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Investigating Nonapoptotic Cell Death Using Chemical Biology Approaches

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Nonapoptotic cell death is important for human health and disease. Here, we show how various tools and techniques drawn from the chemical biology field have played a central role in the discovery and characterization of nonapoptotic cell death pathways. Focusing on the example of ferroptosis, we describe how phenotypic screening, chemoproteomics, chemical genetic analysis, and other methods enabled the elucidation of this pathway. Synthetic small-molecule inducers and inhibitors of ferroptosis identified in early studies have now been leveraged to identify an even broader set of compounds that affect ferroptosis and to validate new chemical methods and probes for various ferroptosis-associated processes. A number of limitations associated with specific chemical biology tools or techniques have also emerged and must be carefully considered. Nevertheless, the study of ferroptosis provides a roadmap for how chemical biology methods may be used to discover and characterize nonapoptotic cell death mechanisms.

Introduction

Cell death is a terminal fate resulting in elimination of the cell, either through physical disintegration or, more commonly *in vivo*, engulfment by a neighboring cell. Because cell death results in elimination of the cell itself, it is not trivial to capture dying cells and study the molecular mechanisms that regulate these processes. The acuteness of chemical tools (e.g., natural products, synthetic small molecules), therefore, provides exceptional advantages in the study of cell death, as they can be used to initiate or inhibit cell death at a precise time. The inherent portability of these tools between systems is also invaluable for the study of cell death. An early illustration of these advantages was provided by J.R. Tata in 1966, who showed that the stereotypical regression of *Xenopus* tail explants induced by the addition of thyroid hormone was blocked by the acute addition of actinomycin D or cycloheximide, natural product inhibitors of transcription and protein synthesis, respectively, that had only recently been discovered (Kerridge, 1958; Reich et al., 1961; Tata, 1966). Tata's classic study was one of the first to suggest that cell death could be a molecularly regulated process and helped establish chemical tools as key enablers of cell death research.

Discovery of Regulated Nonapoptotic Cell Death

Cells can perish in a number of ways that can be distinguished on the basis of morphological, genetic, and biochemical criteria. One fundamental distinction is between regulated and unregulated cell death, and a second distinction is between apoptosis and all forms of nonapoptotic or necrotic cell death. Regulation is evident if the cell death phenotype under observation is morphologically stereotypical and can be suppressed by a specific genetic or chemical manipulation (Kerr et al., 1972; Wolpaw et al., 2011) (Figure 1). By contrast, unregulated cell death, as occurs in response to extreme physical stresses, nonspecific chemically reactive compounds, or detergents, cannot be modified by any specific molecular intervention. At one time, regulated cell death was synonymous with apoptosis and unregulated cell death with necrosis. However, over the last 20 years this simple dichotomy has been shattered by the finding that nonapoptotic cell death can also occur in a regulated fashion (Galluzzi et al., 2018). Chemical biology approaches were central to establishing this new paradigm, as exemplified by the study of necroptosis.

The cytokine tumor necrosis factor alpha (TNF-α) is a potent trigger for apoptosis, but over two decades ago it was observed that cells treated with TNF-α and a small-molecule caspase inhibitor, such as zVAD-fmk, still succumbed to cell death and, in fact, appeared to do so more readily (Vercammen et al., 1998). The thioxo-imidazolidinone necrostatin-1 (Nec-1) was identified from a small-molecule screen as a specific inhibitor of cell death under these conditions (Degterev et al., 2005) (see Table 1, selected small-molecule structures). This inhibitor blocked receptor-interacting serine/threonine kinase 1 (RIPK1) function, implying that cell death was regulated by phosphorylation (Degterev et al., 2008). Nec-1 analogs also suppressed pathological cell death in the nervous system, demonstrating the *in vivo* relevance of this process (Degterev et al., 2005). This form of cell death was rechristened necroptosis, a portmanteau of necrosis and apoptosis, highlighting that it was a regulated form of cell death distinct from apoptosis. Small-molecule screening subsequently helped identify additional important regulators of this process, including mixed-lineage kinase domain-like pseudokinase (MLKL), which is inhibited by the small-molecule necrosulfonamide (Sun et al., 2012). Thus, the necroptosis field provided a conceptual road map for how new nonapoptotic cell death pathways could be discovered using chemical biology approaches. The full power of chemical biology-driven approaches to pinpoint and characterize new forms of nonapoptotic cell death is, however, perhaps best demonstrated by the example of ferroptosis.

Ferroptosis is one of several new nonapoptotic cell death processes to be recognized in the past two decadesrecently (Conrad and Pratt, 2019; Galluzzi et al., 2018). This process can be

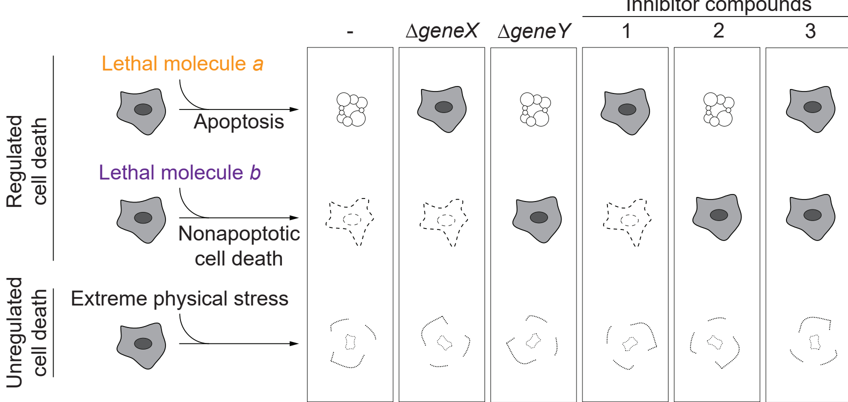

Figure 1. Cell Death Pathways Can Be Regulated by Different Genes and Compounds

Cell death can be triggered by various lethal stimuli and lead either to the activation of a regulated apoptotic or nonapoptotic cell death process, or to unregulated cell death. Regulation is evident if cell death involves stereotypical dead/dying cell morphology and can be modulated by specific genetic (e.g., gene deletion) or chemical perturbations. Here, two lethal compounds (*a* and *b*) induce regulated cell death, either apoptosis (*a*) or a form of nonapoptotic cell death (*b*). Cell death induced by *a* and *b* can be inhibited by deletion of specific genes (*X*, *Y*) or addition of specific inhibitors (1, 2, or 3). Cell death induced by extreme physical stress, such as heat, cold, or detergent treatment, cannot be inhibited by any specific genetic or chemical intervention (i.e., is unregulated).

triggered by inactivation of the system x_c^- cystine/glutamate antiporter (leading to depletion of intracellular glutathione), direct inhibition of the glutathione-dependent lipid hydroperoxidase GPX4, and possibly other mechanisms. GPX4 inhibition ultimately leads to the iron-dependent accumulation of lipid peroxides which eventually kill the cell (Cao and Dixon, 2016; Stockwell et al., 2017). While ferroptosis was named in 2012, similar processes have been observed for decades in various contexts where glutathione metabolism is disrupted (Hirschhorn and Stockwell, 2018; Lewerenz et al., 2018). What crucially enabled ferroptosis to be recognized as a distinct mode of nonapoptotic cell death was the discovery and characterization of potent and specific small-molecule inducers and inhibitors of this process (Table 1). These highly portable tools have been leveraged to investigate ferroptosis using a host of chemical biology approaches in a wide array of models.

Below, we describe how different chemical biology approaches enabled ferroptosis to be identified and studied (summarized in Figure 2). We provide examples of how early discoveries catalyzed a virtuous cycle of innovation leading to new chemical methods and probes to study ferroptosis. This progress has not occurred without some hiccups along the way, and we therefore point out some limitations of current methods. We conclude by considering how chemical biology approaches could help elucidate additional nonapoptotic cell death mechanisms.

Chemical Biology and Ferroptosis

Phenotypic Small-Molecule Screening

Small-molecule phenotypic screening is a powerful means to discover compounds with novel mechanisms of action (Wagner and Schreiber, 2016). Here, libraries of small molecules are searched in an unbiased manner for those that can produce a given cellular or organismal phenotype of interest. Advantageously, such screens have the potential to unveil new and unexpected biochemical mechanisms that underlie the phenotype being studied.

Erastin and 1*S*,3*R*-RSL3 (hereafter RSL3) are prototypic inducers of ferroptosis, originally discovered in large-scale (∼25–50,000 compound) phenotypic screens designed to identify compounds that were selectively lethal to engineered tumor cells expressing oncogenic mutant $HRAS^{V12}$ (Dolma et al., 2003; Yang and Stockwell, 2008). Serendipitously, the use of a generic metabolism-based cell viability readout made these screens agnostic to the nature of the cell death process that was activated by a given lethal small molecule. Although not apparent initially, detailed characterization of the erastin and RSL3 mechanisms of action eventually led to the discovery that these compounds triggered ferroptosis (Dixon et al., 2012). Had the original phenotypic screens been designed to report specifically on the induction of apoptosis, these ferroptosis-inducing small molecules would have gone undiscovered. Subsequently, 14 additional lethal molecules, including ML162 and ML210, were identified from over a million compounds tested for $HRAS^{V12}$-selective lethality and shown to trigger ferroptosis (Weïwer et al., 2012; Yang et al., 2014). Other phenotypic screens, looking for cell death without caspase activation, or enhanced cell death after glutathione depletion, identified additional inducers of ferroptosis, including sorafenib and FIN56 (Dixon et al., 2014; Lachaier et al., 2014; Shimada et al., 2016b).

Phenotypic chemical suppressor screens, where cell death induced by one molecule is blocked by the action of a second molecule, have helped dissect the ferroptosis mechanism and identify specific inhibitors of this process. From a library of over 2,000 annotated bioactive compounds, antioxidants (e.g., α-tocopherol, β-carotene, butylated hydroxytoluene) and iron chelators (e.g., deferoxamine) were found to block cell death induced by erastin (Yagoda et al., 2007). This study first illuminated the fundamental oxidative, iron-dependent nature of this process. Further chemical suppressor screening using large unannotated compound libraries tested in erastin-treated cancer cells or inducible $Gpx4^{-/-}$ mouse embryonic fibroblasts pinpointed the potent and specific ferroptosis inhibitors ferrostatin-1 and liproxstatin-1 (Dixon et al., 2012; Friedmann Angeli et al., 2014). These two compounds and derivatives thereof have been used in a range of mechanistic and animal model studies to establish the fundamental oxidative nature and in vivo relevance of ferroptosis (Dixon et al., 2012; Fang et al., 2019; Friedmann Angeli et al., 2014; Li et al., 2017; Linkermann et al., 2014).

Chemoproteomics

Identifying the target(s) of small molecules isolated in phenotypic screens is an important and often arduous task. A classic approach involves the use of affinity analogs, purification, and protein identification using mass spectrometry. An important success using this approach was the identification of GPX4 as

Table 1. Chemical Modulators of Nonapoptotic Cell Death

Pathway	Effect of Target Inactivation on Death	Target	Effect of Small Molecule on Target Activity	Small Molecule Name	Structure	References
Necroptosis	suppress	RIPK1	inhibitor	necrostatin-1		Degterev et al. (2005)
		MLKL		necrosulfonamide		Sun et al. (2012)
Ferroptosis	induce	GPX4	inhibitor	RSL3		Yang and Stockwell (2008)
				ML210		Weïwer et al. (2012)
				ML162		Weïwer et al. (2012), Yang et al. (2014)
		SQS	activator	FIN56		Shimada et al. (2016b)
		system x_c^-	inhibitor[a]	erastin		Dolma et al. (2003), Dixon et al. (2012)
				sorafenib		Dixon et al. (2014)
				compound 4		Taylor et al. (2019)
	suppress	lipid peroxyl radicals	inhibitor	ferrostatin-1		Dixon et al. (2012)
				liproxstatin-1		Friedmann Angeli et al. (2014)
		iron	chelator	deferoxamine		Yang and Stockwell (2008)

(*Continued on next page*)

Table 1. *Continued*

Pathway	Effect of Target Inactivation on Death	Target	Effect of Small Molecule on Target Activity	Small Molecule Name	Structure	References
Methuosis	induce	unknown	unknown	compound 17		Sun et al. (2017)
				compound 13		Huang et al. (2018)
		PIKFYVE, other(s)	inhibitor	MOMIPP		Robinson et al. (2012), Cho et al. (2018)
CIL56-induced death	induce	unknown	unknown	CIL56		Shimada et al. (2016b)

Examples of synthetic small molecule and natural product inducers and inhibitors of different nonapoptotic cell death pathways. For only some molecules have targets been established. RIPK1, receptor interacting serine/threonine kinase 1; MLKL, mixed lineage kinase domain-like pseudokinase; GPX4, glutathione peroxidase 4; SQS, squalene synthase; PIKFYVE, phosphoinositide kinase, FYVE-type zinc finger containing.
[a]Target inhibition is based on biochemical activity, not direct evidence of target engagement.

a direct target of RSL3 (Yang et al., 2014). Two features of RSL3 aided the success of the chemoproteomic approach here. First, RSL3 contains a chloroacetamide "warhead" that reacts covalently with GPX4. Second, only the 1*S*,3*R* diastereomer of RSL3 is active, making it possible to identify GPX4 as a protein uniquely bound to active (1*S*,3*R*) but not inactive (1*R*,3*R*) analogs. A similar chemoproteomic strategy has recently been successfully deployed to identify the small antioxidant protein thioredoxin (TXN) as a covalent target of the ferroptosis-inducing compound ferroptocide (Llabani et al., 2019). Mutagenesis studies suggest that ferroptocide binds to the active site cysteine residues of thioredoxin as well as to an adjacent cysteine residue. How thioredoxin inhibition triggers ferroptosis is not clear but could involve enhanced lipid peroxidation due to disruption of the thioredoxin/thioredoxin reductase cycle (Bjornstedt et al., 1995).

Although powerful in principle, affinity chemoproteomics has not always yielded straightforward answers. These methods, used together with both lethal and non-lethal analogs of erastin, identified mitochondrial voltage-dependent anion channel (VDAC) 2 and 3 as targets of this molecule (Yagoda et al., 2007). Functionally, erastin reduces the permeability of VDAC1 and VDAC2 when these proteins are expressed in yeast (Yagoda et al., 2007), and can alter VDAC function, stability, and interaction with other proteins in mammalian cells (Maldonado et al., 2013; Yang et al., 2020). However, the ability of erastin to induce ferroptosis is best explained by inhibition of the cystine/glutamate antiporter system x_c^-, leading to depletion of glutathione and other metabolites (Dixon et al., 2014) (and see below). This link between erastin and system x_c^- was not discovered using chemoproteomics, but rather by comparing the lethal effects of erastin to other small molecules suspected to trigger oxidative cell death (Dixon et al., 2012). Chemoproteomic results are therefore not a panacea, perhaps especially when dealing with compounds that engage targets non-covalently with relatively low affinity.

Chemical Genetic Screens

Lethal small molecules can be combined with genetic screening to identify genes that normally promote or suppress cell death. These methods have been applied successfully to the study of several nonapoptotic cell death pathways, including ferroptosis. Short hairpin RNAs (shRNAs), short interfering RNAs (siRNAs), insertional mutagenesis in human haploid cells, and CRISPR/Cas9-based technologies have all been used in loss-of-function ferroptosis suppressor screens (Table 2). Perhaps, as could be anticipated from previous chemical suppressor screens, these genetic screens have identified genes that govern lipid metabolism (e.g., *ACSL4*, *LPCAT3*), iron homeostasis (e.g., *IREB2*, *ATM*), and redox metabolism (*CARS*, *KEAP1*) (Chen et al., 2019; Dixon et al., 2012, 2015; Doll et al., 2017; Hayano et al., 2015; Zou et al., 2019). Disruption of these genes limits ferroptosis by reducing the levels of oxidizable membrane lipids, lowering intracellular free iron, or increasing the antioxidant capacity of the cell.

Remarkably, with one exception (i.e., *ACSL4*), the hits recovered in these genetic suppressor screens are largely non-overlapping. This could be explained by technical differences between the screens. For example, partial gene silencing using shRNA/siRNA may allow for the role of essential genes, such as the cysteinyl-tRNA synthetase *CARS*, to be identified more easily than when using complete knockouts. Different screens have typically used different cell line models. Cell type-specific differences in ferroptosis regulatory networks may therefore also contribute to the

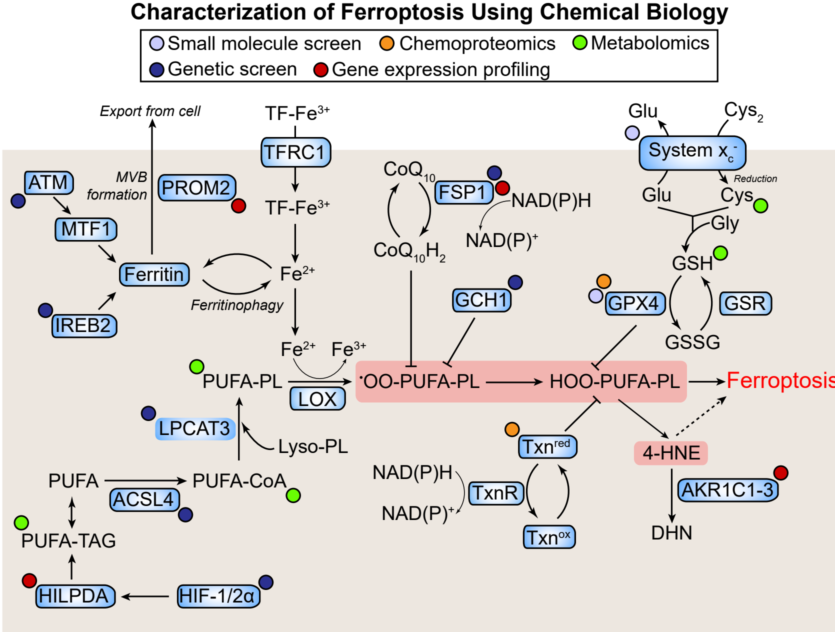

Figure 2. Characterization of Ferroptosis Using Chemical Biology Approaches

Key molecules involved in ferroptosis are annotated to indicate how they were discovered using different chemical biology techniques. Proteins are denoted by rounded rectangles and metabolites are indicated by text only. Colored circles denote chemical biology techniques used to discover the role of key molecules in this pathway. A few important biochemical details are shown in simplified form, especially the process of lipid peroxidation (red highlight), which involves repeated cycles of initiation, propagation, and termination. The role of lipoxygenase (LOX) enzymes in ferroptosis initiation remains controversial, and initiation of lipid peroxidation can occur with or without these enzymes. Abbreviations: 4-HNE, 4-hydroxynonenal; CoQ_{10}, coenzyme Q_{10}; DHN, 1,4-dihydoxy-2-nonene; LOX, lipoxygenase enzyme; PL, phospholipid; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol; TF, transferrin; TFRC1, transferrin receptor 1; TXN, thioredoxin; TxnR, thioredoxin reductase.

different results obtained between screens. It is possible that the execution of ferroptosis: (1) in mouse embryonic fibroblasts is especially dependent upon autophagy-related genes (e.g., *Ulk1*, *Becn1*, *Atg4d*) for the degradation of intracellular ferritin and the liberation of iron (Gao et al., 2016); (2) in renal cell carcinoma cells is especially sensitive to modulation of the hypoxia inducible factor pathway, which controls expression of the candidate lipid metabolic enzyme HILPDA (Zou et al., 2019); and (3) in triple-negative breast cancer cells is highly dependent upon regulation of intracellular labile iron levels by an ATM kinase-dependent mechanism (Chen et al., 2019). Alternatively, differences in the design of each screen may have favored the detection of only a subset of genes from a larger shared regulatory network common to all cells.

Apart from suppressor screens, CRISPR/Cas9-mediated gene disruption has also been used in a loss-of-function ferroptosis *sensitizer* screen, resulting in the identification of ferroptosis suppressor protein 1 (FSP1, formerly AIFM2), which normally prevents ferroptosis (Bersuker et al., 2019). A novel cDNA overexpression screen likewise pinpointed FSP1 as a potent suppressor of ferroptosis (Doll et al., 2019). FSP1 acts as a reductase for the endogenous lipid electron carrier coenzyme Q_{10}. This metabolite limits lipid reactive oxygen species accumulation in the plasma membrane in parallel to GSH-dependent GPX4 activity. Another antioxidant mechanism was discovered using a CRISPR activation screen, finding that enhanced GTP cyclohydrolase-1 expression can suppress ferroptosis by boosting the synthesis of tetrahydrobiopterin/dihydrobiopterin, which are metabolites that can act as direct radical trapping antioxidants (Kraft et al., 2020).

Future genetic screens could yield additional regulators of ferroptosis, although it seems likely that most genes will ultimately be found to impinge in some way upon iron, lipid, or antioxidant metabolism. In fact, ferroptosis screens could be a great way to identify new or unexpected genes involved in these three processes. Likewise, iron or lipid metabolism genes identified in large-scale screens originally designed for other purposes can be tested for links to ferroptosis regulation , with a good chance of finding a connection (Alvarez et al., 2017; Cao et al., 2019; Garcia-Bermudez et al., 2019).

Gene Expression Profiling

Compound treatment can alter gene expression in informative ways, providing candidate molecular markers and potentially leading to new mechanistic insights. Unbiased RNA sequencing identified *CHAC1* as a highly upregulated gene in cells treated with erastin *in vitro*, an effect confirmed in tumors exposed to an erastin analog *in vivo* (Dixon et al., 2014; Zhang et al., 2019). *PTGS2* is another gene expression marker of ferroptotic cells, discovered from a biased analysis of 83 candidate oxidative stress-sensitive genes, that can likewise be upregulated in cells undergoing ferroptosis *in vitro* and *in vivo* (Yang et al., 2014; Zhang et al., 2019). Therefore, increased *CHAC1* and *PTGS2* expression can serve as molecular markers for ferroptosis-inducing drug exposure, although neither is likely to be specific enough to exclusively mark ferroptotic cells.

Gene expression profiling in response to compound treatment can help define mechanisms of cell death resistance. For example, resistance to erastin is correlated with overexpression of *AKR1C1-3* (Dixon et al., 2014; Gagliardi et al., 2019). These genes encode aldo-keto reductases that may suppress ferroptosis by detoxifying reactive carbonyls that form downstream of phospholipid oxidation, such as 4-hydroxynonenal (Burczynski et al., 2001). In certain breast cancer cells, cell detachment from the extracellular matrix can induce ferroptosis (Brown et al., 2017, 2018). Cells that resist this process rapidly upregulate *PROM2*, encoding a transmembrane pentaspanin protein, that promotes exosomal release of iron-loaded ferritin from the cell (Brown et al., 2019). As demonstrated by the example of *PROM2*, which was not previously linked to iron metabolism, it is essential to couple gene expression profiling with functional studies to deduce the nature of the regulatory connection.

Differences in basal gene expression between cells can also yield important insights into the regulation of cell death. For

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Table 2. Summary of Chemical Genetic Screens for Regulators of Ferroptosis and Related Processes

Model	Focus/Scope	Method	Genes	Hits	Reference
HT-1080, Calu-1 + erastin	mitochondrial genes	arrayed shRNA	1,087	*IREB2*, *RPL8*, *ACSF2*, others	Dixon et al. (2012)
MEFs AA starvation + FBS	signaling genes	pooled shRNA	4,625	*Tfrc*, *Aco1*, *Ulk1*, *Becn1*, *Atg4d*, others	Gao et al. (2015)
HT-1080 + erastin	genome-wide	siRNA	21,687	*CARS*, others	Hayano et al. (2016)
MDA-MB-231 + low cystine	kinome	siRNA	715	*ATM*, *ATR*, *TTK*, *SYK*, others	Chen et al. (2019)
HAP1 haploid + RSL3 or ML162	genome-wide	saturating retroviral insertional mutagenesis	~20,000	*ACSL4*, *LPCAT3*, *AGPAT3*, *HSD17B11*	Dixon et al. (2015)
Pfa1 MEFs + RSL3, erastin	genome-wide	CRISPR/Cas9	~20,000	*ACSL4*	Doll et al., 2017
786-O cells + ML210	genome-wide	CRISPR/Cas9	~18,000	*ACSL4*, *KEAP1*, *EPAS1*, *EP300*, others	Zou et al. (2019)
U-2 OS + RSL3	apoptosis and cancer genes	CRISPR/Cas9	3,015	*FSP1*	Bersuker et al. (2019)
Pfa1 MEFs + MCF7 cDNA + tamoxifen (to induce *Gpx4* deletion)	cDNA library	cDNA overexpression	unknown	*GPX4*, *FSP1*	Doll et al. (2019)
Pfa1 MEFs + IKE, RSL3, or tamoxifen (to induce *Gpx4* deletion)	genome-wide	CRISPR activation	~20,000	*GCH1*, others	Kraft et al. (2020)

Important chemical genetic screens performed to isolate genes that regulate ferroptosis sensitivity in different contexts. Only the top or best validated hits from each screen are listed; in many cases there were other hits that were reported but not validated in detail. shRNA, short hairpin RNA; siRNA, short interfering RNA; MEF, mouse embryonic fibroblast.

example, correlating cell viability and basal gene expression data from the NCI-60 cell line panel identified an association between ferroptosis-inducing small molecules and genes regulating NAD(P)H metabolism (Shimada et al., 2016a). Mechanistically, high NAD(P)H levels correlate with ferroptosis resistance, perhaps due to enhanced activity of one or more NAD(P)H-dependent anti-ferroptotic proteins, such as TXN (Llabani et al., 2019) or FSP1 (Bersuker et al., 2019; Doll et al., 2019). In a similar vein, basal gene expression and dose-dependent compound sensitivity data for more than 700 cancer cell lines (Basu et al., 2013; Rees et al., 2016) demonstrate that ferroptosis sensitivity is linked to: (1) a mesenchymal gene expression state (Viswanathan et al., 2017); (2) gene expression changes associated with melanoma dedifferentiation (Tsoi et al., 2018); and (3) low expression of *FSP1* or the lipid metabolic enzyme *ACSL3* (Bersuker et al., 2019; Magtanong et al., 2019). It is possible that further analysis of this resource, available online through the Cancer Therapeutics Response Portal, will yield additional new regulators of ferroptosis.

Metabolomic Analysis

Ferroptosis is fundamentally the result of dysregulated iron, lipid, and antioxidant metabolism. Thus, combining ferroptosis-inducing small molecules with the direct measurement of individual metabolites using mass spectrometry-based techniques has been highly informative. Consistent with the notion that erastin induces ferroptosis by inhibiting cystine import and thereby preventing *de novo* GSH synthesis, polar metabolites, including cysteine and GSH, are among the most significantly depleted in erastin-treated HT-1080 cells (Skouta et al., 2014; Tarangelo et al., 2018; Yang et al., 2014). Intriguingly, depletion of the thiol-containing metabolite coenzyme A (CoA) contributes essentially to the induction of ferroptosis downstream of cystine deprivation and in parallel to GSH depletion (Badgley et al., 2019; Leu et al., 2019). How CoA normally inhibits ferroptosis is unclear but could involve a direct antioxidant mechanism or effects on CoA-dependent lipid metabolism. The need for simultaneous depletion of GSH and CoA to induce ferroptosis likely explains why a direct GSH biosynthetic inhibitor, buthionine sulfoximine, is a relatively poor inducer of ferroptosis alone (Cao and Dixon, 2016). The relative contribution of different antioxidant metabolites (e.g., GSH, CoA, tetrahydrobiopterin, NADPH) to the regulation of ferroptosis sensitivity in different cells and contexts will be important to clarify in future studies.

Ferroptosis involves oxidation of specific polyunsaturated phospholipids and the analysis of this process has been greatly aided by developments in the analysis of non-polar lipid metabolites. In cells undergoing ferroptosis numerous polyunsaturated fatty acid (PUFA) species (e.g. C20:4n6, C22:6n3, C20:5n3) are depleted, while various lysophospholipids accumulate, consistent with cleavage of oxidized acyl chains from existing phospholipids (Skouta et al., 2014; Yang et al., 2014). Combining mass spectrometry with specific ferroptosis inhibitors has led to the suggestion that oxidation of PUFA-containing phosphatidylethanolamines (PUFA-PEs) is especially critical for the initiation of ferroptosis, both *in vitro* and *in vivo* (Anthonymuthu et al., 2018; Kagan et al., 2017; Kenny et al., 2019; Doll et al., 2017). This may not be universal, however, as pronounced depletion of other lipid species, including phosphatidylcholines, is observed in many ferroptosis-inducing conditions (Gaschler et al., 2018a; Kraft et al., 2020; Magtanong et al., 2019; Zhang et al., 2019). How the oxidation of these or other phospholipids

may lead to membrane "blistering" (Magtanong et al., 2019) and eventual lethal membrane permeabilization is mostly unclear.

Leveraging Chemical Biology for New Tools and Mechanistic Insights

Chemical biology-driven analyses have yielded numerous small-molecule activators and inhibitors of nonapoptotic cell death. These initial reagents have subsequently been leveraged to discover additional chemical tools and further our understanding of lethal mechanisms. Below, we focus on examples from the ferroptosis field showing how this virtuous cycle can work in practice.

Expanding the Constellation of Small-Molecule Ferroptosis Modulators

The discovery of the specific inhibitors ferrostatin-1 and liproxstatin-1 makes it straightforward to test whether any given lethal molecule of interest can induce ferroptosis. This has helped identify synthetic small molecules (e.g., $FINO_2$), natural products or natural product derivatives (e.g., withaferin A, ironomycin, ferroptocide, open chain epothilone analogs), and other substances (e.g., ultrasmall silica nanoparticles) as novel ferroptosis-inducing molecules (Abrams et al., 2016; Hassannia et al., 2018; Kim et al., 2016; Llabani et al., 2019; Mai et al., 2017; Taylor et al., 2019).

First-generation ferroptosis inhibitors have also served as the basis for the development of improved analogs, typically with chemical properties more suitable for use *in vivo*, such as the improved ferrostatin-1 analogs SRS16-86 and UAMC-3203 (Devisscher et al., 2018; Linkermann et al., 2014; Skouta et al., 2014). Knowledge of the ferrostatin-1 and liproxstatin-1 mechanism of action has also facilitated the rational design of synthetic compounds with appropriate radical trapping properties, such as a new series of 1,8-tetrahydronaphthyridinols (Zilka et al., 2017). Further developments along these lines may yet yield potent ferroptosis modulators suitable for use in humans.

New Chemical Methods and Chemical Reporters

Understanding that lipid peroxidation and lipid radical formation are important for ferroptosis has catalyzed efforts to develop new methods to study this process. The fluorescence-enabled inhibited autoxidation approach uses the reporter STY-BODIPY to assess the potency of putative radical trapping antioxidant inhibitors of ferroptosis, and represents a significant improvement over the classic 2,2-diphenyl-1-picrylhydrazyl assay (Haidasz et al., 2016; Shah et al., 2019). Application of this method led to a new explanation for the potency of diarylamine-based ferroptosis inhibitors, such as ferrostatin-1, versus phenolic ferroptosis inhibitors: unlike diarylamine-based ferroptosis inhibitors, the phenolic inhibitors form hydrogen bonds with phospholipid head groups that limit their reactivity with lipid radicals.

Small-molecule inhibitors and inducers of ferroptosis have also aided the development of new chemical probes of the ferroptosis mechanism. For example, an alkyne-tagged ferrostatin-1 analog was localized to the endoplasmic reticulum, mitochondria, and lysosomes using stimulated Raman scattering microscopy, suggesting that inhibition of lipid peroxidation in one or more of these locations is required to block ferroptosis (Gaschler et al., 2018b). Ferroptosis inducers have also been useful to characterize a new specific fluorescent probe for intracellular glutathione (RealThiol) (Jiang et al., 2017) and a FRET-based probe for labile iron (FIP-1) (Aron et al., 2016). Interestingly, using FIP-1 it was found that the intracellular labile iron pool is increased by erastin analog treatment and that this may contribute directly to cell death, since the increase is prevented by cotreatment with a protective iron chelator. These new probes allow for key features of ferroptosis to be more easily examined in diverse settings.

Challenges with the Analysis of Nonapoptotic Cell Death Using Chemical Biology Approaches

Chemical biology methods and cell culture-based approaches are powerful but have limitations that can unintentionally introduce confusion or uncertainty into the analysis of nonapoptotic cell death. Consideration of these limitations may help avoid common pitfalls and, in the fullness of time, lead to useful reinterpretation of existing data. Below we illustrate some common limitations using examples from the ferroptosis field.

Limitations of Cell-Based Assays

Ferroptosis is a metabolic process and is therefore highly sensitive to cell growth conditions (Stockwell et al., 2017). For example, differences in the concentration of individual metabolites in the culture medium (e.g., monounsaturated fatty acids, selenium) can significantly alter ferroptosis sensitivity (Magtanong et al., 2019; Vande Voorde et al., 2019). Ferroptosis sensitivity within a population of cells is also sensitive to cell density and cell-cell contact, likely in a cell-type-specific manner (Brown et al., 2018; Wenz et al., 2019; Wu et al., 2019). Control over medium composition and cell seeding density is largely lacking between studies and, combined with genetic drift between cell stocks (Ben-David et al., 2018), could easily contribute to differences in results between studies. Moreover, the measurement of cell viability or death *in vitro* at a single arbitrary time point (e.g., 48 or 72 h), as remains common in the cell death field generally, may overestimate or fail to capture the contribution of a given biochemical process to ferroptosis. For example, overexpression of the glutathione efflux pump MRP1 or stabilization of the tumor suppressor p53 attenuates ferroptosis, but only over short timescales (~hours) (Cao et al., 2019; Tarangelo et al., 2018). These effects are readily apparent from time-lapse imaging but difficult to detect using single endpoint measures.

Small-Molecule Off-Target Effects

Small molecules are rarely entirely specific, and both erastin and RSL3 bind to more than one protein (Gao et al., 2018; Yagoda et al., 2007). The induction of ferroptosis by these agents could therefore conceivably involve the combined inhibition of multiple targets (Yang et al., 2020). This concern is somewhat mitigated by the fact that *Gpx4* deletion alone is sufficient to induce cell death that is completely inhibited by liproxstatin-1 (Angeli et al., 2014; Seiler et al., 2008), while direct deprivation of the system x_c^- substrate cystine is sufficient to trigger ferroptosis that is suppressed by ferrostatin-1 (Magtanong et al., 2019; Tarangelo et al., 2018). Nonetheless, the development of more selective ferroptosis inducers, such as the more selective GPX4 inhibitor ML210, will further help address this issue (Eaton et al., 2019).

More concerning are off-target effects associated with small-molecule ferroptosis inhibitors. Early studies reported that the first-generation MEK1/2 inhibitor U0126 potently inhibited ferroptosis (Dixon et al., 2014; Yagoda et al., 2007). However, U0126 has a cryptic off-target effect as a radical trapping

antioxidant that likely explains its ability to block ferroptosis (Gao et al., 2015). Likewise, several small-molecule lipoxygenase (LOX) inhibitors in common use, including NDGA, zileuton, and PD146176, can suppress ferroptosis by acting as radical trapping antioxidants, independent of effects on LOX enzymes (Shah et al., 2018). Although LOX enzymes do promote ferroptosis in some contexts (Dar et al., 2018; Wenzel et al., 2017), results obtained using putative small-molecule LOX inhibitors must be interpreted cautiously. Of special note, the original Nec-1 molecule can inhibit ferroptosis at high doses, independent of effects on RIPK1 (Angeli et al., 2014). This is potentially problematic for mechanistic studies, but dual necroptosis/ferroptosis inhibitors could conceivably be highly desirable for the treatment of pathological cell death events that involve both processes.

Looking ahead: Discovery and Characterization of Additional Nonapoptotic Cell Death Pathways

As illustrated by the history of necroptosis and ferroptosis, chemical biology-driven approaches have provided a fruitful means to discover and characterize nonapoptotic cell death mechanisms. Chemical biology-driven approaches are likewise providing evidence for several additional nonapoptotic lethal mechanisms, possibly distinct from known pathways. For example, a number of structurally distinct molecules, including an ursolic acid derivative (Sun et al., 2017), a synthetic azaindole-based compound (Huang et al., 2018), and the indole-based chalcone MOMIPP (Robinson et al., 2012) (Table 1), can all induce a nonapoptotic cell death process in cultured cells termed methuosis. Methuosis is characterized by the perturbation of endomembrane trafficking and accumulation of cytoplasmic vacuoles derived from macropinosomes (Maltese and Overmeyer, 2015). Lethal compound treatment accelerates macropinosome and late endosome formation and inhibits the fusion of late endosomes and autophagosomes with lysosomes. This leads to altered protein trafficking, impaired glucose uptake, and activation of the c-Jun N-terminal kinase pathway, which conspire together to trigger caspase-independent cell death, although the precise coup de grâce remains somewhat murky (Li et al., 2019; Mbah et al., 2017). The application of chemoproteomic and chemical genetic methods to identify key targets of the above lethal molecules and the pathways that regulate methuosis sensitivity is likely to be informative and currently in its infancy (Cho et al., 2018; Li et al., 2019).

Another distinct lethal mechanism is triggered by the synthetic oxime-containing small molecule caspase-independent lethal 56 (CIL56) (Shimada et al., 2016b) (Table 1). CIL56-induced cell death does not require key regulators of apoptosis, necroptosis, or ferroptosis, or involve immediate membrane permeabilization or bioenergetic disruption (Ko et al., 2019). Genome-wide shRNA chemical genetic screening identified two genes, zinc finger DHHC-type containing 5 (*ZDHHC5*) and Golgin A7 (*GOLGA7*), as being essential for CIL56-induced death. These two proteins together form a novel palmitoyl S-acyltransferase complex that appears to promote nonapoptotic cell death in CIL56-treated cells through effects on retrograde membrane trafficking from the plasma membrane, a completely novel mechanism. The use of chemoproteomic methods to identify the target(s) of CIL56 would help understand how this lethal mechanism is activated.

Studies of methuosis and CIL56-induced cell death may help unveil new cell death mechanisms and associated biochemical regulatory networks. Agents that induce these forms of cell death could be of use in the treatment of cancer, where the ultimate goal is cell death by any means necessary. Indeed, methuosis inducers and CIL56 (also known as CA3) can both attenuate xenograft tumor growth *in vivo* (Huang et al., 2018; Song et al., 2018). It is currently unclear whether methuosis-inducing small molecules or CIL56 mimics any physiological stimulus that a cell would normally experience *in vivo*. The identification of specific chemical suppressors for these pathways would enable roles for these pathways to be examined *in vivo* (e.g., during pathological cell death), as has been done sucessfully using necrostatins or ferrostatins/liproxstatins. Further application of chemical biology approaches will allow for a deeper exploration of these new cell death mechanisms and may enable discovery of additional, as-yet-unknown nonapoptotic cell death mechanisms.

SIGNIFICANCE

Chemical biology approaches have been central to the discovery and characterization of multiple nonapoptotic cell death pathways. The acuteness and portability of chemical tools make them mainstays of cell death research. These tools can be productively combined with genetic and biochemical methods to help characterize nonapoptotic cell death mechanisms and be further used to discover additional chemical regulators of cell death. Small-molecule off-target effects must be recognized and accounted for in all studies. Broader application of chemical biology methods could lead to the discovery of additional new forms of nonapoptotic cell death.

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DECLARATION OF INTERESTS

S.J.D. is on the scientific advisory board of Ferro Therapeutics, has consulted for Toray Industries and AbbVie Inc., and is an inventor on patents related to ferroptosis.

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