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Necroptosis, necrostatins and tissue injury

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Abstract

Cell death is an integral part of the life of an organism being necessary for the maintenance of organs and tissues. If, however, cell death is allowed to proceed unrestricted, tissue damage and degenerative disease may ensue. Until recently, three morphologically distinct types of cell death were recognized, apoptosis (type I), autophagy (type II) and necrosis (type III). Apoptosis is a highly regulated, genetically determined mechanism designed to dismantle cells systematically (*e.g.* cells that are no longer functionally viable), *via* protease (caspase) action, and maintain homeostasis. Autophagy is responsible for the degradation of cytoplasmic material, *e.g.* proteins and organelles, through autophagosome formation and subsequent proteolytic degradation by lysosomes, and is normally considered in the context of survival although it is sometimes associated with cell death. Necrosis was formerly considered to be an accidental, unregulated form of cell death resulting from excessive stress, although it has been suggested that this is an over-simplistic view as necrosis may under certain circumstances involve the mobilization of specific transduction mechanisms. Indeed, recently, an alternative death pathway, termed necroptosis, was delineated and proposed as a form of 'programmed necrosis'. Identified with the aid of specific inhibitors called necrostatins, necroptosis shares characteristics with both necrosis and apoptosis. Necroptosis involves Fas/tumour necrosis factor-α death domain receptor activation and inhibition of receptor-interacting protein I kinase, and it has been suggested that it may contribute to the development of neurological and myocardial diseases. Significantly, necrostatin-like drugs have been mooted as possible future therapeutic agents for the treatment of degenerative conditions.

Keywords: cell death • necroptosis • tissue damage • necrostatin

Introduction

Cell death and pathophysiology

Cell death is a crucial process in the development of an organism and in the maintenance of tissue integrity through the elimination of redundant or damaged cells [1]. Apart from its role in normal cellular processing, however, cell death is known to make substantial contributions to a variety of pathological conditions including cancer [2], neurodegenerative disorders [3], autoimmune diseases [4] and ischemia-reperfusion (I/R) injury [5]. In a recent report from the Nomenclature Committee on Cell Death [6], information on an impressive array of cell death modalities was presented, these modalities being classified according to cellular morphology, enzymological features, functional criteria and immunological characteristics. Nevertheless, it was until recently generally accepted that three principal forms of cell death existed, namely apoptosis, autophagy and necrosis based on well-defined morphological features. Several years ago, however, another distinct type of cell death was described. Termed necroptosis, it

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shares features with necrosis and apoptosis, and has elicited considerable interest, particularly with respect to clinical conditions such as myocardial infarction and stroke [7].

Apoptosis

Apoptosis, type-I (active) cell death or, as it was originally known, 'shrinkage necrosis' [8] is a genetically programmed mechanism that is associated with the activation of cysteine-dependent aspartate-specific proteases called caspases and is involved in development and homeostasis [9]. The term apoptosis was originally coined by Kerr and colleagues who focused on the specific morphological features that develop during the course of this process [8, 10]. Apoptotic cell death is characterized by rounding up of the cell, cellular shrinkage, plasma membrane blebbing, pseudopod retraction, chromatin condensation and DNA degradation, and ultimately phagocytosis.

Autophagy

Autophagy or type-II cell death takes three forms, macroautophagy [11, 12], microautophagy [13] and chaperone-mediated autophagy [14]. It is a bulk degradation mechanism which is involved in the dismantling of normal and aggregated proteins, and organelles, including mitochondria. Macroautophagy, which predominates in mammalian cells, is associated with the sequestration of cellular components by double-membrane enclosed vesicles, termed autophagosomes, which then fuse with lysosomes to form autolysosomes where proteolytic degradation occurs [15–17]. Importantly, unlike apoptosis and necrosis, autophagy is essentially a survival mechanism that is activated in response to stress such as starvation, hypoxia, mitochondrial dysfunction and infection [18, 19]. It promotes tissue survival by generating amino acids and fatty acids necessary for maintaining normal cellular function during times of nutrient deficiency, or by removing damaged organelles and intracellular pathogens [20]. Autophagy may, however, promote cell death if uncontrolled leading to excessive degradation of cellular components and the development of pathological conditions [21]. It has been reported that interactions may occur between the ubiquitin–proteasome system, which is the principal non-lysosomal system responsible for the degradation of normal and abnormal intracellular proteins [22] and apoptosis [23]. Interactions between autophagic and apoptotic pathways have also been observed [24, 25]. Thus, it would appear that in order to ensure efficient disposal of non-viable cells and maximize tissue survival crosstalk between the different cell death pathways may be necessary.

Necrosis

Necrosis or type-III cell death is morphologically characterized by cellular swelling, swelling of organelles, plasma membrane rupture and loss of intracellular contents [26]. Until recently necrosis was considered to be a non-programmed, unregulated form of cell death occurring as a consequence of some overwhelming stress. Evidence has, however, been accumulating which indicates that this may be an over-simplistic view and that necrosis, under certain circumstances, involves the mobilization of specific signal transduction mechanisms [27, 28]. Poly [ADP-ribose] polymerase (PARP)-1, for example, a nuclear enzyme that plays a key role in maintaining genomic stability, has been implicated in a form of regulated cell death occurring in glycolytic cells. It has been shown that following DNA alkylation PARP-1 activation leads to depletion of cytosolic nicotinamide adenine dinucleotide $(NAD)^+$ and subsequent necrotic cell death through 'energy collapse' [29, 30]. In neuronal cells PARP-1 also mediates cell death induced by secondary DNA damage associated with acute neuronal injury [31, 32]. In a study by Xu *et al.* [33] PARP-1 mediated mitochondrial dysfunction and subsequent cell death was shown to involve the participation of c-Jun N-terminal kinase (JNK), receptor-interacting proteins (RIP) (see section below on RIP kinases) and tumour necrosis factor (TNF) receptor-associated factor-2. Experiments involving genetic knockouts indicated that RIP-1 and TNF receptor-associated factor-2 operate upstream of JNK-1 in PARP-1 hyperactivated cells.

Over the last few years evidence has been presented indicating that a form of cell death which exhibits features of both apoptosis and necrosis may occur. This has now come to be known as necroptosis and is now discussed below.

Necroptosis

Necroptosis is a newly identified type of cell death that has attracted considerable attention over the last few years and been suggested to represent a form of programmed necrosis or regulated non-apoptotic cell death [34]. It was originally described by Degterev *et al.* [7], although it should be noted that Formigli *et al.* [35] previously reported on a phenomenon which shares dynamic, molecular and morphological features with apoptosis and necrosis, which they named aponecrosis. In their study, Degterev and coworkers [7] demonstrated that treatment of cultured cells (including U937 and Jurkat cells) with TNF-α, which induces apoptosis and activates the death-domain receptor (DR), leads to necrotic or non-apoptotic cell death in the presence of caspase inhibitors (*e.g.* zVAD.fmk) or caspase-8 mutations, or in the absence of Fas associated death domain (FADD). It was inferred from these experiments that although necroptosis and apoptosis were activated by the same stimulus (*i.e.* TNF-α), the morphological changes occurring with necroptosis, *i.e.* organelle swelling, rapid mitochondrial dysfunction, plasma membrane permeabilization and lack of nuclear fragmentation were characteristic of necrosis, which had up until this point been assumed to represent uncontrolled cell death occurring as a consequence of overwhelming stress.

Apart from the necrosis-like morphological changes observed, necroptosis was also found to be associated with activation of autophagy. Thus, electron microscopic examination of necroptotic Jurkat cells revealed the presence of electron-dense double-membrane enclosed vesicles characteristic of autophagy, *i.e.*

autophagosomes [15–17]. Employing phosphatidylethanolamine-conjugated microtubule-associated protein 1 light chain 3 (LC3-II) as a measure of autophagy, the induction of autophagy was demonstrated in FADD-deficient Jurkat cells and L929 cells treated with TNF-α, BALB/c 3T3 cells treated with TNF-α and zVAD.fmk or FasL and zVAD.fmk, and U937 cells treated with TNF-α and zVAD.fmk [7]. Necroptosis was found to occur normally when cells were challenged with the inhibitor of autophagy, 3-methyladenine, and it was concluded that autophagy occurred downstream of necroptosis, rather than contributing to its development. In another study [36], evidence was obtained indicating that autophagy may also counteract necroptotic cell death. Thus, in murine fibrosarcoma cells and U937 cells zVAD-induced cell death was found to be blocked by rapamycin, an autophagy inducer, and enhanced by the lysosomal enzyme inhibitor, chloroquine. The induction of autophagy by serum starvation resulted in significant protection against zVAD-induced cell death, whereas knockdown of autophagy-related protein genes or Beclin 1 enhanced zVAD-induced death. Autophagy-related protein gene knockdown was also found to abolish the protective effect of serum starvation in zVAD treated cells. These various observations provide some support for the idea, outlined above, that cooperation between cell death pathways may occur in order to yield a satisfactory outcome. The picture in this respect, however, remains to be clarified.

More recently, using L929sAhFas and L929sACrmA cells treated with various inducers of cell death (TNF, H_2O_2 and anti-Fas), Vanden Berghe and coworkers [37] obtained evidence indicating that necroptosis, necrosis and secondary necrosis although representing different forms of cell death, ultimately result in similar cellular morphologies such as rounding of the cell, cellular swelling, rupture of the cell membrane and release of intracellular contents. It was found that where these forms of cell death differed, however, was with respect to the timing of these subcellular disintegration events and the fact that TNF-induced necroptosis depended on RIP-1 kinase activation and mitochondrial complex 1 and cytosolic phospholipase A_2 activation, although H_2O_2-induced necrosis relied on iron-dependent Fenton reactions.

The importance of necroptosis in the clinical context was highlighted recently in a study by Bonapace *et al.* [38]. Resistance to chemotherapeutic agents, including glucocorticoids, is a strong predictor of poor outcome in children with acute lymphoblastic leukaemia. It was suggested that modulation of cell death regulators might represent a strategy for counteracting drug resistance in this condition [38]. Using obatoclax, a putative inhibitor of members of the BCL-2 family, it was demonstrated that glucocorticoid resistance was reversed in childhood acute lymphoblastic leukaemia cells through activation of autophagy-dependent necroptosis, a mechanism which bypassed the block in mitochondrial apoptosis characteristic of this condition. The induction of cell death was associated with the dissociation of beclin-1 from the anti-apoptotic BCL-2 family member myeloid cell leukaemia sequence 1 and a reduction in mammalian target of rapamycin (mTOR) activity, providing a mechanism of autophagy induction. It was also dependent on the expression of RIP-1 kinase (discussed below) and cylindromatosis (turban tumour syndrome), which have been reported to be key regulators of necroptosis. Inhibition of RIP-1 and cylindromatosis restored glucocorticoid resistance completely.

Necrostatin and inhibition of cell death

In their characterization of this novel cell death pathway Degterev and coworkers [7] were aided substantially by the use of a small tryptophan-based compound, they termed necrostatin-1 (Nec-1). Nec-1 was identified following the screening of a chemical library of some 15,000 compounds for inhibitors of necrotic cell death induced by TNF-α in the presence of zVAD.fmk. Significantly, the drug was found to inhibit necrosis mediated through DR mobilization in the presence of caspase inhibition in all previously described cellular models of necrosis [7]. Thus, Nec-1 inhibited necrosis in (*i*) Jurkat cells treated with FasL, cycloheximide and zVAD.fmk, (*ii*) Jurkat cells expressing dimerizable FADD incubated with the dimerizer AP2017 and zVAD.fmk and (*iii*) BALB/c 3T3, SV40-transformed MEF, HT-29, IEC-18 and HL-60 cells treated with TNF-α and zVAD.fmk. It should be noted that in Jurkat cells treated with FasL/cyclohexamide/zVAD.fmk an inactive form of Nec-1, designated Nec-1i, failed to prevent cell death.

Focusing on the specificity of Nec-1, comparative studies were performed in which its effects on DR-induced apoptosis and necrosis were evaluated [7]. Nec-1 was not found to influence FasL-/cyclohexamide-induced staining of cells with the fluorescent dyes (and markers of apoptosis) annexin-V and propidium iodide, indicating that apoptosis does not represent its cellular target. Further evidence that Nec-1 did not influence apoptosis was provided by the observation that apoptotic morphology, such as cellular shrinkage, plasma membrane blebbing and chromatin condensation, was not altered by Nec-1 treatment. Contrasting with the aforesaid findings, Nec-1 was found to strongly influence various features commonly associated with necrosis. The drug, for example, prevented the loss in plasma membrane integrity and mitochondrial membrane potential seen in FADD-deficient Jurkat cells treated with TNF-α and wild-type Jurkat cells incubated with FasL/cyclohexamide/zVAD.fmk. In addition, Nec-1 inhibited the development of necrotic morphology, including cellular and organellar swelling, plasma membrane rupture and loss of intracellular contents. A number of variants of the necrostatin chemical structure have now been described, including Nec-1, Nec-3 and Nec-5 [7, 39–42]. It has been suggested that some of these molecules could eventually find application as treatments in degenerative disease. Recently, it was proposed that necrostatins should be considered as representing key agents for confirming the presence of necroptosis in cells and tissues [42].

Following on from the initial work of Degterev and colleagues [7], it was reported that macrophage death induced by the plant sterol sitosterol, which underlies a condition called sitosterolemia and has been linked to premature atherosclerosis, was caspase independent and blocked by Nec-1 [43]. In addition, Nec-1 inhibition was found to be associated with the accumulation by macrophages of autophagic vacuoles, providing further evidence

for interactions between necroptosis and autophagy. Meanwhile, shikonin, a naturally occurring naphthoquinone, was found to induce a form of cell death in MCF-7 and HEK-293 cells, which are drug-sensitive cancer cell lines, that was distinct from apoptosis and necrosis in nature [44]. Thus, shikonin-induced cell death was associated with morphological changes characteristic of necrosis, loss of membrane integrity and mitochondrial potential, activation of autophagy as a downstream consequence of cell death and increases in reactive oxygen species (ROS) levels that did not contribute to cell death, and which was prevented by Nec-1. The authors put forward the novel suggestion that inducers of necroptosis, like shikonin, could find application as agents for the treatment of drug-resistant cancers. Subsequently, these same workers reported that Nec-1 treatment resulted in shikonin-induced necroptosis reverting to apoptosis in HL-60 and K562 cells [45]. It was suggested that this process involved Nec-1 altering mitochondrial inner membrane permeability and outer membrane permeability in favour of the latter, with the result that Bax translocation to mitochondria (a feature of apoptosis) was increased.

In studies with rat myoblastic H9c2 cells Nec-1 was found to protect against chemically-induced ischemia [46]. Thus, Nec-1 reduced cell death, as measured using an MTS proliferation assay, in H9c2 cells treated with iodoacetate, an irreversible inhibitor of glycolysis.

Studies in Chinese hamster ovary K1 cells revealed that Nec-1 protects against necrotic death triggered by cadmium (Cd) [47]. This protective effect did not involve the modulation of intracellular calcium, calpain activity or ROS production, all of which had previously been reported to be enhanced by Cd [48], but attenuation of the decrease in the mitochondrial membrane potential. Additionally, Nec-1 was found to elevate NF-κB activity which promotes cell survival and is suppressed by ROS. Comparing the data obtained with Chinese hamster ovary K1 cells with those obtained with U937 cells treated with TNF-α/zVAD-fmk and DLD-1 cells treated with etherynic acid, it was deduced that in addition to protecting against DR-mediated cell death, Nec-1 protects against necrosis by rescuing cells with reduced mitochondrial membrane potential. The authors concluded that the mitochondrion might represent a major site of action for Nec-1. In another study in which Rainbow trout cell lines were treated with Cd, Nec-1 was shown to improve plasma integrity, as measured using propidium iodide, and reverse decreases in cellular ATP contents [49].

In cultured HT-22 hippocampal cells, Nec-1 was found to protect against glutamate-induced cytotoxicity/oxytosis through a mechanism that involved an increase in the cellular levels of glutathione (GSH), as well as a reduction in ROS [50]. Furthermore, it was shown that Nec-1 blocked the nuclear translocation of apoptosis-inducing factor, a marker of caspase-independent programmed cell death, and inhibited the integration of Bcl-2/adenovirus E1B 19 kD interacting protein-3, a potent inducer of cell death. Astrocyte inflammation may contribute to the severe neurological injury occurring following intracerebral haemorrhage. In studies conducted with mouse cortical astrocytes further evidence was obtained indicating that necroptosis in the nervous system involves abnormalities relating to GSH levels [51]. Thus, peroxidative injury induced by hemin, the haemoglobin oxidation by-product, was shown to be associated with intracellular GSH depletion, leading to lipid peroxidation and cell death which was blocked by Nec-1 but not its inactive control (Nec-1i) or z-VAD-fmk. The authors concluded that GSH depletion may play a role in necroptosis after haemorrhagic injury and suggested that drugs which target necroptosis could prove valuable in the treatment of stroke.

In another study it was established that necroptosis contributed to N-methyl-D-aspartic acid (NMDA)-induced excitotoxicity, which has been linked to stroke/ischemia, epilepsy and some neurodegenerative diseases, in rat cultured cortical neurons [52]. Treatment with Nec-1 was found to produce dose-dependent reductions in NMDA-stimulated cell death and lactate dehydrogenase efflux, and totally inhibited the NMDA-induced rise in cellular Ca^{2+}. It was, however, concluded that necroptosis may only make a small contribution to cell death associated with NMDA-induced excitotoxicity.

Aluminium (Al) has been implicated in neurodegenerative disease, including Alzheimer's disease. *In vivo* and *in vitro* studies have suggested that Al induces its neuropathological effects *via* apoptosis and necrosis. More recently, in a study by Zhang *et al.* [53], cellular alterations consistent with necroptosis were also observed. SH-SY5Y neuroblastoma cells subjected to Al stress in the presence of Nec-1 were found to exhibit decreased levels of necrosis and its associated morphological changes. Additionally, Nec-1 treatment inhibited the Al-induced decrease in the mitochondrial membrane potential and reduced ROS production and autophagosome numbers.

As outlined earlier, PARP-1 has been implicated in necrosis [29–33]. In a study carried out by Hitomi and coworkers [54], L929 cells treated with TNF-α and zVAD.fmk were screened for genes required for necroptosis. This resulted in the identification of 432 genes that regulate necroptosis, 32 genes that act downstream and/or function as modulators of RIP-1 kinase (see section below on RIP kinases), 32 genes that mediate DR-mediated apoptosis and 7 genes involved in apoptosis. In addition, PARP-2, which shares 60% homology with PARP-1 in the catalytic domain, was identified as being a gene required for necroptosis. Oligomerization of PARP-1 and PARP-2 stimulates PARP catalytic activity in promoting DNA repair. PARP-2 is cleaved in apoptosis but with a delayed time course relative to PARP-1, possibly indicating that PARP-2 is not the preferred substrate for caspases compared to PARP-1. The expression of a caspase-resistant form of PARP-1 has been found to promote necrosis [55], whereas activation of PARP-1 which results in NAD^+ hydrolysis has been suggested as a mechanism leading to reduced NAD^+ levels and energy failure in necrosis [56]. The findings described led Hitomi *et al.* [54] to propose that when PARP-2 catalytic activity is elevated for sustained periods resistance to caspase action may occur resulting in necroptotic pathways being initiated. More recently, the role of PARP activation in necroptosis was examined in HT-22 cells [57]. It was demonstrated that PJ34, a potent and specific inhibitor of PARP, completely blocked oxidative stress-induced necroptosis. Interestingly, although Nec-1 was found to reduce PARP activity it did not influence PARP-1 expression in glutamate-treated cells or protect against cell death mediated by the PARP activator *N-methyl-N′-nitro-N-nitrosoguanidine* (MNNG). By

contrast, PJ34 protected against MNNG cytotoxicity. From these studies it was concluded that Nec-1 is not a direct inhibitor of PARP-1 and that its site of action lies upstream of PARP.

Necroptosis, necrostatin and RIP-1 kinase

The RIPs are a family of serine/threonine kinases that function as sensors of cellular stress and mediate both survival and death-inducing cellular mechanisms [58–61]. The members of the RIP kinase family, which include RIP-1, RIP-2, RIP-3 and RIP-4, modulate extracellular stress signals initiated through activation of a variety of receptors, and also intracellular stress signals. Factors which cause increased RIP expression include various pathogens, inflammation, T cell receptor stimulation and DNA damage. Interestingly, although these stimuli activate different cell signalling pathways, their actions ultimately terminate on similar mechanisms, such as the activation of the transcription factors NF-κB and activator protein 1 [58–61]. The RIP kinases share a homologous kinase domain but express different recruitment domains [58–61]. These recruitment domains are responsible for directing the kinases to their appropriate signalling pathways. RIP-1 has been implicated in cell death associated with trauma such as stroke and myocardial infarction [42, 58–61] and studies in RIP-1-deficient mice have indicated that it represents a key factor determining cellular survival following exposure to stress signals [62]. It possesses a C-terminal death domain that is important for binding to DR such as TNF-receptor 1, TRAIL (TNF-related apoptosis-inducing ligand)-receptor 1 and TRAIL-receptor 2, and to the death domain-containing adaptor proteins such as TNF-receptor-associated death domain and FADD [58–61].

Necrosis induced by TNF-α in T cells has been reported to be dependent on serine/threonine kinase activity associated with RIP-1 [63]. In another study focusing on the relative contributions made by apoptotic and non-apoptotic pathways to TNF-α-induced cell death, non-apoptotic cell death was found to occur through a receptor-mediated process involving the activation of an intracellular signalling complex incorporating RIP-1 [64]. More recently, employing wild-type and RIP-deficient Jurkat cells, Degterev *et al.* [65] identified RIP-1 kinase as being the principal kinase involved in the activation of necroptosis and the primary target for the anti-necroptotic actions of the necrostatins. In the case of Nec-1 its actions on necroptosis were shown to relate specifically to its inhibitory effects on RIP-1 kinase activity (as indicated by RIP-1 autophosphorylation) and occurred in a concentration-dependent manner. Significantly, evidence was obtained that the inhibitory actions of Nec-1 focused on the activation segment (T loop) of RIP-1. Although two other necrostatins, namely Nec-3 and Nec-5, also targeted RIP-1 kinase, it was found that the inhibitory actions of these agents were mediated *via* mechanisms distinct from those modulated by Nec-1. Degterev *et al.* [65] concluded that their data indicated that the necrostatins represent the first-in-class inhibitors of RIP-1 kinase, which they suggested was the major upstream kinase involved in the mobilization of necroptosis.

Oxidative damage is a feature of many neurological conditions and recently Kim *et al.* [66] reported that oxidative death of oligodendrocyte precursors induced by arachidonic acid, which is elevated during brain ischemia and inflammation, was blocked by Nec-1 by dint of RIP-1 kinase inhibition. Using an Alamar Blue assay arachidonic acid was shown to induce cell death through ROS production and activation of JNK, with JNK inhibition resulting in protection. The treatment of oligodendrocyte precursors with Nec-1 abolished arachidonic acid-induced ROS production and JNK activation, and it was concluded that these processes occurred as a result of upstream RIP-1 activation.

Evidence has also been presented indicating that RIP-3 plays a role in necroptosis. Employing T cells (including T cells lacking RIP-3) Cho *et al.* [67] demonstrated that cell death induced by anti-CD3 antibody and TNF was dependent on RIP-3 phosphorylation and subsequent downstream activation of RIP-1. Furthermore, programmed necrosis (necroptosis) induced by TNF in combination with cyclohexamide and zVAD was found to involve RIP-1–RIP-3 association which could, like necrosis-specific RIP-3 phosphorylation, be blocked by necrostatin.

Vandenabeele *et al.* [68] have reviewed the evidence that the kinases RIP-1 and RIP-3 are involved in TNF-induced cell death, including necroptosis. These workers have suggested that the regulation of RIP-1 and RIP-3 and their downstream signalling pathways may represent therapeutic targets in conditions characterized by cellular destruction, such as I/R injury and neurodegeneration.

Necrostatin and tissue injury

A body of evidence now exists indicating that necrostatin protects against tissue injury induced by both chemical and physical insults. This evidence has implications for two of the principal causes of death, namely neurological and cardiovascular diseases.

Necrostatin and cerebrocortical injury

In the original study by Degterev *et al.* [7] it was demonstrated that, apart from inhibiting TNF-α induced death in cultured cells, Nec-1 markedly reduced tissue injury and improved neurological scores in animals, as indicated by behavioural measures. Employing a cerebrocortical model of I/R injury Nec-1, administered by intracerebroventricular injection, was shown to significantly reduce infarct volume after middle cerebral artery occlusion. By contrast, the inactive form of Nec-1, Nec-1i, which differs from Nec-1 by a single methyl group, was found to produce only marginal decreases in infarction, providing further evidence for the specificity of its action. The Nec-1 analogue, 7-Cl-Nec-1 also yielded reductions in infarct volume and improvements in neurological score. The specificity of 7-Cl-Nec-1 *in vivo*, with respect to its inhibitory action on necroptosis, was confirmed when it was shown that it did not inhibit caspase-3 activation as a result of cerebrocortical I/R injury, whereas zVAD.fmk did.

An observation with implications for the possible future use of Nec-1 clinically was that its protective effects still occurred in animals when administered after the induction of I/R injury [7]. Thus, it was found that Nec-1 given 6 hrs after the onset of middle cerebral artery occlusion induced injury significantly reduced infarct volume. Furthermore, Nec-1, given 4 and 6 hrs after occlusion, also blocked the induction of LC3–11, the marker of autophagy, which was found to occur during necroptosis in cultured cells and reached its maximal level in brain tissue 8 hrs after occlusion. Northington *et al.* [69] have reported that Nec-1 administered after hypoxia-ischemia (HI) reduced injury in the forebrain and thalamus of neonatal mice. Nec-1 treatment appeared to decrease necrotic cell death but increase apoptosis, block RIP-1–RIP-3 complex formation and inhibit RIP-3–FADD interaction. Nec-1 also reduced HI-induced oxidative damage to proteins and decreased the levels of inflammatory markers. NF-κB and caspase 1 activities and Fas-associated death-domain-like IL-1β converting enzyme-inhibitory protein expression were also reduced. It was speculated that the neuroprotective effects of Nec-1 occurred *via* interruption of RIP-1–RIP-3-driven oxidative injury and inflammation. In a study by You *et al.* [70] Nec-1 was found to reduce the tissue damage resulting from controlled cortical impact (CCI) in mice. Previously, it had been reported that tissue damage was reduced and functional outcome improved following CCI in mice deficient in TNF-α and Fas [71]. As a consequence, it was suggested that as these factors feature prominently in necroptosis necrostatin would prove protective. Thus, it was, indeed, confirmed that Nec-1 but not the inactive analogue, Nec1i, administered intra-ventricularly resulted in reduced traumatic brain injury (TBI), as indicated by improvements in histopathological measures and cognitive and motor parameters. In relation to lesion size, Nec-1 pre-treatment produced reductions at 14 and 35 days after CCI. Importantly, Nec-1 also reduced infarct size when administered 5 min. and 15 min. after CCI. Plasmalemmal permeability, as assessed by propidium iodide labelling, was reduced when Nec-1 was given both before and after CCI, whereas neuroinflammation (neutrophil count and microglial activation) was decreased 48 hrs after CCI in mice pre-treated with Nec-1. With respect to motor and cognitive performance Nec-1 administration pre- but not post-CCI yielded benefit. It was concluded from these studies that necroptosis may play a key role in cell death and neurological dysfunction following TBI, and that Nec-1 and other necrostatins might prove of value in the treatment of TBI patients.

Zhu *et al.* [72] have obtained data suggesting that Nec-1 ameliorates symptoms in the R6/2 transgenic mouse, a model of Huntington's disease. Having demonstrated that Nec-1 inhibited RIP-1-mediated necroptosis in a ST14A striatal cell line, these workers went on to show that it also yielded benefit in R6/2 mice when administered intracerebroventricularly *via* an osmotic pump. Thus, in animals treated with Nec-1 for 6 weeks the onset of disease was delayed, as indicated by measures of motor function and body weight.

In an interesting study by Xu *et al.* [73] synergistic neuroprotective effects were observed between Nec-1 and Gly(14)-humanin (HNG), an apoptosis inhibitor, on hypoxia and I/R injury. Studies were conducted with cultured mouse cortical neurons subjected to oxygen-glucose deprivation (OGD) and in mice that underwent middle cerebral artery occlusion. Nec-1 or HNG alone were found to reduce OGD-induced cell death to similar extents with Nec-1/HNG combined treatment leading to larger reductions. *In vivo* HNG or NEC-1 reduced cerebral infarct volume to similar extents whereas combined treatment with HNG and Nec-1 decreased infarct volumes further and improved neurological scores. As a consequence of these studies the authors suggested that combination treatment with anti-apoptotic and anti-necroptotic agents might eventually find application in the treatment of stroke.

Necrostatin and myocardial injury

Nec-1 was shown to reduce infarct size in a murine *in vivo* model of myocardial I/R injury when administered at reperfusion, whereas Nec1i was ineffective [74]. Intriguingly, in Langendorff isolated perfused mouse hearts both Nec-1 and Nec1i reduced infarct size, perhaps highlighting differences that can occur with regard to the data generated when examining drug action under *in vivo* and *in vitro* conditions. Another interesting finding as regards the Langendorff studies was that although Nec-1 at 30 μM protected against infarction, raising its concentration to 100 μM enhanced infarct size. It was concluded that at higher concentrations Nec-1 may produce non-specific or toxic actions that potentiate apoptotic and necrotic mechanisms, culminating in enhanced myocardial infarction. Apart from demonstrating that necrostatin protected against myocardial infarction *in vitro* and *in vivo*, Nec-1 was found to reduce peroxide-induced cell death in C2C12 and H9c2 myoblasts [74]. Additionally, Nec-1 delayed the opening of the mitochondrial permeability transition pore (MPTP), a proposed key determinant of tissue injury, in rat cardiomyocytes [74]. In an *in vivo* murine study conducted by Chua *et al.* [75], Nec-1 was shown to reduce infarct size when administered both prior to ischemia and after the initiation of reperfusion. Nec-1 was also found to reduce plasma troponin-I levels and induce additional protection in the presence of zVAD.fmk. The authors concluded form their studies that Nec-1 inhibits necroptosis in myocardial infarction and that its cardioprotective effect is mediated *via* a caspase-independent mechanism.

Further and, perhaps, more concrete evidence that Nec-1 protects against myocardial IR injury by modulating MPTP opening at reperfusion, was obtained in murine *in vivo* experiments [76]. In a comparative study involving wild-type mice and mice lacking cyclophilin-D (cyclophilin-D knockout, Cyp-D$^{-/-}$), a key component of the MPTP, the cardioprotective effects of Nec-1 were shown to be lost in Cyp-D$^{-/-}$ animals. It was concluded that although it had been proposed that Nec-1 acts through inhibition of a novel death pathway [7], its cardioprotective actions appeared to operate *via* established mechanisms involving Cyp-D and the MPTP. The precise mechanisms by which Nec-1 inhibits MPTP opening in the heart and protects against myocardial I/R injury have yet to be delineated. It has been postulated that MPTP inhibition occurs as a consequence of activation of the so-called reperfusion injury salvage kinase pathway [77], which incorporates

Table 1 Cells and tissues in which necrostatin produces protective actions

Cells/tissues	Inducer of cell death	References
Jurkat, BALB/c 3T3, SV40-transformed MEF, HT-29, IEC-18, HL-60 cells	TNF-α	[7, 64, 65]
Macrophages	Sitosterol	[43]
MCF-7, HEK-293, HL-60, K562 cells	Shikonin	[44, 45]
H9c2 myoblasts	Iodoacetate	[46]
Chinese hamster ovary K1 cells, Rainbow trout cells	Cd	[47, 49]
HT-22 hippocampal cells	Glutamic acid	[50]
Mouse cortical astrocytes	Hemin	[51]
Rat cortical neurons	NMDA	[52]
SH-SY5Y neuroblastoma cells	Al	[53]
Oligodendrocyte precursors	Arachidonic acid	[66]
T cells	Anti-CD3 antibody, TNF-α	[67]
Mouse cerebral cortex (*in vivo* I/R model)	Middle cerebral artery occlusion	[7]
Mouse forebrain, thalamus (*in vivo* HI model)	Right common carotid ligation	[69]
Mouse cerebral cortex (*in vivo* model)	CCI	[70]
ST14A striatal cells;	zVAD-fmk	[72]
Mouse R6/2 *in vivo* HD model		
Mouse cerebral cortex (*in vivo* I/R model); mouse cortical neurons (*in vitro*)	Middle cerebral artery occlusion (*in vivo*); OGD (*in vitro*)	[73]
Murine myocardium (*in vitro* and *in vivo* I/R models); C2C12 and H9c2 myocytes	Global ischemia (Langendorff isolated heart); LAD ligation (*in vivo*); peroxide (myocytes)	[74]
Murine myocardium (*in vivo* I/R model)	LAD ligation	[75, 76]
Rat retina (*in vivo* I/R model)	Intraocular pressure elevation	[78]

HD: Huntington's disease; HI: hypoxia-ischemia; I/R: ischemia-reperfusion; LAD: left anterior descending artery; OGD: oxygen-glucose deprivation.

the pro-survival kinases phosphatidyl-inositol 3-OH kinase cellular Akt/protein kinase B (Akt) and p44/42 mitogen-activated protein kinase (MAPK) extracellular signal-regulated MAPK (Erk1/2). Activation of the reperfusion injury salvage kinase pathway appears to be a prerequisite for cardioprotection, whether induced by treatments such as pre- and post-conditioning or by pharmacological agents [77]. Future studies will establish whether the cardioprotective actions of necrostatin also involve this pathway. As described earlier the beneficial effects of necrostatin have been reported to rely on inhibition of RIP-1 kinase [65]. We have confirmed by Western blotting that RIP-1 is expressed in cardiac tissue (unpublished findings) and suggest that the role played by this pathway in myocardial injury needs to be investigated. In addition, it is important that it is established whether the actions of Nec-1 on the heart operate through RIP-1, as this will provide further insights into the mechanisms underlying myocardial protection.

Necrostatin and retinal injury

Nec-1 has been shown to reduce neuronal damage in a retinal I/R injury model [78]. Sprague-Dawley rats were subjected to raised intraocular pressure and received intravitreal injections of Nec-1 or its inactive analogue, Nec-1i. Seven days after ischemia electroretinograms were performed and then the eyes enucleated for histological analysis, propidium iodide and TUNEL staining, and Western blotting using an anti-LC-3 antibody. Nec-1 pre-treatment of animals was found to preserve the thickness and histoarchitecture of the inner retina, whereas Nec-1i was ineffective. Post-treatment with Nec-1 attenuated the electroretinogram b-wave reduction compared with ischemic controls. Nec-1 was not found to influence the numbers of caspase or TUNEL-labelled cells but did reduce the induction of LC-3 and the number of propidium iodide positive cells after ischemia. The authors concluded that necroptosis may play an important role in neuronal death following retinal I/R and involves autophagy.

Concluding remarks

Without doubt the recent identification of necroptosis has generated considerable excitement and yielded important new information concerning the mechanisms underlying cell death. It has also provided us with a potential target for therapeutic manipulation in the context

of degenerative disease. In this regard the necrostatins are obvious candidates for possible future therapeutic use given that they have been shown to produce beneficial effects in various experimental systems and, importantly, in animal models of stroke and myocardial infarction (Table 1). Clearly, further extensive testing is required before these agents can be considered for clinical use. Significantly, however, it was recently reported that the necrostatins have been licensed by a US-based biopharmaceutical company which specializes in drugs that modulate cell death, and that toxicity testing in animals at various research centres is underway. It has been proposed that this class of drugs could find eventual application in the treatment of various conditions, including acute and chronic neurodegenerative conditions and myocardial infarction [40].

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Conflict of interest

We declare no conflict of interest.

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