Programmed necrosis in cardiomyocytes: mitochondria, death receptors and beyond

Junxia Zhang, Dairu Liu, Mao Zhang and Yan Zhang
State Key Laboratory of Membrane Biology, Institute of Molecular Medicine, Peking University, Beijing, China

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.
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; Degtirev 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 Ca2+-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, Ca2+-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 Ca2+ 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 H2O2-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 (ΔΨm), redistribution of solutes and ions across the IMM and destruction of the
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, lipooxygenases and sphingomyelinsases 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 (Remijen 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 necrotic 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 necrotic 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 H2O2-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.
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(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/Ca2+/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/CaMKII 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 (Bergsakken 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 cristae (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.

NEToxis. NEToxis is a form of cell death in neutrophils, apart from apoptosis and necrosis (Volker et al., 2004; Guimaraes-Costa et al., 2009). When NEToxis happens, neutrophils generate extracellular fibres, or neutrophil extracellular traps (NETs), which are structures composed of granule and nuclear constituents that disarr 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 immunologial 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 NEToxis 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 [Ca²⁺] then increases due to the excess Na⁺ handled by the Na+/Ca²⁺ exchanger. A further elevation of intracellular [Ca²⁺] results from Ca²⁺-induced Ca²⁺ 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/Ca²⁺-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 necrotic 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 cardiomyocytes, 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 cytoxic 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 Ca2+ 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<sup>−/−</sup> 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 attenuates 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 to a 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<sub>50</sub> of between 1 and 4 nM in human cardiomyocytes.

**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.
| 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 necrotic 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·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 necrotic 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/zVAD/poly (1 : C)-triggered death in vitro | Mandal et al. (2014)                                                                                 |
| GSK843           | RIP3 inhibitor  | Prevented LPS-induced cell death by LPS/TNF-α/zVAD/poly (1 : C)-triggered death in vitro | Mandal et al. (2014)                                                                                 |
| GSK872           | RIP3 inhibitor  | Prevented LPS-induced cell death by LPS/TNF-α/zVAD/poly (1 : 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 necrotic cell 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 Ca²⁺ 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 Ca²⁺ 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 TNF/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 sevral 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 necrotic 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

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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 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 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) |
| | 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 cyclophilin 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., 2007). 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 supplants 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 intracellular 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.

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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 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 (Giordano 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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