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Title: ~~Protection or harnessing~~The role of necroptosis ~~for~~ in the treatment of diseases

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Running Title: Necroptosis-associated targets and ~~specific~~ small molecules

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ABSTRACT

Necroptosis is ~~a cell death mode mechanism~~an emerging form of programmed cell death~~of~~ occurring via active and well-regulated necrosis, distinct~~ive~~ from apoptosis ~~in~~ morphologically, and biochemically ~~and molecular features~~. Necroptosis is mainly unmasked when apoptosis is compromised in response to cell stress. Unlike apoptotic~~sis undergoing~~ cells, which are cleared by macrophages or neighboring cells, necrotic cells release danger signals, ~~provoking~~ triggering inflammation, and ~~further severe~~exacerbating tissue damage ~~to tissue~~. ~~It has been growing e~~Evidence increasingly suggests that programmed necrosis is not only associated with pathophysiology of~~ical~~ disease~~s~~, but also ~~provides~~ induces innate immune response to viral infection. Therefore, necroptotic cell death plays both physiological and pathological roles. Physiologically, necroptosis ~~can~~ induce an innate immune response~~s~~ as well as premature assembly of viral~~us~~ particles in cells infected with virus that ~~can~~ abrogates host apoptotic machinery. On the other hand, necroptosis ~~by itself has well been thought to be~~per se is detrimental, causing various diseases such as sepsis, neurodegenerative diseases and ischemic reperfusion injury. This review ~~will deal with~~ discusses the signaling pathways leading to necroptosis, ~~its~~ associated necroptotic proteins with target-specific inhibitors and ~~implicated~~ diseases involved. ~~Many~~ Several~~researches~~ studies ~~of necroptosis~~ currently focus on protective approaches to ~~block~~ inhibit necroptotic cell death. In cancer biology, however, anticancer drug resistance severely hampers the efficacy of chemotherapy based on apoptosis. Pharmacological switch~~ing~~ of cell ~~demise~~ death ~~can encourage~~ finds therapeutic application ~~to overcome~~ in drug-resistant cancers ~~with drug resistance~~. Therefore, ~~future~~ the possible clinical ~~use~~ role of necroptosis ~~for~~ in cancer control will be discussed in brief.

INTRODUCTION

Cell survival is constantly in dynamic equilibrium with cell death for tissue homeostasis. Aside from cell survival, cell death takes placeoccurs in various modes such as programmed way or not random mechanisms upon exposure to various stresses. One of pProgrammed cell death or apoptosis is activated in anvia intrinsic or extrinsic fashion mechanisms in response to a diverse of death stimuli. Both pathways are characterized by involvement of a series of signaling molecules with activated caspases and is so calledtermed caspase-dependent cell death. Meanwhile, necroptosis is classified as type III programmed cell death with apoptotic and necrotic features. It is also coined known as programmed necrosis or caspase-independent cell death according to morphological or molecular features. Initially, necroptosis has been known to bewas considered as unwanted undesirable passive cell death. However, but it is currently considered as specialized cell death modality since it is induced in an orchestrated way under a caspase-compromised condition. Like Similar to apoptosis, necroptosis has its ownactsis executed via distinct signaling pathway mechanism comprising composed of a cascade of specified proteins, consequently leading toresulting in regulated necrotic cell death. Though it's underlyingDespite unknown mechanisms and pathological significances remain unknown as compared towith apoptosis, the discovery of pharmacological inhibitors targeting necroptosis has been extensively pursued. Here, we seek to introduce the concept of necroptotic cell death induced under various pathophysiological conditions —and delineate the pathological mechanisms by whichunmasking necroptosis is unmasked in a well-orchestrated fashion. In this review, necroptosis-associated diseases and its the underlying pathogenesis will be discussed.

SIGNALING PATHWAYS LEADING TO NECROPTOSIS INDUCTION AND BIOCHEMICAL CHANGES

Typically, necroptosis occurs in cells exposed to extrinsic stimuli such as TNFα, FASL, and TRAIL in combination with compromised caspase-8 (C-8). C-8 inhibition is ~~caused~~ induced~~from~~ by genetic defects, viral proteins, and treatment with a pan-caspase inhibitor zVAD ~~treatment~~ (1-3). ~~Identification and biological validation of n~~Necroptosis~~-~~-regulating proteins have been identified and biologically validated comprehensively ~~been pursued~~. ~~From g~~Genome-wide siRNA screening yielded a~~, some~~ few candidate genes associated with necroptosis ~~were proposed~~ (4). ~~Since~~ Based on the~~disclosure~~ role of RIP1 as a necroptosis regulator, ~~identification of~~ RIP3, mixed lineage kinase domain-like protein (MLKL) and PGAM5 were ~~followed~~ identified (5-8). ~~Illustrated is the~~The signaling pathway leading to necroptosis is illustrated in Fig. 1. ~~Upon~~ TNFα ligation to its cognate receptor~~, it drives~~ triggers caspase-dependent cell death as a default cell death ~~mechanism~~mode. ~~Under c~~Caspase-compromised conditions~~, it directs~~ drive cell death ~~fate into~~via necroptosis with accompanying RIP1 activation (9). Upon stimulation, RIP1 interacts with RIP3 ~~to form~~leading to the formation of a necrosome complex via RIP homotypic interaction motif (RHIM) within cells (10). MLKL pseudokinase was identified as a substrate of RIP3 ~~when~~ under necroptotic condition~~sis is triggered~~. Phosphorylated RIP3 ~~can recruit to~~induces ~~phosphorylate~~ MLKL phosphorylation at serine or threonine sites. The~~n,~~ RIP3-MLKL complex translocates to mitochondria~~-~~-associated membrane mediated via~~, in which~~ phosphoglycerate mutase 5 (PGAM5)~~, are involved. Phosphoglycerate mutase 5 (PGAM5) was later discovered as~~ which is a necrosome-associated protein ~~which in turn can~~ regulating~~e~~ dynamic-related protein (Drp1) in turn (8). Finally, Drp1 ~~regulates~~ induces mitochondrial fragmentation, a crucial event for necroptosis (11).

~~When~~Biochemical changes during the early stages of necroptosis ~~is triggered, biochemical changes at an early stage~~ include ATP depletion, ROS generation, calcium overload, and loss of mitochondrial permeability transition (12). At the cellular level, necroptosis is characterized by loss of plasma membrane integrity as well as organelle swelling, finally leading to cellular collapse (10). ~~More strikingly, n~~Notably, necroptotic cells released various damage-associated molecular patterns (DAMPs) such as HMGB1, cytokines and histones into extracellular media. Particularly, a disulfide form of HMGB1 by oxidation ~~can~~ contributes to the inflammatory responses (13). ~~Released~~ HMGB1 ~~can~~ binds to a cognate receptor on endothelial cells and macrophages to transduce cellular responses such as release of proinflammatory cytokines and chemotactic cell migration (14).

NECROPTOSIS: TARGET PROTEINS AND INHIBITORS

As described above, a series of necroptosis-associated proteins ~~was~~ were identified and further validated as targets of necroptosis. ~~After then~~Subsequently, ~~some~~ a few small molecules ~~have been~~were successfully discovered, specifically targeting ~~modulate~~ necroptosis proteins. ~~Discovered t~~Target-specific small molecules that can modulate ~~in~~ necroptosis are listed in Table 1.

RIP-1 inhibitors (~~N~~necrostatin-1 and derivatives): Necrostatin-1 (Nec-1), a 5-(1H-indol-3-ylmethyl)-2-thiohydantoin 1, was first discovered ~~through~~ by screening of ~~for~~ necroptosis inhibitors and later identified as an allosteric inhibitor of RIP1 via stabilization of an inactive conformation of the kinase domain (KD) (15). It has been widely validated in various necroptosis-associated animal models (16-19). Accordingly, Nec-1 plays a crucial role in prevention or ~~mitigation~~ alleviation of necroptotic damage caused ~~from~~ by various stimuli via ~~by~~ targeting RIP1. Another potent Nec-1 derivative Nec-1s (7-Cl-O-Nec-1) ~~has even more~~showed greater ~~selective~~ specificity to RIP1 ~~over~~ compared with other kinases. Other inhibitors have been developed as ~~the~~ RIP1 inhibitors, including furo[2,3-d]pyrimidines and

GSK'963 (20, 21).

RIP3 inhibitors: Recent studies proposed that necroptosis is mediated in a RIP1- or RIP3-dependent manner (22, 23). Unlike RIP1, RIP3 is ~~so~~ essential for necroptosis, but not apoptosis, and ~~that~~ targeting RIP3 ~~seems~~ appears to be more specific ~~for~~ in controlling necroptosis. ~~More i~~Interestingly, silencing of RIP3 ~~silencing~~ substantially ~~rescues~~protected cells ~~or~~ and tissues ~~from~~ against necroptosis ~~inducing conditions by~~ (24). ~~Some s~~Small molecules ~~(~~such as GSK'840, GSK'843 and GSK'872~~)~~ have been reported to suppress RIP3-dependent necroptosis (25). Mechanistically, these~~ose~~ molecules form a complex with RIP3 to restore caspase activity. Consequently, RIP3 inhibitors potentially protect cells from diverse stimuli than RIP1 inhibitors.

MLKL inhibitors: Necrosulfonamide ((E)-N-(4-(N-(3-methoxypyrazin-2-yl) sulfamoyl) phenyl)-3-(5-nitrothiophene-2-yl) acrylamide, NAS) ~~can~~ protects cells ~~from~~ against TNF-induced necroptosis via covalent modification ~~with~~ of a cysteine ~~of~~ in MLKL (7). A compound TC13172 is an MLKL inhibitor with single nanomolar potency. It ~~was demonstrated to cause~~induced covalent inhibition at Cys-86 of MLKL (26). In addition, GW806742X targets the pseudo-kinase domain of MLKL with ~~a~~ nanomolar inhibitory activity, protecting against necroptosis ~~derived cell death~~, although it ~~has exhibits~~shows~~es~~ off-target ~~activities~~ effects against other kinases (26).

~~Besides listed above, some~~Additional agents have been developed as inhibitors of necroptosis ~~though their~~acting directly via ~~direct necroptosis~~ targets that have ~~still not been~~yet to be designated. Ponatinib and pazopanib (27) are~~: T~~ two anti-cancer agents ~~were~~ identified from a representative panel of United States Food and Drug Administration (FDA)-approved drugs through phenotypic screening. Both drugs ~~abolish~~ abrogate phosphorylation ~~activation~~ of ~~mixed lineage kinase domain-like protein (~~MLKL~~)~~ during TNFα-induced necroptosis, ~~proposing~~ suggesting ~~that those drugs can~~ interference with the signaling proteins upstream of

MLKL. However, theyose do not rescue cells from apoptosis. Interestingly, ponatinib affects inhibits both RIP1 and RIP3 while pazopanib exerts acts against preferential inhibitor of RIP1. AlsoFurther, sorafenib is a multi-targeting kinase inhibitor with potent inhibitory activity against B-RAK, and then is widely used for the treatment of acute leukemia. It is proposed that it can blocks signaling target proteins upstream of MLKL such as RIP1 and RIP3, although its real target remains elusive (28). Therefore, sorafenib can be harnessed for fine-tuning of necroptosis-inducing agents.

TPCK: N-tosyl-l-phenylalanine-chloromethyl ketone (TPCK), a serine protease inhibitor, protectsed cells from TNF-mediated necroptosis. Through a screen uUsing a TPCK probe, HtrA2/Omi has been identified as a target and it acts functions as an activator of ubiquitin C-terminal hydrolase (UCH-L1) for execution ofresulting in necroptosis (29) .

ROS scavengers: Reactive oxygen species (ROS) play a role in diverse of signaling pathways, as those aredue to highly reactivityeto against biomolecules such as proteins, DNA and lipids. When Upon exposure ofL929 cells wereexposed toTNFα, necroptotic signaling generates ROS accumulationthroughvia mitochondrial transport chain but not cytosolic enzyme (30), which. This notion was strongly supported by the reports suggesting that butylated hydroxyanisole blocks ROS accumulation and cell death (31). An NADPH oxidase inhibitor diphenyleneiodonium (DPI) protects renal tubular epithelial cells from against necroptosis by blocking ROS generation (32).

PHYSIOLOGICAL AND PATHOLOGICAL SIGNIFICANCE OF NECROPTOSIS

Necroptosis is was originally considered as unwanted undesirable cell death upon exposure to stimuli, causing inducing detrimental tissue damage to tissues. Moreover Furthermore, it is the alternateiveive cell death mechanismtype that can be induced under conditions of defectivewhen default cell death apoptosis is blocked or defective. However, there are growing evidences suggests that necroptosis is mediated through via an orchestrated and specialized

pathway (33-35). Well-regulated cell death ~~plays~~ occurs in a ~~diverse~~ range of biological ~~functions~~ phenomena ~~like~~ such as development, immunology and differentiation. ~~Besides~~ Further, extrinsic apoptosis and necroptosis contribute to ~~a~~ host defense mechanism against microbial infection. ~~Some v~~ Viruses ~~like~~ such as adenoviruses, poxviruses, and herpesviruses ~~disarm~~ inactivate ~~v~~ ad host apoptosis machinery ~~of host~~, being ~~u~~ an ~~b~~ ad virus and ~~would~~ perpetuate if host cells ~~could~~ only execute ~~d~~ caspase-dependent default cell death (36-38). For instance, vaccinia viruses encode a caspase 8 inhibitor to block apoptotic cell death upon infection. Under this caspase-compromised condition, cells are committed to alternative necroptosis (6). The resulting necroptosis is vital to provoke innate immune response by killing virus-infected cells and ~~releasing~~ eliminating releasing danger signals from host cells into external milieu. ~~Moreover~~ Furthermore, ~~it has been reported that~~ necroptosis in T cells regulates antigen-activated T-cell proliferation ~~of T cells~~ and survival. Caspase-8 negatively regulates necroptosis, promoting survival of activated T cells under physiological conditions. In mice lacking caspase-8, T cells fail to show immune response when infected with murine hepatitis virus (39).

Pathologically, ~~there is a growing body of reports~~ evidence suggests that necroptotic cell death leads to various diseases ~~are derived from necroptotic cell death. Representatively, s~~ Sepsis is mainly caused by Gram-negative bacteria, which release endotoxin ~~of which~~ that elicits systemic inflammation ~~through~~ via release of TNFα and IL-1 inflammatory cytokines. Necroptosis ~~is manifested~~ occurs in ~~i~~ Ischemia-reperfusion (I/R) injury and neurodegenerative diseases. During restoration of blood flow into tissues, ~~it leads to~~ tissue damage occurs with severe neutrophil infiltration and cytokine production. Furthermore, necroptosis is ~~demonstrated to be~~ involved in traumatic brain ~~injury~~ and ~~traumatic~~ spinal cord injuries ~~y~~ (19, 40). Excitotoxicity, Huntington's disease and retinal degeneration are closely associated with necroptosis. ~~Involvement of necroptosis can be validated by using~~ Exposure to a specific RIP inhibitor Nec-1 ~~, which can~~ effectively protects cells from necroptosis. Nec-1 protects hippocampal HT-22 cells ~~from~~ against glutamate-induced oxytosis (41). ~~Also, i~~ Inhibition of

RIP1 kinase or RIP3 silencing significantly rescues necroptotic cell death (42). ~~Moreover~~Furthermore, Nec-1 reduces or delays necroptotic damage in transgenic ~~mouse~~ mice ~~bearing~~ expressing mutant Huntingtin protein, astrocytes from amytrophic lateral sclerosis, and retinal pigment epithelium (42-44). Interestingly, microbial infection ~~can otherwise~~ induces necroptosis in host cells. Microbial proliferation occur organism perpetuate its progeny by circumventing host's default program mode cell death in the host. Necroptosis are is induced during by viral infections ~~-like~~ such as vaccinia virus (VV). VV expresses an inhibitor of caspase-1 and -8, diverting the host response toward necroptosis in a RIP3-dependent pathway (45). ~~Also~~Further, S~~s~~endai virus induces necroptosis in neuroblastoma cells (17). Macrophage infected with *S. typhimurium* ~~infection promotes~~ induces necroptotic cell death ~~macrophage expression to execute~~ in a RIP1- and RIP3-dependent ~~pathway~~ manner.

PERSPECTIVES OF NECROPTOSIS AND CONCLUSIONS

~~Specialized cell death n~~Necroptosis has been recognized as an alternative ~~cell death~~ to apoptosis when cells are ~~challenged~~ exposed to various stimuli ~~with~~ under~~a~~ specific conditions. Necroptosis has been ~~known to be~~considered unwanted cell death ~~and to be unleashed~~occurring under specific conditions. As mentioned above, it ~~has been causing~~induces a variety of pathological conditions including septic shock, acute pancreatitis and neuronal degeneration. Apop~~o~~totic death of ~~D~~damaged cells ~~dying of apoptosis are cleared~~is followed by phagocytosis ~~of~~ in macrophages or neighboring cells. Conversely, ~~Since m~~because~~L~~ loss of membrane integrity occurs in ~~of~~ cells undergoing necroptosis~~ loses.~~, ~~on the contrary Conversely~~, intracellular ~~danger molecules~~substances including heat-shock proteins and HMGB1 are released~~t burst~~ into extracellular media~~,~~ provok~~c~~ing inflammation and immune responses. ~~Against~~ In response to infectious viruses or intracellular bacterial pathogens ~~to~~ escaping~~e~~ apoptotic cell death, host cells actively switch ~~apoptosis~~ to necroptosis, causing premature assembly of virus particles or bacteria progeny and releas~~cing~~ of critical components ~~to~~ triggering an~~invoke the~~ immune response (46-48).

~~Likewise~~Similarly, necroptosis ~~is~~ was demonstrated ~~in~~ under conditions of chronic sterile inflammation such as alcoholic-induced liver injury and atherosclerosis (18, 49). Under these pathological ~~contexts~~conditions, necroptotic cells release DAMPS to trigger sterile inflammation, via unknown mechanism ~~of which remains to be elucidated~~.

A potent and specific ~~R~~regulation of necroptosis is therefore, needed~~, therefore, need to be achieved with potency and specificity in order to be suitable to prevention or harness.~~. In~~deed fact~~, pharmacological blockade of necroptosis is of primary concern ~~for~~ in the treatment of ~~its associated~~various diseases. Further studies will be extensively undertaken to ~~encourage~~ identify~~ication of~~ the target molecules mediating the~~, build up of~~ signal transduction~~ing pathway~~ leading to necroptosis and facilitate the discovery of mechanism-based inhibitors.

메모 포함[오전1]: Please mention a few such intracellular molecules here.

On the other hand, necroptosis induction can actively be actively harnessed through via molecular switching to or unmasking it. Generally, cancer cells can grow in an uncontrolled way manner and can further acquire evasion machinery mechanisms to default evade cell death pressure intrinsically or extrinsically. Chemotherapy or radiotherapy is mainly based on apoptosis through caspase activation. However, many cancers have developed various strategies to disarm such apoptotic machinery, including dysregulated apoptosis, activation of pro-survival signaling pathways and upregulation of drug transporters. Of these parameters, aAlteredations in apoptosis contributes to drug resistance of cancer during chemotherapy. To overcome drug-resistant cancers, alternative cell demise death mechanisms such aslike necroptosis or autophagy can be taken into consideredation. In previous reports, cancer cells that are refractory to apoptoticsis agents was were shown to succumb to necroptosis--inducing agents (50). It is conceivable that resistance to apoptosis resistance can be overcome by necroptosis, because necroptotic pathway is quite distinct from apoptotic machinerymechanisms. MoreoverFurthermore, there have been growingseveral reports suggest that necroptosis contributes to suppression of tumorigenesis. Particularly, mutations in the *CYLD* gene aggressively facilitate carcinoma through via upregulation of angiogenic factors (51). AlsoFurther,polymorphismsin*RIP3*genepolymorphismsarefoundoccurinnon-Hodgkin'slymphomaandlevelsofaspliced variant RIP3-γ is are relatively high in colon and lung cancers (52, 53).

Practically, a few inducers of necroptosis inducers have been reported to execute trigger necroptotic cell death in malignant cancers although their targets remain to be identified. By mechanism, cCaspase activation is a prerequisite to triggerfor apoptosis induction, so thatand apoptotic cell death does not properly undergofails if caspases are blocked or compromised by unknown mechanisms. Under those conditions, cells activate alternative cell death necroptosis in response to cell death stimuli. In fact, a natural product shikonin induces necroptotic cell death in MCF-7 breast cancer cells that express Bcl-2 or Bcl-xL, which can acquire multidrug

resistance. Obatoclax, an antagonist of Bcl-2 family members, ~~can~~ induced necroptosis ~~dependent~~ based on autophagy in acute lymphoblastic leukemia resistant to glucocorticoids. In addition, combined treatment of pancaspase inhibitors with 5-fluorouracil ~~can~~ drives necroptosis in colorectal cancer (54). ~~To make it better~~Furthermore, necroptotic cells ~~can~~ trigger adaptive immunity in dendritic cells (DCs), which in turn ~~can~~ activate $CD8^+$ T cells for antitumor immunity (55).

However, necroptosis-based cancer therapy still remains elusive. It has been demonstrated that necroptosis in ~~the~~ tumor microenvironment contributes to inflammation and cancer metastasis (16). Furthermore, the low expression of key modulators of necroptosis ~~modulators is too low~~ in ~~some~~ specific cancers fails to induce necroptosis (56-58), ~~indicating~~ resulting in cancer evasion~~that some cancers can evade from necroptosis~~. ~~–~~

In conclusion, suppression or enhancement of necroptosis ~~can be~~is therapeutically ~~utilized by~~effective ~~either suppressing or triggering it~~ in~~, depending on~~ specific disease~~s specific contexts~~. ~~Understanding~~ A comprehensive~~ly~~ insight into ~~its~~ the underlying mechanisms ~~will~~ is needed to ~~provide useful information for~~facilitate the diagnosis, biomarkers, and drug development ~~for treatment of~~in necroptosis-associated diseases. Most ~~of researches~~studies have focused on the identification of specific inhibitors targeting necroptosis, and ~~its~~ their underlying mechanisms of ~~inhibitors targeting necroptosis~~ regulation~~ors~~. As a result, ~~some~~ a few small molecules have been discovered from the chemical library, and ~~then~~ optimized for further clinical use.

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CONFLICTS OF INTEREST

The authors have no conflict of interests.

FIGURE LEGENDS

Figure 1:~~.~~ Necroptosis regulators and signaling pathways ~~to~~ in ~~activate~~ necroptosis activation. Upon TNF receptor ligation, a ~~series of~~ signal transduction mediated via ~~including~~ RIP1, caspase activation, and tBID cleavage ~~is ensued~~occurs ~~to mediate~~, ~~–~~resulting in apoptotic cell death. Active caspase-8 inhibits necroptosis ~~through~~ via cleavage of RIP1 and RIP3. ~~Under the situation when~~Inhibition of caspase ~~is inhibited, However,~~ leads to the formation of a physical complex of RIP1 ~~forms physical complex~~ with RIP3 to trigger ~~consecutive~~ downstream signaling events including MLKL and PGAM5 recruitment~~s~~, ~~which~~ and transduction of~~mit~~ cytosolic death signals to mitochondria, ~~leading~~ resulting~~to~~ in necroptosis.

Table 1:~~.~~ Target proteins associated with necroptosis and their specific inhibitors.

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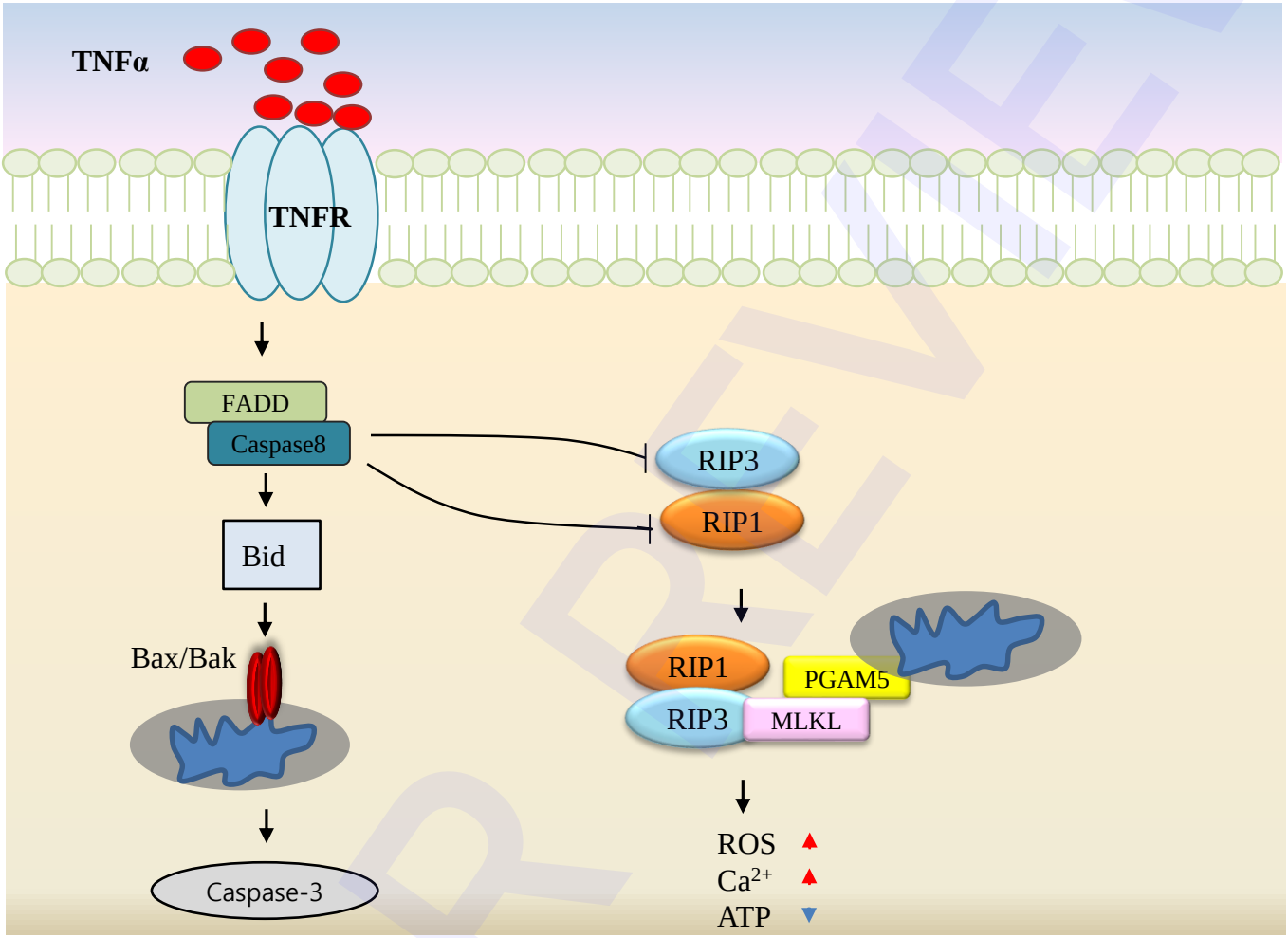
Fig. 1
Cho YS



Apoptosis
(PCD type I)

Necroptosis
(PCD type III)

Table 1. Target proteins associated with necroptosis and their specific inhibitors.

Necroptosis Target proteins	Inhibitors	Mode of action/function
RIP-1	Nec-1 Furo[2,3-d]pyrimidine derivatives GSK'963	Allosteric inhibitor SAR reported >10 000-fold selective for RIP1
RIP-3	GSK'840, GSK'843, GSK'872	Caspase activity activated
MLKL	Necrosulfonamide TC13172 GW806742X	Covalent binding Covalent binding (nanomolar potency) Binds to pseudokinase domain (ATP-mimetic)
Others	Ponatinib & pazopanib Sorafenib TPCK Hydroxyanisole Diphenyleneiodonium (DPI)	Interfere with signaling proteins upstream of MLKL Target unknown UCH-L1 activator ROS scavenger ROS suppression