



Review Paper

Methodology of drug screening and target identification for new necroptosis inhibitors

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ABSTRACT

Apoptosis has been considered as the only form of regulated cell death for a long time. However, a novel form of programmed cell death called necroptosis was recently reported. The process of necroptosis is regulated and plays a critical role in the occurrence and development of multiple human diseases. Thus, the study on the molecular mechanism of necroptosis and its effective inhibitors has been an attractive field for researchers. Herein, we introduce the molecular mechanism of necroptosis and focus on the literature about necroptosis drug screening in recent years. In addition, the identification of the critical drug targets of the necroptosis is also discussed.

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1. Introduction of necroptosis

Programmed cell death is of great significance in the physiological process, such as maintaining the human homeostasis, eliminating the damaged cells and improving the ability of anti-infection [1]. For a long time, people maintain the perception that apoptosis is the sole form of regulated cell death, while necrosis is unregulated, and the work giving an insight to the necrosis mechanism has no value. Nevertheless, along with the investigation of the cellular mechanisms of necrosis, researchers find that besides apoptosis, there exist several cell-death types which are also regulated by certain signaling molecules, such as pyroptosis, ferroptosis, and autophagic cell death [2–8]. These discoveries change the conventional ideas about the classification of cell death, which may offer a different and effective therapy for necrotic diseases. Among these programmed cell deaths, necroptosis is a notable form of regulated necrotic cell death which is caspase-independent. As shown in Fig. 1, necroptosis can be triggered by multiple stimuli like tumor necrosis factor (TNF), TNF-related apoptosis-inducing TRAIL ligand, Fas ligand, interferons (IFN) and other signals when apoptosis is prevented [9]. Although the signaling molecules are shared partly in apoptosis and necroptosis, their results are totally distinct [10]. The changes of morphology

are similar between necroptosis and necrosis [11]. TNF-induced necroptosis requires the kinase activity of receptor interacting protein kinase 1 (RIPK1), which was first identified by Holler and colleagues [12]. After the activation of RIPK1, the second key protein, receptor interacting protein kinase 3 (RIPK3), is recruited and phosphorylated by the RIPK1 [13–15]. In some cases, RIPK3 can be activated without RIPK1, for instance, the execution of necroptosis induced by the murine cytomegalovirus (MCMV) infection is RIPK1-independent [16]. After phosphorylation of RIPK3, the downstream critical protein mixed lineage kinase domain-like protein (MLKL) is activated and forms oligomers, then the trimerized MLKL translocates to the plasma membrane, so the calcium ion channel protein and sodium ion channel protein are activated, and the ions flow into the cell, resulting in the increase of cell osmotic pressure and cell lysis [17–23]. Another model of plasma membrane disruption caused by MLKL is that the activated protein will combine with lipids and break the integrity of cell membrane [24]. As the cell swelling and plasma membrane rupturing, the intracellular contents and danger associated molecular patterns (DAMPs) are released into the interstitial fluid and blood, which will lead to an array of human necrotic diseases, including liver injury [25,26], cold hypoxia-reoxygenation injury [27], renal ischemia/reperfusion injury [28–30], acute necrotizing pancreatitis [31,32], systemic inflammatory response syndrome [33,34], stroke [35,36], and other clinical diseases. Hence, it is essential to investigate the mechanism and discover the inhibitors of the necroptosis.

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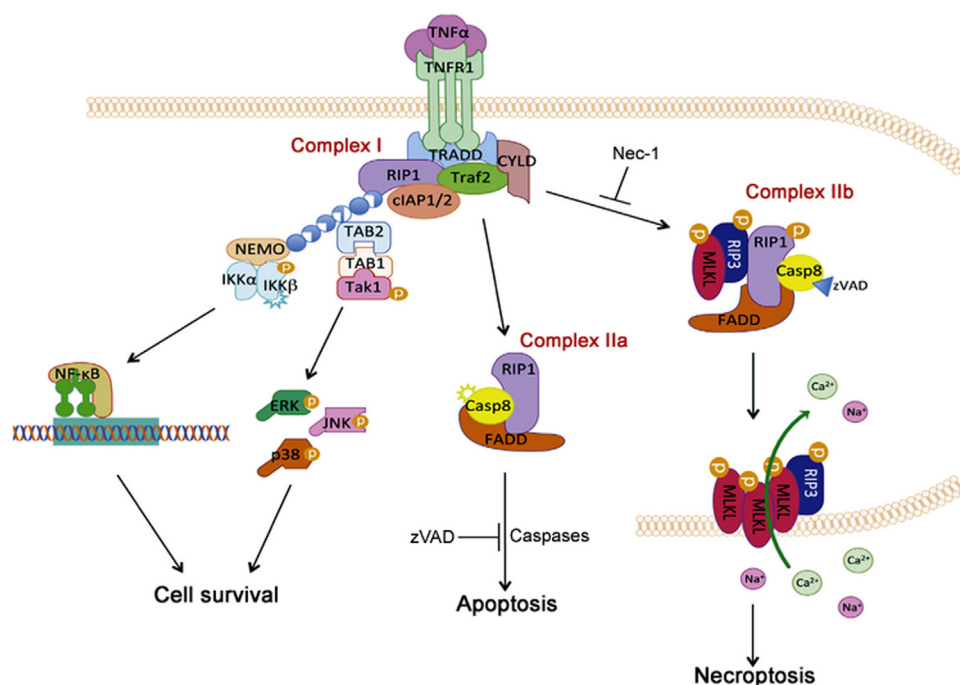


Fig. 1. Necroptotic pathways. After TNF stimulation, TNFR1 recruits TNF-receptor associated death domain (TRADD), cellular inhibitor of apoptosis proteins-1 and -2 (cIAP1/2), TNF receptor associated factor-2 (TRAF2) and RIPK1 to form complex I. K63-linked polyubiquitination of RIPK1 by cIAP1, leads to the recruitment of I- κ B kinase (IKK) complex and transforming growth factor- β activating kinase-1 (TAK1), activating the nuclear factor κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) survival pathways. Complex IIa consisting of RIPK1, FADD and caspase-8 activates apoptosis in the absence of cIAP1. RIPK1 interacts with RIPK3 and MLKL to form complex IIb, which mediates necroptosis in the presence of caspase-8 inhibitor (zVAD) [17].

2. Screening of necroptosis inhibitors

2.1. Screening based on cell level

This kind of drug screening is objective owing to the similarity to the human physiological process. The cells should be cultured *in vitro* before we start the experiment. Researchers can get an intuitive evaluation of the drug effect through the observation of the cell viability, morphology, growth and migration, *etc.* Fauster et al. [37] employed the Fas-associated protein with death domain (FADD)-deficient Jurkat cells to undergo cell death, which were treated with the second mitochondrial-derived activator of caspases (Smac) mimetic and the caspases inhibitor z-Val-Ala-Asp-(OMe)-Fluoromethyl Ketone (z-VAD-FMK) 30–60 min before the addition of TNF- α in the concentration of 10 ng/mL, and then was seeded onto the drug plates which was at two concentrations of 1.5 μ M and 0.5 μ M, respectively. Cell viability was assessed using a luminescence-based Cell Titer-Glo. The screening procedure is demonstrated in Fig. 2. The result indicated that ponatinib and pazopanib were capable of blocking the process of necroptosis. Rodriguez et al. reported a newly found RIPK3 inhibitor, GW440139B, which was screened from the library containing more than 8900 compounds. This molecule provided a tool for preventing the phosphorylation of MLKL, thus blocking the necroptotic pathway [38]. According to the study by Degterev's laboratory [39], necroptosis of human monocytic U937 cells triggered by TNF- α in the presence of caspase inhibitor was used to conduct a cell based screen and the inhibitor of RIPK1, necrostatin-1, was first identified from a chemical library and demonstrated that it had a relatively high selectivity to suppress necroptosis. However, as reported by Degterev et al. [40], in view of the condition that Nec-1 could inhibit another enzyme activity named indoleamine 2,3-dioxygenase (IDO) [41], as well as the poor pharmacokinetics, the feasibility of the compound in the clinic was reduced. A different type of RIPK1 inhibitor, termed GSK963, was

identified by Berger et al. This compound presented a high inhibitory activity for RIPK1 *in vitro* [42]. And a natural product kongensin A (KA) was also identified via a cell-based assay from a chemical library and it has a potential value to inhibit necroptosis efficiently [43]. The detailed information of representative necroptosis inhibitors screened by different labs is shown in Table 1. However, the excessive cost and complicated operation of the screening promote researchers to seek more simple methods, especially in modern pharmaceutical studies, because of the huge amounts of compounds.

2.2. Structure and fragment-based drug discovery

In contrast to the former screening, structure-based drug discovery can reduce the time and accelerate the experimental process since it utilizes the understanding of the three-dimensional structure of the target during the screening, and conduct further experiments with compounds which bind well. Molecular docking technique is a virtual drug design aided by the computer [44–47]. Fayaz et al. obtained the cocrystal structure of RIPK3 and analyzed the binding site in order to identify the novel inhibitors of RIPK3. Three compounds that exhibited an effective ability to interfere with the process of cerebral ischemia were discovered by the structure-based method. Further, these compounds can be optimized to design new inhibitors by the replacement of chemical groups [48]. According to the principle of induced fit theory, we can obtain a series of small molecule compounds with large binding force through screening of the compound libraries, and the application of X-ray diffraction enables researchers to get the three-dimensional structure of the small molecule compounds targeting the key kinases. Finally, the small molecule compounds can be optimized by adding substituents to have stronger potency and higher selectivity [2]. For instance, Xie et al. [49] displayed the crystallization of necrostatins/RIPK1 domain, and proved that these inhibitors all possessed a similar binding pocket, which

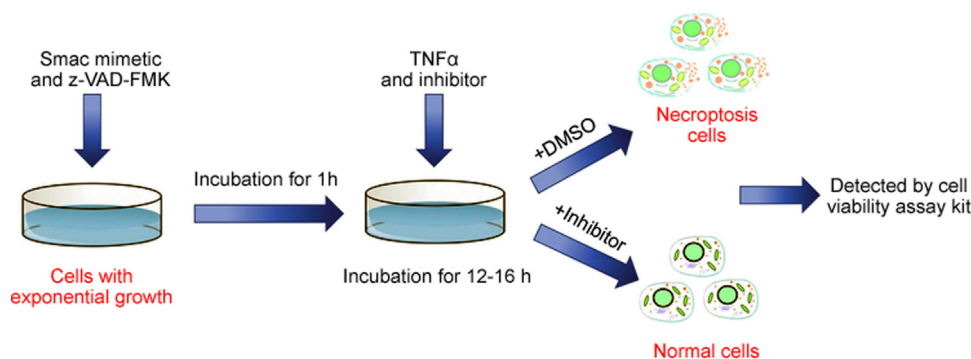


Fig. 2. A cell-based screening workflow of necroptosis inhibitors.

offered a structure basis of RIPK1 inhibition by necrostatins. And the proceeding of the lead optimization and drug discovery can be facilitated due to the structural analysis. Ponatinib-based selective RIPK1 inhibitors were designed as described by Najjar et al. [50]. They found that ponatinib can inhibit dual activity of RIPK1 and RIPK3 in vitro, which may limit its clinic application considering the safety. Therefore, in order to enhance its selectivity and retain its excellent binding affinity to kinases, they exploited molecular docking to get the three-dimensional combining model of ponatinib with RIPK1 on the basis of cocrystal structure of ponatinib/RIPK2 analog and designed a set of ponatinib chemical structures (CS) analogs by introduction of different groups on phenyl ring in consideration of the differences between the binding pocket of RIPK1 and other kinases, referred to as DLG (Asp-Leu-Gly) and DFG (Asp-Phe-Gly) motif, respectively. The results displayed that CS6 can selectively inhibit RIPK1 over RIPK3 with a mild reduction in activity. Additionally, they also synthesized a new type of inhibitors termed ponatinib-Nec-1 (PN) hybrids with the expectation of combining the advantage of high potency of ponatinib and prominent selectivity of necrostatin-1. PN10, one of the hybrid inhibitors. It was testified to achieve a favorable combination of the two agents mentioned above. Another similar approach, fragment-based drug discovery, also contributes to the drug design and development. Firstly, creating a molecular fragments library, then screening the activity of molecular fragments and using nuclear magnetic resonance (NMR) or X-ray diffraction to get its conformation and binding sites, finally, optimizing the molecular fragment on the micro to get a lead compound [51–54]. The method consists of activity screening, molecular modeling, and the structure-activity relationship analysis. To certain extent, fragment-based drug screening is more valuable than the structure-based drug discovery due to the small molecular weight of the obtained compound, which can conduct a further optimization to improve its activity and selectivity.

2.3. DNA-encoded library screening

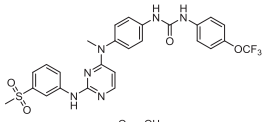
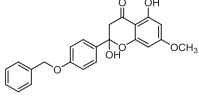
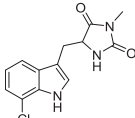
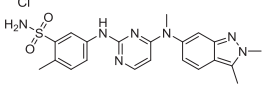
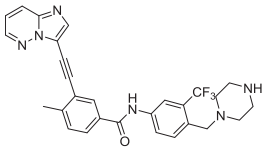
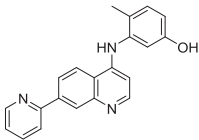
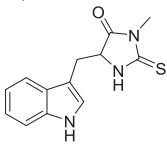
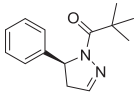
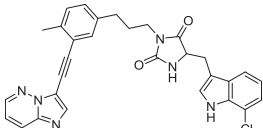
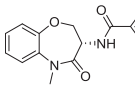
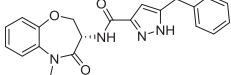
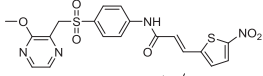
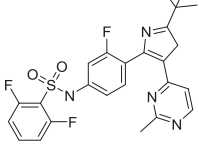
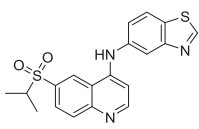
Recently, DNA-encoded library screening has been attracting more and more attention from pharmaceutical researchers and major pharmaceutical companies. It is foreseeable that DNA-encoded library screening would be a primary method in drug discovery [55–57]. This concept was first proposed by Brenner and Lerner in 1992 [58], then Nielsen et al. [59] and Needels et al. [60] adopted this approach to conduct their research, respectively. In fact, encoding and selection methods are very significant to DNA-encoded library screening, which shows the efficiency of the approach. The first step of DNA-encoded library screening is to design a compound library via combinatorial chemistry, and the number of compounds can reach into billions of above, then every specific compound would be covalently connected with a unique

sequence of DNA tag which is used as an identifier for the compound, so the DNA can be regarded as a medium for chemistry library. After selected by the targets, researchers can obtain the corresponding compound structure information and synthesize the compound through DNA sequencing. And finally, researchers can verify the activity of the compound to the target via cell proliferation assay. In conclusion, DNA-encoded library screening can be divided into five steps containing split-and-pool synthesis, affinity-based selection, PCR amplification, DNA sequencing and resynthesis without DNA. The chemical space of DNA-encoded library is a few orders of magnitude larger than traditional screening methods [61]. Therefore, the problems of time wasting and compounds insufficient in traditional high-throughput screening could be resolved efficiently [62], providing a more rapid method for drug screening. Harris et al. [63] established a library using a split-pool method with three cycles of building blocks, which contains 7.7 billion compounds. And they got a series of high-selective, potent inhibitors of RIPK1 through the drug screening from the library. Experiments showed that the compounds selected by the above method have higher activity and more excellent selectivity versus the known RIPK1 inhibitors in the previous literature, and they subsequently identified another compound, GSK2982772, by optimizing the former compound. It possesses a similar chemical structure, but the pharmacokinetic property of the compound is more prominent than that of GSK481. The agent is recognized as a first-in-class inhibitor of RIP1 and now is in clinic trials [64]. Hence, DNA-encoded library screening would play a critical role in discovering the lead compounds.

3. Target identification

Degtrev et al. [2] has reported that RIPK1 is the direct cellular target of necrostatins. According to the previous studies, phosphorylation of RIPK1 requires its own kinase activity. Thereby, the determination of phosphorylation of RIPK1 content can reflect the activity of RIPK1. To identify the target of necrostatins, they chose the 293T cells transfected with pcDNA3-FLAG-RIPK1 vector to express the protein, then anti-FLAG M2 agarose beads were applied to enrich RIPK1, and the beads were incubated in the reaction buffer with different concentrations of necrostatins. Afterwards, immunoblotting was performed to obtain the RIPK1 band. Finally, the phosphorylation of RIPK1 band was visualized by autoradiography (Fig. 3). The results showed that necrostatins can inhibit the autophosphorylation of RIPK1 in a dose-dependent fashion, which indicated that RIPK1 was the molecular target of necrostatins. And they designed a molecular docking model of RIPK1/Nec-1 complex using molecular modeling techniques. And the result of the structure-activity relationship of Nec-1 further suggested that RIPK1 was the direct target of Nec-1. Besides,

Table 1
Information of representative necroptosis inhibitors.

NO.	Name	Target	EC ₅₀ /IC ₅₀	Structure	Ref
1	Compound1	MLKL (KD=9.3 μM)	IC ₅₀ < 0.05 μM (MDFs cell)		[22]
2	6E11	RIPK1 (KD=0.13 μM)	EC ₅₀ = 4.6 μM (Jurkat cell)		[27]
3	7-Cl-O-Nec-1	RIPK1 (KD=0.026 μM)	EC ₅₀ = 0.1 μM (Jurkat cell)		[27]
4	Pazopanib	RIPK1 (KD=0.26 μM)	EC ₅₀ = 0.254 μM (Jurkat cell)		[37]
5	Ponatinib	RIPK1 (KD=0.037 μM), RIPK3	EC ₅₀ = 0.089 μM (Jurkat cell)		[37]
6	GW440139B	RIPK3	EC ₅₀ = 0.0736 μM (NIH 3T3 cell)		[38]
7	Necrostatin-1	RIPK1 (IC ₅₀ = 2 μM)	EC ₅₀ = 0.494 μM (Jurkat cell) IC ₅₀ = 1 μM (L929 cell) IC ₅₀ = 2 μM (U937 cell)		[39,42]
8	GSK963	RIPK1 (IC ₅₀ = 0.029 μM)	IC ₅₀ = 0.001 μM (L929 cell) IC ₅₀ = 0.004 μM (U937 cell)		[42]
9	ponatinib-Nec-1 (PN10)	RIPK1	IC ₅₀ = 0.01 μM (Jurkat cell)		[50]
10	GSK481	RIPK1 (IC ₅₀ = 0.01 μM)	IC ₅₀ = 0.01 μM (U937 cell)		[63]
11	GSK2982772	RIPK1 (IC ₅₀ = 0.001 μM)	IC ₅₀ = 0.0063 μM (U937 cell)		[64]
12	Necrosulfonamide (NSA)	MLKL	IC ₅₀ = 0.124 μM (HT29 cell)		[67]
13	Dabrafenib	RIPK3 (KD=0.0265 μM)	EC ₅₀ = 0.75 μM (HT29 cell) EC ₅₀ = 0.65 μM (U937 cell)		[68]
14	GSK872	RIPK3 (IC ₅₀ = 0.0018 μM)	EC ₅₀ = 0.7 μM (HT29 cell) EC ₅₀ = 0.12 μM (PECs cell)		[70]

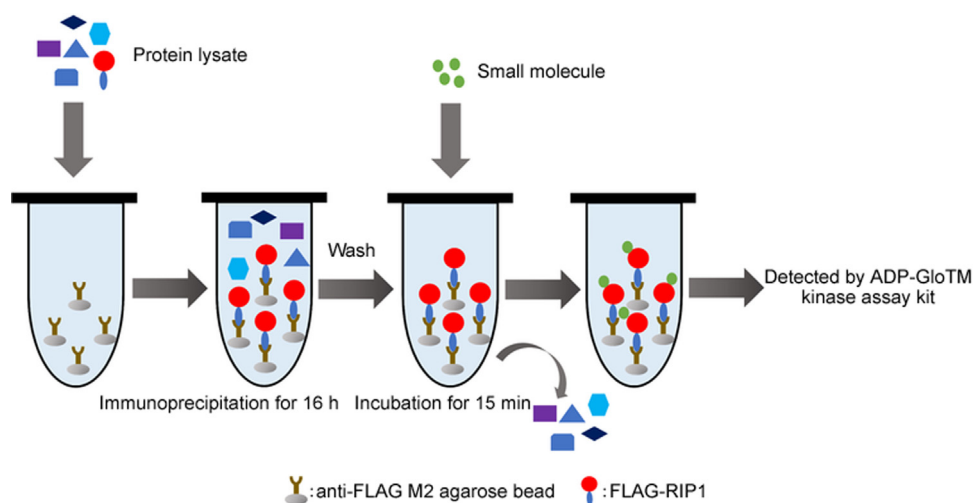


Fig. 3. The experimental workflow of cell-based RIP1 kinase activity assay for small molecules.

Ren et al. [65] discovered a new compound via screening the library and made an optimization to get higher efficiency and selectivity. To identify the target of compound, they monitored the phosphorylation of RIPK1 and RIPK3 through western blotting and evaluated the activities of RIPK1 and RIPK3 by ADP-Glo assay. The results showed that RIPK1 was the target of the novel compound. Martens et al. [66] found that the anti-cancer drug sorafenib can inhibit the TNF-induced cell death at a lower concentration with non-toxic effect. To identify the target of sorafenib, they applied the biotinylated sorafenib to make a pull-down experiment. First, different concentrations of biotinylated sorafenib were pre-incubated with streptavidin agarose beads respectively, and then the beads were washed with lysis buffer twice. After that, the beads were incubated with the cell lysates. After incubation, the $2 \times$ Laemli buffer was used to gather the samples, then the samples were boiled and analyzed via immunoblotting with the indicated antibodies. The results showed that sorafenib can inhibit the activity of RIPK1 and RIPK3 with a dose-dependent effect. Through the similar method, Sun et al. [67] confirmed that the direct target of necrosulfonamide (NSA) was MLKL, one of the critical signaling proteins in the necroptosis process. In addition, Li et al. [43] found that chaperone heat shock protein (HSP90) inhibited RIPK3-dependent necroptosis and was the cellular target of natural product KA.

In addition, the research of Li et al. [68] identified dabrafenib, an anticancer drug used in the clinic, was a new inhibitor of RIPK3. They established a non-radioactive luminescent kinase assay to screen the inhibitors of RIPK3, and the surface plasmon resonance (SPR) assay was used to demonstrate that dabrafenib can bind to the RIPK3. The recombinant RIPK3 was immobilized to the CM5 chip, then the drug dabrafenib was flowed into the sensor chip with a rate of $30 \mu\text{L}/\text{min}$ and used buffer to make it dissociation.

4. Conclusion and outlook

Researchers find that necroptosis is involved in multiple inflammatory diseases, which carries a new therapeutic direction. And most of these diseases exist in a substantial number of patients, so big pharma and labs concentrate mainly on the discovery of the inhibitors of RIPK1, RIPK3 and MLKL to suppress the process of necroptosis [69]. Unfortunately, none of the compounds discovered in recent decades meet the expectation of clinical application, and the limitations of these compounds impel researchers to continuously screen new drugs with higher

selectivity and potency. Furthermore, the molecular mechanism of necroptosis is not fully understood. Thus, we may explore novel key transducers of necroptosis during the study period and screen the corresponding compounds to block the necroptosis pathway, as well as to alleviate diseases associated with cell necrosis.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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