lipoxygenase targeting [6, 8, 29]. Thus, it remains to be seen whether the development of specific lipoxygenase or pan- lipoxygenase inhibitors are a suitable and effective path for novel drugs aimed at efficiently inhibiting ferroptosis.

Radical-Trapping Antioxidants

Radical-trapping antioxidants (RTAs) – also referred to as chain-breaking antioxidants – are molecules which react with chain-carrying radicals, and thus break the autoxidation chain reaction [37, 38]. They have long been included as additives to petroleum-derived products (e.g. oils, fuels, plastics, rubbers) to inhibit their autoxidation. The recognition of their role in protecting biological hydrocarbons (i.e. lipids) from autoxidation is comparatively recent, ushered in largely based on characterization of the RTA activity of α–tocopherol (the most biologically-active form of Vitamin E - VitE) as among the best ever documented [39], please see Box1. This prompted the widely held belief that antioxidants would be a panacea for a wide array of cardiovascular and neurodegenerative diseases. However, it has since become evident that antioxidant activity can vary wildly in different contexts, many of which do not

translate from *in vitro* to *in vivo*. As such, there has been some general misbelief in the beneficial effects conveyed by antioxidants, and thus, little success so far in the development of such compounds specifically in the context of disease prevention. Nevertheless, this appears to be the consequence of our limited understanding rather than a lack of beneficial effects, particularly since deficiency in VitE has been linked to premature onset of neurodegeneration, a ferroptosis-linked disease [40, 41].

BOX1: Vitamin E, Nature's most efficient ferroptosis inhibitor

The discovery of vitamin E (α-tocopherol - α-Toc) dates to almost 100 years ago [42], and it was found to be required to prevent fetal resorption in pregnant, VitE-deficient rats that received oxidizable diets. VitE is taken up via the diet in eight different forms, four (α-, β-, γ-, and δ-) tocopherols and four (α-, β-, γ-, and δ-) tocotrienols. All of the congeners possess RTA activity, but α-Toc is both the most reactive in vitro [39], and possesses the greatest biological activity in vivo. The greater biological activity of α-Toc compared to the other congeners is mostly due to the presence of mechanisms that retain α-Toc and metabolize and excrete the other ones [43]. This preference is largely based upon the presence of α-tocopherol transfer protein (αTPP), which preferentially binds α-Toc and re-distributes it to tissues. The tocopherols (and other phenols) are capable of "trapping" two peroxyl radicals, i.e. the stoichiometric factor for peroxyl radical capture, *n*, for all phenols is 2.0 [37]. α-Toc fulfills the classical role as an antioxidant as it efficiently "traps" peroxyl radical with a rate constant, k_{inh} of 3.2 x 10^6 $M^{-1}s^{-1}$, efficiently outcompeting the oxidation of other lipid substrates by peroxyl radicals (10^2 $M^{-1}s^{-1}$). Importantly, it was recently suggested an alternative function for VitE, that is direct inhibition of lipoxygenases. This proceeds via a "corking" mechanism, where different VitE analogs compete for the substrate-binding site of lipoxygenase thus suppressing the formation of putative specific lipid death signals [8]. Nonetheless, the importance of α-Toc in ferroptosis has been demonstrated by several groups that have consistently shown that α-Toc suppresses ferroptosis *in vitro*. Perhaps more important was the work done by Wortmann and colleagues [18], who showed that endothelial cells devoid of *Gpx4* are normal except when mice were fed a VitE low diet, whereupon the animals developed early thrombotic events probably caused by increased ferroptosis rates in endothelium. Reciprocally, VitE supplementation in conditional

Gpx4 knockout mice was able to restore antiviral and antiparasitic T-cell responses [4] and rescue hepatocellular degeneration [17]. Hence, these studies have put forward two important and highly relevant points regarding the role of VitE in ferroptosis. First is that some cell types can survive without Gpx4 in the presence of α-Toc, whereas others like neurons and kidney tubular epithelial cells cannot. Secondly, most if not all animal studies are conducted under conditions of supra-physiological VitE concentrations that exceed the level in humans by at least two orders of magnitude, suggesting that contribution of ferroptosis in some mouse models of disease can be masked by the exceptionally ("non-physiologically") high concentrations of α-Toc. Whether these effects are due to its RTA activity or its recently proposed lipoxygenase inhibitory effect remains to be seen.

Vitamin E Analogs

Ever since the recognition of α-Toc's privileged position as nature's premier lipid-soluble RTA, researchers have developed with success synthetic compounds with increased reactivity towards peroxyl radicals. Of the various synthetic compounds that have been developed, the tetrahydronaphthyridinols (THNs) are arguably the most promising [44]. Their structures were designed following extensive studies of the structure-reactivity relationships of classical phenolic RTAs, which revealed that incorporation of nitrogen atoms in the aromatic rings of phenolic antioxidants at the 3 and/or 5 positions relative to the hydroxyl group greatly stabilizes electron-rich phenols to autoxidation [45, 46]. Substitution of these compounds with strongly electron-donating groups (e.g. N,N-dialkylamino) to weaken their O-H bond and accelerate their rates of reaction towards peroxyl radicals was then possible without comprising their stability to autoxidation. The THNs are a specific subset of aza-phenols that we believed would possess the best balance of reactivity and stability; they react almost 30-times faster with peroxyl radicals than α-Toc in organic solution and liposomes [47], making them among the most potent RTAs reported to date. The excellent RTA activity of these compounds and the recognition that oxidative lipid degradation is essential to ferroptosis suggests that THNs might also suppress ferroptosis. Indeed, when assaying a small library of THNs with different chain lengths it was shown that THNs with alkyl chains ranging from 12-15 conferred an optimal inhibitory effect and were even slightly

more potent than the classical ferroptosis inhibitors ferrostatin-1 (Fer-1) and Lip-1 [48]. Another interesting feature of THNs is their ability to efficiently bind to αTPP [49], whereby - in theory - they should become protected from liver metabolism, allowing efficient tissue distribution.

Liproxstatins and Ferrostatins

Liproxstatins and ferrostatins are the result of high throughput screening approaches to identify compounds that are able to efficiently inhibit ferroptosis. Liproxstatins were initially identified in a screen using compounds that suppress cell death in an inducible *Gpx4* knockout mouse cell line [6], while ferrostatins were identified in a model where ferroptosis was elicited using cystine uptake inhibition by erastin [7]. Lip-1, the first identified molecule of the liproxstatin class, suppresses ferroptosis in the low nanomolar range and demonstrates good pharmacological properties including a half-life of 4.6 hours in plasma [6]. Importantly, this molecule ameliorates acute renal failure in a genetic model of *Gpx4* deficiency, strongly suggesting an *in vivo* anti-ferroptotic activity. Similarly, modifications to the original ferrostatin-1 (Fer-1) scaffold using classical medicinal chemistry led to second-generation compounds that present sufficient *in vivo* stability [19]. Importantly, both types of compounds preserved tissue function in both hepatic and kidney IRI. Although very little was initially known regarding their mechanism of action, initial investigations suggested that Fer-1 may work as RTA [26]. Recently, to better understand their mode of action, we set out to explore in detail their RTA activity. Indeed, we could provide evidence at least in the test tubes that although their intrinsic reactivities towards peroxyl radicals are lower than that of α-Toc, they are better inhibitors in lipid bilayers probably due to better dynamics within the lipid and weaker unproductive interactions with the phosphatidylcholine headgroups and/or aqueous surroundings at the interface [48]. Moreover, Fer-1 and Lip-1 were found to possess a greater radical-trapping capacity compared to typical phenolic RTAs, which we propose to be due to their subsequent transformation to nitroxides. Although nitroxides are commonly thought to exhibit superoxide dismutase activity in cells, we have shown that nitroxides are good RTAs [50], and recent work by us as well as Wipf and Stockwell [51] have shown that they can indeed inhibit ferroptosis in *in vitro* cellular systems.

Deuterated Lipids

One of the classic examples of quantum mechanical tunnelling in enzyme catalysis is that which is observed for the oxidation of linoleic acid by soybean lipoxygenase. Thus, linoleic acid, wherein the H-atoms of the bis-allylic methylene group have been replaced with D-atoms, are significantly poorer substrates than the naturally occurring isotopologue. Thus, it appears that the enzyme has evolved to maximize the rate of linoleic acid oxidation by taking advantage of quantum mechanical tunnelling. It was recently shown that tunnelling is also likely key to the rapid propagation step of PUFA autoxidation; the propagation step of the autoxidation of 11,11-d_2-linoleic acid is ca. 20-fold slower than for linoleic acid [52]. Based on these observations, supplementation of cell media with deuterated linoleic acid was investigated, and has proven beneficial in several cell models of lipid peroxidation-related neurological disease including PD [53] and Friedreich's ataxia [54]. Additionally, it was also recently shown that cells exposed to deuterated linoleic acid (LA) presented a marked resistance to ferroptosis. Nevertheless, it remains to ascertain whether the administration of deuterated PUFAs is a viable intervention *in vivo* given the significant amounts of material that would be required and whether this would adversely impact the physiological processes that are controlled by PUFA oxidation.

ACSL4 Inhibitors

A recent report suggests that ferroptosis occurs with preferential oxidation of phosphatidylethanolamines (PE) containing arachidonic (AA) and adrenic acid (AdA) [8]. The enzyme responsible for channeling AA and AdA to this PE-oxidizable pool was identified to be acyl-CoA synthetase long-chain family member 4 (ACSL4) [55]. Moreover, *ACSL4* knockout cells are indeed refractory to ferroptosis induced by genetic or pharmacological inactivation of Gpx4 [55, 56], suggesting that Acsl4 may be a target for ferroptosis inhibition. Indeed thiazolidinediones (TZNs), which were reported to specifically inhibit ACSL4 over other ACSL isoforms [57], were found to suppress ferroptosis [55]. TZNs have been developed and marketed as insulin sensitizers, acting as peroxisome proliferator-activated receptor gamma (PPARγ) agonists, but the effect of TZNs on ferroptosis was shown to be independent of PPARγ and could be ascribed exclusively on their inhibition of ACSL4. Importantly, treatment of animals with

rosiglitazone, a member of the TZN class of compounds, was able to mitigate and retard the mortality rate due to acute renal failure in a genetic model of ferroptosis [55]. Thus, pharmacological inhibition of ACSL4 provides a new paradigm in preventing ferroptosis and also other lipid peroxidation-driven processes by halting lipid peroxidation not at the radical level but by decreasing the substrates that are essential to fuel the lipid peroxidation process. Importantly, genetic studies using *Acsl4* knockout animal should provide answers if *Acsl4* may have deleterious effect by impacting other essential AA-dependent process.

Concluding Remarks

Our understanding of the ferroptosis process is still in its early stages, although during the last four years seminal discoveries have advanced knowledge, particularly in the area of the contribution of lipid peroxidation to the process. This improved understanding has proven to be relevant as in association with the long-standing knowledge of the chemistry of lipid autoxidation has already put forward new molecules such as the THN that are potent inhibitors of ferroptosis. Additionally, the recent recognition that ACSL4 can be pharmacologically inhibited and that this translates to an increased resistance to ferroptosis should encourage the identification of more potent and more selective Acsl4 inhibitors. This deeper understanding of the ferroptosis process has so far enabled us to interfere at several points in the ferroptosis cascade of events. Yet recent studies have also proposed that inhibition of other cellular events can suppress ferroptosis. Among the recently proposed targets for inhibition are glutaminolysis [58], ferritinophagy [59, 60], lysosomal activity [61] and MEK signaling [7]. Nevertheless, these events are still inadequately characterized and more studies are required to evaluate their true anti-ferroptotic potential. Finally, studies into additional pathological settings should put to test what is the optimal approach to interfere with the ferroptosis *in vivo* (see Outstanding Questions), with the ultimate goal to preserve tissue homeostasis in disease-relevant settings.

Acknowledgments

This work was in part supported by grants from the Deutsche Forschungsgemeinschaft (DFG) CO 291/2-3 and CO 291/5-1 to M.C., the Human Frontier Science Program (HFSP) RGP0013 to M.C., and the Natural Sciences and Engineering Research Council of Canada, the Canada Foundation for Innovation to D.A.P. DAP and RS acknowledge support from the Canada Research Chairs program and the NSERC Post-Graduate Scholarship program, respectively.

Conflict of Interest

M.C filed patent applications for some of the molecules described in the article all the other authors declare no conflict of interest.

List of abbreviations

αToc, α-tocopherol; AA, arachidonic acid; AdA, adrenic acid; Acsl4/ACSL4, acyl-CoA synthetase long chain family member 4; Alox/ALOX, lipoxygenase; CNS, central nervous system; Fer-1, Ferrostatin-1; GSH, glutathione; GPX4/Gpx4, glutathione peroxidase; HD, Huntington's disease; HTT, huntingtin; IRI, ischemia/reperfusion injury; LA, linoleic acid; Lip-1, Liproxstatin-1; oxPL, oxidized phospholipids; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid; RTA, radical trapping antioxidant; THN, tetrahydronaphthyridinols; TZN, thiazolidinediones; VitE, vitamin E

References

1 Conrad, M., *et al.* (2016) Regulated necrosis: disease relevance and therapeutic opportunities. *Nat Rev Drug Discov* 15, 348-366

2 Jennis, M., *et al.* (2016) An African-specific polymorphism in the TP53 gene impairs p53 tumor suppressor function in a mouse model. *Genes Dev* 30, 918-930

3 Jiang, L., *et al.* (2015) Ferroptosis as a p53-mediated activity during tumour suppression. *Nature* 520, 57-62

4 Matsushita, M., *et al.* (2015) T cell lipid peroxidation induces ferroptosis and prevents immunity to infection. *J Exp Med* 212, 555-568

5 Do Van, B., *et al.* (2016) Ferroptosis, a newly characterized form of cell death in Parkinson's disease that is regulated by PKC. *Neurobiol Dis* 94, 169-178

6 Friedmann Angeli, J.P., *et al.* (2014) Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice. *Nat Cell Biol* 16, 1180-1191

7 Dixon, S.J., *et al.* (2012) Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* 149, 1060-1072

8 Kagan, V.E., *et al.* (2017) Oxidized arachidonic and adrenic PEs navigate cells to ferroptosis. *Nat Chem Biol* 13, 81-90

9 Kagan, V.E., *et al.* (2005) Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nat Chem Biol* 1, 223-232

10 Yang, W.S., *et al.* (2014) Regulation of Ferroptotic Cancer Cell Death by GPX4. *Cell* 156, 317-331

11 Yant, L.J., *et al.* (2003) The selenoprotein GPX4 is essential for mouse development and protects from radiation and oxidative damage insults. *Free Radic Biol Med* 34, 496-502.

12 Seiler, A., *et al.* (2008) Glutathione Peroxidase 4 Senses and Translates Oxidative Stress into 12/15-Lipoxygenase Dependent- and AIF-Mediated Cell Death. *Cell Metab* 8, 237-248

13 Wirth, E.K., *et al.* (2010) Neuronal selenoprotein expression is required for interneuron development and prevents seizures and neurodegeneration. *FASEB J* 24, 844-852

14 Wirth, E.K., *et al.* (2014) Cerebellar hypoplasia in mice lacking selenoprotein biosynthesis in neurons. *Biol Trace Elem Res* 158, 203-210

15 Chen, L., *et al.* (2015) Ablation of the Ferroptosis Inhibitor Glutathione Peroxidase 4 in Neurons Results in Rapid Motor Neuron Degeneration and Paralysis. *J Biol Chem* 290, 28097-28106

16 Ueta, T., *et al.* (2012) Glutathione peroxidase 4 is required for maturation of photoreceptor cells. *J Biol Chem* 287, 7675-7682

17 Carlson, B.A., *et al.* (2016) Glutathione peroxidase 4 and vitamin E cooperatively prevent hepatocellular degeneration. *Redox Biol* 9, 22-31

18 Wortmann, M., *et al.* (2013) Combined deficiency in glutathione peroxidase 4 and vitamin e causes multiorgan thrombus formation and early death in mice. *Circ Res* 113, 408-417

19 Linkermann, A., *et al.* (2014) Synchronized renal tubular cell death involves ferroptosis. *Proc Natl Acad Sci U S A* 111, 16836-16841

20 Cardoso, B.R., *et al.* (2016) Glutathione peroxidase 4: a new player in neurodegeneration? *Mol Psychiatry*

21 Bazinet, R.P. and Laye, S. (2014) Polyunsaturated fatty acids and their metabolites in brain function and disease. *Nat Rev Neurosci* 15, 771-785

22 Joshi, Y.B., *et al.* (2015) The 12/15-lipoxygenase as an emerging therapeutic target for Alzheimer's disease. *Trends Pharmacol Sci* 36, 181-186

23 Deas, E., *et al.* (2016) Alpha-Synuclein Oligomers Interact with Metal Ions to Induce Oxidative Stress and Neuronal Death in Parkinson's Disease. *Antioxid Redox Signal* 24, 376-391

24 Abeti, R., *et al.* (2016) 'Mitochondrial energy imbalance and lipid peroxidation cause cell death in Friedreich's ataxia'. *Cell death & disease* 7, e2237

25 Paul, B.D., *et al.* (2014) Cystathionine gamma-lyase deficiency mediates neurodegeneration in Huntington's disease. *Nature* 509, 96-100

26 Skouta, R., *et al.* (2014) Ferrostatins inhibit oxidative lipid damage and cell death in diverse disease models. *J Am Chem Soc* 136, 4551-4556

27 Mason, R.P., *et al.* (2013) Glutathione peroxidase activity is neuroprotective in models of Huntington's disease. *Nat Genet* 45, 1249-1254

28 Li, Y., *et al.* (1997) A role for 12-lipoxygenase in nerve cell death caused by glutathione depletion. *Neuron* 19, 453-463

29 Yang, W.S., *et al.* (2016) Peroxidation of polyunsaturated fatty acids by lipoxygenases drives ferroptosis. *Proc Natl Acad Sci U S A* 113, E4966-4975

30 Kuhn, H., *et al.* (2015) Mammalian lipoxygenases and their biological relevance. *Biochim Biophys Acta* 1851, 308-330

31 Haeggstrom, J.Z. and Funk, C.D. (2011) Lipoxygenase and leukotriene pathways: biochemistry, biology, and roles in disease. *Chemical reviews* 111, 5866-5898

32 Schneider, C., *et al.* (2007) Control of oxygenation in lipoxygenase and cyclooxygenase catalysis. *Chem Biol* 14, 473-488

33 Yin, H., *et al.* (2011) Free radical lipid peroxidation: mechanisms and analysis. *Chemical reviews* 111, 5944-5972

34 Gilbert, N.C., *et al.* (2011) The structure of human 5-lipoxygenase. *Science* 331, 217-219

35 Brutsch, S.H., *et al.* (2015) Expression of inactive glutathione peroxidase 4 leads to embryonic lethality, and inactivation of the alox15 gene does not rescue such knock-in mice. *Antioxid Redox Signal* 22, 281-293

36 Brutsch, S.H., *et al.* (2016) Male Subfertility Induced by Heterozygous Expression of Catalytically Inactive Glutathione Peroxidase 4 Is Rescued in Vivo by Systemic Inactivation of the Alox15 Gene. *J Biol Chem* 291, 23578-23588

37 Ingold, K.U. and Pratt, D.A. (2014) Advances in radical-trapping antioxidant chemistry in the 21st century: a kinetics and mechanisms perspective. *Chemical reviews* 114, 9022-9046

38 Ingold, K.U. (1961) Inhibition of the Autoxidation of Organic Substances in the Liquid Phase. *Chemical reviews* 61, 563-589

39 Burton, G.W. and Ingold, K.U. (1986) Vitamin E: application of the principles of physical organic chemistry to the exploration of its structure and function. *Accounts of Chemical Research* 19, 194-201

40 Ulatowski, L.M. and Manor, D. (2015) Vitamin E and neurodegeneration. *Neurobiol Dis* 84, 78-83

41 Harding, A.E., *et al.* (1982) Spinocerebellar degeneration secondary to chronic intestinal malabsorption: a vitamin E deficiency syndrome. *Ann Neurol* 12, 419-424

42 Evans, H.M. and Bishop, K.S. (1922) On the Existence of a Hitherto Unrecognized Dietary Factor Essential for Reproduction. *Science* 56, 650-651

43 Brigelius-Flohe, R. and Traber, M.G. (1999) Vitamin E: function and metabolism. *FASEB J* 13, 1145-1155

44 Valgimigli, L. and Pratt, D.A. (2015) Maximizing the reactivity of phenolic and aminic radical-trapping antioxidants: just add nitrogen! *Acc Chem Res* 48, 966-975

45 Pratt, D.A., *et al.* (2001) 5-Pyrimidinols: novel chain-breaking antioxidants more effective than phenols. *J Am Chem Soc* 123, 4625-4626

46 Wijtmans, M., *et al.* (2003) 6-Amino-3-pyridinols: towards diffusion-controlled chain-breaking antioxidants. *Angew Chem Int Ed Engl* 42, 4370-4373

47 Nam, T.G., *et al.* (2007) Tetrahydro-1,8-naphthyridinol analogues of alpha-tocopherol as antioxidants in lipid membranes and low-density lipoproteins. *J Am Chem Soc* 129, 10211-10219

48 Zilka, O., *et al.* On the Mechanism of Cytoprotection by Ferrostatin-1 & Liproxstatin-1 and the Role of Lipid Peroxidation in Ferroptotic Cell Death. *submitted*

49 Li, B., *et al.* (2013) Besting vitamin E: sidechain substitution is key to the reactivity of naphthyridinol antioxidants in lipid bilayers. *J Am Chem Soc* 135, 1394-1405

50 Haidasz, E.A., *et al.* (2016) Acid Is Key to the Radical-Trapping Antioxidant Activity of Nitroxides. *J Am Chem Soc* 138, 5290-5298

51 Krainz, T., *et al.* (2016) A Mitochondrial-Targeted Nitroxide Is a Potent Inhibitor of Ferroptosis. *ACS Cent Sci* 2, 653-659

52 Shchepinov, M.S. (2007) Reactive oxygen species, isotope effect, essential nutrients, and enhanced longevity. *Rejuvenation Res* 10, 47-59

53 Shchepinov, M.S., *et al.* (2011) Isotopic reinforcement of essential polyunsaturated fatty acids diminishes nigrostriatal degeneration in a mouse model of Parkinson's disease. *Toxicol Lett* 207, 97-103

54 Cotticelli, M.G., *et al.* (2013) Insights into the role of oxidative stress in the pathology of Friedreich ataxia using peroxidation resistant polyunsaturated fatty acids. *Redox Biol* 1, 398-404

55 Doll, S., *et al.* (2017) ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid composition. *Nat Chem Biol* 13, 91-98

56 Dixon, S.J., *et al.* (2015) Human Haploid Cell Genetics Reveals Roles for Lipid Metabolism Genes in Nonapoptotic Cell Death. *ACS Chem Biol* 10, 1604-1609

57 Kim, J.H., *et al.* (2001) Expression and characterization of recombinant rat Acyl-CoA synthetases 1, 4, and 5. Selective inhibition by triacsin C and thiazolidinediones. *J Biol Chem* 276, 24667-24673

58 Gao, M., *et al.* (2015) Glutaminolysis and Transferrin Regulate Ferroptosis. *Mol Cell* 59, 298-308

59 Hou, W., *et al.* (2016) Autophagy promotes ferroptosis by degradation of ferritin. *Autophagy* 12, 1425-1428

60 Gao, M., *et al.* (2016) Ferroptosis is an autophagic cell death process. *Cell research* 26, 1021-1032

61 Torii, S., *et al.* (2016) An essential role for functional lysosomes in ferroptosis of cancer cells. *Biochem J* 473, 769-777

Figure Legends

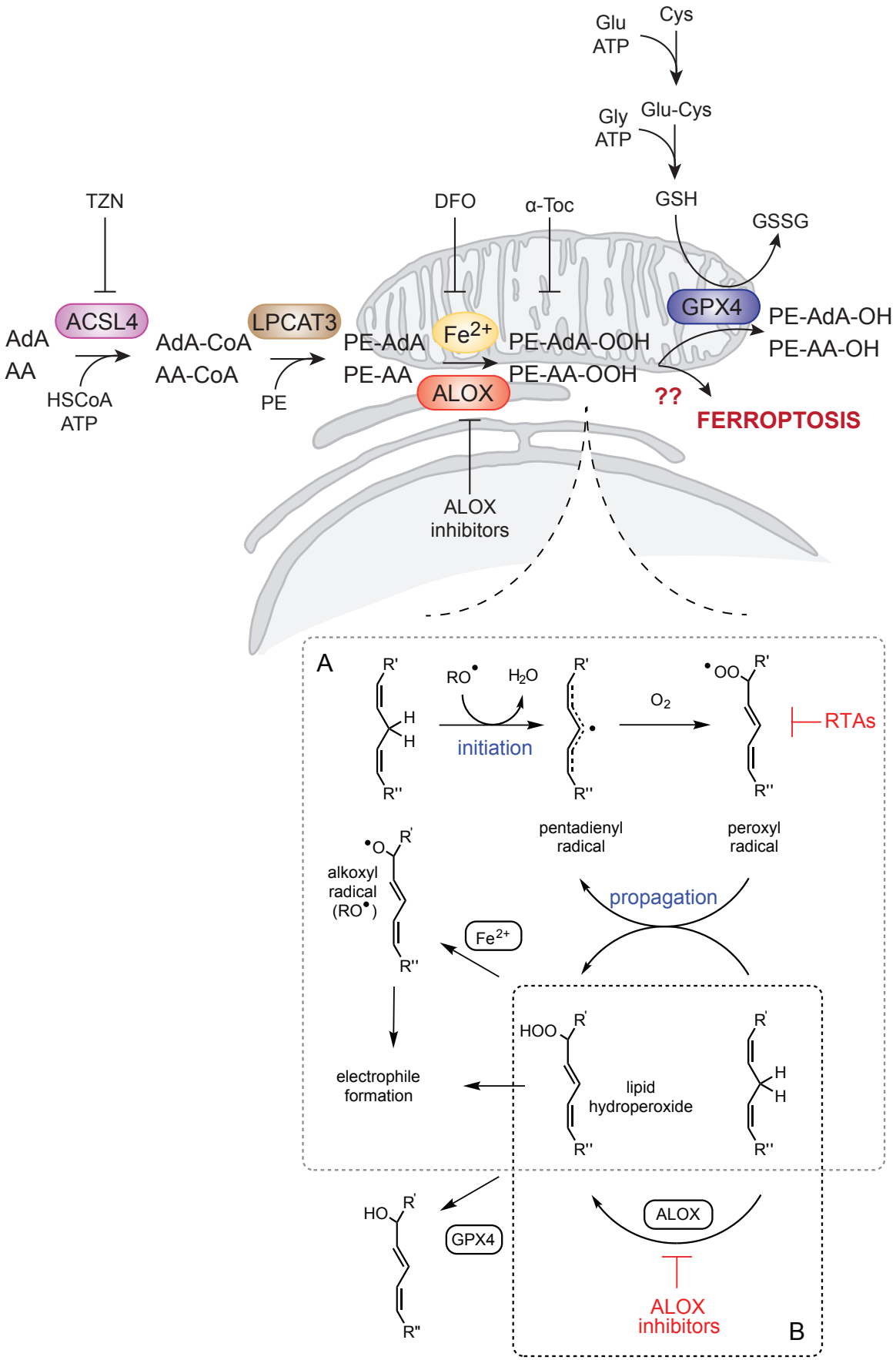
Figure 1: Molecular Pathways of Ferroptosis Regulation.

Fatty acid substrates (arachidonic acid, AA; adrenic acid, AdA) are activated by Acyl-CoA synthetase long chain family member 4 (ACSL4) generating AA-CoA and AdA-CoA which are then esterified via a Lysophosphatidylcholine Acyltransferase (LPCAT3) dependent mechanism into anionic phospholipids. Upon ferroptosis induction, these substrates undergo oxidation through an ALOX- or iron-dependent mechanism, thus driving ferroptosis execution. This system is kept in check by the heterodimeric cystine–glutamate antiporter system Xc−, which exchanges one molecule of extracellular cystine for one molecule of intracellular glutamate. Once taken up by cells, cystine is reduced and subsequently used for protein and glutathione (GSH) synthesis. Glutathione peroxidase 4 (GPX4) is the key GSH utilizing enzyme and responsible for preventing lipoxygenase (ALOX) overactivation and lipid peroxidation. GPX4 preferentially reduces phospholipid hydroperoxide represented here as the ferroptosis relevant phosphatidylethanolamine (PE-OOH) to its corresponding alcohol phospholipid hydroxide (PL-OH), using two molecules of GSH. Inlets show differences between non-enzymatic (A) and enzymatic (B) lipid peroxidation (Abbreviations: TZN, thiazolidinedione; DFO, deferoxamine; α-Toc, α-tocopherol, GSSG, oxidized glutathione; HS-CoA, coenzyme A; RTA, radical trapping antioxidants).

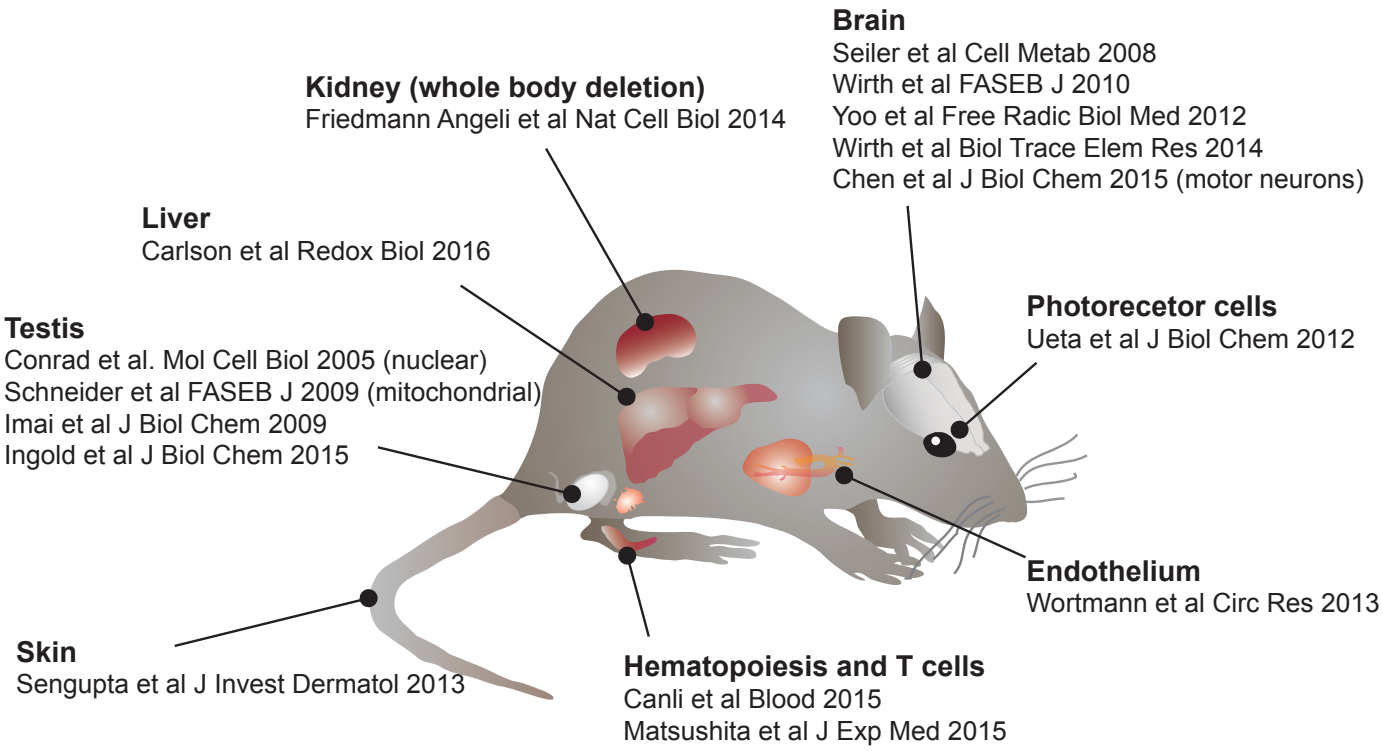
Figure 2: Genetic studies on Gpx4 knockout animals. Representative illustration of tissues where conditional deletion of Gpx4 was performed. Numbers indicate the respective citations as given in the text.

Figure 3: Chemical structures and mechanism of action of ferroptosis inhibitors.

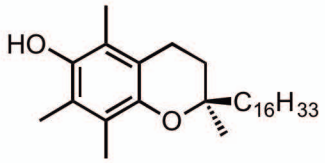
Angeli_Figure 1



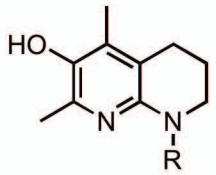
Angeli_Figure 2



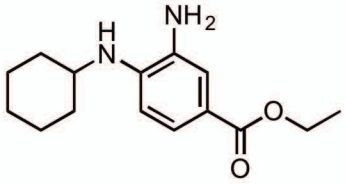
Radical-Trapping Antioxidants



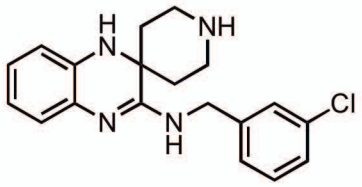
α-Tocopherol



Tetrahydronapthyridinols (THNs)

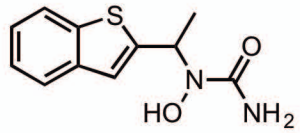


Ferrostatin-1

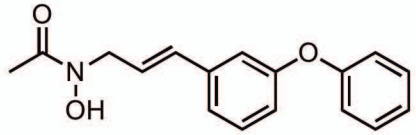


Liproxstatin-1

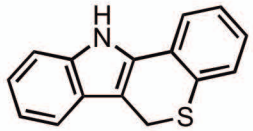
Lipoxygenase Inhibitors



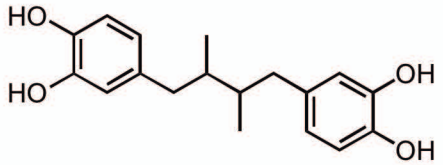
Zileuton



BW A4C

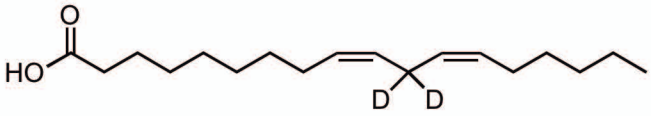


PD146176



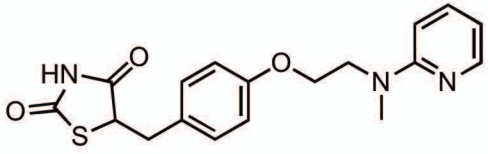
NDGA

Deuterated Lipids

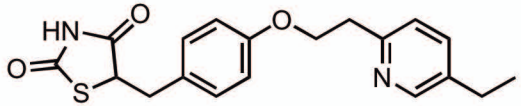


11,11-d_2-Linoleic Acid

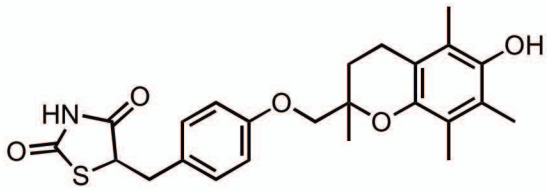
Acsl4 Inhibitors



Rosiglitazone



Pioglitazone



Troglitazone