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Small molecule probes for cellular death machines

Ying Li[1,*], Lihui Qian[1,*] and Junying Yuan[1,2]

The past decade has witnessed a significant expansion of our understanding about the regulated cell death mechanisms beyond apoptosis. The application of chemical biological approaches had played a major role in driving these exciting discoveries. The discovery and use of small molecule probes in cell death research has not only revealed significant insights into the regulatory mechanism of cell death but also provided new drug targets and lead drug candidates for developing therapeutics of human diseases with huge unmet need. Here, we provide an overview of small molecule modulators for necroptosis and ferroptosis, two non-apoptotic cell death mechanisms, and discuss the molecular pathways and relevant pathophysiological mechanisms revealed by the judicial applications of such small molecule probes. We suggest that the development and applications of small molecule probes for non-apoptotic cell death mechanisms provide an outstanding example showcasing the power of chemical biology in exploring novel biological mechanisms.

Addresses
[1] Interdisciplinary Research Center on Biology and Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 26 Qiuyue Rd, Pudong, Shanghai 201210, China
[2] Department of Cell Biology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115, United States

Corresponding author: Yuan, Junying (jyuan@hms.harvard.edu)
[*] These authors contributed equally to this work.

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Introduction

Apoptosis has been established as an evolutionarily conserved programmed cell death mechanism involved in sculpting the development of metazoan [1–3]. On the other hand, necrosis had been traditionally considered to be an uncontrolled process triggered by overwhelming environmental stress or acute injury. In the past decade, this opinion has been challenged by the identification of molecular mechanisms involved in regulating necrotic cell death [4••,5••,6,7]. In this regard, the development of small molecule modulators of necrotic cell death has played major roles in the discovery of cellular machinery that controls cell death beyond apoptosis. The combination of chemical biology and traditional genetics has made it possible to vigorously define novel cell death mechanisms as well as establish their physiological and pathological significance. Here we will provide a critical review of the field and discuss future prospective and applications in understanding the molecular mechanisms of cell death beyond apoptosis.

Small molecules are not only core players in pharmaceutical industry for drug development, but also have become powerful tools for investigating the function of proteins and biological mechanisms in academic research. Small molecules provide several advantages for exploring biological functions in mammalian systems: easy to be applied in cell culture systems, accessible to precise temporal control and possibility to be tested in animal models of human diseases. Furthermore, small molecules provide the possibility to selectively target one particular function of a multifunctional protein, which used to be only possible in traditional genetic model systems such as fruit fly Drosophila and nematode *C. elegans*. In this review, we will discuss two non-apoptotic forms of cell death mechanisms, of which the discovery and development were tightly connected with the application of small molecule tools.

Necroptosis: a programmed necrotic cell death

The ability of TNFα, a very important pro-inflammatory cytokine, to induce necrotic cell death of certain cultured cell lines was noted in the late 1980s and early 1990s [8,9]. In this regard, Vercammen *et al.* demonstrated that inhibition of caspases not only did not inhibit, as one would expect if caspases mediated this type of cell death, but sensitized the death of L929 cells treated with TNFα [8]. Various mechanisms were speculated as to how TNFα might be able to induce necrosis, including the induction of ROS after mitochondrial damage, or aggregated TNFα to punch holes directly on cell membrane. Holler *et al.* extended these studies to demonstrate that activated primary T cells and Jurkat cells, a T cell line, could also undergo this type of necrotic cell death when stimulated by FasL with inhibition of caspases. By complementing RIPK1 deficient Jurkat cells with a kinase inactive mutant, Holler *et al.* showed that the kinase activity of RIPK1 was required for Jurkat cells to die when induced by FasL, TNFα and TRAIL under caspase deficient condition [10]. However, the scope of this study was limited as it was unclear if this mechanism applied to any other cell types or *in vivo*, as which would require making RIPK1 kinase dead mutations in each cell line and in animal models. Without such evidence, one cannot establish a common necrotic cell death mechanism regulated by RIPK1.

Degterev *et al.* conducted a phenotypic high throughput screen for small molecule inhibitors of U937 cell necrosis

induced by TNFα and zVAD.fmk, a pan caspase inhibitor [4••], which led to the identification of necrostatin-1 (Nec-1). Nec-1 was able to inhibit a wide array of necrosis induced by ligands of death receptors in 10 different cell cultured models of necrosis including that induced by the dimerization of RIPK1 kinase domain. The ability of Nec-1 to inhibit this diverse list of necrotic cell death models made it possible to propose the existence of a common regulated necrotic cell death mechanism termed 'necroptosis' [4••]. Nec-1 was shown to be an inhibitor of RIPK1 [11••]. The ability of Nec-1 to inhibit diverse examples of necrosis induced by ligands of death receptor family demonstrated the critical role of RIPK1 kinase activity in mediating necroptosis as a common regulated necrosis mechanism [12,13].

The ease of applying a small molecule RIPK1 inhibitor, Nec-1, made it possible to widely test the role of RIPK1 and necroptosis in many cell culture systems and animal models as a tool to study the involvement of necroptosis [12,13]. The discovery and application of Nec-1 provided a prominent example of the power of chemical biology in exploring unknown biological mechanisms and has led to the human clinical trials for developing RIPK1 inhibitors as treatments of human inflammatory and neurodegenerative diseases from colitis and rheumatoid arthritis to amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD). The success of Nec-1 also stimulated subsequent chemical biological studies of necroptosis and led to the development of not only inhibitors of RIPK1, but also RIPK3 and MLKL [14,15,16•] (Figure 1).

The rapid development in the past decade has led to the understanding of the molecular mechanism that mediates necroptosis [17]. When cells are stimulated by TNFα under caspase deficient condition, the intracellular domain of TNFR1 recruits a complex that includes RIPK1 to promote its activation. Activated RIPK1 in turn promotes necroptosis by interacting with RIPK3 to lead to its activation. Activated RIPK3 then mediates the phosphorylation of MLKL, a catalytically inactive pseudokinase [14,15,18] to promote cell lysis [16•,19–22]. Emerging *in vitro* and *in vivo* evidence has demonstrated necroptosis as a common intrinsic cell death mechanism in response to the activation of death receptors, toll-like receptors [23,24] and viral infections [25,26] in various cell and animal models. Necroptosis has been established as a form of regulated cell death involved in ischemia-reperfusion injury [27,28], inflammatory diseases [29], pathogen infection [30,31], tumor metastasis [32] and neurodegenerative diseases [33,34].

RIPK1 kinase inhibitors

The definitive evidence for RIPK1 as the target of Nec-1 and its improved analog Nec-1s (also called 7N-1) was provided by Degterev *et al.* [11••] (Table 1). Although the original isolate Nec-1, methyl-thiohydantoin-Trp (MTH-Trp), has been noted to be a low affinity inhibitor of indoleamine 2,3-dioxygenase (IDO) (IC50 ~ 100 μM), an improved analogue of necrostatin-1, 7-Cl-*O*-Nec-1 (=Nec-1 s or 7N-1) (5-(7-chloro-1H-indol-3-yl)methyl)-3-methylimidazolidine-2,4-dione) [35,36], has no IDO inhibitory activity. Nec-1s is a highly specific and CNS permeable inhibitor of RIPK1 [37,38]. Since the kinase activity of RIPK1 is involved in mediating most, if not all, of the deleterious functions downstream of TNFR1 upon stimulation by TNFα, inhibition of RIPK1 may provide much of the beneficial effects of anti-TNFα therapy without the side effects associated with removing TNFα. Thus, the ability of small molecule inhibitors of RIPK1 such as Nec-1s to block its kinase activity without affecting its scaffolding function may selectively block the deleterious effects of TNFα without removing TNFα [4••]. This prediction is supported by the normal physiology of mice carrying genetic knockin mutations that inactivate RIPK1, including D138N and K45A, and their resistance against systemic inflammatory syndrome induced by TNFα as well as animal models of neurodegeneration [38,39••,40,41].

RIPK1 kinase has been recognized as an important therapeutic target for the treatment of inflammatory and degenerative human diseases. GlaxoSmithKline (GSK) has identified a set of RIPK1 inhibitors using fluorescence polarization (FP) assay. Different from Nec-1, which occupies a hydrophobic pocket in close proximity to the activation loop of RIPK1 kinase as a type III inhibitor [42], these GSK compound series (1-aminoisoquinolines, pyrrolo[2,3-b]pyridines and furo[2,3-d]pyrimidines, represented by Cpd27) bind to the DLG-out inactive conformation as typical type-II inhibitors [43]. GSK has also developed a type III RIPK1 inhibitor GSK2982772, an optimized derivative of benzoxazepinone, and advanced this compound into human clinical trials targeting psoriasis, rheumatoid arthritis, and ulcerative colitis [44,45]. In addition, Denali Therapeutics has advanced a RIPK1 inhibitor into Phase I human clinical trial targeting ALS and AD.

Multi-targeted type II receptor tyrosine kinase inhibitors Ponatinib, Rebastinib and Pazopanib were reported as pan-RIP family kinase inhibitors and blocked necroptotic cell death efficiently. A hybrid molecule named PN10 designed as a fusion of the scaffold of ponatinib and Nec-1s robustly and selectively inhibited RIPK1 kinase and protected against necroptosis in cellular and mouse models [46]. Exploring novel chemical space on RIPK1 may be an important future direction for developing potent RIPK1 inhibitors.

RIPK3 kinase inhibitors

RIPK3 is a homolog of RIPK1 and contains a N-terminal kinase domain like other members of RIP family, but lacks a C-terminal death domain as that of RIPK1. The kinase activity of RIPK3 is important for mediating TNFα induced necroptosis [15,47]. The RHIM domain