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Targeting Inhibitors of Apoptosis Proteins (IAPs) For New Breast Cancer Therapeutics

Shaomeng Wang*, Longchuan Bai, Jianfeng Lu, Liu Liu, and Chao-Yie Yang

Comprehensive Cancer Center and Departments of Internal Medicine, Pharmacology and Medicinal Chemistry, University of Michigan, 1500 E. Medical Center Drive, Ann Arbor, MI 48109, USA

Abstract

Apoptosis resistance is a hallmark of human cancer. Research in the last two decades has identified key regulators of apoptosis, including inhibitor of apoptosis proteins (IAPs). These critical apoptosis regulators have been targeted for the development of new cancer therapeutics. In this article, we will discuss three members of IAP proteins, namely XIAP, cIAP1 and cIAP2, as cancer therapeutic targets and the progress made in developing new cancer therapeutic agents to target these IAP proteins.

Apoptosis and human cancer

Apoptosis, or programmed cell-death, is a fundamental cellular process to remove damaged or unwanted cells in multiple cellular organisms. Improper regulation of apoptosis is therefore linked in many human diseases, including cancer, autoimmune diseases, inflammation and neurological diseases.^{1–3} In fact, defective regulation of apoptosis is a hallmark of human cancer.⁴

Basic apoptosis pathways

Apoptosis is a tightly regulated process. Several major apoptosis pathways have been identified and characterized in the last two decades, although these pathways often have extensive cross-talks. The intrinsic and extrinsic apoptosis pathways are two of the best studied (Figure 1).⁵

The intrinsic, or mitochondria, apoptotic pathway integrates a variety of cell stress signals and is initiated by permeabilization of the outer membrane of mitochondria and loss of mitochondrial potential. On the molecular level, the intrinsic pathway involves the translocation and oligomerization of Bax or Bak, members of the Bcl-2 family proteins, which forms a pore in the outer member of mitochondria and leads to the release of pro-apoptotic molecules such as cytochrome C. Upon its release from mitochondria into cytosol, cytochrome C, together with dATP, Apaf-1 and procaspase-9, forms the apoptosome, which processes the procaspase-9 zymogen into the active form of caspase-9. Caspase-9 then cleaves and activates caspase-3, -6 and -7, which leads to further processing of downstream cell-death substrates, and ultimately apoptosis.

The extrinsic, or death-receptor, apoptotic pathway, is initiated by the binding of death ligands such as Fas/Apo-1, TNF-alpha, Apo2L/TRAIL, and Apo3L ligands to their cognate cell-surface receptors, FasR, TNFR1, DR4/DR5 and DR3, respectively. The binding of these

cytokines to their receptors results in recruitment of the death-inducing signaling complex (DISC) to the cytoplasmic domain of the death receptors. The DISC contains an adapter protein, which recruits procaspase-8 into the complex and results in autoactivation of caspase-8. Caspase-8 further cleaves and activates caspase-3, leading to apoptosis.

Apoptosis in both the extrinsic and intrinsic pathways is caspase-dependent. Additionally, there is also a caspase-independent apoptosis, which is mediated by AIF (apoptosis-inducing factor).⁶ When mitochondria are damaged, AIF is released from the outer membrane of mitochondria into the cytosol and then translocates into the nucleus, where it binds to DNA and triggers caspase-independent apoptosis.

Inhibitor of apoptosis proteins (IAPs) as a class of key regulators of apoptosis

Apoptosis is regulated at multiple levels and the inhibitor of apoptosis proteins (IAPs) are a class of key negative regulators of apoptosis for both the intrinsic and extrinsic pathways.⁷⁻¹¹

IAP proteins were first discovered in baculoviruses by Lois Miller and colleagues¹² and are defined by the presence of one to three domains known as baculoviral IAP repeat (BIR) domains. A total of eight IAP proteins have been identified in mammalian cells and four of them, namely XIAP, cIAP1, cIAP2 and ML-IAP, have a direct role in regulation of apoptosis.¹⁰ Structurally, XIAP contains three BIR (BIR1-BIR3) domains, followed by a UBA (ubiquitin-associated domain), and a RING domain (Figure 2). In addition to all these functional domains in XIAP, cIAP1 and cIAP2 contain a CARD (caspase recruitment domain), whereas ML-IAP has only a single BIR domain and a RING domain (Figure 2).

These IAP proteins suppress apoptosis by either directly or indirectly inhibiting the activity of caspases (Figure 1). XIAP is the only member that inhibits caspase activity through direct binding to caspases, whereas other IAP proteins inhibit caspase activity indirectly. XIAP binds to three different caspases, namely two executioner caspases, caspase-3 and caspase-7, and one initiator caspase, caspase-9, and inhibits their activity through two distinct BIR domains.⁸ While XIAP binds to caspase-9 through its BIR3 domain, it binds to caspase-3/-7 through a short linker between BIR1 and BIR2.⁸ Since caspase-3 and caspase-7 play a key role in execution of apoptosis in both the extrinsic and intrinsic pathways, and caspase-9 is a critical mediator of the intrinsic pathway, XIAP effectively inhibits apoptosis in both pathways (Figure 2).¹¹ cIAP1 and cIAP2 were discovered through their binding to tumor necrosis factor associated factor 2 (TRAF2). TRAF2 recruits these IAP proteins to TNF receptor 1- and 2-associated complexes where they suppress caspase-8 activation and death-receptor-mediated apoptosis (Figure 2). ML-IAP was discovered by analysis of protein sequence homologous to BIR domains of known IAP proteins and is found to be overexpressed in melanoma.¹³ ML-IAP inhibits apoptosis, not by directly binding to caspases, but by binding to Smac, an endogenous antagonist of IAPs.

IAPs are not just regulators of apoptosis

Although these IAP proteins were initially investigated primarily as inhibitors of apoptosis, recent studies have revealed their role in modulation of other cellular processes.^{10, 14} For example, XIAP physically associates with survivin to drive NF κ B activation, which promotes tumor cell invasion *in vitro* and metastasis *in vivo*.¹⁴ Of note, the regulation of cancer cell invasion and metastasis by XIAP is independent of its function of caspase inhibition. cIAPs also play a key role in regulation of both the canonical and non-canonical NF κ B pathways.¹⁴ In the canonical pathway, binding of TNF α to TNFR1 results in the assembly of complex I, consisting of TRADD, TRAF2/5, cIAP1/2 and RIPK1. This leads to

cIAP-mediated ubiquitylation of the components of complex I through their RING domain and ultimately drives the activation of NF κ B and expression of NF κ B-targeted genes. Hence, in the presence of cIAP1/2, TNF α stimulates NF κ B, leading to cell survival and inflammation. Furthermore, XIAP and cIAP1/2 are also targeted genes for the transcription of NF κ B and activation of NF κ B increases the expression of these IAPs, creating a positive feed-back mechanism for cell survival and inflammation.

Therefore, targeting these IAPs not only promotes apoptosis in tumor cells, but also may have additional therapeutic benefit through suppression of the activity of NF κ B.

Expression of IAP proteins in human breast cancer

The genomic amplification of 11q22, which contains genes encoded cIAP1 and cIAP2, occurs at high frequency in hepatocellular carcinoma (HCC), lung and several types of human cancer,¹⁴ but has not been detected in human breast tumor samples. Instead, these IAPs, particularly XIAP, are found to be overexpressed in human breast cancer cell lines and tumor tissues.

By immunoblot, XIAP and cIAP1 proteins were found to be expressed in most of the NCI panel of 60 human tumor cell lines, including several human breast cancer cell lines, with substantial variability in their relative levels, whereas cIAP2 protein was found to be infrequently overexpressed but was overexpressed in the breast cancer MCF-7 cell line.¹⁵

Jaffer, *et al.*, examined the expression of XIAP in mammary carcinoma tissues by immunohistochemistry (IHC)¹⁶ and showed that XIAP is most strongly and commonly detected in grade 3 ductal carcinoma, suggesting a possible role of XIAP in the more aggressive clinical behavior of grade 3, compared with lower-grade ductal carcinomas. In another study of 42 cases of invasive ductal breast cancer with triple-negative phenotype, expression of XIAP protein is significantly correlated with a more aggressive tumor phenotype and decreased overall and disease-free survival,¹⁷ suggesting a prognostic value of XIAP for invasive ductal breast cancer with triple-negative phenotype.

Foster, *et al.*, examined the expression of XIAP and cIAP1/2 in a number of human breast cancer biopsies representing major breast cancer subtypes.¹⁸ XIAP has an elevated level in the majority of human breast cancer biopsies but is not detected in normal breast tissues. cIAP1 is expressed in both normal and tumor tissues at relatively equal levels. Interestingly, cIAP2 has a higher level of expression in normal tissues than in breast tumor tissues.

Parton, *et al.*, evaluated the coordinate expression of apoptosis-associated proteins in clinical breast cancer specimens before and during chemotherapy¹⁹ and concluded that XIAP may maintain tumor cell survival in the face of caspase activation. Yang, *et al.*, examined the levels of apoptotic effectors and inhibitors in human tumor cell lines, which included four breast cancer cell lines, and normal cell lines as well as in breast carcinoma tissue specimens and normal tissues,²⁰ and showed that the human breast tumor cells expressed high levels of XIAP. When the activity of XIAP was blocked by expression of dominant-negative XIAP-associated factor 1, apoptosis was induced in tumor cells, but not in normal cells. Furthermore, stable transfection with XIAP siRNA in MDA-MB-231 cells or treatment with an antisense XIAP sensitized cancer cells to TRAIL and taxane but not to carboplatin and doxorubicin.

Collectively, these data suggest that human breast cancer tissues have higher levels of expression of XIAP than normal tissues and XIAP plays a key role in mediating apoptosis resistance of breast cancer cells to a variety of apoptosis stimuli, including those from chemotherapeutic agents.

Smac/DIABLO as an endogenous antagonist of IAP proteins

In 2000, the laboratories of Xiaodong Wang and David Vaux independently and simultaneously discovered and characterized Smac (*Second Mitochondria-derived Activator of Caspases*)/DIABLO (*Direct IAP-Binding protein with Low PI*) protein as an endogenous antagonist of XIAP and cIAP1/2 proteins.^{21, 22} Smac is released from mitochondria into the cytosol when mitochondria are damaged by apoptotic stimuli such as UV radiation.^{21, 22} It contains a 55-residue mitochondria-targeting sequence at its *N*-terminus, which is proteolytically cleaved during its release from mitochondria, allowing its subsequent interaction with IAP proteins and exerting its caspase promoting activity.^{21–23}

Soon after the discovery of Smac, the crystal structure of Smac protein complexed with the XIAP BIR3 domain was determined by Yigong Shi's group²³ and the solution structure of Smac peptide complexed with the XIAP BIR3 domain was solved by Fesik and his colleagues at Abbott Laboratories.²⁴ The crystal structure showed that Smac protein forms an elongated homodimer (Figure 3A).²³ Both the crystal and solution structures revealed that the four *N*-terminal residues (Ala1-Val2-Pro3-Ile4) in Smac bind to a well-defined groove on XIAP BIR3 through extensive hydrogen bonding interaction and hydrophobic contacts (Figure 3B). In particular, the free amino group of Ala1 in Smac has strong hydrogen bonding interaction with the Glu314 and Gln319 residues of XIAP, which explains why the first 55-residues in Smac must be proteolytically removed in order for Smac to interact with IAP proteins. These experimental structures have provided the structural basis for the design of small-molecule mimetics of Smac proteins as antagonists of IAP proteins and as a new class of anticancer drugs in the last decade.

Design of small-molecule Smac mimetics as antagonists of IAPs

The interaction between Smac and XIAP BIR3 is mediated by the AVPI 4-residue motif from Smac and a well-defined groove in XIAP and has a submicromolar binding affinity, suggesting the possibility that druglike, small molecules can be designed to mimic Smac protein to target this protein-protein interaction. In the last several years, a number of classes of small-molecule Smac mimetics have been designed and a number of them have been advanced into clinical development for cancer treatment.^{11, 25}

Although Smac-based peptides can bind to XIAP BIR3 with relatively high affinities ($K_d = 0.5 \mu\text{M}$), they are not cell-permeable and lack activity in cell-based assays. Oost and colleagues at Abbott Laboratories have designed a set of potent and cell-permeable peptidomimetics, such as compound **1** (Figure 4), which binds to XIAP BIR3 with a K_d value of 16 nM.²⁶ Using a cell-free functional assay, compound **1** was shown to effectively antagonize XIAP BIR3-mediated inhibition of caspase activity, providing direct evidence for its functional antagonism against XIAP BIR3. Compound **1** potently inhibits cell growth with an IC_{50} value of 13 nM in the MDA-MB-231 breast cancer cell line and effectively induces activation of caspase-3 and cell death in the cells. In addition to the MDA-MB-231 cell line, compound **1** also inhibits cell growth in a subset of the NCI 59 cancer cell lines, including the BT-549 breast cancer cell line. Compound **1** also shows modest activity in inhibition of tumor growth in the MDA-MB-231 xenograft model in mice. These *in vitro* and *in vivo* data for compound **1** provided the first preclinical evidence that small-molecule Smac mimetics may have a therapeutic potential for the treatment of human breast and other types of cancer as a single agent.²⁶

Although compound **1** potently inhibits cell growth and induces apoptosis in the MDA-MB-231 cell line, it only shows modest antitumor activity in the MDA-MB-231 xenograft model in mice, suggesting that this compound has less than optimal pharmacokinetic properties for drug development. A number of groups have reported the discovery and

development of small-molecule Smac mimetics with high *in vitro* potency and superior *in vivo* antitumor activity.^{11, 25}

Our group has reported the design of several classes of Smac mimetics.^{11, 25} Among them, SM-406 has a potent antitumor activity *in vitro* and *in vivo* and is orally active (Figure 4).²⁷ SM-406 binds to XIAP, cIAP1 and cIAP2 with K_i values of 66.4, 1.9, and 5.1 nM, respectively. SM-406 antagonizes XIAP BIR3 protein in a cell-free functional assay and induces rapid degradation of cellular cIAP1/2 proteins. SM-406 inhibits cell growth against the MDA-MB-231 breast cancer cell line with an IC_{50} value of 144 nM and induces dose- and time-dependent apoptosis. SM-406 has good oral bioavailability of 38–55% in rodents and non-rodents. SM-406 effectively inhibits tumor growth in the MDA-MB-231 xenograft model at 30 mg/kg oral dosing and completely inhibits tumor growth at 100 mg/kg oral dosing.

Scientists from Genentech have reported the design of a series of Smac mimetics, such as GDC-0152 (Figure 4).²⁸ GDC-0152 binds to the XIAP BIR3 domain, the BIR domain of ML-IAP, and the BIR3 domain of cIAP1 and cIAP2 with K_i values of 28, 14, 17, and 43 nM, respectively. GDC-0152 decreases viability of breast cancer cells without affecting normal mammary epithelial cells. GDC-0152 induces activation of caspase-3/7 and apoptosis in tumor cells. GDC-0152 inhibits tumor growth when dosed orally in the MDA-MB-231 breast cancer xenograft model.

Novartis has developed a class of potent Smac mimetics, such as LBW242 (Figure 4).^{29, 30} LBW242 binds to XIAP and cIAP1 with IC_{50} values of 200 nM and 5 nM, respectively, as measured in competitive ELISA assays using a biotinylated Smac peptide and recombinant IAP proteins. Similar to other Smac mimetics, LBW242 has a single-agent antitumor activity in a subset of human cancer cell lines, including the MDA-MB-231 breast cancer cell line. Oral administration of LBW242 modestly inhibits tumor growth in a mouse model of multiple myeloma.³¹

Compound **1**, AT-406, GDC-0152 and LBW242 were all designed to mimic a single Smac AVPI binding motif for interaction with IAP proteins. Since Smac protein forms a homodimer and concurrently interacts with both the BIR2 and BIR3 domains in XIAP,²³ small molecules have been designed to mimic the dimeric form of Smac (bivalent Smac mimetics).^{26, 27} The Harran and Wang Laboratories from the University of Texas Southwestern Medical Center were the first to report the discovery of such compounds.³¹ Compound **2** contains two AVPI mimetics, tethered together with a linker (Figure 4). Compound **2** binds to XIAP containing both BIR2 and BIR3 domains with a K_d value of 0.3 nM and activates caspase-3 with a potency equal to that of Smac protein *in vitro*. In T98G human glioblastoma tumor cells, while compound **2** has no single-agent activity, it synergizes with both TNF α and TRAIL to induce caspase activation and apoptosis at concentrations as low as 0.1 nM. In addition to its strong binding to XIAP, compound **2** also binds with cIAP1 and cIAP2 in cell lysates. Similar to monovalent Smac mimetics, compound **2** was subsequently shown to be effective in cell growth inhibition and apoptosis induction in a subset of human cancer cell lines as a single agent.³²

Although compound **2** was not advanced into clinical development, its impressive potency in both biochemical assays and in cell-based assays has inspired other research groups to design and develop bivalent Smac mimetics.^{11, 25} Our group has designed a series of bivalent Smac mimetics and has performed extensive evaluation of one such compound, SM-164 (Figure 4).^{33, 34} Similar to compound **2**, SM-164 binds to XIAP containing BIR2-BIR3 with a K_i value of 0.5 nM.^{33, 34} NMR, gel filtration and mutation studies have also firmly established that SM-164 currently interacts with both the BIR2 and BIR3 domains in

XIAP, providing a basis for its very high affinity to XIAP.³⁵ SM-164 binds to cIAP1 and cIAP2 with low nanomolar affinities.³⁶ SM-164 induces activation of caspases, and strong apoptosis, in MDA-MB-231 and other cancer cell lines as a single agent at concentrations as low as 1 nM. In comparison, bivalent SM-164 is 100–1000 times more potent than its corresponding monovalent Smac mimetics.^{35, 36} SM-164 induces robust apoptosis in the MDA-MB-231 xenograft tumor tissues in mice with a single, intravenous dose and achieves tumor regression.³⁶ These preclinical data on SM-164 suggested that bivalent Smac mimetics may indeed have a therapeutic potential for cancer treatment. Indeed, to date, two bivalent Smac mimetics have been advanced into clinical development.

Development of Smac mimetics as single agents for cancer treatment

Smac mimetics were initially not expected to achieve single-agent antitumor activity since IAP proteins were thought to function as apoptosis brakes. However, Abbott scientists showed that Smac mimetics compounds can activate caspases and induce apoptosis in a small subset of human cancer cell lines among the 59 NCI cancer cell lines,²⁶ which was subsequently confirmed by several groups using different Smac mimetics in tumor cell lines of diverse tumor types. It is now clear that Smac mimetics induce apoptosis as single agents in tumor cells through a TNF α -dependent mechanism.^{32, 35, 36} Once entering cells, Smac mimetics bind to cIAP1 proteins and induce auto-ubiquitination of cIAP1, followed by rapid cIAP degradation in a proteasomal-dependent manner.³⁶ Subsequent studies showed that cIAP1 exists predominantly as an inactive monomer as an E3 ligase and its BIR domains inhibits the dimerization and activation.^{37, 38} Smac mimetics promote dimerization of cIAP1 by binding to the BIR domain and inducing a conformational change and dimerization formation, promoting its autoubiquitination and subsequent degradation.³⁷ Degradation of cIAP1 by Smac mimetics takes place in cell lines both responsive and resistant to Smac mimetics, indicating that the degradation of cIAP1 is necessary but not sufficient for apoptosis induction.³⁶ In tumor cells that produce endogenous TNF α , caspase-8 is activated *via* the formation of complex II consisting of caspase-8, RIP1K and FADD upon degradation of cIAP1, which leads to activation of down-stream caspases and apoptosis.^{35, 36} Of note, not all tumor cells that produce TNF α are sensitive to apoptosis induction by Smac mimetics, indicating additional blockade(s) other than cIAP1 for TNF α -induced apoptosis.³⁹

Screening of a panel of 50 non-small cell lung cancer cell lines with a bivalent Smac mimetic showed that 14% of them are sensitive in a cell growth assay. Our screening of 51 human breast cancer cell lines using SM-406 and SM-164 showed that while these two compounds have different potencies, their patterns of sensitivity are similar (unpublished observation). Both compounds are effective in inhibition of cell growth in 15% of these 51 breast cancer cell lines. Interestingly, basal-like breast cancer cell lines appear to be much more responsive to cell growth inhibition and apoptosis induction by Smac mimetics than other subtypes of breast cancer cell lines. The *in vitro* activity for SM-406 has been demonstrated in a number of xenograft models in mice. These preclinical data suggest that Smac mimetics may have a single-agent activity for the treatment of a subset of human cancer.

Development of Smac mimetics in combination with other agents for cancer treatment

Since IAP proteins effectively suppress apoptosis from both intrinsic and extrinsic pathways and many anticancer drugs work, at least in part, by inducing apoptosis in tumor cells, Smac mimetics have been found to effectively enhance the antitumor activity of a variety of cancer therapeutic agents in breast and other types of human cancer.

Fulda, *et al.*, were the first to demonstrate that a short Smac peptide tethered to a carrier peptide for improving intracellular delivery enhanced apoptosis in tumor cells *in vitro* by TRAIL, Fas/CD95 ligand, doxorubicin, cisplatin and VP-16.⁴⁰ Significantly, the Smac peptide, when administered intratumorally in combination with TRAIL, achieved eradication of established tumors without detectable toxicity to normal brain tissue in an intracranial malignant glioma xenograft model *in vivo*, while both agents alone had minimal antitumor activity.⁴⁰ Similarly, Arnt, *et al.*, showed that a cell-permeable Smac peptide can increase apoptosis induction and long term antiproliferative effects of paclitaxel, etoposide, 7-ethyl-10-hydroxycamptothecin and doxorubicin in MCF-7 and other breast cancer cell lines.⁴¹ Yang, *et al.*, further demonstrated that a cell-permeable Smac peptide selectively reversed the apoptosis resistance of H460 lung cancer cells and, in combination with taxol and cisplatin, regressed the tumor growth *in vivo* with little toxicity to the mice.⁴² Although these Smac-based peptides are not suitable for drug development due to their modest cellular potencies and expected poor *in vivo* pharmacological properties, their *in vitro* and *in vivo* data have provided evidence that Smac mimetics may be developed in combination with other anticancer drugs as effective therapeutic strategies for cancer treatment.

Indeed, small-molecule mimetics have shown strong synergy with TRAIL and TNF α ligands at much lower concentrations than Smac-based peptides. Xiaodong Wang's group showed that the bivalent Smac mimetic **2** can effectively promote apoptosis of TRAIL and TNF α in glioma cells.³¹ Subsequently, compound **2** was shown to sensitize TRAIL and etoposide in breast cancer cell lines.⁴³ The synergy between Smac mimetics and TRAIL/TNF α is very broad. While TNF α was unable to induce apoptosis against any of 51 cancer cell lines of diverse tumor types, it effectively induced apoptosis when combined with a Smac mimetic against 48% of the cell lines.³⁹ Similarly, although only 20% of the 51 cancer cell lines were sensitive to TRAIL for apoptosis induction, 55% of the cell lines became responsive when combined with the Smac mimetic.³⁹

We showed that SM-164 greatly potentiates the antitumor activity of TRAIL in >50% of 19 breast, prostate and colon cancer cell lines in a cell growth assay.³⁴ The strong synergistic activity of SM-164 with TRAIL is observed in both TRAIL-sensitive and -resistant cell lines. The combination of SM-164 and TRAIL achieves tumor regression in the MDA-MB-231 (subclone 2LMP) breast cancer xenograft model without toxicity to normal mouse tissues, while both agents lack significant activity as single agents. Mechanistic studies show that both XIAP and cIAP1 are non-redundant inhibitors of TRAIL and SM-164 achieves strong synergy with TRAIL by concurrently targeting XIAP and cIAP1. Smac mimetics were also shown to enhance apoptosis of TRAIL in chronic lymphocytic leukemia, including subgroups with resistant disease or unfavorable prognosis, in pediatric acute lymphoblastic leukemia, and in pancreatic carcinoma models *in vitro* and *in vivo*.⁴⁴⁻⁴⁶

Smac mimetics also potentiate apoptosis induction by chemotherapeutic agents, including paclitaxel, etoposide, SN-38, 5-FU and cisplatin, in a panel of human cancer cell lines of diverse tumor types.⁴⁷ Mechanistic studies revealed that the synergistic interaction between Smac mimetics and chemotherapeutic agents is due to canonical NF- κ B activation, and production of TNF α and activation of the extrinsic apoptosis pathway.⁴⁷ In addition to conventional chemotherapeutic agents, Smac mimetic **2** was shown to increase apoptosis in response to ErbB antagonists, including Trastuzumab, Lapatinib or Gefitinib, in Her2-overexpressing breast cancer BT-474 cells, or Gefitinib in EGFR-overexpressing breast cancer MDA-MB-468 cells.⁴⁸

Studies of the resistant mechanisms of Smac mimetics have also provided important clues for the development of combination strategies for cancer treatment. It has been shown that cancer cell lines resistant to the combination of a Smac mimetic with TNF α become

sensitive when cFLIP is down-reregulated, suggesting that agents which can down-regulate cFLIP can effectively enhance apoptosis induction by Smac mimetics.³⁹ Our own study showed that when tumor cells initially sensitive to Smac mimetics become resistant, LRIG1 (leucine-rich repeats and immunoglobulin-like domains protein 1) is down-regulated.⁴⁹ Knocking-down of LRIG1 attenuates TNF α gene expression induced by Smac mimetics and increases the activity of multiple RTKs, including c-Met and Ron.⁴⁹ Furthermore, the combination of Smac mimetic SM-164 with multitargeted tyrosine kinase inhibitors Crizotinib or GSK1363089 is effective against resistant cells *in vitro* and *in vivo*, suggesting the development of a rational combination of Smac mimetics with certain RTK inhibitors.

Taken together, these preclinical data provide evidence that Smac mimetics may be developed in combination with conventional chemotherapeutic agents, TRAIL and TNF α , which target death-receptors, as well as antagonists targeting certain tyrosine kinase receptors.

Smac mimetics in clinical development for cancer treatment

To date, four monovalent and two bivalent Smac mimetics have been advanced into clinical development for evaluation of their safety, maximum tolerated dose, pharmacokinetics, pharmacodynamics and/or initial efficacy in patients with advanced solid tumors, lymphomas and leukemia.⁵⁰

GDC-0152 from Genentech was the first Smac mimetic to enter a Phase I human clinical trial in June 2007 (Figure 4).²⁸ Although GDC-0152 was orally bioavailable in animals, it was administered intravenously to patients with locally advanced or metastatic malignancies. GDC-0152 was shown to exhibit linear pharmacokinetics over the dose range (0.049 to 1.48 mg/kg) tested but no other information was reported from the Phase I trial.²⁸ More recently, another Smac mimetic from Genentech, GDC-0197, has entered Phase I clinical trials as an oral agent.

LCL161 from Novartis is an orally bioavailable Smac mimetic (Figure 4) and has been evaluated in Phase I clinical trials at a weekly dosing schedule.⁵¹ LCL161 is well tolerated in patients with advanced cancer and no dose-limiting toxicity was found at up to 1800 mg weekly dosing. At doses equal to or greater than 320 mg of LCL161, cIAP1 levels are reduced consistently in skin punch biopsies 8 h after the first dose, and in a tumor biopsy after 24 h. cIAP1 levels in peripheral blood mononuclear cells (PMBCs) are decreased 2 h post-dose and recover by the following week. Circulating markers of cell death peak on day 2 following doses equal to or greater than 320 mg, and circulating cytokines, including MCP-1 and IL-8, increase 4 h post-dose equal to or greater than 900 mg. To date, no objective responses have been observed, but the Phase I study provides evidence that Smac mimetics can effectively achieve target inhibition, as demonstrated by induction of cIAP1 degradation and induction of cytokines without toxicity to patients. Another Phase I clinical trial is being conducted to test the weekly combination of LCL161 with Paclitaxel in adults with advanced solid tumors.⁵⁰ Additionally, a randomized, Phase II, neoadjuvant clinical trial is planned to test weekly dosing of Paclitaxel with or without weekly dosing of LCL161 in triple negative breast cancer before surgery.⁵⁰

AT-406 (SM-406) (Figure 4) was discovered in our laboratory at the University of Michigan²⁷ and has been licensed by Ascenta Therapeutics/DebioPharm for clinical development. AT-406 is being evaluated as an oral agent in two Phase I trials, in patients with advanced solid tumors and lymphomas, as a single agent and in combination with Daunorubicin and Cytarabine in patients with poor-risk acute myelogenous leukemia (AML).⁵⁰

Bivalent Smac mimetic TL-32711 from TetraLogics is being evaluated in four Phase I clinical trials, with a weekly intravenous dosing-schedule, as a single agent or in combination with chemotherapeutic agents.⁵⁰ In the first-in-men Phase I clinical trial in patients with refractory solid tumors or lymphoma, TL-32711 is very well tolerated at a weekly intravenous dose as a single agent.⁵² TL-32711 induces rapid and sustained cIAP1 degradation in both PBMCs and tumor biopsies. TL-32711 causes caspase activation and cleavage of cytokeratin-18 in serum, evidence for apoptosis induction. Patients resistant to conventional chemotherapeutic agents and treated with TL-32711 have experienced stable disease.

Another bivalent Smac mimetic, known as AEG40826/HGS1029, was discovered by Aegera Therapeutics and licensed by Human Genome Sciences for clinical development.⁵³ HGS1029 was found to be well tolerated in patients with advanced solid malignancies in a weekly, intravenous schedule up to 3.4 mg. Similar to TL-32711, HGS1029 induces rapid and sustained reduction of cIAP1 levels after a single dose of administration and shows evidence of apoptosis induction in patients. Tumor regression was observed in one patient with colon cancer and 2 patients with NSCLC. Dose-limiting toxicities for HGS1029 include severe fatigue, elevated amylase and lipase.

Taken together, these initial clinical data provide evidence that Smac mimetics are well-tolerated, induce rapid and sustained cIAP degradation, and have antitumor activity as single agents in patients with advanced cancer.

Other therapeutic strategies to target IAP proteins

In addition to the design and development of small-molecule Smac mimetics to target IAPs, other strategies have been used to identify small-molecule antagonists of IAP proteins, particularly XIAP.

XIAP binds to and inhibits caspase-3/-7 through a linker immediately preceding its BIR2 domain, suggesting a possible site for the design of small-molecule antagonists of XIAP. Using a biochemical functional assay and by screening of a chemical library of 160,000 compounds, Wu, *et al.*, identified a number modestly active small-molecules that can restore the caspase-3 activity in the presence of recombinant XIAP but have no effect on caspase-3 activity in the absence of XIAP.⁵⁴ Further structural elaboration on one class of compounds to improve physiochemical properties and potencies yielded several compounds, including TWX006 and TWX024 (Figure 5), which have IC₅₀ values of 10 and 25 μ M, respectively, in the same *in vitro* caspase-3 biochemical assay. TWX024 was further characterized due to its superior aqueous solubility. TWX024 was shown to specifically block the interaction of active caspase-3 and recombinant XIAP BIR1-BIR2 protein in a series of coprecipitation experiments. In 293 cells, TWX024 is capable of antagonizing XIAP and restoring apoptosis induction by ectopic expression of CD95 death receptor. While TWX024 is synergistic with TRAIL to induce apoptosis in HCT116 cells lacking BAX, TWX024 does not show a single-agent activity.

Using a similar biochemical assay, Schimmer, *et al.*, screened a chemical library of one-million small molecules and identified a class of polyphenylureas with XIAP-inhibitory activity (Figure 5).⁵⁵ Further characterization showed that these active polyphenylureas specifically block the interaction of XIAP and caspase-3/-7 and have no effect on the interaction between XIAP and Smac peptide, where caspase-9 binds. NMR experiments provided evidence that these polyphenylureas may bind to the linker between BIR1 and BIR2, which mediates the interaction between XIAP and caspase-3/-7. In contrast to the XIAP inhibitor identified by Wu, *et al.*, these polyphenylurea XIAP inhibitors are capable of inducing apoptosis in a variety of human cancer cell lines and in leukemia cells from

patients. One such polyphenylurea also showed significant antitumor activity in a xenograft model of prostate cancer. Additionally, these XIAP inhibitors showed synergistic activity in combination with TRAIL and conventional anticancer drugs, including Etoposide, Doxorubicin and Paclitaxel, in a variety of cancer cell lines.

Through computational structure-based virtual screening of natural products from a traditional herbal medicine three-dimensional structure database, followed by biochemical testing of selected candidate compounds, our laboratory has identified embelin (Figure 5) as a small-molecular weight inhibitor.⁵⁶ Embelin binds to the XIAP BIR3 domain with an IC₅₀ value of 4.1 μ M, comparable to the potency of a natural Smac peptide. An NMR analysis showed that embelin interacts with several crucial residues in the XIAP BIR3 domain with which Smac and caspase-9 bind. Embelin inhibits cell growth, induces apoptosis, and activates caspase-9 in prostate cancer cells with high levels of XIAP, but has a minimal effect on normal prostate epithelial cells with low levels of XIAP. Embelin overcomes the protective effect of XIAP to apoptosis and enhances the etoposide-induced apoptosis in Jurkat cells stably transfected with XIAP, and has a minimal effect in Jurkat cells transfected with vector control.

Despite the promising anticancer activity from these initial reports, none of these XIAP inhibitors has progressed into clinical development.

Concluding Remarks

IAPs (XIAP, cIAP1, cIAP2 and ML-IAP) are key regulators of apoptosis and block apoptosis from both the intrinsic and extrinsic pathways. In human breast cancer cell lines and tissues, one or more of these IAPs is overexpressed, which would protect tumor cells from apoptosis induction from agents targeting both the intrinsic and extrinsic pathways. Therefore, it has been proposed that small-molecule antagonists of these IAP proteins may have a therapeutic potential for the treatment of human cancer.

Although several approaches have been employed to design and discover small-molecule antagonists of IAPs, the most successful approach to date has been the design of small-molecule Smac mimetics. Smac interacts with XIAP, cIAP1/2 and ML-IAP *via* its AVPI tetra-peptide binding motif and these IAP proteins have a well-defined groove, suitable for the design of high-affinity small-molecules. Indeed, potent, small-molecule Smac mimetics with good pharmaceutical properties (solubility and pharmacokinetic properties) have been designed and developed. In preclinical studies, Smac mimetics show single-agent activity in a subset of human breast cancer cell lines *in vitro* and *in vivo*. In particular, triple-negative subtype of human breast cancer appears to be particularly sensitive to Smac mimetics. Furthermore, Smac mimetics show broad synergistic activity in combination with agents targeting the death-receptors such as TNF α and TRAIL, as well as with conventional chemotherapeutic agents and certain receptor-tyrosine kinase inhibitors. To date, six Smac mimetics have been advanced into clinical development. Initially, clinical data suggest that these compounds are well tolerated in patients with advanced cancer and demonstrate initial evidence of antitumor activity, including tumor regression and stable diseases. Additional clinical trials are being conducted to test their combination with conventional anticancer drugs in patients with triple-negative breast cancer and other types of solid tumors and acute leukemia.

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Figure 1.

Functional domains of mammalian inhibitor of apoptosis proteins (IAPs). BIR: baculoviral IAP repeat domain; UBA: Ubiquitin - associated domain; CARD: caspase recruitment domain; RING: Really interesting new gene finger domain.

Figure 2.
Basic apoptosis pathways and regulation of apoptosis by IAP proteins.

Figure 3.

(A) Crystal structure of the dimeric Smac protein in complex with two XIAP BIR3 proteins (PDBID: 1G73). The AVPI motifs are shown in ball models. (B) Detailed interactions between the AVPI binding motif and XIAP BIR3 residues. Oxygen and nitrogen atoms are colored in red and blue colors. Hydrogen bonds are depicted in dash lines. Electrostatic surfaces of XIAP BIR3 are shown where the red, grey and blue colors denote negative, neutral and positive charged regions. The figures are prepared using the PyMOL and APBS programs.

Figure 4.
Chemical structures of representative small-molecule Smac mimetics.

TWX024 **1396-12** **Embelin**

Figure 5.
Chemical structures of representative XIAP inhibitors.