Smac mimetics as new cancer therapeutics

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The recent discovery of Smac and the elucidation of its structure and function have led to the rapid development of Smac mimetics, comprising Smac derivative and mimicking molecules, for use in cancer treatment. Smac is an endogenous proapoptotic protein that resides in the mitochondria and is released when a cell is triggered to undergo programmed cell death. One of the mechanisms by which Smac promotes apoptosis is through its ability to inhibit inhibitors of apoptosis (IAPs), by direct inhibition and/or proteasomal degradation of some members of the IAP family, and therefore disinhibit caspases. Thus, the use of Smac mimetics as anticancer agents follows a rational approach in cancer therapeutics. This approach directly targets dysregulated, neoplastic cells that overexpress IAPs or underexpress Smac. Although Smac mimetics are able to elicit an anticancer response when used alone, these molecules can also function effectively and synergistically when combined with other therapeutic agents. A variety of Smac mimetic types comprising peptides, polynucleotides, and compounds have been studied both *in vitro* and *in vivo*. This discussion addresses the current status of Smac mimetics in cancer research.
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Introduction

Cancers resistant to conventional radio-chemoimmune interventions continue to be a challenge in oncology. Novel anticancer interventions are required to overcome tumors with resistant phenotype to increase the therapeutic response and to minimize systemic side effects of a given treatment modality with poor efficacy. As the vast majority of anticancer interventions elicit their response by enhancing the cell's natural machinery of cell suicide [programmed cell death (apoptosis)], the various pathways of programmed cell death have rapidly become targets for chemotherapeutic interventions, largely by enhancing the process of apoptosis as a result of increased cellular stress in the rapidly dividing tissue. For instance, cisplatin causes formation of DNA binding adducts, which prevent DNA replication and lead to increases in p53 and BAX culminating in apoptosis. Small molecules that are capable of penetrating the plasma membrane and bind to antiapoptotic molecules, thereby removing inhibitory signals that render survival, are particularly advantageous. These molecules may act as single agents or in synergy to current therapeutics, which augment their efficacy while minimizing side effects of single modality therapy. Smac mimetics are molecules that echo the action of Smac/direct IAP binding protein with low isoelectric point (pI) (DIABLO) and were introduced in 2004 by Li *et al.* [1]. This report is a summary of the available evidence of the anticancer properties of Smac mimetics and their potential use in cancer therapeutics.

Smac is a mitochondrial protein

Cysteine-aspartic acid proteases (caspases) are central players in cellular apoptosis, and their activation leads to DNA fragmentation, which is the ultimate hallmark of programmed cell death [2]. Thus, their activation is under a substantial degree of regulatory feedback mechanisms. Caspases can be activated by external or internal stimuli through the classical extrinsic or intrinsic apoptotic pathways, respectively [3]. The extrinsic pathway involves the stimulation of death receptors by extracellular ligands resulting in the formation of the death-inducing signaling complex and activation of caspase 8, whereas the intrinsic pathway involves the release of cytochrome *c* from the mitochondria by selective alterations of mitochondrial outer membrane permeability. Once cytochrome *c* release occurs, assembly of the apoptosome ensues by the synergistic interaction of cytochrome *c*, apoptosis protase activating factor-1, caspase 9, and ATP [4]. Both the death-inducing signaling complex and the apoptosome are capable of activating downstream caspases (caspases 3, 6, 7 – executioner caspases), which otherwise rest in the cytosol in the form of an inactive procaspase [5].

As executioner-caspase activation typically leads to programmed cell death, their functional activity is highly regulated by multiple and interrelated pathways. Inhibition of caspases 3, 6, and 7 might occur by the direct action of a family of proteins collectively known as

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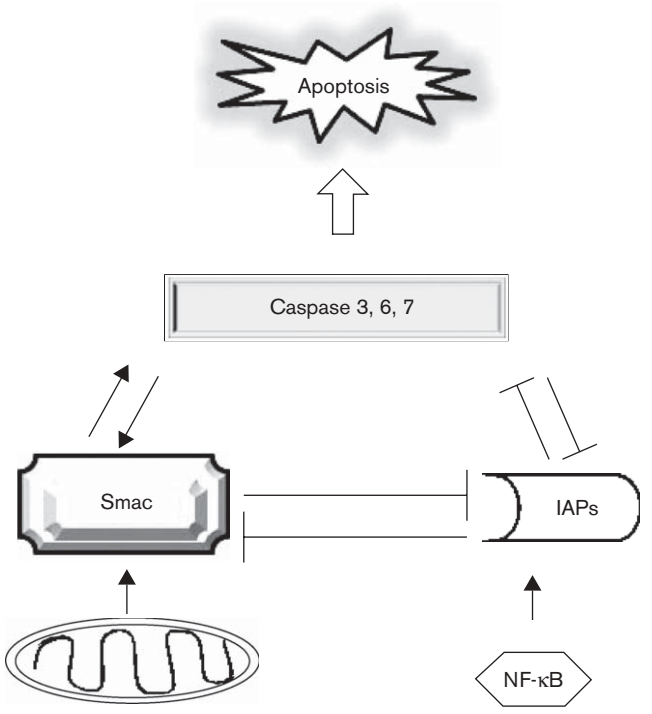
inhibitors of apoptosis (IAPs), which are under the synthetic transcription activity of NF-κB [6]. IAPs function by directly binding to and inhibiting caspases, preventing their proapoptotic protease function [7]. Smac (second mitochondria-derived activator of caspase), a mitochondrial protein, is capable of inhibiting IAPs, therefore freeing IAP interaction with caspases and permitting their activity to progress to apoptosis through disinhibition (Fig. 1) [8].

The discovery of Smac was first published by Du *et al.* [9] in 2000 and, at the same time, by Verhagen *et al.* [9,10], who named the protein DIABLO. Hence, the name Smac/DIABLO is typically assigned to the molecule to credit the work of both groups. For simplicity, we will refer to this molecule as Smac. Smac resides within the mitochondrial intermembrane space, which is subsequently released into the cytosol upon the induction of apoptosis. Smac and other mitochondrial proteins, such as Omi, adenylate kinase-2, cytochrome *c*, and apoptosis-inducing factor, are released into the cytosol through semiselective permeability of the mitochondrial membrane. Bcl-family proteins are pivotal in this process as they may facilitate (i.e. Bid [9,11], Bax and/or Bak [9,12]) or inhibit (such as Bcl-2 or Bcl-x_L [9,13]) the release of apoptotic mediators.

Fig. 1



Interaction between the inhibitors of apoptosis (IAPs), which are under the regulation of NF-κB, and Smac, which is secreted into the cytosol from the intermitochondrial membrane. IAPs bind and inhibit caspases. By binding the IAPs, Smac releases the caspase inhibition leading to stimulation of apoptosis.

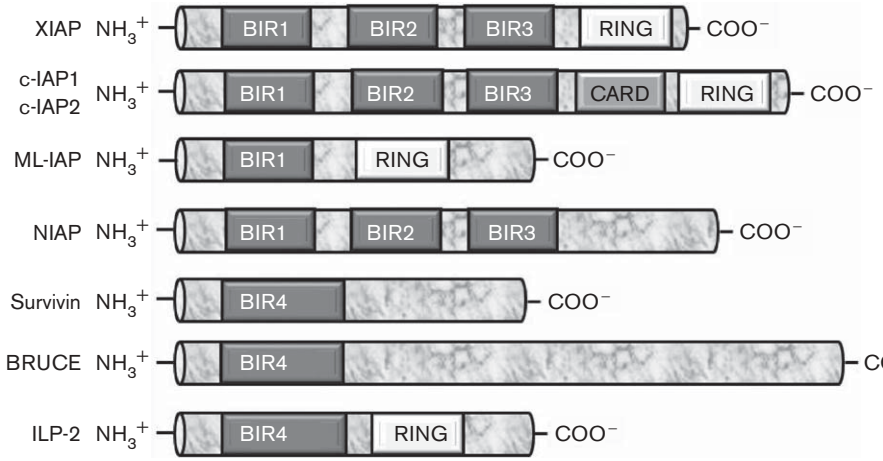
Although mitochondrial membrane alteration can lead to the co-incidental release of Smac and cytochrome *c* [9,14], the interaction of these mitochondrial proteins is also significantly interrelated, as cells deficient in cytochrome *c* are unable to release Smac from the mitochondria even in the presence of Bax [15]. Conversely, Smac release may be necessary for the efficient release of cytochrome *c* [16]. Furthermore, although Smac and cytochrome *c* release from the mitochondria occurs in the intrinsic pathway of apoptosis, complete functioning of the extrinsic pathway may require Bax-stimulated release of Smac as well [17]. This is because of the ability of Smac to remove the inhibitory effects of IAPs on caspases, which may lead to alterations of upstream caspases and upregulation of extrinsic pathway receptors [18]. Thus, although IAPs inhibit apoptosis, mitochondrial release of Smac serves to promote apoptosis by blocking the effects of the IAPs on caspases (Fig. 1) [7].

Proapoptotic mechanisms of Smac

IAPs suppress apoptosis through the inhibition of caspases. This family of proteins generally contains three baculovirus IAP repeat (BIR) domains, and a really interesting gene (RING) zinc finger domain (Fig. 2) [6]. The first and third BIR domains, BIR1 and BIR3, are not required for the inhibition of caspases 3 and 7, whereas the BIR2 domain alone is sufficient in suppressing these caspases, at least for X-linked inhibitor of apoptosis (XIAP) (Fig. 3) [19]. It appears that the linker region between BIR1 and BIR2 plays an important role in the differential inhibition of caspases 3 and 7 [20]. This linker region competitively inhibits both caspase 3 and 7, whereas the BIR2 domain further inhibits caspase 7 in a noncompetitive manner. In contrast, the BIR3 domain is required for effective inhibition of caspase 9 by XIAP [21]. Unlike the direct inhibition of the active site in caspases 3 and 7 by BIR1–2 linker, the BIR3 domain of XIAP blocks caspase 9 activity by binding its inactive monomeric form, without interacting with its active site [22].

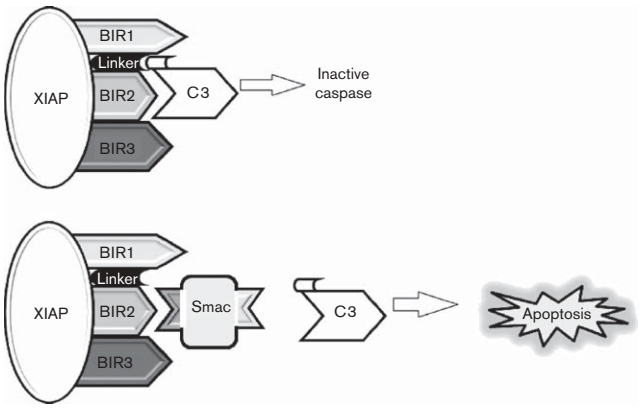
The IAP domains comprising the BIR1–2 linker, BIR2, and BIR3 are integral in their ability to interact with and subsequently inhibit caspases. These regions can also interact with Smac. As Smac can bind many different IAPs (i.e. XIAP, c-IAP1, c-IAP2, and survivin), its cytosolic presence provides a substantial contribution to the apoptotic response [9]. Smac is able to interact with IAPs by binding to BIR2 and BIR3 domains, but not to BIR1 [8]; its aminoterminal segment appears to be indispensable in this interaction with the various BIR domains, particularly for BIR3 [23]. As binding of caspase 9 or Smac to XIAP at its BIR3 domain is mutually

Fig. 2



Schematic representation of the different domains of inhibitors of apoptosis (IAP). IAPs have several tandem repeats known as the baculoviral IAP repeat (BIR), these are separated by a spacer region. Some IAPs also contain a really interesting gene (RING) residue at the carboxy terminal. BIR domains are responsible for caspase interaction and binding of Smac. c-IAP1 and c-IAP2 contain a caspase-recruitment domain (CARD), which is important in caspase activation. BRUCE, BIR containing ubiquitin conjugate enzyme; ILP-2, inhibitor of apoptosis protein (IAP)-like protein-2; ML-IAP, melanoma inhibitor of apoptosis; NIAP, neuronal inhibitor of apoptosis; XIAP, X-linked inhibitor of apoptosis.

Fig. 3



Inhibitors of apoptosis (IAPs) such as X-linked inhibitor of apoptosis (XIAP) inactivate executioner capases such as caspase 3 (C3) through baculovirus IAP repeat (BIR) 2 interaction as well as a linker between BIR1 and BIR2. Smac has a similar binding site to the BIR2 domain and when present in the cytoplasm, it blocks the activity of XIAP on C3 such that it can then initiate apoptosis. Caspase 9 inhibition by XIAP occurs by direct biding of the BIR3 domain to caspase 9. Smac removes this inhibition in a similar manner as for C3.

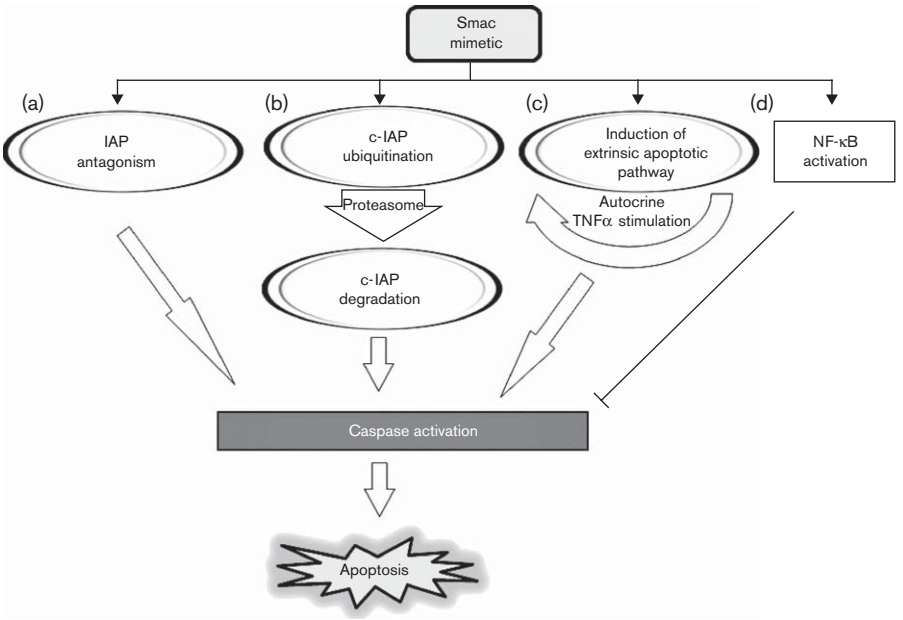
exclusive, Smac interaction with BIR3 prevents XIAP from binding to caspase 9, thus freeing this caspase from inhibition [24]. Smac monomer is only able to interact with BIR2 or BIR3 but not both simultaneously [8]. Effective disinhibition of caspases by Smac still requires the presence of both BIR2 and BIR3 domains on a single XIAP molecule, however, which allows a dimer of Smac to efficiently inhibit XIAP in a 2:1 ratio [25]. Without both of these domains on XIAP, BIR2 or BIR3 fragments are still able to inhibit caspases even in the presence of Smac.

Determination of the specific biochemical structures involved in the IAP–Smac interaction has facilitated the development of Smac-like molecules. The crucial *N*-terminus of Smac has four residues (Ala-Val-Pro-Ile) that contact the BIR3 domain of XIAP, and this structure is stabilized by electrostatic and hydrophobic forces [26,27]. Once this structure was elucidated, the development of Smac mimetics, including effective nonpeptic mimetics that have enhanced bioavailability and stability [26,28], provided a variety of molecules that could serve as IAP antagonists.

In addition to simply binding and inhibiting IAPs, Smac and its monovalent or bivalent mimetics may even alter levels of certain IAPs. For example, IAP antagonists enhance autoubiquitination of c-IAP1 and c-IAP2 and result in proteasomal degradation of these two inhibitors of apoptosis (Fig. 4) [29,30]. However, levels of other molecules such as XIAP remain unchanged until apoptosis is induced and caspases such as 3 and 8 are activated. Degradation of c-IAP1 and c-IAP2 requires the presence of BIR2 and BIR3 domains for IAP antagonist binding, and also of the RING domain for ubiquitin E3 ligase activity. Mutations in any of these regions render IAP antagonists incapable of altering levels of c-IAP1 or c-IAP2 through this ubiquitination-elimination pathway.

Furthermore, Smac and Smac mimetics increase tumor necrosis factor alpha (TNFα) mRNA expression as well as expression of other NF-κB-regulated genes such as

Fig. 4



Smac mimetics function through multiple mechanisms: (a) binding and antagonizing inhibitors of apoptosis (IAPs) is the classical pathway by which Smac inhibits IAPs; (b) promoting autoubiquitination and proteasomal degradation of c-IAPs is another mechanism that accounts for IAP elimination culminating in stimulation of the caspases; (c) activation of the extrinsic pathway of apoptosis occurs by increased activity of tumor necrosis factor alpha (TNFα) and autocrine secretion, which then activates the death receptor pathway of apoptosis leading to downstream caspase activation; (d) paradoxically, Smac mimetics also stimulate the survival pathway of NF-κB, depending on the phenotype of the cell, the pathways that predominantly dictate the magnitude of the apoptotic response either by administration of Smac mimetics alone or in combination with other compounds.

MCP-1 and IL-8. The upregulation of NF-κB is accomplished by activation of both canonical and noncanonical NF-κB pathways [29,30]. IAP antagonists disinhibit NF-κB by recruiting RIPK1 to TNF receptor-1 (TNF-R1), which results in phosphorylation and proteasomal degradation of IκB, which would otherwise inhibit nuclear translocation of NF-κB. IAP antagonists also disinhibit NF-κB by preventing c-IAP degradation of NIK. Increased levels of NIK activates NF-κB through the noncanonical pathway. Thus, NF-κB increases with IAP antagonists in a dose-dependent manner and results in increased TNFα production. The proapoptotic effects of IAP antagonists depend specifically on functional TNFα death receptors, as opposed to other death receptors such as DR5 or Fas (Fig. 4) [29,30]. Suppressing any level of the TNFα signaling pathway – inhibiting TNF-R1, TNFα, or NF-κB – blocks induction of apoptosis by IAP antagonists. Ultimately, the molecules responsible for the apoptosis that follows autocrine TNFα secretion include RIPK1, FADD, and caspase 8 [31]. These three components form a single RIPK1–FADD–caspase-8 complex that is required for cell death because of IAP antagonists and TNFα. This dependence on the extrinsic rather than intrinsic pathway of apoptosis is highlighted by the fact that cells treated with IAP antagonists are rescued by small interference RNA knockdown of caspase 8 but not caspase 9 [32].

In summary, Smac and its mimetics cause rapid degradation of c-IAP1 and c-IAP2, which allows activation of NF-κB and associated production of TNFα. Autocrine TNFα signaling promotes RIPK1–FADD–caspase-8 complex formation and subsequent activation of caspase 8 and downstream executioner caspases resulting in apoptosis. IAP antagonists result in apoptosis by multiple converging mechanisms. The various mechanisms that lead to caspase activation are depicted in Fig. 4. The current state of Smac mimetic use in cancer therapy is the focus of this review.

Smac expression in cancer

The role of Smac in cancer has been reviewed earlier [33]. Although cancer does not always involve defects in the apoptotic pathway with direct upregulation of IAPs and/or downregulation of Smac, there are some cases where such dysregulation certainly plays a role [34]. For instance, when there is upregulation of the IAPs with concomitant Smac downregulation, as it occurs in human lung cancer H446 cells after treatment with fibroblast growth factor 2 (FGF-2), apoptosis is significantly inhibited [35]. FGF-2 is thought to reduce apoptosis in these cancer cells, in part, by inducing expression of survivin and by blocking mitochondrial release of Smac into the cytoplasm. In addition, elevated serum concentration of basic FGF, suggesting decreased Smac release,

predicted poor prognosis in patients with small lung cell cancer [36].

The prognostic value of downregulated Smac has also been observed in renal cell carcinoma (RCC), where low or no Smac expression predicted an unfavorable prognosis [37]. Other IAP antagonists released from the mitochondrion, such as Omi/HtrA2, were not associated with outcomes in patients with RCC, which underscored the predictive potential of Smac independent of other proapoptotic molecules released by the mitochondria [38]. Overexpression of Smac in gastric cancer sensitized cells to chemotherapeutic agents [39]. Similarly, increased levels of Smac sensitized osteosarcoma cells and hepatocellular cancer cells to chemotherapeutic drug-induced apoptosis [40]. NSAID-mediated apoptosis was prevented by silencing the Smac gene by small interference RNA studies in HTC116 colon cancer cells [41]. Cervical cells (HeLa) transfected with Smac were more sensitive to apoptosis induced by X-ray irradiation compared with control [39]. Smac sensitized neuroblastoma, glioblastoma, and pancreatic cancer cells to γ-irradiation-induced apoptosis without involvement in the DNA damage/repair mechanisms or activation of p53, p21, or NF-κB mechanisms [42]. Complete eradication of HepG2 xenografts was observed by in-vivo interference of Smac with an adenovirus Smac followed by 5-FU [40]. The prognosis of patients with lung cancer was worse in a cohort of smokers with low expression of Smac as determined by real-time reverse-transcriptase PCR [43]. Smac expression inversely correlated with the grade of RCC. Patients with RCC expressing Smac had a longer postoperative disease-specific survival than those without Smac expression at a 5-year follow-up [37]. When the chemotherapeutic doxorubicin was administered to the multiple myeloma cell line RPMI 8226 cells, there was a rise in apoptotic cell death stimulated by a gradual decline in IAP expression, upregulation of Smac, and increased association of Smac with existing IAPs [44]. Doxorubicin-resistant multiple myeloma DDR cells, however, did not show a significant decrease in IAP expression and did not have increased IAP suppression by Smac after doxorubicin treatment. Thus, the ability of chemotherapeutic drugs to modify levels of Smac can be a critical factor in the treatment of some forms of cancers with particular alteration of the NFκB-IAP axis.

Current status of Smac mimetics cancer therapy

Over the past few years, evidence for the use of Smac mimetics in the treatment of cancer continues to accumulate. Preclinical evidence *in vitro* and *in vivo* is beginning set the stage for Smac mimetics to enter phase I clinical trials, though currently there are none reported in *ClinicalTrials.gov*. At this stage, the use of Smac mimetics is still considered experimental. Several lines of evidence support the current utility of various types of Smac mimetics – (i) peptides, (ii) polynucleotides, and (iii) compounds – as potential anticancer agents in multiple cancers from hematologic malignancies to solid tumors (Table 1). One of the advantages of using Smac mimetics is that they can assist in overcoming resistance to conventional anticancer therapies, especially when resistance occurs through alterations in the NFκB-IAP pathway. Smac mimetics can induce cell death when used alone, and combination therapy often results in additive and synergistic activation of proapoptotic molecules. However, the response of different cancer types is varied and not all cancers are successfully treated with Smac mimetics. Their effectiveness may be primarily limited to cancers with aberrant apoptotic pathways. This section will describe the application of Smac-mimetic peptides, polynucleotides, and compounds to a variety of cancer types and discuss how each cancer responds to these molecules.

Smac mimetics: peptides

A fusion peptide of the last four to eight *N*-terminal residues of Smac combined with penetratin, a carrier peptide from *Drosophila* with little toxicity to cells, inhibited IAP interaction with caspases 3, 7, and 9 and enhanced apoptosis induced by paclitaxel, etoposide, SN-38, and doxorubicin in MCF-7 breast cancer cells [45]. Mutational alterations of the *N*-terminal alanine residue precluded sensitization by the Smac-mimetic fusion peptide. Administration of this mutated form of Smac did not induce apoptosis in breast cancer cells. The combined treatment of Smac mimetic and paclitaxel increased apoptosis than treatment with paclitaxel alone. Clonogenic assays revealed that there was a dose-dependent response, where higher doses resulted in greater percent apoptosis, and that a six Smac-residue fusion peptide was the most effective length. These Smac mimetics were effective in other cell lines as well, such as T47D breast cancer cells [45]. Pretreatment with 80 μg/ml of the eight-Smac-residue form of the fusion peptide followed by 25 nmol/l paclitaxel resulted in significant induction of apoptosis in T47D cells.

Similarly, treatment with 10 μmol/l of the *N*-terminal 4 or 7 amino acids of Smac augmented epothilone B and Apo-2L/tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-induced apoptosis and caspase protease activity in human acute leukemia Jurkat T cells [46]. This increase in apoptosis was accompanied by down-regulation of XIAP, c-IAP, and survivin, and commensurate activation of caspases 3 and 8.

Autoubiquitination and proteasomal degradation of IAPs, c-IAP1, and c-IAP2 in cervical cancer HeLa cells occurred when the *N*-terminus of Smac bound to the BIR domains of these IAPs [47]. Smac-6, a hexapeptide comprising the

Table 1 **Smac-mimetic design and utility**

Name/description	Cancer	Dose	Combined with
Peptides			
4–8 *N*-terminal residues of Smac fused to penetration	MCF-7 breast cancer cells	20, 40, or 80 μg/ml	Paclitaxel, etoposide, SN-38, or doxorubicin
4 or 7 *N*-terminal residues of Smac	Human acute leukemia Jurkat T cells	10 μmol/l	Epothilone B derivative (BMS 247550) or Apo-2L/TRAIL
6 *N*-terminal residues of Smac	HeLa cervical cancer cells	0.1–6.0 μmol/l	None
7 *N*-terminal residues of Smac	L428 and L540 human Hodgkin lymphoma cells	10 μmol/l	Granzyme B
7 *N*-terminal residues of Smac with antenopedia sequence	Caki-1 renal carcinoma cells	200 μmol/l	None or cisplatin
7 *N*-terminal residues of Smac conjugated to 8 arginine residues	Suit-2, CFPAC-1, Panc1, and BxPC-3 pancreatic cancer cells	50 μmol/l	TRAIL
	(a) H460 non-small-cell lung cancer, (b) H460 xenograft mouse model	(a) 10, 20, 50 μmol/l, (b) 10 μmol/l	(a) Cisplatin or taxol, (b) cisplatin
7 *N*-terminal residues of Smac conjugated to the protein transduction domain of TAT protein	(a) SHEP/Bcl-2 neuroblastoma, SH-SY5Y neuroblastoma, Mel-HO melanoma, Panc1 pancreatic carcinoma, (b) U87 MG human malignant glioma xenograft mouse model	(a) 500 μmol/l, (b) 1 mg	(a) TRAIL or doxorubicin, (b) TRAIL
Polyvalent naïve phage-derived peptide sequences based on *N*-terminus of Smac with natural and unnatural amino acid substitutions	ML-IAP-overexpressing melanoma cell lines	3–5 nmol/l	None
Polynucleotides			
Flag-C-tagged mature Smac pcDNA3.1	K562 and CEM human leukaemic cells	6 μl for 2e6 cells	Ultraviolet light or TRAIL
Flag-C-tagged full-length Smac pcDNA3.1	NC65 and Caki-1 renal carcinoma cells	0.5 ml	TRAIL or cisplatin
	Human acute leukemia Jurkat T cells	0.5–1.0 μg for 2–3e6 cells	Epothilone B derivative (BMS 247550) or Apo-2L/TRAIL
Compounds			
Compound 3 Bivalent, small molecule mimic of the 4 *N*-terminal residues of Smac	(a) T98G glioblastoma cells, (b) HeLa cervical cancer cells	(a) 100 nmol/l, (b) 100 nmol/l	(a) TRAIL, (b) TNFα
	H460 and SW1573 non-small-cell-lung cancer cells	1 μmol/l	Cisplatin
	(a) A2780, A2780/DDP, and A2780/ADR ovarian cancer cells, (b) cells from 10 patients	(a) 100 nmol/l, (b) 100 nmol/l	TRAIL
	MDA-MB-231 mammary gland adenocarcinoma cells	IC_{50} – 3.8 nmol/l	None, TRAIL, or etoposide
	(a) 50 non-small-cell lung cancer cells, (b) HCC461 xenograft mouse model	(a) 100 nmol/l for sensitive cell lines (HCC44 and HCC461), (b) 5 mg/kg	None
	HCT116, HT29, and DLD1 colorectal cancer cells	100 nmol/l	NSAIDs
Compounds 2 and 11 Capped tripeptide compounds resembling the *N*-terminus of Smac and containing unnatural amino acids	(a) MDA-MB-231 mammary gland adenocarcinoma cells, (b) MDA-MB-231 xenograft mouse model	(a) 1 μmol/l or 10 nmol/l, (b) compound 2–40 mg/kg/day, compound 11–20 mg/kg/day	None
SM-164 Bivalent, nonpeptide, cell-permeable	HL-60 human leukemia cells	IC_{50} – 1.39 nmol/l	None
LBW242 Low-molecular-weight oligopeptide	(a) Murine pro-B Ba/F3 cells, (b) FLT-3-ITD-Ba/F3 xenograft mouse model	(a) IC_{50}–0.5 to >1 μmol/l, (b) 50 mg/kg/day	None or PKC412
	(a) MM.1S, U266, RPMI-8226, OPM2, MM.1R, and Dox-40 multiple myeloma cells, (b) purified patient tumor cells who relapsed after prior therapy, (c) MM.1S xenograft mouse model	(a) IC_{50}–8–30 μmol/l, (b) 20 and 30 μmol/l, (c) 35 mg/kg orally	None, TRAIL, melphalan, dexamethasone, bortezomib, or salinosporamide
	SKOV3 ovarian cancer cells	20 μmol/l	None
Compound A Designed from crystal structure of Smac-bound XIAP	(a) Kym1 human rhabdomyosarcoma cells, (b) SKOV3 ovarian cancer cells	(a) 5 nmol/l, (b) 500 nmol/l	None
MV1 and BV6 Novel monovalent and bivalent IAP antagonists	EVSA-T and MDA-MB-231 breast cancer and A2058 melanoma cells	IC_{50}–5 μmol/l for MV1, 14 nmol/l for BV6	None

IAP, inhibitor of apoptosis; IC_{50}, half maximal inhibitory concentration; TNFα, tumor necrosis factor alpha; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; XIAP, X-linked inhibitor of apoptosis.

six *N*-terminal residues of Smac, enhanced ubiquitination of c-IAP1, producing a dose-dependent response using intervals ranging from 0.1 to 6.0 μmol/l for 2 h of incubation, and mutation of the alanine residue in the conserved AVPI sequence inhibited this enhancement. Deletion of BIR1–3 domains or mutation of the RING zinc finger domain of c-IAP1 also decreased the ability of Smac to promote ubiquitination of c-IAP1. These results suggested that the BIR domains normally inhibited the RING domain's E3 activity, and this inhibition was removed upon binding of the Smac *N*-terminus to the BIR domains. Once the IAPs were targeted for degradation, caspase-mediated apoptosis was facilitated [47].

Granzyme B-induced apoptosis was restored by incubating the otherwise resistant L428 and L540 Hodgkin lymphoma cell lines with Smac N7, the seven *N*-terminal residues of Smac peptide [48]. These two cell lines were resistant to granzyme B released from cytotoxic T lymphocytes because of XIAP overexpression coupled with dysfunctional mitochondrial release of Smac. Cytosolic Smac N7 treatment overcame this resistance and resensitized the Hodgkin lymphoma cells to killing by cytotoxic T lymphocytes thereby reversing the phenotype related to altered Smac and XIAP in these cells [48].

Caki-1 renal cancer cells were resistant to chemoradiotherapy, but were sensitized to cisplatin-induced apoptosis using concurrent treatment of a Smac mimetic [49]. Of the renal cell carcinoma cell lines used, the Caki-1 cells had the highest levels of XIAP. This elevated expression of XIAP was overcome by exposing the renal cancer cells to Smac-Ant peptide, a Smac mimetic comprising the last seven residues of the *N*-terminus of Smac and an antenopedia sequence to assist in cellular incorporation. Treatment with this peptide alone decreased cell viability significantly in a dose-dependent manner, and there was an even greater effect when this Smac mimetic was combined with cisplatin.

A Smac mimetic comprising the seven *N*-terminal residues of Smac conjugated to eight arginine residues, which enhanced cell membrane translocation, significantly increased TRAIL-induced apoptosis over control group in multiple pancreatic cancer cells lines [50]. In this experiment, a TRAIL-resistant cell line, AsPC-1, which overexpresses FLICE-inhibitory protein (FLIP)-S was not sensitized after Smac adminstration. There was enhancement, however, when FLIP antisense was added in the presence of TRAIL and the Smac mimetic to the resistant AsPC-1 cells, and this increased apoptosis was greater than the simple addition of the effects of FLIP antisense alone and the effects of Smac mimetic alone [50]. This finding underscores the role of the extrinsic pathway modulation by Smac mimetics (Fig. 4).

The same 8-arginine-conjugated Smac mimetic was able to promote apoptosis in chemoresistant non-small-cell lung cancer H460 cells and *in vivo* [51]. H460 cells showed normal expression of apoptosome constituents, but additionally had overexpression of XIAP that inhibited caspase 9 activity. Smac-mimetic treatment reversed this inhibition and sensitized H460 lung cancer cells to cisplatin or taxol-induced apoptosis *in vitro* in a dose-dependent manner. Similarly, Smac-mimetic treatment *in vivo* sensitized xenografts in nude mice to cisplatin-induced apoptosis without any noticeable side effects in mice receiving combination treatement. Cotreatment with the Smac mimetic and taxol was more effective than single modality treatment [51].

These finding have been replicated in other xenograft models, where the right striatum of CD1 nu/nu athymic mice was stereotactically implanted with U87MG malignant glioma cells. In this model, treatment with a Smac mimetic resulted in sensitization of the tumor cells to TRAIL-induced apoptosis. Complete eradication of implanted tumors was achieved in addition to an increased mouse survival without any evidence of neurotoxicity [52]. In this experiment, the Smac mimetic was synthesized by Interactiva GmbH (Sedanstr, Ulm, Germany) to consist of seven *N*-terminal residues of Smac conjugated to the TAT-protein domain responsible for protein transduction, to assist intracellular delivery, and was administered twice locally.

In addition, cell lines resistant to apoptosis because of aberrant apoptotic signaling pathways, including SHEP neuroblastoma cells (Bcl-2 overexpressing), SH-SY5Y neuroblastoma cells (caspase 8 deficient), Mel-HO melanoma cells (apoptosis protase activating factor-1 deficient), and Panc1 pancreatic carcinoma cells (Ras/PI3 Kinase/Akt mutated), were sensitized to TRAIL or doxorubicin treatment in the presence of the TAT-conjugated Smac mimetic [52]. Treatment of these cells with the Smac mimetic alone did not result in apoptosis. This emphasized that overcoming resistance in cells with altered apoptotic pathways required the synergistic activity of a second proapoptotic agent in addition to the Smac mimetic. Yet, sensitization was possible in these highly resistant cells.

Phage-derived Smac mimetics that resemble the *N*-terminus of Smac can selectively target the upregulated melanoma inhibitor of apoptosis (ML-IAP). As its name implies, overexpression of this IAP occurs, almost exclusively, in melanoma cell lines [53]. Analysis of a library designed from naïve peptides showed that certain peptide sequences had different biding affinity and specificity to the single BIR domain of ML-IAP versus the BIR3 domain of XIAP. This library was created using a phagemid vector and *E. coli* and additional peptides were synthesized with various natural and unnatural amino acid substitutions. In certain cases, changing the proline residue of the Smac-based peptide sequence AVPIAQKSE to (3S)-methyl-proline gave this new peptide 100 times more specificity for ML-IAP when compared with XIAP and also increased its affinity for ML-IAP. Polarization-based competition experiments involved the addition of 1 μmol/l of ML-IAP-BIR, 1–2 μmol/l of XIAP-BIR3, or 30 μmol/l of XIAP-BIR2 to 3–5 nmol/l of Smac-derived peptides that were conjugated to a 5-carboxyfluorescein-label (Smac-FAM).

This type of library design underscores the ability of developing targeted specific therapies. As ML-IAP is not expressed in most normal cells, these Smac resembling

peptides decrease the possibility of nonspecific cytotoxicity and allow for selective targeting of melanoma cells that overexpress XL-IAP.

Smac mimetics: polynucleotides

In leukemia cell lines K562 and CEM, transfection with 6 μl of *E. coli* plasmid DNA containing mature Smac increased their sensitivity to both UV light-induced and TRAIL-induced apoptosis and increased activation of caspases 3 and 9 [54]. This enhancement was the result of Smac activation on both the extrinsic and intrinsic pathways of apoptosis. In addition, mature Smac-transfected leukemia cells showed decreased proliferation and increased G0/G1 arrest of the cell cycle – determined by colony formation, Ki-67 protein expression, and BrdU incorporation – suggesting an interrelated mechanism between apoptosis and cell cycle proteins.

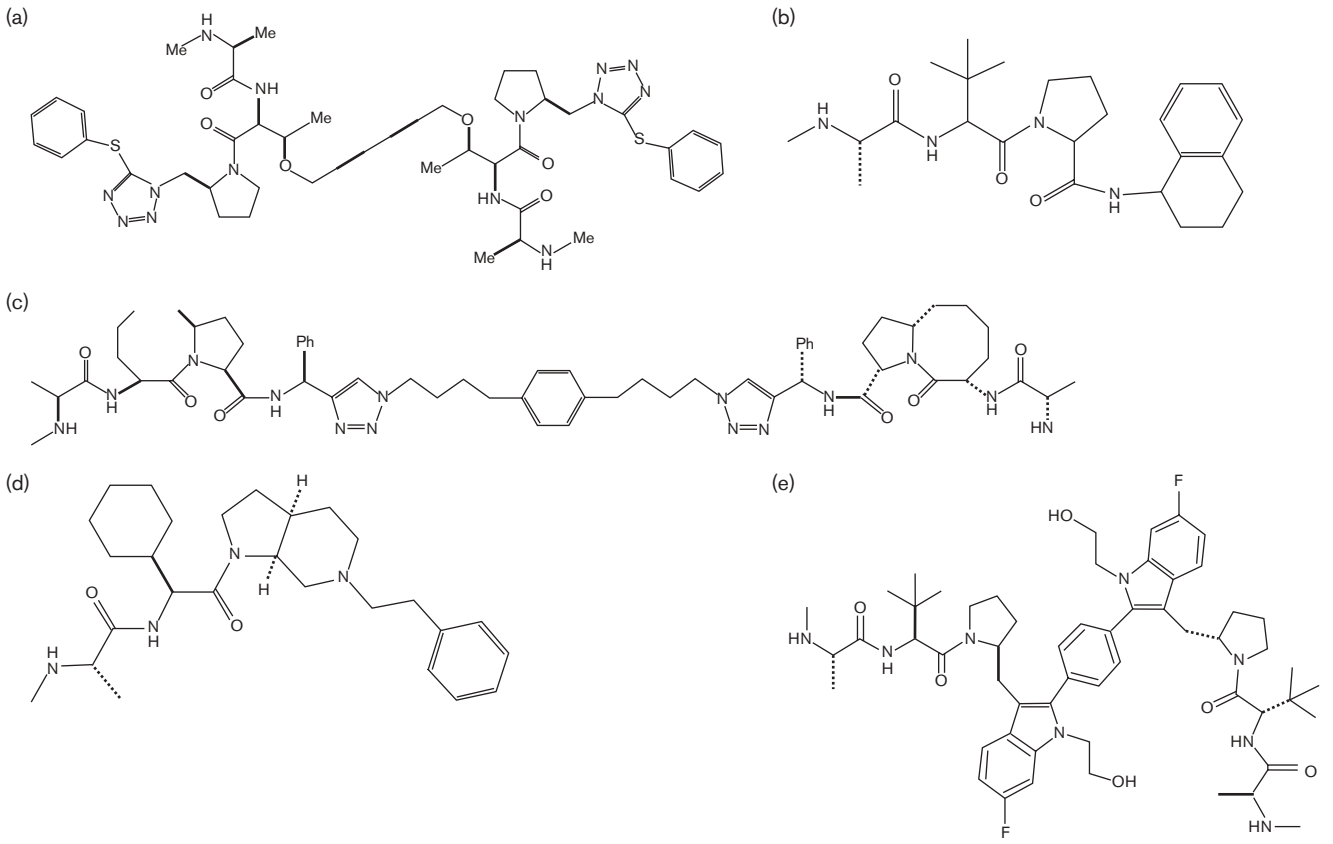
Transient transfection of renal carcinoma NC65 and Caki-1 cells with 0.5 ml of pcDNA3.1-Smac using LipofectAMINE 2000 sensitized both of these cell lines to TRAIL-induced apoptosis, and NC65 cells to cisplatin-induced apoptosis. Transfection of Smac cDNA alone, however, did not produce an effect on growth.

Similarly, transfection of human acute leukemia Jurkat T cells (using LipofectAMINE PLUS reagent) with 0.5–1.0 μg of plasmid DNA containing full-length Smac produced effects similar to adding Smac mimetic peptides to these cells, that is, it enhanced epothilone B and Apo-2L/TRAIL induction of apoptosis and caspase protease activity [46]. Caspases 3 and 8 were activated with associated suppression of XIAP, c-IAP, and survivin.

Smac mimetics: small molecules

Compound 3 is a bivalent, small-molecule symmetric diyne that resembles a Smac dimer and mimics the four *N*-terminal residues of Smac (Fig. 5a) [1]. Human glioblastoma T98G cells had a higher degree of apoptosis when treated with compound 3 and TRAIL compared with TRAIL alone. A similar effect was observed in cervical cancer HeLa cells, where co-treatment with

Fig. 5



Schematic representation of Smac mimetic: small molecules. Compound 3 (a) is a bivalent, small-molecule symmetric diyne that resembles a Smac dimer and mimics the four *N*-terminal residues of Smac (a). Compound 2 is shown in (b). Compound 11 has the same chemical structure but has a cyclohexyl group instead of a tert-butyl group (b). SM-164 is a small-molecule mimic of Smac that is nonpeptic and has cell-permeable capabilities (c). LBW242 is a low-molecular-weight Smac mimetic (d). Compound A (e) is a Smac mimetic, designed from the crystal structure of the baculovirus IAP repeat (BIR) 3 of X-linked inhibitor of apoptosis (XIAP) bound to the four amino acids of Smac, which is capable of binding the BIR2 and BIR3 domains of XIAP, c-IAP1, and c-IAP2 (e).

compound 3 and TNFα synergistically induced apoptosis [1]. For both the T98G and HeLa cell lines, compound 3 was administered 4 h before the addition of either TRAIL or TNFα, respectively.

Compound 3 also enhanced cisplatin-induced apoptosis in H460 and SW1573 non-small-cell lung cancer cell lines [55]. The combination of cisplatin and Smac mimetic (1 μmol/l) resulted in an augmentation of apoptosis in a dose-dependent manner with regard to cisplatin. The apoptotic response was more pronounced with the combination of cisplatin and Smac mimetic versus cisplatin alone or Smac mimetic treatment alone. The increase in apoptosis was the result of an augmentation in the activity of caspase 3, without affecting the role of caspase 9 from XIAP disinhibition, which underscored the various mechanisms of action of Smac mimetics (i.e. autoubiquitination of cIAPs).

Compound 3 induced apoptosis both alone and in combination with TRAIL in the ovarian cancer cell lines A2780, A2780/DDP, and A2780/ADR [56]. The combination of both molecules produced an effect greater than single modality treatment. In addition, the combination of Smac mimetic and TRAIL produced significant activation of caspases 3, 8, and 9 and cleavage of PARP-1. Similarly, when these two molecules were applied for 24 h to ovarian cancer cells isolated from 10 different patients, the combination of 100 nmol/l Smac mimetic and 50 ng/ml TRAIL produced significantly more apoptosis when compared with either treatment alone. Although the overall effect was significant, there was notable heterogeneity in the rate of induced-apoptosis among the 10 patient tumor isolates. Thus, the individual phenotype of the cell dictates both the magnitude of the response and the mode of action of the Smac mimetic (i.e. caspase 3 mediated vs. extrinsic pathway or a combination of these as noted by activation of both caspases 8 and 9).

Similar observation with compound 3 occurred in MDA-MB-231 breast cancer cell, where viability was decreased by single modality treatment or in combination with either TRAIL or etoposide [57]. Treatment of MDA-MB-231 cells with 0.1–100 nmol/l for 24 h using the Smac mimetic alone suppressed cell growth in a dose-dependent manner, with an half maximal inhibitory concentration (IC_{50}) of 3.8 nmol/l. When these cells were treated with either 30 ng/ml of TRAIL alone or 5 μmol/l of etoposide alone, no obvious apoptosis resulted. However, addition of small concentrations of compound 3 (2.5 nmol/l) to TRAIL or etoposide produced significant DNA fragmentation and apoptosis through caspase 3 activation [57].

Although small doses of compound 3 effectively synergized the activity of TRAIL in high-IAP-expressing MDA-MB-231 cells, low-IAP-expressing breast cancer (T47D and MDA-MB-453) cells showed greater resistance and were not inhibited by the Smac mimetic even at substantially higher doses of compound 3 (50 nmol/l) [57]. Only mild synergy was observed in low-IAP-expressing breast cancer cells with the combination of compound 3 and either etopopside or TRAIL. In MDA-MB-453 cells, relatively higher synergy was produced by the TRAIL and Smac mimetic combination, whereas less synergy was produced by etoposide combined with Smac mimetic. This finding indicated that, in this system, the extrinsic pathway of apoptosis induced by TRAIL played a more prominent role compared with the direct role of Smac mimetics in IAP inhibition.

Compound 3 induced apoptotic cell death in human non-small-cell lung cancer cells when used alone [31]. Of 50 lung cancer cell lines examined, cell survival assay for IC_{50} determination showed that *22%* had a positive response to up to 100 nmol/l of Smac-mimetic treatment, whereas the remaining 78% had no response even at the maximal 100 nmol/l concentration.

In Harlan Athymic Nude-Foxnlnu mice with HCC461 xenografts, Smac-mimetic injections significantly reduced tumor size. Xenografts of nonresponsive HCC15 cells were resistant to Smac-mimetic injections. Autocrine-secreted TNFα in these cancer cells, which would normally enhance their survival through the NF-κB pathway, can be reversed to a proapoptotic molecule by Smac mimetics by the formation of the RIPK1–FADD–caspase-8 complex that promotes caspase 8 activation and therefore apoptosis [31].

Colon cancer HCT116, HT29, and DLD1 cells were sensitized to NSAID-induced apoptosis by compound 3 and by GT-T, an independently synthesized Smac mimetic with properties similar to compound 3 [58]. The control compounds that differed only by a single residue did not show this enhancement. Treatment of these colon cancer cells with either low-dose NSAIDs or the Smac mimetic alone did not yield significant apoptotic effects. However, combination treatment using 100 nmol/l Smac mimetic was able to potentiate NSAID-induced apoptosis and also inhibited long-term survival in these cells, as determined by colony formation assays. Furthermore, NSAID resistance in Smac-knockout or Bax-knockout cells could be overcome by Smac-mimetic treatment. These effects of the Smac mimetic were a result of binding and inhibition of IAPs thereby allowing for caspase 3 activation. Caspase 3 activity was subsequently necessary for Smac-mediated cytochrome *c* release.

In addition to compound 3, treatment with capped tripeptide Smac mimetics, compounds 2 and 11 (Fig. 5b), which resemble the *N*-terminus of Smac and contain

unnatural amino acids, resulted in increased caspase 3 activity and apoptosis by binding to BIR3 of XIAP in mammary gland adenocarcinoma MDA-MB-231 cells [59]. This response was dose dependent as demonstrated by the decreased survival of breast cancer cells with increasing doses of Smac-mimetic compounds. Cells were incubated for up to 30 h (showing maximal caspase 3 activation after 12–18 h) after exposure to 1 or 10 nmol/l concentrations of the BIR3-binding Smac mimetic (compound 11; Fig. 5b). Compound 2 (Fig. 5b), also a BIR3 ligand, showed a similar activity to compound 11 but with slightly less potency. Compounds 11 and 2 promoted cell death in 58 human cancer cell lines.

In right flank, MDA-MB-231 inoculated, female C.B-17-*Prkdc*^{scid} mice, Smac-mimetic treatment was able to significantly reduced tumor growth rate. Doses of 40 mg/kg/day for compound 2 and 20 mg/kg/day for compound 11 were used over the dosing period of days 14–30. Both compounds significantly inhibited tumor growth in the mouse model, with compound 2 being slightly less potent than compound 11. The decrease in tumor growth rate was observed only during treatment and tumor growth resumed after terminating therapy.

SM-164 is a small-molecule mimic of Smac that is nonpeptic and has cell-permeable capabilities (Fig. 5c). This molecule has been shown to effectively cause apoptosis in the HL-60 (human promyelocytic leukemia) cell line independently without the need for another apoptosis inducer [60]. SM-164 initiated apoptosis and decreased leukemia cell growth in a dose-dependent manner (IC_{50} value of 1.39 nmol/l) by strongly inhibiting XIAP and promoting caspase activation. SM-164 is bivalent and thus binds to both BIR2 and BIR3 domains. This characteristic confers higher potency to SM-164 when compared with monovalent counterparts that bind to only BIR2 or BIR3 individually but not both.

The low-molecular-weight Smac-mimetic LBW242 (Fig. 5d) was able to inhibit cellular proliferation (IC_{50} values from 0.5 to > 1 μmol/l depending on the cell line) and acted with positive synergy with the protein tyrosine kinase inhibitor PKC412 in murine pro-B Ba/F3 cells with mutated Fms-like tyrosine kinase-3, a mutation in acute myelogenous and acute lymphoblastic leukemia patients [61]. Both PKC412-sensitive cell lines and PKC412-resistant Ba/F3 cell lines were treated with LBW242 and PKC412 across dose ranges of effective dose 50–90, where higher doses tended to produce additive or synergistic effects. In addition, male NCr-nude mice inoculated with FLT-3-ITD-Ba/F3 cells through tail vein injection were orally administered Smac-mimetic LBW242 at 50 mg/kg/day, PKC412 at 40 mg/kg/day, or both LBW242 and PKC412 over 10 days, which showed that tumor burden, determined by bioluminescence, was least for the combined treatment [61].

Moreover, treatment using LBW242 over a 48-h period induced apoptosis in the MM.1S, U266, RPMI-8226, and OPM2, as well as in the conventional therapy resistant MM.1R and Dox-40, multiple myeloma cell lines [62]. Cell viability decreased in a dose-dependent manner (IC_{50} ranging from 8 to 30 μmol/l) where higher doses resulted in the fewest viable cells. In addition, purified multiple myeloma cells taken from four patients who were refractory to various therapies (dexamethasone, bortezomib, or thalidomide) also showed decreased cell viability because of LBW242-induced apoptosis. LBW242 used in combination with TRAIL, melphalan, dexamethasone, bortezomib, or salinosporamide resulted in an additive or synergistic apoptotic effect in multiple myeloma cells [62].

Normal lymphocytes were analyzed to determine toxicity. These cells were not affected by low doses (0–10 μmol/l) of LBW242, but showed decreased viability at higher doses. Using an in-vivo xenograft mouse model, where male triple immune-deficient beige-nude-scid mice were subcutaneously inoculated with MM.1S cells, LBW242 treatment inhibited tumor growth and prolonged mouse survival without significant weight loss or neurologic changes [62].

Ovarian cancer SKOV3 cell line has defects in the intrinsic pathway of apoptosis and treatment with LBW242 (20 μmol/l) induced caspase-8 mediated cell death, independent of caspase 9 [32]. Small interference RNA experiments showed that caspase 8, TNFα signaling molecules (TNFα, TNF-RI, and RIPK1), and XIAP were required for the proapoptotic effects of LBW242. LBW242 enhanced TNFα expression 30 times greater than background levels in this ovarian cancer cell line. LBW242-induced cell death was prevented with the introduction of TNFα antagonists such as Etanercept. In this experiment, LBW242 caused a rapid proteosome-dependent decrease in the levels of c-IAP1 but not XIAP. TNFα-mediated apoptosis was negatively controlled by c-IAP1, and therefore its degradation enhanced TNFα production and apoptosis. However, XIAP was required for TNFα production and cells with reduced XIAP expression were resistant to the effects of LBW242.

Compound A is a Smac mimetic, designed from the crystal structure of the BIR3 of XIAP bound to the four amino acids of Smac, which is capable of binding the BIR2 and BIR3 domains of XIAP, c-IAP1, and c-IAP2 (Fig. 5e) [30]. Compound A is able to induce significant apoptosis in Kym1 human rhabdomyosarcoma and SKOV3 ovarian cancer cell lines at doses of 5 and 500 nmol/l,

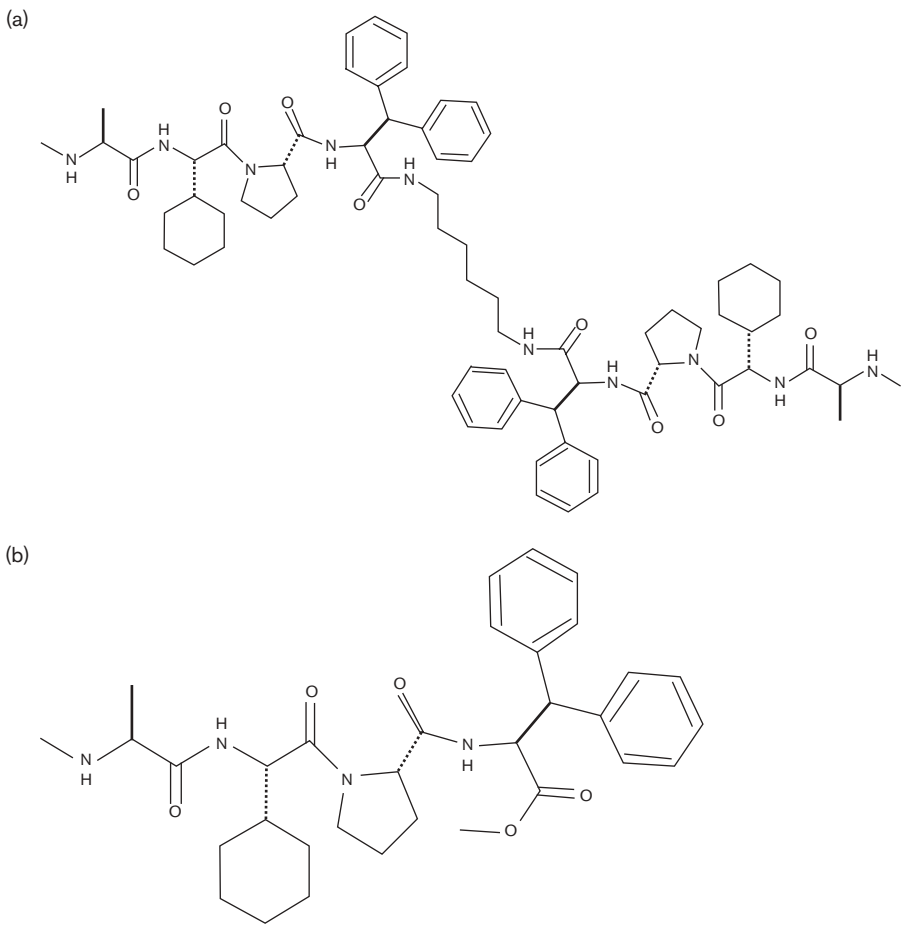
respectively. This induction seems to be mediated by the extrinsic pathway of apoptosis. The proapoptotic effects of compound A were blocked by inhibiting NF-κB-driven TNFα production with a super-repressor form of IκB. Apoptosis was also diminished by inhibiting TNFα with TNFα-blocking antibodies, and inhibiting caspase 8 with crmA. Compound A resulted in rapid proteasome-dependent degradation of c-IAP1.

Structure-based design allowed the development of monovalent (MV1) and bivalent (BV6) Smac-mimetic compounds (Fig. 6a and b) that were capable of interfering with the binding of caspase 9 by XIAP [29]. Application of these two compounds as a single agent to EVSA-T and MDA-MB-231 breast cancer cells and A2058 melanoma cells resulted in caspase 8-dependent cell death. The IC_{50} values in the sensitive EVSA-T cells were 5 μmol/l for MV1 and 14 nmol/l for BV6. MV1 and BV6 also lead to autoubiquitination and subsequent proteasomal degradation of c-IAP1 and c-IAP2, activation of canonical and noncanonical NF-κB pathway activation, and TNFα de-novo synthesis and signaling [29].

Future therapeutic use of Smac mimetics

The use of Smac mimetics in cancer therapy has proven to be effective in a variety of cancer cell types. Much of their usefulness is because of the ability of these Smac-like molecules to bind to inhibitors of apoptosis, such as XIAP, and therefore promote the apoptotic response. As IAPs may be upregulated in the progression of cancer, Smac mimetics are able to provide directed toxicity to neoplastic cells while relatively sparing normal tissues. Smac mimetics can be combined with proapoptotic regimens such as ionizing radiation or chemotherapeutic agents to potentiate their effects in an additive or synergistic manner. Application of the Smac mimetic alone, however, might be sufficient to elicit a therapeutic response. In addition to facilitating apoptosis by directly binding to IAPs, Smac mimetics can use other mechanisms such as cell cycle arrest, promoting autoubiquitination

Fig. 6



Schematic representation of Smac mimetic: small molecules developed by structure-based design. MV1 is a monovalent compound (a), whereas BV6 is bivalent (b).

of IAPs, or activating the extrinsic pathway of apoptosis through paracrine TNFα stimulatory mechanisms. Animal models have shown many of the anticancer properties of Smac mimetics. Treatment with these drugs can decrease tumor growth and prolong survival *in vivo*. These outcomes are primarily because of the proapoptotic effects of Smac mimetics. Smac mimetics have shown limited in-vivo toxicity and good therapeutic response, especially when used in conjunction with other therapies. Additional in-vivo studies are required before the implementation of these compounds in human clinical trials. To facilitate this progression, Smac-mimetic design needs to move from simple peptides used for basic science research toward more drug-like peptidic and nonpeptidic mimetics of clinical value. For example, bivalent Smac mimetics have been shown to be significantly more potent than monovalent mimetics. However, because of the higher molecular weight of bivalent mimetics, they likely have much reduced oral bioavailability when compared with monovalent forms and may require parenteral administration [63]. The application of Smac mimetics as a new class of cancer therapeutics holds great potential, but much basic and clinical research is required to precisely identify the future impact of these drugs.

References

1 Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X, Harran PG. A small molecule Smac mimic potentiates T. *Science* 2004; **305**:1471–1474.
2 Nagata S. Apoptotic DNA fragmentation. *Exp Cell Res* 2000; **256**:12–18.
3 Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene* 2006; **25**:4798–4811.
4 Huerta S, Goulet EJ, Huerta-Yepez S, Livingston EH. Screening and detection of apoptosis. *J Surg Res* 2007; **139**:143–156.
5 Fuentes-Prior P, Salvesen GS. The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochem J* 2004; **384**: 201–232.
6 Deveraux QL, Reed JC. IAP family proteins–suppressors of apoptosis. *Genes Dev* 1999; **13**:239–252.
7 Huerta S, Heinzerling JH, Anguiano-Hernandez YM, Huerta-Yepez S, Lin J, Chen D, *et al*. Modification of gene products involved in resistance to apoptosis in metastatic colon cancer cells: roles of Fas, Apaf-1, NFkappaB, IAPs, Smac/DIABLO, and AIF. *J Surg Res* 2007; **142**:184–194.
8 Chai J, Du C, Wu JW, Kyin S, Wang X, Shi Y. Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature* 2000; **406**: 855–862.
9 Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 2000; **102**:33–42.
10 Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, *et al*. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 2000; **102**:43–53.
11 Li S, Zhao Y, He X, Kim TH, Kuharsky DK, Rabinowich H, *et al*. Relief of extrinsic pathway inhibition by the Bid-dependent mitochondrial release of Smac in Fas-mediated hepatocyte apoptosis. *J Biol Chem* 2002; **277**:26912–26920.
12 Kandasamy K, Srinivasula SM, Alnemri ES, Thompson CB, Korsmeyer SJ, Bryant JL, *et al*. Involvement of proapoptotic molecules Bax and Bak in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial disruption and apoptosis: differential regulation of cytochrome c and Smac/DIABLO release. *Cancer Res* 2003; **63**:1712–1721.
13 Sun XM, Bratton SB, Butterworth M, MacFarlane M, Cohen GM. Bcl-2 and Bcl-xL inhibit CD95-mediated apoptosis by preventing mitochondrial release of Smac/DIABLO and subsequent inactivation of X-linked inhibitor-of-apoptosis protein. *J Biol Chem* 2002; **277**:11345–11351.
14 Munoz-Pinedo C, Guio-Carrion A, Goldstein JC, Fitzgerald P, Newmeyer DD, Green DR. Different mitochondrial intermembrane space proteins are released during apoptosis in a manner that is coordinately initiated but can vary in duration. *Proc Natl Acad Sci U S A* 2006; **103**:11573–11578.
15 Hansen TM, Smith DJ, Nagley P. Smac/DIABLO is not released from mitochondria during apoptotic signalling in cells deficient in cytochrome c. *Cell Death Differ* 2006; **13**:1181–1190.
16 Yu J, Wang P, Ming L, Wood MA, Zhang L. SMAC/Diablo mediates the proapoptotic function of PUMA by regulating PUMA-induced mitochondrial events. *Oncogene* 2007; **26**:4189–4198.
17 Deng Y, Lin Y, Wu X. TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO. *Genes Dev* 2002; **16**:33–45.
18 Verhagen AM, Coulson EJ, Vaux DL. Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. *Genome Biol* 2001; **2**:1–10.
19 Takahashi R, Deveraux Q, Tamm I, Welsh K, Assa-Munt N, Salvesen GS, *et al*. A single BIR domain of XIAP sufficient for inhibiting caspases. *J Biol Chem* 1998; **273**:7787–7790.
20 Suzuki Y, Nakabayashi Y, Nakata K, Reed JC, Takahashi R. X-linked inhibitor of apoptosis protein (XIAP) inhibits caspase-3 and -7 in distinct modes. *J Biol Chem* 2001; **276**:27058–27063.
21 Sun C, Cai M, Meadows RP, Xu N, Gunasekera AH, Herrmann J, *et al*. NMR structure and mutagenesis of the third Bir domain of the inhibitor of apoptosis protein XIAP. *J Biol Chem* 2000; **275**:33777–33781.
22 Shiozaki EN, Chai J, Rigotti DJ, Riedl SJ, Li P, Srinivasula SM, *et al*. Mechanism of XIAP-mediated inhibition of caspase-9. *Mol Cell* 2003; **11**:519–527.
23 Srinivasula SM, Datta P, Fan XJ, Fernandes-Alnemri T, Huang Z, Alnemri ES. Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. *J Biol Chem* 2000; **275**: 36152–36157.
24 Srinivasula SM, Hegde R, Saleh A, Datta P, Shiozaki E, Chai J, *et al*. A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature* 2001; **410**:112–116.
25 Huang Y, Rich RL, Myszka DG, Wu H. Requirement of both the second and third BIR domains for the relief of X-linked inhibitor of apoptosis protein (XIAP)-mediated caspase inhibition by Smac. *J Biol Chem* 2003; **278**:49517–49522.
26 Liu Z, Sun C, Olejniczak ET, Meadows RP, Betz SF, Oost T, *et al*. Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. *Nature* 2000; **408**:1004–1008.
27 Wu G, Chai J, Suber TL, Wu JW, Du C, Wang X, *et al*. Structural basis of IAP recognition by Smac/DIABLO. *Nature* 2000; **408**:1008–1012.
28 Wist AD, Gu L, Riedl SJ, Shi Y, McLendon GL. Structure-activity based study of the Smac-binding pocket within the BIR3 domain of XIAP. *Bioorg Med Chem* 2007; **15**:2935–2943.
29 Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, Garg P, *et al*. IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. *Cell* 2007; **131**:669–681.
30 Vince JE, Wong WW, Khan N, Feltham R, Chau D, Ahmed AU, *et al*. IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. *Cell* 2007; **131**:682–693.
31 Petersen SL, Wang L, Yalcin-Chin A, Li L, Peyton M, Minna J, *et al*. Autocrine TNFalpha signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis. *Cancer Cell* 2007; **12**:445–456.
32 Gaither A, Porter D, Yao Y, Borawski J, Yang G, Donovan J, *et al*. A Smac mimetic rescue screen reveals roles for inhibitor of apoptosis proteins in tumor necrosis factor-alpha signaling. *Cancer Res* 2007; **67**: 11493–11498.
33 Anguiano-Hernandez YM, Chartier A, Huerta S. Smac/DIABLO and colon cancer. *Anticancer Agents Med Chem* 2007; **7**:467–473.
34 Ren Y, Akyurek N, Schlette E, Rassidakis GZ, Medeiros LJ. Expression of Smac/DIABLO in B-cell non-Hodgkin and Hodgkin lymphomas. *Hum Pathol* 2006; **37**:1407–1413.
35 Xiao D, Wang K, Zhou J, Cao H, Deng Z, Hu Y, *et al*. Inhibition of fibroblast growth factor 2-induced apoptosis involves survivin expression, protein kinase C alpha activation and subcellular translocation of Smac in human small cell lung cancer cells. *Acta Biochim Biophys Sin (Shanghai)* 2008; **40**:297–303.
36 Ruotsalainen T, Joensuu H, Mattson K, Salven P. High pretreatment serum concentration of basic fibroblast growth factor is a predictor of poor prognosis in small cell lung cancer. *Cancer Epidemiol Biomarkers Prev* 2002; **11**:1492–1495.
37 Mizutani Y, Nakanishi H, Yamamoto K, Li YN, Matsubara H, Mikami K, *et al*. Downregulation of Smac/DIABLO expression in renal cell carcinoma and its prognostic significance. *J Clin Oncol* 2005; **23**:448–454.
38 Kempkensteffen C, Hinz S, Christoph F, Krause H, Magheli A, Schrader M, *et al*. Expression levels of the mitochondrial IAP antagonists Smac/DIABLO

and Omi/HtrA2 in clear-cell renal cell carcinomas and their prognostic value. *J Cancer Res Clin Oncol* 2008; **134**:543–550.
39 Zheng LD, Tong QS, Wang L, Liu J, Qian W. Stable transfection of extrinsic Smac gene enhances apoptosis-inducing effects of chemotherapeutic drugs on gastric cancer cells. *World J Gastroenterol* 2005; **11**:79–83.
40 Zhao J, Jin J, Zhang X, Shi M, Dai J, Wu M, *et al*. Transfection of Smac sensitizes tumor cells to etoposide-induced apoptosis and eradicates established human hepatoma in vivo. *Cancer Gene Ther* 2006; **13**: 420–427.
41 Kohli M, Yu J, Seaman C, Bardelli A, Kinzler KW, Vogelstein B, *et al*. SMAC/Diablo-dependent apoptosis induced by nonsteroidal antiinflammatory drugs (NSAIDs) in colon cancer cells. *Proc Natl Acad Sci U S A* 2004; **101**:16897–16902.
42 Giagkousiklidis S, Vogler M, Westhoff MA, Kasperczyk H, Debatin KM, Fulda S. Sensitization for gamma-irradiation-induced apoptosis by second mitochondria-derived activator of caspase. *Cancer Res* 2005; **65**: 10502–10513.
43 Sekimura A, Konishi A, Mizuno K, Kobayashi Y, Sasaki H, Yano M, *et al*. Expression of Smac/DIABLO is a novel prognostic marker in lung cancer. *Oncol Rep* 2004; **11**:797–802.
44 Abe S, Hasegawa M, Yamamoto K, Kurata M, Nakagawa Y, Suzuki K, *et al*. Rapid induction of IAP family proteins and Smac/DIABLO expression after proapoptotic stimulation with doxorubicin in RPMI 8226 multiple myeloma cells. *Exp Mol Pathol* 2007; **83**:405–412.
45 Arnt CR, Chiorean MV, Heldebrant MP, Gores GJ, Kaufmann SH. Synthetic Smac/DIABLO peptides enhance the effects of chemotherapeutic agents by binding XIAP and cIAP1 in situ. *J Biol Chem* 2002; **277**:44236–44243.
46 Guo F, Nimmanapalli R, Paranawithana S, Wittman S, Griffin D, Bali P, *et al*. Ectopic overexpression of second mitochondria-derived activator of caspases (Smac/DIABLO) or cotreatment with N-terminus of Smac/DIABLO peptide potentiates epothilone B derivative-(BMS 247550) and Apo-2L/TRAIL-induced apoptosis. *Blood* 2002; **99**:3419–3426.
47 Yang QH, Du C. Smac/DIABLO selectively reduces the levels of c-IAP1 and c-IAP2 but not that of XIAP and livin in HeLa cells. *J Biol Chem* 2004; **279**:16963–16970.
48 Kashkar H, Seeger JM, Hombach A, Deggerich A, Yazdanpanah B, Utermohlen O, *et al*. XIAP targeting sensitizes Hodgkin lymphoma cells for cytolytic T-cell attack. *Blood* 2006; **108**:3434–3440.
49 Bilim V, Yuuki K, Itoi T, Muto A, Kato T, Nagaoka A, *et al*. Double inhibition of XIAP and Bcl-2 axis is beneficial for retrieving sensitivity of renal cell cancer to apoptosis. *Br J Cancer* 2008; **98**:941–949.
50 Mori T, Doi R, Kida A, Nagai K, Kami K, Ito D, *et al*. Effect of the XIAP inhibitor Embelin on TRAIL-induced apoptosis of pancreatic cancer cells. *J Surg Res* 2007; **142**:281–286.
51 Yang L, Mashima T, Sato S, Mochizuki M, Sakamoto H, Yamori T, *et al*. Predominant suppression of apoptosome by inhibitor of apoptosis protein in non-small cell lung cancer H460 cells: therapeutic effect of a novel polyarginine-conjugated Smac peptide. *Cancer Res* 2003; **63**:831–837.
52 Fulda S, Wick W, Weller M, Debatin KM. Smac agonists sensitize for Apo2L/T. *Nat Med* 2002; **8**:808–815.
53 Franklin MC, Kadkhodayan S, Ackerly H, Alexandru D, Distefano MD, Elliott LO, *et al*. Structure and function analysis of peptide antagonists of melanoma inhibitor of apoptosis (ML-IAP). *Biochemistry* 2003; **42**: 8223–8231.
54 Jia L, Patwari Y, Kelsey SM, Srinivasula SM, Agrawal SG, Alnemri ES, *et al*. Role of Smac in human leukaemic cell apoptosis and proliferation. *Oncogene* 2003; **22**:1589–1599.
55 Checinska A, Hoogeland BS, Rodriguez JA, Giaccone G, Kruyt FA. Role of XIAP in inhibiting cisplatin-induced caspase activation in non-small cell lung cancer cells: a small molecule Smac mimic sensitizes for chemotherapy-induced apoptosis by enhancing caspase-3 activation. *Exp Cell Res* 2007; **313**:1215–1224.
56 Petrucci E, Pasquini L, Petronelli A, Saulle E, Mariani G, Riccioni R, *et al*. A small molecule Smac mimic potentiates TRAIL-mediated cell death of ovarian cancer cells. *Gynecol Oncol* 2007; **105**:481–492.
57 Bockbrader KM, Tan M, Sun Y. A small molecule Smac-mimic compound induces apoptosis and sensitizes T. *Oncogene* 2005; **24**:7381–7388.
58 Bank A, Wang P, Du C, Yu J, Zhang L. SMAC mimetics sensitize nonsteroidal anti-inflammatory drug-induced apoptosis by promoting caspase-3-mediated cytochrome c release. *Cancer Res* 2008; **68**:276–284.
59 Oost TK, Sun C, Armstrong RC, Al-Assaad AS, Betz SF, Deckwerth TL, *et al*. Discovery of potent antagonists of the antiapoptotic protein XIAP for the treatment of cancer. *J Med Chem* 2004; **47**:4417–4426.
60 Sun H, Nikolovska-Coleska Z, Lu J, Meagher JL, Yang CY, Qiu S, *et al*. Design, synthesis, and characterization of a potent, nonpeptide, cell-permeable, bivalent Smac mimetic that concurrently targets both the BIR2 and BIR3 domains in XIAP. *J Am Chem Soc* 2007; **129**:15279–15294.
61 Weisberg E, Kung AL, Wright RD, Moreno D, Catley L, Ray A, *et al*. Potentiation of antileukemic therapies by Smac mimetic, LBW242: effects on mutant FLT3-expressing cells. *Mol Cancer Ther* 2007; **6**:1951–1961.
62 Chauhan D, Neri P, Velankar M, Podar K, Hideshima T, Fulciniti M, *et al*. Targeting mitochondrial factor Smac/DIABLO as therapy for multiple myeloma (MM). *Blood* 2007; **109**:1220–1227.
63 Sun H, Nikolovska-Coleska Z, Yang CY, Qian D, Lu J, Qiu S, *et al*. Design of small-molecule peptidic and nonpeptidic Smac mimetics. *Acc Chem Res* 2008; **41**:1264–1277.