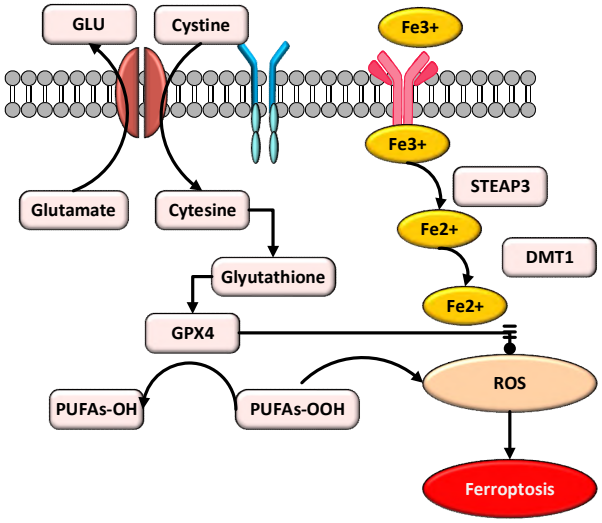

FERROPTOSIS AND CANCER

March 2025

ABSTRACT
We discuss ferroptosis a form of cell death that has recently attracted attention in cancer therapy.
Terrence McGarty
TGL 214

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Communications relating to these documents and these should be sent to:
mcgarty@alum.mit.edu.

Access to prior Technical Notes can be made via Research Gate,
https://www.researchgate.net/profile/Terrence-Mcgarty/research

Contents

1 INTRODUCTION

There are variety of cell death methods. Apoptosis is one which has been well studied. Ferroptosis is a recently understood process where excess iron can impact mitochondria and cell survival. We examine some recent work on ferroptosis and its implications for cancer therapeutics. We focus on the application in prostate cancer (PCa).

As Dixon et al (2012) noted:

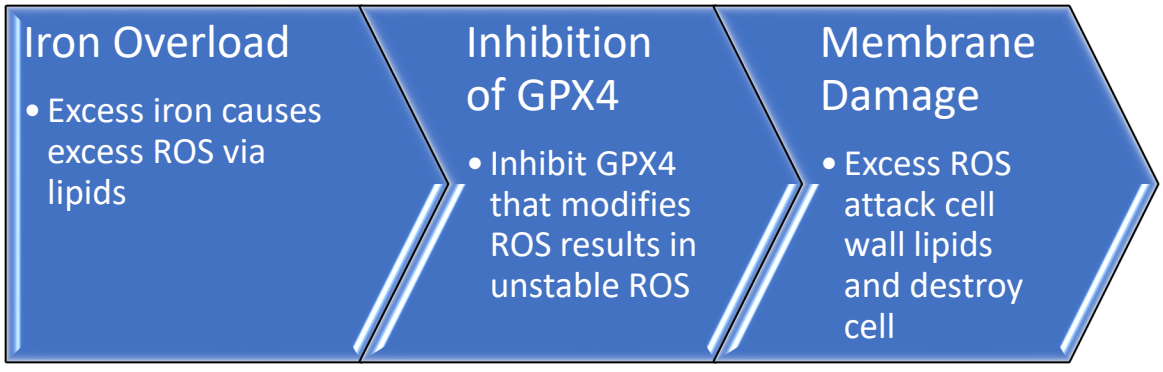
Nonapoptotic forms of cell death may facilitate the selective elimination of some tumor cells or be activated in specific pathological states. The oncogenic RAS-selective lethal small molecule erastin triggers a unique iron-dependent form of nonapoptotic cell death that we term ferroptosis.

Ferroptosis is dependent upon intracellular iron, but not other metals, and is morphologically, biochemically, and genetically distinct from apoptosis, necrosis, and autophagy.

We identify the small molecule ferrostatin-1 as a potent inhibitor of ferroptosis in cancer cells and glutamate-induced cell death in organotypic rat brain slices, suggesting similarities between these two processes. Indeed, erastin, like glutamate, inhibits cystine uptake by the cystine/glutamate antiporter…., creating a void in the antioxidant defenses of the cell and ultimately leading to iron dependent, oxidative death.

Thus, activation of ferroptosis results in the nonapoptotic destruction of certain cancer cells, whereas inhibition of this process may protect organisms from neurodegeneration.

The general principal of ferroptosis simplified are shown below:



Namely in the above we see that iron overload can attack the cell, and depending on normal controls to limit ROS elements created the result is destruction of cell wall and death of cell. This is the simples terms of ferroptosis. There are details we will examine from various perspectives.

Multiple authors have argued that ferroptosis can be used to attack cancer cells. We examine this hypothesis but the main concern is specificity in targeting. The proposals demonstrate how ferroptosis can be effected. The question is; how does this process target organ specific malignant cells? It is not at all clear how this mechanism if any works.

Our approach is as follows:

1. Examine the process of creating reactive oxygen species wherein the species are derived from lipoproteins inside the cell. ROS are highly reactive and can in turn cause massive cellular damage.

2. Examine the ferroptosis cell elements including drivers such as iron and ROS creation. Also examine the mitigating cell elements which turn ferroptosis off to protect the cell.

3. Broadly examine the putative use of ferroptosis to attack cancer cells.

4. Consider the therapeutic methods to effect ferroptosis and thus the destruction of malignant cells.

5. Consider in detail the application to prostate cancer.

2 REACTIVE OXYGEN SPECIES

As Halliwell and Gutteridge had noted reactive oxygen species, or oxygen radicals, oxidants, are species derived from oxygen but more reactive. Free radicals and the like can result in damage to DNA and multiple other cellular elements.

Let us first present a few basic principles.

1. Assume some lipid structure RH and some ionized metal M^{n+} . Then we have the reaction[1]:

$$\mathbf{RH + M^{n+} \rightarrow R^{\cdot} + M^{(n-1)} H}$$

Which creates the radical lipo.

2. Now this propagates with oxygen as follows:

$$\mathbf{R^{\cdot} + O_2 \rightarrow ROO^{\cdot}}$$

Then

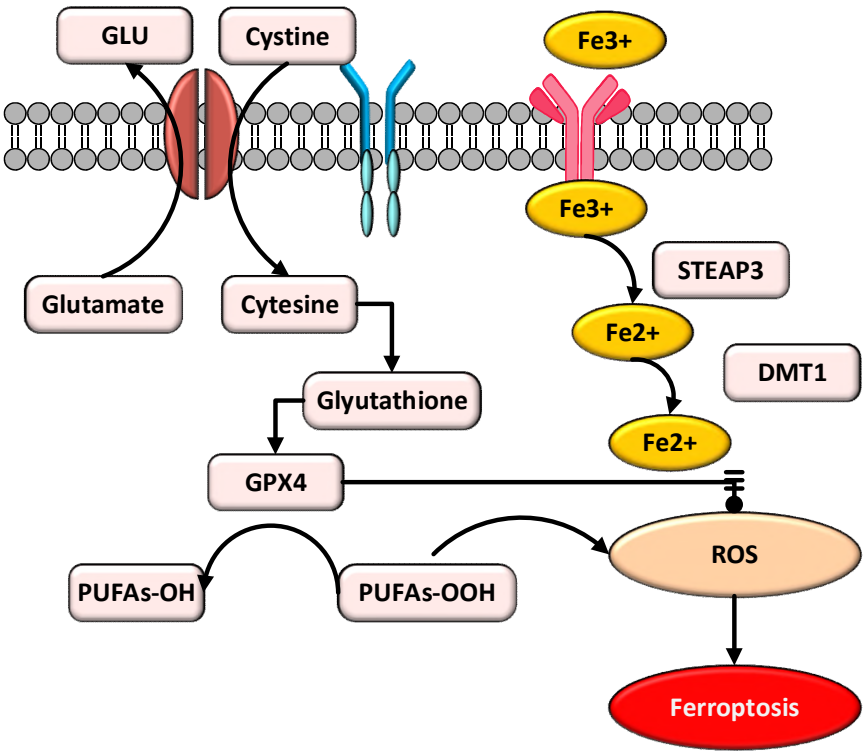
$$\mathbf{ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}}$$

Now free radicals containing oxygen are called reactive oxygen species, ROS. It is these free radical types that driven by metals cause the toxic environment resulting in ferroptosis. Specifically the metal driver here is Fe^{3+} .

2. Now these radical can be mitigated by glutathione so a balance is obtained.

3. However in certain cells this is blocked and the result in ferroptosis where the ROS attack the cell lipids degrading the cell.

[1] See pp 219-220, Rodwell, Biochemistry, McGraw Hill, 2015.



2.1 FUNDAMENTALS

As Thannickal and Fanburg have noted:

Molecular oxygen (dioxygen; O_2) is essential for the survival of all aerobic organisms. Aerobic energy metabolism is dependent on oxidative phosphorylation, a process by which the oxidoreduction energy of mitochondrial electron transport (via a multicomponent NADH dehydrogenase enzymatic complex) is converted to the high-energy phosphate bond of ATP. O_2 serves as the final electron acceptor for cytochrome-c oxidase, the terminal enzymatic component of this mitochondrial enzymatic complex, that catalyzes the four-electron reduction of O_2 to H_2O. Partially reduced and highly reactive metabolites of O_2 may be formed during these (and other) electron transfer reactions.

These O_2 metabolites include superoxide anion (O22z) and hydrogen peroxide (H_2O_2), formed by one- and two-electron reductions of O_2, respectively. In the presence of transition metal ions, the even more reactive hydroxyl radical (OHz) can be formed.

These partially reduced metabolites of O_2 are often referred to as "reactive oxygen species" (ROS) due to their higher reactivities relative to molecular O2. ROS from mitochondria and other cellular sources have been traditionally regarded as toxic by-products of metabolism with the potential to cause damage to lipids, proteins, and DNA.

ROS are the major elements that can cause genetic changes in a cell.

To protect against the potentially damaging effects of ROS, cells possess several antioxidant enzymes such as superoxide dismutase (which reduces O22z to H2O2), catalase, and glutathione peroxidase (which reduces H2O2 to H2O). Thus oxidative stress may be broadly defined as an imbalance between oxidant production and the antioxidant capacity of the cell to prevent oxidative injury.

Oxidative stress has been implicated in a large number of human diseases including atherosclerosis, pulmonary fibrosis, cancer, neurodegenerative diseases, and aging.

Yet the relationship between oxidative stress and the pathobiology of these diseases is not clear, largely due to a lack of understanding of the mechanisms by which ROS function in both normal physiological and disease states.

Accumulating evidence suggests that ROS are not only injurious by-products of cellular metabolism but also essential participants in cell signaling and regulation.

Although this role for ROS is a relatively novel concept in vertebrates, there is strong evidence of a physiological role for ROS in several nonmammalian systems. In bacteria, the OxyR protein functions as a transcriptional regulator of H2O2-inducible genes and has been shown to be directly activated by oxidation.

2.2 CANCER AND ROS

Cancer and ROS have been associated with each other for a long time. ROS can mutate key genes and thus initiate a malignant process. From Panieri and Santoro:

Cancer is one of the leading causes of death worldwide. Despite extensive research and considerable efforts for developing targeted therapies, many tumors are still characterized by poor prognosis and high mortality. For this reason, novel strategies to improve the outcome of patients suffering from aggressive or therapy-resistant malignancies are critically needed. Recent evidences indicate that altered redox balance and deregulated redox signaling, which are two common hallmarks of tumors, can be strongly implicated in malignant progression and resistance to treatment.

It has been long postulated that cancer cells exhibit persistently high reactive oxygen species (ROS) levels as a consequence of genetic, metabolic and microenvironment-associated alterations.

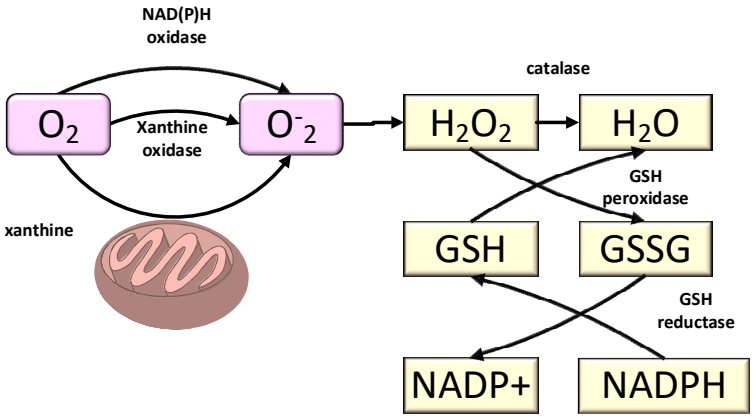
The question is; do normal cell having ROS excess result in cancer, and then do the cancer cell not only maintain high ROS but continue to grow the ROS levels. They continue:

These are then compensated by an increased antioxidant ability from these cancer cells. Although seemingly paradoxical, this pro-oxidant shift can promote tumor growth by inducing DNA damage and genomic instability which then activate an inflammatory response, stabilizing the hypoxia inducible factor-14 and thus reprogramming metabolism.

Due to the selective pressure induced by sustained ROS production, cancer cells have developed an efficient mechanism of ROS detoxification that presents a selective advantage over and upholds its survival under prooxidizing conditions. Therefore, the dependency of cancer cells from their antioxidant systems represents a specific vulnerability that must be exploited to induce targeted cell death.

This can be achieved by increasing oxidative stress above the toxicity threshold, sparing normal cells, which are characterized by having lower intracellular ROS levels. Due to their dualistic nature, ROS can act as 'good' and 'bad' molecules, and regulate cellular physiology or induce cytotoxicity depending on the magnitude, duration and site of their generation. Hence, strategies aimed at altering redox signaling events in tumor cells and intend to disable key antioxidant systems in the presence of ROS inducers might represent promising new anticancer treatments.

We demonstrate these issues graphically below.



The most relevant radicals in biological regulation are superoxide and NO (see Table 1). These radicals are formed by two groups of enzymes, i.e., the NAD(P)H oxidase and NOS isoforms, respectively. Many regulatory effects are mediated by hydrogen peroxide and other ROS that are chemically derived from superoxide

From Droge

2.3 FLAVINOIDS

Antioxidants have been a mainstay of attempts to reduce the impact of ROS. Plant flavonoids are a large class of these antioxidants. As Grotewold noted:

Diets high in flavonoids, fruits, and vegetables are protective against a variety of diseases, particularly cardiovascular disease and some types of cancer.

Antioxidants and dietary fiber are believed to be the principal nutrients responsible for these protective effects. Reactive oxygen species (ROS) are formed in vivo during normal aerobic metabolism and can cause damage to DNA, proteins, and lipids, despite the natural antioxidant defense system of all organisms.

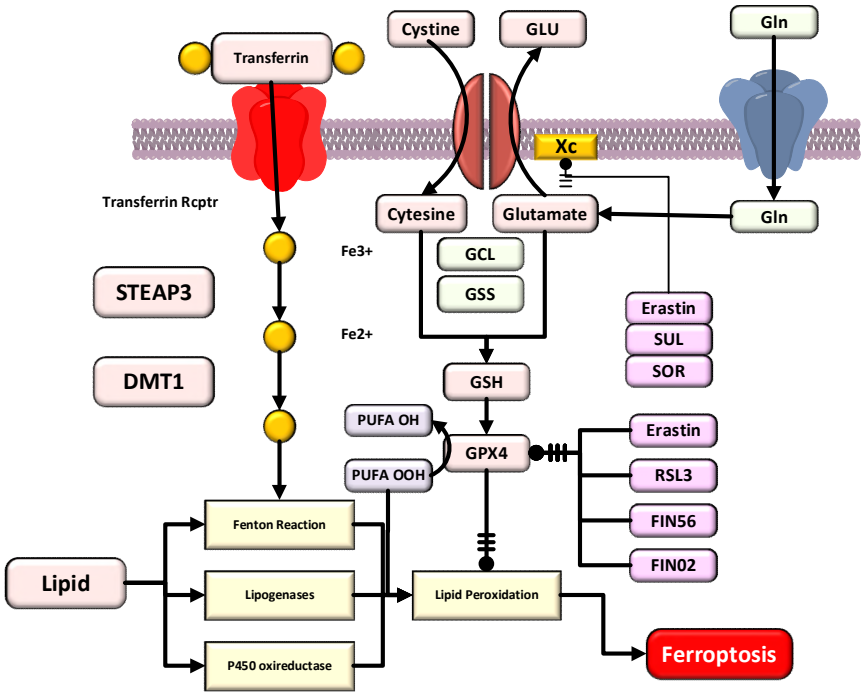
ROS contribute to cellular aging, mutagenesis, carcinogenesis, and coronary heart disease possibly through the destabilization of membranes, DNA damage, and oxidation of low-density lipoprotein (LDL).

Many in vitro studies have demonstrated the potent peroxyl radical scavenging abilities of flavonoids, which contribute to inhibiting lipid peroxidation and oxidation of LDL . Since oxidation of LDL is implicated in the pathogenesis of coronary heart diseases through its ability to decrease the susceptibility of LDL to oxidation, a number of researches have undertaken investigations examining the activity of dietary agents rich in flavonoids in inhibiting LDL oxidation…

The question is, however, can anti-oxidants overcome massive ROS cell degradation. Are they are best supportive. Can they be preventative?

3 FERROPTOSIS

We now consider several approaches to defining and understanding ferroptosis. It is interesting to see the various approaches and which elements take precedence. The Figure below is a comprehensive presentation of the process which we will get to again. Note the complex control mechanisms that result in ferroptosis. It requires iron to create the ROS and blockage of the GPX4 to prevent the blockage of the ROS. As the ROS proliferate then we end up destroying the cell wall. This is a simplified view. We now present multiple other views of this process.



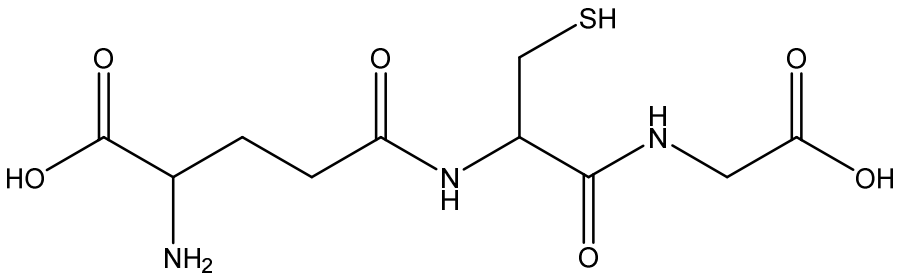
First, from Brown et al:

Ferroptosis is a form of cell death driven by iron- dependent damage to lipids, the building blocks of cellular membranes.

Note that the "iron damage" is secondary to processes on lipids.

Specifically, lipids that contain polyunsaturated fatty acids readily undergo oxidation, causing damage to membranes. If not eliminated by intracellular repair mechanisms, such as the enzyme glutathione peroxidase 4 (GPX4[2]), high concentrations of oxidized lipids can lead to membrane rupture and ultimately to cell death.

[2] https://www.ncbi.nlm.nih.gov/gene/2879 **GPX4** The protein encoded by this gene belongs to the glutathione peroxidase family, members of which catalyze the reduction of hydrogen peroxide, organic hydroperoxides and lipid hydroperoxides, and thereby protect cells against oxidative damage. Several isozymes of this gene family exist in vertebrates, which vary in cellular location and substrate specificity. This isozyme has a high preference for lipid hydroperoxides and protects cells against membrane lipid peroxidation and cell death. It is also required for normal



glutathione

Where the neutralizing reaction is:

2 Glutathione+Lipid-hyperoxide → Glutathione disulfide + Lipid-alcohol +Water

Ferroptosis is regulated by multiple cellular processes, including iron regulation, lipid metabolism, and antioxidant defense systems. It has been implicated in a range of pathologies, including neurodegeneration, cancer, and organ injury. These and other conditions bearing signatures of ferroptosis—including iron accumulation, an increase in reactive oxygen species, and lipid oxidation—may be amenable to ferroptosis-modulating treatments

Now in contrast, Bogdan et al note:

Iron is deeply linked to cell death; traditionally, iron is thought to contribute to cell death pathways through ROS production.

Again, they stipulate the iron as being the driving factor.

Recently, an ROS-independent role for iron in modulating apoptosis (programmed cell death) was defined.

In addition, an entirely new mode of iron-dependent cell death, termed 'ferroptosis', was described. Iron Inhibits Alternative Splicing of Fas and Facilitates Production of a Pro-Apoptotic Isoform

This is a bit confusing. First the iron independent and second the iron dependent. Ferroptosis is iron dependent by definition. It turns the lipids into ROS which in turn cause the damage if not modulated.

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sperm development; thus, it has been identified as a 'moonlighting' protein because of its ability to serve dual functions as a peroxidase, as well as a structural protein in mature spermatozoa. Mutations in this gene are associated with Sedaghatian type of spondylometaphyseal dysplasia (SMDS). This isozyme is also a selenoprotein, containing the rare amino acid selenocysteine (Sec) at its active site. Sec is encoded by the UGA codon, which normally signals translation termination. The 3' UTRs of selenoprotein mRNAs contain a conserved stem-loop structure, designated the Sec insertion sequence (SECIS) element, that is necessary for the recognition of UGA as a Sec codon, rather than as a stop signal. Transcript variants resulting from alternative splicing or use of alternate promoters have been described to encode isoforms with different subcellular localization

The cell death receptor Fas exists in two isoforms that are generated by alternative splicing. Fas is a proapoptotic receptor with a transmembrane domain encoded by exon 6. Alternative splicing of Fas pre-mRNA results in exclusion of exon 6 and generates a soluble, anti-apoptotic Fas isoform. A recent genome-wide screen identified iron as a crucial suppressor of serine/arginine-rich splicing factor 7 (SRSF7[3], also known as 9G8), an RNA splicing regulator and important mediator of Fas exon 6 exclusion. Iron inhibits SRSF7 RNA-binding activity, without affecting overall SRSF7 expression or localization, by competing with zinc in the RNA-binding zinc-knuckle domain of SRSF7.

*Through this mechanism, iron inhibited **SRSF7** binding to Fas pre-mRNA and subsequent exon 6 exclusion, leading to an increase in the expression of the pro-apoptotic, exon 6-containing Fas isoform. Further studies will be necessary to confirm the suppression mechanism of SRSF7 splicing activity as well as the regulation of other alternative splicing events by iron. With the recent identification of ZIP8 and ZIP14 as iron transporters, there exists a potential relationship between ZIP transporters and Fas-induced apoptosis.*

Given that ZIP14[4] is crucial for the development of hepatic iron overload in hereditary hemochromatosis, ZIP14-mediated iron transport may be a contributing factor to liver cell death in hemochromatosis patients by enhancing expression of the pro-apoptotic Fas isoform. A thorough characterization of any relationship between ZIP transporters and Fas-induced apoptosis might yield another link between cellular iron homeostasis and cell death.

Ferroptosis Is a Novel Form of Iron-Dependent Cell Death Ferroptosis, an iron-dependent cell death pathway that is non-apoptotic, non-necroptotic, and non-autophagic, was first described in 2012. Ferroptosis is characterized by lipid peroxidation that is generated at least partially by ROS from heme-containing NADPH oxidase (NOX) family enzymes.

Ferroptosis can be inhibited by iron chelation as well as by several novel small molecules (e.g., ferrostatin-1 and liproxstatin-1) but not by inhibitors of apoptosis (zVAD) or necroptosis (necrostatin-1).

Although the exact role of iron in ferroptosis is not fully understood, there is considerable evidence that iron is a necessary component. IRP2 was identified as an essential gene for the

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[3] https://www.ncbi.nlm.nih.gov/gene/6432 **SRSF7** The protein encoded by this gene is a member of the serine/arginine (SR)-rich family of pre-mRNA splicing factors, which constitute part of the spliceosome. Each of these factors contains an N-terminal RNA recognition motif (RRM) for binding RNA and a C-terminal RS domain for binding other proteins. The RS domain is rich in serine and arginine residues and facilitates interaction between different SR splicing factors. In addition to being critical for mRNA splicing, the SR proteins have also been shown to be involved in mRNA export from the nucleus and in translation. Multiple transcript variants encoding different isoforms have been found for this gene

[4] https://www.ncbi.nlm.nih.gov/gene/23516 **ZIP14** his gene encodes a member of the SLC39A family of divalent metal transporters that mediates the cellular uptake of manganese, zinc, iron, and cadmium. The encoded protein contains eight transmembrane domains, a histidine-rich motif, and a metalloprotease motif, and is expressed on the plasma membrane and the endocytic vesicle membrane. It is an important transporter of nontransferrin-bound iron and a critical regulator of manganese homeostasis. Naturally occurring mutations in this gene are associated with neurodegeneration with brain iron accumulation and early-onset parkinsonism-dystonia with hypermanganesemia.

induction of ferroptosis in HT-1080 cells, likely because of its role in iron accumulation and regulation of iron metabolism genes through the IRE–IRP system. Recently, a study identified a role for the serum iron-carrier protein transferrin in ferroptosis induction.

This study noted that, whereas MEFs grown in serum- and amino-acid-free media showed only modest apoptosis, replenishing the media with serum caused very extensive cell death that was neither apoptotic nor necroptotic.

Further investigation determined that two serum components, transferrin and glutamine, were necessary to induce the cell death phenotype. Iron chelation, immunodepletion of transferrin from the serum, and RNAi knockdown of TfR1 all independently prevented cell death. In addition, recombinant iron-loaded (holo-) but not iron-free (apo-) transferrin enhanced cell death. Erastin, a synthetic small molecule that induces ferroptosis required both transferrin and glutamine to effectively kill cells, while ferrostatin 1 (ferroptosis inhibitor) blocked cell death induced by amino acid starvation, suggesting that this form of cell death is indeed ferroptosis.

Third, as Alvarez-Meythaler et al note:

Ferroptosis is a novel type of programmed cell death characterized by iron and lipidic ROS/peroxides accumulation.

This description seems to be more balanced. It is iron plus ROS.

It has been proposed that cancer cells from different tissues show different degrees of ferroptosis sensitivity. Even so, some authors have shown that ferroptotic reagents can induce cancer cell death that could be rescued by ferroptosis inhibitors.

This iron- and oxidative-mediated cell death is activated through excessive levels of iron production by Fenton reaction and through the loss of balance in ROS production and cell glutathione (GSH)-dependent antioxidants, which protect cells from lipid peroxidation.

Glutathione peroxidase 4 (GPX4) is a crucial enzyme for the elimination of lipid ROS continuously generated by the cell.

Its inhibition can induce ferroptosis even with normal levels of the cofactor GSH. Besides, depletion of GSH or its precursor, cysteine (Cys), constitutes an indirect way to activate ferroptosis.

Ferroptosis is characterized, contrary to other regulated cell death mechanisms, by cell membrane integrity, normal nucleus size, and dense small mitochondria.

Recent studies have described a direct contribution of autophagy in ferroptosis initiation, arguing the presence of a specific autophagic cell death called ferritinophagy. After Cys suppression, autophagy is activated to sequester and degrade ferritin, a cell iron storage protein, by the selective autophagy cargo receptor NCOA4, inducing ROS accumulation and the consequent ferroptotic cell death.

Inhibition of the expression of autophagic proteins such as ATG5, ATG7, and NCOA4 reduces ferritin elimination, iron levels, lipid peroxidation, and ferroptosis activation.

Furthermore, autophagy pathway activation by Tat-Beclin-1, a direct autophagicmechanism inducer, selectively promotes ferroptotic cell death in tumor cells. Other studies demonstrate that ferroptosis stimulation also induces autophagy, evidenced by an intensification in the conversion of mature LC3 and autolysosome assembly, demonstrating a close interplay between both signaling mechanisms

Fourth from Mu et al:

Ferroptosis is programmed necrosis mainly triggered by extra-mitochondrial lipid peroxidation arising from an iron-dependent ROS accretion. Excessive iron originally from aberrant iron metabolism or maladjustment of two major redox systems (lipid peroxidation and thiols) was the main incentive factors of ROS production. Glutathione (GSH), a thiol-containing tripeptide, synthesis is determined by the constant import of cysteine (Cys2) by the cell surface Cys2/glutamate antiporter xCT

One can see the varying focus on what ferroptosis is and how it occurs. Fundamentally the process results from iron being introduced into cells with poorly controlled limits on ROS production. The excess ROS result in cell destruction.

3.1 THE PROCESS

The ferroptosis process can best be explained below. Basically is starts with damage to intracellular elements. The result is intracellular oxidation processes to eliminate the damage. That sets of a cycle whereby iron if in abundance outside the cell may be admitted and the result is a breakdown of the cell itself.

As Yin et al noted specific details of this process as follows:

Ferroptosis is inherently the result of cellular damage <u>caused</u> by dysregulation of the intracellular oxidative and antioxidant balance.

Iron is a constituent of many intracellular metalloproteins and participates in processes such as oxygen transportation, ATP production, DNA synthesis and repair and substance metabolism.

However, excessive intracellular iron could generate ROS via the Fenton reaction, leading to cell damage[5].

[5] See https://www.sciencedirect.com/topics/chemistry/fenton-reaction

The Fenton reaction is the Fe(III/II)-catalyzed oxidation of organic substrates, RH, by H_2O_2

$$\mathbf{Fe2{+}aq{+}H2O2 \rightleftarrows Fe2{+}H2O2 \rightarrow Fe3{+}aq{+}OH^{-}{+}OH\bullet}$$

Therefore, maintaining normal iron homeostasis is indispensable for cell survival

Extracellular iron could bind to transferrin in the form of trivalent iron ions, which are then recognised and bound by transferrin receptors located on the cell membrane. These complexes develop endosomes, which in turn are transported to the lysosome. In the acidic environment within the lysosome, trivalent iron ions dissociate from transferrin and subsequently get reduced to divalent iron ions by the six transmembrane epithelial antigen of prostate family member 3 (STEAP3[6]).

Through the divalent metal transporter protein 1 (DMT1[7]), divalent iron ions travel across the lysosomal membrane into the cytoplasm, where these highly chemically reactive and redox-active iron ions form a ***labile iron pool (LIP).*** *Extracellular divalent iron could be directly entered into the cytoplasm through the transmembrane DMT1; furthermore, cytoplasmic haemoglobin could be degraded to release divalent iron as well, jointly participating in the composition of the LIP. Iron in LIP could be transported out of the cell via ferroportin, a membrane iron transport protein located on the cell membrane, or be stored by binding to ferritin, thereby maintaining the balance of cellular iron ions.*

Ferritin could in turn undergo NCOA4-mediated degradation, which increases the amount of unstable intracellular iron. When LIP levels exceed the iron homeostatic range, abundant ***unstable irons generate ROS via the Fenton reaction****. In the presence of endogenous ROS generated by other metabolic processes, these ROS can lead to lipid peroxidation of* ***phospholipids containing polyunsaturated fatty acids (PUFA[8])****, thereby initiating ferroptosis.*

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or

RH+OH•→R•+H2O

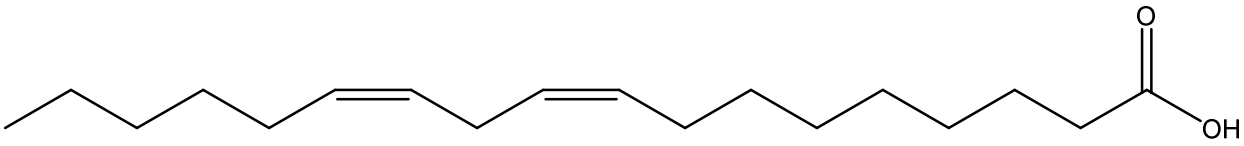
Also note from Yan et al:
Electrons may escape from oxidation-reduction reaction and be captured by O2 to form superoxide (O2•), peroxides (H2O2 and ROOH), and free radicals (HO• and RO•).69 The oxidation by Fe2+ and H2O2, which is called Fenton reaction, would provide hydroxyl radicals that subtract hydrogen (H) from lipid to form a lipid radical (L•) as the start of the non-enzymatic reaction of lipid peroxidation.70 Lipid radicals combine with O2 to form lipid peroxyl radical (LOO•), which then snatches hydrogen adjacent PUFA to form LOOH and a new lipid radical, and develops another oxidation reaction

[6] https://www.ncbi.nlm.nih.gov/gene/55240 **STEAP3** This gene encodes **a multipass membrane protein that functions as an iron transporter. The encoded protein can reduce both iron (Fe3+) and copper (Cu2+) cations**. This protein may mediate downstream responses to p53, including promoting apoptosis. Deficiency in this gene can cause anemia. Alternative splicing results in multiple transcript variants

[7] https://www.ncbi.nlm.nih.gov/gene/4891 **DMT1** (or SLC11A2) This gene encodes a member of the solute carrier family 11 protein family. The product of this **gene transports divalent metals and is involved in iron absorption**. Mutations in this gene are associated with hypochromic microcytic anemia with iron overload. A related solute carrier family 11 protein gene is located on chromosome 2. Multiple transcript variants encoding different isoforms have been found for this gene

[8] https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/polyunsaturated-fatty-acid
Polyunsaturated fatty acids UFAs) are a class of fatty acids that include n-6 and n-3 fatty acids. The n-3 fatty acid α-linolenic acid is a precursor for the long-chain products docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). PUFAs have been shown to decrease the risk of heart disease when consumed in lieu of SFAs in both epidemiological.

Note that PUFA, polyunsaturated fatty acids are double bonded fats with methyl groups as shown in the example below.

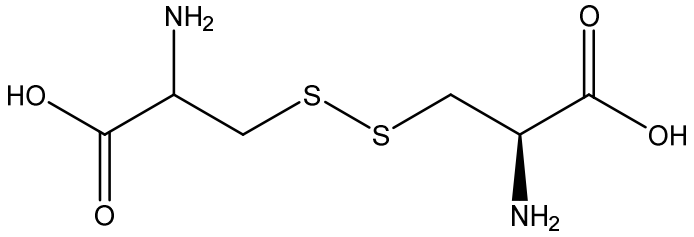


linoleic acid

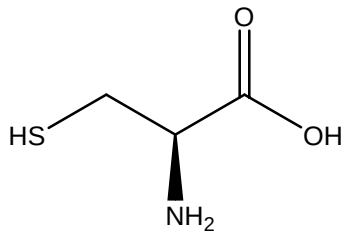
Lipid peroxidation is the core process of ferroptosis, in which PUFAs serve as the main substrate engaged in cellular lipid peroxidation. Acyl-CoA synthetase long-chain family member 4 (ACSL4) and Lysophosphatidylcholine Acyltransferase 3 (LPCAT3) catalyse free arachidonic acid (AA) and adrenic acid (AdA) to form AA/AdA-membrane phosphatidylethanolamine (PE), which subsequently undergoes lipid peroxidation via either enzymatically or non-enzymatically.

Enzymatic processes are primarily mediated by arachidonic acid lipoxygenases (ALOXs), while non-enzymatic processes are dominated by ROS. Together, these reactions enable the accumulation of LPO and the damage to the plasma membrane, ultimately causing ferroptosis. Ordinarily, several mechanisms allow cells to prevent peroxidation reactions from occurring. System Xc- and glutathione peroxidase 4 (GPX4) are two major components of the antioxidant process, and their inactivation is directly involved in the initiation of ferroptosis. System Xc- Transporter proteins could carry glutamate out of the cell and transport cystine into the cell simultaneously.

We show the cystine and cysteine structures below. They are key elements in this process.



cystine



cysteine

Cystine could be reduced to cysteine, which then participates in the biosynthesis of reduced glutathione (GSH).

GPX4 can utilise GSH to reduce PUFAs-OOH to PUFAs-OH, ***thereby preventing the accumulation of LPO and the inhibition of ferroptosis.***

Therefore, the repression of system Xc- could reduce GSH production by decreasing cystine input, thus inhibiting the function of GPX4 and resulting in the accumulation of LPO.

Several other factors, such as the apoptosis inducing factor alpha-related 2 (AIFM2)-Coenzyme Q10 (CoQ 10) pathway, the GTP cyclohydrolase-1 (GCH1)-tetrahydrobiopterin (BH4) pathway, the dihydroorotate dehydrogenase (DHODH)- CoQH2 pathway and some tumour suppressors are also involved in the regulation of ferroptosis.

Another description of the process is from Chen (2023) as below:

Iron overload is necessary for the occurrence of ferroptosis.

Iron is an essential trace element for the human body and participates in a variety of life processes, such as nucleic acid metabolism, cell cycle and enzyme synthesis, etc. Dietary sources of iron mainly include heme iron and non-heme iron. Non-heme iron can be derived from iron ions or ferritin, etc. ***Iron ions are usually obtained from food in ferrous (Fe2+) or ferric (Fe3+) form. Fe3+ is reduced to Fe2+ by different ferrireductases in the intestine such as duodenal cytochrome B (DCYTB), and then Fe2+ enters enterocytes via divalent metal transporter 1 (DMT1) on the apical membrane****.*

Ferritin iron and heme iron *can enter enterocytes via* ***ferritin receptor and CD91****, respectively. In enterocytes, Fe2+ not involved in biological processes can be bound to ferritin for storage. As required, Fe2+ can leave enterocytes via ferroportin 1 (FPN1) and be oxidized to Fe3+ by ferroxidase hephaestin (HEPH) for transport.*

Transferrin (TF) produced from the liver is responsible for transporting Fe3+, and one TF can bind two Fe3+.14 Fe3+-TF transports to target cells and binds to its receptor transferrin receptor (TFR1), then subsequently transports Fe3+ to endosomes.

In the acidic environment of endosomes, Fe3+ is reduced to Fe2+ by metalloreductases such as six transmembrane epithelial antigen of the prostate 3 (STEAP3) and subsequently transported into the cytoplasm via DMT1.

<u>Intracellular Fe2+ needs to be strictly regulated.</u>

Intracellular Fe2+ deficiency restricts various biological processes.

Intracellular ***Fe2+ excess leads to the Fenton reaction-excess Fe2+ reacts with hydrogen peroxide (H2O2). Fenton reaction generates ROS, and iron overload and ROS accumulation can lead to ferroptosis.***

Excess Fe2+ also promotes lipid peroxidation by participating in the catalytic subunit of lipoxygenase (LOX).

Excess intracellular Fe2+ can be oxidized to Fe3+ by ferritin and stored as ferritin heavy chain 1 (FTH1) or ferritin light chain (FTL).

This iron-binding ferritin can be degraded by nuclear receptor coactivator 4 (NCOA4) and release free iron.

Cells can also expel excess Fe2+ via FPN1. FPN1 and its regulator hepcidin are essential for iron regulation, and FPN1 is currently the only iron export pathway. Abnormal expression or function of these proteins can lead to increased intracellular labile iron.

Iron-dependent lipid peroxidation is another important process of ferroptosis.

Phospholipid (PL) is one of the main components of cell membranes.

PLs can bind different fatty acyl chains to their sn1 and sn2 sites to increase their diversity.

Polyunsaturated fatty acyl (PUFA) can combine with the sn2 site of PL to form PUFA-PLs after being catalyzed by acyl-coenzyme A synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3). ACSL4 or LPCAT3 knockout results in a marked reduction in PUFA-PLs production*.*

PUFAPLs can increase the fluidity of cell membranes and maintain the normal physiological function of cells.

However, PUFA-PLs are also important substrates of lipid peroxidation.

PUFAs contain bis-allylic, which can be easily stripped a hydrogen atom by strong oxidants and form a ***phospholipid radical (PL•),*** *and subsequently bind to an oxygen molecule to form a phospholipid peroxyl radical (PLOO).*

Importantly, PLOO• can rob a hydrogen atom from the bis-allylic of another PUFA-PL, leading to the formation of PUFA-PL-OOH and the formation of another PL

The continuous production and accumulation of PUFA-PL-OOH driven by the labile iron pool and ROS will disrupt the integrity of the cell membrane and lead to ferroptosis.

LOXs can also drive PUFA-PLs peroxidation and promote ferroptosis.

3.2 ERASTIN

Erastin is a mitigating factor in ferroptosis. From Sato et al:

System xc− was recently described as the most upstream node in a novel form of regulated necrotic cell death, called ferroptosis.

In this context, the small molecule erastin was reported to target and inhibit system xc−, leading to cysteine starvation, glutathione depletion and consequently ferroptotic cell death.

Although the inhibitory effect of erastin towards system xc− is well-documented, nothing is known about its mechanism of action.

Therefore, we sought to interrogate in more detail the underlying mechanism of erastin's pro-ferroptotic effects. When comparing with some well-known inhibitors of system xc−, erastin was the most efficient inhibitor acting at low micromolar concentrations. Notably, only a very short exposure of cells with low erastin concentrations was sufficient to cause a strong and persistent inhibition of system xc−, causing glutathione depletion. These inhibitory effects towards system xc− did not involve cysteine modifications of the transporter.

More importantly, short exposure of tumor cells with erastin strongly potentiated the cytotoxic effects of cisplatin to efficiently eradicate tumor cells*.*

Hence, our data suggests that only a very short pre-treatment of erastin suffices to synergize with cisplatin to efficiently induce cancer cell death, findings that might guide us in the design of novel cancer treatment paradigms. …

System xc− is one among many ***amino acid transporters*** *expressed in the plasma membrane of mammalian cells.*

This transporter is composed of ***xCT (SLC7A11),*** *which is the substrate-specific subunit, and 4F2 heavy chain (****SLC3A2****).*

xCT was shown to be responsible for the specific function of system xc−, whereas 4F2 heavy chain, which had been known as one of surface antigens (CD98), is the common subunit of some other amino acid transporters.

System xc− exchanges intracellular glutamate with extracellular cystine at a 1:1 molar ratio. Recently, we have demonstrated that cystathionine is also a physiological substrate, which can be exchanged with glutamate, and that system xc− plays an essential role for maintaining cystathionine in immune tissues like thymus and spleen.

Cystine taken up via system xc− is rapidly reduced to cysteine, which is used for synthesis of protein and glutathione (GSH), the major endogenous antioxidant in mammalian cells.

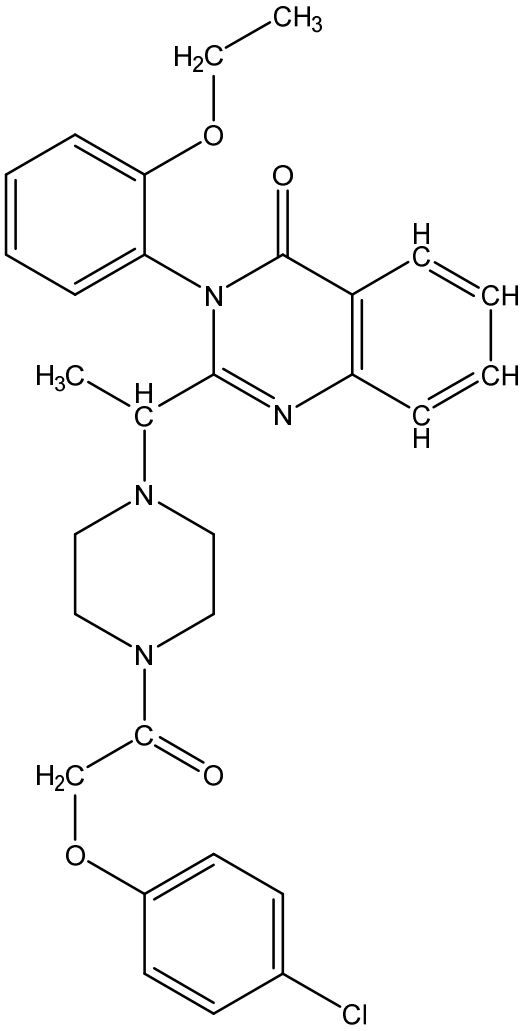
Some part of cysteine is released via neutral amino acid transporters, thus contributing to maintain extracellular redox balance, and a cystine/cysteine redox cycle which can act independently of cellular GSH. Inhibition of system xc− causes a rapid drop of intracellular glutathione level and cell death in most of cultured cells

From MCE[9]:

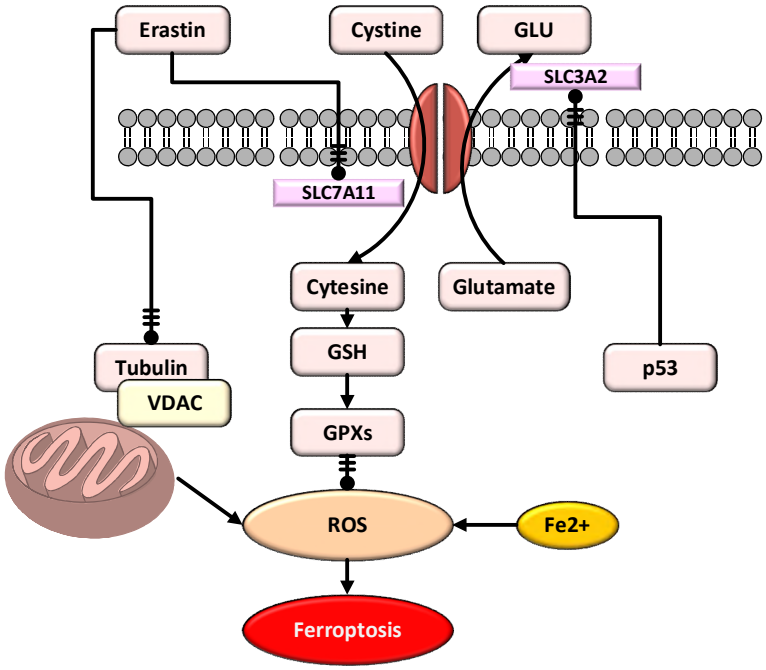
Erastin is a ferroptosis inducer. Erastin exhibits the mechanism of ferroptosis induction related to ROS and iron-dependent signaling. Erastin inhibits voltage-dependent anion channels (VDAC2/VDAC3) and ***accelerates oxidation, leading to the accumulation of endogenous reactive oxygen species.***

Erastin also disrupts ***mitochondrial permeability transition pore*** *(mPTP) with anti-tumor activity. Furthermore, Erastin can block the uptake of cystine mediated by SLC7A11 and also spares UMRC6-EV and -C91A cells from disulfidptosis under glucose starvation.*

9 https://www.medchemexpress.com/Erastin.html?srsltid=AfmBOor89WHd9uMCc2Z8pPzklEwzHbIk3jOgPuuGvesPqcFNcAgP9gYM



Simply stated, the Figure below shows that Erastin performs two functions. First it blocks SLC7A11 thus blocking the channel, and second it blocks tubulin thus in activating the channel promoting ROS. This may be an effective means to blocking ferroptosis.



3.3 XC- TRANSPORTER

One of the key elements in effecting ferroptosis is the activation of a specific cell transporter membrane. This is called the XC- system. As Zhao et al note:

System XC− is a reverse transporter located in the plasma membrane.

It is a heterodimer composed of a light chain subunit, xCT, encoded by SLC7A11[10], and functioning as the substrate-specific subunit, and a heavy chain subunit 4F2[11], encoded by SLC3A2[12], which is common to other amino acid transporters.

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[10] https://www.ncbi.nlm.nih.gov/gene/23657 SLC7A11 This gene encodes a member of a heteromeric, sodium-independent, anionic amino acid transport system that is highly specific for **cysteine and glutamate**. In this system, designated Xc(-), the anionic form of cysteine is transported in exchange for glutamate. This protein has been identified as the predominant mediator of Kaposi sarcoma-associated herpesvirus fusion and entry permissiveness into cells. Also, increased expression of this gene in primary gliomas (compared to normal brain tissue) was associated with increased glutamate secretion via the XCT channels, resulting in neuronal cell death.

[11] https://www.ncbi.nlm.nih.gov/gene/6520 4F2 This gene is a member of the solute carrier family and encodes a cell surface, transmembrane protein. The protein exists as the **heavy chain of a heterodimer, covalently bound through di-sulfide bonds to one of several possible light chains**. The encoded transporter plays a role in regulation of intracellular calcium levels and transports L-type amino acids. Alternatively spliced transcript variants, encoding different isoforms, have been characterized.

[12] https://www.ncbi.nlm.nih.gov/gene/6520 SLC3A2 This gene is a member of the solute carrier family and encodes a cell surface, transmembrane protein. **The protein exists as the heavy chain of a heterodimer, covalently bound through di-sulfide bonds to one of several possible light chains.** The encoded transporter plays a role in regulation of intracellular calcium levels and transports L-type amino acids. Alternatively spliced transcript variants, encoding different isoforms, have been characterized.

System XC− transfers glutamate out of cells and cystine into cells at a ratio of 1:1.

Upon transfer into the cell, cystine is rapidly reduced to cysteine, which is then used in the synthesis of glutathione (GSH), a tripeptide composed of cysteine, glutamate, and glycine.

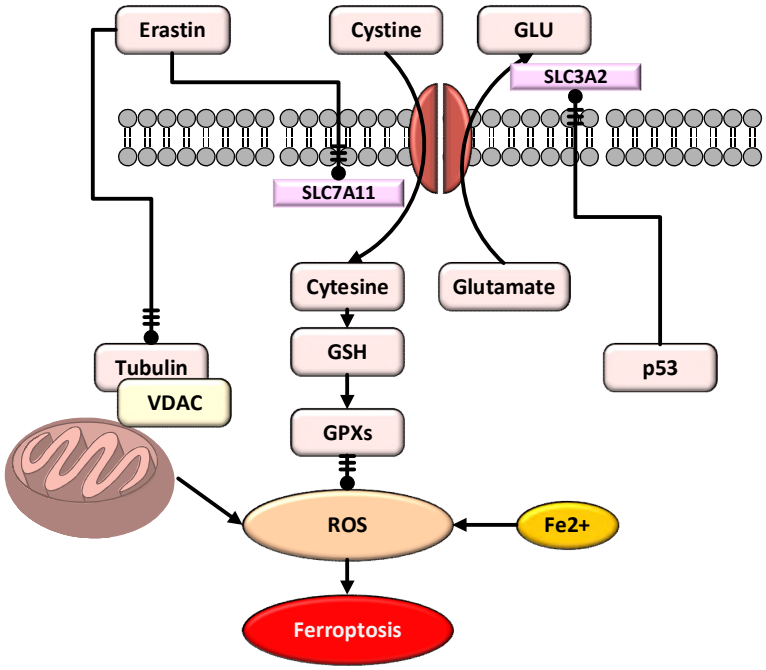
The sulfhydryl structure contained in GSH can be oxidized and dehydrogenated, making GSH an important antioxidant and free radical scavenger in the body.

GPX is a peroxide-degrading enzyme, and GSH is an essential cofactor in its activation.

GPX plays a significant role in maintaining redox homeostasis and protecting cells from lipid oxidative stress leading to death. A variety of ferroptosis inducers can inhibit cystine absorption by inhibiting system XC−, resulting in reduced GPX activity.

The consequence of this is a reduction in the cell's antioxidant capacity and hence increased L-ROS, ultimately leading to ferroptosis.

Therefore, inhibition of the cystineglutamate transporter system XC− is an important pathway to induce ferroptosis.



3.4 COMPARISONS

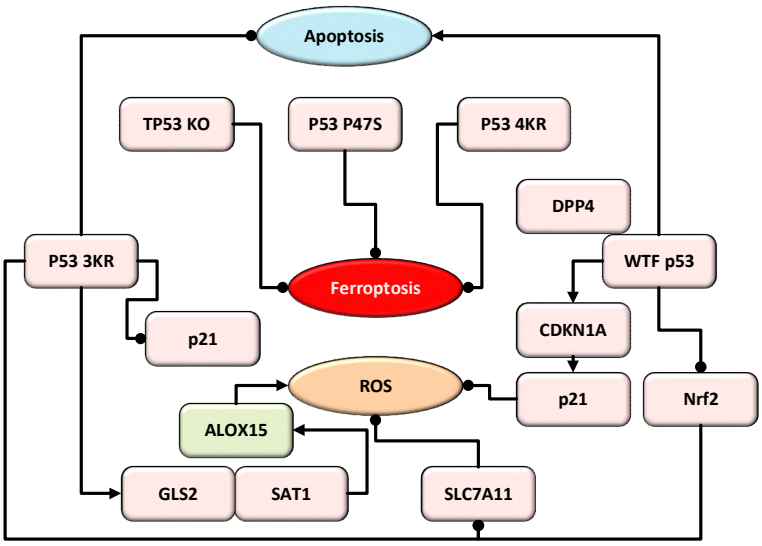
We now present a summary of the comparisons between the primary types of cell death. This is from Mu et al:

Cell death	Ferroptosis	Apoptosis	Autophagy	Necroptosis	Pyroptosis
Biochemical features	Inhibition of xCT and reduced GSH, inhibition of GPX4. Iron accumulation and lipid peroxidation	Activation of caspases oligonucleosomal DNA fragmentation	Increased lysosomal activity	Drop in ATP levels; activation of RIP1, RIP3, and MLKL	Dependent on caspase-1 and proinflammatory cytokine releases
Morphological features	Small mitochondria with condensed mitochondrial membrane densities, reduction or vanishing of mitochondria crista, as well as outer mitochondrial membrane rupture	Plasma membrane blebbing; cellular and nuclear volume reduction; nuclear fragmentation	Formation of double-membraned autolysosomes	Plasma membrane rupture; organelle swelling; moderate chromatin condensation	Karyopyknosis, cell edema and membrane rupture
Key genes	GPX4, Nrf2, LSH, TFR1, xCT	Caspase, P53, Fas, Bcl-2, Bax	ATG5, ATG7, DRAM3, TFEB	LEF1, RIP1, RIP3	Caspase-1, IL-1 (3, IL-18
Regulatory pathways	xCT and Gpx4, MVA, HSF1-HSPB1, p62-Keap1-Nrf2 pathway, LSH signal pathway	Death receptor, Mitochondrial, Endoplasmic reticulum pathway Csapase, P53, Bcl-2 mediated signaling pathway	PI3K-AKT-mTOR,MAPK-ERK1/2-mTOR signal pathway	TNFa,TNFR1,TLR3, TRAIL, FasL, ROS, PKC-MAPK-AP-1-mediated signaling pathway	Caspase-1, NLRP3-mediated signaling pathway.
Released DAMP	HMGB1	Ecto-CRT, HMGB1, and ATP	HMGB-1	DNA and IL-6	HMGB1, ATP, IL-1p, and IL-18

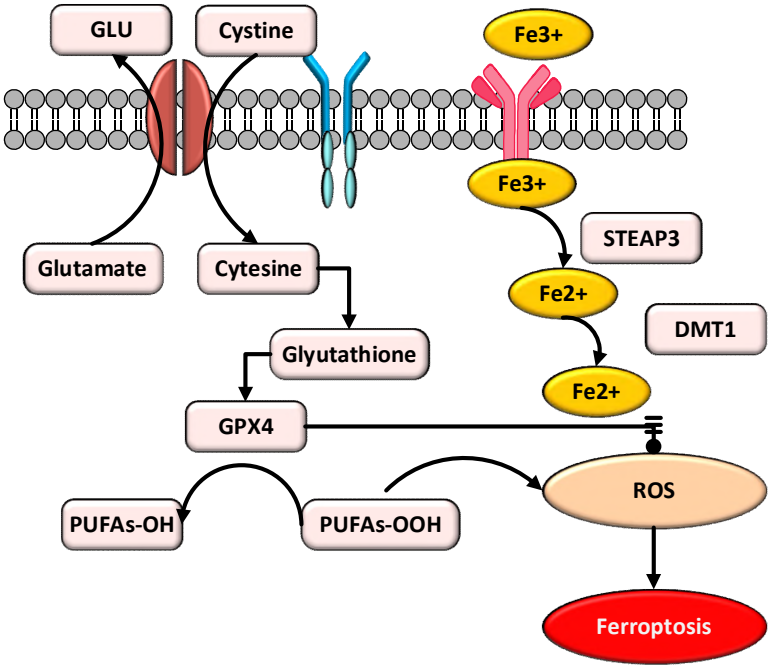
Cell death	Ferroptosis	Apoptosis	Autophagy	Necroptosis	Pyroptosis
Immune features	Pro-inflammatory	Mostly antiinflammatory	Mostly antiinflammatory	Mostly pro-inflammatory	*pro-inflammatory*
Inducers	Erastin,DPI2, BSO, SAS, lanperisone, SRS, RSL3, DPI7, DP110, FIN56, sorafenib, artemisinin	FASL, DCC, UNC5B	Rapamycin, lithium, sodium, valproate, carbamazepine, C2-ceramide, rapamycin	TNFa, zVAD-fmk, PAMPS	ZnO—NPs, Ivermectin
Inhibitors	Desferoxamine, vitamin E, U0126, ferrostatin-1, SRS, CA-1, cycloheximide, aminooxyacetic acid Liproxstatin-1 HCl	XIAP, C-IAP1, C-IAP2, ILP-2, ML-IAP/livin, NAIP, Z-VADFMK	3-ME, LY294002, wortmannin, PIK-III, compound 31, SAR 405, Vps34-In1,MRT68921, Spautin-1, Bafilomycin A1,	Nec-1, NSA, Kongensin- A	Necrosulfonami-de

3.5 PATHWAY SUMMARIES

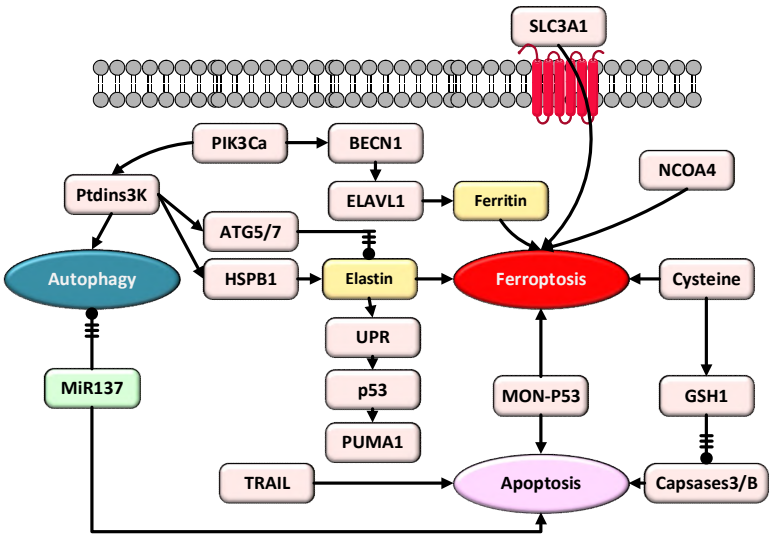
We now present a set of summaries of the various pathways. The first below shows the relationship between apoptosis and ferroptosis. There are some interacting paths with p53 being one intermediary. However, ferroptosis is fundamentally different in how it kills the cells and how it can be mitigated.



A second example is shown below. We have considered one like this previously but this is a simplified version showing the iron input and the GPX4 control function. We have shown a more complete version earlier.



The diagram below demonstrates the relationship to autophagy and apoptosis. This diagram tends to demonstrate the related pathways. Again the level of detail is lacking in all three paths the results are for summary only.



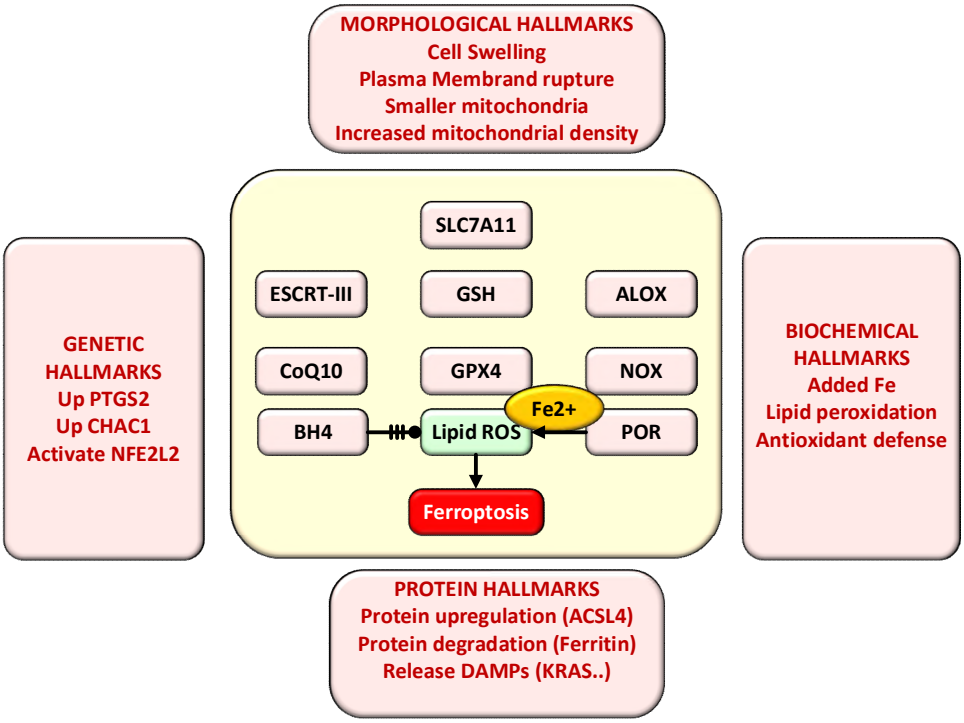
Now regarding the specifics, Chen et al (2024) have noted:

When compared to other types of programmed cell death, including necrotic apoptosis, autophagy, and apoptosis, ferroptosis cells exhibit several unique morphological and biochemical features. These characteristics include changes in the cellular ultrastructure, such as an unbroken nucleus, an increase in mitochondrial density, a decrease in mitochondrial volume and cristae, and an interruption of the outer mitochondrial membrane.

Lipid peroxidation, elevated ferric ions, the buildup of intracellular reactive oxygen species (ROS), reduction of GSH, decreased GPX4 activity/levels, and decreased SLC7A11 production are biochemical indicators of this process. Iron response element-binding protein 2 (IREB2), citrate synthase (CS), ATP synthase F0 complex subunit C3 (ATP5G3), acyl-CoA synthetase family member 2 (ACSF2), tetratricopeptide repeat domain 35 (TTC35), and ribosomal protein L8 (RPL8) are the six genes that, at the molecular level, are upregulated during ferroptosis.

4 CANCER MITIGATION

There have been several studies regarding the use of ferroptosis in the treatment of various cancers. Our initial focus is on prostate cancer and then we briefly present other cancers as well. The graphic below demonstrates some of the key metrics of ferroptosis. Some of these become targetable by therapeutics. Organ specific applications of ferroptosis has just begun to be examined. We now follow with a few examples.



4.1 PCA

There has been recent work regarding ferroptosis and prostate cancer, PCa[13]. As Chen et al (2024) have noted:

The PI3K/AKT pathway is engaged in 70% of advanced PCa, and PTEN loss happens often with human PCa development.

The androgen receptor (AR) transcription factor activity is regulated by PTEN loss, which in turn inhibits androgenresponsive gene expressions.

By reducing the expression of the androgen-responsive gene Fkbp5 and blocking PHLPP-mediated AKT inhibition, conditional AR deletion in the epithelium increases the proliferation of PTEN null cancer cells. ***Possible significant implications for PCa pathogenesis and therapy***

[13] https://www.researchgate.net/publication/264960277_Prostate_Cancer_A_Systems_Approach

were highlighted … found PI3K and AR pathway crosstalk as a mechanism of CRPC formation.

As a downstream player of the PI3K/AKT signaling cascade, mTOR regulates biological processes in tumors.

High levels of cholesteryl ester have been associated with an increased risk of PCa in multiple investigations.

… stimulation of the PI3K/AKT/mTOR pathway can generate the buildup of cholesteryl ester, ***which supports PCa progression*** *… PCa cells could activate downstream sterol regulatory element-binding protein 1 (SREBP1) and its transcriptional target stearoyl-CoA desaturase-1 (SCD1) through the PI3K/ AKT/mTORC1 pathway to mediate monounsaturated fatty acids production. What's more, mTORC1 can mediate nuclear factor erythroid 2-related factor 2 (NRF2) signaling, acting as an important regulator of cellular redox homeostasis.*

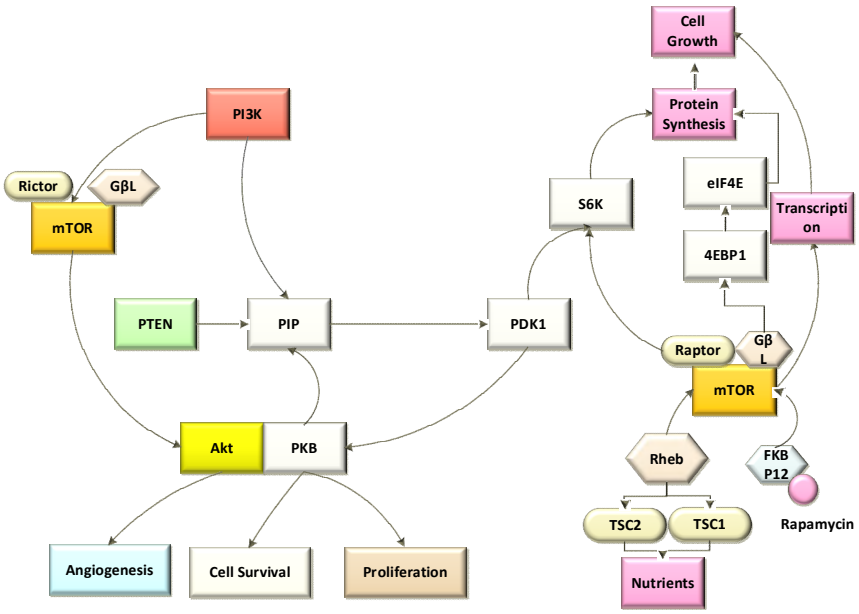
… prevents lipids from being peroxidized, thus preventing ferroptosis in cancer cells. To sum up, mTORC1 is an antioxidant and a negative regulator of ferroptosis; it also inhibits ferroptosis in PCa cells by interfering with lipid metabolism. In a nutshell, increased cell proliferation and castration resistance are all outcomes of PTEN loss or activation of the PI3K/AKT/mTOR pathway.

Mou et al have noted that a molecule of erastin can target ferroptosis:

Erastin induces ferroptosis in Ras-carrying human prostate adenocarcinoma cells. The phosphorylation of HSF1-dependent HSPB1 contributes to the ferroptosis resistance to erastin through inhibiting lipid ROS accumulation and iron uptake [70]. HSPB1 inhibition specifically increased erastin-induced ferroptosis by facilitating iron accumulation from the upregulation of TFR1 and slight reduction of FTH1 expression.

Chen et al present several targets. The following are putative negative regulators.

1. ***Activation of PI3K/AKT/mTOR*** *leads to the development of CRPC by inhibiting ferroptosis. The PI3K/AKT pathway is engaged in 70% of advanced PCa, and PTEN loss happens often with human PCa development. The androgen receptor (AR) transcription factor activity is regulated by PTEN loss, which in turn inhibits androgenresponsive gene expressions. By reducing the expression of the androgen-responsive gene Fkbp5 and blocking PHLPP-mediated AKT inhibition, conditional AR deletion in the epithelium increases the proliferation of PTEN null cancer cells. Possible significant implications for PCa pathogenesis and therapy were highlighted by Mulholland et al., who found PI3K and AR pathway crosstalk as a mechanism of CRPC formation*



2. ***Overexpression of DECR1[14]*** *inhibits CRPC cells from ferroptosis. The rate-limiting enzyme in PUFA oxidation, 2,4-Dienoyl-CoA reductase 1 (DECR1), is shown to be elevated in prostate tumor samples and castration-resistant mice models [22]. Decreased DECR1 expression significantly inhibits PCa cell growth through PUFA buildup, heightened lipid peroxidation vulnerability, and ferroptosis induction, according to recent studies. It is intriguing that temporarily repressing the DECR1 gene improved the inhibitory impact of enzalutamide on PCa cells. On the flip side, temporary overexpression of the DECR1 gene boosted cancer cell proliferation. Additionally, CRPC cells become more susceptible to ferroptosis when DECR1 is downregulated which raises GPX4 expression. In addition, it is highly important for prognosis prediction that increased DECR1 expression is associated with shorter disease-free survival in PCa patients.*

3. ***PANX2[15] promotes PCa progression*** *via suppressing ferroptosis. Pannexin 2 (PANX2) is a protein that forms a cell-specific channel and is mostly found in postnatal neural precursor cells and mature neurons. A study discovered that PANX2 expression levels were notably increased in PCa cells and showed a favorable correlation with the Gleason score in PCa. In this study, disrupting PANX2 expression notably raised Fe2+ levels in cancer cells. PCa cells can upregulate iron metabolism via PANX2, enabling them to evade ferroptosis, promoting the progression of PCa. On the flip side, PCa cells are unable to proliferate, migrate, or invade*

[14] https://www.ncbi.nlm.nih.gov/gene/1666 **DECR1** Enables 2,4-dienoyl-CoA reductase (NADPH) activity; NADPH binding activity; and identical protein binding activity. Involved in fatty acid beta-oxidation. Located in cytosol; mitochondrion; and nucleoplasm. Part of catalytic complex

[15] https://www.ncbi.nlm.nih.gov/gene/56666 **PANX2** The protein encoded by this gene belongs to the innexin family. Innexin family members are the structural components of gap junctions. This protein and pannexin 1 are abundantly expressed in central nervous system (CNS) and are coexpressed in various neuronal populations. Studies in Xenopus oocytes suggest that this protein alone and in combination with pannexin 1 may form cell type-specific gap junctions with distinct properties. Multiple transcript variants encoding different isoforms have been found for this gene.

when PANX2 is silenced. The study found that PANX2 may promote the growth and progression of PCa cells by controlling NRF2, a significant controller of intracellular oxidative balance and effectively prevents ferroptosis by collaborating with the antioxidant transcription factor STAT3 to enhance SLC7A11 expression. PANX2 functions as a suppressor of ferroptosis by specifically affecting iron metabolism and promoting the progression of PCa through the up-regulation of SLC7A11 levels.

4. ***Up-regulation of SLC7A11*** *may lead to docetaxel-resisitance. The inhibition of ferroptosis and the acceleration of PCa progression may result from up-regulating the levels of SLC7A11. Zhang et al. found that long non-coding RNA OIP5-AS1 expression was greatly elevated in PC3 and DU145 cells upon chronic Cd exposure, which targets and enhances the miR-128-3p/SLC7A11 signaling, and finally promotes cell growth and suppresses ferroptosis. As a result, PCa cells migrate and form colonies. In addition, Jiang X and colleagues observed that lncRNA-PCAT1 competes for miR-25-3p, which increases the stability of c-Myc through physical interactions. This transcriptionally activates SLC7A11 expression, leading to an increase in docetaxel resistance, which is recommended to be used for CRPC. Furthermore, lncRNA-PCAT1 knockdown promotes ferroptosis, effectively impairing docetaxel resistance.*

5. ***HSPB1[16] may inhibit erastin-induced ferroptosis*** *of PCa. As a member of heat shock protein family B, HSPB1 can degrade unfolded or misfolded proteins, which it uses to protect cells from death. The upregulation of HSPB1, which has been linked to extremely aggressive cancer and bad clinical results, suggests that it is an important regulator of PCa cell activity. …. erastin-induced ferroptosis was greatly amplified in PCa cell lines when HSPB1 was knocked down, but inhibited when HSPB1 was overexpressed. Phosphorylation of HSPB1 by protein kinase C could prevent cancer cell ferroptosis by blocking cytoskeletal-mediated iron absorption and lipid ROS generation. To sum up, HSPB1 suppresses erastin-induced PCa ferroptosis and acts as a negative regulator of ferroptosis*

4.2 LIVER

From Mou et al:

Ferroptosis was one of the underlying mechanisms in sorafenib treating HCC.

HCC cells with the retinoblastoma (RB) protein deficiency had 2–3 times higher death rate more than that of cells with a normal level of RB protein.

This susceptibility of HCC with deactivated RB protein to ferroptosis was due to the augment of oxidative stress response in cells from increased reactive oxygen concentration in mitochondria.

[16] https://www.ncbi.nlm.nih.gov/gene/3315 HSPB1 This gene encodes a member of the small heat shock protein (HSP20) family of proteins. In response to environmental stress, the encoded protein translocates from the cytoplasm to the nucleus and functions as a molecular chaperone that promotes the correct folding of other proteins. This protein plays an important role in the differentiation of a wide variety of cell types. Expression of this gene is correlated with poor clinical outcome in multiple human cancers, and the encoded protein may promote cancer cell proliferation and metastasis, while protecting cancer cells from apoptosis. Mutations in this gene have been identified in human patients with Charcot-Marie-Tooth disease and distal hereditary motor neuropathy

Metallothionein-1g (MT-1G) is a novel negative regulator of ferroptosis in HCC. MT-1G knockdown contributed to sorafenib-induced ferroptosis by increasing lipid peroxidation and GSH depletion. CDGSH iron sulfur domain 1 (CISD1) and ACSL4 inhibition promote erastin-induced ferroptosis in HCC. Low-density lipoprotein (LDL)–docosahexaenoic acid (DHA) nanoparticles cause cell death in HCC cells through the ferroptosis pathway. The p62-Keap1-Nrf2 pathway plays a vital role in saving HCC cells from ferroptosis, and Ras/Raf/MEK pathway is reported to be a critically important target for ferroptosis in treating HCC

4.3 THYROID

Yin et al have recently noted the following regarding the application to thyroid cancer[17]:

Thyroid cancer (TC) is a prevalent endocrine malignancy, with a significant increase in incidence worldwide.

Ferroptosis is a novel form of programmed cell death, primarily caused by iron overload and reactive oxygen species (ROS)-dependent accumulation of lipid peroxides.

The main manifestations of cellular ferroptosis are rupture of the outer membrane, crumpling of the mitochondria and shrinkage or disappearance of the mitochondrial cristae, thus leading to cell death. Ferroptosis is an important phenomenon in tumour progression, with crosstalk with tumour-associated signalling pathways profoundly affecting tumour progression, immune effects and treatment outcomes.

The functions and mechanisms of ferroptosis in TC have also attracted increasing attention, mainly in terms of influencing tumour proliferation, invasion, migration, immune response, therapeutic susceptibility and genetic susceptibility. However, at present, the tumour biology of the morphological, biological and mechanism pathways of ferroptosis is much less deep in TC than in other malignancies. Hence, in this review, we highlighted the emerging role of ferroptosis in TC progression, including the novel mechanisms and potential opportunities for diagnosis and treatment, as well as discussed the limitations and prospects. Ferroptosis-based diagnostic and therapeutic strategies can potentially provide complementary management of TCs …

The mechanisms of TC progression, metastasis and recurrence remain a point of interest in the current field of tumour therapy. PCD-related death modalities, especially ferroptosis, are closely connected with multiple biological behaviours of TC. Here, we focus on elucidating the functional, mechanistic, diagnostic and therapeutic aspects of ferroptosis in TC. Nevertheless, there are still many urgent issues that need to be tackled in this area. Mechanistically, ferroptosis is currently far less explored in TC than in other malignant tumours.

Many cancer-related genes and signalling pathways can regulate ferroptosis, and some signalling molecules generated by ferroptosis can also be the source of activation of other pathways, which in turn constitutes a complex positive or negative feedback network. NcRNAs

[17] https://www.researchgate.net/publication/331935614_Thyroid_Cancer_Seek_and_Ye_Shall_Find

are also gradually recognised as key mediators in the regulation of ferroptosis. NcRNAs are still mainly regulated in TC through the ceRNA mechanism to regulate gene expression and reshape tumour behaviour.

However, the known quantity of miRNAs, lncRNAs and circRNAs in TC is still relatively small, and all of them also basically remain in sample validation and cell and animal exploration. In addition, multiple forms of PCD exist in TC, including apoptosis, pyroptosis, necroptosis and autophagy. There exists a close interplay mode between these modes of death, especially autophagy and ferroptosis, which can profoundly and complexly affect the course of TC. Compared to other forms of PCD, the study of ferroptosis in TC is still in its early stages. How to decipher this complex interaction needs to be studied in depth. Ferroptosis significantly interacts with the immune system, influencing the progression and treatment of various cancer types, including TC.

The release of damage-associated molecular patterns (DAMPs) during ferroptosis can stimulate immune to response, thus attracting immune cells to the tumour microenvironment.

This is an interesting observations. DAMPs are good targets and are often produced when cells are destructed. The problem of course is destroying the right cells.

For instance, ferroptotic cells can release high-mobility group box 1 (HMGB1), which activates DCs and promotes the presentation of tumour antigens to T cells, thereby enhancing anti-tumour immunity.

Moreover, the ferroptosis-induced release of lipid peroxidation products can modulate the activity of macrophages and NK cells, further influencing the immune landscape of TC.

Macrophages can shift into different phenotypes based on the stimulated signals from the tumour microenvironment, and ferroptosis can promote the polarization of macrophages towards an M1 phenotype, associated with pro-inflammatory and anti-tumour activities.

NK cells, on the other hand, can recognise and kill ferroptotic cells, adding another layer of immune surveillance. The interplay between ferroptosis and the immune system also has implications for immunotherapy. Immune checkpoint inhibitors, such as anti-PD-1 and anti-CTLA-4 antibodies, can be combined with ferroptosis inducers to enhance therapeutic efficacy.

By promoting ferroptosis in tumour cells, these combination therapies can increase the immunogenicity of the tumour, making it more susceptible to immune-mediated destruction.

Despite its potential, the association between ferroptosis and immune cells like CD8+ T cells, macrophages, NK cells and DC cells in TC has not received enough attention. Improving the specificity of ferroptosis inducers and strictly controlling their dosage is essential to enhance the immunocidal effect and reduce adverse effects on normal tissues.

Understanding these interactions can provide new insights into developing combination therapies that harness both ferroptosis and the immune response, potentially improving treatment outcomes for TC patients.

5 THERAPEUTICS

The approach to therapeutics using ferroptosis is the understand the pathways and then act to activate them in cancer cells. As Jiang et al noted:

The research field of ferroptosis has been enjoying exponential growth over the past few years, since the term was coined in 2012. This unique modality of cell death, driven by iron-dependent phospholipid peroxidation, is regulated by multiple cellular metabolic events, including redox homeostasis, iron handling, mitochondrial activity, and metabolism of amino acids, lipids and sugars, in addition to numerous signaling pathways relevant to disease. Intriguingly, therapy resistant cancer cells, particularly those of the mesenchymal state and prone to metastasis, are exquisitely vulnerable to ferroptosis.

Further, numerous organ injuries and degenerative pathologies are driven by ferroptosis. As such, pharmacological modulation of ferroptosis, via both its induction and inhibition, holds great potential for the treatment of drug-resistant cancers, ischemic organ injuries, and other degenerative diseases linked to overwhelming lipid peroxidation.

In this Review, we seek to provide an extensive and critical analysis of the current understanding of the molecular mechanisms and regulatory networks of ferroptosis, the potential physiological functions of ferroptosis in tumor suppression and immune surveillance, and its pathological roles and potential for therapeutics. Importantly, as in all rapidly evolving new research areas, issues and confusions exist due to misconceptions and inappropriate use of experimental tools – this Review also tries to address these issues and to provide practical guidelines.

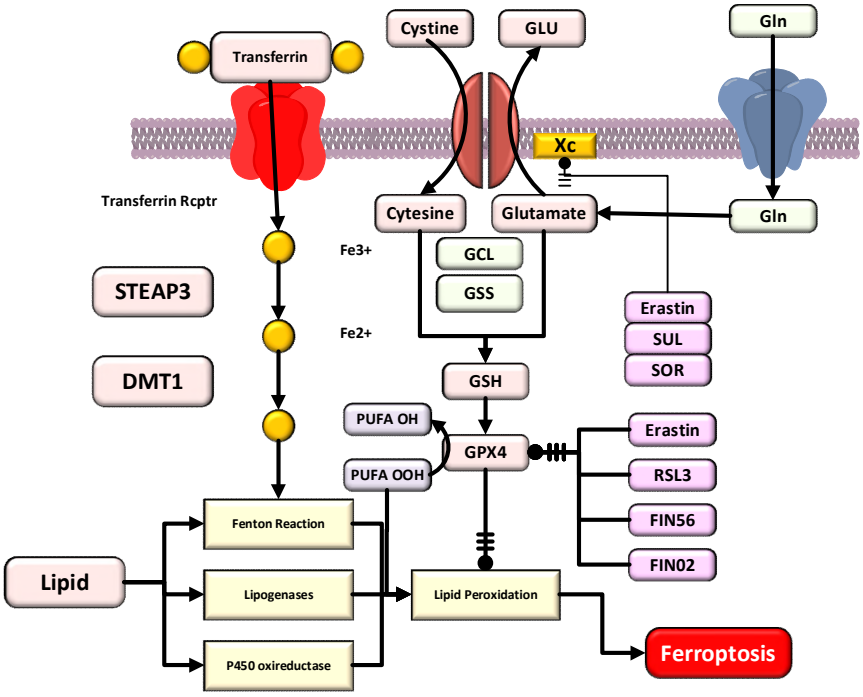
Finally, we discuss important concepts and pressing questions that should be a focus of future ferroptosis research …

Recent years have witnessed rapid progress in the mechanistic understanding of ferroptosis. Through the initial discovery of the role of the cystine-import-GSH-GPX4 machinery in suppressing ferroptosis, the role of phospholipid hydroperoxides (PLOOHs) as the executioners of ferroptosis is now established. More recently, GPX4-independent ferroptosis surveillance pathways have been identified. Furthermore, the mechanisms of PLOOH synthesis, particularly the synthesis and activation of polyunsaturated fatty acids (PUFAs), the precursor of PLOOHs, have been extensively investigated in the context of ferroptosis.

Importantly, all these studies converge on cellular metabolism and have revealed an intimate relationship between ferroptosis and metabolic pathways

5.1 STRATEGY

The intent of a therapeutic is to induce ferroptosis. The flow below demonstrates multiple paths of approach.



The above demonstrates the initiation of ferroptosis as well as control points.

1. As for initiation it starts with lipids and injected iron that through the Fenton reaction produces lipid peroxidases.

2. However to allow ferroptosis to continue GPX4 must be blocked.

3. The lipid peroxidases can be mitigated by GPX4 if it is operational and thus ferroptosis would be blocked. That can be done with erastin and other blockers.

4. Moreover, GSH can be blocked by blocking the Xc channel by the noted channel blockers.

5.2 Some Options

We now consider some specific options. As Yin et al note:

Anlotinib *is a small-molecule inhibitor of the multi-target tyrosine kinase receptor and can effectively curb tumour growth and angiogenesis…. demonstrated that anlotinib could effectively inhibit the activity of some human ATC cell lines, including KHM-5 M, C643 and 8505C. In mechanistic explorations, anlotinib significantly reduced ferroptosis-associated markers, including transferrin, ferritin light chain (FTL) and GPX4, but not pyroptosis, apoptosis and necroptosis. Moreover, protective autophagy was initiated under anlotinib stimulation, and autophagy interruption potentiated anlotinib-mediated ferroptosis and tumour-killing responses in vivo and in vitro. This study fully substantiated the potential role of autophagy-ferroptosis in the inhibition of tumour and angiogenesis in ATC by amlotinib, and may therefore contribute to the development of novel therapeutic strategies for ATC treatment.*

Neferine *is a bisbenzylisoquinoline alkaloid isolated from lotus seed, possessing a wide range of pharmacological effects such as antihypertensive, antiarrhythmic, anti-inflammatory, anticancer and antioxidant.146,147 Neferine enhanced the apoptosis of IHH-4 and CAL-62 cells and potentiated the ferroptosis effect of these cells.148 Further animal experiments showed that neferine could effectively promote the ferroptosis effect in tumours and consequently kill TC cells by inhibiting the Nrf2/HO-1/NQO1 pathway.*

Vitamin C*, an ascorbic acid vitamin, is recognised for its ability to regulate immune function, act as an epigenetic modulator, regulate programmed cell death and tumoricidal properties. Vitamin C significantly inhibited the growth of ATC cells by inducing free iron release, ROS generation and sustained lipid peroxidation, which ultimately led to the ferroptosis effect of ATC.150 BRAFV600E is a frequent mutation type in PTC, and its relevant inhibitors have been widely developed and applied, but the associated drug resistance is a growing challenge.*

Diaryl *ether derivative capable of targeting and inhibiting the expression level of GPX4, decreasing mitochondrial polarization and effectively inducing the ferroptosis effect in TC cells by reducing mitochondrial polarization and effectively inducing ferroptosis. This compound was recognized by the authors*

5.3 ALTERNATIVES

As Mou et al have noted:

Sulfasalazine *SSZ is recently recognized as a system xc- inhibitor. xCT expression has circadian rhythm and the expression of TFR1 was affected by the circadian organization of molecular clock. Bmal1 and the clock regulate the circadian rhythm of xCT expression. The clock-controlled gene c-Myc rhythmically activated the transcription of the TfR1 gene. SSZ has been reported to disrupt the circadian rhythm of transferrin receptor 1 gene expression and thus it was plausible that SSZ may affect iron metabolism … sulfasalazine inhibits Cys2 uptake via system xc-, resulting in ferroptosis in glioma cells. Based on the circadian rhythm of xCT, SSZ has different effects on inducing ferroptosis at various times. But some argued that ferroptosis was not observed in the mouse embryonic fibroblasts treated with sulfasalazine [86]. One reasonable interpretation was the discrepancy of different cell lines on the sensitivity to ferroptosis.*

Artesunate *ART and its derivatives can produce ROS and cause oxidative stress in cancer cells. In pancreatic ductal adenocarcinoma, head and neck cancers (HNCs), and ovarian cancer cells, the mechanism underlying the antitumor effect of ART was ferroptosis-induction. However, because of the activation of Nrf2-antioxidant response element signaling pathway, the ferroptosis induction of artesunate can be partially attenuated in some cisplatin-resistant HNCs . So Nrf2 inhibition via silencing Keap1 helps the reversal of ferroptosis resistance to artesunate in HNC cells.*

Temozolomide *TMZ markedly induces system xc- expression via the activation of activating transcription factor 4 (ATF4) and Nrf2 pathway in glioblastoma multiforme (GBM) cells [89].*

Cystathionine γ-lyase (CTH), an enzyme in the transsulfuration pathway, is induced after temozolomide treatment, which can supply Cys when system xc- is blocked. Based on the finding of erastin facilitating ferroptotic cell death to temozolomide, thus, the combination of TMZ and erastin maybe a promising therapy in GMB treatment.

Cisplatin *From the screening among five chemotherapeutic drugs, cisplatin was found as a ferroptosis-inducer. Cisplatin exerts its cytotoxic effects on A549 and HCT116 cells to undergo ferroptosis by reduced GSH depletion together with GPXs inactivation. Targeted agents*

Sorafenib: *Sorafenib was first identified as a ferroptosis-inducer in HCC cell lines. System xc- inhibition and GSH depletion, the accomplices for ROS accumulation, were the main mechanism for sorafenib-induced ferroptosis*

Haloperidol*, as a sigma receptor 1 antagonist, can bolster erastin and sorafenib-induced ferroptosis by stimulating cellular iron accumulation, GSH depletion, and lipid peroxidation. But the overactive p62-keap1-Nrf2 pathway will weaken the ferroptosis process, owing to the target genes of Nrf2 including heme oxygenase-1 (HO-1), FTH1, and quinone oxidoreductase-1 (NQO1) which can directly inhibit ROS accretion. Nrf2 inhibition using genetic tools or drugs could remarkably reinforce the anti-tumor effect of sorafenib [93]. Lapatinib and BAY87–2243 Lapatinib is a tyrosine kinase inhibitor. It can incite ferroptosis in breast cancer cells when it was used together with* ***siramesine****. BAY87-2243, a robust inhibitor of NADH-coenzyme Q oxidoreductase, can promote ferroptosis in a dose-dependent manner on a series of BRaf (V600E) melanoma cell lines.*

Lanperisone: *Lanperisone promotes ROS production to kill K-Ras-mutant mouse embryonic fibroblasts in ferroptotic ways. And it also induces lung cancer cell ferroptotic death by inhibiting Cys2 uptake in the mouse model*

6 OBSERVATIONS

We now consider several observations regarding ferroptosis.

6.1 NECROPTOSIS COULD BE AN ALTERNATE

Meier et al note:

Most metastatic cancers remain incurable due to the emergence of apoptosis-resistant clones, fuelled by intratumour heterogeneity and tumour evolution. To improve treatment, therapies should not only kill cancer cells but also activate the immune system against the tumour to eliminate any residual cancer cells that survive treatment. While current cancer therapies rely heavily on apoptosis — a largely immunologically silent form of cell death — there is growing interest in harnessing immunogenic forms of cell death such as necroptosis. Unlike apoptosis, necroptosis generates second messengers that act on immune cells in the tumour microenvironment, alerting them of danger.

This lytic form of cell death optimizes the provision of antigens and adjuvanticity for immune cells, potentially boosting anticancer treatment approaches by combining cellular suicide and immune response approaches. In this Review, we discuss the mechanisms of necroptosis and how it activates antigen-presenting cells, drives cross-priming of CD8+ T cells and induces antitumour immune responses. We also examine the opportunities and potential drawbacks of such strategies for exposing cancer cells to immunological attacks.

6.2 MALIGNANT CELL TARGETING ID CRITICAL

One of the dominant challenges of cancer therapeutics is the targeting of the malignant cells and not others. Collateral damage is often worse than the disease. Thus when considering ferroptosis one needs to consider this fact. Just treating ferroptosis qua ferroptosis as a means to an end may result in a vast set of sequellae. In a sense it can be akin to classic chemotherapy.

6.3 CELL ACCESS IS NECESSARY

As we have considered elsewhere, accessing the cancer cells is not as simple as it may appear. Cancer cells have developed mechanisms that protect and enhance their proliferation[18]. Namely such things as reduced vascularization, resistant extravasation, tumor micro environments (TME) are just a few examples. A therapeutic may work in vitro against naked tumor cells but in vivo is ineffective.

6.4 COLLATERAL DAMAGE

All therapeutics have the disadvantage of some form of collateral damage to other cells. As Jiang et al noted:

[18] https://www.researchgate.net/publication/385654147_Cancer_Therapeutic_Options

In addition to cancer and ischemic organ injuries, ferroptosis has been implicated in the pathogenesis of a growing list of other diseases, such as neurodegeneration, liver and lung fibrosis, autoimmune diseases. Mycobacterium-tuberculosisinduced tissue necrosis, cigarette-smoking-associated chronic obstructive pulmonary disease, and a rare genetic neurological disorder called Pelizaeus-Merzbacher Disease.

While this long list speaks to the clinical relevance and therapeutic potential of ferroptosis-modulating approaches, further investigation is required to determine if there is indeed a causative role of ferroptosis in these diseases. For example, in most cases the general observation is that non-apoptotic cell death was observed in the disease tissue, and that a ferroptosis inhibitor, often a lipophilic RTA, could mitigate the observed cell death and in some cases, alter the severity of the symptom.

However, lipid peroxides can regulate immunity and inflammation, processes that play important roles in all the listed diseases. Therefore, caution is needed to distinguish whether the observed effect of lipophilic RTA is via modulation of inflammation or ferroptosis, or both.

A detailed mechanistic interrogation of tissue cell death associated with the disease, including examining specific in vivo ferroptosis biomarkers (which the field sorely miss), will be crucial for this purpose.

Thus ferroptosis has a broad sweep, not only the putative targeting of cancer cells but the targeting of a wide variety of other cells as well.

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