International Journal of Sciences: Basic and Applied Research (IJSBAR)

ISSN 2307-4531
(Print & Online)

http://gssrr.org/index.php?journal=JournalOfBasicAndApplied

Discovery of Hits That Can Specifically Inhibit Necroptosis but Not Apoptosis

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Abstract

Necroptosis or programmed necrosis is a specialized and regulated necrosis, and is unmasked when apoptotic machinery for death stress is defective. Initially, it was proposed that necroptotic cell death was pathologically associated with ischemic brain injury and retinal disorders. In contrast, it plays a beneficial significance in innate immune response to viral infection that can evade host's apoptotic surveillance. Also, it has been therapeutically emerging as the strategy to overcome the cancers with acquired anticancer drug resistance. Presently, a few small molecules to interfere with signaling pathways for necroptosis have been disclosed since necrostatin-1 (Nec-1) was for the first time identified as an inhibitor of receptor interacting protein 1 (RIP1), a key necroptosis regulator. In an effort to discover hits that can selectively inhibit necroptotic cell death, in this study, we screened in-house and in silico chemical libraries in a cell-based format. Eventually, 7 hits were identified from in-house chemical library while 2 hits were from computer modeling. Most hits less protected cells from tumor necrosis factor alpha (TNFα)- and zVAD-mediated necroptosis than a reference compound necrostatin-1, without affecting apoptotic cell death induced in HeLa. Interestingly, a few of hits had preferential protective effects on zVAD or TNFα while Nec-1 exhibited EC_{50} values at the similar concentrations against both necroptosis inducers, suggesting that chemicals deduced in our study can discriminate signaling pathways leading to receptor or nonreceptor-mediated necroptotic cell death. Therefore, some potent hits will be further improved to use for the treatment of necroptosis-associated disorders.

Keywords: Necroptosis; Screening; Cell-based assa; Hit; Apoptosis.

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1. Introduction

Necroptosis is an emerging mode of cell demise that has been gaining great attention over the last few years, and it is also referred to as programmed necrosis or regulated nonapoptotic cell death [1]. Its concept was initially suggested as the cell death can be induced by a ligand such as TNFα (tumor necrosis factor alpha) that is bound to a cognate receptor like TNFα receptor (TNFR), and is later strongly supported by other signaling necroptosis-associated proteins and their specific inhibitors [2]. Generally, necroptosis is unmasked only under the specific condition that apoptotic machinery is defective in response to death stress [2]. It implies that cell death modalities between autophagy, apoptosis and necroptosis crosstalk each other for decision of cell fate. In fact, it has been suggested that a combined condition of TNFα stimulation with caspase inactivation can drive necroptosis through the formation of RIP1-RIP3 complex, an early key event following TNFR ligation [3-5]. Thereafter, it has been further discovered that mixed lineage kinase domain like protein (MLKL) and mitochondrial protein phosphatase (PGAM5) are crucial to execute necroptosis [6]. The formation of RIP1/RIP3 necrosome complex dephosphorylates and then inactivates mitochondrial fission factor Drp1, which in turn causes mitochondrial fragmentation at an early stage of necroptosis. As a result, PGAM5 links an extracellular death signal to mitochondrial process, leading to necroptosis. Since the molecular understanding of necroptotic cell death, the discovery and optimization of small molecules with potently inhibitory activity against it have been explored for therapeutic uses. The first successful outcome is a series of hydantoin containing indole derivatives, referred to as potent necrostatins [7]. Out of hydantoin scaffold-bearing compounds, a chemical necrostatin-1 (Nec-1) exhibits so modest pharmacokinetic profiles as to be delivered to CNS following intravenous administration [8]. RIP1 was later found to be a targeting molecule of Nec-1 through *in vitro* kinase assay [9]. Since then, discovery of tricyclic derivatives [10] and substituted 3H-thienol[2,3-d]pyrimidin-4-ones [11] has been followed. More recently, a hit has been identified through screening a chemical library of 200,000, exhibiting a protective activity against necroptosis. A selected hit compound (E)-N-(4-(N-(3-methoxypyrazin-2-yl)sulfamoyl)phenyl)-3-(5-nitrothiophene-2-yl)acrylamide is referred to as necrosulfonamide (NSA) and is reported to be more potent than Nec-1, with an EC_{50} of less than 1 μM under the necrosis-inducing context. Finally, mixed lineage kinase domain-like (MLKL) protein was finally revealed to be a specific target of NSA, which can modify covalently Cys 86 residue within N-terminal CC domain of MLKL [12]. Physiologically, necroptosis contributes to various pathophysiological phenotypes, including ischemic brain injury, neurodegenerative disease, acute pancreatitis [8, 13, 14]. Also, retinal detachment-induced photoreceptor cell death is found to be associated with activation of RIP kinases, key proteins of necroptosis, when caspases are compromised [15]. Moreover, necroptosis has been reported to occur in the terminal ileum of patients with Crohn's disease, suggesting a critical role of necroptotic cell death in the pathogenesis of this inflammatory disease [16]. With growing knowledge of necroptosis-associated diseases, needs for therapeutic drugs are being pursued to protect or improve the progression of necroptosis. As a part of such efforts, there has been a growing body of researches that the inhibition of RIP1 by Nec-1 exerts cytoprotective effects against neurodegeneration, adult brain ischemia, myocardial infarction and photoreceptor loss-associated retinal disorders [15, 17, 18]. Contrariwise, necroptosis inducers can be applicable for overcoming cancers resistant to drugs based on the apoptosis [19, 20]. In fact, targeting weak point of cancers resistant to apoptosis by promoting susceptibility of necroptosis is attracting as the significant approach of chemotherapy [21]. In pursuit of exploring hits that would

rescue cells from necroptotic stimuli, we set out to screen in-house chemical library (6800 chemicals) and computer modeling-predicted compounds in a necroptosis-prone cell line L929. Either TNFα or zVAD promoted necroptosis via receptor or non-receptor, respectively as reported previously [22]. This study was aimed at new chemical entities that could suppress necroptotic cell death via nonreceptor. To this end, we set out cell based assay format that can differentiate necroptotic cell death via nonreceptor or receptor. In our previous article, intriguingly, it was shown that RIP3 or RIP1 is required for TNFα- or zVAD-mediated necroptosis[22]. Some possible candidates selected through primary screening were further examined if those chemicals might protect cells from undergoing apoptosis. Finally, 8 hit compounds were primarily suggested to protect necroptosis but not apoptosis although EC_{50}s of some were largely 10 times higher than a RIP1 inhibitor Nec-1. It is of note that some hits had differential effects on nonreceptor (zVAD)- and receptor (TNFα)-mediated necroptosis. Taken together, we present structures of 9 hits that can potently and specifically inhibit necroptosis. To outline structural moieties responsible for anti-necroptotic activity, study on hits optimization will be further undertaken.

2. Materials and Methods

2.1. Cell lines and culture

NIH3T3, HeLa and L929 cells were obtained from American Type Culture Collection, ATCC (Manassas, VA). Cell lines were grown in high glucose-Dulbecco's modified Eagle's medium (DME) media supplemented with 1% penicillin/streptomycin and 10% FBS, and allowed to incubate at 37°C in a 5% CO_2 incubator. Cells were maintained under an optimal condition by dissociation and subculture of cells at low density.

2.2. Reagents

Tumor necrosis factor alpha (TNFα) was obtained from eBioscience (San Diego, CA, USA). RIP1 and RIP3 antibodies were from BD Pharminigen (San Diego, CA, USA) and Prosci (Poway, CA, USA), respectively. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cell proliferation assay kit was bought from Promega (Madison, WI, USA). Cycloheximide (CHX), zVAD and necrostatin-1(Nec-1) were purchased from Sigma (St. Louis, MO, USA). Chemical resources were a representative chemical library from chemical bank (Korea Research Institute of Chemical Technology, Daejeon, South Korea), and compounds with anti-necroptosis activity were screened through an *in vitro* cell based assay and computer modeling. Other chemicals were of analytical grade.

2.3. Induction and protection of cell death

L929, HeLa and NIH3T3 cell lines were induced to apoptosis or necroptosis under specific death conditions. In brief, L929 cells were stimulated with mouse TNFα (10 ng/ml) or zVAD (10 μM) to die in a necroptotic way for 24 h. HeLa cells were grown in a combined condition of 1 μg/ml cycloheximide (CHX) with 10 ng/ml human TNFα in the absence or presence of zVAD (10 μM). Additionally, NIH3T3 was exposed to 10 ng/ml mouse TNFα and 1μg/ml CHX along with or without 10 μM zVAD. To discriminate between apoptotic and

necroptotic cell death, a RIP1 specific inhibitor Nec-1 was used. Cell death was evaluated by measuring cell viability of treated group relative to non-treated control. Cell viability was determined by MTS according to a protocol as described before [23].

2.4. Immunoblotting

Cells harvested from L929, HeLa and NIH3T3 were disrupted in a lysis buffer and the resulting cytosolic proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins resolved on gel were transferred to nitrocellulose (NC) membranes, which were further processed to do immunoblot with RIP1 or RIP3 antibody. To verify equal loading, actin was probed with antibody as a reference protein.

2.5. Cell-based assay for screening of antinecroptotic hits

To identify hits with antinecroptotic potency, cell based assay using L929 cells, which are prone to undergo necroptotic cell death upon TNFα stimulation, was primarily employed. Compounds with protective activity of greater than 50% in the assay were primarily chosen as hit candidates unless otherwise stated. For the 2nd round screening, EC_{50}s of some selected small molecules were further calculated from dose-response curves against zVAD or TNFα. To check the specificity of chemicals against cell death types such as apoptosis and necroptosis, moreover, we examined if they could affect the apoptotic cell death induced in HeLa stimulated with TNFα.

2.6. Computer modeling

To select the compounds our in-house library, we carried out the similarity analysis based on the molecular properties of Nec-1. An analysis of the physicochemical properties of Nec-1 resulted in the following filter criteria: molecular weight < 400, H-bond donors < 2, H-bond acceptors < 5, 2<Alog P < 4, number of rings including aromatic rings <3, and fingerprint (FCFC4)-based similarity (DiscoveryStudio4.5) > 0.60. These filters helped to reduce by 235 compounds from an in-house chemical library, including 8,000 compound species.

3. Results

3.1. A schematic chart for exploring hits that could protect cells from undergoing programmed necrosis

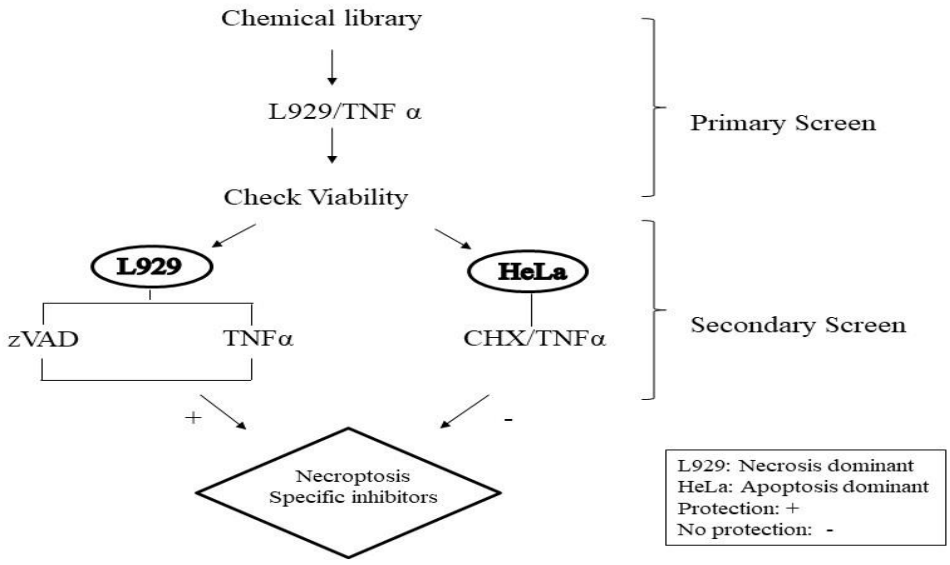
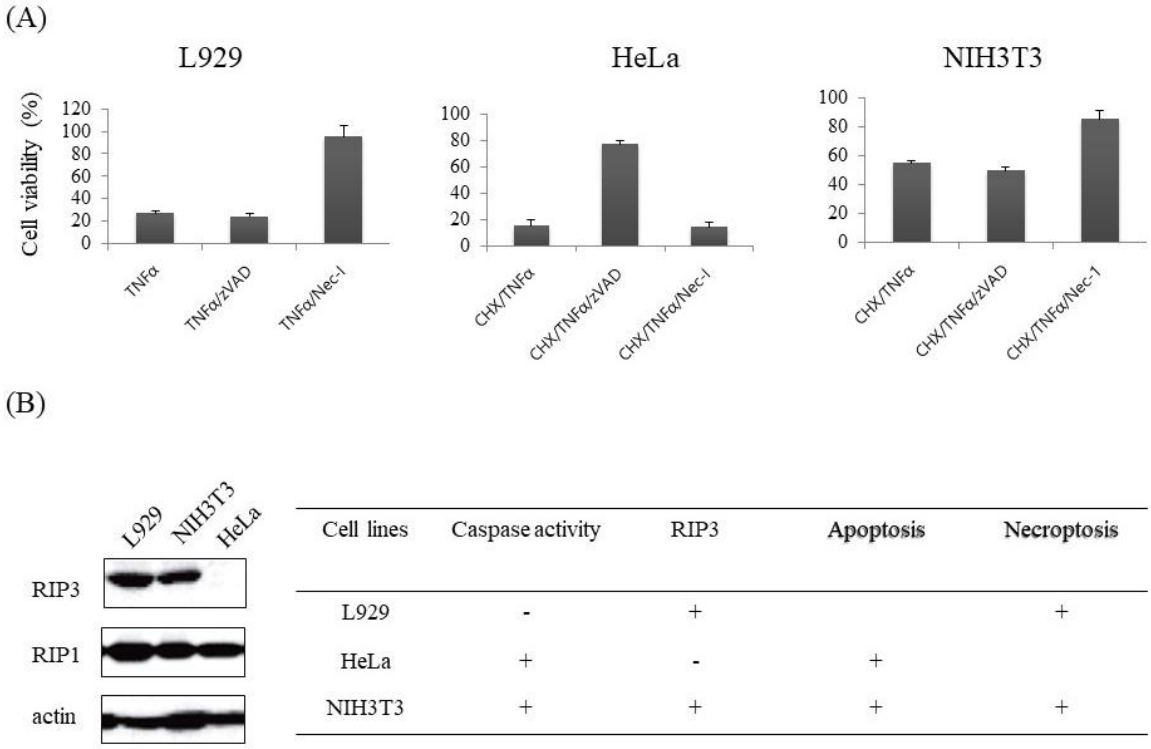


Figure 1: A schematic flow chart for isolation of hits with antinecroptotic activity selectively.To find hits that can specifically protect cells from necroptotic stress, L929 cells, which were pretreated with various compounds out of a chemical library for 1 h, were stimulated with TNFα to induce necroptotic cell death. Compounds with protective activity of more than 50% were further subjected to 2nd round screening that could test if compounds would be potent against either receptor (TNFα receptor)- or nonreceptor (zVAD)-mediated necroptosis in L929 cells. Moreover, some selected compounds were examined if those compounds could prevent TNFα-mediated apoptosis in HeLa cell line. Finally, through this schematic procedure, chemical profiles were determined with respect to protective potency against programmed cell death, necroptosis and apoptosis. Test chemicals were available from a chemical library classified by structural diversity and novelty, and computational modeling. Protective potency of chemicals was primarily tested in a cell based assay, in which L929 cell line is prone to necroptosis upon TNFα stimulation. Compounds that could exhibit potent protective activity against necroptosis were subjected to the 2nd round of screening. At this step, necroptosis was mediated according to receptor or nonreceptor death context, named TNFα or zVAD. Potent hits with EC_{50} around μM concentration were further investigated if those would negatively or positively affect apoptotic cell death in HeLa cells. Finally, plausible necroptosis specific inhibitors were successfully deduced and then their structures were then identified from chemical database.

3.2. Cellular and molecular foundations of cell based assay systems suitable for execution of apoptosis or necroptosis



Cell lines	Caspase activity	RIP3	Apoptosis	Necroptosis
L929	-	+		+
HeLa	+	-	+	
NIH3T3	+	+	+	+

Figure 2: Cell-based assays suitable for apoptosis and programmed necrosis (necroptosis). Induction and protection of cell death were tested in 3 different cell lines including L929, HeLa and NIH3T3 under the various conditions of cell death stimuli (A). Specific conditions used in this experiments were described in detail in Materials and Methods. L929 cells were treated with TNFα in the presence or absence of zVAD. Also, Cells were subjected to TNFα stimulation in combination with Nec-1. For the induction of cell death in HeLa, both CHX and TNFα were used. Under the condition of inducing cell death, HeLa cells were preincubated with either zVAD or Nec-1 to see whether cell death could be affected by those inhibitors. Finally, combined treatment of CHX with TNFα was required to activate cell death in NIH3T3. As with HeLa, cells were treated with either zVAD or Nec-1 under the cell death context of TNFα/CHX. Expression levels of RIP1 and RIP3, key necroptotic regulators, were investigated in L929, HeLa and NIH3T3 (B). Cell lysates (10 μg) extracted from each cell were run on SDS-PAGE and then transferred to nitrocellulose (NC) membrane. Transferred proteins were probed with antibody against RIP1or RIP3 and then visualized by chemiluminescence. For verification of equal loading, expression levels of actin were compared between samples. Cell death induced by death conditions was summarized in 3 different cells depending on caspase activity and expression level of RIP3 on the right side of immunoblot result. Some representative cell lines were investigated in responses to an inflammatory cytokine TNFα, and their cytotoxic responses to it were evaluated by MTS cell proliferation assay (Fig. 2A). Remarkably, L929 exerted cytotoxic responses to zVAD or TNFα, that is, necroptosis but not apoptosis. Either TNFα or zVAD killed cells very extensively, and their cytotoxic effects were also neutralized by Nec-1. In contrast, HeLa cells exhibited an apoptotic response to TNFα, and were significantly protected from TNFα-driven apoptosis by zVAD. However, Nec-1 did not affect TNFα-induced cell death, indicating that TNFα induces apoptotic cell death. NIH3T3 cells have differential cell death types depending on the death contexts. Particularly, apoptosis was induced when stimulated with TNFα in the presence of CHX. Intriguingly,

treatment of zVAD, a pan-caspase inhibitor, along with TNFα and CHX caused cells to commit an alternative cell type, necroptosis. As expected, however, TNFα-induced necroptotic cell death was successfully rescued by Nec-1, a specific RIP1 inhibitor. To seek out molecular correlation of necroptotic proteins to cell death modes, expression levels of two key necroptosis regulators RIP1 and RIP3 were primarily compared among cell lines (Fig. 2B). Both L929 and NIH3T3 expressed highly both two RIP proteins while HeLa cells did RIP1 with little RIP3 levels. It has been well known that caspase activity is not activated in response to TNFα stimulation. Therefore, L929 cells were driven to necroptosis when stimulated with TNFα due to inert caspase whereas HeLa cells with low expression of RIP3 committed to apoptotic cell death, but not necroptosis. Unlike L929 and HeLa cells, NIH3T3 cells were induced to default cell death apoptosis upon TNFα stimulation, but were diverted from apoptosis to necroptosis in the presence of zVAD.

3.3. Screening hits from chemical library or modeling-designed candidates

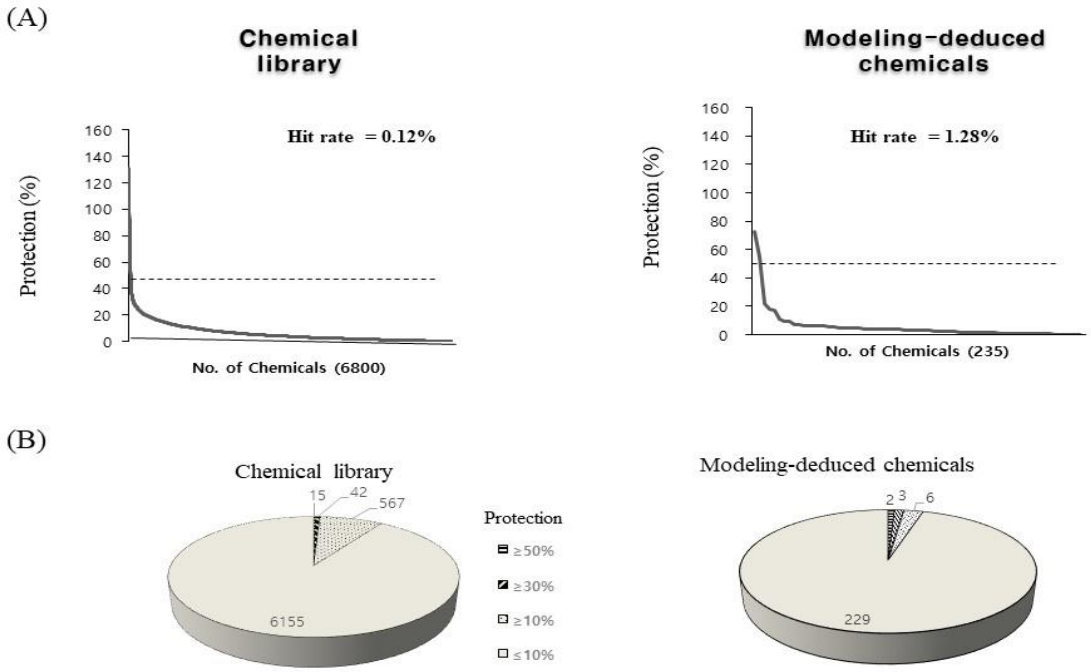


Figure 3: Summary of screening outcomes for discovery of hits with antinecroptotic potency. The dotted line represents protection cutoff (50%) drawn for the primary screen of hit candidates. Compounds suggested from a chemical library or computer modeling were assayed for protective activity against TNFα-mediated necroptosis in L929 cells. Each compound from chemical sources was sorted in the decreasing order of protective potency (A). Probability of selecting compounds with protective activity of more than 50% are approximately 0.12% and 1.28%, respectively, from a chemical library and computer modeling-deduced chemicals. When compounds were sorted by protective percentage (B), a few of compounds effectively protected cells from TNFα-mediated necroptosis, representing that only 15 and 2 compounds, respectively, exerted protective activity of more than 50% out of chemicals from different sources.

We carried out the cell-based assay using L929 cell line/TNFα stimulation in order to choose small molecules that can effectively protect cells from TNFα stimulation. Protective potencies of 6500 and 235 compounds, suggested from chemical library and computer modeling, were expressed as percent protection of each chemical against TNFα toxicity relative to a non-stimulated group (Fig. 3A). Compounds at a fixed concentration of 1 μM were sorted in a decreasing order as to how much those compounds could rescue L929 cells from TNFα exposure. Those protective potencies were widely ranged from about 70% to nothing. When plotting the graph by chemical numbers per % protective activity, hits with protective activity of greater than 50 % accounted for at most 0.1% and 1% out of a chemical library and modeling compounds, respectively (Fig. 3B).

3.4. Molecular docking

We carried out docking experiment using 4ITH (pdb code) structure, showing Nec-1 derivative's binding mode against RIP1K [24]. LigandFit method in DiscoveryStudio4.5 program was used for docking. As shown in Fig 4, compound 9 well docked having H-bond with D156 residue.

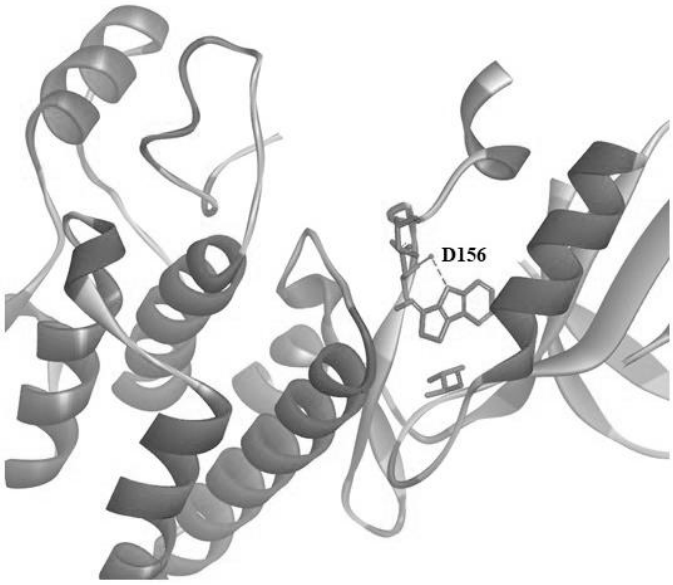


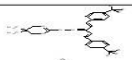
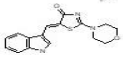
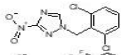
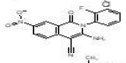
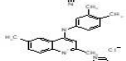
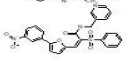
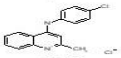
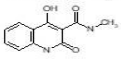
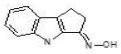
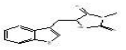
Figure 4: The binding mode of compound 9 against RIP1. The green lines represents H-bond.

3.5. Structure of hits, and their comparative antinecroptotic activities (Table 1)

Finally, disclosed were structures of 9 compounds over 50% cut-off level through the primary screening. The selected compounds with protective activities against necroptosis were investigated for receptor- or nonreceptor-mediated necroptosis with preferential selectivity for necroptosis over apoptosis (Table 1). Dose-response curves of selected compounds revealed that their EC_{50} values against TNFα were largely calculated to 2- 10 μM, a little higher than those of Nec-1. In response to zVAD stimulation, compound **1**, **2**, **4** and **6** did not show protective effects at the highest concentration used (20 μM), but the rests were potent, presenting EC_{50} values of 0.1-3 μM. In contrast, all the hits did not affect apoptotic cell death of HeLa treated with TNFα at the concentrations used. Intriguingly, compound **9** which showed high similarity score (FCFC4 > 0.7) is

comparable or superior to Nec-1 with respect to the protective potency against zVAD-mediated necroptosis.

Table 1: Antinecroptotic activities of hits selected from screening of in-house chemical library and computer modeling-deduced chemicals. In-house chemical library was from KRICT, including about 6800 chemicals classified by representative chemical moiety. Also, some chemicals were suggested from database of chembank (KRICT) through computer modeling. Nine compounds selected from primary screening were further assayed to plot dose-response curves against TNFα- or zVAD-driven necroptosis, and then EC_{50} of each compound was calculated using GraphPad Prism software.

Compound No.	Structure	EC_{50} (μM)		Apoptosis**
		TNFα	zVAD*	
1		2.8	-	-
2		5.4	3.6	-
3		6.0	-	-
4		5.0	2.0	-
5		3.0	-	-
6		4.8	2.0	-
7		12.0	-	-
8		6.2	0.6	-
9		2.0	0.1	-
Nec-1		0.7	0.3	

* and ** represent no significant protection at a concentration of 50 μM in the following cell based assays of zVAD-mediated necroptosis in L929 and TNFα-induced apoptosis in HeLa, respectively.

4. Discussion

Since physiological significances of necroptosis were emphasized, a few molecular targets for necroptosis and specific inhibitors have been extensively explored. As a result, a cascade of signaling pathway and underlying molecular mechanism leading to necroptosis has been in part established. Particularly, some antinecroptotic chemical probes can be useful for not only drug development for treatment of neurodegenerative diseases, but also identification and validation of target molecules associated with necroptosis. In fact, discovery of Nec-1 and necrosulfonamide (NSA) makes it possible to identify a corresponding specific molecular target in L929 cells stimulated with TNFα [9, 12]. To this end, a suitable assay system whatever it may be based on target molecules or phenotypes is prerequisite. To date, a cell based format is conveniently used for primary screening of chemicals due to incompatibility of enzymatic activity of necroptosis-related proteins to high throughput screening (HTS). In an attempt to improve a cell based assay for screening chemicals against necroptosis, 3 different cell lines were examined. Out of cell lines derived from normal or cancer tissues, only NIH3T3 committed the fate of both apoptosis and necroptosis when stimulated with TNFα in the absence or presence of

zVAD. Consistent with a previous article, in our results, L929 cells exhibited an atypical response to TNFα, mediating necroptosis rather than a default programmed cell death apoptosis when stimulated with it [25]. Later, our study suggests that zVAD and TNFα caused necroptosis in a RIP1- and a RIP3-dependent pathway, respectively. It can be explained by unmasking of necroptosis resulting from defects in the activation of caspases upon TNF stimulation [22]. In HeLa cell line, TNFα induced so extensively apoptosis that its cell death could completely reversed by zVAD, a caspase inhibitor. RIP1 and RIP3 are proximal necroptosis regulators following TNFR ligation, and their complex formation between RIPs is required for driving necroptosis. Interestingly, there was generally high expression of RIP1 in 3 cell lines under investigation, but notably little expression of RIP3 in HeLa. Therefore, TNFα-stimulated HeLa cells could not transduce death signals to necroptotic cell death due to RIP3 insufficiency and then failure in complex formation of RIP1/RIP3. Based on molecular foundations associated with cell death, flow chart can be made for subsequent procedures to discover hits with antinecroptotic activity. That is, inhibitors with protective potency more than 50% were primarily selected out of chemical compounds in TNFα-driven necroptotic prone cell line L929. EC_{50}s of selected compounds were calculated from dose-response curves against zVAD or TNFα, respectively. Some potent chemicals were further examined for anti-apoptotic activity in HeLa cells, apoptosis-prone cells. Through these screening processes, 9 hits were finally deduced and then their structures were identified. Interestingly, some hits (**2, 4 and 6**) exhibited protective activity against zVAD and TNFα with EC_{50} around 3 μM although most hits are less effective than a reference compound Nec-1. Some (**compound 3, 5 and 7**) had preferential inhibitory activity against TNFα over zVAD. In contrast, EC_{50} values of **compound 8 and 9** for zVAD had 10~20 times lower than those for TNFα. Chemical probes with differential inhibitory profiles make it very useful to interfere with the signaling pathways at the specific targets. Thereafter, hits will be chemically modified to improve protective activity and selectivity based on the predicted binding mode.

5. Conclusions

Small molecules that protect cells from necroptotic cell death were screened through cell-based assay from a representative chemical library and computer modeling-proposed candidates. As a result, nine hits were demonstrated to have a protective activity against necroptosis but not apoptosis. All the hits were not comparable to Nec-1 with respect to protective potency against TNFα or zVAD, but some hits showed specifically antinecroptotic activity for zVAD over TNFα, indicating that necroptosis may be mediated via different routes, receptor or nonreceptor ligand. This study supported the notion that zVAD or TNFα could execute cell death in a RIP1- or RIP3-dependent way. Moreover, it introduced the cell based assay that could effectively screen potent antinecroptotic small molecules for therapeutic uses. Therefore, this study highlights that hits have a potency to protect effectively necroptotic stresses from zVAD and TNFα. However, deduced hits are so chemically different from known antinecroptosis chemicals that those compounds have structural versatility to get them modified. Consequently, novel molecular targets for hits will additionally be identified to fill in the gaps between necroptotic molecules leading to signaling pathway associated with necroptosis.

6. Conflict of Interest

The author declares no directly or indirectly conflict of interest.

Acknowledgements

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Author resposnses to reviwer's Comments

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Accept As Is:	
Requires Minor Corrections:	X
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