

Combinatorial cancer immunotherapy strategies with proapoptotic small-molecule IAP antagonists

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ABSTRACT Members of the inhibitor of apoptosis (IAP) family control several critical aspects of innate immunity, cell death, and tumorigenesis. Small molecule antagonists that target specific IAP oncoproteins, primarily cIAP1 and cIAP2, but potentially also XIAP and Livin, modulate distinct immune signal transduction pathways that can lead to an increased sensitivity of tumors cells to cytokine-mediated apoptosis. These antagonists are based on the structure of an endogenous cellular IAP inhibitor called Smac. Smac is normally sequestered within the mitochondria and is released into the cytoplasm upon cell death stimuli, thereby overcoming the anti-apoptotic action of the IAPs. The therapeutic usefulness of recombinant tumoricidal cytokines to treat cancer patients is principally limited due to their unacceptable adverse side effects. Therefore, investigators have sought to develop alternative regimens that do not rely on exogenously delivered death ligands. These approaches include the stimulation of the immune system with oncolytic virus-based agents or Toll-like receptor agonists in combination with Smac mimetics. Similarly, preclinical combination immunotherapy studies reveal that recombinant interferon synergizes with Smac mimetics to kill cancer. This strategy opens up new therapeutic avenues for anti-cancer therapy by modulating specific immune-mediated death pathways employing unique dual-pronged combinatorial approaches.

KEY WORDS: *Smac mimetic, XIAP, cIAP1, cIAP2, TNF α , interferon, oncolytic virus, TLR agonist, virotherapy, apoptosis*

Introduction: IAPs are multi-faceted proteins

The cellular inhibitor of apoptosis (IAP) gene family is defined by the presence of a canonical zinc-finger domain motif called baculovirus IAP repeat (BIR). The cellular IAPs also possess other domains that confer additional properties and functions (Budhidarmo and Day, 2015, Reed *et al.*, 2004). The IAPs are found in baculoviruses and in all animal species. There are eight human IAP members with varied roles in cell division, cell motility, cell signaling, innate immunity, and apoptosis (LaCasse *et al.*, 2015, and associated articles). Several IAPs are proto-oncogenes with transforming or tumor-supporting genetic alterations found in various cancers (Fulda and Vucic, 2012). The cancer promoting activities of the IAPs are counter-balanced by several tumor suppressors, some of which are direct IAP antagonists. Some IAP members possess RING and UBA functional domains, targeting other proteins with ubiquitin moieties in various configurations (Gyrd-Hansen and Meier, 2010). This post-translational modification typically results in either proteasomal-mediated degradation or in the formation of

signaling complexes that recruit other key transducers or amplifiers of the signaling pathway. There may be additional changes in protein function arising from IAP-mediated ubiquitination, such as protein re-localization within the cell. The IAPs are typically viewed as suppressors of programmed cell death (apoptosis) as some family members can inhibit or suppress specific cell death proteases, the caspases. However, other IAP members critically regulate cell division and innate immunity, and this function is central to the role of the IAPs in cancer.

Abbreviations used in this paper: IAP, inhibitor of apoptosis; cIAP1, cellular inhibitor of apoptosis 1; cIAP2, cellular inhibitor of apoptosis 2; XIAP, X-linked inhibitor of apoptosis; BIR, baculovirus IAP repeat; TLR, Toll-like receptor; ILP, isolated limb perfusion; BCG, bacillus Calmette-Guerin; IFN, interferon; TNF α , tumor necrosis factor alpha; NF- κ B, nuclear factor kappa-B; SMC, Smac mimetic compound; DR, death receptor; PBLs, peripheral blood leukocytes; HL, Hodgkin lymphoma; NK, natural killer; CIK, cytokine-induced killer; NPC, nasopharyngeal carcinoma; DCs, dendritic cells.

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IAPs regulate life and cell death signaling by TNF superfamily members

Two very similar and functionally redundant IAPs, cIAP1 and cIAP2, are ubiquitin ligases that control TNF α and related cytokine member signaling through the classical or alternative NF- κ B pathways (Beug *et al.*, 2012). cIAP1 and cIAP2 bind to TRAF1 or TRAF2 and can be coupled to TNF superfamily receptors or effector kinases via TRAF1 or 2 bridging interactions, in some cases involving TRAF3. The cIAPs activate the classical NF- κ B pathway by ubiquitinating RIP1, thereby forming the TNFR-RIP1 signalosome. The signalosome then recruits other ubiquitin ligases and kinases to activate classical NF- κ B signaling, which leads to the induction of survival, proliferative and inflammatory gene products (Fig. 1A). Conversely, in the absence of the cIAPs, TNF α signaling promotes the formation of RIP1-containing death complexes (lacking ubiquitin moieties on RIP1) called the ripoptosome and in some cases can lead to the formation of the necrosome (involving RIP3). These IAP-regulated death complexes can induce either caspase-8-mediated apoptosis or necroptosis, respectively (Fig. 1B). Hence, by suppressing the activation of the ripoptosome or preventing necroptosis and by stimulating NF- κ B-responsive genes, the cIAPs represent critical decision points in determining life and death upon TNF α stimulation. In a cIAP-deficient context, tumor cells frequently exhibit a predisposition to apoptosis over normal cells. Another

IAP with ubiquitin ligase activity, X-linked IAP (XIAP), can also influence TNF α signaling and NF- κ B activation. XIAP binding to TAB1 enhances the kinase TAK1 activation of NF- κ B. Notably, XIAP is also a potent direct inhibitor of the effector caspase-3 and -7, which are activated by caspase-8. Therefore, XIAP can also control, at a distal nexus, the induction of apoptosis mediated by TNF α . Moreover, XIAP is a potent suppressor of TRAIL-induced cell death (Albeck *et al.*, 2008, Cummins *et al.*, 2004).

Both cIAP1 and cIAP2 suppress the alternative NF- κ B pathway induced by additional TNF α superfamily ligands, such as CD40L, RANKL or TWEAK (Beug *et al.*, 2012, Varfolomeev *et al.*, 2012). The cIAPs continuously target the cytosolic kinase NIK for proteasomal-mediated degradation. However, upon ligand binding to the cognate TNFR superfamily member, the cIAPs and TRAFs are recruited to the receptor, which results in NIK stabilization and IKK α signaling. As a result, NF- κ B-induced proliferative, survival and inflammatory genes are expressed in specific cell types.

IAP antagonists sensitize tumors to TNF- and TRAIL-mediated cell death

The commitment of a cell to death occurs in part with the release of a large processed mitochondrial protein, called second mitochondrial activator of caspases (Smac) (Du *et al.*, 2000). Smac binds to multiple cytoplasmic IAPs and targets them for either

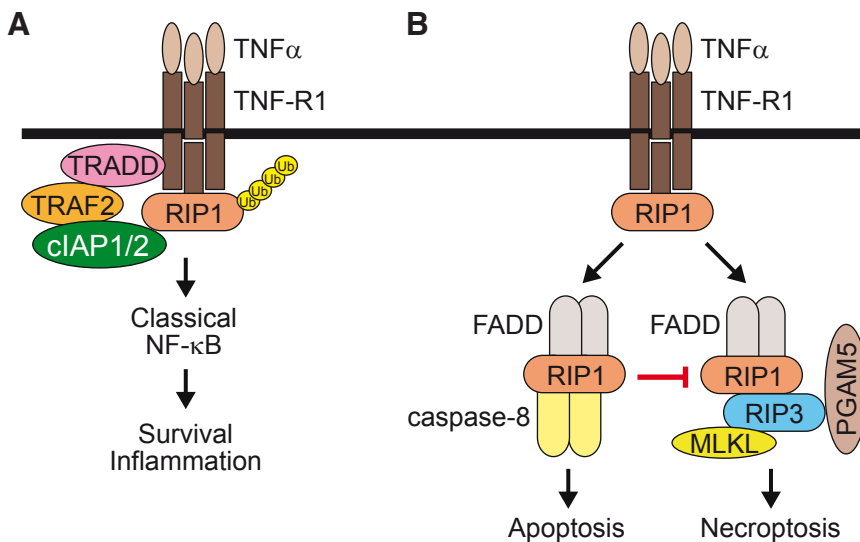


Fig. 1. Regulation of TNF-TNFR1 survival and death pathways in cancer cells by cIAP1, cIAP2 and Smac mimetic compounds. Shown is a simplified model of the pathways activated by TNF α binding to its receptor TNFR1 on a cancer cell (A). Normally receptor engagement by the ligand triggers recruitment of the cIAPs, cIAP1 and/or cIAP2 via TRAF2 and TRADD, to the TNFR1 and induces the K63-branched polyubiquitination of RIP1. This ubiquitination of RIP1 creates a signaling scaffold to recruit other kinases and ubiquitin ligases. This ultimately results in classical (or canonical) NF- κ B activation and signaling. The NF- κ B heterodimers enter the nucleus to induce the expression of genes related to cell survival, proliferation or inflammation (B). Alternatively, under conditions for which the cIAPs are absent, such as chemically-induced depletion with a Smac mimetic compound (SMC), RIP1 is no longer ubiquitinated and forms a default death complex, called the ripoptosome, which activates the initiator cell-death caspase-8 that can activate effector caspases leading to apoptosis. In addition, if caspase-8 is lost, RIP1 can form another death-inducing complex with RIP3, called the necrosome, which can lead to an alternate form of cell death called regulated necrosis or necroptosis. In either case, SMC and TNF α addition results in the death of a cancer cell.

ubiquitin-mediated proteasomal degradation (in the case of cIAP1 and cIAP2) or competes with caspase binding and inhibition (in the case of XIAP). The interaction between the exposed new N-terminal sequence of Smac with the IAPs maps to a four amino acid sequence, AVPI, which is conserved in several other IAP-binding proteins such as caspase-9. This peptide binds to surface grooves on the BIR2 and BIR3 domains of the IAPs. Chemical synthetic mimetics of the Smac tetrapeptide structure have been developed to increase stability and cell penetration characteristics. We will refer to these as Smac mimetic compounds (SMCs) hereafter. SMCs recapitulate many of the actions of the Smac polypeptide. For example, the SMC-mediated loss of cIAPs specifically sensitizes tumor cells to apoptosis by extrinsic death ligands from the immune system, such as TNF α (Fig. 1B), TRAIL, LT α and FasL (*e.g.*, Li *et al.*, 2004). As single agents, SMCs are toxic to 5-15% of tumor cell lines, as those cell lines can produce TNF α endogenously. However, SMC cytotoxicity can be increased to 50% or more of cancer cell lines with the addition of exogenous recombinant TNF α or TRAIL (Benetatos *et al.*, 2014, Cheung *et al.*, 2009).

At least seven structurally different SMCs have entered clinical trials for cancer therapy to date, and these compounds have proven to be safe and well tolerated in phase 1 studies (Table 1) (Bai *et al.*, 2014, Dhuria *et al.*, 2013, Erickson *et al.*, 2013, Infante *et al.*, 2014, Wong

et al., 2012). SMCs can be broadly classified as either monovalent or bivalent structures based on the number of IAP binding moieties present, either one or two units, respectively. The bivalent structures represent homodimers of monovalent units tethered together through a linker region. As stand-alone agents, SMCs demonstrate limited efficacy in the highly refractory and relapsed cancer patient population enrolled in phase 1/2 clinical studies. This finding is not unexpected given that most tumor cell lines require an additional death ligand for killing by SMCs to occur *in vitro*. Therefore, for SMCs to be effective in the clinic, they will likely require an endogenous or exogenous source of death ligand. We discuss multiple approaches as to how this can be clinically achieved based on results from preclinical studies.

Smac mimetic-mediated synergistic killing of cancer cells with death ligands induced by various immune triggers

Smac mimetic-mediated induction of cytokines and chemokines

Interestingly, SMCs themselves mediate the induction of systemic cytokines (such as IL8, TNF α) and chemokines (such as MCP1, RANTES, CXCL1) when administered to animals or humans at very high doses (*e.g.*, Kearney *et al.*, 2013). The dose-limiting toxic effects observed *in vivo* for SMCs relate primarily to TNF α production and its consequences (Bai *et al.*, 2014, Erickson *et al.*, 2013, Infante *et al.*, 2014). Therefore, dosing has been reduced in clinical trials to avoid issues related to cytokine release syndrome. However, the production of TNF α may be needed to achieve anti-tumor responses in SMC clinical trials, and therefore a proper balance between acceptable toxicity and efficacy is likely required. Alternatively, approaches that rely on the induction of TNF α locally, within the tumor environment, may improve potency with overall less adverse side effects.

High-dose SMC treatment may have unintended consequences that can lead to cytokine release syndrome or to blunting of tumor responses to the death ligands. Mice genetically deleted in the hematopoietic compartment of the three IAPs, cIAP1, cIAP2 and XIAP, which are the targets of SMCs, demonstrate a pronounced cytokine release syndrome (Wong *et al.*, 2014). This is attributed to the ability of these IAPs to restrain RIP1- and RIP3-dependent cytokine production. In addition, mice deficient in XIAP can also demonstrate a hyperinflammatory state in response to certain

pathogens, such as *Candida albicans* (Hsieh *et al.*, 2014). These XIAP-deficient mice mirror responses seen in male humans that carry inactivating mutations in XIAP, a primary immunodeficiency syndrome, called X-linked lymphoproliferative disease type 2 (XLP2) (Rigaud *et al.*, 2006). XLP2 individuals experience treatment refractory Crohn's-like colitis due to immune defects in NOD1/2-RIP2 signaling (Pedersen *et al.*, 2014). The disease is also associated with inappropriate macrophage activation. Altogether, these findings suggest that SMC targeting of XIAP at high doses may be responsible for some of the observed effects in humans. Further study is warranted to clarify this issue.

Smac mimetic synergy with chemo- or radio-therapy mediated induction of cytokines

Several chemotherapeutic agents, such as Doxorubicin and Irinotecan, are known to weakly activate NF- κ B and to induce TNF α and other cytokines. Combinations of SMCs and chemotherapeutics have shown efficacy *in vitro* and in animal tumor models and some are being currently evaluated in the clinic (Table 1). Other chemotherapeutic agents also have additional immunomodulatory properties, such as cyclophosphamide (Wu and Waxman, 2014). Cyclophosphamide is being used in conjunction with a monovalent SMC in a clinical trial for multiple myeloma, based on highly promising data employing a transgenic mouse model (Chesi *et al.*, 2013). Alternatively, the focal delivery of radiation to a tumor provokes a localized inflammatory response, which together with an SMC may provide the necessary cell death signal (Hill *et al.*, 2011). However, in the presence of an SMC, it is unreasonable to assume that these somewhat indiscriminate approaches will be able to induce the proper level of cytokines required to adequately reduce overall tumor burden, particularly in patients with metastatic disease. To augment the tumoricidal effect of SMCs, novel approaches have been studied in preclinical models of cancer. These strategies enhance the delivery of death ligands within dispersed tumor microenvironments.

Smac mimetic synergy with virus infection

We hypothesized that viral infection of tumor-bearing animals may provide the necessary induction of local and systemic cytokines to act in combination with SMCs. Replicating oncolytic viruses are being developed as a potentially safe and targeted tumor immunotherapeutic approach (Lichty *et al.*, 2014). We therefore evaluated several candidate oncolytic viruses with

TABLE 1

SMAC MIMETIC COMPOUNDS IN THE CLINIC

Smac mimetic compound (alternate names)	Structural class	Developing company (partners)	Combination agent trials* (accessory drugs shown for listed trials)	References
RG7419 (GDC-0152)	monovalent	Roche-Genentech	?	(Erickson <i>et al.</i> , 2013, Flygare <i>et al.</i> , 2012, Wong <i>et al.</i> , 2012)
CUDC-427 (GDC-0917)	monovalent	Curis (Genentech)	?	(Wong <i>et al.</i> , 2013)
AE640826 (HGS1029)	bivalent	Aegera/Pharmascience	?	
LCL161	monovalent	Novartis	Paclitaxel, Cyclophosphamide, Gemcitabine	(Dhuria <i>et al.</i> , 2013, Infante <i>et al.</i> , 2014)
Debio1143 (AT-406)	monovalent	DebioPharma (Ascenta)	Daunorubicin + Cytarabine, Cisplatin, Carboplatin + Paclitaxel	(Cai <i>et al.</i> , 2011)
APG1387 (SM1387)	bivalent	Ascentage (Ascenta)	?	
Birinapant (TL32711)	bivalent	Tetralogic	5-Azacididine, Gemcitabine, Conatumumab, Antiviral therapy** (Tenofovir or Entecavir)	(Benetatos <i>et al.</i> , 2014, Condon <i>et al.</i> , 2014)

*As identified at <https://clinicaltrials.gov/>; **for a trial against Hepatitis B virus infection;?, not known.

SMCs *in vitro* for their combined anti-cancer effects (Beug *et al.*, 2014). We found that members of the rhabdovirus family were particularly effective at synergizing with SMCs to kill tumor cells *in vitro* and *in vivo*, including models refractory to virus-mediated cancer killing (Beug *et al.*, 2014). This effect is mediated by the systemic induction of type I interferons, such as IFN β , and by TNF α in mice in a manner that does not require infection of all the tumor cells. A diffusible cytokine storm induced by virus infection results in bystander death of non-infected tumor cells in the presence of an SMC. The synergy was remarkable and depending on the model, up to 10,000-fold increased sensitivity toward virus-mediated death was observed. The combination was well tolerated by the animals – SMCs did not hinder the anti-viral responses *in vivo* and the infections were well controlled in immunocompetent animals as expected for these attenuated oncolytic vectors. The excellent activity of the rhabdoviruses in combination with SMCs is postulated to be due to the capability of the virus to induce a strong type I IFN response, especially for attenuated variants, which carry mutations that enhance the host IFN response (such as those mutations in the VSV Matrix protein). This study was also the first report to show that type I or II IFNs could synergize with SMCs, likely via their secondary induction of TNF α *in vivo* (Beug *et al.*, 2014). However, other death-inducing effects of IFN are possible and may play a role in the combination effects.

Smac mimetic synergy with ligands for Toll-like receptors

Viral infection stimulates the production of cytokines through Toll-like receptor (TLR) pathways and other IFN-producing pathways. We asked whether synthetic ligands that activate TLRs could replace the virus in our models, and would thus synergize with SMCs. TLR3 agonists such as poly(I:C), which mimic viral double stranded RNA, and TLR9 agonists such as CpG oligodeoxynucleotides, which mimic bacterial DNA, effectively synergize with SMCs in various *in vitro* and *in vivo* tumor models without the need for a 'live' virus infection (Beug *et al.*, 2014). Previous reports also demonstrate that SMCs can kill tumor cells *in vitro* in combination with TLR agonists in a caspase8-dependent manner (Estornes *et al.*, 2012, Friboulet *et al.*, 2010, Friboulet *et al.*, 2008, Verillaud *et al.*, 2012, Weber *et al.*, 2010). Enhanced killing *in vitro* is observed for poly(I:C) and bivalent SMC combination for EBV-associated nasopharyngeal carcinomas (NPCs), which commonly overexpress cIAP2 (Friboulet *et al.*, 2008). This synergy allowed for lower concentrations of poly(I:C) to be used. In addition, this group demonstrated that poly(I:C) induces cIAP2 expression in a variety of other cancer cells, and that the bivalent SMC and TLR3 agonist combination cooperatively inhibit the clonogenic growth in a large fraction of carcinoma and melanoma cell lines (Friboulet *et al.*, 2010). Sensitization towards poly(I:C)-induction of apoptosis in melanoma cells is also achieved with a monovalent SMC (Weber *et al.*, 2010). The more selective TLR3 agonist, poly(A:U), can also synergize with a bivalent SMC to kill NPCs (Verillaud *et al.*, 2012). Lebecque and colleagues established in part the mechanistic details by which poly(I:C) stimulation of TLR3 induces apoptosis in lung cancer cells in conjunction with a SMC, and how this differs with TNF α induced killing (Estornes *et al.*, 2012). They describe a caspase-8 death complex that associates with ligated TLR3; this reaction requires RIP1, which is negatively regulated by cIAP2-TRAF2-TRADD.

Smac mimetic synergy with an infectious agent vaccine administration

Vaccines consisting of inactivated, non-infectious or infectious viruses or bacteria are also capable of safely inducing cytokines in a host. Prophylactic vaccination strategies to provide immunity against pathological viruses have been administered routinely and safely for millions of adults and children over several decades, and vaccines could be engineered to work in conjunction with SMCs for cancer therapy. The level of multi-TLR stimulation by a vaccine may not be as strong compared to an infectious 'live' virus because of the lack of efficient TLR binding which is mostly intracellular (endosomal) based. Recently, a novel approach employs the use of highly bioactive non-replicating rhabdovirus-derived particles (NRRPs), which apart from direct tumoricidal properties, are also known to induce the release of a plurality of cytokines which synergize with SMCs (Batenchuk *et al.*, 2013). This agent retains cell-entry capacity, thus endosomal localization and efficient TLR ligation likely explains its cytokine induction profile. As NRRPs exhibit enhanced therapeutic index over live virus counterparts, the combinatorial use of SMCs and NRRPs represents a promising strategy to treat disseminated cancers. In addition, vaccines can induce localized TNF α production, such as the tuberculosis vaccine BCG, which is used to treat bladder cancer by instillation of BCG into the bladder to cause TNF-mediated cancer cell death (Vacchelli *et al.*, 2012). BCG can also synergize with SMCs to kill bladder cancer cells *in vitro* using conditioned media from immune cells (Jinesh *et al.*, 2012). This study reveals that TNF α secreted from BCG-stimulated neutrophils is the key mediator of the SMC anticancer action for this combination immunotherapy. Therefore, other vaccine approaches, which could provide systemic or local cytokine release, may show benefit in combination with an SMC to treat cancer.

Smac mimetic synergy with adoptive immune-cell therapy and cancer vaccines

SMCs demonstrate pleiotropic effects on the immune system due to the many roles IAPs play in cell signalling pathways involved in activation, survival, apoptosis and differentiation. For example, SMCs can augment the anti-tumor activity of a melanoma cell cancer vaccine approach in mice by enhancing T-cell responses against the tumor (Dougan *et al.*, 2010). This study showed that SMCs can augment both prophylactic and therapeutic antitumor vaccines *in vivo*, and that SMCs have co-stimulatory properties that lead to T-cell proliferation and activation. Another group demonstrated that SMCs can sensitize resistant hematological cancers and sarcomas to cytokine-induced killer (CIK) cell adoptive therapy (Rettinger *et al.*, 2014). CIKs are peripheral blood leukocytes (PBLs), which are activated and expanded *ex vivo* by timed addition of cytokines to produce cells that have properties similar to natural killer (NK) cells. However, these researchers note that the bivalent SMC had an inhibitory effect on CIK and normal PBL survival. Therefore, improved tumor-targeted killing occurred when these cancers were preincubated with SMCs, with the drug removed prior to the addition of the CIKs. SMCs also potentiate Hodgkin lymphoma (HL) cell death by NK cells (Brinkmann *et al.*, 2014). In a separate study, a monovalent SMC induced the expression of ligands for the activating immune receptor NKG2D on HL cells, and enhanced the susceptibility of HL cells to NKG2D-dependent lysis by NK cells (Sauer *et al.*, 2013).

Additional immunomodulatory effects of SMCs

SMCs demonstrate divergent immunomodulatory effects on human immune cell subsets, with implications for combination immunotherapy and tumor growth. Cebon and colleagues demonstrated that T-cells treated with a monovalent SMC enhanced cytokine secretion upon activation, with little effect on helper or cytotoxic T-cell survival or proliferation (Knights *et al.*, 2013). Furthermore, they showed that a monovalent SMC treatment of peripheral blood mononuclear cells enhanced priming of naïve T-cells with peptides *in vitro* yet intriguingly the dendritic cells (DCs) compartment underwent phenotypic maturation with a reduced capacity to cross-present a tumor antigen-based vaccine. Another group previously showed that a bivalent SMC could induce maturation of monocyte-derived DCs (Muller-Siennerth *et al.*, 2011). This likely relates to the need for cIAP1-dependent degradation of TRAF2 to allow CD40L-mediated differentiation of monocytes into macrophages (Dupoux *et al.*, 2009), an effect that can be mimicked by SMC addition. It is also well established that chronic high-dose administration of an SMC can lead to monocyte/macrophage cell death *in vitro* and *in vivo*. For example, a bivalent SMC induced the death of freshly isolated human monocytes *in vitro*, while T-cells, DCs and macrophages were largely protected against SMC-induced killing (Muller-Siennerth *et al.*, 2011). A different bivalent SMC induced apoptosis of murine bone-marrow derived macrophages *in vitro* and lead to the death of murine macrophages *in vivo* upon repeated high dose intraperitoneal injections (McComb *et al.*, 2012). Interestingly, intraperitoneal administration of a bivalent SMC in murine ascites models of human ovarian carcinoma, or a murine sarcoma, caused a rapid inflammatory burst of TNF α , IL1 β and IFN γ that was associated with the reversion of tumor-associated macrophages from a pro-tumoral M2 phenotype to a pro-inflammatory M1-like phenotype (Lecis *et al.*, 2013). Another study with intraperitoneal injection of a bivalent SMC, followed by a TNF α injection, demonstrated an enhanced immune cell recruitment and inflammatory cytokine/chemokine burst *in vivo* at early time points (Kearney *et al.*, 2013). This is associated with a massive recruitment of neutrophils to the peritoneum due to the accumulation of tumorous debris. SMC treatment leads to the elimination of ascites and prolonged survival in the ovarian cancer model and was curative in some cases for the murine sarcoma model (Lecis *et al.*, 2013). These two tumor models

are unresponsive to SMC treatment *in vitro*, demonstrating the need for immune cell recruitment and cytokine production for an effective SMC-mediated anti-tumor response. In a separate study, a monovalent SMC impaired the *in vivo* cytotoxic T-cell response of mice to LCMV virus infection (Gentle *et al.*, 2014). In the same study, *in vitro* activated helper and cytotoxic T-cells were killed by the monovalent SMC, while naïve T-cells were not affected. XIAP inhibition was required for SMC-induced T-cell death, which is congruent with the well-known role that XIAP plays in preventing activation- and FasL-induced death in T-cells. In contrast, several studies have shown that SMCs do not impair oncolytic VSV control (Beug *et al.*, 2014, Liu *et al.*, 2013). Further work is required to validate and elucidate the mechanisms behind these apparent virus-specific effects.

Smac mimetic synergy with recombinant cytokines (or other biologics)

Recombinant IFNs are currently used for specific cancer, antiviral and multiple sclerosis therapy. Therefore, based on the virus and SMC combination therapy results, IFN could potentially be combined with SMCs to more effectively and broadly treat cancer. The combination cancer immunotherapy approach utilizing oncolytic rhabdoviruses in preclinical models relies clearly on IFN production for its anti-cancer effects (Beug *et al.*, 2014). Recently, combinations of recombinant IFN α and a bivalent SMC were synergistically effective in killing acute myeloid leukemia (AML) cells (Bake *et al.*, 2014), without untoward activity against normal peripheral blood lymphocytes. SMC and IFN α act in concert to stimulate the expression of TNF α ; its secretion into the tissue culture media mediate the AML-specific cell death.

Recombinant TNF α therapy is currently restricted to isolated-limb perfusion (ILP) in combination with chemotherapy, which is used to treat mainly unresectable limb melanoma and sarcoma (Deroose *et al.*, 2012). The TNF α -ILP approach avoids systemic toxicity while improving early drug delivery to the tumor. Subsequent tumor-specific vascular endothelium damage augments the overall tumoricidal effect. ILP combinations with SMCs could likewise be applied to increase the tumor-killing effect of TNF α . TRAIL receptor death ligands and receptor agonistic monoclonal antibodies, such as Conatumumab (AMG 655) are under clinical development for cancer; their combinatorial use with SMCs also represents interesting strategies to explore (Table 1).

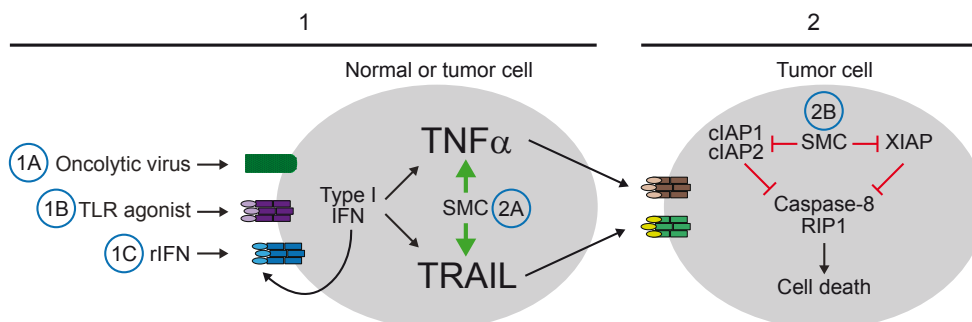


Fig. 2. One-two punch combination immunotherapy model involving IAP antagonists. The summary model shown illustrates the two-step approach for the combination immunotherapy with a Smac mimetic compound (SMC). In the first step (1), a tumor-bearing animal is given either a virus-derived agent (1A) or a TLR agonist (1B) to stimulate type 1 IFN production from normal cells (e.g. macrophage or other) or tumor cells. The induced IFN can then stimulate the production of death ligands such as TNF α or TRAIL, and this effect may be enhanced in the presence of an SMC (2A). Alternatively, the need for an immune trigger can be substituted with the direct administration of recombinant interferon (rIFN) (1C). In the second phase (2), the addition of an SMC results in the death of a tumor cell in the presence of death ligands, as the loss of the cIAPs switches TNF α signaling from a survival to a death pathway (2B). For those SMCs which also target XIAP, there may be added benefit to tumor cell killing by relieving the caspase-3/7 inhibitory effects of XIAP. The sequence of drug addition steps can be reversed or can occur simultaneously.

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Conclusion: future prospects for IAP antagonism and combination cancer immunotherapy

Several animal tumor models have shown that combination therapy using SMCs alongside novel or already-approved innate immune stimulants to induce cytokine production is a potent, highly feasible and broadly applicable anti-cancer strategy. We liken this combinatorial immunotherapeutic approach to the “one-two punch” in boxing. The first punch stuns the opponent leaving them vulnerable to the greater devastation caused by the second blow (Fig. 2). Experience to date in the clinic has shown that neither agent, SMCs or the immune stimulant, is highly effective on its own at disabling its adversary; however, in combination they complement each other by providing the necessary cellular death cues to achieve a decisive ‘knock-out’ in preclinical models. Future combination clinical trial studies will ultimately reveal the utility of such an approach.

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