# Perspectives on the therapeutic modulation of an alternative cell death, programmed necrosis (Review)

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**Abstract.** In response to overwhelming external stimuli, cells are forced to die in different ways. By default, cells are prone to apoptosis, a programmed cell death through the activation of the caspase cascade. However, this process would be blocked if one of the proteins involved in executing apoptosis was genetically impaired or chemically inhibited, or if the apoptotic machinery was not properly operated under specific conditions, such as ischemia and microbial infection. To address these issues, the paradigm of programmed cell death needs to be revised; thus, an alternative form of cell death, programmed necrosis (termed necroptosis, through the caspase-independent pathway), which is distinct from apoptosis in many aspects, has recently been adopted. There is much interest in programmed necrosis as it is very closely associated with pathophysiological conditions, such as stroke, heart attack and septic shock. In an effort to identify target molecules of small compounds interfering with the signaling downstream of tumor necrosis factor-α (TNF-α), necrostatin-1 (Nec-1) was first identified to be a selective allosteric inhibitor of the death domain receptor-associated adaptor kinase, receptor interacting protein 1 (RIP1) *in vitro*. Since then, some novel scaffolds with selective and distinctive activity have been proposed. In this review, the significant advancement and state-of-art direction for the development of small molecules that can control programmed necrosis is discussed. Furthermore, the perspectives on novel strategies harnessing therapeutic targets identified thus far, are also discussed.

**Contents**



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## 1. Overview of programmed necrosis (necroptosis)

Over the past 40 years, apoptosis has been generally thought as the only prototype of programmed cell death. By contrast, another cell demise termed 'necrosis' is simply described as a passive and unwanted cell death in response to the overexposure of chemical, physical or radioactive stress. The third cell death mode, autophagy, is not discussed in this review as there is still some controversy as to its role in cell survival and cell death. Based on morphology, biochemistry and physiology, necrotic cell death has unique characteristics, distinct from those of apoptosis and autophagy. Some typical features discriminating between apoptosis and necrosis are listed by criteria in Table I. Of note, the loss of membrane integrity in cells undergoing necrosis induces the release of intracellular debris, referred to as danger signals into the microenvironmental niche, consequentially eliciting inflammatory responses (1). Although little attention has previously been paid to necrotic death, its pathophysiological significance coupled to inflammatory response has recently been emphasized.

Apart form apoptotic stress in nature, there exist a variety of physical, chemical and biological stimuli causing necrosis. These include high energy irradiation, DNA alkylating agents and cytokines (2-4). Of the death stimuli listed above, tumor necrosis factor (TNF)-α is a pleiotropic inflammatory cytokine, and it initiates survival or programmed cell death, apoptosis through the TNF-α receptor and a cascade of downstream executioners (5). However, under specific conditions in which the apoptotic machinery is blocked by a pan-caspase inhibitor (zVAD peptide) or viral infection, the cells themselves redirect the apoptotic cell demise into an alternative cell death (Fig. 1). Since a unified nomenclature on such a backup cell death program has not been withdrawn, it is therefore referred to as necroptosis, programmed necrosis, or caspase-independent cell death. In spite of ambiguous nomenclature, there is a growing body of evidence indicating that programmed necrosis is a backup cell death program that is activated when caspase-driven cell death is blocked (4,6). More precisely, programmed necrosis contributes to N-methyl-D-aspartate (NMDA)-induced

Table I. Representative characteristics discriminating between apoptosis and necroptosis.

| Cell death modes/ characteristics[a] | Apoptosis | Programmed necrosis |
|---|---|---|
| Membrane integrity | Retained | Disintegrated |
| Caspase requirement | Yes | No |
| Nuclear morphology | Shrinkage | Swelling |
| DNA cleavage pattern | Laddering | Smearing |
| RIP1 involvement | No | Yes |
| ROS generation | No | Yes |

[a]Criteria designating cell death modes in terms of morphology, molecular marker and biochemical readout. RIP1, receptor interacting protein 1.

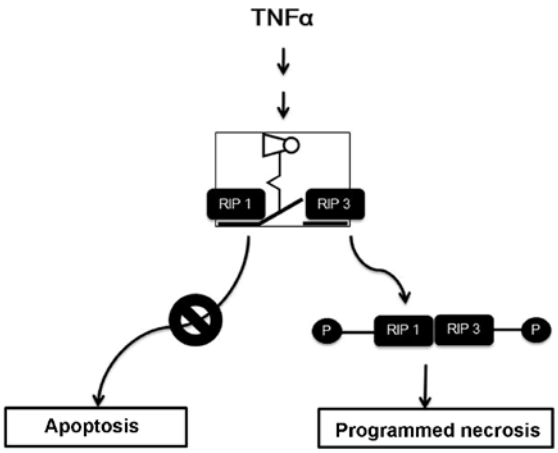


Figure 1. Schematic illustration of the molecular switch of cell demise in response to tumor necrosis factor-α (TNF-α) stimulation under the condition of caspase inhibition. Once cells are stimulated with TNF-α, a default cell death apoptosis occurs through the caspase cascade. When apoptosis is blocked or hindered by chemical or biological factors, cells activate a backup cell death, programmed necrosis, in an active and ordered fashion through the formation of the receptor interacting protein (RIP)1-RIP3 necrotic complex.

excitotoxicity in neurons, as well as heavy metal poisoning and chemical-induced toxicity (7-9). The precise manipulation of cell death modes makes it possible to consequently define a new paradigm of another cell death mode by analyzing the biochemical and molecular parameters. As a result, Hitomi *et al* demonstrated that a variety of proteins were related to necroptosis through a genome-wide analysis (10). Thereafter, proteins responsible specifically for programmed necrosis have been extensively investigated and consequently, some promising therapeutic proteins are discussed in this review. The identification of some specific programmed necrotic proteins and the development of small molecules specifically targeting receptor interacting protein 1 (RIP1) make it conceivable that necrotic cell death is not only an independent and specialized form of cell death, but that it is also a part of an orchestrated signaling network.

## 2. Therapeutic target molecules identified for mediating necroptosis

Since necrosis has long been thought to be a passive and unwanted cell response to devastating external stresses, the identification of novel proteins responsible for necrotic death has not been completed. However, accumulating evidence indicates that DNA alkylating agents, shikonin and heavy metals induce programmed necrosis-like cell death, distinct from necrosis or apoptosis (Fig. 2) (11-13). Specifically, DNA alkylation-induced DNA damage is repaired through the activation of poly(ADP-ribose)polymerase-1 (PARP-1), which is a nuclear enzyme that catalyzes the covalent linkage of long branched chains of PAR to a variety of nuclear DNA-binding proteins, including PARP-1 (14,15). However, massive and intolerable DNA damage to cells mediates necrosis through he excessive activation of PARP-1, which depletes the ATP energy supply of the cells supply and subsequently results in metabolic catastrophe (16).

RIP1 was first identified as a regulator of programmed necrosis upon TNFR stimulation (17). The activity of the RIP1 death domain kinase is required for death receptor- and zVAD.fmk-mediated necroptosis in murine and human cells. Later, three individual research groups identified another novel protein, RIP3, as a critical protein for the activation of programmed necrosis when default cell death (apoptosis) is hindered (18-20). Furthermore, it has been demonstrated that the pronecrotic complex formation between RIP1 and RIP3 is required for programmed necrosis, indicating that downstream or upstream signaling networks of the RIP1-RIP3 complex are plausible and can be tightly regulated by a cascade of proteins. It has also been published that the NAD-dependent deacetylase, SIR2, is involved in the regulation of TNF-mediated programmed necrosis (21). It not only recruits RIP3, but also catalyzes the deacetylation of RIP1 to allow it to be in a stable conformation, forming a necrotic complex.

Recently, the mixed lineage kinase domain-like protein (MLKL) was identified as a RIP3 substrate, as well as a biological target of the hit compound against necroptosis by a combined approach of chemical biology and biochemistry (22). Unlike both RIP1 and RIP3 proteins, MLKL does not possess kinase activity due to its absence of a phosphate-binding loop and key amino acids for kinase. However, its binding to RIP3 through kinase-like domain leads to an increased RIP3 kinase activity through the formation of a stable complex. Subsequently, MLKL is phosphorylated as a RIP3 substrate to form a necrosis-inducing signaling complex, termed the necrosome, which includes RIP1, RIP3 and MLKL. In addition, other kinases and metabolism-related proteins have been disclosed by using interference RNAs (18), and are putatively expected to be involved in creating the signaling network of programmed necrosis.

Apart from cytosolic proteins described above, mitochondrial proteins have been suggested to be putative candidates for mediating necroptosis. Pro-death Bcl2 proteins, which have been documented to play a decisive role in the intrinsic apoptotic pathway, have also been suggested to be invovled in necrotic death (23,24). For instance, Bax, Bmf, BNIP3 and Nix are candidate mitochondrial proteins responsible for specific necrotic death. In light of mitochondrial function, cyclophilin-D (CyP-D) and mitochondrial permeability transition (MPT)

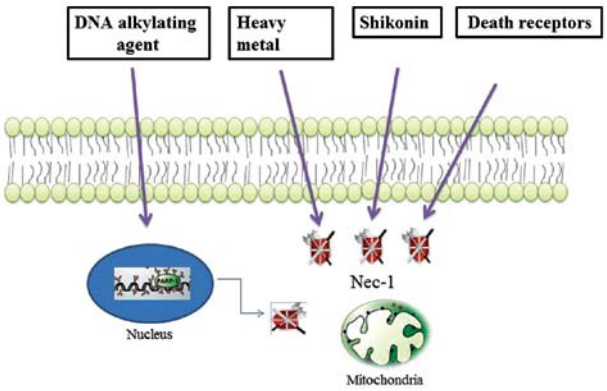


Figure 2. Putative programmed necrosis-inducing agents. Various agents derived from organic or inorganic chemicals have been suggested to induce necrotic cell death, but not apoptosis. This is supported by the fact that all the necrosis inducers above have been rescued by necrostatin-1 (Nec-1), a receptor interacting protein 1 (RIP1) specific inhibitor. Alkylating agents cause excessive activation of PARP proteins to repair extensive DNA damage and eventually lead to the depletion of $NAD^+$, inducing an energy crisis and necrotic cell death. The inflammatory cytokine, tumor necrosis factor-α (TNF-α), exerts a prototype of necrotic cell death by binding to TNFR in cells with defective apoptotic machinery. Also, there is a growing body of evidence that heavy metals and chemicals induce necrotic cell death although their underlying mechanisms remain to be clarified. A variety of mitochondrial proteins is involved in regulating apoptosis and necrosis although the regulatory mechanisms remain elusive. Interactive communications between the nucleus, cytosol and mitochondria may be crucial for sensing, processing and responding to death signals.

pore have been gaining attention as the emerging targets to modulate necrosis effectively. Of note, RIP3, being activated by a cascade of events following TNFR ligation, has been suggested to interact with the mitochondrial protein, glutamate dehydrogenase 1 (GLUD1), therefore linking the signaling pathway from extracellular stimulation, intracellular events, to mitochondria. Recently, the signaling downstream of RIP1/RIP3 complex has been extensively explored. Accordingly, the interaction of MLKL with the RIP1/RIP3 complex recruits the mitochondrial protein phosphatase, PGAM5, functioning as the convergent point for multiple necrosis pathways (25).

## 3. Small molecules that protect cells from programmed necrosis, but not apoptosis

Small molecules that protect cells from undergoing programmed necrosis are listed by scaffold in Fig. 3. Since the introduction of the necroptosis concept to cell demise, the discovery and optimization of small molecules with potent inhibitory activity against it have been pursued for therapeutic use. The first successful outcome is a series of hydantoin compounds containing indole derivatives (Fig. 3, scaffold 1) (25,26), which are potent necrostatins. A structure-activity relationship (SAR) analysis indicated that several positions of the indole moiety were very vulnerable to chemical modification, apart from electron-donating or -withdrawing substituents at the 7-position, and that the hydantoin ring was also very sensitive to structural modifications. In fact, the substitution of the amide nitrogen and removal of a carbonyl group led to a complete loss of activity. Also, steric bulk and extension of the linker between the indole and hydantoin ring are found to be detrimental for their inhibitory activity against necroptosis. Out of this class bearing scaffold 1, a chemical necrostatin-1 (Nec-1) has so modest pharmacokinetic profiles to be delivered to the central nervous system (CNS) following intravenous administration (27). Subsequently, an extensive exploration for target molecules of this scaffold resulted in the identification of RIP1 as an interacting molecule of Nec-1 (28). Mechanistically, Nec-1 inhibits RIP1 in an ATP-competitive manner.

Since then, the discovery of a series of tricyclic derivatives (Fig. 3, scaffold 2) (29) and substituted 3H-thieno[2,3-*d*] pyrimidin-4-ones (Fig. 3, scaffold 3) (30) were ensued. SAR of scaffold 2 demonstrates that the (3*R*, 3a*R*)-rel-diastereomers are more potent than the corresponding (3*R*, 3a*S*)-rel-diastereomers. The replacement of fluorine or methoxy at the 8-position of the tricyclic ring enhances the protective activity, whereas that at the 6-, 7- and 9-positions is fatal. Also, the introduction of a methoxy group to the 4-position of the phenyl ring improves activity, while the location of the methoxy at the 2-position of it deteriorates its potency; the placement of amides at the 2-position in the tricyclic ring part shows the best activity. Notably, in contrast to a hydantoin-indole necrostatin, these derivatives do not protect cells from zVAD-induced programmed necrosis in L929 cells, suggesting that there is a mechanistic distinction between the two series of compounds (29). In scaffold 3 (Fig. 3), the thioethylcyanide moiety on the α-position of fused pyrimidone-4 part is required for the inhibition of necroptosis. The presence of the -OMe group in the para-position of the benzene ring bonded to pyrimidone nitrogen is found to be critical for its protective activity. The introduction of aliphatic rings, such as cyclopentyl, cycloheptyl or benzene at the position of thiophene ring exhibits some variable activities. It is apparent that derivatives with the methyl group at the α- and β-position of the thiophene ring exhibit significant activities. With increasing size of the aliphatic ring, their inhibitory activities are detrimental. By contrast, substitution of the phenyl ring for the cyclohexane ring keeps its active. Furthermore, [1,2,3] thiadiazole derivatives (Fig. 3,scaffold 4) drawn through high throughput screening have been found to effectively protect cells from necroptosis (31). Through SAR analysis, it has been demonstrated that secondary 2,6-dihalo substituted benzyl amides are required for their antagonizing effects on necroptosis. When the methyl group is located in the benzylic position, the (*S*)-enantiomeric configuration has its own ability to interfere with necroptosis. Small branched or cyclic alkyl groups are favorable in the 4-position of [1,2,3] thiadiazole. Of note, the replacement of [1,2,3] thiadiazole with a variety of thiophene derivatives is tolerable. Through the extensive optimization of necrostatins, a novel necrostatin, Nec-7, bearing thiazole exerts differential biological activity from structurally diverse necrostatins, such as Nec-1, Nec-3, Nec-4 and Nec-5 (32). A series of Nec-7 derivatives suppresses TNF-α-induced necroptosis in the FADD-deficient variant of human Jurkat T cells, but have no RIP1 inhibitory activity, suggesting that they may target other necroptosis proteins. SAR analysis showed that various substituents at the phenyl 4-position are essential, that the para-position of the phenyl ring is tolerable to substituents and that the pyrazole ring is susceptible to structural modification (32).

With the discovery of new targets, a hit compound has been identified through the screening a library of 200,000 compounds for chemicals that protect necrosis. The compound,

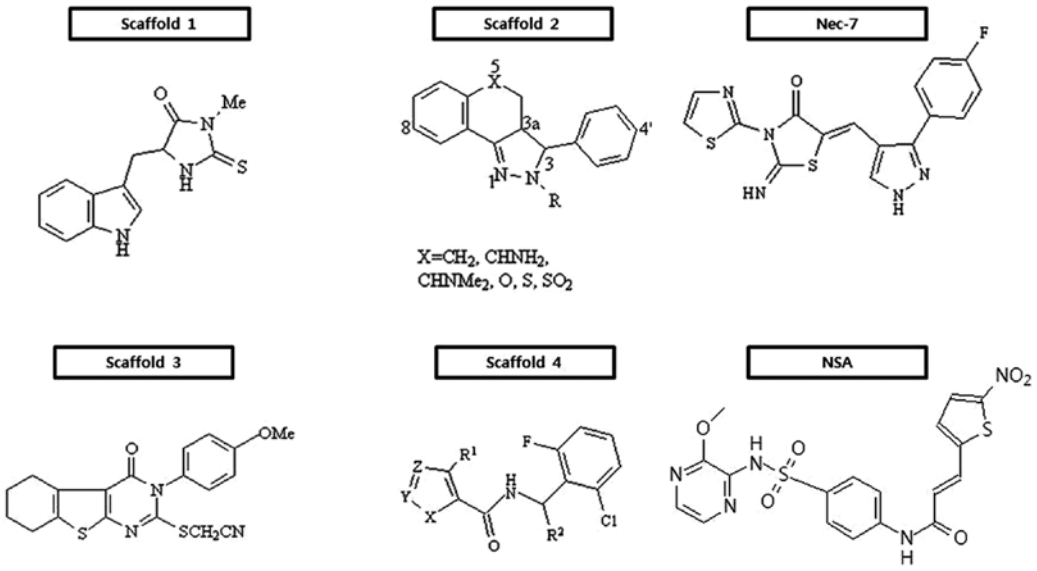


Figure 3. The novel scaffolds that exhibit protective activity against tumor necrosis factor-α (TNF-α)-mediated necroptosis. Chemical entities with antagonizing activities against necroptosis are withdrawn through high-throughput screening of the chemical library. Out of the scaffolds listed, necrostatin-1 (Nec-1) and necrosulfonamide (NSA) target RIP1 and mixed lineage kinase domain-like protein (MLKL), respectively, effectively protecting cells from necrotic stimulus but not apoptotic stress.

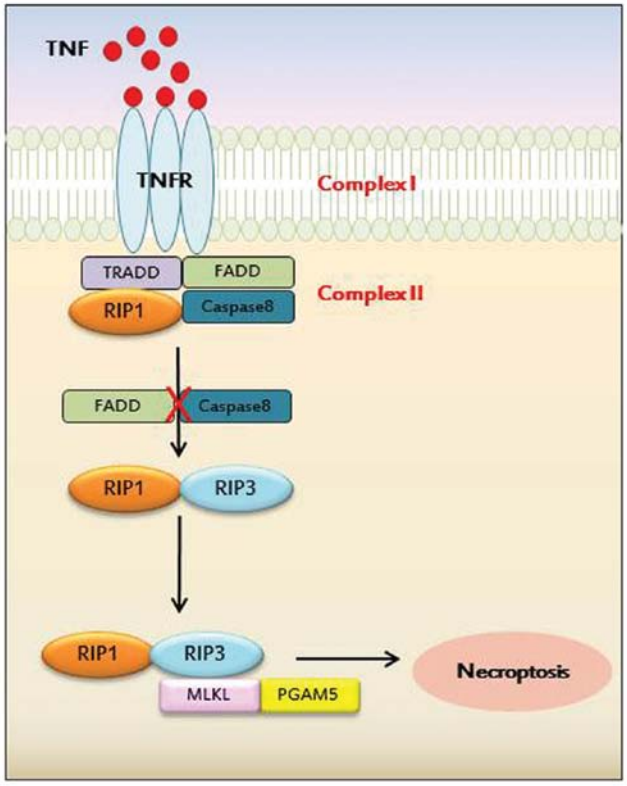


Figure 4. Schematic summary of the signaling linking of cytosolic events to the mitochondria during tumor necrosis factor-α (TNF-α)-mediated necroptosis. Upon TNFR ligation, its intracellular domain following trimerization of receptor is exposed to recruit a death-domain containing adaptor protein, TRADD, which acts as a scaffold protein for TRAF2 and RIPK1 to form a complex I. In a second step, bound proteins are eventually dissociated from the receptor when TNFR1 is engulfed into the cytosol. TRADD and receptor interacting protein 1 (RIP1) integrate FADD and caspase-8, forming a cytoplasmic complex (complex II) in which caspase-8 is activated to mediate apoptosis. In the situation when caspase is inhibited, RIP1 interacts physically with RIP3 to trigger consecutive downstream signaling events including mixed lineage kinase domain-like protein (MLKL) and PGAM5 recruitments, which transmit cytosolic death signals to the mitochondria. In summary, cell itself diverts actively apoptotic cell death to necrotic cell death by switching molecular interactions depending on the cellular context.

(E)-N-{4-[N-(3-methoxypyrazin-2-yl)sulfamoyl]phenyl}-3-(5-nitrothiophene-2-yl) acrylamide, is commonly referred to as necrosulfonamide (NSA) and has been reported to be more potent than Nec-1, with an $IC_{50}$ of >1 $\mu$M under the necrosis-inducing context. In an effort to reveal its target, MLKL has finally been proven to be a specific target of NSA which can modify covalently the Cys 86 residue within the N-terminal CC domain of MLKL and consequently interfere with the induction of necrosis (22).

Among those scaffolds listed above, scaffold 1 has shown *in vivo* activity in some mouse models, such as middle artery occlusion (MCAO) (27), ischemic/reperfusion heart injury (33) and traumatic brain injury (TBI) (34). Apart from efforts on the development of necroptosis inhibitors, very little attempts have been made to develop therapeutic drug targeting unregulated cell death, literally necrosis. Recently, LG Life Sciences, Inc. (Seoul, Korea) identified a series of necrosis inhibitors, referred to as NecroX™, which has been of interest for therapeutic candidates of liver diseases and fibrosis, ischemia and neurodegenerative diseases (35). However, it acts specifically as a scavenger of mitochondrial ROS, so that its action mechanism is totally different from that of necroptosis inhibitors. In a study from my group (unpublished data), NecroX™ was not effective against TNF-α-mediated necrosis, thus suggesting that death-causing ROS are differentially derived from death modes.

## 4. Control of diseases related to programmed necrosis

Physiological outcomes of programmed necrosis during viral infection are of significance as an innate immune defense mechanism. In such a case that viruses or intracellular bacteria encode caspase inhibitors, host cells themselves fail to operate the quality control death program (apoptosis) enough to get rid of infected cells, leading to the propagation of infectious agents. In light of the pathophysiological aspects, however, an alternative cell death to apoptosis may rather induce serious damage

to tissues, such as ischemic brain and heart tissue. Nerve cell death occurs in neurodegenerative disorders, with a continuum of apoptosis and necrosis being central to acute and chronic degenerative events (36). In addition, it has been known that chemical-induced pancreatitis is associated with programmed necrosis (37). Clinically, parenchymal necrosis is a key complication of pancreatitis, the severity of which depends on the cell death modes. Conversely, caspase induction protects from necrotizing pancreatitis (38). Sepsis is also thought to be derived from cell death caused by acute uncontrolled microbial infection. For instance, the pore-forming α-toxin from *C. septicum* triggers a multifaceted necrotic cell death response that is distinctively found in myonecrosis and sepsis (39). However, the detailed process by which cell death is linked to pathogenic outcomes remains elusive; programmed necrosis but not apoptosis may provide some insight into its pathogenesis. Decisively, Nec-1 can significantly delay the brain necrotic lesions induced by arterial occlusion, implying clearly that there occurs necrotic cell death controlled by RIP1-specific inhibitor in an ischemic setting. There is a growing need that a series of novel small molecules be developed to treat inflammatory-related diseases mentioned above. This may rather be based on the programmed necrosis-targeting strategy beyond an effective suppression of apoptosis. In fact, under an ischemic condition, the penumbra of the injured brain are the battle ground for stroke treatment, and immediate action should be taken for treatment by restoring perfusion to the ischemic area (40). Accordingly, there is at least an urgent need to develop safe and potent neuroprotective drugs that can mitigate the damage of cells in the penumbra shortly after onset and prior to hospital arrival.

A new paradigm of cell death makes it difficult to delineate cell damage inflicted by extracellular stimuli, whether it may be derived from microbial infection or chemotherapy. Although it is not generally admitted, imatinib has been suggested to induce cell death through a mixture of necrotic and apoptotic death. Thus, it may be meaningful to address which type of cell death will have a physiological effect over a treatment period.

## 5. Conclusions

In this review, the backup form of cell death to apoptosis has on its own significant meaning in biology. Cells cope actively with TNF-α-mediated cell death and consequently divert an apoptotic force into an alternative one. More significantly, the switch of death modes such as this have differential effects on physiological outcomes according to the stimuli and tissue niche. In this review, inflammatory diseases associated with programmed necrosis were briefly discussed to demonstrate the identification of small molecules against necroptosis. A variety of efforts in the search for chemical entities have been made since the first identification of RIP1. Presently, Nec-1 is the only small molecule being developed for targeting a specific molecule, RIP1. Thereafter, some necrotic proteins have been extensively explored and signaling networks between molecules have been partly unveiled. The identification of a novel programmed necrosis regulator, RIP3, and elucidation of its signaling pathway will set out to adopt new strategies, such as the suppression of RIP3 kinase activity or the dissociation of the pronecrotic RIP1-RIP3 complex (Fig. 4). Therefore, the discovery of novel small molecules which specifically and selectively inhibit RIP1 or RIP3 will not only clearly elucidate its molecular mechanisms, but may further translate into drug development pipelines. Taken together, this review provides insight into the molecular entity of small molecules against therapeutic target proteins governing programmed necrosis.

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