A roadmap to creating ferroptosis-based medicines

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Ferroptosis is a regulated form of non-apoptotic cell death implicated in pathological settings. To be exploited clinically, ferroptosis requires reagents that unequivocally detect ferroptosis in human and animal tissues. Such tools may enable development of ferroptosis-based medicines for diverse diseases.

Cell death is a critical physiological process that can occur through unregulated necrosis or in regulated manner[1]. The past two decades have uncovered a variety of regulated cell death modalities that are distinct from apoptosis[2]. Ferroptosis, discovered in 2012, is a regulated form of cell death that is initiated by iron-dependent peroxidation of phospholipids with polyunsaturated fatty acyl tails[3]. Over the past decade since its discovery, ferroptosis has become a critical and growing field of research. Many investigators have revealed key regulators of this process, including ferroptosis-inhibitory processes (*i.e.,* the system-x_c-/glutathione/GPX4 axis, the FSP1/ubiquinol axis, and the GCH1/DHFR/tetrahydrobiopterin axis), cellular processes inducing lipid peroxidation, iron dependency, and metabolic pathways that modulate ferroptosis[4-12]. These findings have shaped our understanding of the molecular actions driving and blunting ferroptosis and have highlighted its potential importance in diverse pathological settings, including neurodegeneration, stroke, traumatic brain injury, ischemia-

reperfusion injury, cardiomyopathy, and kidney degeneration[13,14]. In addition, induction of ferroptosis is potentially useful as a novel strategy to attack specific cancers[15-17].

Studies in the past decade have largely concentrated on (i) mechanistic discoveries of ferroptosis-regulating pathways, and (ii) generating small molecule modulators to interrogate ferroptosis in cell models (**Figure 1**).

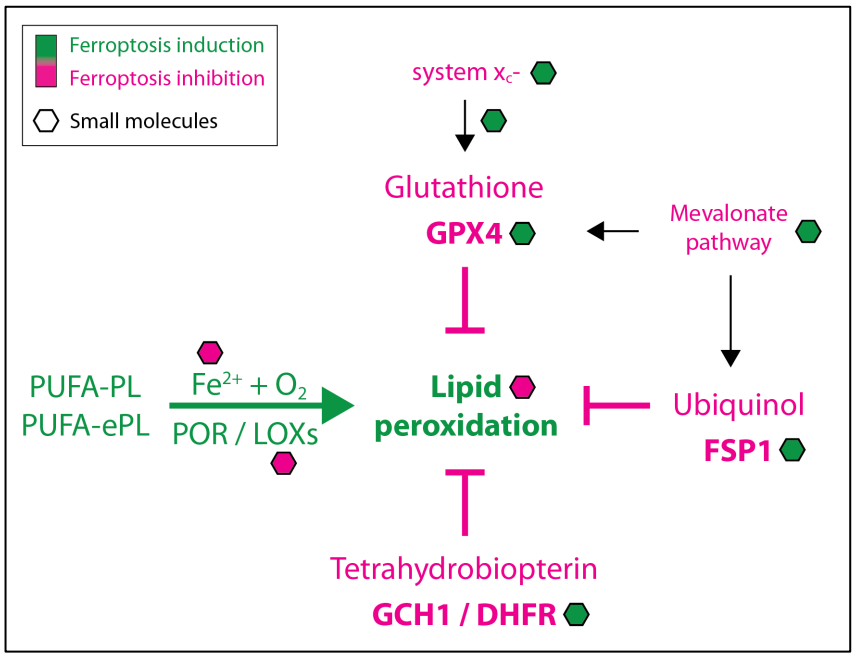

Figure 1: Major ferroptosis regulators and small molecule modulators

Yet, in order to rigorously evaluate the therapeutic potential of ferroptosis modulation, a major remaining challenge is to reliably detect and classify ferroptosis in animal and human tissues under pathological conditions. Currently available detection reagents[12] such as probes (*e.g.,* C11-BODIPY, TBARS and LiperFluo) and assay systems (*e.g.,* FENIX and metabolomics/lipidomics) work well in cell culture models, but have limitations for rapid and widespread use for the analysis of tissues. Thus, biomarkers and associated detection modalities are needed to unambiguously identify ferroptotic cell death in disease tissues. In line with this, a recent study determined that antibodies against transferrin receptor 1 (TfR1)

detect ferroptosis in cells and *in vivo*[18]. However, application of this reagent to tissue contexts has not yet been fully explored, and specificity versus other contexts needs to be defined. Hence, additional tools for specifically detecting ferroptosis are needed. Alongside reagent and technology development, small molecule and large molecule regulators of distinct ferroptosis targets are required to clinically modulate ferroptosis disease-relevant settings. A few demonstrated examples with efficacy in animal models[19,20] represent foundational approaches for ultimately creating drug-like ferroptosis-regulating medicines.

To translate ferroptosis biology into new therapeutics, we recommend the following two strategies to fully leverage the last decade of ferroptosis research:

(i) Generating a toolkit (biomarkers, reagents, and analysis methods) for specifically detecting ferroptosis in animal models and human patient samples

(ii) Creating *in vivo* compatible drug candidates that selectively modulate ferroptosis-relevant targets in animal models

We describe below the rationale and potential of each of these approaches.

Ferroptosis biomarker and detection modalities

The most important parameters for reagent development in order to classify ferroptosis *in vivo* are specificity and selectivity for ferroptosis. In line with this notion, for instance neurodegenerative diseases such as Alzheimer's and Parkinson's disease have been suggested to be caused by apoptosis, ferroptosis, or necroptosis[21]. Hence, it is critical to selectively discriminate between cell death modalities in the same disease setting in order to be able to develop disease-modifying medicines.

Two hallmarks of ferroptosis are oxidation of polyunsaturated-fatty-acid-containing phospholipids (PUFA-PLs) and redox-active iron[22]. These cellular entities have been central elements of reagent development to detect ferroptosis in cell culture models[12]. However, their application for detecting ferroptosis in tissues of patient or animal origin is of limited impact to

date due to several key issues: (i) these tools cannot be used on formalin-fixed paraffin-embedded (FFPE) tissue samples, which represents the major type of available tissue sources; (ii) most existing assays are technically complex and difficult for routine application in clinical practice; (iii) products of lipid peroxidation, namely malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), are good proxies for assessing lipid peroxidation, but not exclusive to ferroptosis, as they can also be produced upon general oxidative stress; and (iv) in tissue settings, it is technically challenging to specifically connect iron redox state to ferroptotic cell death. Together, current biomarkers and detection technologies are not sufficient for interrogating the existence of ferroptosis in animals or patients.

Hence, reagents that can be utilized to unequivocally detect ferroptosis and not oxidative stress in tissue samples is of high demand and currently a barrier towards creating ferroptosis-based medicines. The community needs to tackle this challenge and firstly identify novel biomarkers with high ferroptosis specificity over other cell death modalities, as well as contexts that trigger oxidative stress without inducing ferroptosis, and vice versa. Such biomarkers can be of diverse nature, and would include mRNAs, metabolites, lipids, proteins, enzymes as well as membrane proteins and receptors. For example, several studies have already identified candidate ferroptosis biomarkers, such as *CHAC1*, *PTGS2*, and *HMOX1*, which can be explored and validated in additional tissue settings. To address the challenge of biomarker discovery, a set of exploratory studies should be initiated that dissect the nature and molecular details of cell death modalities, as well as overlapping features with oxidative stress pathways. In particular, we need side-by-side comparisons of transcriptomics, metabolomics, lipidomics, proteomics and phospho-proteomics that specifically map critical components of and responses to individual cell death pathways. Only if we have a comprehensive picture of the ingredients of distinct cell death and oxidative stress pathways, we will be able to wisely choose unambiguous biomarkers of ferroptosis. Once a collection of selective ferroptosis biomarkers is defined, subsequent studies need to explore their consistency in detecting ferroptosis within cell populations. It is important to analyze their presence on a single-cell

level to choose highly consistent markers. Such studies will include single-cell transcriptomics, single-cell phenotyping by high-content image analysis, as well as recently developed ultra-sensitive single-cell proteomics[23] and single-cell lipidomics/metabolomics[24].

Following this procedure, the field should be able to nominate highly specific biomarkers of ferroptotic cell death within the next three-year span of research.

As a next step, suitable reagents that selectively recognize ferroptosis biomarkers in tissue samples need to be created. This will form the next generation of tools to precisely detect ferroptosis in animal models and human patient samples. It will be important to develop a broad spectrum of tools for various detection technologies in order to be able to monitor ferroptosis in fixed animal as well as human tissues, frozen samples, and for live patient imaging. Such tools include (i) chemical probes that specifically detect previously specified ferroptosis markers in mouse and human FFPE tissue sections, (ii) probes suitable for patient imaging by magnetic resonance tomography (MRI), optoacoustic imaging, computer tomography (CT), or position emission tomography (PET) to detect lipid peroxides or other relevant biomarkers *in vivo*, and (iii) antibodies, as well as fluorescently tagged single-chain antibodies and nanobodies to detect ferroptosis biomarkers on the surface of cells, which can be used for tissue imaging in immunohistological studies.

These three lines of reagent development should be a key future research area to advance the transition of ferroptosis biology into medicines.

Use of artificial intelligence to detect ferroptosis

Application of specific probes, fluorescent agents or antibodies to detect ferroptosis biomarkers in tissues and primary cells generates large quantities of imaging data that need proper analysis in order to be able to classify samples as containing or lacking ferroptosis. Analyses of such images can be carried out manually to choose distinct features and quantify its presence in a specific sample. This analysis strategy is tedious, limited in terms of speed and throughput, and most importantly prone to bias by individual researchers. In contrast,

image analysis can be done using artificial intelligence (AI), including machine learning (ML), tools in an unbiased way to extract a large set of image features that human eyes would not necessarily perceive or select, and use this information to advance image analysis performance[25], and thus ferroptosis classification.

ML-based analysis routines can be performed with supervised learning strategies, where the ML algorithm learns from a defined training data set to classify labels, which can subsequently be used to assign test sample images to one of the previously classified labels. Importantly, this analysis method needs (i) extracted features from labeled training data sets, and (ii) *a priori* knowledge of the outcome (*e.g.,* distinct morphological understanding of different cell death pathways). Thus, this analysis strategy can only be used in settings where suitable controls are available that can be used to define cell death modalities. However, in many cases the researcher is not aware of the outcome, *e.g.*, which type of cell death modality is the cause of a certain disease in patient tissue samples. Hence, supervised ML limits the ability to gain novel and unexpected biological insights.

Deep learning (DL) algorithms can overcome the need for *a priori* knowledge by classifying directly from raw image inputs. The DL method uses neural networks (*e.g.,* convolutional neural networks) to extract features from the raw images of various conditions without seeing the labels, then learns differences between these conditions and eventually classifies the output[25]. This type of analysis routine is perfectly suited to distinguish larger, as well as subtle, differences between diverse cell death and oxidative stress pathways. Current image-based ML/DL methods largely concentrate on analysis of cell culture models in high-content imaging approaches. However, recent advances have also started to apply ML/DL tools to analyzing tissue images, especially by applying DL algorisms.

The combination of novel tools (probes, reagents, antibodies, etc.) for tissue sample staining, together with unbiased in-depth image analysis techniques by machine learning and deep

learning will be fundamental innovation drivers to specifically detect ferroptosis in animal and human tissues (**Figure 2**). Especially, when it comes to distinguishing ferroptosis from other cell death modalities (*e.g.*, apoptosis, necroptosis, and pyroptosis), artificial intelligence tools will be key to dissect hidden, but significant characteristics of these cell death phenotypes. In addition to phenotypic specification, ML/DL algorithms will be important to define yet unknown temporal and spatial differences between cell death pathways, which will add additional opportunities to specify novel ferroptosis biomarkers. With all these knowledge, researchers may be able to detect diseases that involve ferroptosis and eventually generate and evaluate ferroptosis-modulating medicines.

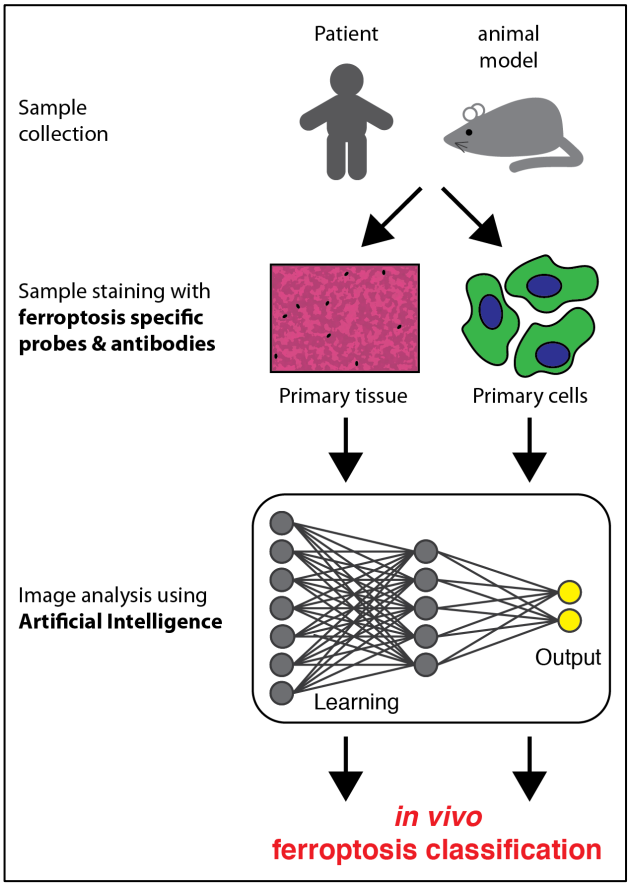

Figure 2: Sample staining and artificial intelligence methods for specific detection of ferroptosis in primary tissues and primary cells

Advances in ferroptosis-based therapeutics

The discovery of ferroptosis in 2012 fueled the investigation of diverse small molecule modulators of this pathway. These modulators range from ferroptosis inhibitors that may be used to treat degenerative diseases, to ferroptosis inducers that may be applied to treatment of specific cancer subtypes. Notable ferroptosis inhibitors are radical-trapping antioxidants, deuterated PUFAs, or iron-chelating molecules. When evaluating these ferroptosis inhibitors, it is obvious that currently available ferroptosis inhibitors lack particular cellular targets, but act through general mechanisms to remove hallmarks of ferroptosis, namely lipid peroxides and iron. In contrast, inducers of ferroptosis have largely been developed against components of the system-x_c-/glutathione/GPX4 axis (*e.g.,* imidazole ketone erastin, buthionine sulfoximine, and RSL3). While these are target-driven strategies, they have utilized the potential of only a narrow margin of ferroptosis regulation. A recent review by Conrad and colleagues summarized the progress of the most widely used ferroptosis modulators towards becoming medicines[13]. Although some of these small molecules have shown useful *in vivo* effects, most of them have not (yet) passed the stage of preclinical development and only very few have entered clinical testing.

Hence, to advance successful ferroptosis-modulating clinical candidates, current and future research efforts should employ target-based therapeutic strategies. This is important because having defined targets will facilitate: (i) lead optimization as well as preclinical validation to accelerate the path of novel candidate molecules towards clinical evaluation; (ii) *in vivo* target engagement and pharmacodynamics studies, and (iii) specificity towards unambiguously targeting ferroptosis. Based on a number of excellent discoveries of recent years, the ferroptosis community is now in a comfortable situation to nominate encouraging targets from diverse ferroptosis-regulating pathways to induce or block ferroptosis. Here, we highlight best examples of innovative target-based therapeutic strategies with clinical potential, which we describe in the following and summarize in **Table 1**.

As described before, until recently the system-x_c-/glutathione/GPX4 axis was believed to be the only inhibitory pathway of ferroptosis, which has resulted in many inhibitors of this axis to drive ferroptosis. However, it is now understood that two other gatekeepers specifically counteract ferroptosis in a GPX4-independent manner, namely the **FSP1/ubiquinol axis**[4,5] and the **GCH1/DHFR/tetrahydrobiopterin axis**[6,7]. Targeting these enzymes and their associated pathways with selective inhibitors holds promise for effective cancer treatment. Further targeted approaches to facilitate lipid peroxidation and ferroptosis in cancer are activation of the E3 ligases **MDM2/MDMX**, inhibition of the kinase **AMPK** and activation of the **YAP** transcription factor. Together, generating small molecule modulators of these targets will expand the therapeutic repertoire beyond the system-x_c-/glutathione/GPX4 axis and thereby elevate chances to attack ferroptosis-sensitive tumors in future clinical settings.

In order to inhibit ferroptosis, clearly the process of iron-dependent lipid peroxidation needs to be hampered. As an alternative to radical-trapping agents, target-based strategies to attenuate or block lipid peroxidation are the inhibition of the **cytochrome P450 oxidoreductase (POR)** or **MDM2/MDMX E3 ligases**. Importantly, inhibition of defined targets to block lipid peroxidation may increase specificity of ferroptosis-related medicine. Undoubtedly, future research will illuminate additional targets from genetic and chemical screens to counteract ferroptosis in degenerative diseases.

Besides small molecule drug development, the field of ferroptosis medicines should step into the generation of **large molecule therapeutics,** including antibodies, nanobodies, and single-chain antibodies to activate or repress ferroptosis. Although the number of large molecule therapeutics in *e.g.* cancer or immune therapies has skyrocketed in recent years, the ferroptosis field has barely entered this therapeutic space to explore the potential of an alternative set of promising targets. Notably, large molecule approaches will have their highest potential in specifically targeting extracellular-oriented surface proteins and receptors. The biggest advantage of large molecule ferroptosis medicines will be the high specificity of these

drug candidates towards their targets. As an example, antibodies against TfR1, which recently were developed to visualize ferroptosis[18], may be used to block transferrin-mediated iron import and hence inhibit ferroptosis. Given the immense success of large molecule drug discovery in the past decade, the ferroptosis field could benefit from this opportunity. Therefore, we propose that large molecule drug discovery becomes an essential segment of ferroptosis-based medicines.

In summary, to accomplish clinical translation of ferroptosis, the community needs to develop selective small or large molecule therapeutics against ferroptosis-regulating targets that have a favorable set of pharmacokinetics and ADME parameters, alongside demonstrated efficacy, and safety.

Table 1: Examples of emerging small molecule ferroptosis-regulating strategies

Ferroptosis outcome	Drug phenotype	Targeting strategy	Disease context
Induction	Reduction of **ubiquinol**	Inhibition of **FSP1**	Cancer
Induction	Reduction of **tetrahydrobiopterin**	Inhibition of **GCH1** or **DHFR**	Cancer
Induction	Induction of lipid peroxidation	Inhibition of **AMPK**	Cancer
Induction	Induction of lipid peroxidation	Activation of **MDM2/MDMX**	Cancer
Induction	Induced lipid peroxidation through **ACSL4** upregulation	Activation of **YAP** transcription factor	Cancer
Inhibition	Inhibition of lipid peroxidation	Inhibition of **Cytochrome P450 oxidoreductase (POR)**	Degenerative diseases
Inhibition	Inhibition of PPARα-mediated lipid remodeling	Inhibition of **MDM2/MDMX**	Degenerative diseases

Conclusion

In this commentary, we argue for the urgent need of technology advances to specifically detect ferroptosis *in vivo* as well as therapeutics that act through novel ferroptosis-regulating targets

in order to ultimately generate ferroptosis-based medicines. Thus, we propose the following two strategies be explored as the core of future efforts to translate ferroptosis research from bench to bedside:

(i) Development of **REAGENTS** and **ARTIFICIAL INTELLIGENCE TOOLS** to label and classify ferroptosis in human primary tissues.

(ii) Development of small molecule and large molecule **THERAPEUTICS** capable of modulating ferroptosis in animal models and human patients.

Importantly, these aims are interconnected and only if we advance in both will we have the chance to leverage a decade of intense ferroptosis research for clinical benefit. It is safe to say that this endeavor is only feasible if the field brings together researcher from multiple disciplines and scientific as well as medical backgrounds to cooperatively tackle the wide-ranging tasks needed to create ferroptosis-based medicines.

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