Molecular Pathways: Targeting Death Receptors and Smac Mimetics

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Abstract
Inhibitor of apoptosis (IAP) proteins are overexpressed in multiple human malignancies, an event that is associated with poor prognosis and treatment resistance. Therefore, IAP proteins represent relevant targets for therapeutic intervention. Second mitochondrial activator of caspases (Smac) is a mitochondrial protein that is released into the cytosol upon the induction of programmed cell death and promotes apoptosis by neutralizing IAP proteins. On the basis of this property, a variety of small-molecule inhibitors have been developed that mimic the binding domain of the native Smac protein to IAP proteins. Evaluation of these Smac mimetics in preclinical studies revealed that they particularly synergize together with agents that trigger the death receptor pathway of apoptosis. Such combinations might therefore be of special interest for being included in the ongoing evaluation of Smac mimetics in early clinical trials. Clin Cancer Res; 20(15); 3915–20. ©2014 AACR.

Background
Programmed cell death plays a critical role in the control of tissue homeostasis and is typically disturbed in human cancers (1). Apoptosis, one of the best characterized forms of programmed cell death, can be initiated via two major signaling pathways, i.e., via the extrinsic (death receptor) pathway through binding of death receptor ligands to corresponding death receptors on the cell surface, e.g., TRAIL to TRAIL receptors or, alternatively, via the intrinsic (mitochondrial) pathway through the release of mitochondrial proteins such as cytochrome c and Smac into the cytosol (2). Smac promotes apoptosis by neutralizing inhibitor of apoptosis (IAP) proteins (3). IAP proteins are a family of eight antiapoptotic proteins comprising, for example, X chromosome–linked IAP (XIAP), cellular IAP1 (cIAP1), and cellular IAP2 (cIAP2; reviewed in ref. 3). IAP proteins such as XIAP block programmed cell death pathways at a central node by inhibiting activation of caspases, which are critical effector molecules of apoptosis (2).

IAP proteins are expressed at high levels in various human neoplasms (3). Because they contribute to tumor progression and treatment resistance, IAP proteins have been exploited as cancer drug targets (3). In particular, nonpeptidic small-molecule inhibitors have been developed (3). As these IAP antagonists mimic the endogenous IAP antagonist Smac, they are also referred to as Smac mimetics and these two terms are used interchangeably in this review.

Smac mimetics promote activation of caspases by binding to and antagonizing XIAP (3; Fig. 1). In addition, they favor activation of noncanonical NF-κB signaling by binding to cIAP proteins (Fig. 1), thereby enhancing their E3 ligase activity, autoubiquitination and proteasomal degradation, which leads to accumulation of NF-κB–inducing kinase (NIK), an upstream kinase of the noncanonical NF-κB pathway (4–7). NF-κB is a key transcription factor and signals via two major pathways, i.e., the canonical (classical) and the noncanonical (alternative) NF-κB pathway (8). The Smac mimetic–mediated stimulation of NF-κB and subsequent upregulation of NF-κB target genes such as the death receptor ligand TNFα have been shown to constitute critical events for the single-agent activity of Smac mimetics, because they engage an autocrine/paracrine cell death signaling loop via the TNFα/TNF-R1 ligand/receptor system (4–7, 9). This triggers the formation of a cytosolic complex containing receptor-interacting protein (RIP) 1, FAS-associating via death domain (FADD), and caspase-8 as well as activation of caspases and apoptosis. Besides TNFα, TRAIL has recently been demonstrated to be upregulated as another NF-κB target gene upon Smac mimetic treatment and to be required for Smac mimetic–triggered apoptotic activity under certain conditions (10). Besides their antiapoptotic function, IAP proteins have also been implicated in several nonapoptotic processes, for example differentiation, migration, invasion, metastasis, and immunomodulation (11, 12), which, however, will not be discussed in this review.

Clinical–Translational Advances
Independent studies using a large panel of cancer cell lines revealed that Smac mimetics as monotherapy exhibit

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cytotoxic activity in a relatively small percentage (6, 13). This highlights the need to develop rational combination therapies to maximally exploit the potential of Smac mimetics as cancer therapeutics. Combinations of Smac mimetics together with compounds that stimulate the extrinsic pathway of apoptosis are particularly interesting in light of the fact that Smac mimetics facilitate signaling via the death receptor pathway by stimulating autocrine/paracrine death receptor/ligand loops.

From the standpoint of clinical translation of basic discoveries into clinical application, it is important to note that data from preclinical studies point to a possible therapeutic window that could be exploited to prime neoplastic, but not normal cells for cell death by Smac mimetics, because they were described to preferentially sensitize various cancer cells for death receptor–induced apoptosis without the same sensitization of nonmalignant cells or normal tissues (14–17). However, this assumption needs to be vigorously tested in the future, especially in clinical trials. Also, the underlying reasons for the preferential sensitization of malignant versus nonmalignant cells for death receptor–induced apoptosis by Smac mimetics have not yet been unraveled and remain subject to future investigations.

**Smac mimetics in combination with TRAIL**

**Glioblastoma.** In a first proof-of-concept study, a cell-permeable Smac peptide mimicking the N-terminal four amino acids of Smac was reported to sensitize glioblastoma cells to TRAIL-induced apoptosis in vitro and also strongly enhanced the antitumor activity of TRAIL in an intracranial mouse model of glioblastoma in vivo, resulting in complete eradication of established tumors and survival of mice without detectable toxicity to normal brain tissue (18). Furthermore, the Smac mimetic compound 3 and TRAIL were shown to synergistically induce caspase activation and apoptosis in glioblastoma cells (19). Recently, small molecules were identified that target TRAIL-R2 and interact with Smac mimetics to induce apoptosis in glioblastoma cells (20).

**Hematologic malignancies.** In chronic lymphocytic leukemia (CLL), IAP inhibitors have been described to act in concert with TRAIL to trigger caspase activation and caspase-dependent apoptosis in a synergistic manner (21). Of note, this synergistic drug interaction was observed in CLL subgroups independent of prognostic factors, including patients with poor prognosis because of 17p deletion, p53 mutation, unmutated V(H) genes, or chemoresistant disease (21). Similarly, combined treatment with the Smac mimetic compound A and TRAIL was shown to significantly increase apoptosis in CLL cells compared to treatment with either agent alone, including samples from patients with poor prognostic parameters (22).

In acute lymphoblastic leukemia (ALL), subtoxic concentrations of IAP antagonists but not a structurally related control compound were reported to act in concert with TRAIL to synergistically induce apoptotic cell death (14). The tumor-selective activity of IAP antagonists was supported by parallel experiments in peripheral blood lymphocytes isolated from healthy donors which remained refractory to the combination treatment (14). Importantly, IAP antagonists acted in concert with TRAIL against primary leukemic blasts that were obtained from children diagnosed with ALL as well as in a patient-derived ALL mouse model (14). Intriguingly, IAP antagonists bypassed the Bcl-2–conferring resistance by switching type II cells, which depend on mitochondrial signaling for TRAIL-triggered apoptosis, toward type I cells that undergo apoptosis independently of mitochondrial factors (14). In addition, a series of Smac
mimetics were demonstrated to sensitize leukemia cells to TRAIL-triggered apoptosis (23). Also, treatment with the Smac peptide N7 or overexpression of Smac were shown to enhance TRAIL-induced caspase activation and apoptosis in acute leukemia cells (24). Furthermore, cotreatment with the Smac mimetic LBW242 and TRAIL resulted in cooperative induction of apoptosis in multiple myeloma cells (25).

**Gastrointestinal malignancies.** Small-molecule IAP inhibitors were shown to synergize with TRAIL to induce apoptosis and to inhibit long-term clonogenic survival of pancreatic carcinoma cells, whereas they did not reverse the lack of toxicity of TRAIL on nonmalignant cells in vitro or normal tissues in vivo, pointing to a therapeutic index (15). In addition, IAP inhibitors acted in concert with TRAIL to suppress tumor growth in parallel with caspase activation and the induction of apoptosis in a mouse xenograft model of pancreatic carcinoma (15). Moreover, IAP inhibitors were shown to preferentially act in concert with the TRAIL-R2 agonistic antibody mapatumumab to trigger caspase-dependent apoptosis in pancreatic carcinoma cells, including primary cultured pancreatic carcinoma cells isolated from tumor specimens, whereas the TRAIL-R1 agonistic antibody lexitumumab was found to require cross-linking for maximal antitumor activity (26). The cooperative antitumor activity of IAP inhibitor and mapatumumab was also observed in an in vivo model of pancreatic cancer (26). Furthermore, cell-permeable Smac peptides containing the first four or seven N-terminal residues of Smac were reported in several independent studies to enhance TRAIL-induced apoptosis in pancreatic carcinoma cells (18, 27, 28).

In addition to pancreatic cancer, IAP inhibitors or Smac mimetics were shown to act in concert with TRAIL or TRAIL-R2 antibody in preclinical in vivo models of colon carcinoma (15, 29, 30). The synergistic interaction of the Smac mimetic BV6 and the agonistic TRAIL-R2 antibody drozitumab was described to occur without the need for autocrine/paracrine TNFα signaling, which has been reported to be critically required for single-agent activity of Smac mimetics (30). Gillissen and colleagues demonstrated that the Smac mimetic LBW242 circumvented TRAIL resistance of Bax/Bak double-deficient colon carcinoma cells (31), supporting previously reported findings showing that genetic or pharmacologic inhibition of XIAP eliminates the requirement of a mitochondrial contribution to TRAIL-triggered apoptosis (14, 15, 30). Furthermore, the Smac mimetic SM-164 was described to cooperate with TRAIL to induce apoptosis in hepatocellular carcinoma cells (32). In cholangiocarcinoma, the Smac mimetic JP1584 was shown to counteract TRAIL-stimulated migration and invasion of tumor cells in vitro as well as metastasis formation in an in vivo model, while no cooperative cytotoxicity of Smac mimetics and TRAIL was found (33).

**Gynecologic malignancies.** Several independent studies demonstrated the antitumor activity of Smac mimetics together with TRAIL in preclinical models of breast cancer. Lu and colleagues reported that the Smac mimetic SM-164 enhanced TRAIL-induced apoptosis in both TRAIL-sensitive and TRAIL-resistant breast cancer cell lines and caused tumor regression together with TRAIL in vivo in a breast cancer xenograft model (29). Furthermore, the Smac mimetic BV6 was described to act in concert with the agonistic TRAIL-R2 antibody drozitumab to trigger apoptosis in breast carcinoma cells and to suppress tumor growth in an in vivo xenograft mouse model (30). Also, this BV6/drozitumab combination treatment triggered apoptosis independently of intact mitochondrial signaling (30), supporting the notion that neutralization of IAP proteins can bypass the mitochondrial pathway to activate effector caspases. Also, synergistic cytotoxicity was described for the Smac mimetic AT-406 and the TRAIL-R2 antibody TRA-8 (34). Bockbrader and colleagues showed that the Smac mimetic compound 3 interacted with TRAIL to stimulate caspase activation and apoptosis in breast carcinoma cells (35). In addition, the Smac peptide N7 or overexpression of Smac was shown to enhance TRAIL-induced cell death using in vitro models of breast carcinoma (36).

In ovarian cancer, the Smac mimetic compound 3 or LBW242 was demonstrated to synergize with TRAIL or the agonistic TRAIL-R2 antibody lexitumumab to stimulate apoptosis in cell lines and primary tumor cells (37, 38). Similarly, the Smac mimetic SM83 and the Smac peptide N7 were shown to sensitize ovarian cancer cells to TRAIL-induced apoptosis and potentiated the antitumor activity in combination with TRAIL in vivo in xenograft mouse models of ovarian cancer (39, 40).

**Additional solid cancers.** The cooperative interaction of Smac mimetics together with TRAIL agonists was also reported in several additional solid cancers. Evaluation of the Smac mimetic AEG40730 in a panel of 51 cancer cell lines revealed that AEG40730 enhanced the sensitivity toward TRAIL across different cancer entities (13). In neuroblastoma and rhabdomyosarcoma, the synergistic activity of IAP inhibitors together with the TRAIL-R1 or TRAIL-R2 antibodies mapatumumab or lexitumumab was described to occur via a RIP1/FADD/caspase-8–containing cell death complex that drives caspase-8 activation and caspase-mediated apoptosis (41, 42). Furthermore, Smac mimetics were demonstrated to prime head and neck squamous cell carcinoma, nasopharyngeal carcinoma, bladder carcinoma or melanoma cells for TRAIL-triggered apoptosis (43–46).

**Smac mimetics in combination with CD95**

Besides acting together with TRAIL receptor agonists, Smac mimetics have been reported to sensitize cancer cells for CD95-induced apoptosis. For example, in ALL subtoxic concentrations of IAP inhibitors were described to synergistically induce apoptosis and to reduce clonogenic survival in combination with agonistic anti-CD95 antibodies or MegaFas ligand, a hexameric form of CD95 ligand, whereas they failed to sensitize normal peripheral blood lymphocytes for CD95-mediated apoptosis (47). The clinical relevance of this combination strategy was underscored by experiments performed in primary leukemic blasts freshly isolated from children with ALL, which similarly showed a synergistic induction of apoptosis of IAP inhibitors and MegaFas ligand (47). Also, the Smac mimetic BV6 was
shown to sensitize Jurkat T-ALL cells that express key components of the death receptor pathway such as FADD or caspase-8 to CD95-mediated apoptosis, whereas FADD- or caspase-8–deficient cells remained resistant to this combination (48). Furthermore, the Smac mimetic BV6 was demonstrated to synergistically stimulate cell death in cancer cells in combination with CD95 ligand independently of TNFα or mitochondrial signaling (30). Also, Geserick and colleagues reported that the Smac mimetic compound A sensitizes squamous cell carcinoma cells to CD95-triggered cell death independently of TNFα signaling, whereas the long isoform of cFLIP protected cells from cell death (49). Moreover, it was demonstrated that the XIAP antagonist 1540-14 significantly enhanced CD95-triggered apoptosis in CLL cells (50).

**Smac mimetics in combination with TNFα**

Smac mimetics as single agents have been shown to engage an autocrine/paracrine TNFα loop via depletion of cIAP proteins and subsequent activation of NF-kB in addition to eliminating the XIAP-imposed inhibition of caspase activation (4–6, 9). Consistently, exogenous application of TNFα was found to act in concert with Smac mimetics to trigger cell death in a variety of cancer cell lines (4–6, 9). A survey of the multitumor activity of the Smac mimetic AEG40730 together with TNFα in a panel of 51 cancer cell lines demonstrated cooperative cytotoxicity by the combination of AEG40730 and TNFα in a substantial proportion of cases (13). In addition, this study showed the key role of cellular FLICE (FADD-like IL1β-converting enzyme)-inhibitory protein (cFLIP) in regulating the synergistic induction of apoptosis by Smac mimetics and death receptor ligands (13). The Smac mimetic birinapant in combination with TNFα was reported to inhibit the growth of melanoma, including a BRAF inhibitor-resistant cell line (51). When birinapant was tested against the panel of childhood leukemia cell lines of the Pediatric Preclinical Testing Program, it was found to prime ALL as well as some solid cancer cell lines for TNFα-induced cell death (52).

In addition to the Smac mimetic–mediated sensitization for apoptosis in combination with TNFα, Smac mimetics have also been reported to potentiate TNFα–induced necroptosis in cancer cells that were resistant to apoptosis, for example due to deficiency in key apoptosis regulatory proteins such as FADD or caspase-8 (48, 53, 54). This Smac mimetic/TNFα–triggered necroptotic cell death occurred in a RIP1- and RIP3-dependent manner independently of caspase activity (48, 53, 54). This indicates that IAP antagonists may bypass some forms of apoptosis resistance by triggering alternative cell death modes.

**Clinical development of Smac mimetics**

Smac mimetics are currently evaluated in phase I/II clinical trials in advanced solid tumor or hematologic malignancies, either as single agent or in combination with anticancer drugs (e.g., paclitaxel, carboplatin, gemcitabine, cyclophosphamide, daunorubicin, and cytarabine). For further clinical development, several key questions remain.

First, which type of Smac mimetic is most suitable to elicit cancer cell death? Various chemically distinct Smac mimetics have been developed over the last decade (reviewed in ref. 3). In principle, they can be divided into monovalent versus bivalent compounds and pan-selective versus cIAP-selective inhibitors. Monovalent compounds are composed of one Smac-mimicking unit, whereas bivalent or dimeric compounds comprise two monovalent Smac-mimicking motifs that are connected via a chemical linker. Bivalent Smac mimetics exhibited a higher potency in vitro and in vivo than monovalent compounds, which has been attributed to their higher binding affinities as well as their ability to bind in parallel to the BIR2 and BIR3 domains of XIAP, leading to simultaneous activation of caspase-3 and -7. This increased potency of bivalent Smac mimetics may also yield more toxic side effects. However, both monovalent (i.e., LCL-161, AT-406, and CIIDC427) and bivalent (i.e., birinapant, HGS1029) compounds were so far found to be well tolerated in clinical trials (55–57), suggesting that both variants may be safe in the clinic. Most Smac mimetics that were developed are pan-selective inhibitors that simultaneously neutralize several IAP proteins such as XIAP, cIAP1, and cIAP2. Antagonizing XIAP-imposed caspase inhibition is considered to be required for potent activation of cell death effector pathways in particular in combination studies. Depletion of cIAP proteins is critical for single-agent activity by engaging an autocrine/paracrine TNFα signaling loop. Indeed, concomitant targeting of several IAP proteins by pan-selective inhibitors was shown to be superior over cIAP-selective compounds for inducing cancer cell death (58).

Second, which Smac mimetic–based combination regimen yields the strongest synergistic effects? Combinations of Smac mimetics together with death receptor ligands such as TNFα, TRAIL, or CD95 ligand proved to be particularly potent to elicit apoptosis in various cancer types. One possible explanation is that Smac mimetics promote death receptor signaling via at least two distinct mechanisms. First, Smac mimetics facilitate activation of caspase-3, -7, and -9 by neutralizing XIAP. Second, Smac mimetics enhance the formation of a cytosolic cell death complex composed of RIP1, FADD, and caspase-8 via reduced ubiquitination of RIP1 upon Smac mimetic–stimulated depletion of cIAP proteins. Although Smac mimetics have been shown to cooperate with standard-of-care treatments such as chemotherapeutics and irradiation, these combinations in general turned out to be less effective compared with death receptor ligands.

Third, which cancer entities are most susceptible to Smac mimetics? Although preclinical studies demonstrated that Smac mimetics inhibit tumor growth in a variety of human cancers, they did not lead to the identification of one or few cancers that exhibit exquisite sensitivity toward Smac mimetics. The identification of Smac mimetic–sensitive cancer entities is also hampered by the current lack of suitable biomarkers that may help to select patients for enrollment into clinical trials.
In summary, Smac mimetics represent promising cancer therapeutics in particular in combination with agents that target the death receptor pathway such as TRAIL agonists. Although this premise has been tested in a variety of preclinical studies, it still remains to be evaluated in clinical trials. Thus, it will be worthwhile to also consider death receptor–targeted agents for the design of future clinical trials with Smac mimetics.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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