Communication

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# Structure-Based Design of Potent, Conformationally Constrained Smac Mimetics

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The second *m*itochondria-derived *a*ctivator of *c*aspase or *d*irect *IA*P *b*inding protein with *l*ow pI (Smac/DIABLO) is a recently identified pro-apoptotic protein released from mitochondria in response to apoptotic stimuli.[1,2] Smac/DIABLO promotes apoptosis in cells, at least in part, by directly interacting with anti-apoptotic inhibitors of apoptosis proteins (IAPs) and functioning as a potent endogenous cellular antagonist of IAPs.[1,2] Structural[3,4] and biological[5] studies have demonstrated that Smac binds to a surface groove in the third baculovirus IAP repeat (BIR3) of X-linked IAP (XIAP) through its N-terminally exposed four residues alanine-valine-proline-isoleucine (A1-V2-P3-I4). Short Smac peptides fused to a carrier peptide for intracellular delivery have been shown to overcome resistance of cancer cells with high levels of IAPs, to enhance the activity of anticancer drugs in vitro and in vivo,[6−8] and to have little toxicity to normal cells and to normal tissues.[6−8] These studies suggest that potent Smac mimetics may have promising therapeutic potential as a new class of anti-cancer drugs.

Smac peptides have several intrinsic limitations (e.g., poor cell-permeability and poor in vivo stability and bioavailability) as potentially useful therapeutic agents. Therefore, our laboratories,[9] as well as others[10,11] are interested in designing Smac peptidomimetics and nonpeptidic mimetics with improved binding affinities, cell-permeability, and in vivo stability and bioavailability. In this communication, we report the successful structure-based design of a class of highly potent, conformationally constrained nonpeptidic Smac mimetics.

The experimental 3D structures of Smac in complex with XIAP BIR3 show that the hydrophobic side chain of I4 in Smac AVPI peptide (**1**) binds to a hydrophobic pocket in XIAP BIR3 but the carbonyl group of the I4 residue has no specific interactions with the protein.[3,4] We therefore replaced the I4 residue with a benzylamine (Figure 1) and determined that **2** binds to XIAP BIR3 protein twice as potently as **1** (Figure 2) in our fluorescence-polarization (FP)-based binding assay.[12]

Analysis of the structures of the Smac/XIAP BIR3 complex showed that the isopropyl group of V2 and the five-membered ring of P3 may be cyclized to form two stereoisomeric bicyclic lactam ring systems (**3** and **4**, Figure 1) without severely distorting the binding conformation of **2**. Modeling studies showed that **3** can essentially maintain both hydrogen-bonding and hydrophobic interactions with the protein as **2** does, while **4** deviates substantially from **2** in its binding conformation. Compound **3** was determined to have a $K_i$ value of 4.47 $\mu$M and **4** is totally inactive ($K_i$ >100 $\mu$M).[9] Hence, the binding data of **3** indicated that while this cyclization strategy is feasible, **3** is substantially less potent than

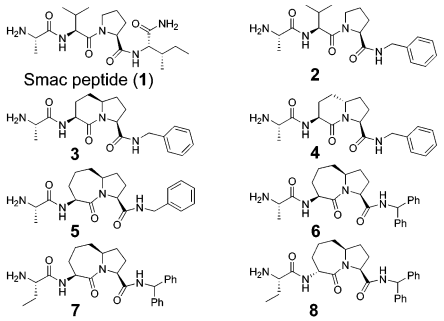


***Figure 1.*** Smac peptide and conformationally constrained mimetics.

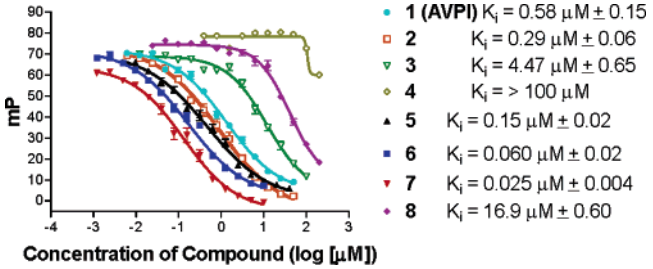


***Figure 2.*** Competitive binding curves of Smac mimetics to XIAP BIR3 protein as determined in an FP-based binding assay and their $K_i$ values and standard deviations. For each compound, 3−5 independent experiments were performed according to the optimized assay conditions,[12] and the $K_i$ values were calculated using a mathematical model developed for FP-based assays.[12]

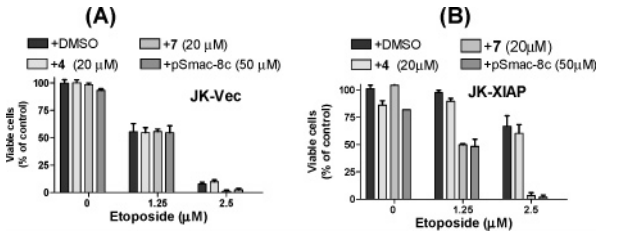


***Figure 3.*** Inhibition of cell growth in Jurkat cells tranfected with a vector control (JK-Vec) (A) or transfected with XIAP (JK-XIAP) (B) by etoposide alone or in combination with **4**, **7**, and pSmac-8c.

**2**, likely due to the conformational differences when bound to XIAP (Supporting Information).[9] We therefore investigated the possibility of inserting one additional carbon atom into the six-membered ring of **3**, which led to the design of **5** (Figure 1). Modeling studies showed that **5** perfectly mimics the binding conformation of **1** and **2** (Supporting Information) and predicted that **5** should be much more potent than **3**. Compound **5** was synthesized and determined to have a $K_i$ value of 150 nM (Figure 2). Hence, **5** is 4-, 2-, and 30 times more potent than **1**, **2,** and **3**, respectively, confirming our

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design strategy. Additional structure-based design and modifications of **5** to further improve its interactions with XIAP BIR3 led to compounds **6** and **7**. It was determined that **6** and **7** have $K_i$ values of 60 and 25 nM, respectively (Figure 2). Hence, **7** is 23 times more potent Smac peptide **1** and represents a potent, conformationally constrained, non-peptidic Smac mimetic.

**8** was designed to confirm the stereospecificity of **7**. Compound **8** has a $K_i$ value of 16.9 $\mu$M (Figure 2) and is 680 times less potent than **7**, indicating that the binding of **7** to XIAP BIR3 is highly stereospecific. The chemical synthesis of **5**, **6**, **7**, and **8** is provided in the Supporting Information.

Figure 2 provides the direct competitive binding curves of Smac AVPI peptide (**1**), and Smac mimetics **2**−**8** for their ability to bind to XIAP BIR3 and compete with a fluorescence-labeled Smac peptide using our established FP-based binding assay.[12] Of note, these compounds do not have any fluorescence.

To gain a further insight into the binding mode of **7** to XIAP BIR3, we performed a nuclear magnetic resonance (NMR) analysis using the heteronuclear single quantum coherence (HSQC) method (Supporting Information). The HSQC spectrum was recorded with a sample containing the $^{15}$N-labeled human XIAP BIR3 protein with different concentrations of **7** and of the Smac AVPI peptide **1**. Analysis of the HSQC spectra showed that **7** induced chemical shifts (identical to those caused by **1**) in several residues in the XIAP BIR3, indicating that **7** and the Smac peptide interact with same set of residues in XIAP BIR3. Furthermore, it was found that **7** is more potent than the Smac AVPI peptide, on the basis of the NMR spectra, consistent with the FP-based binding data.

To directly test the basic mechanism of Smac mimetics in cells, we have employed Jurkat leukemia T cells stably transfected with XIAP (JK-XIAP) or with vector control (JK-Vec).[13] JK-Vec cells have a low level of XIAP protein, while JK-XIAP cells have a very high level of XIAP protein.[13] In the cell growth assay, chemotherapeutic agent, etoposide, potently inhibited cell growth in JK-Vec cells.[13] In our hands, 1.25 and 2.5 $\mu$M of etoposide respectively inhibited 50% and 90% cell growth (Figure 3A). In comparison, in JK-XIAP cells, no growth inhibition was observed at 1.25 $\mu$M of etoposide, and only 30% of growth inhibition was observed at 2.5 $\mu$M of etoposide (Figure 3B). Overexpression of XIAP also potently protects cells from etoposide-induced apoptosis.[14] These data thus show that overexpression of XIAP effectively protects the cells from etoposide-induced cell growth inhibition and apoptosis. Since JK-XIAP and JK-Vec cells have the same genetic background except for the expression of XIAP, they provide us with excellent models to study the molecular mechanism of action of Smac mimetics. Using JK-XIAP and JK-Vec cells, we have tested **7** for its ability to overcome the protective effect of XIAP. Compound **4** was used as an inactive control, and a cell-permeable Smac peptide (pSmac-8c), which was used in a previous study,[7] was a positive control. The results are provided in Figure 3.

A combination of **4**, **7**, or pSmac-8c with etoposide in JK-Vec cells has no significant difference as compared to etoposide alone (Figure 3A), indicating that there is no interactive effect between these compounds with etoposide in cells with low levels of XIAP protein. In contrast, in JK-XIAP cells, while the combination of the inactive control **4** with etoposide has no significant difference as compared to etoposide alone, both **7** and pSmac-8c were able to completely overcome the protective effect of XIAP to etoposide (Figure 3B). Importantly, **4**, **7**, and pSmac-8c do not cause cell death on their own at the concentrations tested. Our data suggested that **7** and pSmac-8c specifically target XIAP in JK-XIAP cells.

In summary, we have successfully designed and synthesized a class of highly potent conformationally constrained nonpeptidic Smac mimetics based upon the experimental 3D structures of Smac in complex with XIAP BIR3. The most potent compound **7** has a $K_i$ value of 25 nM to XIAP BIR3 and is 23 times more potent than natural Smac peptide. Furthermore, the NMR HSQC spectra conclusively confirm that compound **7** binds to the same binding groove in the XIAP BIR3 where Smac peptide and protein bind. Moreover, by using Jurkat cells transfected with XIAP, we provide convincing data to show that compound **7** specifically interacts with XIAP in cells and overcomes the protective effect of XIAP to etoposide-induced cell death. Further optimization and biological evaluation of this class of Smac mimetics may ultimately lead to the development of an entirely new class of anti-cancer drugs that target the anti-apoptotic activity of IAP proteins and overcome apoptosis resistance of cancer cells to current therapeutic agents.

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**Supporting Information Available:** Experimental section including the information on synthesis for compounds **5**, **6**, **7**, and **8**, detailed modeling results on the predicted binding models for **3**, **4**, **5**, and **7**, the experimental procedure for fluorescence polarization-based binding assay, the NMR HSQC experiments with and without compound **7**, and details on the cellular assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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