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Review article

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The therapeutic potential of targeting regulated non-apoptotic cell death

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Abstract

Cell death is critical for the development and homeostasis of almost all multicellular organisms. Moreover, its dysregulation leads to diverse disease states. Historically, apoptosis was thought to be the major regulated cell death pathway, whereas necrosis was considered to be an unregulated form of cell death. However, research in recent decades has uncovered several forms of regulated necrosis that are implicated in degenerative diseases, inflammatory conditions and cancer. The growing insight into these regulated, non-apoptotic cell death pathways has opened new avenues for therapeutic targeting. Here, we describe the regulatory pathways of necroptosis, pyroptosis, parthanatos, ferroptosis, cuproptosis, lysozincrosis and disulfidptosis. We discuss small-molecule inhibitors of the pathways and prospects for future drug discovery. Together, the complex mechanisms governing these pathways offer strategies to develop therapeutics that control non-apoptotic cell death.

Sections

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Introduction

In multicellular organisms, both cell division and cell death are essential to maintaining normal tissue homeostasis. The classical notion has been that there are only two types of cell death modalities: apoptosis, which occurs through a regulated process, and accidental necrosis, which is not regulated. Apoptosis was discovered in the 1970s[1] as a genetically and developmentally programmed and regulated form of cell death. Intrinsic or extrinsic inducers activate caspases (cysteine proteases specific for aspartate-containing sequences), which in turn execute apoptotic cell death – a process characterized by DNA and organelle fragmentation, cell shrinkage, cytoskeleton collapse and formation of apoptotic bodies[2] (Fig. 1). However, the past two decades have revealed that a number of non-apoptotic cell death modalities are also executed in a regulated fashion and, in some cases, are genetically and developmentally programmed. These cell death pathways are therefore referred to as 'regulated non-apoptotic cell death' or 'regulated necrosis'. These pathways are regulated by diverse upstream signals such as cytokines and bacterial or viral components and are independent of caspases 3 and 7, which are required for apoptosis[3,4].

Regulated non-apoptotic cell death modalities include necroptosis, ferroptosis, oxytosis, pyroptosis, parthanatos, NETosis, cuproptosis, lysozincrosis, disulfidptosis and autophagic cell death[4]; the extent to which each of these modalities is consistent with the classic notion of necrosis remains unclear. These modes of cell death are involved in distinct cellular and tissue processes, but share some similar characteristics, as well as having clear differences (Box 1). The defining characteristics of each pathway include the mitochondrial status, the level of DNA and chromatin fragmentation, the involvement of membrane rupture, and whether cell contents are released. Key characteristics of several of the best-studied cell death pathways are summarized in Fig. 1. Interestingly, the similarities between the pathways indicate that one mode of cell death might represent a backup mechanism for another cell death pathway. Different modes of regulated cell death can induce varying levels of immunogenicity. For instance, apoptosis is generally immunologically silent, due to the formation of apoptotic bodies and prevention of intracellular content release, whereas necroptosis and pyroptosis are pro-inflammatory processes that release damage-associated molecular patterns (DAMPs) and cytokines[2,4].

Regulated cell death pathways are important in various disease settings. For example, a plethora of degenerative diseases are intimately linked to the death of essential cells, and cell death can occur by more than one pathway in a particular disease (Table 1). On the other hand, many aberrant cell types, such as neoplastic, fibrotic and inflammatory cells, become resistant to specific cell death pathways. Therefore, a comprehensive understanding of cell death modalities should enable specific modulation of cell death for therapeutic benefit by preserving critical cells in degenerative contexts and eliminating problematic cells in contexts such as cancers.

Apoptotic signalling pathways have been a focus of drug discovery efforts for several years. Induction of apoptosis in the context of cancer treatment has previously been realized by targeting microtubules, oxidative phosphorylation, RNA synthesis, topoisomerase II and distinct kinases. However, there is a high incidence of resistance developing with these therapies. Hence, broadening our understanding of regulated cell death pathways beyond apoptosis is now fuelling the discovery of a further rich set of potential drug targets; small molecules that modulate these pathways could have an impact in many disease settings (Table 2). Importantly, non-apoptotic cell death pathways have the greatest potential for treating degenerative diseases, and combination therapies that target multiple pathways at a time could prove beneficial to overcoming resistance mechanisms.

In this Review, we explain key mechanistic features of the regulated necrosis pathways of necroptosis, pyroptosis, parthanatos,

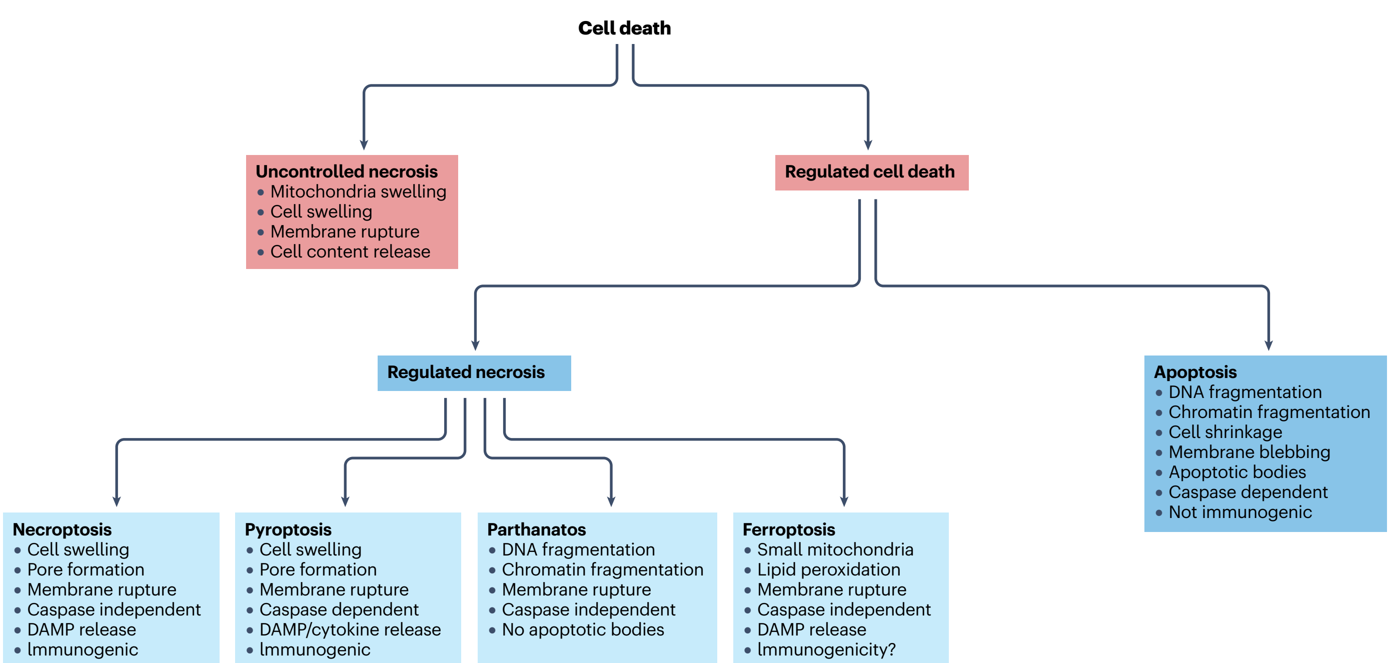


Fig. 1 | Overview of cell death modalities. Key characteristics of various cell death modalities, illustrating similarities and differences. DAMP, damage-associated molecular pattern.

Box 1

Similarities and differences between cell death modalities

Cell death modalities have their unique characteristics but also share similarities. Key characteristics of several cell death modalities are depicted in Fig. 1. Here, we describe selected similarities and differences in pairwise comparisons for distinct cell death pathways.

Apoptosis versus necroptosis

- Similarities: TNFR activation leads to the formation of complex I, which is a step common to the initiation of both apoptotic and necroptotic cell death. This complex contains RIPK1 and undergoes various essential ubiquitination events performed by cIAP1, cIAP2 and LUBAC[9] (Fig. 2).
- Differences: active caspase 8 is a pre-requisite for apoptosis execution; in contrast, inactivation or depletion of caspase 8 is needed to drive necroptosis. Furthermore, DNA and organelle fragmentation together with apoptotic bodies are hallmarks of apoptosis, whereas regulated lysis and content release through MLKL pores are critical to the execution of necroptosis[2] (Fig. 2). Hence, apoptosis is generally immunologically silent, whereas necroptosis has a pro-inflammatory function[2].

Necroptosis versus pyroptosis

- Similarities: necroptosis and pyroptosis can be triggered by pathogen components and host signalling molecules; importantly, both cell death modalities release damage-associated molecular patterns (DAMPs) to facilitate the inflammatory response[68]. Lytic pore formation is an integral part of cell death execution — MLKL-derived pores are generated in necroptosis[27,28] (Fig. 2), whereas N-GSDMD-derived pores are formed in pyroptosis[79,80] (Fig. 4a).
- Differences: necroptosis is initiated by TNFR or TLR activation and is dependent on RIPK1 and RIPK3 activation along with caspase 8 inhibition (Fig. 2); pyroptosis is dependent on inflammasome (NLRP3–ASC–pro-caspase 1) formation upon sensing of pathogen-associated molecular patterns and DAMPs, leading to caspase 1 activation[2] (Fig. 4a). Activation of MLKL in necroptosis occurs through phosphorylation[25,26], whereas activation of N-GSDMD is dependent on proteolytic events[75].

Ferroptosis versus oxytosis

- Similarities: ferroptosis and oxytosis are suppressed by system x_c^- and GPX4; hence, depletion of GSH leads to loss of GPX4 activity and accumulation of lipid hydroperoxides to trigger cell death[225]. Both cell death modalities can be inhibited by ferrostatins, liproxstatins and α-tocopherol[225] (Fig. 5). Oxytosis execution critically depends on calcium levels upon calcium influx; recent studies indicate that calcium levels are also important in some contexts for ferroptotic cell death[226,227].
- Differences: oxytosis, which is induced by excess glutamate, has been demonstrated in neuronal cells[228]; in contrast, ferroptosis occurs in almost all tissues as well as in responsive cancers[189]. Furthermore, the role of AIF seems restricted to oxytosis but not ferroptosis[137,139,229].

ferroptosis, cuproptosis, lysozincrosis and disulfidptosis. In addition, we describe diseases that are caused by deregulation of these pathways and how these cell death modalities could be targeted for therapeutic development. Several small-molecule modulators that can either induce or inhibit non-apoptotic cell death are discussed. Thus, we provide a roadmap for the emerging landscape of controlling cell death for therapeutic gain, which we expect to be a rapidly growing sector of the pharmaceutical and biotechnology industries.

Necroptosis

Necroptosis is a regulated form of cell death in response to infection that is independent of caspases[5]. Importantly, necroptosis can occur in conditions where apoptosis is inhibited and therefore represents a potential backup cell death strategy. However, unlike apoptosis, necroptosis is a pro-inflammatory pathway, in which cytokines and DAMPs are eventually released to trigger inflammation[6]. Necroptosis was first discovered two decades ago as a cell death modality that can be inhibited with the small molecule necrostatin 1 (Nec1)[7,8]. The discovery of Nec1 – a catalytic inhibitor of receptor-interacting serine–threonine protein kinase 1 (RIPK1) – helped define necroptosis as a regulated type of necrosis rather than as a passive form of cell death. The major physiological role of necroptosis is to induce an innate immune response upon sensing of bacterial and viral components through the Toll-like receptor (TLR)–TIR domain-containing adapter inducing interferon-β (TRIF) signalling cascade. Alternatively, an inflammatory response is induced through the tumour necrosis factor (TNF)–RIPK1 signalling cascade (Fig. 2). Although both apoptosis and necroptosis can be initiated by TNF signalling, apoptosis is dependent on active caspase 8, whereas necroptosis occurs when caspase 8 is inactive.

Regulators of necroptotic cell death

TNF signalling can induce necroptosis or apoptosis, depending on downstream signalling events. TNF binds to TNF receptor 1 (TNFR1), which leads to the recruitment of TNF receptor type 1-associated DEATH domain protein (TRADD), TNF receptor-associated factor 2 (TRAF2), RIPK1 and cellular inhibitor of apoptosis protein 1 (cIAP1) and cIAP2, thereby forming the multi-protein complex I[9] at the plasma membrane (Fig. 2). Subsequently, a series of ubiquitination and phosphorylation events modify complex I to trigger downstream signal transduction events that result in apoptosis, necroptosis, or activation of NF-κB and proliferation[2].

First, the E3 ligases cIAP1 and cIAP2 ubiquitinate RIPK1 with Lys63-linked chains[10], which are platforms for binding of the linear ubiquitin chain assembly complex (LUBAC) – consisting of HOIL1, HOIP and SHARPIN – and the TAK1–TAB complex. LUBAC then modifies RIPK1 with linear ubiquitin chains that recruit NF-κB essential modulator

(NEMO) and inhibitor of NF-κB kinase (IKK). The TAK–TAB complex and the IKK complex ultimately activate NF-κB and mitogen-activated protein kinase (MAPK) pathways, which drive proliferation and inflammation[11], thus preventing cell death events.

Second, in apoptosis-competent cells, reduced NEMO–NF-κB signalling activates TNF-driven apoptosis[12,13]. Apoptotic cell death can be dependent upon RIPK1 or independent of it; importantly, both types of apoptosis induction are mediated by caspase 8 (refs. 14,15).

Third, in the event of inactive caspase 8, RIPK1 can also stimulate necroptosis, but this requires RIPK1 to be deubiquitinated. Linear ubiquitin chains are removed from RIPK1 by the deubiquitinases CYLD or SPATA2 (refs. 16–18) and OTULIN[19,20], whereas Lys63-linked chains are removed by the deubiquitinase A20 (refs. 18,21). Deubiquitinated RIPK1 is released from complex I into the cytosol, where it autophosphorylates and becomes activated. Subsequently, RIPK1 binds to RIPK3, which also becomes activated via autophosphorylation[22], and the two proteins form the necrosome, a microfilament-like complex of RIPK1–RIPK3 hetero-oligomeric amyloids or RIPK3 homo-oligomeric amyloids[23,24]. Activated RIPK3 phosphorylates and activates mixed lineage kinase domain-like (MLKL), which polymerizes and forms pores that disrupt the membrane and execute necroptosis[25–28] (Fig. 2).

In addition to TNF signalling, the TLR–TRIF signalling cascade is another pathway that can induce necroptosis. Viral RNAs binding to TLR3 or lipopolysaccharide (LPS) binding to TLR4 initiate recruitment of TRIF to the TIR domain of the TLRs. Subsequently, TRIF interacts with RIPK3, which becomes activated through autophosphorylation. This process also activates MLKL via phosphorylation, which again executes necroptosis by disrupting the plasma membrane[29] (Fig. 2).

Table 1 | The involvement of regulated necrosis modalities in selected diseases

	Necroptosis	Pyroptosis	Parthanatos	Ferroptosis
Alzheimer disease	x	x	x	x
Parkinson disease	x		x	x
Huntington disease			x	x
Amyotrophic lateral sclerosis	x			x
Multiple sclerosis	x			x
Stroke	x		x	x
Acute kidney failure	x			x
Acute heart failure	x			x
Necrotic liver injury		x		x
Acute respiratory distress	x			x
COPD	x			x
Systemic inflammation	x			x
Atherosclerosis		x		x
Bacterial infections		x		x
Multiorgan dysfunction				x
Inflammatory bowel disease	x			

The in vivo (mouse models or human patient samples) occurrence of necroptosis, pyroptosis, parthanatos and ferroptosis in selected diseases. COPD, chronic obstructive pulmonary disease.

Necroptosis in disease

Necroptotic cell death is implicated in diverse disease settings, including neurological disorders, heart failure, inflammation and pulmonary diseases (Table 1). A large body of evidence supports a role for necroptosis in neurological disorders, including Alzheimer disease (AD), Parkinson disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS) and stroke. For example, an AD-related mouse study by Yang et al. showed that Nec1 inhibits neural amyloid-β (Aβ)-induced cell death through reduction of Aβ plaques as well as by reducing tau hyperphosphorylation and aggregation, which alleviated cognitive impairment in behavioural studies[30]. Moreover, necroptosis is activated in human AD brains and negatively correlates with brain weight[31]. The involvement of necroptosis in PD was shown in a mouse study, where Nec1s counteracted the death of dopaminergic neurons induced by a neurotoxin[32]. Further, Ito et al. provided evidence that enhanced levels of RIPK1 resulting from optineurin (OPTN) deficiency, which is implicated in ALS, mediate axonal degeneration through necroptosis. RIPK1, RIPK3 and MLKL also drive axonal pathology in SOD1-mutant mice, arguing for an involvement of necroptosis in the pathology of ALS[33]. In addition, human pathological samples of MS show activation of RIPK1, RIPK3 and MLKL, indicating the presence of necroptosis. In line with this finding, oligodendrocytes can undergo cell death through TNF-mediated necroptosis, and RIPK1 inhibitors counteract this process in animal models of MS[34]. Finally, necroptosis is implicated in stroke, where the necroptosis inhibitor Nec1 reduces infarct volume in mice[7].

Besides the nervous system, other organs can be affected by necroptosis. Several in vivo studies describe the involvement of necroptosis in pulmonary diseases. A rat model of acute respiratory distress syndrome (ARDS) is associated with upregulation of RIPK1, RIPK3 and MLKL[35]. Conversely, *Ripk3*$^{-/-}$ mice have a reduced disease phenotype in LPS-induced ARDS[36], and Nec1 treatment reduces inflammation and neutrophil infiltration in rats with ARDS[37]. In chronic obstructive pulmonary disease, cigarette smoke induces necroptotic cell death and neutrophil-derived airway inflammation in vivo[38,39]. In addition to pulmonary diseases, necroptosis mediates myocardial infarction. RIPK3 is upregulated upon myocardial infarction, and depletion of *Ripk3* significantly reduces in vivo infarct size[40]. Therapeutically, Nec1 reduces infarct size in a mouse model of myocardial ischaemia–reperfusion[41,42]. Moreover, renal ischaemia–reperfusion injury can be caused by necroptosis in vivo, and acute kidney injury is inhibited by application of Nec1 (refs. 43,44).

Necroptosis can also be detected in inflammatory diseases. TNF-induced systemic inflammatory response syndrome (SIRS) is mediated by necroptosis[45] (although tissue damage is only partly due to necroptosis[46]), and depletion of RIPK3 or inactivation of RIPK1 by Nec1 protects against lethal SIRS in mouse studies[45]. TNF-induced SIRS in mice can also be attenuated by the selective RIPK1 inhibitor PK68 (ref. 47). Furthermore, keratinocyte necroptosis in mice can

Table 2 | Selected small-molecule modulators of regulated necrosis

Compound name	Mode of action	Potency (IC_{50}) (nM)[a]	Cell line	Stage of development	Refs.
Necroptosis inhibitors					
Necrostatin 1s	RIPK1 inhibitor	200	Jurkat	Animal models	7,53
GSK2982772	RIPK1 inhibitor	6	U937	Clinical trials	55
GSK547	RIPK1 inhibitor	32	L929	Animal models	56
6E11	RIPK1 inhibitor	4,600	Jurkat	Cellular models	61
PK68	RIPK1 inhibitor	23	HT-29	Animal models	47
GSK840	RIPK3 inhibitor	100–300	HT-29	Cellular models	64
GSK843	RIPK3 inhibitor	3,000	HT-29	Cellular models	64
GSK2399872	RIPK3 inhibitor	300–500	HT-29	Animal models	64
Necrosulfonamide	MLKL inhibitor	124	HT-29	Animal models	25,65
GW806742X	MLKL inhibitor	589	HT-29	Animal models	28,66
TC13172	MLKL inhibitor	2	HT-29	Cellular models	67
Pyroptosis inhibitors					
MCC950	NLPR3 inhibitor	24	BMDMs	Animal models	97
CY-09	NLPR3 inhibitor	5,000	BMDMs	Animal models	98
OLT1177	NLPR3 inhibitor	1–100	J774A.1	Animal models	99
VX-765/VRT-043188	Caspase 1 inhibitor	870	PBMCs	Clinical trials	94
Parthanatos inhibitors					
Olaparib	PARP1 inhibitor	43	MDA-MB-436	Approved	108,116,117
Niraparib	PARP1 inhibitor	18	MDA-MB-436	Approved	108,116,118
Rucaparib	PARP1 inhibitor	609	Capan-1	Approved	108,116,119
Talazoparib	PARP1 inhibitor	5	Capan-1	Approved	108,116,119
A-966492	PARP1 inhibitor	1	C41	Animal models	122
PJ34	PARP1 inhibitor	30–1,000	Primary neuronal cells	Animal models	123
Ferroptosis inducers					
Erastin	System x_c^- inhibitor	600	BJeLR	Cellular models	191
Imidazole ketone erastin	System x_c^- inhibitor	3	BJeLR	Animal models	191
Sorafenib	System x_c^- inhibitor	18,000	HT-1080	Animal models	200
Buthionine sulfoximine	γGCS inhibitor	4,900	ZAZ	Clinical trials	207,208
(1S,3R)-RSL3	GPX4 inhibitor	10	BJeLR	Cellular models	154
ML210	GPX4 inhibitor	70	LOX-IMVI	Cellular models	202,203
FIN56	GPX4 inhibitor and squalene synthase activator	200	HT-1080	Cellular models	204
$FINO_2$	GPX4 inhibitor and iron oxidation	11,000	BJeLR	Cellular models	206
iFSP1	FSP1 inhibitor	100	Pfa1	Cellular models	163
FSEN1	FSP1 inhibitor	70	$H460^{C}$ $GPX4^{KO}$	Cellular models	213
Ferroptosis inhibitors					
Ferrostatin 1	Lipid peroxidation and PEBP1 inhibitor	45	MEFs	Cellular models	214
UAMC-3203	Lipid peroxidation inhibitor	10	IMR-32	Animal models	216,217
Liproxstatin 1	Lipid peroxidation inhibitor	38	MEFs	Animal models	214
α-Tocopherol	Lipid peroxidation inhibitor	1,800	MEFs	Animal models	214
Tetrahydrobiopterin	Lipid peroxidation inhibitor	21,000	HT-1080	Animal models	165
Deferoxamine	Iron depletion	100,000	HT-1080	Animal models	126
Ciclopirox	Iron depletion	5,000	HT-1080	Animal models	126

BMDMs, bone marrow-derived macrophages; MEFs, mouse embryonic fibroblasts; PBMCs, peripheral blood mononuclear cells. [a]The IC_{50} values can vary depending on the cell line being used.

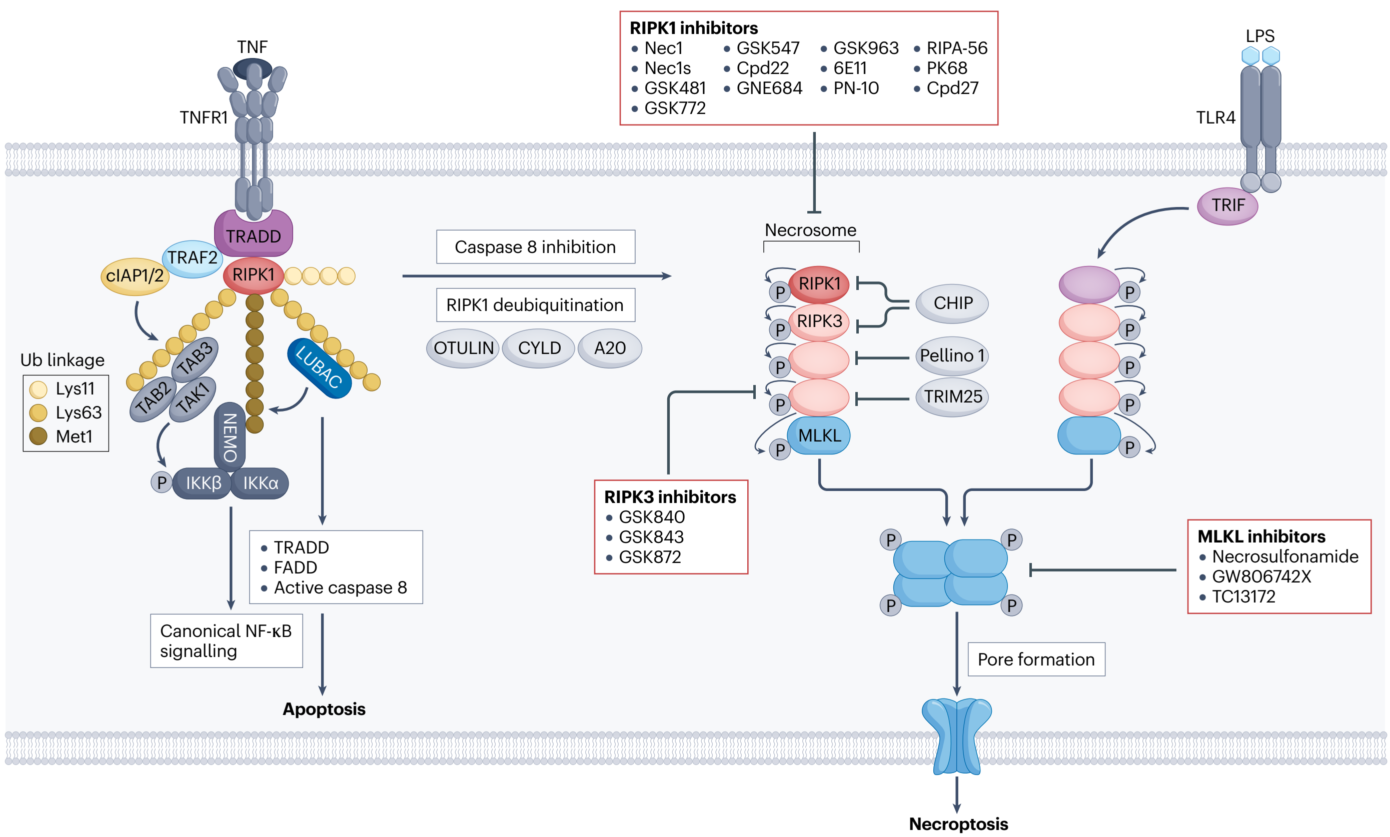


Fig. 2 | Pathways inducing necroptosis. TNF binding to TNFR1 leads to the formation of complex I, consisting of TRADD, TRAF2, RIPK1, cIAP1 and cIAP2. Ubiquitination and phosphorylation events can trigger apoptosis, necroptosis or NF-κB activation. Lys63-linked chains on RIPK1 generated by cIAP1 and cIAP2 form a scaffold for the LUBAC complex and the TAK1–TAB complex to bind. Linear ubiquitination of RIPK1 by LUBAC recruits the NEMO–IKK complex. Inhibition of NEMO–NF-κB signalling activates TNF-driven cell death. Necroptosis is initiated by inhibition of caspase 8 together with RIPK1 deubiquitination by the deubiquitinases CYLD and OTULIN, which remove linear chains, as well as the deubiquitinase A20, which removes Lys63-linked chains. Subsequently, RIPK1 is released from complex I into the cytosol, where it autophosphorylates to become activated and interacts with RIPK3, which is also activated via autophosphorylation. RIPK1 and RIPK3 generate the necrosome. Finally, RIPK3 phosphorylates MLKL, which stimulates pore formation and membrane disruption to execute necroptosis. The E3 ligases CHIP, Pellino 1 and TRIM25 ubiquitinate RIPK1 or RIPK3 to inhibit necroptosis. Activation of TLRs also induces necroptosis. Viral RNAs binding TLR3 or lipopolysaccharide (LPS) binding TLR4 recruits TRIF to the TIR domain of the receptor. The TRIF–RIPK3 interaction activates RIPK3 through autophosphorylation and also activates MLKL, leading to necroptosis. Small-molecule inhibitors of RIPK1, RIPK3 and MLKL reduce necroptotic cell death.

cause skin inflammation[48,49]. Necroptosis also plays a crucial role in pancreatitis, where acinar cell death in vivo could be prevented by Nec1 or *Ripk3* depletion[50]. Finally, necroptosis has a critical role in the in vivo pathology of inflammatory bowel disease (IBD)[51,52].

Inhibitors of necroptosis

To inhibit necroptosis in the aforementioned diseases, three major targets have been exploited — RIPK1, RIPK3 and MLKL (Figs. 2 and 3a and Table 2). Following the first necroptosis inhibitor, Nec1 (refs. 7,8), a more stable analogue, Nec1s, was developed that specifically inhibits RIPK1 and has a better in vivo efficacy profile[7,53]. GlaxoSmithKline (GSK) has generated several RIPK1 inhibitors, including GSK2882481 (GSK481)[54], GSK2982772 (GSK772)[55] and GSK547 (ref. 56). GSK772 has entered clinical testing[57] and demonstrated a favourable safety profile; it is currently in various phase Ib and phase II trials for ulcerative colitis, rheumatoid arthritis and psoriasis. Furthermore, the RIPK1 inhibitor compound 22 (Takeda) demonstrated preclinical efficacy in a MS mouse model[58], and GNE684 (Genentech)[59] reduced the arthritis index in a mouse model[57]; both compounds are benzoxazepinone-derived RIPK1 inhibitors similar to GSK481 and GSK772. Moreover, there are a number of tool compounds inhibiting RIPK1: GSK963, a selective and potent inhibitor for in vitro and in vivo use[60]; 6E11, a natural product derivative with exquisite selectivity and inhibition in cell models[61]; PN-10, a hybrid molecule of ponatinib and Nec1 with high potency and selectivity, also for in vivo use[62]; and RIPA-56 (ref. 63) and PK68 (ref. 47), both potent and selective inhibitors with in vivo applicability. These tool compounds can be applied to interrogate the RIPK1-mediated necroptosis pathway.

The small molecules GSK840, GSK843 and GSK2399872 (GSK872) (developed by GSK) are RIPK3 inhibitors[64]. Although these compounds inhibit necroptosis, they also induce apoptosis and thus have little therapeutic benefit for necroptosis-related degenerative diseases.

Therefore, additional efforts are needed to generate and validate selective RIPK3 inhibitors for in vivo application. Finally, there are few small molecules that inhibit MLKL. Sun et al. identified necrosulfonamide as an inhibitor of necroptosis acting downstream of RIPK3 and used chemical proteomics analysis to identify MLKL as its direct target[25]. Necrosulfonamide treatment of mice with spinal cord injury improved neurological impairment[65]. GW806742X is another MLKL and necroptosis inhibitor[28] that attenuates the pathology in an in vivo asthma model[66]; however, it has off-target activity against RIPK1 and RIPK3 (ref. 67). Moreover, TC13172 is a potent MLKL inhibitor with single digit nanomolar efficacy in cell models and no off-target activity against RIPK1 and RIPK3; thus, it is an exciting molecule for further exploration in preclinical in vivo models. TC13172 covalently binds to cysteine 86 of MLKL[67].

In summary, RIPK1 inhibitors hold the most promise to test the clinical potential of inhibiting necroptosis for the treatment of distinct diseases (Table 1). Notably, beyond the RIPK1 inhibitors listed in Fig. 3a and Table 2, there are additional unpublished RIPK1 inhibitors from Denali Therapeutics that are being tested in clinical phase Ia and Ib–IIa trials for the treatment of diseases such as AD and ALS[57].

Pyroptosis

As part of the innate immune response, pyroptosis is a cellular defence mechanism against extracellular pathogen-associated molecular patterns (PAMPs) and DAMPs in the canonical pathway or against intracellular LPS in the non-canonical pathway[68]. Similarly to necroptosis, pyroptosis releases cytokines to activate immunological processes[2]. The canonical pyroptosis pathway is mediated by caspase 1 (originally named IL-1β-converting enzyme; ICE), whereas the non-canonical pyroptosis pathway is mediated by caspases 4, 5 and 11 (refs. 69–71). Both pathways activate gasdermin D (GSDMD), an effector protein that executes pyroptosis (Fig. 4a).

Regulators of pyroptotic cell death

In the canonical signalling cascade, sensing of cellular stressors (such as bacteria, viruses and toxins) by TLRs leads to the formation of the active inflammasome[72], a supramolecular disc complex composed of NOD-like receptor family pyrin-domain-containing 3 (NLRP3), the bridging factor ASC and pro-caspase 1 (ref. 73). Inflammasome formation subsequently leads to dimerization of pro-caspase 1 and its activation via autocleavage[73,74]. Activated caspase 1 in turn cleaves the effector protein GSDMD to generate the active component GSDMD-N[2,75]. Moreover, caspase 1 cleaves pro-IL-1β and pro-IL-18 to generate their active forms IL-1β and IL-18 (refs. 76–78). Activated GSDMD-N forms membrane pores, which lead to cell lysis by pyroptosis[79,80] and the release of IL-1β and IL-18 (refs. 79,81) (Fig. 4a). In the non-canonical pathway, caspases 4, 5 and 11 are activated when they directly bind and sense intracellular LPS — without the need for inflammasome formation — and they subsequently cleave GSDMD to execute pyroptosis[70,82] (Fig. 4a). IL-1β and IL-18 are not processed nor released.

Pyroptosis in disease

Pyroptosis is implicated in several diseases, including inflammation, cardiovascular diseases and infectious diseases (Table 1). Upon necrotic liver injury in mice, the immune response leads to the recruitment of eosinophils that undergo caspase 1-mediated pyroptosis and release IL-1β and IL-18. This process can be reduced by treating cells extracted from necrotic liver with a caspase 1 inhibitor[83]. In mouse atherosclerotic lesions, cholesterol crystals are identified in necrotic lesions. Here, cholesterol crystals induce an inflammatory response by stimulating NLRP3 inflammasomes and caspase 1 activation, leading to IL-1 secretion[84]. Such cholesterol crystals also induce pyroptosis in mouse endothelial cells[85]. A further mouse study demonstrated that hyperlipidaemia activates caspase 1 and pyroptosis, leading to the destruction of endothelial cells and loss of vascular endothelium function[86]. In a mouse model of AD, NLRP3 is activated together with enhanced caspase 1 activity, which leads to neuroinflammation and pronounced symptoms. Importantly, mice with deletions of *Nlrp3* and *Casp1* in a genetic background of AD (APP/PS1 mice) were largely protected from impaired spatial memory[87]. Moreover, after pyroptosis of microglia, ASC specks are released, which bind Aβ and promote its aggregation, deposition and plaque formation. An *Asc* knockout in the background of APP/PS1 significantly reduced AD pathology in mice[88]. ACS or ACS–Aβ composites released from pyroptotic cells can be incorporated into NLRP3 inflammasomes of neighbouring microglia, facilitating further pyroptosis. This process can act in cycles that amplify cell death and chronic neuroinflammation[89].

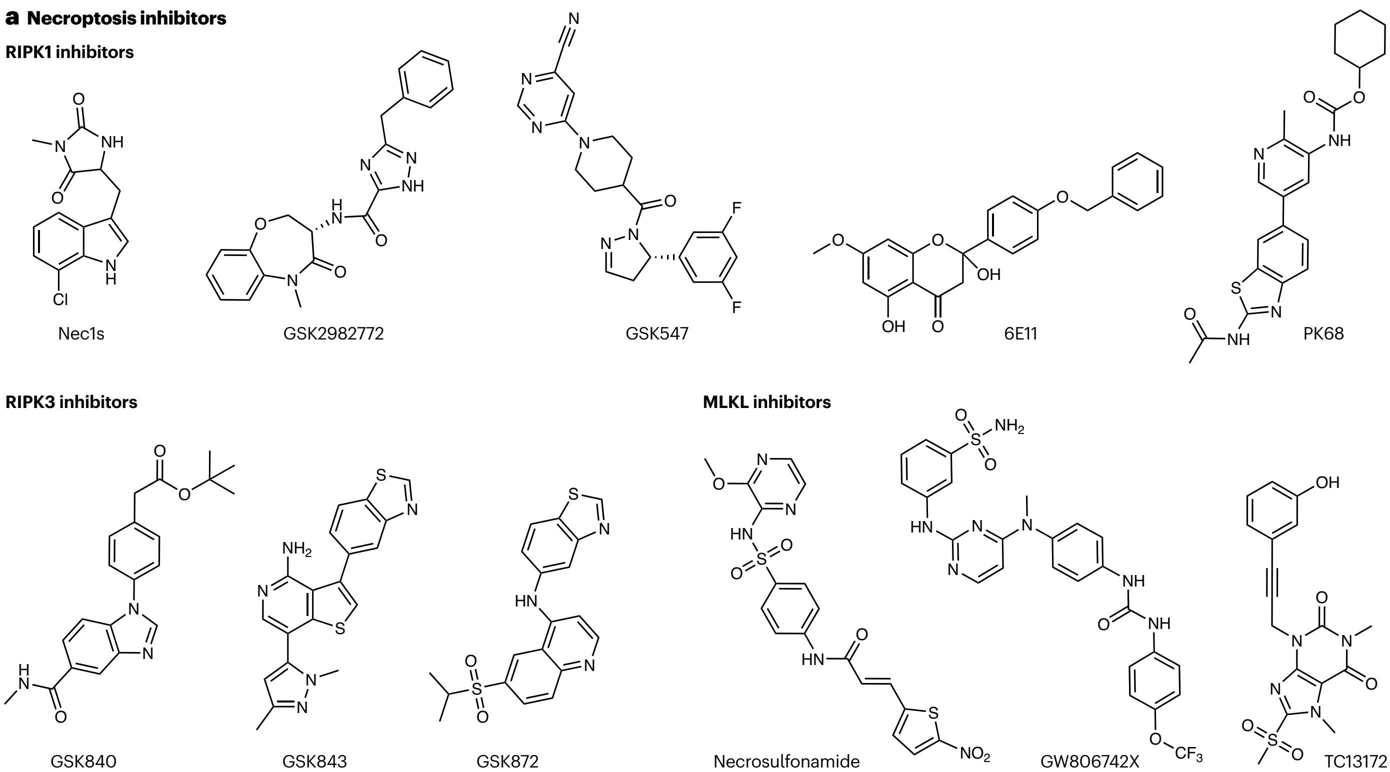
Pyroptosis is also prevalent in diverse infectious diseases. For example, infection of $CD4^+$ T cells with human immunodeficiency virus (HIV) leads to caspase 1-mediated pyroptotic cell death in cell models, as well as in lymph nodes from patients with chronic HIV infection. The death of $CD4^+$ T cells establishes chronic inflammation that leads to further cell death and eventually causes acquired immunodeficiency syndrome. Notably, caspase 1 inhibition interrupted this cycle of death and inflammation[90]. As another example, bacterial infection of hepatocytes in vivo can be cleared by a combination of pyroptosis and IL-18-mediated cytotoxicity by natural killer cells[91].

Pyroptosis might also have an important role in some cancers. Interestingly, pyroptosis can have a dual role in cancer development, on the one hand eliminating certain tumours but, on the other, contributing to a pro-tumour microenvironment supporting tumour growth. A recent review by Yu et al. summarizes aspects of pyroptosis and cancer[71].

Inhibitors of pyroptosis

Several small-molecule inhibitors of caspase 1 and NLRP3 have been developed (Figs. 3b and 4a and Table 2), whereas targeting GSDMD has been a challenge and requires future efforts. Administration of the caspase 1 inhibitor pralnacasan (VX-740; Vertex) reduced levels of IL-1β and IL-18 in a mouse model of IBD and reduced the IBD clinical score with no apparent side effects[92]. In addition, the pro-drug VX-765 is a potent caspase 1 inhibitor, with its derivative VRT-043198 representing the active inhibitory drug[93]. VRT-043198 effectively inhibits the release of IL-1β and IL-18 and has high selectivity towards caspase 1 over caspases 3, 6 and 9, and thus does not inhibit apoptosis. VRT-043198 also has a 16-fold higher selectivity for caspase 1 over caspase 8 (ref. 93). Oral administration of VX-765 had a positive impact on the disease outcome of rheumatoid arthritis and skin inflammation in relevant mouse models[94]. VX-765 also reduced Aβ accumulation, axonal degradation and neuroinflammation, and restored cognitive functions in a mouse AD model[95]. VX-765 has entered clinical testing in patients with epilepsy (clinical trial number NCT01501383).

The first inhibitor of NLRP3 was MCC950, which inhibits NLRP3-mediated inflammasome activation at nanomolar concentrations and reduces IL-1β levels in various in vivo disease models. Mechanistically, MCC950 binds at the NLRP3 ATP-hydrolysis motif to block NLRP3 and inflammasome activation[96,97]. Similarly, CY-09 binds the NLRP3 ATP-binding motif and inhibits its ATPase activity, thus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GW806742X
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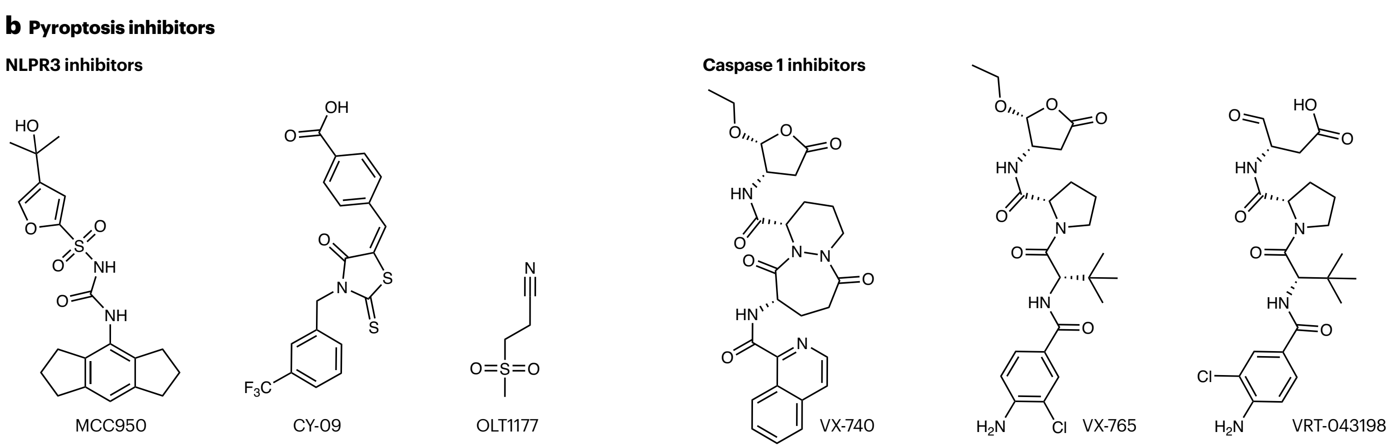
b Pyroptosis inhibitors
NLPR3 inhibitors
MCC950
CY-09
OLT1177
Caspase 1 inhibitors
VX-740
VX-765
VRT-043198

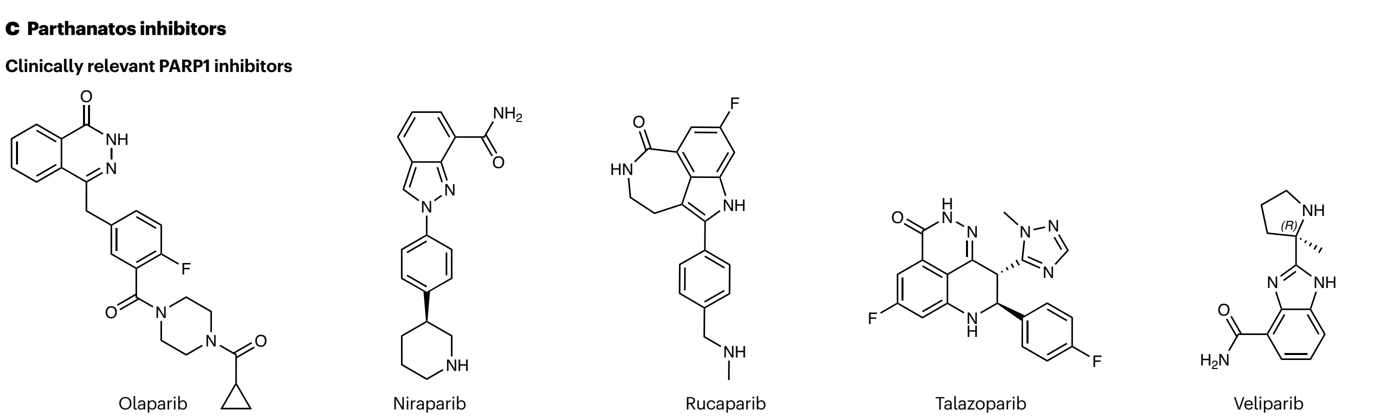
c Parthanatos inhibitors
Clinically relevant PARP1 inhibitors
Olaparib
Niraparib
Rucaparib
Talazoparib
Veliparib

Fig. 3 | Selected inhibitors of necroptosis, pyroptosis and parthanatos. a, Necroptosis inhibitors that target RIPK1, RIPK3 or MLKL. **b**, Pyroptosis inhibitors that target NLPR3 or caspase 1. **c**, Parthanatos inhibitors that target PARP1 and are clinically relevant.

reducing inflammasome activation. CY-09 is effective in inflammatory mouse models[98]. Another inhibitor, OLT1177, inhibits inflammasome oligomerization by counteracting NLRP3–ACS and NLRP3–caspase 1 interactions and inhibits IL-1β and IL-18 release both in vitro and in vivo[99].

In summary, current inhibitors of pyroptosis are mainly at the stage of preclinical development, and either these molecules need further optimization to be developed into clinical candidates or additional candidates need to be generated to progress pyroptosis inhibition into the clinic. The most promising targets are NLRP3 and caspase 1.

Parthanatos

A further regulated non-apoptotic cell death pathway is parthanatos, which is a response to severe and prolonged alkylating DNA damage. Parthanatos is sensed by poly(ADP-ribose) polymerase 1 (PARP1) and is caspase independent[100]. It involves the mitochondria-located apoptosis-inducing factor (AIF) in the process of cell death execution[100]. Similarly to apoptosis, parthanatos leads to DNA fragmentation but, in contrast, it lacks apoptotic bodies because membrane rupture occurs[4].

Regulators of parthanatos

Extensive DNA damage is induced by factors such as nitric oxide (NO)-derived peroxynitrite, reactive oxygen species, DNA-alkylating agents such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), UV light and irradiation. DNA damage leads to hyperactivation of PARP1 and extensive poly(ADP-ribose) (PAR) polymer formation from NAD^+ moieties. These PAR polymers are the major cell death signal[101,102] (Fig. 4b). Importantly, parthanatos is not inhibited by caspase inhibitors, confirming that this cell death pathway is caspase independent[100]. The enzyme poly(ADP-ribose) glycohydrolase (PARG) can hydrolyse PAR and thus counteract PAR-mediated cell death by parthanatos[102,103]. Additionally, ADP-ribosyl hydrolase 3 (ARH3) can hydrolyse protein-free PAR and might protect against parthanatos[100]. Moreover, IDUNA (RNF146)[104] and TRIP12 (ref. 105) are PAR-dependent E3 ligases that ubiquitinate PARP1 to trigger its proteasomal degradation; thus, both E3 ligases protect cells from MNNG-induced parthanatos. Excess PAR translocates as free polymers to the cytoplasm, where it binds with high affinity to AIF localized at the mitochondrial outer membrane. This interaction is necessary to trigger the dissociation of AIF from mitochondria[103,106]. Upon its mitochondrial release, AIF interacts with the macrophage migration inhibitory factor (MIF), an endonuclease that enables translocation of the AIF–MIF complex to the nucleus[107]. Inside the nucleus, the AIF–MIF complex binds DNA and MIF degrades the DNA, thereby driving cell death by parthanatos[107] (Fig. 4b), which can have pathological consequences.

Parthanatos in disease

PARP1 has several cellular functions, including its critical role in DNA repair. Inhibition of PARP1 sensitizes tumour cells towards ionizing radiation and this strategy has been used for anticancer therapy[108]. However, extensive and prolonged activation of PARP1 can also lead to parthanatos[108], which is mainly implicated in brain disorders, including PD[109], AD[110], Huntington disease (HD)[111] and stroke[112,113] (Table 1). Pathological α-synuclein (pre-formed fibrils) activates PARP1 in mouse models of PD, leading to NO-mediated accumulation of PAR and execution of parthanatos — a process that can either be inhibited by PARP1 small-molecule inhibitors or by *PARP1* genetic depletion. Importantly, PAR and pathological α-synuclein form secreted entities that are even more toxic, and thus amplify PD pathogenesis[109]. In cell models of AD, Aβ causes hyperactivation of PARP1, leading to cell death that can be reversed by a PARP1 inhibitor[110]. Moreover, in a study where mice were given 3-nitropropionic acid to model HD, combinatorial treatment with inhibitors against the NMDA receptor and PARP1 reduced immunotoxicity and HD-mediated neurodegeneration[111]. Finally, several studies demonstrate that PARP1 and parthanatos are central to the development of stroke in middle cerebral artery occlusion mouse models — application of PARP1 inhibitors can reduce infarct volume[112,113]. Besides reducing cell death by parthanatos, PARP1 inhibitors might also dampen neuroinflammation by modulating NF-κB expression[112,114].

Inhibitors of parthanatos

PARP1 inhibitors have been developed over the past 40 years (Figs. 3c and 4b and Table 2), and several are in preclinical and clinical development or have been approved by the FDA for use in oncology[108]. For example, pamiparib (BGB-290; BeiGene)[115] is in clinical testing as a cancer therapy. Moreover, FDA-approved PARP1 inhibitors — mainly for cancer treatment — include olaparib (AZD2281; AstraZeneca)[108,116,117], niraparib (MK-4827; Tesaro)[108,116,118], rucaparib (AG-014699; Clovis Oncology)[108,116,119], talazoparib (BMN 673; Pfizer)[108,116,119] and veliparib (ABT-888; AbbVie)[108,116]. Although development of these molecules has primarily been for cancer treatment, they could be evaluated in diseases where extensive PARP1 activation leads to degeneration through parthanatos. For detailed information on the discovery and development of PARP1 inhibitors, we refer readers to the comprehensive review by Curtin and Szabo[108].

There are other less advanced inhibitors that inhibit parthanatos in cell and in vivo models. These include 4′-methoxyflavone (4-MF) and 3′,4′-dimethoxyflavone (DMF)[120], which counteract MNNG-induced parthanatos in cell models by reducing the amount of PAR and subsequent signal progression[120]. Furthermore, AG14361 (ref. 121) is a potent and specific PARP1 inhibitor with cellular and in vivo activity[121]. Similarly, A-966492 (ref. 122) is a highly potent, orally bioavailable PARP1 inhibitor. Both AG14361 and A-966492 have the potential to be used in disease settings where inhibition of parthanatos is desired. The potent PARP1 inhibitor PJ34 is neuroprotective in cells and in in vivo models of stroke[123].

Therefore, although PARP1 inhibitors have not yet been specifically tested on parthanatos-driven degenerative diseases, there is ample opportunity to expand clinical testing of the advanced inhibitors towards such diseases, including their compassionate use in carefully selected circumstances.

Ferroptosis

Ferroptosis is distinct from apoptosis, necroptosis, pyroptosis and parthanatos because it is not induced by a cascade of signalling events, but is rather a metabolicallydriven and regulated cell death process. It occurs through peroxidation of polyunsaturated fatty acyl moieties in lipids in an iron-dependent manner[124,125]. Ferroptosis is important in antiviral immunity and tumour suppression and can also contribute to ageing and be a cause of degenerative diseases[125]. Whether ferroptosis is immunogenic or not is yet to be determined. The concept of ferroptosis,

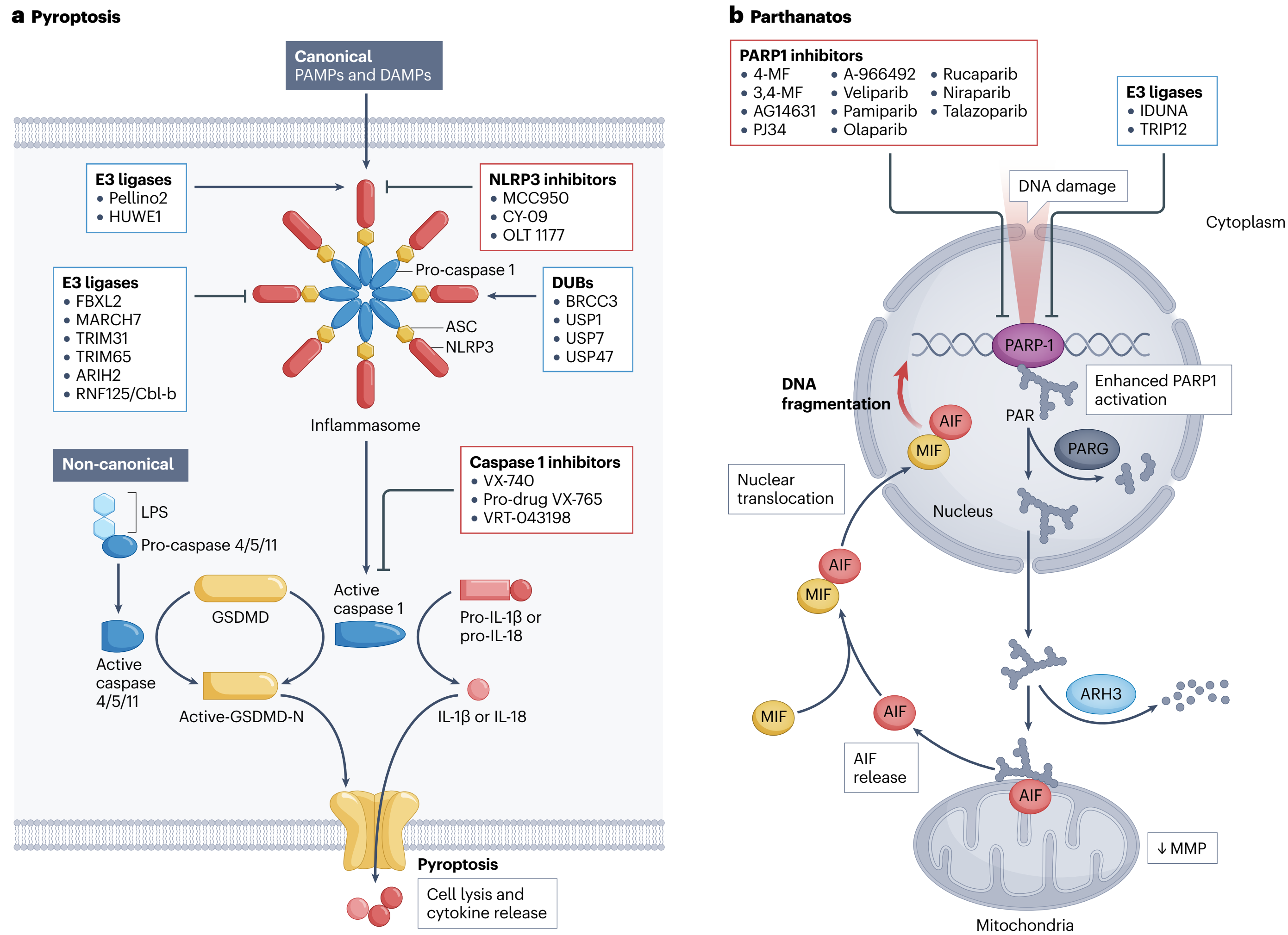

Fig. 4 | Molecular mechanisms of pyroptosis and parthanatos. a, In canonical pyroptosis, the inflammasome – composed of NLRP3, the bridging factor ASC and caspase 1 – is formed upon the sensing of cellular stressors (such as bacteria, viruses and toxins). This leads to autocleavage and activation of caspase 1, which subsequently cleaves GSDMD to form active GSDMD-N. Furthermore, caspase 1 generates active IL-1β and IL-18. GSDMD-N forms membrane pores to execute pyroptosis and to release IL-1β and IL-18. Small-molecule inhibitors of NLRP3 and caspase 1 can counteract pyroptosis. In the non-canonical pathway, caspases 4, 5 and 11 sense intracellular lipopolysaccharide (LPS) and are activated so that they can cleave GSDMD to form active GSDMD-N. Ubiquitination of NLRP3 by the E3 ligases Pellino 2 and HUWE1 promotes inflammasome assembly. In contrast, the E3 ligases FBXL2, MARCH7, TRIM31, TRIM65, ARIH2 and RNF125 together with Cbl-b facilitate degradation of NLRP3. The deubiquitinases (DUBs) BRCC3, USP1, USP7 and USP47 deubiquitinate NLRP3 to enhance inflammasome activity. **b**, DNA damage facilitates hyperactivation of PARP1 and PAR polymer formation from NAD^+ moieties. PARG hydrolyses PAR and thereby inhibits parthanatos. IDUNA and TRIP12 are PAR-dependent E3 ligases that degrade PARP1 and protect cells from parthanatos. Additionally, ARH3 can hydrolyse PAR to counteract cell death. PAR translocates as free polymers to the cytoplasm, where it binds AIF localized in the mitochondrial outer membrane. Interaction of PAR–AIF triggers AIF to dissociate from the mitochondria and interact with the endonuclease MIF, which drives translocation to the nucleus, where MIF degrades DNA, leading to cell death. The PARP1 inhibitors listed can inhibit parthanatos. DAMPs, damage-associated molecular patterns; PAMPs, pathogen-associated molecular patterns.

including the key role of iron, and tools for exploring this new concept were developed by the Stockwell laboratory[126–129]. In parallel, the Conrad team reached similar conclusions from mouse genetic studies[130], in which a conditional knockout of glutathione peroxidase 4 (GPX4) caused a cell death pathway that had not been previously recognized and was dependent on lipid peroxidation[130]; GPX4 is now recognized as a central regulator of ferroptosis. In retrospect, earlier studies in the 1950s observed nutrition needs that protect cell cultures from what we now think of as ferroptosis[131]. Additionally, early work from the Bornkamm laboratory showed that death of Burkitt lymphoma cells is limited upon cysteine uptake, which led to cloning of hydroperoxide GPX as a Burkitt lymphoma growth-promoting enzyme[132,133]. These cell death events are now understood as ferroptosis. The discovery of the chemical probes ferrostatin 1 (ref. 126), liproxstatin 1 (ref. 134), erastin[127] and RSL3 (ref. 129) allowed investigators to identify ferroptosis in diverse systems. An oxidative-stress form of cell death in neurons named oxytosis, which is induced by excess glutamate, was proposed prior to the discovery of ferroptosis[135] and has some similarities (Box 1).

Regulators of ferroptotic cell death

A number of cellular processes induce or suppress ferroptotic cell death (Fig. 5). In order to execute ferroptosis, susceptible lipids need to be oxidized. In particular, polyunsaturated fatty acid (PUFA) phospholipids (PUFA-PLs) are key substrates of lipid peroxidation. PUFAs are first converted to coenzyme A thioesters (PUFA-CoAs) through the enzymatic action of acyl-CoA synthetase long-chain family member 4 (ACSL4)[136] and subsequently processed to PUFA-PLs by lysophosphatidylcholine acyltransferase 3 (LPCAT3)[124,137] before being deposited into membranes. The process of lipid peroxidation is dependent on the Fenton reaction in an iron-dependent manner[126] and on lipoxygenases to catalyse it[137–139]. Cytochrome P450 oxidoreductase (POR) is another enzyme generating lipid hydroperoxides[140,141]. A recent study revealed that PUFA ether PLs produced in peroxisomes might also promote ferroptosis susceptibility[142], thus expanding the repertoire of substrates for the peroxidation reaction. Phosphorylation of ACSL4 at position Thr328 by PKCβII augments its activity and promotes lipid peroxidation, which represents a further level of regulation to enhance ferroptosis[143].

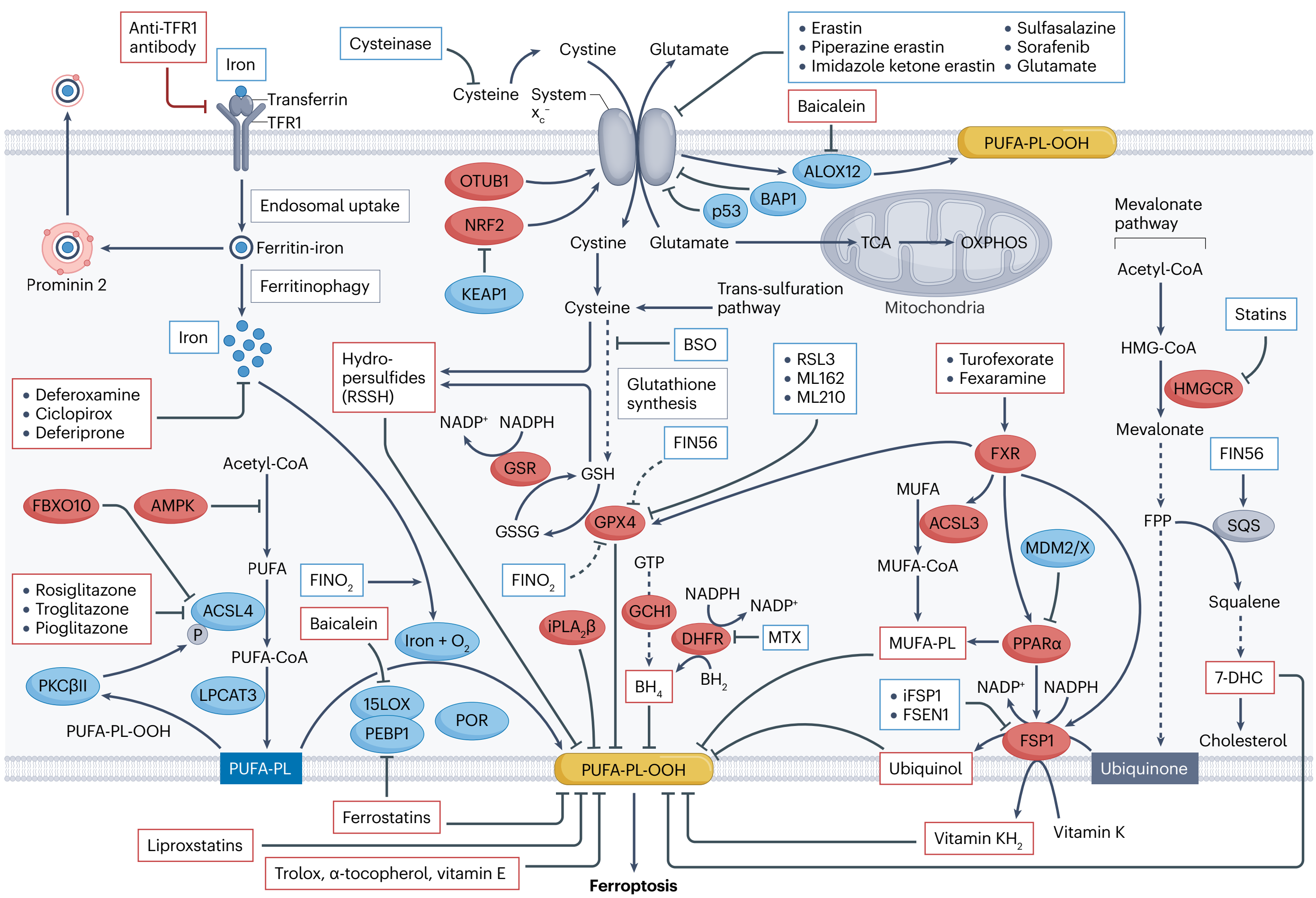

Fig. 5 | Regulatory mechanisms controlling ferroptosis. Ferroptosis is executed upon peroxidation of polyunsaturated fatty acid phospholipids (PUFA-PL). Generation and membrane deposition of PUFA-PLs involves the enzymes ACSL4 and LPCAT3. Lipid peroxidation occurs through the iron-dependent Fenton reaction, is catalysed by lipoxygenases, or is mediated by cytochrome P450 oxidoreductase (POR). Iron is imported through the TFR1 and ferritinophagy pathway, whereas prominin 2 facilitates the export of ferritin-bound iron and reduces labile iron levels and ferroptosis. PKCβII phosphorylates ACSL4 to augment lipid peroxidation and ferroptosis. ACSL4 is inhibited by the E3 ligase FBXO10 or the small molecules rosiglitazone, troglitazone and pioglitazone, which protect from ferroptosis. Energy stress activates AMPK, which counteracts ferroptosis by inhibiting acetyl-CoA carboxylase (ACC). Further, radical-trapping agents, such as ferrostatins, liproxstatins and α-tocopherol (vitamin E) pharmacologically counteract lipid peroxidation. Endogenously, system x_c^-–glutathione (GSH)–GPX4 is the main ferroptosis inhibitory axis. System x_c^- transports cystine into the cell, which is converted to cysteine – a critical building block for GSH synthesis. GPX4 uses GSH to reduce lipid hydroperoxides (PL-OOH) to their alcohol forms. Cysteine can also be produced by the trans-sulfuration pathway. Hydropersulfides (RSSH) can inhibit lipid peroxidation independent of GPX4. OTUB1 and NRF2 stabilize system x_c^-, whereas BAP1 reduces system x_c^- levels. The mevalonate pathway produces ubiquinol or 7-DHC, which are anti-ferroptotic. Other inhibitory mechanisms are regulated by FSP1–ubiquinol, FSP1–vitamin K, GCH1–BH_4–DHFR, FXR and $iPLA_2\beta$. Drugs targeting these enzymes can be used in combination with system x_c^- or GPX4 inhibitors to amplify ferroptosis for cancer treatment. GPX4 activity can also be reduced through cysteine depletion by cysteinases or inhibition of GSH synthesis using buthionine sulfoximine (BSO). Inhibitors marked in blue induce ferroptosis; inhibitors marked in red inhibit ferroptosis. 7-DHC, 7-dehydrocholesterin; BH_4, tetrahydrobiopterin; CoA, coenzyme A; GTP, guanosine triphosphate; MTX, methotrexate; MUFA, monounsaturated fatty acids; NADP, nicotinamide adenine dinucleotide phosphate; OXPHOS, oxidative phosphorylation; SQS, squalene synthase; TCA, tricarboxylic acid cycle.

The Hippo signalling pathway negatively regulates ferroptosis by preventing Yes-associated protein (YAP) from transcriptionally upregulating ACSL4 and transferrin receptor 1 (TFR1). Inactivation of Hippo signalling, which frequently occurs in cancer, allows YAP-mediated gene expression and leads to elevated levels of PUFA-PLs and iron, thereby sensitizing cells to ferroptotic cell death[144]. Additionally, the transcription factor hypoxia-inducing factor 2α (HIF2α) drives ferroptosis by upregulating the expression of hypoxia-inducible lipid droplet-associated protein (HILPDA), which increases PUFA production and subsequent lipid peroxidation in clear cell renal cell carcinoma[145,146]. Ionizing radiation can induce ferroptosis by a variety of mechanisms, including repression of the cystine–glutamate antiporter SLC7A11, glutathione (GSH) depletion and lipid peroxidation[147–149]. Finally, the E3 ligases mouse double minute 2 (MDM2) and MDMX drive ferroptosis in a p53-independent manner by inhibiting peroxisome proliferator-activated receptor-α (PPARα), which regulates levels of anti-ferroptotic monounsaturated fatty acid (MUFA)-PLs as well as ferroptosis suppressor protein 1 (FSP1). Accordingly, small-molecule inhibition of MDM2 and MDMX protects from ferroptosis by augmenting PPARα-induced MUFA-PLs and FSP1 levels[150].

The system x_c^-–GSH–GPX4 axis is the main pathway for ferroptosis inhibition[151] (Fig. 5). System x_c^-, a cystine–glutamate antiporter, consists of two solute carrier (SLC) subunits, SLC7A11 and SLC3A2 (refs. 152,153). Cystine is transported into the cell, then reduced to cysteine, which is a critical building block for GSH synthesis. GPX4 is a key anti-ferroptotic enzyme[130,154] that employs GSH to reduce lipid hydroperoxides (PL-OOH) to their corresponding alcohol forms (PL-OH)[137]. In addition to its active transport, cysteine can be generated through the trans-sulfuration pathway from methionine. Interestingly, two recent studies demonstrated that hydropersulfides based on cysteine or GSH can inhibit ferroptosis independently of GPX4 (refs. 155,156). Thus, persulfides (RSSH) also suppress ferroptosis[155]. These sulfane sulfur species (oxidation state S^0) scavenge lipid peroxyl radical species and act as inhibitors of ferroptosis. Moreover, since the formation of persulfides is dependent on cysteine, this amino acid has a dual mechanism of suppressing ferroptosis through both GSH biosynthesis and persulfide formation. Furthermore, the mevalonate pathway is involved in the generation of selenocysteine that is needed for the selenoprotein GPX4 to form an active enzyme[157]. The mevalonate pathway also leads to the generation of anti-ferroptotic metabolites such as ubiquinol[158] and, as reported in preprints, 7-dehydrocholesterin (7-DHC)[159,160] 7-dehydrocholesterin (7-DHC). Exogenous MUFAs also act as ferroptosis inhibitors. ACSL3 induces the production of MUFA-PLs, which counteract ferroptosis through an unknown mechanism[161].

Ferroptosis surveillance is not only based on the system x_c^-–GSH–GPX4 axis; other cellular regulators are involved in limiting ferroptosis[124,158] and act independently of system x_c^-–GPX4 (Fig. 5). In one axis, FSP1 restores ubiquinol from ubiquinone, and ubiquinol acts as an antioxidant to prevent lipid peroxidation[162,163]. Moreover, FSP1 generates vitamin KH_2, which is a radical-trapping antioxidant[164]. In another axis, GTP cyclohydrolase 1 (GCH1) is the rate-limiting enzyme for the synthesis of tetrahydrobiopterin (BH_4) which prevents lipid peroxidation by its antioxidant effect and promotes lipid remodelling to inhibit ferroptosis execution[165]. Notably, the enzyme dihydrofolate reductase (DHFR) can recycle BH_4 from dihydrobiopterin (BH_2) to inhibit ferroptosis[166]. As a further gatekeeper mechanism, activation of the Farnesoid X receptor (FXR) leads to the reduction of lipid peroxidation by upregulating FSP1, PPARα, GPX4 and genes generating MUFAs[167,168]. Finally, Ca^{2+}-independent phospholipase $A_2\beta$ (iPLA$_2\beta$) can eliminate peroxidized phospholipids to avert ferroptosis[169] and suppress p53-driven ferroptosis[170]. Hence, drugs inhibiting these various enzymes might be used in combination with system x_c^-–GSH–GPX4 inhibitors to potentiate ferroptosis for cancer treatment.

Besides these GPX4-independent ferroptosis-limiting mechanisms, there are additional cellular pathways repressing ferroptosis. Energy stress mediates the activation of AMP-activated protein kinase (AMPK), which, in turn, reduces ferroptosis through inhibition of acetyl-CoA carboxylase (ACC), a critical enzyme for PUFA production[171]. Additionally, mammalian target of rapamycin complex (mTORC) signalling is implicated in ferroptosis inhibition by promoting GPX4 protein synthesis[172] or SREBP1-mediated lipogenesis[173]. In line with this, combinatorial treatment of cancer cells with rapamycin and ferroptosis inducers suppresses cancer expansion[172,173], although mTORC inhibition has also been shown to restrain ferroptosis under certain contexts[174]. A further cellular component that counteracts ferroptosis is prominin 2, which mediates export of ferritin-bound iron and thus reduces the amount of labile iron necessary for the execution of lipid peroxidation[175]. 4-Hydroxynonenal (4-HNE), a product of lipid peroxidation, promotes prominin 2 expression via heat shock factor 1 (HSF1). Specific inhibitors of HSF1 in combination with ferroptosis inducers can potentiate cancer cell death[176].

Together, discoveries in the past two decades have uncovered a wealth of regulatory mechanisms controlling ferroptosis that have opened exciting avenues for drug discovery strategies (Fig. 5).

Ferroptosis in disease

Cell death by ferroptosis is implicated in a variety of degenerative diseases affecting the brain, heart and kidney as well as other organs (Table 1). Among neurodegenerative diseases, ferroptosis has been proposed to drive PD, HD, AD and ALS (Table 1). In PD, a study in induced pluripotent stem cell-derived neurons showed that aggregation of α-synuclein drives ferroptosis by accumulating lipid peroxidation[177]. Furthermore, a PD-associated mutation of iPLA$_2\beta$ showed reduced hydrolysing activity, which led to excess lipid peroxidation and ferroptosis in vitro and in vivo[169]. In a brain slice model of HD, ferrostatins were protective against cell death[178], thereby connecting HD to ferroptosis. An association of ferroptosis with AD was found in a study in mice where GPX4 ablation led to ferroptotic cell death in forebrain neurons (a region associated with AD) and impaired memory. Neuronal cell death in these GPX4-knockout mice was exacerbated by a diet lacking vitamin E (an antioxidant that inhibits ferroptosis) and ameliorated by the ferroptosis inhibitor liproxstatin 1 (ref. 179). Furthermore, Tau hyperphosphorylation – a hallmark of AD – leads to iron overload and increased lipid peroxidation, which can be blocked by α-lipoic acid in vivo[180]. In an ALS mouse model, GPX4 ablation led to motor neuron degeneration and paralysis, which was delayed upon treatment with vitamin E[181], thus linking ALS to ferroptosis. Cell death and damage in traumatic brain injuries, such as stroke, can also be caused by ferroptosis. Intracerebroventricular injection of selenium in mice augmented GPX4 levels to block ferroptosis in stroke[182]. Prokineticin 2, a chemokine present in the brain, reduced neuronal cell death by promoting Fbox10-driven ubiquitination and degradation of ACSL4 in vitro and in vivo[183].

Several mouse model studies also demonstrate an important impact of ferroptosis during ischaemia–reperfusion injury associated with kidney degeneration and heart damage (Table 1). Inactivation of GPX4 causes acute renal failure, and administration of liproxstatin 1 reverts this process[134]. Moreover, ferroptosis is involved in renal tubular cell death in an ischaemia–reperfusion injury mouse model and ferrostatins protect from tubular damage[184]. A recent study further demonstrated that loss of FSP1 or GPX4 promotes renal tubular ferroptosis in vivo[185]. Ferroptotic cell death is also linked to damage to the heart. Knockout of ferritin in mouse cardiomyocytes, in combination with a high-iron diet, induced lipid peroxidation and ferroptosis, leading to severe heart failure. Importantly, this phenotype was reverted by ferrostatin 1 (ref. 186). It has also been shown that inhibiting ferroptosis is protective against ischaemia–reperfusion-mediated cardiomyopathy in mice[187]. Ferroptosis also contributes to cigarette smoke-induced chronic obstructive pulmonary disease[188].

In contrast to degenerative diseases, the induction of ferroptosis is a strategy to target aggressive cancers, as demonstrated in cellular and animal models[189,190]. Intriguingly, there is a large difference in the susceptibility of cancer cells towards ferroptotic cell death, ranging from very sensitive to entirely resistant[165]. Even within certain cancer types, there are cell lines that are either sensitive or resistant to ferroptosis[191]. For example, diffuse large B cell lymphoma cell lines showed differential sensitivity to imidazole ketone erastin (IKE; a system x_c^- inhibitor), which induced ferroptosis in sensitive cell lines and a xenograft mouse[191].

Interestingly, blood, but not the lymphatic system, is important for the execution of ferroptosis in cancer cells. High levels of oleic acid and GSH in the mouse lymphatic system protect metastasizing melanoma cells from ferroptotic cell death. In contrast, melanoma cells that metastasize through the bloodstream of mice are more susceptible to ferroptosis caused by GPX4 depletion or inhibition[192]. However, sterol regulatory element-binding protein 2 (SREBP2) can suppress ferroptosis in blood-circulating melanoma cells by upregulating transferrin expression in vivo[193]. Moreover, enrichment of n-3 and n-6 PUFAs in the acidic tumour microenvironment leads to increased uptake of PUFAs by tumour cells, resulting in ferroptotic cell death by lipid peroxidation in mice[194]. An investigation of the physiological sources of ferroptosis induction demonstrated that $CD8^+$ T cells secrete INFγ, which suppresses SLC7A11 expression in cancer cells and thereby initiates ferroptosis in vivo[195]. More recently, studies in mice showed that INFγ from $CD8^+$ T cells also stimulates ACSL4 expression and, together with arachidonic acid from the tumour microenvironment, leads to enhanced lipid peroxidation and ferroptosis in the tumour[196]. Hence, INFγ secretion by $CD8^+$ T cells has a dual function: firstly, reducing ferroptosis protection by suppressing SLC7A11, and secondly, inducing lipid peroxidation by elevating ACSL4 levels. However, the tumour microenvironment can counteract this mechanism as it enriches oxidized lipids that are imported into $CD8^+$ T cells, where they stimulate lipid peroxidation. Hence, these dysfunctional $CD8^+$ T cells fail to control tumour progression[197]. Renal cancer cells are also able to undergo ferroptosis[145,154]. A study analysing metabolic dependencies identified impaired lipid metabolism in renal cancer cells, which relied on the GSH–GPX4 system to overcome ferroptosis. Hence, inhibition of GSH synthesis reduced renal tumour growth in vivo[145]. Finally, cysteine depletion by SLC7A11 knockout or the application of cysteinases in mice induced ferroptosis in pancreatic ductal adenocarcinoma[198].

Inducers of ferroptosis

As a strategy to induce the ferroptotic elimination of cancer cells, small-molecule inhibitors have been developed that act at various stages of the ferroptosis pathway (Figs. 5 and 6 and Table 2). Other modules (such as FSP1) have also been modulated by small molecules to induce ferroptosis. For targeting the ferroptosis pathway, the most well-studied small molecules inhibit either the system x_c^- cystine–glutamate antiporter (class I inhibitors) or the GPX4 enzyme (class II inhibitors). Both strategies lead to reduced GPX4 activity, accumulation of peroxidized lipids and ferroptotic cell death.

Inhibitors of the system x_c^- antiporter include erastin[126,127], piperazine erastin[154], IKE[191], sulfasalazine[126,199] and sorafenib[200]. These compounds inhibit the cellular import of cystine, which is essential for GSH production. Depletion of GSH reduces the enzymatic activity of GPX4, hence triggering ferroptosis. Erastin was the first compound discovered to induce a specific type of cell death, namely ferroptosis[127]. Its use is mostly restricted to cell culture models as it has low metabolic stability. Piperazine erastin and IKE, both analogues of erastin, have higher potency and improved stability, and IKE can be used in in vivo models[191]. Sulfasalazine is a repurposed drug that activates ferroptosis with low potency in cell culture models[126]. Sorafenib, an inhibitor of multiple kinases, also inhibits system x_c^- and triggers ferroptosis[200], although a recent report showed it does not exclusively induce ferroptosis in a number of cancer cell lines as it likely induces both ferroptosis and other modes of cell death[201].

In addition to system x_c^- inhibitors, a large set of ferroptosis-inducing compounds are inhibitors of GPX4 or affect GSH production. (1S,3R)-RSL3 (refs. 129,154) is a potent and covalent inhibitor of GPX4 (ref. 154) that is widely used in cell culture models but is not applicable in vivo due to its poor pharmacokinetics. ML210 (refs. 202,203) is a pro-drug that undergoes cellular conversion to JKE-1674 to become a potent GPX4 inhibitor and has a favourable proteome-wide selectivity[203]. However, its in vivo applicability needs further evaluation. ML162 is another GPX4 inhibitor with cellular activity[202,203].

In contrast to direct inhibitors of GPX4 activity, FIN56 (ref. 204) depletes GPX4 by an unknown mechanism and activates squalene synthase within the mevalonate pathway, thereby reducing ubiquinol levels[204]. As GPX4 and ubiquinol are key ferroptosis inhibitors, treatment of cells with FIN56 induces ferroptosis. Furthermore, FIN56 can induce autophagy, which contributes to autophagy-dependent degradation of GPX4 and enhanced ferroptosis[205]. $FINO_2$ (ref. 206) inhibits GPX4 albeit in an indirect and not yet elucidated manner; it also oxidizes iron, with both actions favouring lipid peroxidation[206]. Neither FIN56 nor $FINO_2$ has been investigated in vivo. GPX4 activity can also be diminished by strategies that reduce levels of its important cofactor GSH. This can either occur through cysteine depletion by cysteinases[198] or by inhibition of GSH synthesis using, for example, buthionine sulfoximine[207,208].

In summary, although there are a number of specific molecules targeting system x_c^- or the GPX4 enzyme to trigger ferroptosis in cell models, most are not suitable for in vivo application or have not yet been tested in this regard. Thus, future ferroptosis research needs to find additional strategies to develop target-based ferroptosis inducers that are potentially suitable for clinical application. Such strategies could include degradation of key ferroptosis regulators with proteolysis-targeting chimaeras (PROTACs)[209,210] or lysosome-targeting chimaeras[209,211]. Importantly, GPX4-knockout mice have a severe phenotype, which implies that systemic GPX4 inhibition could have considerable side effects. Therapeutic strategies therefore need to consider targeted delivery of GPX4 inhibitors or degraders into the tumour. A few studies have shown the feasibility of such a strategy by delivering IKE[191] or the GPX4 PROTAC dGPX4 (ref. 212) into a tumour using a nanoparticle formulation, which resulted in a reduction of tumour volume.

There are additional targets that, upon inhibition, can contribute to ferroptosis induction. The mevalonate pathway has anti-ferroptotic activity via its involvement in ubiquinol, 7-DHC and selenocysteine production[124]. The enzyme 3-hydroxy-3-methylglutaryl-coenzyme

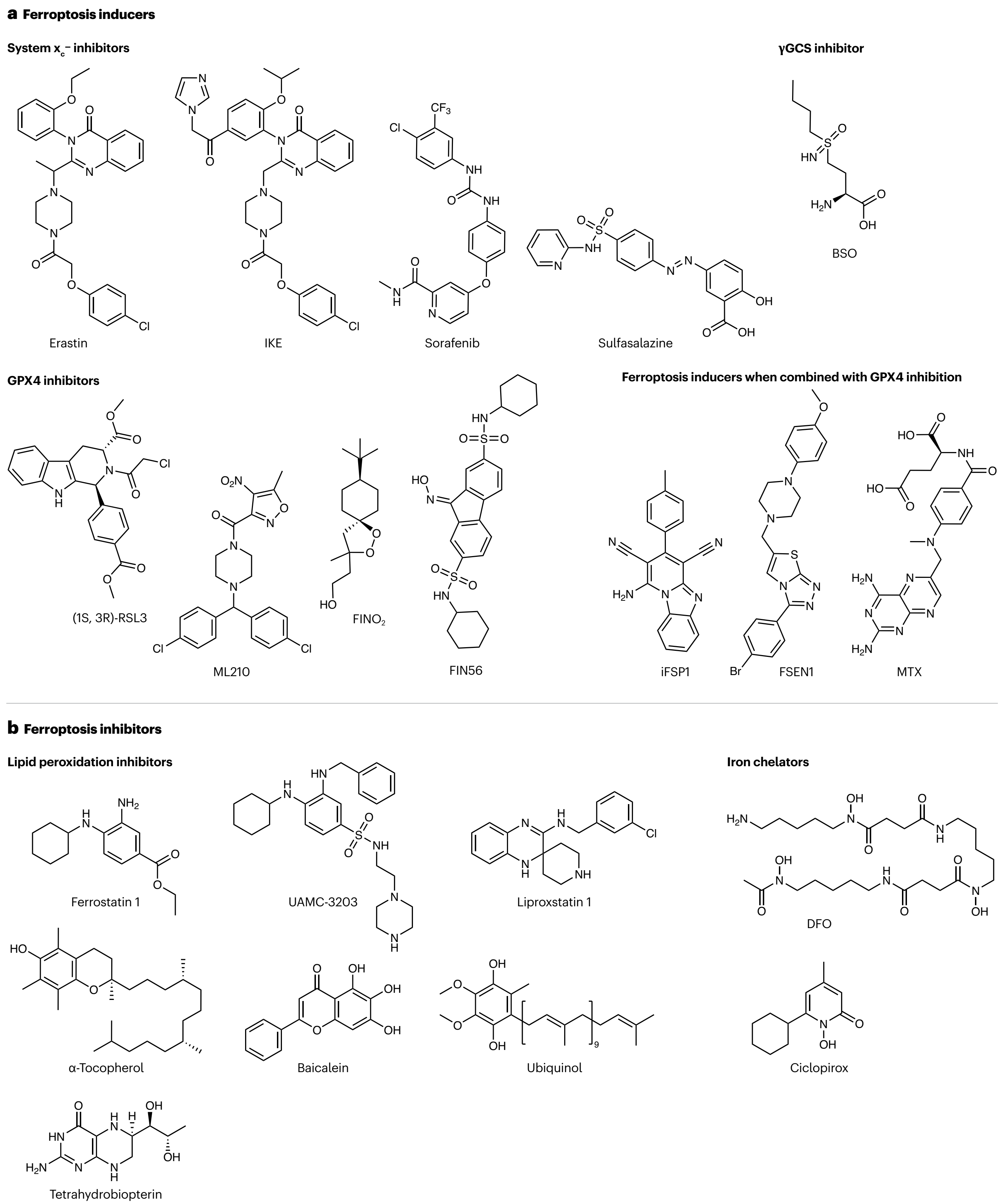
a Ferroptosis inducers
System x_c^- inhibitors
γGCS inhibitor
Erastin
IKE
Sorafenib
Sulfasalazine
BSO
GPX4 inhibitors
Ferroptosis inducers when combined with GPX4 inhibition
(1S, 3R)-RSL3
ML210
$FINO_2$
FIN56
iFSP1
FSEN1
MTX
b Ferroptosis inhibitors
Lipid peroxidation inhibitors
Iron chelators
Ferrostatin 1
UAMC-3203
Liproxstatin 1
DFO
α-Tocopherol
Baicalein
Ubiquinol
Ciclopirox
Tetrahydrobiopterin

Fig. 6 | Selected small-molecule regulators of ferroptosis. a, Ferroptosis inducers that target system x_c^-, γGCS, or GPX4 or that induce ferroptosis when combined with GPX4 inhibition. **b**, Ferroptosis inhibitors that target lipid peroxidation or chelate iron. BSO, buthionine sulfoximine; DFO, deferoxamine; IKE, imidazole ketone erastin; MTX, methotrexate.

A (HMGCR) in the mevalonate pathway is inhibited by statins, which can induce ferroptosis in some contexts and sensitize to ferroptosis in others. Moreover, anti-ferroptotic ubiquinol can be restored by FSP1 action in the plasma membrane[162,163]; therefore, inhibition of FSP1 by the small molecules iFSP1 (ref. 163) or FSEN1 (ref. 213) augments ferroptosis in combination with system x_c^- or GPX4 inhibitors. Finally, recycling of the anti-ferroptotic metabolite BH_4 by DHFR can be prevented by the DHFR inhibitor methotrexate[165,166], which synergizes with system x_c^- or GPX4 inhibitors to enhance ferroptosis induction[166]. Development of inhibitors of GCH1 (which synthesizes BH_4) or of additional DHFR inhibitors could be attractive for combinatorial treatment with system x_c^-–GPX4 inhibitors.

Inhibitors of ferroptosis

To inhibit ferroptosis in degenerative diseases, small molecules have been developed to reduce lipid peroxidation or redox-active iron, two major hallmarks of ferroptotic cell death (Figs. 5 and 6 and Table 2). Radical-trapping agents are the most widely used molecules to counteract lipid peroxidation. Molecules within this group include ferrostatins[126,214], liproxstatins[134,214] and phenoxazine[215]. Liproxstatins display good pharmacological properties for in vivo application and have been used to treat acute renal failure in mice[134]. Liproxstatin 1 and unpublished derivatives are in preclinical development. Initial ferrostatin molecules, such as ferrostatin 1, were less suitable for in vivo use due to their poor metabolic stability. However, improved ferrostatins are now available with enhanced pharmacological stability and can be applied in vivo[178,184]. Additional ferrostatin analogues, especially UAMC-3203, combine good stability and excellent solubility with in vivo efficacy in a mouse model of acute iron poisoning[216,217]. Although ferrostatins function as antioxidants, ferrostatin 1 has additional inhibitory function on the 15-LOX–PEBP1 complex, which generates lipid hydroperoxides, hence expanding the ferroptosis inhibitory effect beyond antioxidant activity[218]. Other potent antioxidants that inhibit lipid peroxidation are α-tocopherol (the most active vitamin E derivative)[126,165], ubiquinol[162,163,204] and BH_2 and BH_4[165]. Furthermore, targeting lipoxygenases with, for example, baicalein[137] can inhibit ferroptosis, and the inhibition of ACSL4 by thiazolidinediones (troglitazone, rosiglitazone and pioglitazone) protects from ferroptosis through reduced availability of PUFA-PLs[136]. The second hallmark of ferroptosis is the presence of redox-active iron to facilitate the peroxidation reaction, and reducing its level can counteract ferroptosis. This can be achieved by the application of iron-chelating agents such as defroxamine[126] or ciclopirox[126]. In contrast to ferroptosis inducers, there are several ferroptosis inhibitors that have shown in vivo efficacy in degenerative models such as of kidney degeneration or neurodegeneration.

Overall, targeting ferroptosis therapeutically has gained attention in the past decade, which has led to the generation of several small molecules. Although many of these small molecules are tool compounds to interrogate ferroptosis biology for mechanistic understanding, some of them have reached the stage of preclinical development. Nevertheless, the field of ferroptosis needs to develop further in vivo compatible inducers and inhibitors, including degrader technologies. With more advanced preclinical small-molecule modulators, it might eventually be possible to clinically evaluate ferroptosis inhibition or induction in the settings of ischaemia–reperfusion injury, transplantation, neurodegeneration and oncology.

Other metal-driven cell death: cuproptosis, lysozincrosis and disulfidptosis

Recent studies propose that, in addition to iron-mediated cell death (ferroptosis), there are other metal-driven cell death modalities, including copper-induced and zinc-induced cell death. First, a copper-dependent mode of cell death was proposed and termed cuproptosis[219]. The copper ionophore elesclomol can trigger cuproptosis, which was shown to depend on mitochondrial respiration and to proceed independently of inhibitors of apoptosis, ferroptosis and necroptosis but to require ferredoxin 1 (FDX1) and protein lipoylation enzymes. Tsvetkov et al.[219] proposed that excess copper binds selectively to lipoylated tricarboxylic acid cycle proteins to induce a toxic gain of function through proteotoxic stress, namely copper-dependent oligomerization of lipoylated proteins, eventually leading to cell death[219]. Second, a lysosomal zinc-mediated cell death process was proposed and termed lysozincrosis[220]. The mucolipin TRP channel 1 (TRPML1) — a Ca^{2+} and Zn^{2+} release channel found in lysosomes and upregulated in certain cancer cells — can be activated with synthetic agonists to induce lysosomal zinc-dependent cell death through mitochondrial swelling and dysfunction. In mouse models of metastatic melanoma, these TRPML1 agonists reduced tumour growth[220]. Of note, normal cells with low levels of TRPML1 are not susceptible to lysozincrosis mediated by a TRPML1 agonist, providing a therapeutic window for selective cancer treatment.

Cuproptosis and lysozincrosis might be controllable by targeting molecular components of these modes of cell death. For example, depletion of FDX1 is sufficient to suppress cuproptosis[219]; thus, development of FDX1 inhibitors could be beneficial in copper-overload diseases such as Wilson disease, Menke disease or environmental copper exposure. Moreover, a recent study showed that the peptide methanobactin produced by *Methylosinus trichosporium* has a high affinity towards copper and can prevent hepatocyte death and liver failure in a rat model of Wilson disease by depleting copper[221]. Thus, methanobactin might be used to alleviate cuproptosis-mediated diseases. Similarly, zinc toxicity through lysozincrosis might be inhibited by targeting TRPML1 with small molecules. On the other hand, it might be possible to leverage cuproptosis and lysozincrosis for therapeutic benefit to eliminate copper-sensitive or zinc-sensitive pathological cells such as some cancer cells.

Recently, the Gan laboratory reported that excess intracellular disulfides accumulating in cells expressing a high level of SLC7A11 and undergoing glucose starvation induce a distinct form of cell death termed disulfidptosis[222]. The excess disulfides cause additional disulfide bonds to form in actin cytoskeleton proteins, resulting in F-actin impairment. Furthermore, actin polymerization and the GTPase Rac regulate this type of cell death. Thus, the actin cytoskeleton appears to be particularly susceptible to disulfide stress, resulting in yet another mode of cell death.

Outlook for potential therapeutics

These regulated necrosis modalities are linked to diverse disease settings, including degenerative diseases and cancer, and their discovery

Box 2

Ubiquitination in regulated cell death pathways

Ubiquitination by E3 ligases and deubiquitination by deubiquitinase (DUB) enzymes have critical roles in protein homeostasis as well as in signalling processes and regulate the stability and activity of many proteins involved in cell death pathways[230]. Over 600 E3 ligases and about 100 DUBs provide a rich source of underexplored possible targets for small-molecule drug development in non-apoptotic cell death pathways. We provide a few examples of cell death regulation by E3 ligases and DUBs below.

Necroptosis

In TNFR signalling, cIAP1 and cIAP2 ubiquitinate RIPK1 to generate a platform for the LUBAC complex to bind. LUBAC subsequently adds linear ubiquitin chains to RIPK1 and NEMO for downstream signalling processes to initiate apoptosis or necroptosis[231]. The DUBs CYLD, OTULIN and A20 remove ubiquitin moieties from RIPK1 to allow its association with RIPK3 and the initiation of necroptosis. Moreover, the E3 ligase CHIP ubiquitinates RIPK3 and RIPK1 to trigger lysosomal degradation of both of these central necroptosis activators[232]. Furthermore, Pellino 1 (ref. 233) and TRIM25 (ref. 234) ubiquitinate RIPK3 to induce its proteasomal degradation (Fig. 2).

Pyroptosis

The E3 ligase Pellino 2 ubiquitinates NLRP3 to activate NLRP3 inflammasomes and lipopolysaccharide-induced lethality[235]. NLRP3 is also ubiquitinated by the E3 ligase HUWE1, which promotes downstream signalling as a defence against bacterial infections[236]. In contrast, the E3 ligases FBXL2 (ref. 237), MARCH7 (ref. 238), TRIM31 (ref. 239), TRIM65 (ref. 240) and ARIH2 (ref. 241) facilitate the degradation of NLRP3. Furthermore, RNF125 and Cbl-b sequentially ubiquitinate NLRP3 to initiate its degradation[242]. Conversely, the deubiquitinases BRCC3, USP1, USP7 and USP47 deubiquitinate NLRP3 to enhance inflammasome activity[243–245] (Fig. 4a).

Ferroptosis

The MDM2–MDMX E3 ligase complex promotes ferroptosis in a p53-independent manner by reducing PPARα levels[150]. Thus, small-molecule inhibition of MDM2–MDMX suppresses ferroptosis[150]. KEAP1 is a pro-ferroptotic regulator, which ubiquitinates the central antioxidative regulator NRF2 for proteasomal degradation[246]. Hence, the KEAP1–NRF2 inhibitor CPUY192018 activates the protective effect of NRF2 and alleviates renal oxidative damage in vivo[247]. In contrast, the F-box protein FBXO10 is an anti-ferroptotic enzyme that ubiquitinates ACSL4 to initiate its degradation, leading to reduced lipid peroxidation[183]. The DUB BAP1 activates ferroptosis by reducing histone 2A ubiquitination on the SLC7A11 promoter, thereby repressing *SLC7A11* gene expression[248]. In contrast, the DUB OTUB1 inhibits ferroptosis by stabilizing protein levels of SLC7A11 (ref. 249) (Fig. 5).

opens up a wealth of targets for drug discovery. To this end, many small molecules that target these cell death pathways have been uncovered in the past two decades (Table 2). Excitingly, several of these small molecules are in clinical trials as drug candidates (such as RIPK1 inhibitors relevant for necroptosis and PARP1 inhibitors for parthanatos) or in preclinical development (such as multiple ferroptosis inhibitors or activators and NLRP3 inhibitors for pyroptosis). However, despite recent successes, several of the discovered compounds have little in vivo potential. Therefore, additional small molecules that are compatible in vivo as well as orally applicable need to be developed in order to translate concepts from bench to bedside. It will also be necessary to identify further critical regulators of these cell death pathways through genetic (such as CRISPR) or chemical genetic approaches in order to uncover novel targets for drug screens. Such targets might emerge from the ubiquitination pathway, including E3 ligases and deubiquitinases, which are intimately involved in regulating cell death pathways (Box 2). There are more than 700 such enzymes, which have largely been under-represented in drug development approaches so far. Importantly, cell death regulators that are not easily 'druggable' could potentially be targeted by changing their protein homeostasis through chemical inhibition of relevant deubiquitinases or E3 ligases. Further, we emphasize that targeted protein degradation technologies, including PROTACs[209,210], lysosome-targeting chimaeras[209,211] and transcription factor-targeting chimaeras[223], as well as protein stabilization technologies such as deubiquitinase-targeting chimaeras[224], should be utilized to modulate protein levels of key cell death regulators and investigate new targets or be applied themselves as potential drugs. These strategies have been underexplored so far.

Notably, several regulated necrosis modalities lead to similar disease phenotypes; for example, both ferroptosis and necroptosis can cause neurological disorders or acute kidney injury (Table 1). Thus, on the one hand, it is important to develop biomarkers and improve detection technologies to unequivocally differentiate between the cell death modalities in a particular disease state in order to select the correct drug. On the other hand, combinatorial drug treatments of two cell death pathways, such as ferroptosis and necroptosis in neurodegeneration, could prove beneficial in overcoming the disease burden.

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Author contributions
Both authors contributed equally to all aspects of the article.

Competing interests
B.R.S. is an inventor on patents and patent applications involving ferroptosis, holds equity in and serves as a consultant to Exarta Therapeutics and ProJenX Inc., holds equity in Sonata Therapeutics, and serves as a consultant to Weatherwax Biotechnologies Corporation and Akin Gump Strauss Hauer & Feld LLP. K.H. is an inventor on a patent application involving ferroptosis.

Additional information
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