INFLAMMATION

SMAC mimetics and RIPK inhibitors as therapeutics for chronic inflammatory diseases

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New therapeutic approaches for chronic inflammatory diseases such as inflammatory bowel disease, rheumatoid arthritis, and psoriasis are needed because current treatments are often suboptimal in terms of both efficacy and the risks of serious adverse events. Inhibitor of apoptosis proteins (IAPs) are E3 ubiquitin ligases that inhibit cell death pathways and are themselves inhibited by second mitochondria-derived activator of caspases (SMAC). SMAC mimetics (SMs), small-molecule antagonists of IAPs, are being evaluated as cancer therapies in clinical trials. IAPs are also crucial regulators of inflammatory pathways because they influence both the activation of inflammatory genes and the induction of cell death through the receptor-interacting serine-threonine protein kinases (RIPKs), nuclear factor κB (NF-κB)–inducing kinase, and mitogen-activated protein kinases (MAPKs). Furthermore, there is an increasing interest in specifically targeting the substrates of IAP-mediated ubiquitylation, especially RIPK1, RIPK2, and RIPK3, as druggable nodes in inflammation control. Several studies have revealed an anti-inflammatory potential of RIPK inhibitors that either block inflammatory signaling or block the form of inflammatory cell death known as necroptosis. Expanding research on innate immune signaling through pattern recognition receptors that stimulate proinflammatory NF-κB and MAPK signaling may further contribute to uncovering the complex molecular roles used by IAPs and downstream RIPKs in inflammatory signaling. This may benefit and guide the development of SMs or selective RIPK inhibitors as anti-inflammatory therapeutics for various chronic inflammatory conditions.

INTRODUCTION

Chronic inflammatory diseases like inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and psoriasis comprise a group of disorders in which deregulation of the immune systems plays a pivotal role in establishing and maintaining disease (*1*). Common to these diseases is an excessive inflammatory response, causing the production and release of inflammatory cytokines and chemokines that accelerate a vicious cycle of inflammation with the immune system unable to resolve this cascade. This abnormal inflammatory response results in tissue destruction and impaired mucosal healing of the gastrointestinal tract in IBD, tissue destruction in joints accompanied by joint pain and swelling in RA, and dermatosis in psoriasis.

The etiology of these inflammatory diseases is not yet fully elucidated, but it is hypothesized that an interplay between genetics and environmental factors exists. However, the initial trigger is as-yet unknown (*2*, *3*) but might include viral infections in some cases of RA (*4*) and trauma, infections, and certain drugs in psoriasis (*5*). Furthermore, a substantial overlap in predisposing genes exists between IBD, RA, psoriasis, and several other immune-mediated chronic inflammatory diseases (*6*), and additionally, the influence of host microbiota in disease development is well established in IBD and of growing interest in the other chronic diseases as well (*5*, *7*).

The mechanisms behind the inflammatory responses in the above-mentioned disorders are believed to be rather similar, however, affecting different locations of the body (that is, intestine, joints, and skin) depending on the specific diagnosis. In particular, an imbalance in proinflammatory T helper 17 (T_H17) cells, which produce the inflammatory cytokine interleukin-17 (IL-17), and immunosuppressive regulatory T cells (T_{reg}) is hypothesized to play a crucial role in establishing the extensive inflammation, and molecules targeting this pathway are therefore being investigated for controlling these diseases (*1*, *8*). The complexity of, and uncertainty about, the etiology of this group of chronic disorders complicates therapeutic strategies, and despite the disorders having many similarities, the pharmacokinetics and pharmacodynamics of current medications are only effective in some, but not all, of the diseases, and the response may even vary individually within the same cohort of patients, most likely due to pathogenic heterogeneity within the diseases as well. One such example is etanercept, a biologic drug that binds to the inflammatory cytokine tumor necrosis factor–α (TNF-α), which reaches the joints but not the intestine (*9*, *10*). However, the mechanism explaining the efficacy of etanercept for treating RA but not for treating IBD is still unknown. Nevertheless, classes of drugs based on mutual mechanisms are used to treat these disorders. Common strategies include the administration of anti-inflammatory agents, such as glucocorticoids, immunomodulators (for example, thiopurines and methotrexate in IBD and RA, respectively), biologics (such as TNF-α inhibitors, anti-integrins, and anti–IL-12/23p40 drugs), and recently marketed small molecules (like jakinibs in IBD, RA, and psoriasis, and sphingosine-1-phosphate receptor modulators in IBD) (*11*–*18*). Still, an unmet need for new therapeutic options exists because many patients are unresponsive to available therapies, become intolerant to therapy over time, or experience severe adverse effects necessitating the discontinuation of therapy.

Inhibitor of apoptosis proteins (IAPs) are E3 ubiquitin ligases that are well known for their ability to directly or indirectly inhibit cell death pathways, and antagonists of these proteins, known as second mitochondria-derived activator of caspases (SMAC, also known as Diablo) mimetics (SMs), are being evaluated in clinical trials for their potential to treat cancers (*19*). In addition to direct interference with cell death pathways, through direct or indirect inhibition of apoptosis-inducing proteases (known as caspases), IAPs additionally

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promote survival through the activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways (*20*, *21*). Apart from affecting cell survival, activation of NF-κB and MAPK leads to the production of inflammatory mediators (*22*). Furthermore, IAPs are key regulators of distinct innate responses, wherein their ubiquitylation activity—especially toward receptor-interacting serine-threonine protein kinases (RIPKs)—plays a crucial role in downstream signaling and in the suppression of RIPK1-mediated cell death by the ripoptosome and necrosome (*20*, *23*, *24*). In addition, the expression and abundance of IAPs have been demonstrated to be increased under chronic inflammatory conditions, including IBD and psoriasis (*25*, *26*). This increase is mainly due to an increased number of infiltrating leukocytes producing large amounts of IAPs but is, in IBD, also caused by an increased expression and abundance of cIAP2 in regenerating intestinal epithelia (*27*, *28*). It is therefore plausible that the inhibition of IAPs or their mediators has the potential to alleviate the excessive immune response observed in various inflammatory disorders, marking IAPs and their downstream effectors, the RIPKs, as potential therapeutic targets for diseases like IBD, RA, and psoriasis (*29*, *30*). Small molecules that inhibit IAPs directly or act as indirect inhibitors of IAPs by blocking death-inducing RIPKs, such as SMs or selective RIPK inhibitors, respectively, may show promise as anti-inflammatory agents (*29*–*31*). However, because apoptosis induction and autocrine cytokine stimulation are known to occur in neoplastic cells upon SM-induced IAP inhibition using high dosages of SMs (*32*–*34*), similar effects in non-neoplastic cells could be a concern for the use of SMs in non-neoplastic diseases.

The aim of this review is to provide an overview of the potential anti-inflammatory functions of SMs and RIPK inhibitors of relevance to the pathogenesis of inflammatory diseases. In addition, clinical data are evaluated, and the potential of SMs and RIPK inhibitors as a new therapeutic strategy in inflammatory diseases is presented. RIPK inhibitors may allow for a more specific blockade of inflammatory events as compared to SMs, which also activate other inflammatory pathways mediated by NF-κB–inducing kinase (NIK) and the non-canonical NF-κB members.

THE ROLE OF IAPs AND RIPKs IN INFLAMMATORY SIGNALING

The production of inflammatory cytokines involves many pathways, including signaling through the TNF receptor (TNFR) superfamily and innate immune signaling through pattern recognition receptors (PRRs) (*35*), and IAPs and RIPKs are important in most of these signaling pathways (*23*, *36*). The roles of IAPs and RIPKs are many and varied and depend on several factors, including the cell type, the signaling pathway, and the specific type of IAP or RIPK in question. Depending on the stimulus and context, IAPs can ubiquitylate RIPK1 or RIPK2, leading to inflammatory signaling by NF-κB and MAPKs, suppress the formation of the RIPK3 necroptosis-inducing complex, or suppress the inflammatory pathways triggered by RIPK1-RIPK3 complex activation.

TLR signaling

The immediate response to an immune challenge is raised by the innate immune system. PRRs detect molecules that are typical of pathogens and cellular damage—pathogen-associated molecular patterns and damage-associated molecular patterns (DAMPs), respectively—and activate the NF-κB and MAPK pathways in response. Such activity initiates the production of proinflammatory cytokines (*37*). IAPs are known to function as positive regulators of the downstream signaling of PRRs, including the cell surface receptors Toll-like receptor 2 (TLR2) and TLR4 (*38*, *39*), and the cytosolic receptors nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and NOD2 (*40*–*42*). Innate signaling through these receptors is believed to play a key role in the immunopathogenesis of IBD (*43*). IAPs have similar proinflammatory effects in RA (*44*) and psoriasis (*25*, *45*). In addition, IAP regulation of retinoic acid–inducible gene I (RIG-I) and nucleotide-binding oligomerization domain leucine-rich repeat and pyrin domain-containing proteins (NLRPs) has been suggested, but data are rather contradictory, and their roles have not yet been fully elucidated (*23*).

TLR4 stimulation results in the recruitment of myeloid differentiation primary response protein 88 (MYD88) or Toll/IL-1 receptor (TIR) domain–containing adaptor-inducing interferon-β (TRIF) to the receptor depending on the receptor stimuli (Fig. 1) (*23*). These two pathways lead to either activation of canonical NF-κB and MAPK pathways with subsequent production of the proinflammatory cytokines IL-6, TNF-α, and IL-12 or to RIPK3-dependent necroptosis. In addition, depending on the stimulus, the TRIF-dependent pathway leads to activation of interferon (IFN) regulatory factors with subsequent production of type 1 IFNs (*23*), a pathway, however, not included in the present review. Notably, only the MYD88-dependent response is regulated by cellular IAPs (cIAPs), because administration of an SM has been shown to decrease the induction of genes encoding proinflammatory cytokines, including *TNFA*, *IL6*, and *IL12*, without any alteration in expression of genes encoding IFNs or IFN-related genes in mice (*38*). The production of IL-6, TNF-α, and IL-12 by immune cells is pivotal for the development and maintenance of inflammation in IBD (*11*, *12*), and disruption of this signaling may therefore be useful in IBD therapy. Furthermore, a study implied that cIAPs only had a regulatory role in MAPK activation but not in the activation of NF-κB (*38*). Upon recruitment of MYD88 to the plasma membrane receptor, TNFR-associated factors (TRAFs) accompany and further facilitate the recruitment of a complex consisting of TAK1-binding protein 2 (TAB2) and TAB3 (TAB2/3) and transforming growth factor–β (TGF-β)–activated kinase 1 (TAK1), which is important for MAPK activation, and the IκB kinase (IKK) complex consisting of IKKα, IKKβ, and IKKγ (also called NEMO), which is important for NF-κB activation (Fig. 1) (*23*). However, activation of TAK1, a prerequisite for MAPK activation, is prevented as long as the entire complex is membrane associated (*38*). Thus, MAPK activation depends on translocation of the membrane complex to the cytoplasm, whereas NF-κB activation does not, because phosphorylation of the IKK complex, a prerequisite for NF-κB activation, does not require TAK1 to be activated by phosphorylation (Fig. 1) (*46*). This translocation is enabled by degradative Lys^{48} (K48)–linked ubiquitylation of the adaptor or bridging factor TRAF3 by cIAPs (*38*). In addition, it has been demonstrated that the bivalent SM birinapant reduces cIAP1 abundance in lipopolysaccharide (LPS)–induced liver injury in mice and reduces the production of TNF-α and IL-1, indicating that SMs are anti-inflammatory in primary immune cells (*47*). Alternatively, SMs may promote TLR4-mediated death of macrophages, thus reducing the production of TNF-α and IL-1, as observed in cIAP2-knockout mice (*39*). For NF-κB activation upon TLR4 stimulation, the ubiquitylating protein of importance is believed to be TRAF6 and not cIAPs, thus emphasizing the role of SMs in affecting only the MAPK pathway in

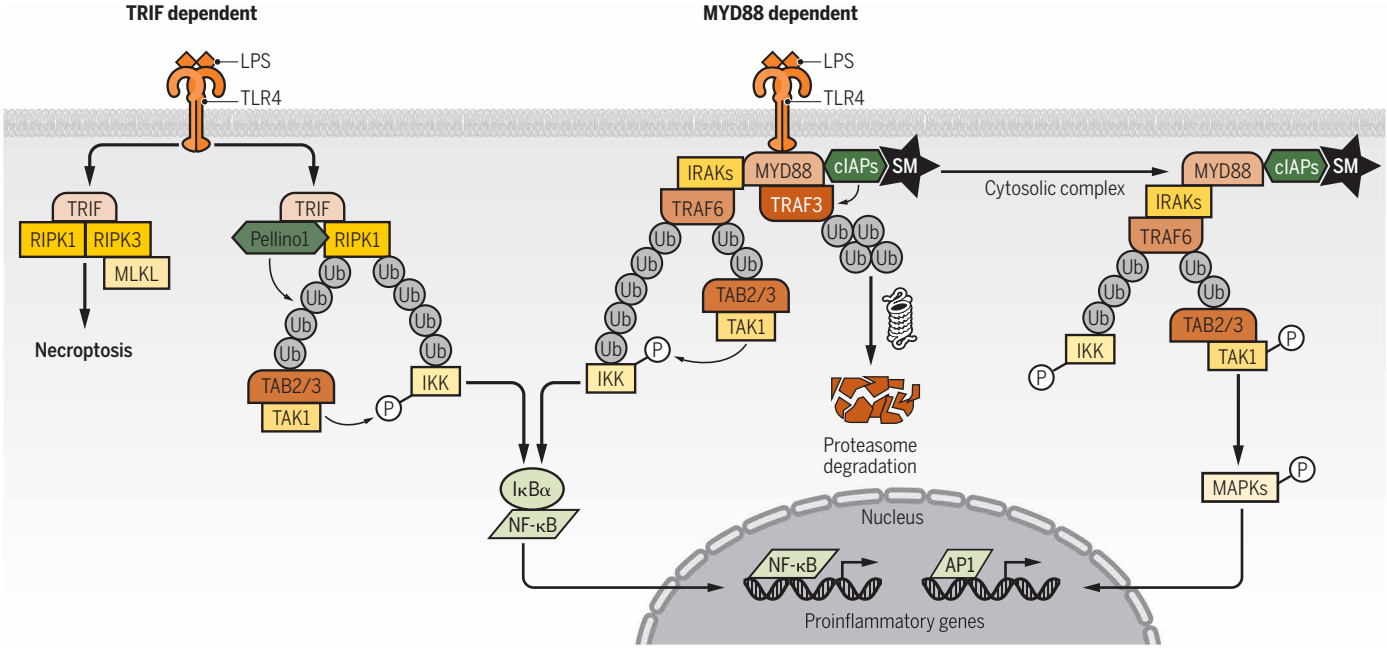

Fig. 1. TLR4-dependent inflammatory signaling. Activation of the PRR TLR4 by LPS initiates downstream signaling through both TRIF- and MYD88-dependent pathways. The adaptor TRIF engages the kinase RIPK1 and the ubiquitin (Ub) ligase Pellino1, and ubiquitylation of RIPK1 by Pellino1 then enables recruitment of the kinase TAK1 and the TAK1-binding proteins TAB2/3 and the IκB kinase (IKK) complex. Consequently, TAK1 phosphorylates IKKβ of the IKK complex, leading to degradation of IκBα and allowing translocation of NF-κB into the nucleus. TRIF may also recruit the necrosome components RIPK1, RIPK3, and MLKL, leading to necroptosis. In MYD88-dependent TLR4 signaling, IRAKs, TRAF3, TRAF6, and cIAPs are recruited to the membrane. Autoubiquitylation of TRAF6 causes it to engage the TAB2/3-TAK1 and IKK complexes, leading to NF-κB activation. Activation of MAPKs and the downstream transcription factor AP1 requires translocation of the membrane complex into the cytosol, an event facilitated by cIAPs through K48-linked ubiquitylation of TRAF3. Administration of SMAC mimetics (SMs) induces the loss of cIAPs, thus disabling the cytosolic translocation of the signalosome and thereby interrupting MAPK activation.

TLR-mediated signaling (Fig. 1), at least in murine macrophages (*38*). Nevertheless, the mechanisms may be different in human macrophages versus murine macrophages because SMs have been shown to inhibit the phosphorylation of crucial MAPKs, as well as implicated in NF-κB signaling, in human macrophages (*30*). This study further demonstrated an inhibition of canonical NF-κB signaling and activation of noncanonical NF-κB signaling, with only the former affecting LPS-induced IL-27 production but not TNF-α production (*30*).

In contrast to the MYD88-dependent response, which does not require RIPKs, the role of RIPK1 is pivotal in the TRIF-dependent response to TLR4 stimulation (Fig. 1). Upon recruitment of TRIF to the membrane, RIPK1 serves as a crucial signaling platform for TAB2/3 and IKK and is needed to activate NF-κB (*48*, *49*), just as TRAF6 acts as this important signalosome component in the MYD88-dependent pathway. The signaling cascade downstream of RIPK1 is controlled by the ubiquitin ligase Pellino1, which provides the necessary Lys^{63} (K63)–linked polyubiquitin chains on RIPK1 (*50*), in contrast to other signaling pathways discussed in this review wherein ubiquitylation of RIPK1 and RIPK2 involves IAPs. In addition, TRIF signaling can lead to necroptosis through the necrosome consisting of RIPK1, RIPK3, and mixed lineage kinase domain–like pseudokinase (MLKL) when caspase-8 is deficient or inactivated in murine bone marrow macrophages (*51*). Adding to the complexity of the IAP-RIPK axis, IAPs are also negative regulators of necroptosis, because the X-linked IAP (XIAP) inhibits LPS-induced necroptosis (*52*).

NOD signaling

The NODs are intracellular receptors that sense bacterial peptidoglycan constituents. The signaling pathway that is induced upon NOD stimulation is rather similar to that induced by the TNFR1, which is described in detail in the next section, except that RIPK2 is the important scaffolding protein ubiquitylated with K63-linked chains by IAPs downstream of NODs instead of RIPK1 (Fig. 2) (*23*). Protein-protein interactions and ubiquitylation patterns in NOD signaling are widely debated, as are the differences between this pathway, which does not induce cell death, and most other proinflammatory signaling pathways, which do (*53*).

The recruitment of IAPs to RIPK2 downstream of NOD activation has been suggested to involve TRAF proteins, as is the case for the TNFR1 pathway as well (see below), although the exact role of TRAFs in the NOD pathway is somewhat controversial (*40*, *41*, *54*). Whereas some studies using pan-IAP–inhibiting SMs suggest that both cIAPs and XIAP are responsible for the crucial RIPK2 ubiquitylation (*40*, *41*, *55*), other investigations using selective XIAP inhibitors have revealed XIAP to be mainly responsible for the downstream MAPK and NF-κB activation and proinflammatory signaling upon NOD stimulation in murine bone marrow–derived macrophages and human peripheral blood mononuclear cells (PBMCs) (*31*, *42*). More specifically, the XIAP-mediated K63-branched ubiquitin chain on RIPK2 enables downstream signaling through the recruitment of linear ubiquitin chain assembly complex (LUBAC), an E3 ubiquitin ligase complex facilitating Met1 (M1)–linked ubiquitin chains, needed for efficient recruitment and activation of downstream kinases, IKK and TAK1

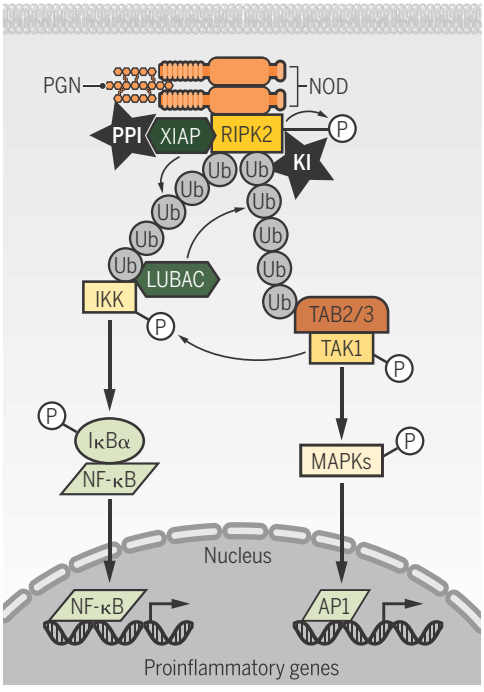

Fig. 2. NOD-dependent inflammatory signaling. After peptidoglycan (PGN) stimulation of NOD, RIPK2 is recruited to the receptor. Subsequently, XIAP is initially engaged and mediates K63-linked ubiquitylation (Ub) of RIPK2. This allows binding of linear ubiquitin chain assembly complex (LUBAC), which further ubiquitylates RIPK2 and other associated proteins, to recruit and activate the kinases IKK and TAK1 (*41*). These events culminate in both degradation of IκBα and hereby nuclear translocation of NF-κB and activation of MAPKs, leading to expression of AP1-responsive genes. Administration of XIAP-selective inhibitors [indicated by protein-protein interaction (PPI)] blocks binding of XIAP to RIPK2 disrupting downstream signaling. In addition, administration of RIPK2 inhibitors [indicated by kinase inhibitor (KI)] blocks the ability of RIPK2 to autophosphorylate and stabilize itself as well as blocking the binding of XIAP and hereby disabling important downstream ubiquitylations.

that activate NF-κB and MAPKs, respectively (*41*). Furthermore, another study uncovered that a cIAP1-dependent interplay exists between NOD2 and TNFR1 signaling (*42*). It has been proposed that NOD2 activation itself results in a low amount of proinflammatory cytokine production and that the TNF-α that is subsequently produced stimulates TNFR1 to amplify cytokine production in vivo (*42*). In this context, it is hypothesized that the TNFR1 pathway does not substitute for NOD2 signaling but instead helps to maintain NOD2 signaling (*42*), a theory supported by the fact that TNF-α stimulates NOD2 and RIPK2 at both the transcriptional and protein levels (*56*). Thus, it is conceivable that SM administration has the potential to affect NOD signaling in an indirect manner through an amplification loop, whereas selective IAP inhibitors, or the RIPK2 inhibitors (discussed later), may have an even greater anti-inflammatory effect on innate pathways.

Evidence for the key role of XIAP in NOD2 signaling also comes from the fact that there are more than 100 reported XIAP-deficient individuals with mutations in *BIRC4*, the gene that encodes XIAP, who demonstrate the primary immunodeficiency called X-linked lymphoproliferative disease type 2 (XLP-2), and of these, about 20% exhibits an early-onset aggressive and fatal form of IBD that is similar to Crohn's disease (CD) and are unresponsive to conventional medication (*57*, *58*). The lymphocytes, Paneth cells, and myeloid cells from XIAP-deficient individuals are severely compromised in NOD2 signaling (*59*). Reconstitution of those XLP-2 IBD patients with a well-functioning immune system from an allogeneic hematopoietic stem cell graft restores gut homeostasis and allows for the complete resolution of symptoms (*58*).

TNF superfamily receptor signaling

NF-κB is an important transcription factor in inflammatory signaling (*60*), and evidence suggests that activation of this protein is increased in IBD, causing a rise in the production of inflammatory cytokines, including IFN-γ and TNF-α (*61*, *62*). TNF-α is a particularly well-known mediator of the detrimental effects associated with chronic inflammatory disorders such as IBD, RA, and psoriasis (*63*, *64*).

NF-κB signaling is divided into canonical and noncanonical pathways (Fig. 3), both of which activate cells and stimulate proinflammatory signaling, and these pathways are further closely connected to the apoptotic and necroptotic cell death pathways that can be triggered by TNF-α (Fig. 4). XIAP, cIAP1, and cIAP2 regulate this complex network of cellular processes and are essential for the balance between cell survival and cell death (*65*). XIAP suppresses TNF-α–induced cell death by acting as a direct caspase inhibitor at the distal nexus of caspase-3 or caspase-7 activation after caspase-8 activation. cIAP1 and cIAP2 are potent suppressors of TNF-α–induced apoptosis or necroptosis because they direct TNF-α signals toward NF-κB activation and suppress the formation of the RIPK1-dependent cell death complexes, the ripoptosome and the necrosome (*24*). RIPKs act as important signaling platforms through their ubiquitylation decorations and are considered as master controllers of inflammation due to their pivotal role in inflammatory signaling (*66*). However, IAPs can be considered as masters of master controllers because they act upstream of RIPKs, orchestrating the crucial initial ubiquitylation signals that then recruit additional ubiquitin ligases, kinases, and other ubiquitin-binding factors.

Canonical NF-κB signaling is triggered by binding of TNF-α to TNFR1, resulting in the formation of a receptor signaling complex (complex I) composed of TNFR1-associated death domain protein (TRADD), TRAF2, RIPK1, and cIAPs (Fig. 3) (*67*, *68*). cIAPs are indispensable for downstream signaling because their ubiquitin ligase activity facilitates the modification of RIPK1, allowing for the engagement of LUBAC, the TAB2/3-TAK1 complex, and IKK (*21*, *69*). The nondegradative K63-branched ubiquitylation of RIPK1 by cIAPs forms the scaffolding platform (or RIPK1 signalosome) for these essential signaling events that ultimately lead to the activation of NF-κB (*20*). The RIPK1 signalosome, stabilized by M1-linked ubiquitin chains catalyzed by LUBAC (*70*), culminates in the phosphorylation and subsequent degradation of NF-κB inhibitor α (IκBα), thus enabling translocation of NF-κB subunits into the nucleus and subsequent activation of prosurvival and proinflammatory genes (*68*). By contrast, if the cIAPs are absent or lost during complex I formation and RIPK1 is not ubiquitylated or is subsequently deubiquitylated through the action of proteins such as the ubiquitin carboxyl-terminal hydrolase CYLD or the A20 ubiquitin-editing complex, then the assembly of complex II (the death complex defined as the ripoptosome) and/or assembly of the necrosome will proceed (*71*). This culminates in apoptosis or necroptosis depending on the presence of caspase-8 or RIPK3, respectively (Fig. 4), an effect that is prevalent in cancer cells (*72*). A similar TNF-α–induced death effect has been shown in some primary cells, such as murine macrophages, wherein administration of high-dose SM induces RIPK3-dependent cell death, but this

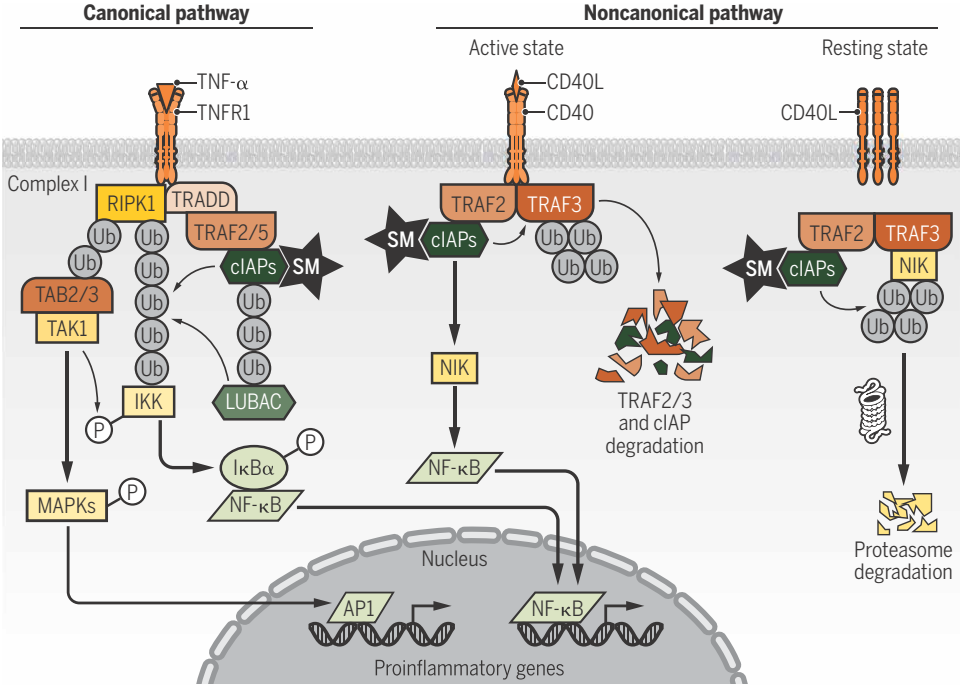

Fig. 3. Canonical and noncanonical NF-κB signaling downstream of TNF superfamily receptors. Activation of TNFR1 by TNF-α induces the assembly of complex I, which includes TRADD, TRAF2 or TRAF5, RIPK1, and cIAPs. K63-linked autoubiquitylation (Ub) of cIAP facilitates the recruitment of LUBAC. cIAP-mediated K63-linked ubiquitylation of RIPK1 recruits the TAB2/3-TAK1 complex and the IKK complex. IKK complex recruitment is further facilitated through LUBAC-mediated, M1-linked ubiquitylation of RIPK1. Subsequently, TAK1 activates both canonical NF-κB and MAPK signaling pathways by phosphorylating IKKβ of the IKK complex and MAPKs, respectively. Other receptors for TNF superfamily ligands, such as the death receptor CD40, induce noncanonical NF-κB signaling. In the resting state, the noncanonical pathway is suppressed by the cytosolic cIAP-TRAF2-TRAF3 complex through cIAP-mediated ubiquitylation and subsequent degradation of NIK. Upon activation of CD40 by CD40L (also called CD154), the cIAP-TRAF2-TRAF3 complex is recruited to the membrane, where cIAP ubiquitylates TRAF3, leading to its degradation, as well as degradation of TRAF2 and cIAPs. Consequently, NIK accumulates and activates NF-κB. Administration of SMs inhibits canonical signaling, because recruitment of essential kinases is disabled. For the noncanonical pathway, administration of SM is predicted to inhibit NF-κB activation in the presence of ligand, because the cIAP-TRAF2-TRAF3 complex cannot be marked for degradation by Ub chains. Oppositely, SM administration is predicted to promote NF-κB activation in the absence of ligand because cIAPs can no longer ubiquitylate NIK for degradation.

effect seems to be highly cell type dependent and is not found in many other primary cells, including T cells (*73*). TNF-α stimulation of TNFR1 can be inhibited by SM administration, leading to either decreased production of proinflammatory cytokines by the canonical pathway, the induction of cell death (by shunting RIPK1 toward those processes), or both, depending on the cell type.

Immune cells also have another TNFR, TNFR2. TNFR2 activation by TNF-α induces canonical NF-κB signaling through some of the same proteins present in TNFR complex I, including TRAFs, cIAPs, and HOIL-1–interacting protein, the catalytic component of LUBAC (*74*). However, the consequential activation of NF-κB differs from that induced by TNFR1, because this pathway, in contrast to the proinflammatory response of TNFR1 signaling, primarily drives immune modulation and tissue regeneration because of the restricted tissue distribution pattern of TNFR2 and possibly due to indirect activation of the noncanonical NF-κB pathway (see below) (*75*). Whereas TNFR1 is present on almost every cell in the human body, TNFR2 is limited to a subset of immune cells, and it is predominantly observed on immunosuppressive T_{reg} cells (*75*). NF-κB activation increases the stability, expansion, and function of T_{reg} cells and inhibits the differentiation of inflammatory T_H17 cells by stimulating *IL2* expression in vivo (*75*, *76*). In addition, polymorphisms in the gene encoding TNFR2 correlate with RA (*77*, *78*) and IBD (*79*, *80*), supporting a role for TNFR2 in balancing the proinflammatory responses that are necessary to counteract infections but must be resolved to avoid chronic inflammatory conditions after the infection has been cleared. Last, unlike TNFR1, TNFR2 does not have a cytoplasmic death domain and is therefore not capable of directly mediating TNF-α–induced cell death. However, cross-talk between the two receptors is evident. TNFR2 is present in much higher amounts (sometimes as much as 100-fold more) than TNFR1 on immune cells, and TNFR2 binds to membrane-bound TNF-α presented by neighboring cells with higher affinity than does TNFR1 (*81*). The strong activation of TNFR2 by TNF-α causes depletion of TRAF2-cIAP1/2 complexes (*81*, *82*). In immune cells, this results in the stabilization of NIK and the activation of noncanonical NF-κB signaling (see below), leading to costimulatory survival and proliferative signals (*81*). Furthermore, the depletion of TRAF2 and cIAP1/2 blocks TNFR1 signalosome formation and can potentially favor ripoptosome or necrosome formation upon high-level or chronic TNF-α stimulation, leading to immune cell death. One study demonstrated TNFR2 activation to sensitize murine macrophages to TNFR1-induced necroptosis through autocrine TNF-α production, because the inhibitory effect of cIAPs on cell death pathways is no longer present, due to the abovementioned depletion of TRAF2-cIAP1/2 complexes by TNFR2 activation (*82*). Moreover, another study demonstrated TNFR1-TNFR2 cross-talk in murine macrophages to involve inflammasome activation, with TNFR2 stimulation acting as an inflammasome priming signal and subsequent TNFR1 activation serving as an activation signal, at least when XIAP is absent (*83*). The resulting inflammatory cytokine production and inflammasome-mediated cell death depended on the kinase activity of RIPK1 and reactive oxygen species (*83*), highlighting RIPK1 as an essential mediator of inflammatory and cell death signaling.

Noncanonical NF-κB signaling is activated mainly through receptors for TNF superfamily members, such as CD40, CD30, 4-1BB, OX40, glucocorticoid-induced TNFR (GITR), fibroblast growth factor–inducible 14, and B cell–activating factor receptor (BAFF-R) (*84*), and indirectly through TNFR2. cIAPs suppress noncanonical NF-κB signaling by constitutively catalyzing K48-branched ubiquitylation of the key signaling enzyme NIK, which leads to proteasomal degradation of NIK, as shown in cancer cell lines, human embryonic kidney 293T cells, and immune cells (Fig. 3) (*33*, *34*, *85*). Nevertheless,

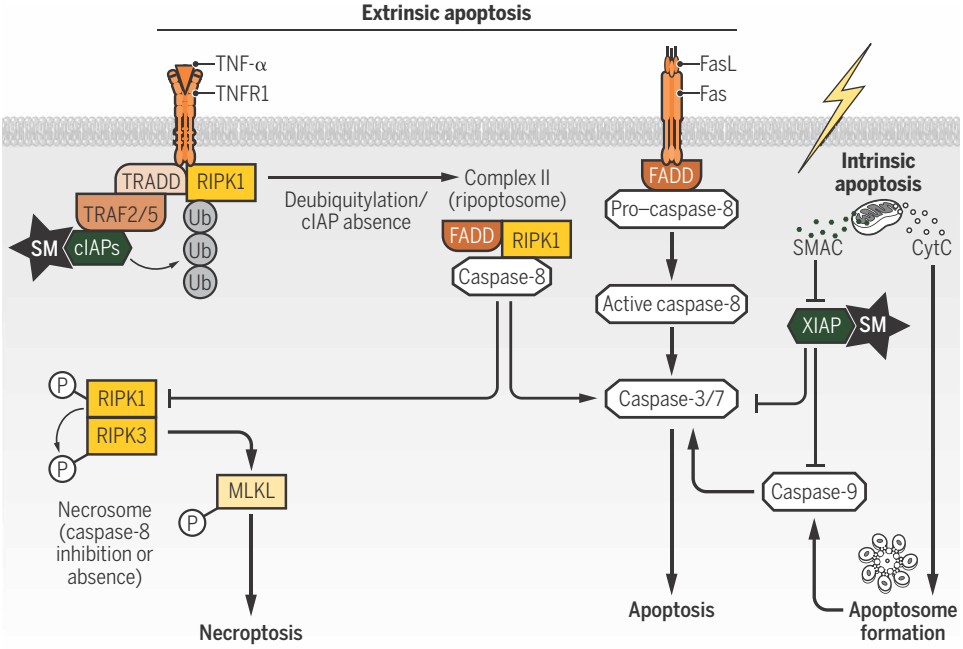

Fig. 4. Cell death pathways. Activation of TNFR1 by TNF-α stimulates canonical NF-κB signaling in a manner that depends on the assembly of complex I (TRADD, TRAF2 or TRAF5, RIPK1, and cIAPs) and on cIAP-mediated ubiquitylation (Ub) of RIPK1 (Fig. 3). If cIAPs are absent or inhibited by an SM or if RIPK1 is deubiquitylated, then stimulation of TNFR1 leads to the formation of complex II (also called the ripoptosome or death complex), resulting in cell death. This complex, comprising FADD, RIPK1, and caspase-8, can stimulate necroptosis or apoptosis. It promotes necroptosis by stimulating the formation of the necrosome (RIPK1, RIPK3, and MLKL), leading to phosphorylation and oligomerization of MLKL and disruption of the plasma membrane. Complex II promotes apoptosis by promoting the activation of caspases. Activation of death receptors, such as Fas, stimulates the recruitment of FADD and pro–caspase-8, which leads to cleavage and activation of caspase-8, thus enabling activation of the effector caspases (caspase-3 and caspase-7) that execute apoptosis. Cytokine death ligand pathways, like those induced by TNF-α or FasL, are known as extrinsic apoptosis. Intrinsic apoptosis, which is triggered by nonreceptor-mediated stimuli, such as DNA damage, involves mitochondrial release of cytochrome C (CytC) and SMAC. CytC is crucial for the formation of the apoptosome, which activates caspase-9, culminating in mitochondrial-mediated apoptosis by caspase-3 and caspase-7. XIAP inhibits caspase-3, caspase-7, and caspase-9 to prevent intrinsic apoptosis; inhibition of XIAP by endogenous SMAC or exogenous SM promotes apoptosis.

when this pathway is stimulated by ligand binding, recruitment of the cIAP-TRAF2-TRAF3 complex to the receptor triggers degradation of TRAF3, which is needed to engage NIK in this inhibition complex, as well as degradation of TRAF2 and cIAPs (Fig. 3) (*84*, *85*). These events prevent degradative ubiquitylation of NIK, which accumulates and activates the noncanonical NF-κB pathway. Notably, cIAPs have opposite effects on the two NF-κB signaling pathways because they are positive regulators of canonical signaling and negative regulators of noncanonical signaling. The noncanonical NF-κB pathway is important in immunity because it provides survival and costimulatory signals triggered by TNF superfamily receptors on B cells (for instance, BAFF), T cells (for instance, CD30, 4-1BB, OX40, and GITR), and dendritic cells (for instance, CD40) (*86*, *87*). In addition, noncanonical constitutive activation seems to be of particular importance in several immune system neoplasms of B cell origin, such as chronic lymphocytic leukemia and multiple myeloma, wherein deletions of TRAF3 or cIAPs, as well as NIK mutations, lead to constitutive NIK and NF-κB activity (*88*). The effect of IAP inhibition on NF-κB signaling therefore depends on the relative importance of these two key NF-κB pathways in a given cell type and the cytokine environment, which may explain, in part, the distinct responses of different cells to SMs, especially those differing between neoplastic and normal primary cells (*32*, *47*, *89*, *90*). However, other factors and mechanisms that still remain to be fully explained are also likely at play. For example, the transcription factor specificity protein 3 (SP3) has been identified as a mediator of SM-mediated induction of TNF-α, further providing some evidence for the abundance of SP3 (and the possible functional regulation of SP3 by protein inhibitor of activated signal transducers and activators of transcription 1) accounting for the differential sensitivity of cancer cells versus normal cells to SMs (*91*). Other explanations are also likely, but it is clear that cancer cells demonstrate a profound sensitivity to death induction by SMs [when TNF-α or TNF-related apoptosis-inducing ligand (TRAIL) is present] as compared to primary cells.

It is evident that IAPs constitute a group of functionally complex proteins because they exhibit multiple distinct functions, for instance, the ability to directly inhibit caspases, as seen for XIAP, or to act as E3 ubiquitin ligases tagging proteins either for degradation or signaling, as seen for cIAP1, cIAP2, and XIAP. cIAPs are not solely positive but are also negative regulators of NF-κB activation; their ubiquitylating activity can be degradative (for example, K48-linked ubiquitin of NIK) or function as signaling platforms (like K63-linked ubiquitin for RIPK1 or RIPK2). Some of the functions of the IAPs are redundant, or partially so, whereas some IAPs have unique functions. For example, survivin (encoded by *BIRC5*) plays a role in segregating chromosomes during cell division (*92*), and neuronal apoptosis inhibitory protein (encoded by *BIRC1*) acts in triggering caspase-1 activation and IL-1 production in response to *Legionella* or *Salmonella* infection (*93*, *94*).

USING SMs TO BLOCK INFLAMMATION

Mode of action of SMs

The role of cIAPs in the balance of cell turnover is manifold because they mediate ubiquitylation of many pivotal proteins that favor cell survival versus cell death—for example, by suppressing the formation of death complexes. However, as briefly touched upon above, XIAP is a potent inhibitor of apoptosis because it binds to and directly inactivates the central effector caspases of both the extrinsic and intrinsic apoptotic pathways, caspase-3 and caspase-7 (Fig. 4) (*95*). Furthermore, XIAP directly inhibits caspase-9, the initiator caspase of the intrinsic pathway activated by apoptosome formation, which is triggered by cytochrome c release from mitochondria (*96*). Mammalian cells retain strategies to counteract XIAP-mediated inhibition of caspases, including mitochondrial release of the proapoptotic

IAP antagonist SMAC (*97*). In addition to interfering with XIAP-mediated inhibition of caspases, SMAC also inhibits the anti-apoptotic functions of cIAPs (*98*). The discovery of SMAC has paved the way for the development of pharmacologic SMs, with the goal of inducing apoptosis in cancer cells. These small-molecule antagonists mimic the N-terminal four residues (Ala-Val-Pro-Ile) of endogenous mitochondrially processed SMAC that mediate binding to IAPs (*99*, *100*). Specifically, binding of SMs to XIAP sterically interferes with the binding of caspase-3 and caspase-7 to XIAP (*101*), and SMs compete with caspase-9 for binding to XIAP because they share the same binding site on XIAP (*102*). Both mechanisms relieve caspase inhibition, enabling them to direct apoptotic pathways.

In the case of cIAPs, binding of SMs causes allosteric changes that activate the E3 ubiquitin ligase activity provided by the really interesting new gene (RING) C-terminal domain of the cIAPs (*103*). A defining hallmark of the IAP family is the presence of one or more baculovirus IAP repeat (BIR) domains, which both mediate protein-protein interactions and mediate intramolecular binding to the RING domain to maintain the inactive state (Fig. 5A). However, SMs interrupt this autoinhibition and enable RING-mediated dimerization, which is necessary for ubiquitylation activity because it is required for binding to the E2 that carries ubiquitin, and therefore for the formation of the active state (Fig. 5A) (*103*, *104*).

Upon SM binding, autoubiquitylation of cIAPs leads to degradation of cIAPs, as demonstrated in cancer cells (*33*, *34*), and, as a result, accumulation of NIK initiates noncanonical NF-κB signaling. The TNF-α that is subsequently produced can then feed forward and stimulate TNFR1 in an autocrine or paracrine manner (*33*). In this way, the drug-induced ubiquitylation activity of cIAPs can lead to a transient burst of RIPK1 ubiquitylation, and consequently, brief canonical NF-κB activation as demonstrated in cancer cells (*90*). Ultimately, the canonical NF-κB pathway is shut down as RIPK1 is deubiquitylated, and RIPK1 can then form death complexes (either the ripoptosome or the necrosome depending on the presence of caspase-8; Fig. 4) in response to TNF-α binding to TNFR1. The resulting ripoptosome formation, consisting of Fas-associated protein with death domain (FADD), RIPK1, and caspase-8, activates caspase-8 and promotes apoptosis (Fig. 4) (*32*, *33*). For necroptosis to occur, caspase-8 activity must be absent, leading to triggering of phosphorylation events by RIPK1 and RIPK3 in the necrosome, ultimately resulting in the activation and oligomerization of the pseudokinase MLKL, which induces necroptosis by disrupting the plasma membrane (*68*). In contrast to these responses described in cancer cells, SM treatment alone does not activate canonical or non-canonical NF-κB signaling in primary human cells—except for some immune cells (*85*)—unless that cells are also challenged with LPS, in which case the canonical pathway is inhibited and the noncanonical pathway is activated (*29*, *30*, *38*). Furthermore, Liu and colleagues (*105*) reported a signaling axis between NIK and the sine oculis homeobox (SIX) proteins, SIX1 and SIX2, wherein activation of the noncanonical pathway—and thereby NIK—by the preclinical SM BV6, induces the accumulation of SIX1 and SIX2 in fibroblasts. The SIX proteins then provide negative feedback on noncanonical NF-κB signaling by reducing the expression of various inflammatory genes (*105*). This indirect effect of BV6 administration on SIX proteins further emphasizes the anti-inflammatory potential of SMs in IBD therapy and in RA and psoriasis therapy. This study used a high micromolar SM concentration, and it would therefore be interesting to evaluate whether the same mechanism is observed at lower nanomolar concentrations, because high doses of SM for possible therapeutic strategies should be avoided due to side effects, as discussed below.

SMs are considered "pan-selective" IAP inhibitors, referring to their ability to antagonize several, but not all IAP proteins: They not only target the redundant or fungible cIAPs, cIAP1 and cIAP2, at nanomolar doses, which accounts for their main pharmacological actions, but also target XIAP at higher doses. Nevertheless, SMs differ in their pharmacologic properties, such as binding affinities and potency in inhibiting these key inflammatory signaling proteins (*19*). At present, both monomeric and dimeric compounds have been developed, the former representing one SMAC-mimicking Ala-Val-Pro-Ile–type element [these drugs typically can be administered orally, although one (GDC-0152) cannot], and the latter containing two similar SMAC-mimicking moieties joined by a chemical linker, which is always administered intravenously because of decreased oral bioavailability (although intraperitoneal administration routes are possible in animals) (*106*). Preclinically, dimeric compounds have been shown to be more potent (up to two to three logs in some cases) in inducing caspase activation and cIAP degradation than their monomeric counterparts (*19*, *103*). Dimeric compounds also have the ability to bind not just one but two BIR domains, and intermolecular bridging between BIR domains may result in a greater stimulation of cIAP autoubiquitylation because this should stabilize RING dimerization (Fig. 5A) (*107*). Moreover, for dimeric SMs, concurrent blocking of two BIR domains in XIAP through intramolecular bridging allows for simultaneous activation of caspase-3 and caspase-7 through BIR2 and caspase-9 through BIR3 (Fig. 5B). However, whether this increased potency of dimeric compounds extends to clinical settings has not been established.

Lessons from SMs in clinical trials

Since the discovery of SMAC, research on SMs has gained huge interest, and five monomeric compounds (the peptidomimetics BI891065, CUDC-427, DEBIO 1143, and LCL-161 and the nonpeptidomimetic antagonist ASTX660) and three peptidomimetic dimeric compounds (APG-1387, birinapant, and AEG40826/HGS1029) have completed or are involved in ongoing phases 1 and 2 clinical trials for the management of various distinct malignancies, including breast, ovarian, and fallopian cancers, non–small cell lung cancer, renal cell carcinoma, colorectal and peritoneal cancer, myeloma, and leukemia (Table 1) (*108*–*120*). Of note is the monomeric SM GDC-0152, the first peptidomimetic SM to enter clinical trials, which paved the way for development of more potent SMs, although the clinical trial for this compound was not completed because of reasons unrelated to patient safety (ClinicalTrials.gov identifier NCT00977067). Besides the field of oncology, a trial of birinapant was conducted for its antiviral activity against hepatitis B, but the trial was terminated early due to exacerbated occurrence of reversible Bell's palsy (ClinicalTrials.gov identifier NCT02288208). A similar clinical trial is ongoing for APG-1387 in chronic hepatitis B (ClinicalTrials.gov identifier NCT03585322). However, the intention of this review is not to determine the efficacy of SMs in malignant disorders but rather to evaluate their general tolerability in humans and potential applicability for inflammatory disorders.

In general, the investigated SMs have been well tolerated clinically, both as singly administered agents and as combination therapy with other treatment modalities, including chemotherapeutics. Nevertheless, drug-related severe adverse events like reversible Bell's palsy, which is thought to be caused by inflammation and compression of

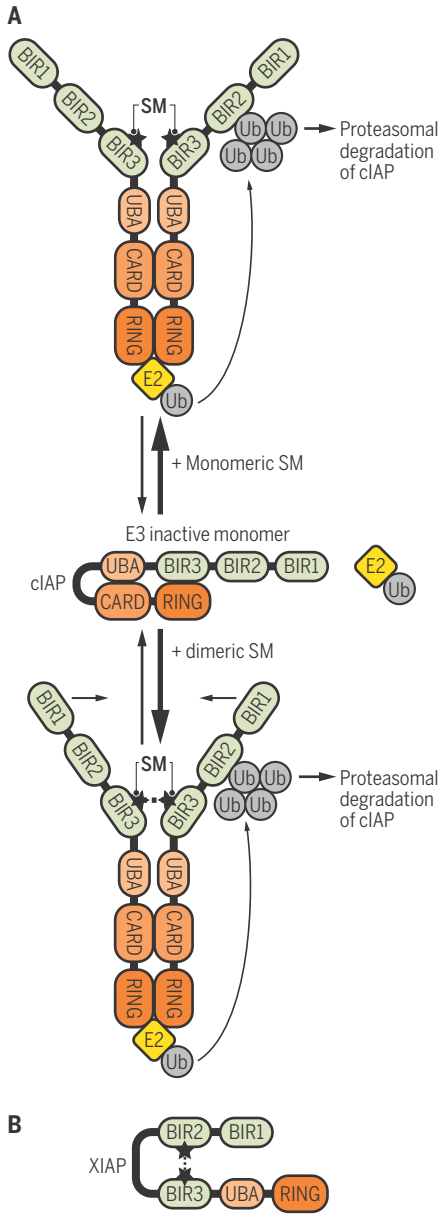

Fig. 5. Mechanism of action of SMAC mimetics. (**A**) Monomeric cIAP is a catalytically inactive E3 ubiquitin ligase due to BIR3-mediated inhibition of the RING domain, which is necessary for dimerization and the recruitment of the E2 ubiquitin–conjugating enzyme. SMs disrupt this autoinhibition by binding to the BIR3 domain, resulting in RING domain dimerization, E2 binding, and catalytic activity. In the absence of a bona fide substrate to accept the ubiquitin (Ub), cIAP itself receives the proteasomal degradation tag. The stability of the active cIAP dimer is higher with dimeric SMs compared to monomeric SMs because intermolecular bridging strengthens the interaction and brings the two IAP molecules into closer proximity. (**B**) Dimeric SMs also mediate intramolecular bridging in XIAP by simultaneously binding to the BIR2 and BIR3 binding grooves, thereby preventing XIAP from binding to caspase-3/7 and caspase-9, respectively. RING, really interesting new gene; CARD, caspase-recruitment domain; UBA, ubiquitin-associated domain; BIR, baculoviral IAP repeat domain.

the seventh intracranial nerve, have been associated with dimeric SMs (*110*, *114*–*116*). This condition is mitigated by glucocorticoids and nonsteroidal anti-inflammatory drugs (NSAIDs) and resolves upon cessation of SM treatment. Dose-dependent cytokine release syndrome (CRS) has been reported upon administration of LCL-161 in 9 of 64 patients (14%) who were mainly treated at doses of ≥1800 mg once per week (*111*, *117*). From the inflammatory disease perspective, this is obviously of major concern and indicates that LCL-161, at least at high doses or in continuous administration, might be inappropriate for use outside oncology. Nevertheless, this CRS effect can be mitigated with premedication or by treatment with glucocorticoids or NSAIDs. In summation, no drug-related fatalities have been reported in an estimated total cohort of 1000 to 2000 patients with cancer (based on published reports, meeting abstracts, or database entries listing the number of patients treated or evaluated) or in a small cohort of normal volunteers previously exposed to one of the SMs in clinical development.

The in vivo pharmacodynamic effects of the SM drugs in humans have been examined to a limited degree by showing efficient target reduction of IAP abundance in PBMCs from treated patients (*108*, *109*, *112*, *114*). IAP abundance also has been measured in peripheral tissue (skin biopsies) and was shown to be potently reduced by SM administration (*111*). Further, three studies included measurements of peripheral cytokines in drug-exposed patients (*111*, *121*, *122*). Upon drug administration, all measured cytokines—TNF-α, IL-8, IL-10, and CC chemokine ligand 2 [also known as monocyte chemoattractant protein-1 (MCP-1)]—were increased at LCL-161 doses higher than 500 mg once per week but returned to normal 4 days after each dose, whereas such an effect on plasma cytokine levels was not observed in the low-dose group despite a reduction in IAP abundance in PBMCs and skin biopsies in these patients (*111*). None of the measured chemokines or cytokines (MCP-1, TNF-α, IL-6, IL-8, and IFN-γ) were found to be induced in serum by CUDC-427, and no cases of CRS were reported with this drug (*121*). Further, patients treated with birinapant showed no statistically significant changes in plasma cytokine levels (IL-6, IL-8, TRAIL, and TNF-α), although a dose-dependent lymphopenia was seen, whereas other white blood cells were unaffected (*122*). However, 5 of 12 patients receiving birinapant demonstrated CRS at doses of 35 mg/m^2 and above (*114*). These studies suggest that SMs might have compound-specific side effects and certainly also potential dose-dependent side effects, albeit at doses higher than would be needed for sufficient blockade of IAP-dependent signaling to reduce inflammation. However, the highest tolerated dose is generally applied for cancer therapy. Nonetheless, the safety profile must be considered on the basis of the limited number of patients exposed and the high degree of comorbidity.

SMs and anti-inflammatory therapeutic capabilities

Using SMs as anti-inflammatory agents is a new strategy that needs to be explored clinically. Clinical research on these small molecules has so far been primarily focused on their potential to act as anti-cancer agents, except for the previously mentioned clinical trials in hepatitis B and an anti-HIV activity observed in preclinical models, a notion that is still under investigation (*123*, *124*). The hypothesis in this area of research is based on the ability of SMs to reactivate latent HIV infection in macrophages and T_H cells through NF-κB stimulation of elements in the viral long terminal repeats, possibly allowing for highly active antiretroviral therapy to eradicate the infection for good.

The response to SM therapy markedly differs between neoplastic and non-neoplastic cells, wherein the latter are not sensitized to

Table 1. Ongoing and completed clinical trials with SMs. A list of all identifiable completed or ongoing clinical trials from searching the ClinicalTrials.gov and ClinicalTrialsRegister.eu databases and studies published as research papers (PubMed) or abstracts (Web of Science) by January 2020. Indications and reported side effects are listed. If a compound has been evaluated for the same indication in different trials, then only the trial representing the highest phase is listed. Studies terminated early are excluded. AML, acute myelogenous leukemia; CRC, colorectal cancer; CRS, cytokine release syndrome; DLT, dose-limiting toxicity; HNSCC, head and neck squamous cell carcinoma; MDS, myelodysplastic syndrome; NSCLC, non–small cell lung cancer; RCC, renal cell carcinoma; SCLC, small-cell lung cancer; TNBC, triple-negative breast cancer; N/A, not available; pts., patients.

Compound	Phase	Type of malignancy	Results	Clinical trial identifier (trial status) (publication)
ASTX660*	1	AML	N/A	NCT04155580 (ongoing)
	1/2	Solid tumors, lymphoma	N/A	NCT02503423 (ongoing)
BI 891065*	1	Solid tumors, NSCLC	N/A	NCT03166631 (ongoing)
CUDC-427* (formerly GDC-0917)	1	Solid tumors	42 pts. Well-tolerated at 5–600 mg/day Decreased cIAP1 abundance in PBMCs	NCT01226277 (completed) (*109*)
DEBIO 1143*	1	Solid tumors, lymphoma	51 pts. Well-tolerated <180 mg/day. At higher dosage alanine aminotransferase was increased in 5% of pts. Decreased cIAP1 abundance in PBMCs	NCT01078649 (completed) (*112*)
	1	Pancreatic cancer, CRC	N/A	NCT03871959 (ongoing)
	1b	Solid tumors, NSCLC	N/A	NCT03270176 (ongoing)
	1/2	HNSCC	N/A	NCT02022098 (ongoing)
	1/2	SCLC, HNSCC, gastrointestinal ovarian, endometrial, peritoneal, and cervical cancer	N/A	NCT04122625 (ongoing)
LCL-161*	1	Solid tumors	71 pts. Well-tolerated up to 1800 mg once/week with 9% experiencing CRS at higher concentrations, and in 6% of pts. as a DLT. cIAP1 degradation in peripheral tissue	NCT01098838 (completed) (*111*)
	2	TNBC	209 pts. Well-tolerated in combination with paclitaxel, although notable toxicity at 1800 mg/week	NCT01617668 (completed) (*113*)
	2	Multiple myeloma	25 pts. Well-tolerated, but CRS observed in 4 of 11 pts. at 1800 mg/week. After dose reduction to 1200 mg/week, no further CRS was observed	NCT01955434 (completed) (*117*)
	1b	CRC, NSCLC, TNBC, RCC	N/A	NCT02890069 (ongoing)
	1/2	SCLC, ovarian cancer	N/A	NCT02649673 (ongoing)
	2	Leukemia	N/A	NCT02098161 (ongoing)
APG-1387†	1/2	Solid tumors, hematologic malignancies	N/A	NCT03386526 (ongoing)

continue to next page

Compound	Phase	Type of malignancy	Results	Clinical trial identifier (trial status) (publication)
Birinapant†	1	Solid tumors, lymphoma	50 pts. Well-tolerated in dosages <35 mg/m^2. At higher doses, CRS was observed in 5 of 12 pts. and Bell's palsy in 2 of 3 pts. receiving 63 mg/m^2. cIAP1 suppressed in PBMCs	NCT00993239 (completed) (*114*)
	1	Ovarian cancer	27 pts. Well-tolerated among 89% of pts. 1 pt. got pancreatitis at an accumulated dose of 78 mg/m^2, and 1 pt. got Bell's palsy at 104 mg/m^2	NCT01940172 (completed) (*115*)
	1/2	Solid tumors	176 pts. Well-tolerated with ascending dose strategy <35 mg/m^2. Bell's palsy was observed in 8% of pts., yet lower risk than in the single-dosing group with no ascending strategy	NCT01188499 (completed) (*110*)
	1/2	MDS	21 pts. Well-tolerated at 13 mg/m^2 when combined with 5-azacitidine with side effects consistent with the disease under investigation. 17% of evaluable pts. developed Bell's palsy	NCT01828346 (completed) (*116*)
	1	HNSCC	N/A	NCT03803774 (ongoing)
	1/2	AML	N/A	NCT01486784 (ongoing)
HGS1029/ AEG40826†	1	Solid tumors	66 pts. Well-tolerated at dosages up to 2.1 mg/m^2 Dose-related decrease of cIAP1 abundance in PBMCs	NCT00708006 (completed) (*108*)

*Monovalent †Bivalent

apoptosis, as demonstrated in various cells, such as epithelial and endothelial cells, fibroblasts, lymphocytes, monocytes, and bone marrow stromal cells (*125*–*127*). Considering this and the finding that inflammatory signaling upon TLR2 or TLR4 activation was disrupted by SM administration in murine macrophages (*38*), SM compounds might additionally be considered advantageous as therapeutic agents for non-neoplastic disorders like inflammatory diseases. One study specifically demonstrated the ability of DEBIO 1143 to inhibit trinitrobenzene sulfonic acid–induced colitis in mice, because administration of this SM reduced infiltration of inflammatory immune cells into the colon and lowered IL-12p40 and IL-6 production by mesenteric lymph node cells (*26*). The ability of SMs to ameliorate symptoms of inflammatory diseases may relate either to their interference with XIAP-dependent NOD2-mediated activation of NF-κB and MAPKs, or the quelling of overactive macrophage activity by inducing macrophage cell death in a TNF-α– and cIAP-dependent manner (*73*, *128*). In addition, the effect of SMs on the balance in the T_H17-T_{reg} axis, for which there is increasing evidence of importance in establishing excessive inflammation (*8*, *129*), is a new approach and indeed relevant for targeting inflammatory diseases. A study investigating cIAP inhibition in an experimental murine model of arthritis demonstrated the preclinical SM GT13072 to inhibit T_H17 responses and reduce production of the pathological cytokine IL-17A (*130*). In addition, a synergistic effect between GT13072 and TNF-α inhibition has been found (*130*), favoring a switch toward expansion of immunosuppressive T_{reg} cells. This highlights the anti-inflammatory potential of SMs and suggests that new combination therapies with clinically approved TNF-α inhibitors may be effective for diseases like IBD and RA.

Inflammatory signaling is an indispensable part of the immune system to protect the host from invading pathogens. Thus, interference with such important inflammatory signaling pathways might result in the establishment of infections. Challenges exist with the current use of biologics; therapy with TNF-α inhibitors for IBD has been linked to serious and opportunistic infections by bacterial, viral, and fungal agents (*131*, *132*). In contrast, IAPs do not seem to be involved in signaling initiated by RIG-I, a cytosolic PRR that senses double-stranded DNA and activates the IFN response (*38*, *133*–*135*). One study, however, suggests that cIAP1 and cIAP2 positively regulate downstream signaling of RIG-I through TRAF3 and TRAF6

ubiquitylation (*89*), but questions have been raised as to whether the data in this study actually support this hypothesis (*23*). Thus, antiviral immunity could be less affected in SM therapy than it is in presently used anti-inflammatory and immune modulatory drugs, including jakinibs, inhibitors of Janus kinases, which seem prone to activate latent viral infections (*136*, *137*). Furthermore, SMs, being small molecules, have shorter half-lives than currently used biologics. This could make potential side effects more rapidly reversible, resulting in a better safety profile.

The design of SMs is extensively geared toward the development of compounds with optimal binding affinity, cell permeability, and in vivo stability (*138*, *139*). Consequently, many chemically distinct compounds with different pharmacokinetic and pharmacodynamic properties have been developed. Depending on the pharmacodynamics of the specific compound, SMs may not all be well suited for anti-inflammatory therapy. In this context, it is of interest that new IAP subtype–specific inhibitors, such as the XIAP-specific inhibitors XB2d89 and XB2B3d78 (Genentech), have been developed to specifically target inflammatory signaling cascades without engaging apoptotic signaling in primary cells (*31*). These compounds certainly could hold promise as new small-molecule drugs for targeted anti-inflammatory therapy, but, compared to SMs, clinical data are lacking.

RIPK INHIBITORS AS ANTI-INFLAMMATORY THERAPEUTICS

Targeting RIPKs to block inflammation

The RIPK family in humans consists of seven serine-threonine protein kinases of which three (RIPK1, RIPK2, and RIPK3) are clearly linked to inflammatory processes and various disorders of inflammation (*140*–*143*). Evidence for the RIPKs being potential prime drug targets comes from the initial discovery of necrostatin through a chemical screen. Necrostatin is a type III allosteric modulator of RIPK1 that blocks necrosis in addition to suppressing inflammation (*144*). Necrostatin acts mainly as an inhibitor of RIPK1, although it can also suppress RIPK3 activation. Further proof for the RIPKs being a drug target for inflammatory diseases originates from the results of genetic experiments in mice that have revealed that RIPK3 and MLKL single-knockout animals are viable, completely normal, and resist inflammatory challenge, whereas dual knockout of cIAP1 and cIAP2 is embryonic lethal (*145*). Knock-in mice expressing a kinase-dead mutant form of RIPK1 are also viable and exhibit blunted inflammatory responses, although they maintain TNF-α–NF-κB signaling functions. Nevertheless, whole-body knockout of RIPK1, which removes both the kinase (cell death) and scaffolding (NF-κB signaling) functions of RIPK1, is lethal (*145*). Therefore, it should be possible to pharmaceutically target the kinase function of RIPK1 or RIPK3 to block necroptosis and inflammation in a specific manner without impeding TNF-α signaling to NF-κB and without causing severe toxicity. The possibility of directly targeting different aspects of RIPK signaling makes the design of inhibitors specific for certain parts of RIPK signaling possible, whereas this might not be possible with inhibitors specific for cIAP1 or cIAP2.

The duality in RIPK function—that is, NF-κB inflammatory and survival signaling, which is kinase independent, and death pathway induction, which is kinase dependent—explains why RIPK antagonists may offer highly selective and efficacious inflammatory inhibition by targeting one and not both of these functions. Accordingly, inhibition of necroptosis might be a rational anti-inflammatory strategy, because necroptosis is highly proinflammatory due to the extracellular release of immune-stimulating DAMPs. The DAMPs establish feed-forward amplification loops with NF-κB–dependent production of inflammatory cytokines (*146*). In contrast, the multifunctionality of cIAPs—the ability to control cell death and inflammation through regulation of TNF superfamily receptor signaling and activation of NF-κB and to suppress the formation of RIPK-containing death complexes (*24*, *147*)—may be less compatible with the application of SMs as anti-inflammatory agents, although this remains to be assessed in clinical settings. As an example of the problem with cIAP inhibition, the SM-mediated proteasomal degradation of cIAPs blocks RIPK1 ubiquitylation and NF-κB activation, but at the same time, it might potentially lead to inflammatory consequences like the induction of necroptotic cell death in response to TNF-α if caspase-8 is deficient or overwhelmed (*147*). In addition, derepression of the activity of cIAPs against NIK might lead to activation of the non-canonical NF-κB pathway with cytokine production and survival of immune cells. Other feasible inflammatory routes mediated by the NLRP3 inflammasome (generating IL-1) or induction of emergency hematopoiesis have been ascribed to the triple antagonism of cIAP1, cIAP2, and XIAP as well (*148*, *149*). As already mentioned, a great concern with SMs as anti-inflammatory drugs originates in the lack of specificity of the compounds, which block all IAPs, and particularly, in their blocking of both cIAPs and XIAP, albeit often at different concentrations (*150*). Newer, more selective SMs that do not target both cIAPs and XIAP in parallel seem to have selective anti-inflammatory effects in animal models (*151*). An example is specifically bivalent SMs, like birinapant, that selectively induce cIAP1 degradation, thereby blocking TNF-α–mediated NF-κB activation, and thus may be useful in TNF-α–dependent inflammatory conditions like IBD (*152*). The differences between different classes of SMs and RIPK antagonists as anti-inflammatory agents, however, remain to be determined and validated in more detail in animal models and in clinical trials.

RIPK1 inhibitors

Preclinical evidence suggests that RIPK1 and RIPK3 are important mediators of inflammation in animal models of inflammatory disease, as discussed above. Drug development programs by pharmaceutical companies like GlaxoSmithKline and a partnership between Sanofi and Denali have initially focused on RIPK1 inhibitors, but candidates targeting RIPK3 are increasing in numbers, although they have yet to be moved into clinical trials.

An important role for RIPK1 has been demonstrated in a mouse model of apoptosis of intestinal crypts, contributing to chronic inflammation and mucosal erosion. In this model, constitutive expression of NF-κB was induced in intestinal epithelial cells to mimic the chronic activation of this important transcription factor as observed in IBD (*153*), and RIPK1 inhibitors selectively inhibited TNF-α–induced death of intestinal epithelial cells without affecting crypt proliferation. Furthermore, the authors observed prevention of TNF-α–induced apoptosis and caspase activation upon sole administration of the antioxidant, butylated hydroxyanisole (*153*). On the basis of the involvement of RIPK1 activation in the production of ROS that further potentiates RIPK1-mediated cell death (*154*–*156*), it is therefore plausible that a combination therapy of RIPK1 inhibition and antioxidants has potential to ameliorate the inflammatory burden in IBD. In addition, targeting RIPK1 in colitis seems effective, because the preclinical RIPK1 inhibitor GNE684 almost completely prevented colitis and ileitis and reduced intestinal epithelial cell

apoptosis in mice in which NEMO was knocked out in the intestinal epithelium to establish the inflammatory model (*157*). RIPK1 seems to be of importance for inflammation in RA and psoriasis as well, because increased immunolabeling for RIPK1 is observed in the epidermis of patients with psoriasis and in the synovium from patients with RA (*157*). Moreover, animal models suggest that targeting RIPK1 might be effective under these conditions given that the administration of GNE684 ameliorates inflammation in experimental models of arthritis and skin inflammation (*157*).

These proof-of-concept studies have led pharmaceutical companies to develop RIPK1 antagonists that have entered clinical trials. So far, the results of these studies have revealed that they are well tolerated (*158*). GlaxoSmithKline has entered two RIPK1 antagonists in clinical trials, GSK2982772 that is now in a phase 2 study for psoriasis, ulcerative colitis, and RA (Table 2), and GSK3145095 that was in phase 1/2 trials for pancreatic cancer but terminated early after an internal review of the company's research and development portfolio (ClinicalTrials.gov identifier NCT03681951). Moreover, Sanofi and Denali have entered a brain-permeant RIPK1 antagonist, DNL747, into a phase 1 study in Alzheimer's disease and amyotrophic lateral sclerosis.

RIPK2 inhibitors

The receptor NOD2 is well known for its risk association with CD (*159*–*161*). This makes it an interesting risk variant in the ongoing search for potent targets in the management of patients with this chronic intestinal disorder or for targeting and suppressing wild-type variants in IBD in general. However, patients with IBD exhibit increased expression of *RIPK2*, as opposed to unchanged or even decreased expression of *NOD2* (*26*). It has been suggested that the pivotal influence of RIPK2 in inflammation is independent of NOD2, because silencing of RIPK2 in NOD2-deficient mice ameliorates experimental colitis (*26*). Moreover, signaling through NOD2 has also been shown to contribute to RA in vivo (*162*), and mononuclear cells from RA patients exhibit increased expression of *NOD2* and *RIPK2* (*163*). Hence, NOD2-RIPK2 signaling might be an appropriate target to dampen inflammatory diseases in general. This is additionally supported by the finding that NOD2-RIPK2 promotes the production of pathogenic IL-17A, one of the key cytokine drivers of inflammation in the disorders discussed in this review (*1*, *164*), in experimental arthritis (*162*).

Upon ubiquitylation of RIPK2 by XIAP, the ubiquitin ligase LUBAC and protein kinases are recruited to mediate inflammatory NF-κB and MAPK signaling (Fig. 2) (*23*). The interaction between XIAP and RIPK2 occurs through the BIR2 domain of XIAP, a region that mediates SM binding as well (*55*, *165*). This could imply that SMs act through blockage of XIAP-mediated RIPK2 ubiquitylation. However, SMs prevent RIPK1 ubiquitylation by depleting cIAP1 and cIAP2 but have, so far, shown little, if any, direct effect on RIPK2 ubiquitylation. SMs bind specifically to grooves in the BIR3 and BIR2 domains of cIAP1, cIAP2, and XIAP, with a generally higher affinity for BIR3 over BIR2 and an increased avidity for cIAP1 and cIAP2 over XIAP (*21*, *106*). Accordingly, SMs can be designed to selectively target the BIR2 domain of XIAP, which might enable SMs to directly affect RIPK2 ubiquitylation. These XIAP antagonists have recently been shown to disrupt XIAP-RIPK2 interactions and to prevent RIPK2 ubiquitylation and subsequent downstream NOD2 signaling (*31*).

Table 2. Ongoing and completed clinical trials with RIPK inhibitors. A list of all identifiable completed or ongoing clinical trials from searching the ClinicalTrials.gov and ClinicalTrialsRegister.eu databases and studies published as research papers (PubMed) or abstracts (Web of Science) by January 2020. Indications and reported side effects are listed. If a compound has been evaluated for the same indication in different trials, then only the trial representing the highest phase is listed. Early terminated studies have been excluded. ALS, amyotrophic lateral sclerosis; IBD, inflammatory bowel disease; RA, rheumatoid arthritis; UC, ulcerative colitis.

Compound	Target	Phase	Indications	Results	Clinical trial identifier (trial status) (publication)
GSK2982772 (GSK'772)	RIPK1	1	Intended for IBD	79 healthy participants. Well-tolerated up to 240 mg/day. Adverse events of mild intensity (most commonly contact dermatitis and headache).	NCT02302404 (completed) (*158*)
	RIPK1	1	Intended for autoimmune diseases	*	NCT03266172 (completed)
	RIPK1	1/2	RA	*	NCT02858492 (completed)
	RIPK1	2	Psoriasis	*	NCT02776033 (completed)
	RIPK1	2	UC	Not yet disclosed	NCT02903966 (completed)
DNL747	RIPK1	1	Alzheimer's	Not yet disclosed	NCT03757325 (completed)
	RIPK1	1	ALS	N/A	NCT03757351 (ongoing)

*Observations for pharmacokinetic studies available at ClinicalTrials.gov.

Nonselective tyrosine kinase inhibitors, such as the approved drug ponatinib, can block RIPK2 autophosphorylation and ubiquitylation (*166*). These kinase inhibitors attenuate NOD-mediated inflammatory signaling without affecting the TLR4 pathway, as demonstrated in a study using ponatinib on primary human monocytes (*166*). Supporting the anti-inflammatory potential of RIPK2 inhibition, ponatinib inhibits NF-κB activation in vitro and abrogates NOD2-dependent TNF-α production in human primary monocytes (*166*). Likewise, the use of RIPK2-selective kinase inhibitors has proven to be efficacious in blocking NOD signaling in vivo as well, with a statistically significant reduction of IL-6 and IL-8 in the colon and IL-6 and TNF-α in the blood (*167*). This inhibition of NOD2 downstream signaling has been demonstrated to arise from blockage of XIAP binding to RIPK2 and not from the abolishment of RIPK2 kinase activity per se (*168*).

The mechanism of action of specific RIPK2 inhibitors has been demonstrated to be linked to delayed RIPK2 ubiquitylation and NF-κB activation rather than a complete abrogation of these events (*169*). The preclinical RIPK2 inhibitor, WEHI-345, inhibits transcription and secretion of TNF-α and IL-6 in murine bone marrow–derived macrophages, despite the molecular markers of NF-κB and MAPK being activated in contradiction of this observation (*169*). However, activation of NF-κB and MAPK was found to be delayed. This may result in an asynchronous activation of several transcription factors downstream of NOD signaling that might explain the nearly complete abrogation of TNF-α and IL-6 production (*169*). Other studies have demonstrated reduced inflammation upon RIPK2 inhibitor administration in different animal models of experimental colitis and a model of lung inflammation (*170*, *171*). Together, these studies strongly support the concept of RIPK2 inhibitors as potent anti-inflammatory therapeutics. GlaxoSmithKline entered the RIPK2 inhibitor GSK2983559, intended for IBD, in a phase 1 clinical trial, but the trial was terminated early due to nonclinical toxicology findings and reduced safety margins (ClinicalTrials.gov identifier NCT03358407).

Introduction of both XIAP-selective antagonists and RIPK2 inhibitors is a new approach to reduce specific NF-κB activation in innate immune signaling, as opposed to RIPK1 modulators like SMs that are more directly linked to the TNFR pathway. In general, available preclinical results point to chemical and pharmacological modulation of the kinases controlled by IAPs, like RIPKs, as potential targets to control inflammatory responses to microbial infections and to prevent collateral tissue injury (*141*).

CONCLUSIONS AND OUTLOOK FOR THE FUTURE

Despite the potential of SMs and RIPK inhibitors as anti-inflammatory therapeutics, studies on human primary cells—and especially human cells of importance for inflammation—are warranted. Such studies shall enlighten the complex interplay between these small-molecule antagonists and IAPs in inflammatory pathways relevant to inflammatory diseases, including MAPK and NF-κB signaling downstream of TNFR1 or PRRs like NOD1, NOD2, and TLR4. Most published data in the field of SM therapy focus on their effects in malignancies—primarily with emphasis on NF-κB signaling—but other properties may, from an anti-inflammatory perspective, be of potential interest as well. For instance, it has been shown that administration of SM impairs leukocyte extravasation in vitro and in vivo, thereby affecting the recruitment of cells involved in establishing an inflammatory environment (*29*), a mode of action that, however, needs further elucidation.

The growing interest in more specific targeting of IAPs through XIAP-selective antagonists or inhibition of RIPKs requires further mechanistic studies to reveal new strategies for inhibiting PRR pathways (*31*). IAPs and RIPKs represent a complex and intertwined network of signaling cascades, but as already described, RIPK1 and RIPK3 have inflammatory roles that are independent of cIAPs, for example, in TRIF-mediated TLR4 signaling, although cIAPs are engaged in TLR4 signaling through MYD88 activation. Therefore, it will be interesting to specifically explore the contributions of these two separate pathways in LPS-TLR4 signaling with small molecules or small interfering RNAs to define the specific contexts in which targeting of one or both of these pathways might be beneficial. It is also important to extend the research to other PRRs and to unravel the specific molecular role of each IAP in PRR-dependent inflammatory pathways. This may facilitate the development of IAP antagonist–based therapies applicable to PRR-mediated inflammatory diseases, including IBD, RA, and psoriasis. Such therapeutic strategies could be used in both acute and chronic disease stages (that is, for the treatment of flares and for maintenance of clinical remission) using a treatment paradigm involving metronomic dosing, for example. As described, several of the effects of IAPs are cell type dependent, and further studies on the effects of SM and RIPK inhibitors on general inflammation models are highly warranted.

In conclusion, IAPs and perhaps even more so the downstream kinases RIPK1 to RIPK3 seem to be promising disease-specific anti-inflammatory targets for several chronic immune-related disorders. IAP targeting might, however, cause unwanted effects due to the diversity of IAP functions in both inflammation and cell death signaling, whereas carefully designed inhibitors of RIPK signaling may be better suited for specific targeting of inflammation-related signaling.

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