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REVIEW ARTICLE

Programmed necrosis in cardiomyocytes: mitochondria, death receptors and beyond

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Excessive death of cardiac myocytes leads to many cardiac diseases, including myocardial infarction, arrhythmia, heart failure and sudden cardiac death. For the last several decades, most work on cell death has focused on apoptosis, which is generally considered as the only form of regulated cell death, whereas necrosis has been regarded to be an unregulated process. Recent findings reveal that necrosis also occurs in a regulated manner and that it is closely related to the physiology and pathophysiology of many organs, including the heart. The recognition of necrosis as a regulated process mandates a re-examination of cell death in the heart together with the mechanisms and therapy of cardiac diseases. In this study, we summarize the regulatory mechanisms of the programmed necrosis of cardiomyocytes, that is, the intrinsic (mitochondrial) and extrinsic (death receptor) pathways. Furthermore, the role of this programmed necrosis in various heart diseases is also delineated. Finally, we describe the currently known pharmacological inhibitors of several of the key regulatory molecules of regulated cell necrosis and the opportunities for their therapeutic use in cardiac disease. We intend to systemically summarize the recent progresses in the regulation and pathological significance of programmed cardiomyocyte necrosis along with its potential therapeutic applications to cardiac diseases.

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Abbreviations

AIF, apoptosis-inducing factor; ANT, adenine nucleotide translocase; CaMKII, Calcium/calmodulin-dependent protein kinase II; cIAP, inhibitor of apoptosis proteins; CypD, cyclophilin D; FADD, Fas-associated protein with death domain; H/R, hypoxia/reoxygenation; HF, heart failure; HSP70, heat shock protein 70; I/R, ischaemia/reperfusion; IDO, indoleamine-2,3-dioxygenase; IMM, inner mitochondrial membrane; MI, myocardial infarction; MLKL, mixed lineage kinase domain-like pseudokinase; mPTP, mitochondrial permeability transition pore; NETs, neutrophil extracellular traps; NSA, necrosulfonamide; OMM, outer mitochondrial membrane; PAR, poly (ADP-ribose); RIP, receptor-interacting protein; TNFR, TNF receptor; TRADD, TNFRSF1A-associated *via* death domain; VDAC, voltage-dependent anion channel

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Introduction

Cardiomyocytes, as terminally differentiated cells, have a highly limited capacity for regeneration, and excessive death of cardiac myocytes induced by stresses and their pathological effects leads to the development of a variety of cardiac diseases, including myocardial infarction (MI), malignant arrhythmia, heart failure (HF) and sudden cardiac death (Whelan *et al*., 2010; Orogo and Gustafsson, 2013). Hence, for the prevention and treatment of cardiac diseases, it is of great importance to elucidate the mechanisms of cardiomyocyte death and to define inhibitory interventions that can prevent it.

In the last several decades, most of the work on cell death has focused on apoptosis, which is generally considered the only form of regulated cell death and which is amenable to manipulation. However, necrosis, a major pathological feature of various cardiac pathological conditions (Nakagawa *et al*., 2005; Lim *et al*., 2007; Smith *et al*., 2007), was totally ignored because it was believed to be 'unregulated' or 'incidental'. Nevertheless, this concept has been challenged by the findings that necrotic death occurs through conserved cellular processes that occur in the lowly nematode worm *Caenorhabditis elegans*, as well as in mammals (Holler *et al*., 2000; Xu *et al*., 2001; Syntichaki *et al*., 2002; Degterev *et al*., 2005). Furthermore, genetic and biochemical dissection of these processes shows that, depending on the death-initiating stimulus, necrosis is orchestrated and executed by appropriate mechanisms, rather than simply representing a disorganized breakdown of the cell (Syntichaki and Tavernarakis, 2003). While extensive research work has been conducted to define the exact significance and contribution of necrosis to the development of disease, recent findings have indicated the existence and importance of programmed necrosis in various pathophysiological processes, especially in cardiac diseases (Baines *et al*., 2005; Nakagawa *et al*., 2005). Mitochondrial-dependent (intrinsic pathway) and death receptor-dependent (necroptosis, extrinsic pathway) necrotic cell death are the two major forms of programmed necrosis. Furthermore, some types of cell death that are consistent with the morphological definition of the programmed necrosis have also been identified, including pyroptosis (Chen *et al*., 1996), ferroptosis (Dolma *et al*., 2003; Yang and Stockwell, 2008), parthanatos (Virag and Szabo, 2002) and NETosis (Brinkmann *et al*., 2004). This review focuses on the regulatory mechanisms that have come to light as a result of recent progress in understanding the regulation and pathological significance of programmed cardiomyocyte necrosis, together with its potential therapeutic applications in cardiac diseases.

Mechanisms of programmed cardiomyocyte necrosis

Mitochondria and programmed cardiomyocyte necrosis

Mitochondria play a major role in coupling substrate catabolism to ATP production and are also involved in programmed forms of cell death. The inner mitochondrial membrane (IMM) of a healthy mitochondrion is impermeable to small molecules and even to protons, resulting in a chemical and electrical gradient between the intermembrane space and the mitochondrial matrix. The gradient is necessary for the conversion of ADP to ATP during respiration (Kung *et al*., 2011). Therefore, the maintenance of IMM integrity is critical for mitochondrial function. Unlike the imbalance of the outer mitochondrial membrane (OMM) that occurs during apoptosis, necrosis usually destroys the IMM, thereby inducing the opening of mitochondrial permeability transition pores (mPTPs) (Figure 1) (Goldenthal, 2016).

Several proteins, including **adenine nucleotide translocase (ANT),** voltage-dependent anion channels (VDAC), cyclophilin D (CypD, a peptidyl-prolyl *cis–trans* isomerase) and phosphate carriers in the IMM, and peripheral benzodiazepine receptors and hexokinase in the OMM, have been proposed to be the components of mPTPs. However, the exact components of the mPTPs have not been delineated. The involvement of ANT in mPTPs is supported by the decreased sensitivity of Ca^{2+}-induced mPTP opening induced by the binding of adenine nucleotides to ANT (Pestana *et al*., 2010). In contrast, genetic experiments raise significant questions concerning the necessity of ANT for mPTP function (Rodic *et al*., 2005). Thus, rather than being a critical component of mPTPs, ANT may play a regulatory role in their action. VDAC, the most abundant protein in the OMM, was observed to co-purify with ANT (McEnery *et al*., 1992), suggesting that these proteins may interact at the contact sites between the OMM and the IMM. However, Ca^{2+}-induced and oxidative stress-induced mPTP opening is not affected by the deletion of all three mouse VDAC genes (VDAC1, VDAC2 and VDAC3), indicating that VDAC is dispensable for mPTP function (Baines *et al*., 2007). CypD, which is encoded by the nuclear gene *Ppif*, is a peptidyl-prolyl *cis–trans* isomerase that resides in the mitochondrial matrix (Halestrap and Davidson, 1990; Connern and Halestrap, 1992). The absence of CypD protects cells against necrotic stimuli both *in vitro* and *in vivo*, and overexpression of CypD does the opposite (Baines *et al*., 2005; Nakagawa *et al*., 2005), indicating that CypD is a key regulator of mPTP and necrosis. However, the fact that mPTP opening occurs in the absence of CypD argues strongly against the theory that it has an essential structural role in the pore.

The opening of mPTPs is triggered primarily by elevated matrix Ca^{2+} concentration and by oxidative stress (Figure 1) (Nakayama *et al*., 2007; Whelan *et al*., 2012; Goldenthal, 2016). Recently, double-knockout genetic experiments confirmed and extended the involvement of Bax and Bak, which are known as pro-apoptotic proteins, in mPTP action and in necrotic cell death (Karch *et al*., 2013). Data suggested that the activation of the **PI3K**/**Akt**/FoxO3a/Bnip3L pathway in H9C2 cardiomyocytes plays an important role in H_2O_2-induced necrosis and mitochondrial dysfunction (Chen *et al*., 2016). Subsequently, activated Bnip3 was shown to trigger fragmentation, mitophagy and necrosis by targeting mitochondria (Dhingra *et al*., 2017).

mPTP opening has several immediate consequences, including cessation of respiration-driven ATP synthesis, reversal of the **FoF1-ATP synthase** activity due to the collapse of the mitochondrial membrane potential ($\Delta\Psi m$), redistribution of solutes and ions across the IMM and destruction of the

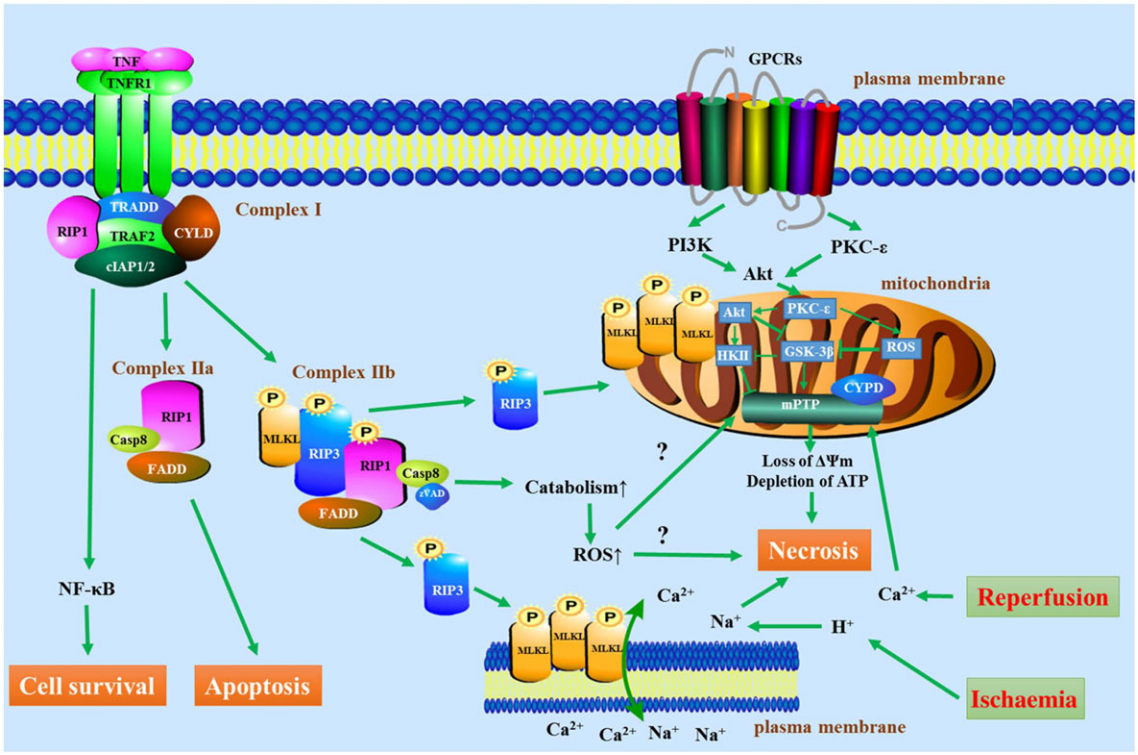

Figure 1

Main signalling pathways of programmed necrosis in cardiomyocytes. Association of TNFR1 with the TNF trimer leads to the formation of complex I, consisting of TRADD, TRAF2, RIP1, CYLD and cIAP1/cIAP2, at the cytoplasmic membrane. K63-linked polyubiquitination of RIP1 by cIAP1/cIAP2 leads to the recruitment of critical proteins and the activation of survival pathways. In the absence of cIAP1/cIAP2, RIP1, FADD and caspase-8 form cytosolic complex IIa, which activates the caspase cascade and induces apoptosis. Under conditions in which caspase-8 activity is inhibited genetically or pharmacologically (zVAD), RIP1 interacts with RIP3 and MLKL to form complex IIb, which is involved in the mediation of necroptosis. The kinase activity of RIP1 is essential for complex IIb action. RIP3 and MLKL are phosphorylated in complex IIb and translocate to the plasma membrane or to mitochondria-associated membranes, where the complex mediates membrane permeabilization. Phosphorylated MLKL changes the permeability of the plasma membrane, resulting in ion exchange (Na^+ and Ca^{2+}) across the membrane. RIP1 and RIP3 undergo a complex set of phosphorylation events, and necrosis ensues through unclear mechanisms. One potential mechanism, as shown, may involve the activation of catabolic pathways and ROS production. A second necrotic pathway involves the mPTP in the IMM and its regulation by CypD. mPTP may be opened by increased Ca^{2+} concentration, oxidative stress, decreased ATP levels and other stimuli that occur during I/R and HF. Furthermore, mitochondrial protein kinases such as PKC-1, Akt/HKII and GSK-3β have been suggested to be recruited from the cytosol to mitochondria in response to the activation of GPCR receptors. As described in the text, mPTP opening results in profound alterations in mitochondrial structure and function; these changes result in decreased ATP levels and loss of ΔΨm. Furthermore, ischaemia leads to increased [Na^+], which directly induces necrosis, and reperfusion leads to increased [Ca^{2+}], which induces mPTP opening. No definitive connection between death receptors and mitochondrial necrosis pathways has been delineated. A possible connection is RIP3-induced ROS generation (see text for details).

osmotic gradient due to the entry of large amounts of water into the solute-rich matrix (Figure 1). The entry of water results in matrix swelling and expansion of the redundant IMM, leading to rupture of the OMM and the release of apoptogens (i.e. cytochrome *c*) into the cytosol. The release of apoptogens triggers both apoptosome assembly and caspase activation (Baines *et al.*, 2005; Nakayama *et al.*, 2007). More recently, Casey *et al.* (2007) demonstrated that lipid peroxidation is also an important factor that contributes to necrotic death resulting from both opened mPTPs and oxidative stress. In addition, the opening of mPTPs results in a decrease in ATP levels. Although severe ATP depletion and loss of plasma membrane integrity are primarily responsible for cell death in necrosis, it is possible that activation of downstream apoptotic signalling also contributes (Kung *et al.*, 2011).

Recent studies have shown that 'mitochondrial protein kinases', such as Akt, **PKC-1**, **extracellular-regulated kinases (ERKs),** glycogen synthase kinase-3β (**GSK-3β**) and hexokinases (HK) I and II (Figure 1), in addition to their main pools in the cytosol, are located in mitochondria and that they receive signals from cytosolic molecules, thereby determining the death or survival of the cell (Miura *et al.*, 2010; Miura and Tanno, 2012). These kinases regulate mPTP opening through the formation of complexes with each other and with subunit proteins of the mPTP (Miura *et al.*, 2010; Miura and Tanno, 2012). Accumulating evidence indicates that phosphorylation of GSK-3β and HK in mitochondria directly modifies mPTPs in such a way as to elevate their threshold for opening, although the molecular structure of the mPTP and the details of its modification by GSK-3β and HK remain unclear (Kuno *et al.*, 2008). Furthermore, recent

studies have suggested that the signalling pathways of mitochondrial protein kinases are modified in the presence of concurrent cardiovascular disease (Kobayashi *et al*., 2008; Kuno *et al*., 2008; Zhai *et al*., 2011; Miura and Tanno, 2012). HK2, an Akt substrate, binds to mitochondria and inhibits mPTP opening mainly through its interactions with OMM proteins associated with mitochondrial fission [e.g. dynamin 1-like protein (Drp1)] and apoptosis (**Bcl-2** family members) during ischaemia/reperfusion (I/R) injury. Considering the role of HK2 binding in stabilizing contact sites between the OMM and IMM, it is likely to be a pharmacological target (Halestrap *et al*., 2014).

Death receptor-dependent cardiomyocyte necrosis (necroptosis)

Studies have shown that death receptor stimulation under apoptosis-deficient conditions (i.e. caspase inhibition) still induces cell death with the morphological features of necrosis in certain cell types, supporting the existence of programmed necrosis (Kawahara *et al*., 1998; Vercammen *et al*., 1998; Kitanaka and Kuchino, 1999). This type of necrotic cell death was referred to as 'necroptosis' by Degterev *et al*. (2005). Recent data have shown that death receptors, such as the TNF receptor (TNFR, comprising **TNFR1** and **TNFR2**), TNF-related apoptosis-inducing ligand and the **Fas ligand receptor**, which usually activate the apoptotic machinery, can also stimulate necroptosis (Chan *et al*., 2003; Kung *et al*., 2011) (Figure 1). Among these receptors, the most extensively characterized pathway leading to necroptosis is initiated by ligation of TNFR1 (Vandenabeele *et al*., 2010). The binding of TNF to TNFR1 stimulates the formation of complex I, which also includes the adaptor TNF receptor superfamily 1A-associated *via* death domain (TRADD), the serine/threonine kinase receptor-interacting protein 1 (**RIP1**), TNF receptor-associated factor 2 (TRAF2) and inhibitor of apoptosis proteins **cIAP1** and **cIAP2**, which possess the ability to stimulate the expression of multiple survival genes by activating NF-κB (Wilson *et al*., 2009; Pasparakis and Vandenabeele, 2015), an important transcription factor for cell survival. Complex I is converted to complex II through a series of changes that includes endocytosis of complex I, dissociation of TNFR1, deubiquitination of **RIP1** by CYLD (cylindromatosis) and A20, and recruitment of Fas-associated protein with death domain (FADD) and procaspase-8 (Micheau and Tschopp, 2003; Hitomi *et al*., 2008; Wang *et al*., 2008). If **caspase-8** is not inhibited, it can cleave RIP1 (Chan *et al*., 2003; Ea *et al*., 2006) and thereby stimulate the expression of multiple survival genes; this stimulation increases in the presence of ROS (Zhang *et al*., 2009). In the signalling pathway of TNF/z-VADfmk-induced necroptosis, the RIP1/**RIP3** complex appears to be dispensable due to their kinase activities (Lin *et al*., 1999; Holler *et al*., 2000). Identification of downstream targets will further define this pathway (Temkin *et al*., 2006), and other possible pathways parallel to RIP1–RIP3 may also be important.

Identifying the signalling events downstream of the initiation of programmed necrosis is important for determining how necrosis is executed and for developing potential therapeutic reagents targeting specific events after an initial insult (Figure 1). The events in this pathway that occur downstream of RIP1 and RIP3 are incompletely understood, but they include the phosphorylation by RIP3 of mixed lineage kinase domain-like protein (**MLKL**) (Sun *et al*., 2012), phosphoglycerate mutase 5 (PGAM5, a mitochondrial phosphatase) (Wang *et al*., 2012) and certain catabolic enzymes (glutamate dehydrogenase 1, glutamate ammonia ligase and glycogen phosphorylase). The last signalling potentially elicits necroptosis through the generation of ROS (Zhang *et al*., 2009). Other downstream events involved in necroptosis signalling include activation of **calpains**, phospholipases, lipoxygenases and sphingomyelinases and permeabilization of lysosomes LX (Poppe *et al*., 2002; Thon *et al*., 2005; Hara *et al*., 2007; Kim *et al*., 2008; Oikawa *et al*., 2009). However, another report showed that inhibition of PGAM5 or its downstream Drp1 did not markedly protect the mouse fibroblasts from TNF-induced necroptosis (Remijsen *et al*., 2014), suggesting that the downstream effectors of RIP1/RIP3/MLKL necrosomes may be species-specific and cell-type specific.

RIP3 plays a key role in programmed necrosis; however, the specific function of RIP3-dependent necroptosis in the heart remains poorly understood. Luedde *et al*. (2014) showed that RIP3-dependent necroptosis modulates post-ischaemic adverse remodelling in a mouse model of MI. TRAF2, a key component of the TNFR1 signalling complex, is recruited to TNFR1 through its interaction with the adaptor protein TRADD (Hsu *et al*., 1996). Mice with cardiac-restricted expression of low levels of TRAF2 were protected against I/R injury (Burchfield *et al*., 2010). Plasma TNF levels were significantly elevated in mice with cardiac-specific genetic ablation of TNFR1, which largely prevented the pathological cardiac remodelling and dysfunction associated with TRAF2 deletion (Guo *et al*., 2017). Importantly, genetic deletion of RIP3 largely rescued the cardiac phenotype triggered by TRAF2 deletion. Mechanistically, TRAF2 critically regulates RIP1/RIP3/MLKL necroptotic signalling through the adaptor protein TRADD as an upstream regulator and **TAK1** as a downstream effector (Dhingra and Kirshenbaum, 2014). All of this evidence indicates that TRAF2 plays a critical role in necroptotic cardiac cell death, pathological remodelling and HF, thereby providing a promising therapeutic target (Guo *et al*., 2017). Recently, it was shown that in addition to TNF-induced necrosis, FADD participates in H_2O_2-induced necrosis by influencing the formation of RIP1/RIP3 complexes in H9C2 cardiomyocytes (Wang *et al*., 2015). Furthermore, miR-103/107, which is regulated by long noncoding RNA H19, targets FADD directly. Together, these RNAs regulate necrosis in the cellular model as well as MI in a mouse I/R model (Wang *et al*., 2015).

Crosstalk between death receptor and mitochondrial necrosis pathways

The death receptor and mitochondrial necrosis pathways are functionally interconnected through several potential mechanisms. One possible connection is ROS, which are generated by catabolic enzymes that are activated by RIP3 and further increase the sensitivity of mPTP opening (Zhang *et al*., 2009). Second, some unidentified substrates of RIP3 may be components of mPTPs or may regulate these components indirectly. Finally, in response to TNF treatment, RIP1 translocates to the mitochondria and exerts possible effects on ANT

(Temkin *et al*., 2006) (Figure 1), thus providing an opportunity for additional regulation. Furthermore, the interaction between RIP3 and MLKL is required for the translocation of necrosomes to mitochondria-associated membranes, a process that is essential for necroptosis signalling (Chen *et al*., 2013).

Other forms of cardiomyocyte-programmed necrosis or necrosis-like cell death

*RIP3/**CaMKII**-mediated cardiomyocyte necrosis*. Recently, our group identified a novel form of regulated necrosis in cardiomyocytes that is mediated by RIP3/**Ca^{2+}/calmodulin protein kinase II (CaMKII)**/mPTP signalling and is independent of other necrosome components, such as RIP1 and MLKL (Zhang *et al*., 2016). In response to multiple cardiac insults, including I/R injury and **doxorubicin** treatment, RIP3 was up-regulated in cardiomyocytes, and it subsequently activated CaMKII through both direct phosphorylation and indirect oxidation (Zhang *et al*., 2016). Furthermore, neither RIP1 nor MLKL was required for RIP3/CaMKII-mediated cardiomyocyte necrosis (Zhang *et al*., 2016), showing that it is a process that is distinctly different from necroptosis, in which the formation of a RIP1/RIP3/MLKL necrosome is essential (Cho *et al*., 2009; He *et al*., 2009; Zhang *et al*., 2009; Sun *et al*., 2012). In addition to necrosis, the RIP3/CaMKII complex is also involved in cardiomyocyte apoptosis and inflammation, suggesting that this signalling is responsible for multiple forms of cardiac myocyte death and injury. Inhibition of the RIP3/CaMKII pathway thus represents an attractive potential therapeutic target for the treatment of cardiac diseases related to cardiomyocyte death (Feng and Anderson, 2017). On the other hand, RIP3/CaMKII-mediated cardiomyocyte necrosis is another point at which the mitochondria-mediated and death receptor-mediated cell necrotic pathways converge. Whether this form of programmed necrosis exists in other cell types and/or in other cardiac pathological conditions merits further study.

Pyroptosis. Pyroptosis was first reported in mouse macrophages infected with the Gram-negative bacterium *Shigella flexneri* (Chen *et al*., 1996). Pyroptosis is induced by **caspase-1** activation leading to the secretion of potent pro-inflammatory cytokines, inevitably killing the cell (Aglietti *et al*., 2016; Liu *et al*., 2016b). The detailed regulatory mechanisms of pyroptosis have been already reviewed (Mariathasan *et al*., 2004; Aglietti *et al*., 2016; Liu *et al*., 2016b).

In 2001, a highly selective inhibitor of caspase-1, **Ac-YVAD-cmk**, was shown to preserve contractile function in human atrial trabeculae subjected to simulated I/R (Pomerantz *et al*., 2001). Furthermore, caspase-1 overexpression in mice increased infarct size after I/R by 50%, whereas complete knockout of caspase-1 or the use of Ac-YVAD-cmk reduced infarct size (Syed *et al*., 2005; Kawaguchi *et al*., 2011; Koshinuma *et al*., 2014). **NLRP3** inflammasome-activated NLRP3/ASC-dependent inflammatory responses result in the release of significant amounts of caspase-1 and **IL-1β**, and these innate immune responses play an important role in diabetic cardiomyopathy, MI and I/R injury (Jong and Zuurbier, 2013; Sandanger *et al*., 2013; Takahashi, 2013). Moreover, diverse studies have confirmed that caspase-1-mediated pyroptosis is also extensively involved in the development of infectious diseases, nervous system-related diseases, atherosclerosis and other diseases (Chang *et al*., 2013; Tan *et al*., 2014; Li *et al*., 2014b). Thus, identification of additional proteolytic targets of caspase-1 could yield insight into the mechanism of pyroptosis and novel features of this form of cell death (Bergsbaken *et al*., 2009) and provide therapeutic strategies for cardiovascular disease.

Ferroptosis. Ferroptotic cell death was recognized fortuitously during a high-throughput screening process designed to identify molecules that selectively induce the death of isogenic cells carrying a RAS mutant isoform (Yang and Stockwell, 2008). An anticancer compound, erastin, was identified, and interestingly, this compound was found to induce a regulated but non-apoptotic form of cell death that depended on cellular iron stores. As a novel form of cell death, ferroptosis is similar to apoptosis and necrosis in cell morphology, characterized with small normal mitochondria-increased mitochondrial membrane density and reduction/vanishing of mitochondria crista (Dolma *et al*., 2003; Xie *et al*., 2016). The regulatory mechanisms of ferroptosis have been summarized in several previous reviews (Dixon *et al*., 2012; Zheng *et al*., 2017).

Since its discovery, ferroptosis has not only been verified as an attractive anticancer mechanism (Zheng *et al*., 2017) but has also been implicated in a broad range of pathological conditions, including liver (Sun *et al*., 2016), kidney (Krainz *et al*., 2016) and heart (Gao *et al*., 2015) dysfunction. Furthermore, current study showed that **mTOR** overexpression suppressed the cell death induced by ferroptosis inducers. Meanwhile, erastin-induced ROS production was significantly lower in mTOR-transgenic cells than in control cardiomyocytes, and mTOR deletion increased cell death under the same conditions (Baba *et al*., 2018). Taken together, these findings suggest that ferroptosis is a significant type of cell death in cardiomyocytes and that mTOR plays an important role in protecting cardiomyocytes against excess iron and ferroptosis, at least in part by regulating ROS production. It may be that understanding the effects of mTOR in preventing iron-mediated cell death will provide a basis for a new therapy for patients with MI (Baba *et al*., 2018).

Parthanatos. Parthanatos, which is distinct from apoptosis, necrosis or autophagy, is dependent on the generation of poly (ADP-ribose) (PAR) that triggers nuclear translocation of apoptosis-inducing factor (AIF) to result in caspase-independent cell death. **PARP-1**, which is the necessary factor of parthanatos, was originally considered as a 'genome guardian' (Poirier *et al*., 1982). The opening of mPTP and loss of mitochondrial membrane potential is an early event in PARP-1 dependent cell death (Yu *et al*., 2002; Alano *et al*., 2004). The mechanisms of parthanatos have been shown in several previous reviews (Mashimo *et al*., 2013; Wang *et al*., 2016).

Cytoprotection by either pharmacological inhibition or genetic knockdown of PARP-1 indicates that PARP-1 plays a significant role in cellular injury following cardiac I/R (Virag and Szabo, 2002). Currently, PARylation and AIF

translocation were significantly higher in the HF group and correlation to reduced cardiac function and the clinical appearance of chronic heart failure (CHF; Barany *et al.*, 2017). Moreover, oxidative stress causes DNA breaks producing the activation of nuclear PARP-1 enzyme that leads to energy depletion and unfavourable modulation of different kinase cascades (Akt-1/GSK-3β, MAPKs and various PKC isoforms), and thus, it promotes the development of HF (Halmosi *et al.*, 2016). The identification of PAR-binding proteins and their characterization may provide a novel opportunity to understand the PAR-signalling mechanisms and to develop low MW inhibitors to prevent toxic manifestations of parthanatos.

NETosis. NETosis is a form of cell death in neutrophils, apart from apoptosis and necrosis (Volker *et al.*, 2004; Guimaraes-Costa *et al.*, 2009). When NETosis happens, neutrophils generate extracellular fibres, or neutrophil extracellular traps (NETs), which are structures composed of granule and nuclear constituents that disarm and kill bacteria extracellularly in response to inflammatory stimuli (Volker *et al.*, 2004). NETs may serve as a physical barrier preventing further spread of bacteria. However, NETs might also have an adverse effect on the host, because viscous DNA NETs formed when hyperactivated neutrophils expel their chromatin as part of their immunological defence response, occluding the cardiac microcirculation, and decreasing reoxygenation of the tissue (Yang *et al.*, 2015). NETs contribute to endothelial damage, thrombosis and I/R injury, making it a novel player in the development of cardiovascular disease, especially thrombosis and atherosclerosis (Massberg *et al.*, 2010; Brill *et al.*, 2012; Knight *et al.*, 2014). The role of NETs and their components in pathophysiology of thrombosis was further confirmed by DNase and treatment with the anti-NET antibody, which decreased clot formation in a mouse model (Massberg *et al.*, 2010; Brill *et al.*, 2012).

Inhibition of peptidyl arginine deiminase by **Cl-amidine** treatment prevents NET formation and thereby decreases atherosclerotic lesion size and delays carotid artery thrombosis in apolipoprotein $E^{-/-}$ mice receiving a cholesterol-rich diet (Knight *et al.*, 2014). All in all, these findings lend support to a prominent role of NETosis in cardiometabolic diseases.

Programmed cardiomyocyte necrosis in cardiovascular diseases

A large number of studies have shown that cell death is an important component in the pathogenesis of various cardiovascular diseases (Tavernarakis, 2007; Dorn, 2009; Karch and Molkentin, 2015; Zhao *et al.*, 2015). This discussion now mainly focuses on cardiac myocytes, although a variety of other cell types are also involved. The magnitude and kinetics of cell death observed in various cardiac diseases differ substantially. For example, MI is characterized by a large burst of cardiac myocyte death that occurs during the 24 h following the onset of ischaemia (Zhu *et al.*, 2007). In contrast, HF induces ongoing cardiac myocyte death over periods of months to years at levels that, although low, are still 100-fold higher than those seen in healthy subjects (Tannous *et al.*, 2008). The renewed interest in the role of regulated cell death in heart disease has resulted in recognition of the fact that not only apoptosis but also necrosis is tightly regulated. Nevertheless, it is still unclear which cell death processes occur in specific heart diseases and which processes might be therapeutically useful.

Myocardial ischaemic injury

MI, a common presentation of ischaemic heart disease/coronary artery disease, is the leading cause of death worldwide (Sahoo and Losordo, 2014). In clinical treatment, necrosis has traditionally been considered to be the major type of cardiomyocyte death to occur during MI. Furthermore, programmed necrosis has been demonstrated to play a key role in the development of MI.

The best way to prevent cardiac ischaemic injury is to restore the blood flow to myocardial tissues, that is, reperfusion. However, reperfusion of the heart elicits further damage to the cardiac tissue, which is named as I/R injury. Ischaemic injury is often clinically divided into two stages: a reversible stage during which the injury is amenable to repair upon restoration of blood flow and an irreversible stage caused by persistent deprivation of oxygen and metabolic substrates. Cells die primarily through necrosis with extensive mitochondrial dysfunction in the irreversible stage. However, ischaemia alone does not account for all of the observed pathology. Neutrophil infiltration, cytokine production and generation of ROS greatly exacerbate the restoration of blood flow to irreversibly injured ischaemic tissues (Figure 1). *In vivo* studies of I/R injury use animal models with complete occlusion of one of the end arteries to an organ.

The most obvious connection between myocardial ischaemia-induced MI and necrotic cell death is mPTP. Ischaemia results in hypoxia, anaerobic metabolism and intracellular acidosis. In response to acidosis, H^+ is pumped out of the cell by the **Na^+/H^+ exchanger**, which consequently increases intracellular [Na^+]. The level of intracellular [Ca^{2+}] then increases due to the excess Na^+ handled by the **Na^+/Ca^{2+} exchanger**. A further elevation of intracellular [Ca^{2+}] results from Ca^{2+}-induced Ca^{2+} release from the endoplasmic reticulum/sarcoplasmic reticulum and reperfusion (Murphy and Steenbergen, 2008) (Figure 1). Each of these events contributes to the opening of mPTPs. CypD is an important positive regulator of mPTPs. Cells lacking CypD are resistant to oxidative stress/Ca^{2+}-induced cell death but sensitive to apoptotic stimuli. Both gene deletion and pharmacological inhibition of CypD reduce infarct size after I/R in mice (Griffiths and Halestrap, 1993; Clarke *et al.*, 2002; Hausenloy *et al.*, 2003; Argaud *et al.*, 2005; Baines *et al.*, 2005; Nakagawa *et al.*, 2005).

When patients were administered the CypD inhibitor **cyclosporin A**, cardiac injury was significantly reduced, as indicated by reduced serum levels of **creatine** kinase (Piot *et al.*, 2008). However, although significant reductions in infarct size persisted at 6 months post-MI, only a statistically insignificant trend towards preserved cardiac function was observed (Mewton *et al.*, 2010). Thus, further work is needed to assess the efficacy of this cardioprotective strategy in humans. Although the details of RIP1/RIP3 signalling remain obscure, **necrostatin-1**, a low MW inhibitor of

RIP1, reduced infarct size in response to I/R *in vivo* (Lim *et al.*, 2007). Interestingly, the cardioprotective effect of necrostatin-1 was dependent on the presence of CypD, suggesting a connection between RIP1 and mitochondrial necrosis (Lee *et al.*, 2003; Lim *et al.*, 2007). Although the molecular nature of this potential connection is still unclear, the generation of ROS by activation of metabolic pathways by RIP3 during necrosis is one possible explanation (Linkermann *et al.*, 2012). Inhibition of CaMKII by the selective inhibitor **KN-93** profoundly inhibited I/R-induced MI and necrosis due to the inhibition of the RIP3-CaMKII-mPTP myocardial necroptosis pathway (Zhang *et al.*, 2016). Taken together, these studies demonstrate again that, in addition to apoptosis, necrosis also contributes to the pathogenesis of MI.

Recently, Bax and Bak, two biomarkers of apoptosis, have also been shown to regulate necrosis. Deletion of Bax and Bak markedly reduces heart necrotic injury in mice subjected to I/R. These effects occur through a pathway distinct from the regulation of apoptosis by Bax and Bak, as shown by the retained ability of Bax mutants, which cannot support apoptosis, to mediate necrosis (Whelan *et al.*, 2012). In addition, research work in which I/R injury was mimicked in primary rat cardiomyocytes by hypoxia/reoxygenation (H/R) treatment showed that heat shock protein 70 (HSP70) down-regulates cardiomyocyte necroptosis by suppressing autophagy during myocardial I/R, revealing the novel protective mechanism of HSP70 and supplying the connection between regulated necrosis and I/R injury (Liu *et al.*, 2016a).

Currently, microRNAs (miR) have emerged as possible modulators of necroptosis initiation during I/R (Qin *et al.*, 2016). In fact, it has been reported that miR-223 KO mice show enhanced expression of primary necroptotic machinery proteins, whereas the converse is true for transgenic miR-223-overexpressing mice. Similar changes were found to correlate with cardiac resistance to I/R-induced necroptosis. Furthermore, the death receptors, TNFR1 and **death receptor 6**, both of which were previously shown to initiate necroptosis (Degterev *et al.*, 2008), have been identified as the targets of miR-223 (Qin *et al.*, 2016).

Heart failure

Heart failure (HF) is a clinical syndrome in which the heart is unable to pump sufficient blood to meet the needs of the body. HF often results from prior MIs. The progressive loss of cardiac myocytes and the development of cardiac dysfunction are characteristic features of HF. Although questions remain regarding the unambiguous identification of necrotic cells (short of performing time-consuming electron microscopy), the rate of necrosis in cardiomyocytes is elevated in failing human hearts compared with controls and appears to exceed that of apoptosis, which was shown to serve as one critical factor contributing to cell demise in end-stage HF in early studies (Guerra *et al.*, 1999).

In the connection between HF and necrosis, Ca^{2+} handling and mPTP opening may be critically involved. The evidence for this comes from transgenic overexpression of the **L-type Ca^{2+} channel β2a subunit** in cardiac myocytes, which resulted in intracellular Ca^{2+} overload, myocyte necrosis and HF (Nakayama *et al.*, 2007). Importantly, this phenotype was rescued by deletion of CypD but not by overexpression of **Bcl-2**. Similarly, doxorubicin-induced cardiomyopathy was ameliorated by knockout of peptidylprolyl isomerase F (Konstantinidis *et al.*, 2012). The currently recognized importance of the TNFR1-associated pathway of necroptosis in HF was identified because the expression of pro-inflammatory genes, including those encoding TNF, is up-regulated under conditions characterized by pressure/volume overload (Chen *et al.*, 2010; Chen *et al.*, 2011). Furthermore, inflammation-associated cell death mediated by TNF is relevant to the cardiomyocyte stretching observed in volume-overloaded systolic HF and the pressure overload seen in hypertension and aortic stenosis (Sakaguchi *et al.*, 2012). The expression of RIP1, phosphorylated and total RIP3 and active cytotoxic forms of MLKL is elevated in HF groups compared with controls. On the other hand, the subcellular localization of both RIP3 and phosphorylated MLKL was consistent with activation of necroptosis signalling.

The data discussed above provided the first evidence that necroptosis may be involved in the development of human HF, MI or dilated cardiomyopathy. In contrast to MI, the involvement of necrosis in HF is somewhat unexpected. Although this interpretation may be correct, it is important to also consider the recently discovered effects of CypD on cardiac metabolism (Elrod *et al.*, 2010). The magnitude of cardiac myocyte necrosis in failing hearts and the general applicability to pathogenesis of this syndrome will require clarification in future work.

Other cardiac diseases

Myocarditis. Viral myocarditis, especially acute viral myocarditis, with high mortality due to irreversible HF is only associated with cardiogenic shock and cardiac death in certain cases and is most commonly elicited by adenoviruses and enteroviruses, such as the coxsackieviruses (Pollack *et al.*, 2015; Heymans *et al.*, 2016). The main histopathological changes in heart tissue due to viral myocarditis are cardiomyocyte necrosis and inflammatory cell infiltration of the endothelium (Cooper, 2009). Research has shown that RIP1/RIP3 is highly expressed in cardiomyocytes in the acute viral myocarditis mouse model induced by CVB3; **Nec-1**, a specific blocker of the necroptosis pathway, dramatically reduced myocardial damage in this model by down-regulating the expression of RIP1/RIP3 (Zhou *et al.*, 2018). These findings provide evidence that necroptosis plays an important role in cardiomyocyte death and is a major form of cell death in acute viral myocarditis. Thus, blocking the necroptosis pathway may serve as a new therapeutic option for the treatment of acute viral myocarditis.

Sepsis-induced cardiac injury. Sepsis-induced cardiac dysfunction, one of the major causes of death in intensive care units, is induced by overwhelming of the inflammatory response and unrestrained cell death. Septic cardiac dysfunction is frequently associated with an imbalance in the production of pro-inflammatory cytokines (Haveman *et al.*, 1999; Oberholzer *et al.*, 2001), including TNF-α and **IL-6**, which leads to myocyte death, myocardium microlesions and cardiac dysfunction (Rudiger and Singer, 2007; Furian *et al.*, 2012). The decrease in inflammatory cytokine production attenuates cardiac dysfunction in

sepsis (Carlson *et al.*, 2005), highlighting the critical role of inflammation in the treatment of cardiac dysfunction in sepsis. In recent decades, **PPAR-γ,** a ligand-activated transcription factor that is involved in cell proliferation, lipid metabolism and inflammation (Zingarelli and Cook, 2005), has been proven to be cardioprotective in sepsis. Activation of PPAR-γ by rosiglitazone pretreatment decreased the levels of necrosis-associated proteins, including RIP1, RIP3 and MLKL, thereby improving the survival of septic rats. In contrast, inhibition of PPAR-γ further exacerbated the condition, decreasing the survival rate to close to 0% (Peng *et al.*, 2017). In conclusion, PPAR-γ activation, by reducing pro-inflammatory cytokines, apoptosis and necroptosis in the myocardium, prevents septic myocardial dysfunction.

Metabolism-associated injury. Hypercholesterolaemia, which is associated with increased morbidity and mortality, is still the leading risk factor for heart disease (Roger *et al.*, 2012). A number of mechanisms for the association of high cholesterol-induced oxidative/nitrosative stress with subsequent myocardial dysfunction have been proposed, including lipotoxicity, mitochondrial damage and intracellular Ca^{2+} mishandling. In addition, recent studies have revealed that inflammation and oxidative stress are closely associated with a type of necrosis termed cholesterol-induced necroptosis, a recently described type of programmed necrosis that is involved in cardiac impairment (Osipov *et al.*, 2008). Importantly, a high-cholesterol mouse model was shown to display significantly increased myocardial ROS and nuclear DNA damage and to lead to the activation of gene expression of TNF and RIP3 mRNA. These changes contributed to the elucidation of cholesterol-induced necroptosis (Chtourou *et al.*, 2015). Furthermore, it was confirmed that the positive interaction between necroptosis and ROS is due to injury induced by high glucose levels and inflammation in H9C2 cardiac cells (Liang *et al.*, 2017).

Transplant rejection. Anti-donor immune responses result in tissue damage caused by the death of heart myocytes. This occurs as an active molecular process and ultimately leads to rejection. Although recent advances in therapeutic immunosuppression have allowed adequate control of host immune cell-mediated acute rejection, the overall prognosis is not positive (Christie *et al.*, 2012).

It is well known that necroptosis leads to the release of inflammatory molecules and the expression of high mobility group box 1 (HMGB1), both of which can activate host immune cells (Vercammen *et al.*, 1998; Al-Lamki *et al.*, 2009; Kaczmarek *et al.*, 2013). The first evidence that myocyte necrosis is closely related to inflammation but independently varies with the grade of transplant rejection, was presented in 1989. Cardiomyocyte necrosis was detected by measuring donor heart antibody uptake and shown to be related to the grade of rejection (Allen *et al.*, 1989). However, a prospective study involving 64 consecutive patients who underwent orthotopic heart transplantation recently demonstrated that there is no association between measured myocardial cell death, necrosis and apoptosis markers in donor myocardium and primary graft dysfunction in allograft recipients (Szarszoi *et al.*, 2016), suggesting that more detailed investigations of the role of cardiomyocyte necrosis in transplanted hearts are required.

Hypertension-induced cardiomyopathy. In both humans and animal models, pressure overload induced by various pathological factors (e.g. hypertension) is characterized by a period of compensation in which left ventricular concentric hypertrophy normalizes systolic wall stress and contractile function is preserved. The period of adaptation is followed by a transition to maladaptive cardiac remodelling and HF, that is, hypertension-induced cardiomyopathy. Hypertension-induced cardiomyopathy is mainly due to the changes in the composition of the motor unit and cytoskeleton of cardiomyocytes (Wagoner and Walsh, 1996), alterations in the metabolism of the extracellular matrix (Weber, 1997) and cardiomyocyte loss (Bing, 1994; Li *et al.*, 1993; Ikeda *et al.*, 2002).

Although most of the studies on the cardiomyocyte death in hypertension-induced cardiomyopathy are on apoptosis (Teiger *et al.*, 1996; González *et al.*, 2002; Gonzalez *et al.*, 2003; Gonzalez *et al.*, 2006), cardiomyocyte necrosis is also detected in hypertensive animal models (Ratajska *et al.*, 1994; Matsubara *et al.*, 1999). Cardiomyocyte necrosis induced by hypertension is related to the increased circulating **angiotensin II** and catecholamines (Ratajska *et al.*, 1994; Matsubara *et al.*, 1999). But the role of cardiomyocyte-programmed necrosis in hypertension-induced cardiomyopathy remains largely unknown.

Therapeutic opportunities of regulated necrosis in cardiac diseases

RIP1 inhibitors

RIP1, which belongs to the seven-member RIP serine/threonine kinase family, plays essential roles in cell necroptosis (Cho *et al.*, 2009; He *et al.*, 2009; Zhang *et al.*, 2009). The first specific and potent low MW RIP1 inhibitor, necrostatin-1, was identified in 2005 and shown to inhibit the cell death induced by TNF/death receptor signalling *via* the inactivation of caspase. Its discovery provided the first direct evidence that death receptor signalling triggered a common alternative non-apoptotic cell death pathway that was later termed necroptosis (Degterev *et al.*, 2005). Furthermore, the data showed that necroptosis is a delayed component of ischaemic neuronal injury that involves necrostatin-1 and its derivatives (Degterev *et al.*, 2005).

The action of necrostatin-1 in reducing peroxide-induced cell death, which is accompanied by delayed opening of mPTPs, was examined in cultured C2C12 and H9C2 myocytes (Smith *et al.*, 2007). Necrostatin-1 can also reduce infarct size in isolated perfused and *in vivo* mouse hearts (Smith *et al.*, 2007). Another interesting finding with respect to the above studies is that, although low concentrations of necrostatin-1 protected against infarction, increased concentrations enhanced infarct size (Smith *et al.*, 2007). It was concluded that at higher concentrations, necrostatin-1 may have non-specific or toxic actions that potentiate apoptotic and necrotic mechanisms, culminating in enhanced MI. In

an *in vivo* murine study, necrostatin-1 was shown to reduce infarct size when administered both prior to ischaemia and after the initiation of reperfusion, and its cardioprotective effect is mediated by the inhibition of necroptosis in a caspase-independent mechanism (Chua *et al.*, 2006). Further and perhaps more concrete evidence that necrostatin-1 protects against myocardial I/R injury by modulating mPTP opening at reperfusion was obtained in *in vivo* experiments in mice (Lim *et al.*, 2007). The cardioprotective effects of necrostatin-1 were lost in CypD$^{-/-}$ animals, indicating that its cardioprotection operates *via* inhibition of CypD-mediated mPTP opening.

It has been postulated that mPTP inhibition occurs as a consequence of activation of the so-called reperfusion injury salvage kinase pathway. The precise mechanisms by which necrostatin-1 inhibits mPTP opening in the heart and protects against myocardial I/R injury have yet to be delineated. Treatment with necrostatin-1 attenuated ROS generation and the expression of HMGB1, **IL-23** and **IL-17A** and increased the expression of **NOS2** and **COX-2** in a mouse model (Zhang *et al.*, 2014). Furthermore, together with the fact that the decreased TnT expression induced by necrostatin-1 was blocked by exogenous HMGB1 administration, it was concluded that necrostatin-1 played a protective role in cardiomyocyte I/R injury, and this was associated with inhibition of the HMGB1/IL-23/IL-17 pathway. Administration of necrostatin-1 at the onset of reperfusion inhibits RIP1-dependent necrosis *in vivo* and leads toa reduction of infarct size and preservation of cardiac function in acute MI rats (Oerlemans *et al.*, 2012; Liu and Xu, 2016). Intraperitoneal injection of necrostatin-1 or necrostatin-5 before reperfusion of the isolated rat heart reduced the infarction zone (Dmitriev *et al.*, 2013). Similarly, intravenous administration of Nec-1 prior to reperfusion in swine I/R injury effectively reduced infarction size and preserved left ventricular function (Liu and Xu, 2016).

A derivative of necrostatin-1, **Nec-1s**, also called 7-Cl-O-Nec-1, (Figure 2 and Table 1), which is obtained by chemical modification of necrostatin-1, lacks IDO inhibitory activity and has increased plasma stability and increased specificity for RIP1 over a broad range of kinases (Takahashi *et al.*, 2012), suggesting that Nec-1s would be preferred tool for targeting RIP1 *in vivo*. High-resolution determination of the structure of RIP1 bound to Nec-1s revealed that Nec-1s binds in a relatively hydrophobic pocket between the N and C lobes in close proximity to the activation loop (Xie *et al.*, 2013), making RIP1 unable to phosphorylate RIP3 and consequently unable to assemble the necrosome (Xie *et al.*, 2013). In addition, it was established that cell loss by necroptosis can be prevented by the necrostatin-1 analogues, necrostatin-3 and -4 (Figure 2 and Table 1), and necrostatin-5 and -7 (Degterev *et al.*, 2008; Takahashi *et al.*, 2012).

By means of a fluorescence polarization assay, three classes of compounds (1-aminoisoquinolines, pyrrolo[2,3-*b*] pyridines and furo[2,3-*d*]pyrimidines) that bind to the catalytic site of RIP1 were identified (Harris *et al.*, 2013). Cpd27, one of the compounds in the furo[2,3-*d*] pyrimidine series, showed potent anti-RIP1-kinase activity and blocked TNF-induced lethality in a mouse model of systemic inflammatory response syndrome (Harris *et al.*, 2013) (Figure 2 and Table 1).

GSK963 (Figure 2 and Table 1) is a structurally distinct, 'non-traditional' RIP1 kinase inhibitor that offers several distinct advantages over the other RIP1 inhibitors that have been described to date. It is more potent than Nec-1 in both biochemical and cellular assays, inhibiting RIP1-dependent cell death with an IC_{50} of between 1 and 4 nM in human

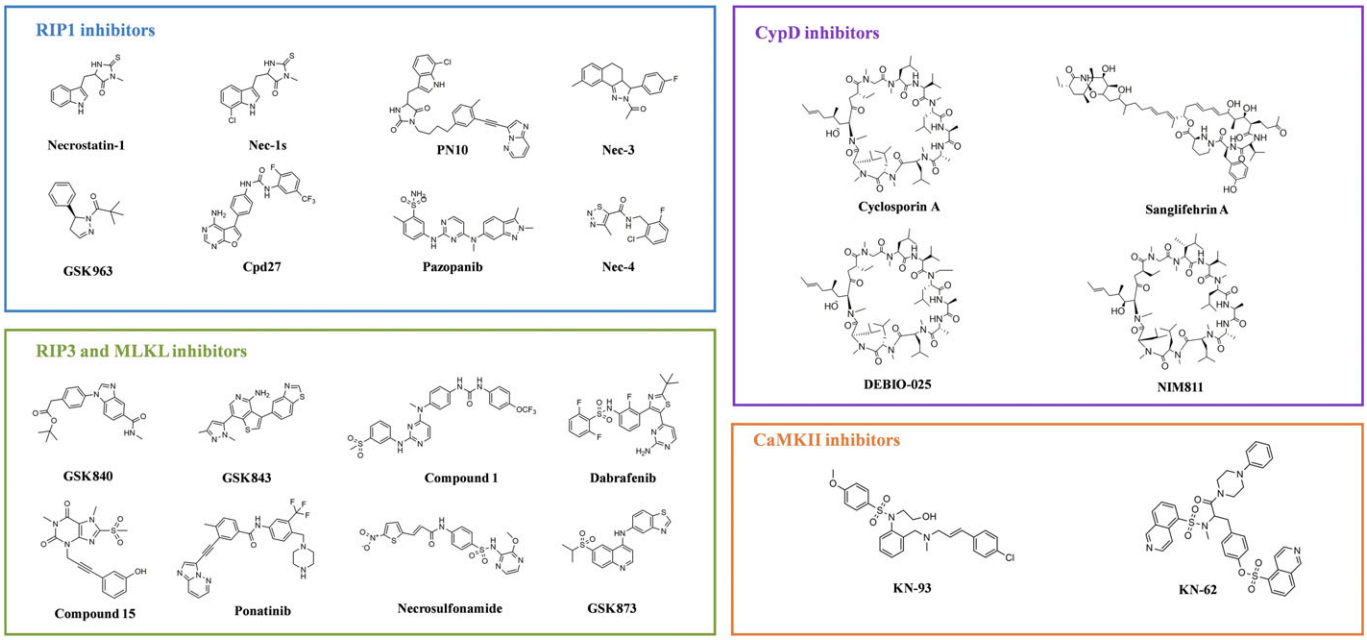

Figure 2

Chemical structures of inhibitors of regulated necrosis. The mechanisms of action and key functions of the inhibitors, along with relevant references are provided in Table 1.

Table 1

Summary of low MW modulators of regulated necrosis in cardiovascular diseases

Compound	Mechanism	Application	Reference
Necrostatin-1	RIP1 inhibitor	Protective effect on myocardial tissue in rats/pigs with acute MI and paraquat-induced cardiac contractile dysfunction in mice	Degterev *et al.* (2013), Koudstaal *et al.* (2015), Liu and Xu (2016), Szobi *et al.* (2016) and Zhang *et al.* (2018)
Nec-1s (R-7-Cl-O-Nec-1)	RIP1 inhibitor	Suppressed necroptosis in I/R hearts	Qin *et al.* (2017)
PN10	RIP1 inhibitor	Blocker of TNF-induced injury *in vivo*	Najjar *et al.* (2015)
Pazopanib	RIP1 inhibitor	Inhibited necroptotic cell death induced by various cell lines, while not protecting from apoptosis	Fauster *et al.* (2015)
GSK'963	RIP1 inhibitor	Inhibited TNF-α/zVAD-induced injury *in vivo* at a dose of 2 $mg \cdot kg^{-1}$	Berger *et al.* (2015)
Cpd27	RIP1 inhibitor	Prevented TNF-induced lethality in a mouse model of SIRS	Harris *et al.* (2013)
Necrostatin-4	RIP1 inhibitor	Suppressed necroptosis *in vivo*	Degterev *et al.* (2008)
Necrostatin-3	RIP1 inhibitor	Suppressed necroptosis *in vivo*	Degterev *et al.* (2008)
Ponatinib	RIP1/RIP3 inhibitor	Inhibited necroptotic cell death induced by various cell lines, while not protecting from apoptosis	Fauster *et al.* (2015)
GSK840	RIP3 inhibitor	Prevented LPS-induced cell death by LPS/TNF-α/z VAD/poly (I : C)-triggered death *in vitro*	Mandal *et al.* (2014)
GSK843	RIP3 inhibitor	Prevented LPS-induced cell death by LPS/TNF-α/z VAD/poly (I : C)-triggered death *in vitro*	Mandal *et al.* (2014)
GSK872	RIP3 inhibitor	Prevented LPS-induced cell death by LPS/TNF-α/z VAD/poly (I : C)-triggered death *in vitro*	Mandal *et al.* (2014)
Dabrafenib	RIP3 inhibitor	Suppressed necroptosis *in vivo* (the only RIPK3 inhibitor tested to date)	Li *et al.* (2014a)
Necrosulfonamide	MLKL inhibitor	Prevented necroptosis induced by TNF-α/zVAD in mouse fibroblasts	Sun *et al.* (2012)
Compound 1	MLKL inhibitor	Inhibited necroptotic death of mouse dermal fibroblasts	Hildebrand *et al.* (2014)
Compound 15	MLKL inhibitor	Inhibited oligomerization and translocation of MLKL to the cell membrane	Yan *et al.* (2017)
Cyclosporin A	CypD inhibitor	Reduced infarct size and improved post-ischaemic recovery of the MI and I/R hearts in mice, rats, rabbits and pigs	Argaud *et al.* (2005), Gomez *et al.* (2005), Devalaraja-Narashimha *et al.* (2009), Boengler *et al.* (2010) and Skyschally *et al.* (2010)
Sanglifehrin A	CypD inhibitor	Protective in several mouse and rat models of I/R injury	Clarke *et al.* (2002) and Linkermann *et al.* (2013)
Debio-025	CypD inhibitor	Reduced the sensitivity of the mPTP to Ca^{2+} and reduced infarct size efficiently (i.e. 48%)	Gomez *et al.* (2007)
NIM811	CypD inhibitor	Blocked mPTP opening and protected diabetic hearts from injury in rats	Sloan *et al.* (2012)
KN-93	CaMKII inhibitor	The most widely used CaMKII inhibitor *in vivo* and effectively suppresses ventricular arrhythmia induced by LQT2 without decreasing TDR in rabbits	Anderson *et al.* (1998), Ke *et al.* (2012), Wang *et al.* (2013) and Hegyi *et al.* (2015)
KN-62	CaMKII inhibitor	Shares similar structural elements and mechanism of action with KN-93 and binds to the holoenzyme and interferes without directly binding to CaM	Okazaki *et al.* (1994) and Narayanan *et al.* (1996)
SMP-114 (rimacalib)	CaMKII inhibitor	A p.o. available CaMKII inhibitor that has already entered clinical phase II trials for the treatment of rheumatoid arthritis and may also be useful in treating cardiac SR Ca^{2+} leakage and its arrhythmogenic cellular correlates in rodents	Gaskin *et al.* (2003) and Neef *et al.* (2017)

continues

Table 1
(Continued)

Compound	Mechanism	Application	Reference
AC3-I	CaMKII inhibitor	A peptide mimicking the autoinhibitory regulatory segment of CaMKIIα, lacks the CaM-binding sequence and protects against myocardial apoptosis induced by MI or isoprenaline administration	Yang *et al.* (2006)

SIRS, systemic inflammatory response syndrome; SR, sarcoplasmic reticulum.

and murine cells (Berger *et al.*, 2015). Although it lacks measurable activity against IDO *in vivo*, GSK963 provides much greater protection against hypothermia at matched doses to Nec-1 in a model of TNF-induced sterile shock, clarifying our current understanding of the role of RIP1 in contributing to disease pathogenesis.

A phenotypic screening of potential low MW inhibitors of TNF-α-induced necroptosis in FADD-deficient Jurkat cells was conducted using a representative panel of FDA-approved drugs (Fauster *et al.*, 2015). In this screening, two anticancer agents were identified as necroptosis inhibitors. **Ponatinib** inhibits both RIP1 and RIP3, while **pazopanib** preferentially targets RIP1. PN10, a highly potent and selective 'hybrid' RIP1 inhibitor based on ponatinib, was developed; this compound combines the favourable properties of two different allosteric RIP1 inhibitors, ponatinib and necrostatin-1 (Najjar *et al.*, 2015).

RIP3 inhibitors

RIP3, another member of the RIP serine/threonine kinase family, has been implicated as a critical regulator of necroptosis and has been shown to be associated with various diseases. Thus, RIP3 inhibitors are promising candidates for clinical use.

The scaffold function of RIP3 is to stabilize RIP1 of complex IIb, thus propagating RIP1-dependent apoptosis; this only occurs at higher protein expression levels. This observation is supported by studies of two RIP3 kinase inhibitors, GSK843 and GSK872; at high concentrations, these inhibitors promote TNF-induced RIP1-dependent apoptosis and caspase-8 activation (Figure 2 and Table 1) (Mandal *et al.*, 2014). Additionally, the recognition that necroptosis could occur independently of RIP1 spurred discussions that RIP3 inhibitors may have an effect, leading to the identification of compounds GSK840, GSK843 and GSK872 (Figure 2 and Table 1) (Mandal *et al.*, 2014). These compounds, especially GSK840, inhibited RIP3 with high specificity within a panel of 300 other human kinases. In contrast to GSK843 and GSK872, GSK840 does not induce RIP1 kinase activity-dependent apoptosis at higher concentrations. Unfortunately, however, GSK840 is unable to inhibit murine RIP3, making it impossible to assess its potential for disease treatment using murine experimental disease models (Mandal *et al.*, 2014).

Recently, a viral inhibitor of RIP3-dependent necroptosis has been reported (Upton *et al.*, 2010). In addition, the findings indicated that murine cytomegalovirus M45, which acts in concert with the viral inhibitor of RIP activation, potentially inhibits RIP-induced necrotic cell death and accelerates viral replication. M45 inhibits TNFR/FasL/TNF-induced necroptosis by inhibiting either RIP1, RIP1–RIP3 complex formation or RIP3 alone (Mack *et al.*, 2008; Upton *et al.*, 2008).

Dabrafenib (Figure 2 and Table 1), a B-RafV600E inhibitor, is an important anticancer drug for metastatic melanoma therapy. Dabrafenib inhibits RIP3 enzymatic activity *in vitro* by competing with the binding of ATP to the enzyme (Li *et al.*, 2014a). Moreover, dabrafenib rescued cells from RIP3-mediated necroptosis by decreasing RIP3-mediated MLKL phosphorylation and disrupting RIP3/MLKL interaction rather than by inhibition of B-Raf. Dabrafenib was further shown to prevent necrosis induced by paracetamol (acetaminophen) *in vivo* and *in vitro* (Li *et al.*, 2014a). The function of RIP3 inhibitors in cardiovascular diseases treatment remains unclear, although RIP3 plays a critical role in sevrla types of cardiac injury, as discussed (Cho *et al.*, 2009; He *et al.*, 2009; Zhang *et al.*, 2009; Luedde *et al.*, 2014). In the clinic, RIP3 inhibitors may represent potential preventive or therapeutic agents for necroptosis-related cardiovascular diseases involving RIP3.

MLKL inhibitors

MLKL, which has been proposed to be the terminal protein in the execution of necroptotic cell death, is up-regulated in some heart diseases (Sun *et al.*, 2012). The first compound reported to inhibit MLKL was (*E*)-*N*-(4-(*N*-(4,6-dimethylpyrimidin-2-yl)sulfamoyl)phenyl)-3-(5-nitrothiophene-2-yl) acrylamide [also named necrosulfonamide (NSA)] (Figure 2 and Table 1) (Sun *et al.*, 2012). Upon induction of necroptosis by TNF, the formation of punctate structures that resembled the amyloid-like structures formed by RIP1/RIP3 interaction could be prevented by necrostatin-1 but not by NSA (Sun *et al.*, 2012). Furthermore, NSA did not affect the RIP3-dependent phosphorylation of MLKL and was able to protect human cells but not murine cells due to the difference in the sequences of human and murine MLKL (Sun *et al.*, 2012). On the NSA scaffold, a new class of MLKL inhibitors based on '**compound 1**' (also known as GW806742X or SYN-1215) circumvent NSA in terms of its specificity by targeting the pseudokinase domain of MLKL (Figure 2 and Table 1) (Hildebrand *et al.*, 2014). These compounds directly block the switch that activates MLKL upon RIP3-mediated phosphorylation, thus preventing MLKL oligomerization and translocation. Somewhat surprisingly, however, recent data have indicated that compound 1 inhibits not only MLKL but also RIP1 (Silke and Vince, 2012; Hildebrand *et al.*, 2013; Newton *et al.*, 2016). Because of its binding to RIP1, compound 1 cannot decisively be used to

implicate MLKL in necroptosis and the specificity of compound 1 deserves further investigation.

Recently, Yan *et al*. (2017) identified a novel MLKL inhibitor, compound 15 (TC13172), by phenotypic screening (Figure 2 and Table 1). This report presented the first example of the use of LC-MS/MS to identify an MLKL inhibitor. Compound 15 inhibits the oligomerization and translocation of MLKL to the cell membrane (Yan *et al*., 2017). The discovery

Table 2

Programmed necrosis in cardiomyocytes and its pharmacological interventions in cardiac diseases

Programmed necrosis	Cardiac disease model	Species	Pharmacological intervention	Outcomes	Reference
mPTP-dependent programmed necrosis	Myocardial I/R injury	Rat	Sanglifehrin A	Reduction of infarct size, only when given at reperfusion	Hausenloy *et al*. (2003)
	Acute MI	Clinical trials	Cyclosporin A	Reduction of infarct size, reduction of creatine kinase and troponin I release	Piot *et al*. (2008)
	Acute ST-segment elevation MI	Clinical trials	Cyclosporin A	Reduction of infarct size measured by MRI	Piot *et al*. (2008)
	Myocardial infarction	Pig	Cyclosporin A	Ambiguous results on the effect of infarct size in different researches	Karlsson *et al*. (2010), Lie *et al*. (2010), Skyschally *et al*. (2010) and Karlsson *et al*. (2012)
Necroptosis	Myocardial I/R injury	Mouse	Nec-1	Reduction of infarct size after I/R	Lim *et al*. (2007)
		Mouse	Nec-1	Reduction of infarct size after I/R and protection of long-term heart function with reduced fibrosis and inflammation	Lim *et al*. (2007) and Oerlemans *et al*. (2012)
		Mouse	Nec-1	Reduction of infarct size	Smith *et al*. (2007)
		Mouse	Nec-1	No additional infarct size in $CypD^{-/-}$ mice	Lim *et al*. (2007)
		Mouse	Nec-1	Reduction of cell death and deletion of mPTP opening	Smith *et al*. (2007)
		Human CMPCs	Nec-1	Reduction of necrosis measured by cytometry	Lim *et al*. (2007)
RIP3/CaMKII-mediated necrosis	Myocardial I/R injury	Mouse	KN-93	Reduction of cardiomyocyte necrosis and infarct size	Zhang *et al*. (2016)
	Dox-induced HF	Mouse	KN-93	Amelioration of cardiomyocyte necrosis and HF	Zhang *et al*. (2016)
Pyroptosis	Myocardial I/R injury	Mouse	Ac-YVAD-cmk	Reduction of infarct size	Syed *et al*. (2005), Kawaguchi *et al*. (2011) and Koshinuma *et al*. (2014)
		Clinical trials	Ac-YVAD-cmk	Protection of contractile function	Pomerantz *et al*. (2001)
Ferroptosis	Myocardial I/R injury	Mouse	Compound 968	Inhibition of glutaminolysis and ferroptosis and reduction of infarct size	Gao *et al*. (2015)
		Mouse	Ferrastatin-1	Inhibition of glutaminolysis and ferroptosis and reduction of infarct size	Gao *et al*. (2015)
Parthanatos	HF	Mouse	AG690/11026014	Protection of AngII-induced cardiac remodelling and improvement of cardiac function	Feng *et al*. (2017)
NETosis	Deep vein thrombosis	Mouse	DNase 1	Protection of DVT after 6 h and also 48 h IVC stenosis	Brill *et al*. (2012)

AngII, angiotensin II; CMPCs, cardiomyocyte progenitor cells; DVT, deep vein thrombosis; IVC, inferior vena cava; Nec-1, necrostatin-1.

of the novel and potent MLKL inhibitor reported here will almost certainly be beneficial in exploring the biological function of MLKL, including its role in necroptosis-related heart disease pathogenesis (Yan *et al.*, 2017).

CypD inhibitors

Mice with deletion of *ppif* (the gene-encoding CypD) are more resistant to I/R damage of the heart than the WT mice (Baines *et al.*, 2005; Nakagawa *et al.*, 2005; Alam *et al.*, 2015; Ikeda *et al.*, 2015). Inhibition of CypD can be achieved pharmacologically through the use of immunophilin-binding ligands (Zhang *et al.*, 2001) or sanglifehrin A (Figure 2 and Table 1). **Cyclosporin A** (Figure 2 and Table 1) binds to CypD and inhibits Ca^{2+}-induced mPTP opening (Millay *et al.*, 2008) during I/R in the kidney (Linkermann *et al.*, 2013), brain (Nighoghossian *et al.*, 2015) and heart (Keogh, 2004). However, it is important to note that despite the encouraging results obtained using these compounds in I/R models, their protective effects cannot be exclusively attributed to the inhibition of CypD-dependent necrosis because cyclosporin A and sanglifehrin A are also immunosuppressants that block the immune response.

Conversely, many results argue strongly against CypD as an essential structural component of the pore. Thus, further evidence is needed to settle this question. In addition, studies have shown that a novel cyclosporin A analogue, Debio-025 (Figure 2 and Table 1), specifically binds to mitochondrial cyclophylin D and reduces the sensitivity of the mPTP to Ca^{2+}, thereby efficiently reducing infarct size (Gomez *et al.*, 2007).

NIM811 (Figure 2 and Table 1), a non-immunosuppressive derivative of cyclosporin A, is very effective at blocking mPTP across a wide range of doses. NIM811 blocks mPTP formation by selectively binding matrix CypD. However, unlike cyclosporin A, it does not bind cyclophilin A. NIM811 was shown to protect diabetic hearts from injury in rats (Sloan *et al.*, 2012).

CaMKII inhibitors

CaMKII is a serine–threonine kinase that is abundant in myocardium and other excitable tissues. Emerging evidence suggests that sustained CaMKII activation plays a central role in the pathogenesis of a variety of cardiac diseases, such as HF (Backs *et al.*, 2009; Ling *et al.*, 2009), arrhythmia (Wu *et al.*, 2002) and other forms of heart disease (Wagner *et al.*, 2015). The programmed cell death evoked by CaMKII includes apoptosis and necroptosis and is one of the key mechanisms underlying the detrimental effect of sustained CaMKII activation (Vila-Petroff *et al.*, 2007; Joiner *et al.*, 2012; Zhang *et al.*, 2016). Development of new inhibitors will enable preclinical proof of concept tests and clinical development of successful lead compounds and will provide improved research tools that can be used to more accurately examine and extend knowledge of the role of CaMKII in cardiac health and disease (Pellicena and Schulman, 2014).

The most widely used inhibitor in the study of the cellular and *in vivo* functions of CaMKII is KN-93 (Figure 2 and Table 1), one of a remarkable number of inhibitors developed by Sumi *et al.* (1991). KN-93 supplanted **KN-62** (Figure 2 and Table 1), with which it shares similar structural elements and a similar mechanism of action (Tokumitsu *et al.*, 1990). Both of these compounds are likely to block the ability of Ca^{2+}/CaM to wrap around the CaM-binding segment and free it from the catalytic domain (Pellicena and Schulman, 2014). Inhibition of CaMKII by KN-93 profoundly inhibits I/R and doxorubicin-induced MI and necrosis and blocks RIP3-induced ROS production (Zhang *et al.*, 2016). Furthermore, identification of the autoinhibitory regulatory segment of CaMKII-α led to the development of long inhibitory peptides (Payne *et al.*, 1988; Malinow *et al.*, 1989) such as autocamtide-3 inhibitor (AC3-I) (Braun and Schulman, 1995) and autocamtide-2 inhibitor proteins (Ishida *et al.*, 1995). However, some caution is warranted in the use of peptide inhibitors that are often optimistically described as 'highly specific inhibitors' when experience or data suggest otherwise (Pellicena and Schulman, 2014). For example, off-target effects of AC3-I can occur when peptides are fused to GFP to increase expression and metabolic stability or when the peptides are modified by the addition of lipids or internalization sequences to allow cell permeation (Patel *et al.*, 1999; Wu *et al.*, 2009; Pellicena and Schulman, 2014). In summary, CaMKII plays a central role in cardiac myocyte death, and thus targeting this kinase is a promising approach for treatment of cardiovascular disease.

Conclusion

The recognition that a substantial proportion of necrotic death is regulated has consequences for many different areas of science and medicine. First, this recognition raises questions about the physiological roles of necrosis, the molecular connections between necrosis and other death processes and the evolutionary relationships among various forms of cell death. In addition, mounting evidence from *in vivo* and *in vitro* studies suggests that cardiac cell death plays an important role in the pathogenesis of cardiac diseases. Thus, inhibition of cardiac myocyte necrosis offers a novel approach to the treatment of cardiac diseases. The relationship between different types of cell death and cardiac disease models, together with the pharmacological intervention approaches and outcomes, is summarized in Table 2.

Although studies using experimental animal models have revealed that inhibition of the cellular components that regulate necrotic cell death is a valuable therapeutic strategy, a number of problems remain unsolved. For instance, whether the proteins that mediate necrotic death have functions that extend beyond their role in necrosis regulation is unclear. As an example, *in vivo* targeting of RIP1 or RIP3 under pathophysiological conditions that involve complex intercellular interactions may affect not only necroptosis but also apoptosis and activation of the inflammasome. Many death regulatory genes are common to more than one mode and, therefore, necrosis should be considered as a network of interconnected pathways comprising of different forms of cell death (Ouyang *et al.*, 2012). This complexity should be taken into account when evaluating the therapeutic activity of drugs in experimental disease models.

In addition, the crosstalk among apoptosis, necrosis, autophagic cell death and other forms of cell death has not been thoroughly characterized to date. Noteworthy, multiple forms of cell death are present in one diseased condition.

Under certain conditions, including cancer (Liu *et al.*, 2012), HF (Zhang *et al.*, 2016), inflammatory bowel disease (Nunes and Bernardazzi, 2014) and hypercholesterolaemia (Li *et al.*, 2015), apoptosis and programmed necrosis can be induced simultaneously. Zhang *et al.* (2016) proposed that approximately 30% of doxorubicin-induced and H/R-induced cell death could be blocked by zVAD (an inhibitor of apoptosis), suggesting that under these conditions, cardiomyocyte necrosis and apoptosis develop at the ratio of 7:3. Myocardial I/R leads to many secondary effects including disruption of cellular energy metabolism, production of ROS and DNA damage. These secondary effects lead to activation of the nuclear repair enzyme PARP1 and the transcription factor p53. Furthermore, activation of these proteins can initiate inflammatory signalling, intrinsic pathways that induce necrosis through opening of the mPTP and apoptotic tubular cell death through permeabilization of the mitochondrial outer membrane (Wolff *et al.*, 2008; Elrod and Molkentin, 2013). Another example is in MI. It is proposed that mPTP, which is composed of the dimers of ATP synthase complex, can be opened by the interaction of CypD with the lateral stalk of the ATP synthase complex. Thus, the opening of mPTP in MI may induce the production of ATP, which subsequently induces the occurrence of pyroptosis (Giorgio *et al.*, 2013). However, the specific relationships among these pathways are ambiguous. Further investigation is required to determine the key factors and the specific biomarkers in cell death and the mechanisms underlying activation of the cell death machinery in cardiac diseases.

Another important issue is the determination of which step to target in each pathway. It is uncertain whether preservation of cell survival by inhibiting effector caspases truly results in preservation of cell function because such inhibition does not preserve mitochondrial integrity. Mitochondria are responsible for providing ATP to the myocytes and are therefore essential for survival.

Finally, before new, effective and safe drugs for the prevention or treatment of cardiovascular diseases can be developed, an increased understanding of the proteins involved in regulating cell death is necessary. For example, there is still ambiguity regarding the molecular mechanism by which RIP1/RIP3/MLKL, the best-established downstream mediator of necroptosis identified so far, mediates the execution of necroptosis.

Although there is still a long way to go, the concept that cell necrosis is a tightly regulated process opens up a brand new direction in the prevention and therapy of human diseases. Future studies aimed at the discovery of new components and regulatory mechanisms in programmed necrosis signalling, as well as translational studies directed at the development of new inhibitors with high specificity and low toxicity that are better suited to clinical realities, are warranted.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b,c).

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Conflict of interest

The authors declare no conflicts of interest.

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