Letters

Structure-Based Design, Synthesis, and Evaluation of Conformationally Constrained Mimetics of the Second Mitochondria-Derived Activator of Caspase That Target the X-Linked Inhibitor of Apoptosis Protein/Caspase-9 Interaction Site

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Abstract: A successful structure-based design of conformationally constrained second mitochondria-derived activator of caspase (Smac) mimetics that target the XIAP/caspase-9 interaction site is described. The most potent Smac mimetic **12d** has a K_i of 350 nM for binding to the XIAP BIR3 domain protein. **12d** is found to be effective in enhancing apoptosis induced by cisplatin in PC-3 human prostate cancer cells.

Apoptosis, or programmed cell death, is an essential cell suicide process[1] that is important for normal development, host defense, and suppression of oncogenesis.[2] Inappropriate control of apoptosis plays a role in many human diseases, including cancer, autoimmune diseases, and neurodegenerative disorders.[2−4] In recent years, key apoptosis regulators have become attractive molecular targets for designing new therapies to treat a variety of human diseases and conditions.[3,4]

Inhibitor of apoptosis proteins (IAPs) were recently discovered as important intrinsic cellular inhibitors of apoptosis, although their functions may not be limited to the regulation of apoptosis.[5,6] XIAP (X-linked IAP) is the most potent inhibitor of apoptosis among all the IAP proteins.[5,6] XIAP protein potently inhibits intrinsic and extrinsic apoptosis pathways.[5,6] XIAP contains three BIR (birculovirus IAP repeat) domains and a C-terminal ring finger domain.[6] The third BIR domain (BIR3) selectively targets caspase-9, an initiator caspase, while the linker region between BIR1 and BIR2 inhibits effectors caspase-3 and -7.[6,7] Although XIAP prevents the activation of all three caspases, it is apparent that the interaction with caspase-9 is the most critical for its inhibition of apoptosis.[8,9]

A recent crystallographic analysis[10] showed that XIAP BIR3 binds to caspase-9 and traps it in its inactive monomeric form. Unlike most other protein−protein interactions, the binding between XIAP and caspase-9 is mediated by a small but well-defined surface groove in the XIAP BIR3 domain and four N-terminally exposed amino acid residues (ATPF) in caspase-9.[10] This surface groove in the XIAP BIR3 domain represents an attractive site for small-molecule inhibitors of XIAP and other IAP proteins whose binding will block the inhibitory activity of IAP proteins to caspase-9 and promote apoptosis in cells.

In fact, the second mitochondria-derived activator of caspase or direct IAP binding protein with low p*I* (Smac/DIABLO), a protein released from mitochondria in response to apoptotic stimuli, directly interacts with the BIR3 domain of XIAP, cIAP-1, and cIAP-2 and a single BIR domain in ML-IAP and functions as a direct endogenous inhibitor of IAP proteins.[11,12] Biological studies[9] and high-resolution experimental structures[13,14] demonstrated that Smac binds to the same surface groove in the XIAP BIR3 domain where caspase-9 binds via its N-terminally exposed four residues (AVPI) in a manner similar to that in caspase-9/XIAP interaction. Consistent with the structural data, Smac peptides as short as four residues derived from Smac protein bind to the recombinant XIAP BIR3 domain protein with the same affinities as the mature Smac protein.[14,15] Thus, binding of Smac protein and peptides to XIAP effectively removes the inhibition of XIAP to caspase-9. Several recent studies showed that short Smac peptides fused to a carrier peptide for intracellular delivery (cell-permeable Smac peptides) overcome resistance of cancer cells with high levels of XIAP protein to apoptosis and enhance the activity of anticancer drugs in vitro and in vivo.[16−18] Of great significance, these cell-permeable Smac peptides have little toxicity to normal cells in vitro and to normal tissues in vivo.[16−18] These studies suggest that cell-permeable Smac mimetics may have great therapeutic potential as a new class of anticancer drugs for overcoming apoptosis resistance of cancer cells with high levels of IAP proteins.

Smac peptides have several intrinsic limitations (e.g., poor in vivo stability and poor bioavailability) as pharmacological tools and as potentially useful therapeutic agents for the treatment of cancer. In this paper, we present, to our knowledge, the first successful example of structure-based design of a class of conformationally constrained Smac mimetics.

The basic design idea is illustrated in Scheme 1 and Figure 1. On the basis of the 3D experimental structures of the XIAP BIR3 domain in complex with Smac protein and peptide,[13,14] the hydrophobic side chain of Ile4 in the Smac AVPI (Ala1-Val2-Pro3-Ile4) binding motif binds to a well-defined hydrophobic pocket in XIAP

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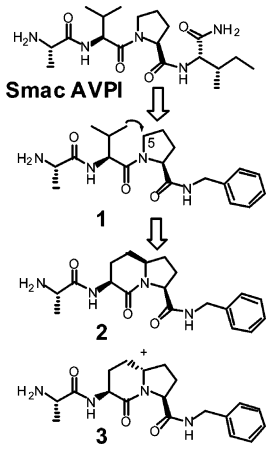
Scheme 1. Design for Deriving Conformationally Constrained Smac Mimetics[a]



[a] Starting from the AVPI peptide, simple Smac mimetic **1** was designed and synthesized. Cyclization of side chain of valine and proline rings of **1** led to conformationally constrained Smac mimetics **2** and **3**.

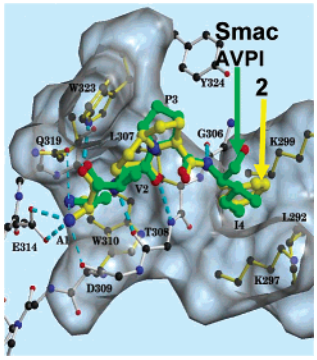
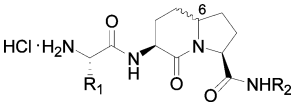


Figure 1. Superposition of modeled structure of **2** in complex with XIAP BIR3 and the X-ray structure of Smac AVPI in complex with XIAP BIR3. Carbon atoms are shown in yellow and green for **2** and Smac peptide, respectively. Oxygen and nitrogen atoms are shown in red and blue, respectively. Hydrogen bonds are shown in light-blue dashed lines.

BIR3 and the carbonyl group of Ile4 does not appear to have specific interactions with the protein. Accordingly we designed **1** (Scheme 1) in which the Ile4 residue was replaced by a benzylamine. In our fluorescence polarization based (FP) binding assay (see Supporting Information for assay details), **1** is as potent as the Smac AVPI peptide (Table 1), confirming that the Ile residue can be replaced by a suitable hydrophobic group. Cyclization of the side chain of Val2 and Pro3 ring in **1** leads to two bicyclic conformationally constrained Smac mimetics (**2** and **3** in Scheme 1). Detailed modeling studies showed that although **2** has some deviations in comparison to the Smac AVPI peptide (e.g., the conformation of the five-membered ring), it quite closely mimics the interactions of Smac/XIAP BIR3 (Figure 1). In contrast, the other stereoisomer **3** is unable to mimic the interaction between Smac/XIAP in both hydrogen bonding and hydrophobic interaction (Supporting Information). Thus, it was predicted that **2** would show good binding affinity to XIAP BIR3, while **3** would be much less potent than **2**. Indeed, an FP-based binding assay determined that while **2** has a K_i of 4.47 μM for the recombinant XIAP BIR3 protein in displacing a fluorescently labeled Smac peptide (Table 1), **3** has a

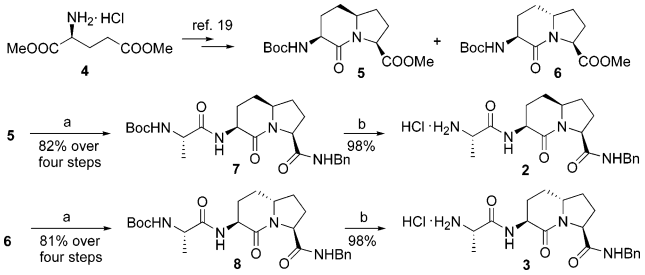
Table 1. Experimentally Determined Binding Affinities by FP-Based Assay



compd	configuration of C6	R_1	R_2	$K_i \pm$ SD,[a] μM
AVPI				0.58 ± 0.15
1				0.29 ± 0.06
2	*R*	Me	Bn	4.47 ± 0.65
3	*S*	Me	Bn	>100
10a	*R*	Et	Bn	1.41 ± 0.16
10b	*R*	*n*-Pr	Bn	>100
10c	*R*	*i*-Pr	Bn	43.11 ± 1.51
12a	*R*	Me	Ph	>100
12b	*R*	Me	2-phenylethyl	22.4 ± 1.87
12c	*R*	Me	diphenylmethyl	2.33 ± 0.68
12d	*R*	Et	diphenylmethyl	0.35 ± 0.01

[a] The K_i values and standard deviation (SD) were determined by three to five independent experiments in triplicate.

Scheme 2. Synthesis of Conformationally Constrained Smac Mimetics[a]



[a] Reagents and conditions: (a) (i) 2 N, LiOH, then 1 N HCl; (ii) benzylamine, EDC, HOBt, *N*,*N*-diisopropylethylamine, CH_2Cl_2, room temp; (iii) 1 N HCl in MeOH; (iv) *N*-(*tert*-butoxycarbonyl)-L-alanine, EDC, HOBt, *N*,*N*-diisopropylethylamine, CH_2Cl_2, room temp; (b) 1 N HCl in MeOH.

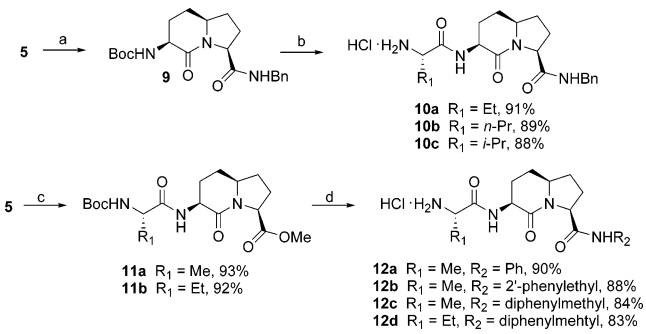
K_i greater than 100 μM, confirming our modeling results and design strategy.

The chemical synthesis of these two compounds is detailed in Scheme 2. Briefly, the key intermediates **5** and **6** were prepared according to the literature method.[19] Hydrolysis of the methyl ester groups in **5** and **6** followed by condensation with benzylamine gave two amides. Removal of the Boc protective groups in these two amides followed by condensation with *N*-*tert*-butoxycarbonyl-L-alanine yielded intermediates **7** and **8**. Removal of the Boc protective groups in these two compounds afforded **2** and **3**.

2 was used as the new template for further design and optimization. On the basis of our modeled complex structure for **2** (Figure 1), the methyl group in Ala1 and the benzyl ring bind to small and large hydrophobic pockets, respectively. The hydrophobic interactions at these two sites have been shown to be important for the interactions between Smac peptides and XIAP BIR3.[13−15] We have therefore designed and synthesized new analogues of **2** with modifications made at these two sites. The synthesis of these new analogues was performed similarly to that for **2** (Scheme 3), and their experimental binding affinities by the FP assay are provided in Table 1.

Our modeling analysis showed that the small hydrophobic pocket occupied by the methyl group in **2** can accommodate a slightly larger hydrophobic group. Con-

Scheme 3. Synthesis of New Analogues of **2**[a]



[a] Reagents and conditions: (a) (i) 2 N LiOH, then 1 N HCl; (ii) benzylamine, EDC, HOBt, *N*,*N*-diisopropylethylamine, CH_2Cl_2, room temp, 92%; (b) (i) 1 N HCl in MeOH; (ii) *N*-(*tert*-butoxycarbonyl)-L-amino acid, EDC, HOBt, *N*,*N*-diisopropylethylamine, CH_2Cl_2, room temp; (iii) 1 N HCl in MeOH; (c) (i) 1 N HCl in MeOH; (ii) *N*-(*tert*-butoxycarbonyl)-L-alanine or *N*-(*tert*-butoxycarbonyl-L-2-aminobutyric acid, EDC, HOBt, *N*,*N*-diisopropylethylamine, CH_2Cl_2, room temp; (d) (i) 2 N LiOH, then 1 N HCl; (ii) amine, EDC, HOBt, *N*,*N*-diisopropylethylamine, CH_2Cl_2, room temp; (iii) 1 N HCl in MeOH.

sistent with the modeling analysis, replacement of the methyl group with an ethyl group improves the binding activity by 3-fold (**10a** vs **2**), but replacement of this methyl group by an *n*-Pr or *i*-Pr (**10b** and **10c**) significantly decreases the binding. The binding data thus confirmed that the hydrophobic pocket occupied by the methyl group can accommodate only a slightly larger group (e.g., an ethyl group) but not an *n*-Pr or *i*-Pr group.

The benzyl group in **2** mimics the hydrophobic side chain of Ile4 in the Smac peptide. Replacement of the benzyl group with a phenyl (**12a**) or with a 2-phenylethyl (**12b**) reduces the binding affinity by more than 20- and 5-fold, respectively, demonstrating the importance of the linker length for optimal hydrophobic interaction at this site.

In the experimental 3D complex structures,[13,14] although the carbonyl group of Ile4 in the Smac AVPI peptide has no specific interaction with the XIAP protein, we hypothesized that it plays a role in enhancing the binding affinity by controlling the orientation and/or reducing the conformational flexibility of the hydrophobic side chain of the Ile4 residue. To test this idea, we designed and synthesized **12c**, in which a second phenyl group was introduced to the α carbon atom of the benzyl group. Compound **12c** has a K_i of 2.33 μM, twice as potent as **2**. In our modeled structure of **12c** in complex with XIAP BIR3, this second phenyl group does not appear to have a direct interaction with the protein, suggesting that the slight improvement in binding affinity of **12c** over **2** is likely due to the conformational restriction of the phenyl ring inserting into the hydrophobic pocket (Figure 1).

Finally, **12d** was designed by incorporating the results obtained for **10a** and **12c** and was determined to have a K_i of 0.35 μM in our FP-based assay. Hence, **12d** is as potent as the Smac AVPI peptide and represents a fairly potent Smac mimetic.

The FP-based binding assay showed that the Smac mimetic **12d** potently displaces the binding of a fluorescently labeled Smac peptide from XIAP BIR, but the assay does not provide direct information on which residues in XIAP **12d** interacts with. To further probe

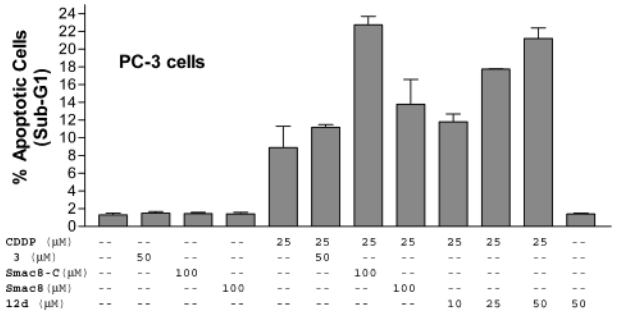


Figure 2. Potentiating cisplatin (CDDP) induced apoptosis in human prostate PC-3 cancer cells by Smac mimetic **12d** and in comparison with an inactive Smac mimetic **3**, a Smac peptide without a carrier peptide (Smac8), and a Smac peptide tethered to a carrier peptide (Smac8-C).

the binding of **12d** to XIAP BIR3, we performed a nuclear magnetic resonance analysis using the heteronuclear single quantum coherence (HSQC) method. The HSQC spectrum was recorded for a sample containing the ^{15}N-labeled human XIAP BIR3 protein with and without **12d**. Analysis of the HSQC spectra showed that **12d** caused induced chemical shifts in several residues in the XIAP BIR3, virtually identical to those caused by the Smac AVPI peptide (Supporting Information), indicating that **12d** binds to the same surface groove where Smac and caspase-9 bind.

Smac peptides tethered to a carrier peptide for intracellular delivery have been shown to potentiate chemodrug-induced apoptosis in cancer cells with high levels of XIAP proteins.[16−18] Accordingly, **12d** was evaluated for its ability to potentiate cisplatin-induced apoptosis in the PC-3 human prostate cells with high levels of XIAP protein (Supporting Information) and compared to Smac peptide without (Smac8) or with (Smac8-C) a carrier peptide derived from the *Drosophila* antennapedia penetratin sequence.[17] Consistent with previous studies,[16−18] Smac8, Smac8-C, and **12d** do not induce apoptosis on their own, while the cytotoxic drug cisplatin (25 μM) significantly induces apoptosis in PC-3 human prostate cancer cells. The combination of cisplatin with 100 μM Smac peptide without a carrier peptide (Smac8 in Figure 2) only slightly increases the cisplatin-induced apoptosis (from 9% to 13%) presumably because of the poor cell permeability of this Smac peptide without a carrier molecule. In contrast, combination of cisplatin with 100 μM Smac peptide with a carrier peptide (Smac8-C in Figure 2) significantly increases the apoptosis (from 9% to 23%). While the combination of cisplatin with 10 μM **12d** only slightly increases cisplatin-induced apoptosis, 25 and 50 μM **12d** significantly increase the cisplatin-induced apoptosis. In fact, 50 μM **12d** is as effective as 100 μM Smac8-C in potentiating the cisplatin-induced apoptosis (Figure 2). **3** (50 μM), which has a K_i greater than 100 μM, is ineffective in potentiating the cisplatin-induced apoptosis. Taken together, our cellular studies suggest that a potent Smac mimetic (**12d**) is as effective as the natural Smac peptide tethered to a carrier peptide (Samc8-C) in increasing apoptosis induced by cisplatin, while an inactive Smac mimetic (**3**) and a Smac peptide without a carrier (Smac8) are ineffective.

In summary, we described a successful structure-based design of a class of conformationally constrained

Smac mimetics. The most potent Smac mimetic **12d** obtained from this study is as potent as the natural Smac peptide binding to the XIAP BIR3 protein. Furthermore, **12d** contains only one unnatural amino acid and no natural amino acid and thus has much reduced peptide characteristics in its chemical structure. As expected from its much reduced peptide nature, **12d** is as effective as the Smac peptide tethered to a carrier peptide[17] in enhancing apoptosis induced by cisplatin in PC-3 prostate cancer cells, while the natural Smac peptide without a carrier peptide is ineffective. Thus, **12d** may be used as a powerful chemical and pharmacological tool to elucidate the roles of Smac and IAP proteins in regulation of apoptosis in cells. Further optimization of **12d** may ultimately lead to the development of an entirely new class of anticancer drugs that specifically target IAP proteins and overcome apoptosis resistance of cancer cells to current chemotherapeutic agents.

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Supporting Information Available: Information on the synthesis of compounds in Table 1, the detailed conditions and experimental procedure for FP based binding assay, the NMR HSQC experiments with and without **12d**, and detailed cellular assays as indicated in Figure 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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