# Nonpeptidic and Potent Small-Molecule Inhibitors of cIAP-1/2 and XIAP Proteins

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A series of compounds were designed and synthesized as antagonists of cIAP-1/2 and XIAP based upon our previously identified lead compound SM-122 (1). The most potent of these (7) binds to XIAP, cIAP-1, and cIAP-2 proteins with  $K_i$  values of 36, <1, and <1.9 nM, respectively. Consistent with its potent binding affinities to IAPs, 7 effectively antagonizes XIAP in a cell-free caspase-9 functional assay, efficiently induces cIAP-1 degradation in cells at concentrations as low as 10 nM, and triggers activation of caspases and PARP cleavage in the MDA-MB-231 breast cancer cell line. Compound 7 potently inhibits cell growth in the MDA-MB-231 cancer cell line with an IC<sub>50</sub> value of 200 nM and is 9 times more potent than compound **1**.

### Introduction

Inhibitor of apoptotic proteins (IAPs<sup>*a*</sup>) are a class of key regulators of apoptosis characterized by one to three baculovirus IAP repeat domains.<sup>1,2</sup> Among these, cIAP-1 and cIAP-2 inhibit death receptor mediated apoptosis<sup>3,4</sup> while XIAP, by binding to effectors caspase-3 and -7 and an initiator caspase-9 and inhibiting the activities of these three caspases, blocks both death receptor-mediated and mitochondria-mediated apoptosis.<sup>5</sup> These three IAPs each have three baculoviral IAP repeat (BIR) domains. The BIR3 domain of XIAP binds to caspase-9 and the BIR2 domain together with the linker preceding it binds to caspase-3 and -7.<sup>6</sup> Biological studies have indicated that cIAP-1/-2 and XIAP confer on cancer cells resistance to various anticancer drugs and, as a consequence, strategies targeting these IAPs have potential as novel anticancer therapies.<sup>7</sup>

The second mitochondria derived activator of caspases (Smac) is an endogenous inhibitor of these IAPs<sup>8,9</sup> and interacts with IAP proteins via its N-terminal AVPI tetrapeptide motif.<sup>10,11</sup> Smac antagonizes cIAP-1 and cIAP-2 by binding to the BIR3 domain of these two proteins and inducing their rapid degradation.<sup>12,13</sup> In comparison, Smac protein, in a dimeric form, binds concurrently to both the BIR2 and BIR3 of XIAP and removes the inhibition of XIAP to caspase-9 and caspase-3/7.<sup>14,15</sup> The AVPI tetrapeptide of Smac has been used as a lead structure for the design of both peptidic and nonpeptidic small-molecule Smac mimetics as antagonists of IAP proteins.<sup>16–21</sup> The design and synthesis of bivalent Smac mimetics to mimic Smac dimer have also been reported.<sup>22,23</sup>

We have previously reported the design, synthesis, and evaluation of SM-122 (1 in Figure 1) as a conformationally

constrained, nonpeptidic Smac mimetic.<sup>20</sup> It potently binds to XIAP, cIAP-1, and cIAP-2, efficiently inhibits cell growth, and induces cell death in a number of cancer cell lines, but the subsequent pharmacokinetic (PK) studies indicated that it has a less-than-ideal PK profile. In order to improve the PK property, we modified its [8,5] bicyclic core structure to produce SM-337 (Figures 1 and 2),<sup>25</sup> in which carbon 5 of the eight-membered ring of 1 was replaced with a nitrogen atom to which a phenylacetyl group was attached through an amide bond. Compound 2 binds to XIAP, cIAP-1, and cIAP-2 proteins with higher affinities than 1 and is more potent than 1 in cell growth inhibition in the MDA-MB-231 cell line.<sup>25</sup> It also has an improved pharmacokinetic profile and is orally bioavailable,<sup>25</sup> suggesting that modifications of the eightmembered ring in 1 can produce new Smac mimetics with improved in vitro and in vivo properties. In this paper, we report the design, synthesis, and evaluation of a series of new Smac mimetics in which a substituted group was introduced to the eight-membered ring in 1. Our efforts have led to novel Smac mimetics that bind to cIAP-1/2 and XIAP with high affinities and are more potent than compound 1 in inhibition of cancer cell growth and in induction of apoptosis.

#### **Results and Discussion**

Our data for compound **2** suggest that introduction of a polar group into the eight-membered ring region in compound **1** may have significant effect on binding affinities of the resulting compounds to XIAP, cIAP-1, and cIAP-2 proteins, as well as on their cellular activities and in vivo properties. To further explore the structure—activity relationship in this region, we have therefore introduced a hydroxyl group on the eight-membered ring in compound **1** to test the influence in binding and cellular activity of the resulting compounds. Compounds **3** and **4** in which a hydroxyl group is attached to carbon 6 of the eight-membered ring in compound **1** were therefore synthesized. In a fluorescence polarization (FP) based binding assay, **3** and **4** are equipotent in binding to XIAP, cIAP-1, and cIAP-2 BIR3 proteins and only slightly less potent than **1**, showing that the introduction of a hydroxyl

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<sup>&</sup>lt;sup>*a*</sup> Abbreviations: IAP, inhibitor of apoptotic protein; XIAP, X-linked IAP; cIAP, cellular inhibitor of apoptotic protein; Smac, second mitochondria derived activator of caspases; BIR, baculoviral inhibitor of apoptotic protein repeat; PK, pharmacokinetic; PARP, poly(ADP-ribose) polymerase; FP, fluorescence polarization; mP, millipolarization units.

group to carbon 6 of the eight-membered ring of 1 is not detrimental to the binding to these IAP proteins (Table 1).



Figure 1. Structures of Smac mimetics.



**Figure 2.** Smac mimetics antagonize XIAP BIR3 in a cell-free caspase-9 functional assay. 500 nM XIAP BIR3 protein achieves 80% inhibition of caspase-9 activity in Caspase-Glo 9 assay kit, and Smac mimetics dose-dependently restore the activity of caspase-9. Caspase-9 activity was measured after incubation with the caspase-9 specific substrate for 1 h.

We then designed compounds **5** and **6** by introducing a phenylacetyl group at carbon 6 with two different configurations at the new chiral center. In the FP-based binding assay, the cis isomer **5** has  $K_i$  values of 108, 1.6, and 4.2 nM to XIAP, cIAP-1, and cIAP-2, respectively, and is thus equipotent with **2** and 2-5 times more potent than **1**. The trans isomer **6** is 2-3 times less potent than **1**, indicating that the cis configuration is more favorable for binding to the BIR3 domains of these IAPs.

On the basis of the more potent compound **5**, we then synthesized **7** by replacement of the diphenylmethyl group in **5** with an *R*-1-tetrahydronaphthyl group. Previously, we have found that this modification can improve both binding and cellular activities.<sup>20</sup> Indeed, **7** binds to XIAP with a  $K_i$  value of 36 nM and is 5 times more potent than **1**. It also binds potently to cIAP-1 and cIAP-2 with  $K_i < 1$  nM and  $K_i < 1.9$  nM, respectively, being 10 times more potent than **1**. Thus, compound **7** is the most potent compound in this series.

Compounds 5, 6, and 7 were evaluated together with 1 and 2 for their ability to antagonize XIAP in a cell-free caspase-9 functional assay (Figure 2). In this assay, XIAP BIR3 protein dose-dependently inhibits the activity of caspase-9 and, at 500 nM, achieves 80% inhibition. All of these new Smac mimetics 5, 6, and 7 can dose-dependently restore the activity of caspase-9. Compound 5 is as potent as 1 and 2, restoring 60% of caspase-9 activity at  $5\mu$ M, while 6 is about 3 times less potent than 1, 2, or 5. Consistent with its more potent binding affinity to XIAP BIR3, 7 is 3 times more potent than 1, restoring 60% of caspase-9 activity at 1.5  $\mu$ M.

Our previous study showed that 1 and 2 can effectively inhibit cell growth and induce apoptosis in the MDA-MB-231 human breast cancer cell line.<sup>25</sup> Therefore, compounds 5, 6, and 7, together with 1 and 2, were evaluated for their ability to inhibit cell growth in the MDA-MB-231 cancer cell line (Figure 3). It was found that in this assay, 5 is as potent as 2 and 3 times more potent than 1. Although 6 is less potent than 1 in binding to XIAP, cIAP-1, and cIAP-2, it is slightly more



Figure 3. Inhibition of cell growth by Smac mimetics in the MDA-MB-231 cancer cell line. Cells were seeded in 96-well flat-bottom cell culture plates at a density of  $(3-4) \times 10^2$  cells/well and grown overnight, then incubated with Smac mimetics for 4 days. Cell growth was determined using a WST-based assay.

Table 1.	Binding Affinities of	of Smac Mimetics to	XIAP BIR3, cIAP-	1 BIR3, and cIAP-2	2 BIR3 Proteins
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compd	XIAP BIR3		cIAP-1 BIR3		cIAP-2 BIR3	
	$IC_{50} \pm SD (nM)$	$K_{\rm i} \pm { m SD}  ({ m nM})$	$IC_{50} \pm SD (nM)$	$K_{\rm i} \pm { m SD}  ({ m nM})$	$IC_{50} \pm SD (nM)$	$K_{\rm i} \pm { m SD}  ({ m nM})$
1	$635.7 \pm 50.7$	$190.7 \pm 15.2$	$46.2 \pm 8.3$	$6.7 \pm 1.2$	$74.7 \pm 12.1$	$18.3 \pm 2.9$
2	$369.7 \pm 187.3$	$110.9 \pm 56.2$	$9.0 \pm 2.8$	$1.3 \pm 0.4$	$26.3 \pm 6.3$	$6.3 \pm 1.5$
3	$1068 \pm 89.3$	$320.3 \pm 26.8$	$54.5 \pm 4.1$	$7.9 \pm 0.6$	$91.8 \pm 13.2$	$22.5 \pm 3.1$
4	$1102 \pm 98.3$	$330.6 \pm 29.5$	$45.5 \pm 3.4$	$6.6 \pm 0.5$	$99.6 \pm 12.3$	$24.8 \pm 2.9$
5	$361.0 \pm 86.0$	$108.0 \pm 26.1$	$10.9 \pm 4.2$	$1.6 \pm 0.6$	$19.1 \pm 6.2$	$4.2 \pm 1.7$
6	$1017 \pm 95.0$	$305.2 \pm 28.5$	$84.1 \pm 16.6$	$12.2 \pm 2.4$	$195.8 \pm 10.4$	$47.0 \pm 2.5$
7	$125.0\pm9.6$	$36.0\pm2.9$	$2.6 \pm 0.3$	<1	$5.3 \pm 1.5$	<1.9



**Figure 4.** Induction of cIAP-1 degradation, cleavage of PARP, and processing of caspase-3 by compounds **1**, **2**, **5**, and **7** in the MDA-MB-231 cell line. Cells were treated with different concentrations of the compounds for 24 h, and levels of cIAP-1, cleaved PAPR (CL PARP), and cleaved caspase-3 (CL C3) were probed by Western blot analysis.

Scheme 1. Synthesis of Key Intermediates<sup>a</sup>



<sup>*a*</sup> Reagents and conditions: (a) 9-BBN, THF, then  $H_2O_2(35\%$  in water), 3 N NaOH; (b) Dess-Martin periodinane, CH<sub>2</sub>CI<sub>2</sub>, 18% for **12**, and 66% for **13**; (c) Dess-Martin periodinane, CH<sub>2</sub>CI<sub>2</sub>, 86%; (d) NaBH<sub>3</sub>CN, MeOH, H<sub>2</sub>SO<sub>4</sub> (cat.), 89%.

potent than 1 in this cell growth assay with an IC<sub>50</sub> of  $1.1 \,\mu$ M. The most potent compound 7 achieves an IC<sub>50</sub> of  $0.2 \,\mu$ M and is 9 times more potent than 1.

Several reports have shown that Smac mimetics induce rapid cIAP-1 degradation in cancer cells and that cIAP-1 is a direct and critical cellular target for Smac mimetics.<sup>12,13,24</sup> We evaluated **1**, **2**, **5**, and **7** for their ability to induce cIAP-1 degradation and cleavage of caspase-3 and poly(ADP-ribose) polymerase (PARP) in the MDA-MB-231 cell line (Figure 4). We found that **5** and **7** at 10 nM effectively induce cIAP-1 degradation and are as potent as **2** and more potent than **1**. Compounds **5** and **7** also induce robust cleavage of PARP and processing of caspase-3, two biochemical markers of apoptosis, within 24 h and are more effective than **1** (Figure 4).

Synthesis of these new Smac mimetics is shown in Schemes 1 and 2. Compound 8 was prepared by a published method.<sup>26</sup> Hydroboration of the C–C double bond in 8 by reaction with 9-BBN followed by oxidation of the resulting borane by alkaline  $H_2O_2$  gave a mixture of four alcohols, two 5-hydroxy epimers (9 and 10), and two 6-hydroxy epimers (11). The alcohol 9 was separated from the other three isomers by chromatography, and its structure was confirmed by X-ray

analysis (Figure 5). Oxidation of the mixture of the other three isomers by Dess–Martin periodinane yielded two ketones, 12 and 13, which can be separated by chromatography. Oxidation of 9 under the same conditions yielded 12, thus confirming the structures of both ketones. Reduction of the ketone 12 by NaBH<sub>3</sub>CN under the influence of a catalytic amount of  $H_2SO_4$ furnished alcohol 10 as a single isomer.

Hydrogenolysis of the benzyl ester group in **9** and **10** followed by condensation of the resulting two acids with aminodiphenylmethane afforded **15** and **16** (Scheme 2). Removal of the Boc protecting group from **15** or **16** followed by condensation of the resulting ammonium salts with L-*N*-Boc-*N*-methylalanine afforded two amides. Removal of the Boc protecting groups in these two amides yielded **3** and **4**. Condensation of the acid from hydrogenolysis of the benzyl ester group in **10** with (*R*)-1,2,3,4-tetrahydronaphthyl-1amine furnished **17**. Transformation of the hydroxyl group in **15**, **16**, and **17** to an azido group by a Mitsunobu reaction gave three corresponding azides, **18**, **19**, and **20**. Removal of the Boc protecting group from these azides followed by condensation of the resulting ammonium salts with L-*N*-Boc-*N*-methylalanine afforded three azido amides, reducing of the



Figure 5. Crystal structure of the key intermediate 9.

Scheme 2. Synthesis of Smac Mimetics  $3-7^a$ 



<sup>*a*</sup> Reagents and coditions: (a) (i) H<sub>2</sub>, 10% Pd–C, methanol; (ii) aminodiphenylmethane, EDC, HOBt, *N*,*N*-diisopropylethylamine, CH<sub>2</sub>CI<sub>2</sub>; (b) (i) 4 N HCI in 1,4-dioxane, methanol; (ii) L-N-Boc-*N*-methylalanine, EDC, HOBt, *N*,*N*-diisopropylethylamine, CH<sub>2</sub>CI<sub>2</sub>; (iii) 4 N HCI in 1,4dioxane, methanol. (c) (i) H<sub>2</sub>, 10% Pd–C, methanol; (ii) (*R*)-1,2,3,4tetrahydronaphthyl-1-amine, EDC, HOBt, *N*,*N*-diisopropylethylamine, CH<sub>2</sub>CI<sub>2</sub>, 79% over two steps; (d) (i) diethyl azodicarboxylate, DPPA, PPh<sub>3</sub>, THF; (e) (i) 4 N HCI in 1,4-dioxane, methanol; (ii) L-*N*-Boc-*N*-methylalanine, EDC, HOBt, *N*,*N*-diisopropylethylamine, CH<sub>2</sub>CI<sub>2</sub>; (iii) PPh<sub>3</sub>, THF-H<sub>2</sub>O 1:1; (iv) phenylacetyl chloride, *N*,*N*-diisopropylethylamine, CH<sub>2</sub>CI<sub>2</sub>; (v) 4 N HCI in 1,4- dioxane, methanol.

azido group in which with triphenylphosphine in 1:1 THF– $H_2O$  yielded the corresponding amines which were condensed with phenylacetyl chloride to furnish the phenylacetyl amides. Removal of the Boc protecting group from these amides provided our designed compounds **5**, **6**, and **7**.

In summary, we have designed and synthesized a series of new Smac mimetics based on a previously identified nonpeptidic Smac mimetic compound, **1**. The most potent compound **7**  binds to XIAP, cIAP-1, and cIAP-2 with low to subnanomolar affinities. Compound 7 induces cIAP-1 degradation in cancer cells with concentrations as low as 10 nM and antagonizes XIAP in a cell-free functional assay. Compound 7 effectively induces processing of caspase-3 and PARP cleavage, has an IC<sub>50</sub> value of 200 nM in inhibition of cell growth in the MDA-MB-231 breast cancer cell line, and is 9 times more potent than the initial lead compound **1**. On the basis of these findings, additional in vitro and in vivo studies for compound 7 are underway, and the results will be reported in due course.

## **Experimental Section**

**Chemistry. General.** NMR spectra were measured at 300 MHz. <sup>1</sup>H chemical shifts are reported relative to HDO (4.79 ppm) as the internal standard. Final products were purified using a C18 reverse phase semipreparative HPLC column with solvent A (0.1% of TFA in water) and solvent B (0.1% of TFA in CH<sub>3</sub>CN) as eluents. All the target compounds (**3**, **4**, **5**, **6**, and 7) have purities of >95% based upon elemental analysis.

Benzyl (3S,6S,9R,10aR)-6-((tert-Butoxycarbonyl)amino)-9hydroxy-5-oxodecahydropyrrolo[1,2-a]azocine-3-carboxylate (9). To a solution of compound 8 (1.25 g, 3 mmol) in 50 mL of dry THF was added 12 mL of 9-BBN solution (0.5 M in THF, 6 mmol). After the solution was stirred under N<sub>2</sub> at room temperature for 12 h, 2 mL of 3 M NaOH solution and 3 mL of H<sub>2</sub>O<sub>2</sub> solution (35% in water) were added dropwise at 0 °C. The mixture was warmed to room temperature and stirred for 2 h before being extracted with ethyl acetate. The combined organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and then condensed. The residue was purified by chromatography to give compound 9 (320 mg, 25%) and a mixture of three other isomers 10 and 11 (580 mg, 45%). Chemical data for compound 9:  $[\alpha]_D^{20}$  -21.5 (c 1, CHCl<sub>3</sub>). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  7.40–7.28 (m, 5H), 5.43 (brd, J =8.0 Hz, 1H), 5.28, 5.18 (ABq, J = 12.2 Hz, 2H), 4.67 (t, J = 9.3 Hz, 1H), 4.65 (m, 1H), 4.20 (m, 1H), 3.96 (m, 1H), 2.45–1.60 (m, 10H), 1.38 (brs, 9H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ 172.38, 170.83, 155.13, 135.39, 128.58, 128.40, 128.34, 79.70, 70.63, 67.14, 60.13, 56.16, 50.70, 45.22, 32.67, 31.70, 28.35, 27.34. ESI MS: m/z 433.3 (M + H)<sup>+</sup>. HR ESI MS for C<sub>23</sub>H<sub>33</sub>N<sub>2</sub>O<sub>6</sub> required, 433.2339; found, 433.2339.

Benzyl (3S,6S,10aR)-6-((tert-Butoxycarbonyl)amino)-5,9-dioxodecahydropyrrolo[1,2-a]azocine-3-carboxylate (12) and Benzyl (3S,6S,10aR)-6-((tert-Butoxycarbonyl)amino)-5,8-dioxodecahydropyrrolo[1,2-a]azocine-3-carboxylate (13). To a solution of the mixture of 10 and 11 obtained above (570 mg, 1.3 mmol) in 15 mL of CH<sub>2</sub>Cl<sub>2</sub> was added Dess-Martin periodinane (660 mg, 1.56 mmol) at room temperature. The mixture was stirred at the same temperature for 2 h and then condensed. The residue was purified by chromatography to give compounds 12(102 mg, 18%)and 13 (370 mg, 66%). Compound 9 can be oxidized to compound 12 using the same method. Chemical data for compound 12:  $[\alpha]_D^{20}$ -246.6 (*c* 1, CHCl<sub>3</sub>). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ 7.40-7.28 (m, 5H), 5.42 (brd, J = 8.2 Hz, 1H), 5.28, 5.18 (ABq, J = 12.2 Hz, 1H)2H), 4.62 (dd, J = 9.0, 8.2 Hz, 1H), 4.37 (m, 2H), 3.13 (m, 1H), 3.02 (t, J = 12.0 Hz, 1H), 2.50-1.98 (m, 6H), 1.83 (m, 1H), 1.60(m, 1H), 1.38 (brs, 9H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ 211.37, 172.20, 170.94, 154.92, 135.23, 128.56, 128.41, 128.22, 79.81, 67.17, 60.53, 56.06, 53.39, 52.88, 36.79, 32.36, 30.18, 28.22, 27.00. ESI MS: m/z 431.3 (M + H)<sup>+</sup>. HR ESI MS for C<sub>23</sub>H<sub>31</sub>-N<sub>2</sub>O<sub>6</sub> required, 431.2182; found, 431.2170. Chemical data for compound 13:  $[\alpha]_{D}^{20}$  -66.4 (c 1, CHCl<sub>3</sub>). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  7.40–7.20 (m, 5H), 5.49 (brd, J = 7.7 Hz, 1H), 5.17 (s, 2H), 5.09 (m, 1H), 4.52 (t, J = 8.5 Hz, 1H), 4.22 (m, 1H), 3.08 (dd, J = 12.7, 4.5 Hz, 1H), 2.92 (m, 1H), 2.60 (m, 2H), 2.36-1.72(m, 6H), 1.43 (brs, 9H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ 207.72, 170.93, 170.15, 154.74, 135.58, 128.37, 128.30, 128.14, 80.00, 66.67, 60.10, 59.74, 52.13, 48.52, 39.65, 34.18, 32.36, 28.21,

26.90. ESI MS: m/z 431.3 (M + H)<sup>+</sup>. HR ESI MS for C<sub>23</sub>H<sub>31</sub>-N<sub>2</sub>O<sub>6</sub> required, 431.2182; found, 431.2177.

Benzyl (3S,6S,9S,10aR)-6-((tert-Butoxycarbonyl)amino)-9hydroxy-5-oxodecahydropyrrolo[1,2-a]azocine-3-carboxylate (10). To a solution of compound 12 (160 mg, 0.37 mmol) in 15 mL of methanol was added NaBH<sub>3</sub>CN (120 mg, 1.9 mmol) and 3 drops of  $H_2SO_4$  (98%) at -15 °C. After the mixture was stirred at the same temperature for 4 h, 10 mL of water was added and the mixture was extracted with ethyl acetate (30 mL  $\times$  4). The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub> and then condensed. The residue was purified by chromatography to give compound **10** (142 mg, 89%).  $[\alpha]_D^{20}$  -66.5 (*c* 1, CHCl<sub>3</sub>). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  7.30 (brs, 5H), 5.45 (brd, J = 8.2 Hz, 1H), 5.20, 5.10 (ABq, J = 12.0 Hz, 2H), 4.85 (m, 1H), 4.48 (m, 2H), 4.13 (m, 1H),3.16 (brs, 1H), 2.42-1.45 (m, 10H), 1.40 (brs, 9H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ 172.30, 171.03, 155.06, 135.43, 128.49, 128.27, 128.15, 79.36, 67.60, 66.87, 59.74, 54.00, 51.65, 43.62, 32.12, 31.82, 29.30, 28.29, 27.11. ESI MS: m/z 433.3 (M + H)<sup>+</sup>. HR ESI MS for C<sub>23</sub>H<sub>33</sub>N<sub>2</sub>O<sub>6</sub> required, 433.2339; found, 433.2340.

tert-Butyl ((3S,6S,9R,10aR)-9-Azido-3-(benzhydrylcarbamoyl)-5-oxodecahydropyrrolo[1,2-a]azocin-6-yl)carbamate (18). To a solution of compound 10 (430 mg, 1 mmol) in 20 mL of methanol was added 100 mg of 10% Pd-C. After the mixture was stirred under H<sub>2</sub> overnight, the catalyst was removed and the filtration was condensed to give an acid. To a solution of this acid in 20 mL of CH<sub>2</sub>Cl<sub>2</sub> was added aminodiphenylmethane (220 mg, 1.2 mmol), EDC (230 mg, 1.2 mmol), HOBt (160 mg, 1.2 mmol), and 1 mL of N,N-diisopropylethylamine. The mixture was stirred at room temperature overnight and then condensed. The residue was purified by chromatography to furnish 16 (416 mg, 82% for two steps). To a solution of 16 (254 mg, 0.5 mmol) in 20 mL of THF was added diethyl azodicarboxylate (170 mg, 1 mmol), PPh<sub>3</sub> (260 mg, 1 mmol), and diphenylphosphorylazide (350 mg, 1.3 mmol). The mixture was stirred at room temperature overnight and then condensed. The residue was purified by chromatography to yield compound **18** (228 mg, 86%).  $[\alpha]_D^{20}$  34.3 (*c* 0.85, CHCl<sub>3</sub>). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  7.52 (brd, J = 8.6 Hz, 1H), 7.42-7.18 (m, 5H), 6.21 (d, J = 8.6 Hz, 1H), 5.40 (brd, J =8.2 Hz, 1H, 4.71 (t, J = 6.7 Hz, 1H), 4.50 (m, 1H), 4.22 (m, 1H), 3.50 (m, 1H), 2.51 (m, 1H), 2.24 (m, 1H), 2.08 (m, 2H), 1.95–1.75 (m, 2H), 1.48–1.20 (m, 13H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ 171.50, 169.56, 155.02, 141.58, 141.30, 128.62, 127.52, 127.45, 127.38, 127.15, 79.96, 64.14, 60.55, 60.20, 57.20, 57.08, 49.96, 42.25, 32.83, 29.23, 28.32, 25.03. ESI MS: m/z 533.3 (M + H)<sup>+</sup>. HR ESI MS for C<sub>29</sub>H<sub>37</sub>N<sub>6</sub>O<sub>4</sub> required, 533.2876; found, 533.2874

tert-Butyl ((3S,6S,9S,10aR)-9-Azido-3-(benzhydrylcarbamoyl)-5-oxodecahydropyrrolo[1,2-a]azocin-6-yl)carbamate (19). Compound 15 was synthesized using the same method as that for 16 from compound 9 (85% for two steps), and compound 19 was synthesized using the same method as that for 18 from **15**. Chemical data for **19**:  $[\alpha]_D^{20}$  3.2 (*c* 1, CHCl<sub>3</sub>). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  7.76 (brd, J = 8.6 Hz, 1H), 7.38–7.19 (m, 5H), 6.20 (d, J = 8.6 Hz, 1H), 5.55 (brd, J = 7.7 Hz, 1H), 4.70 (t, J = 7.1 Hz, 1H), 4.60 (m, 1H), 4.31 (m, 1H), 3.35 (m, 1H), 4.70 (t, J = 7.1 Hz, 1H), 4.60 (m, 1H), 4.70 (m, 1H), 4.701H), 2.61 (m, 1H), 2.20-1.96 (m, 4H), 1.82-1.43 (m, 3H), 1.42 (brs, 9H), 1.41–1.28 (m, 2H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ 171.48, 169.50, 154.89, 141.68, 141.21, 128.62, 128.59, 127.52, 127.46, 127.39, 127.14, 79.72, 59.57, 59.10, 57.23, 54.73, 51.68, 39.98, 32.77, 31.51, 28.35, 28.23, 23.95. ESI MS: m/z 533.3  $(M + H)^+$ . HR ESI MS for C<sub>29</sub>H<sub>37</sub>N<sub>6</sub>O<sub>4</sub> required, 533.2876; found, 533.2879.

*tert*-Butyl ((3*S*,6*S*,9*R*,10*aR*)-9-Azido-5-oxo-3-(((*R*)-1,2,3,4tetrahydronaphthalen-1-yl)carbamoyl)decahydropyrrolo[1,2-*a*]azocin-6-yl)carbamate (20). To a solution of compound 10 (215 mg, 0.5 mmol) in 20 mL of methanol was added 50 mg of 10% Pd-C. After the mixture was stirred under H<sub>2</sub> overnight, the catalyst was removed and the filtrate was condensed to give an acid. To a solution of this acid in 20 mL of CH<sub>2</sub>Cl<sub>2</sub> was added (*R*)-1,2,3,4-tetrahydronaphthyl-1-amine (90 mg, 0.6 mmol), EDC (115 mg, 0.6 mmol), HOBt hydrate (80 mg, 0.6 mmol), and 0.5 mL of N,N-diisopropylethylamine. The mixture was stirred at room temperature overnight and then condensed. The residue was purified by chromatography to furnish 17 (186 mg, 79% for two steps). To a solution of 17 in 20 mL of THF was added diethyl azodicarboxylate (170 mg, 1 mmol), PPh<sub>3</sub> (260 mg, 1 mmol), and diphenylphosphorylazide (350 mg, 1.3 mmol). The mixture was stirred at room temperature overnight and then condensed. The residue was purified by chromatography to yield compound **20** (155 mg, 79%).  $[\alpha]_{D}^{20}$  54.1 (*c* 1, CHCl<sub>3</sub>). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  7.32 (m, 1H), 7.15 (m, 2H), 7.06 (m, 1H), 6.77 (brd, J = 8.3 Hz, 1H, 5.43 (brd, J = 8.2 Hz, 1H), 5.16 (m, 1H), 4.54 (m, 1H),4.48 (t, J = 7.5 Hz, 1H), 4.25 (m, 1H), 3.52 (m, 1H), 2.80 (m, 2H),2.48-1.50 (m, 14H), 1.42 (brs, 9H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ 171.02, 170.07, 154.94, 137.11, 136.33, 128.98, 128.76, 127.20, 126.23, 79.76, 61.17, 60.35, 56.99, 49.85, 47.47, 42.20, 32.74, 29.84, 29.14, 29.01, 28.25, 26.08, 19.76. ESI MS: m/z 497.3 (M + H)<sup>+</sup>. HR ESI MS for C<sub>26</sub>H<sub>37</sub>N<sub>6</sub>O<sub>4</sub> required, 497.2876; found, 497.2883.

(3S,6S,9R,10aR)-N-Benzhydryl-9-hydroxy-6-((S)-2-(methylamino)propanamido)-5-oxodecahydropyrrolo[1,2-a]azocine-3carboxamide (3). HCl (4 N) in 1,4-dioxane (2 mL) was added to a solution of 13 (120 mg, 0.23 mmol) in MeOH (5 mL). The solution was stirred at room temperature overnight and then concentrated to give an ammonium salt. L-N-Boc-N-methylalanine (60 mg, 0.3 mmol), EDC (57 mg, 0.3 mmol), HOBt hydrate (45 mg, 0.3 mmol), and 0.3 mL of N,N-diisopropylethylamine were added to a mixture of this salt in CH<sub>2</sub>Cl<sub>2</sub> (10 mL). The mixture was stirred at room temperature overnight and then concentrated. The residue was purified by chromatography to yield an amide to a solution of which in MeOH (5 mL) was added 4 N HCl in 1,4-dioxane (2 mL). The solution was stirred at room temperature overnight, then concentrated. The residue was purified by semipreparative HPLC to give 3 as a trifluoroacetate salt. The gradient used ran from 75% of solvent A and 25% of solvent B to 55% of solvent A and 45% of solvent B in 40 min. The purity of the product was confirmed by analytical HPLC to be over 98%. <sup>1</sup>H NMR (300 M Hz,  $D_2O$ )  $\delta$  7.35–7.15 (m, 10H), 5.90 (s, 1H), 4.65 (m, 1H), 4.45-4.28 (m, 2H), 4.05 (m, 1H), 3.82 (m, 1H), 2.55 (s, 3H), 2.35-1.45 (m, 10H), 1.42 (d, J = 7.2 Hz, 3H). ESI MS: m/z493.3  $(M + H)^+$ . Anal.  $(C_{28}H_{36}N_4O_4 \cdot 1.5CF_3COOH)$  C, H, N.

(3*S*,6*S*,9*S*,10*aR*)-*N*-Benzhydryl-9-hydroxy-6-((*S*)-2-(methylamino)propanamido)-5-oxodecahydropyrrolo[1,2-*a*]azocine-3-carboxamide (4). Compound 4 was synthesized from intermediate 14 by the same method used for 3. The purity was confirmed by analytical HPLC as over 98%. <sup>1</sup>H NMR (300 M Hz, D<sub>2</sub>O)  $\delta$ 7.35–7.18 (m, 10H), 5.95 (s, 1H), 4.70 (m, 1H), 4.45–4.32 (m, 2H), 4.05 (m, 1H), 3.85 (m, 1H), 2.58 (s, 3H), 2.32–1.55 (m, 10H), 1.42 (d, *J* = 7.2 Hz, 3H). ESI MS: *m/z* 493.3 (M + H)<sup>+</sup>. Anal. (C<sub>28</sub>H<sub>36</sub>N<sub>4</sub>O<sub>4</sub>·1.3CF<sub>3</sub>COOH) C, H, N.

(3S,6S,9R,10aR)-N-Benzhydryl-6-((S)-2-(methylamino)propanamido)-5-oxo-9-(2-phenylacetamido)decahydropyrrolo-[1,2-a]azocine-3-carboxamide (5). To a solution of 16 (106 mg, 0.2 mmol) in MeOH (5 mL) was added HCl solution (4 N in 1,4dioxane, 2 mL). The solution was stirred at room temperature overnight and then concentrated to give an ammonium salt. L-N-Boc-N-methylalanine (62 mg, 0.3 mmol), EDC (58 mg, 0.3 mmol), HOBt hydrate (44 mg, 0.3 mmol), and 0.3 mL of N,Ndiisopropylethylamine were added to a mixture of this salt in CH<sub>2</sub>Cl<sub>2</sub> (10 mL). The mixture was stirred at room temperature overnight and then concentrated. The residue was purified by chromatography to yield an amide. Triphenylphosphine (80 mg, 0.3 mmol) and H<sub>2</sub>O (3 mL) were added to a solution of this amide in THF (10 mL). The mixture was stirred at room temperature overnight and then partitioned between CH<sub>2</sub>Cl<sub>2</sub> (60 mL) and brine (20 mL). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and then concentrated. The residue was redissolved in CH<sub>2</sub>Cl<sub>2</sub> (10 mL). Phenylacetyl chloride (0.04 mL) and N,N-diisopropylethylamine (0.1 mL) were added to this solution, and the mixture was stirred at room temperature overnight, then concentrated. The residue was purified by chromatography to furnish a phenylacetylamide.

HCl (4 N in 1,4-dioxane, 2 mL) was added to a solution of this amide in MeOH (10 mL), and the solution was stirred at room temperature overnight, then concentrated. The residue was purified by semipreparative HPLC to give compound **5** as a salt with TFA. The gradient used ran from 70% of solvent A and 30% of solvent B to 50% of solvent A and 50% of solvent B in 40 min. The purity was confirmed by analytical HPLC to be over 98%. <sup>1</sup>H NMR (300 M Hz, D<sub>2</sub>O)  $\delta$  7.10–6.90 (m, 10H), 5.85 (s, 1H), 4.55 (m, 1H), 4.34 (m, 1H), 4.22 (m, 1H), 3.80 (m, 1H), 3.65 (m, 1H), 3.30, 3.20 (ABq, J = 8.4 Hz, 2H), 2.55 (s, 3H), 2.10–1.45 (m, 10H), 1.40 (d, J = 7.2 Hz, 3H). ESI MS: m/z 610.3 (M + H)<sup>+</sup>. Anal. (C<sub>36</sub>H<sub>43</sub>N<sub>5</sub>O<sub>4</sub>·1.7CF<sub>3</sub>COOH) C, H, N.

(3*S*,6*S*,9*S*,10*aR*)-*N*-Benzhydryl-6-((*S*)-2-(methylamino)propanamido)-5-oxo-9-(2-phenylacetamido)decahydropyrrolo-[1,2-*a*]azocine-3-carboxamide (6). Compound 6 was synthesized from 17 by the same method used for 5. The gradient used ran from 70% of solvent A and 30% of solvent B to 50% of solvent A and 50% of solvent B in 40 min. The purity was confirmed by analytical HPLC to be over 98%. <sup>1</sup>H NMR (300 M Hz, D<sub>2</sub>O)  $\delta$  7.35–7.18 (m, 10H), 5.92 (s, 1H), 4.70 (m, 1H), 4.55–4.35 (m, 2H), 3.90 (m, 1H), 3.79 (m, 1H), 3.50, 3.40 (ABq, *J* = 8.4 Hz, 2H), 2.58 (s, 3H), 2.35–1.55 (m, 10H), 1.40 (d, *J* = 7.2 Hz, 3H). ESI MS: *m*/*z* 610.3 (M + H)<sup>+</sup>. Anal. (C<sub>36</sub>H<sub>43</sub>N<sub>5</sub>O<sub>4</sub>·1.4CF<sub>3</sub>COOH) C, H, N.

(3*S*,6*S*,9*R*,10*aR*)-6-((*S*)-2-(Methylamino)propanamido)-5-oxo-9-(2-phenylacetamido)-*N*-((*R*)-1,2,3,4-tetrahydronaphthalen-1yl)decahydropyrrolo[1,2-*a*]azocine-3-carboxamide (7). Compound 7 was synthesized from the intermediate 18 using the same method as that for 5 and purified by HPLC using the same gradient. The purity was confirmed by analytical HPLC to be over 98%. <sup>1</sup>H NMR (300 M Hz, D<sub>2</sub>O)  $\delta$  7.35–7.05 (m, 9H), 4.85 (m, 1H), 4.45 (m, 1H), 4.25 (m, 1H), 3.95–3.80 (m, 3H), 3.50, 3.40 (ABq, *J* = 8.5 Hz, 2H), 2.70 (m, 2H), 2.60 (m, 3H), 2.30–1.50 (m, 14H), 1.42 (d, *J* = 7.2 Hz, 3H). ESI MS: *m*/*z* 574.3 (M + H)<sup>+</sup>. Anal. (C<sub>33</sub>H<sub>43</sub>N<sub>5</sub>O<sub>4</sub>·1.2CF<sub>3</sub>COOH) C, H, N.

Fluorescence Polarization Based Binding Assays for XIAP, cIAP-1, and cIAP-2 Proteins. A set of sensitive and quantitative fluorescence polarization (FP) based assays were used to determine the binding affinities of our designed Smac mimetics to XIAP BIR3, cIAP-1 BIR3, and cIAP-2 BIR3 proteins.

**Protein Expression and Purification.** Human XIAP BIR3 (residues 241-356) was cloned into a pET28 vector (Novagen) containing an N-terminal 6xHis tag. Protein was produced in *E. coli* BL21(DE3) cells grown at 37 °C in 2xYT containing kanamycin to an OD<sub>600</sub> of 0.6.

Protein expression was induced by 0.4 mM IPTG at 27 °C for 4 h. Cells were lysed by sonication in buffer containing Tris, pH 7.5 (50 mM), NaCl (200 mM), ZnOAc (50  $\mu$ M), 0.1%  $\beta$ ME, and leupectin/aprotin protease inhibitors. Protein was purified from the soluble fraction using Ni-NTA resin (QIAGEN) followed by gel filtration on a Superdex 75 column in Tris, pH 7.5 (20 mM), NaCl (200 mM), ZnOAc (50  $\mu$ M), and dithiothreitol (DTT, 1 mM). After purification, DTT was added to a final concentration of 10 mM. Human cIAP-1 BIR3-only (residues 253–363) and cIAP-2 BIR3-only (residues 238–349) were cloned into pHis-TEV vector, produced, and purified using the same method as for the XIAP protein.

**FP-Based Binding Assays.** A fluorescently labeled Smac mimetic (Smac-2F) was used as the tracer in FP assays to determine the binding affinities of our designed Smac mimetics to XIAP, cIAP-1, and cIAP-2 proteins. The  $K_d$  values of Smac-2F to XIAP BIR3, cIAP-1 BIR3, and cIAP-2 BIR3 were determined to be  $4.4 \pm 0.7$ ,  $0.7 \pm 0.5$ , and  $1.9 \pm 1$  nM by monitoring the total fluorescence polarization of mixtures composed with fluorescent tracer at a fixed concentration and different IAP proteins with increasing concentrations up to full saturation. Fluorescence polarization values were measured using the Infinite M-1000 plate reader (Tecan U.S., Research Triangle Park, NC) in Microfluor 2 96-well, black, round-bottom plates (Thermo Scientific). In the saturation experiments, Smac-2F (2, 1, and 1 nM for experiments with XIAP BIR3, cIAP-1 BIR3,

and cIAP-2 BIR3, respectively) and increasing concentrations of proteins were added to each well to a final volume of  $125 \,\mu$ L in the assay buffer (100 mM potassium phosphate, pH 7.5, 100  $\mu$ g/mL bovine  $\gamma$ -globulin, 0.02% sodium azide, Invitrogen, with 4% DMSO). Plates were mixed and incubated at room temperature for 2–3 h with gentle shaking to ensure equilibrium. The polarization values in millipolarization units (mP) were measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Equilibrium dissociation constants ( $K_d$ ) were then calculated by fitting the sigmoidal dose-dependent FP increases as a function of protein concentrations using Graphpad Prism 5.0 software (Graphpad Software, San Diego, CA).

For competitive experiments, the  $K_i$  value of the tested compound was determined in a dose-dependent competitive binding experiment. Mixtures of 5  $\mu$ L of the tested compound in different concentrations in DMSO and 120  $\mu$ L of preincubated protein/tracer complex in the fixed concentrations in the assay buffer were added into assay plates and incubated at room temperature for 2-3 h with gentle shaking. Final concentrations of proteins and tracers in the competitive assays were 10 and 2 nM for XIAP BIR3, 3 and 1 nM for cIAP-1 BIR3, and 5 and 1 nM for cIAP-2 BIR3, respectively. Negative controls containing protein/tracer complex only (equivalent to 0% inhibition) and positive controls containing only free tracers (equivalent to 100% inhibition) were included in each assay plate. FP values were measured as described above. IC<sub>50</sub> values were determined by nonlinear regression fitting of the competition curves. The  $K_i$ values of the tested compound to these IAP proteins were calculated using the measured IC<sub>50</sub> values, the  $K_d$  values of the tracer to different IAP proteins, and the concentrations of the proteins and tracers in the competitive assays.<sup>27</sup>

**Cell-Free Caspase-9 Functional Assay.** For caspase-9 activity assay, the enzymatic activity of active recombinant caspase-9 from Enzo Life Sciences was evaluated by the Caspase-Glo 9 assay kit from Promega. An amount of 2.5  $\mu$ L of compound solution in caspase assay buffer (CAB, 50 mM HEPES, 100 mM NaCl, 1 mM EDTA with 0.1% CHAPS, and 10% glycerol, pH 7.4) containing 20% DMSO was mixed with 7.5  $\mu$ L of XIAP BIR3 protein and preincubated for 15 min, followed by addition of 2.5  $\mu$ L of active caspase-9 solution in CAB. This mixture was incubated at room temperature for 15 min. Luminogenic Z-LEHD substrate was added with 1:1 ratio to give final concentrations of XIAP and caspase-9 as 500 nM and 2.5 unit/ reaction (according to the manufacturer instruction), respectively. Luminescence from the substrate cleavage was monitored by Tecan Infinite M-1000 multimode plate reader for 1 h.

Cell Growth Inhibition Assay. The MDA-MB-231 breast cancer cell line was purchased from the American Type Culture Collection (ATCC). Cells were seeded in 96-well flat bottom cell culture plates at a density of  $(3-4) \times 10^2$  cells/well and grown overnight, then incubated with or without Smac mimetics for 4 days. Cell growth inhibition was determined with a LDH based WST-8 assay (WST-8; Dojindo Molecular Technologies Inc., Gaithersburg, Maryland). Briefly, when treatment of cells was finished, WST-8 was added to each well to a final concentration of 10%, and then the plates were incubated at 37 °C for 2–3 h. The absorbance of the samples was measured at 450 nm using a TECAN ULTRA reader. Concentration of the compounds that inhibited cell growth by 50% (IC<sub>50</sub>) was calculated by comparing the absorbance in the untreated cells and the cells treated with the compounds.

Western Blot Analysis. Treated cells were harvested and washed with cold PBS. Cell pellets were lysed in double lysis buffer (DLB; 50 mmol/L Tris, 150 mmol/L sodium chloride (1 mmol/L EDTA, 0.1% SDS, and 1% NP-40) in the presence of PMSF (1 mmol/L) and protease inhibitor cocktail (Roche) for 10 min on ice, then centrifuged at 13 000 rpm at 4 °C for 10 min. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories). Proteins were electrophoresed onto a 4–20% gradient SDS–PAGE (Invitrogen) and then

transferred to PVDF membranes. Following blocking in 5% milk, the membranes were incubated with a specific primary antibody, washed three times, and incubated with horseradish peroxidase-linked secondary antibody (Amersham). The signals were visualized with a chemiluminescent HRP antibody detection reagent (Denville Scientific). When indicated, the blots were stripped and reprobed with a different antibody. Primary antibodies against cleaved caspase 3, PARP, and  $\beta$ -actin were purchased from Cell Signaling Technology, and primary antibody against cIAP-1 was purchased from R&D System.

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