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Design, Synthesis and Evaluation of Potent, Non-Peptidic Mimetics of Second Mitochondria-derived Activator of Caspases

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Abstract

A series of new Smac mimetics have been designed, synthesized and evaluated. The most potent compound **10** binds to XIAP, cIAP-1 and cIAP-2 BIR3 proteins with K_i values of 3.9, 0.37 and 0.25 nM, respectively. Compound **10** antagonizes XIAP in a cell-free functional assay and induces rapid cIAP-1 degradation in cancer cells. Compound **10** inhibits cell growth in the MDA-MB-231 cancer cell line with an IC_{50} value of 8.9 nM.

Apoptosis is a critical cell process in normal development and homeostasis of multicellular organisms to eliminate unwanted or damaged cells. Evasion of apoptosis is now recognized as a hallmark of all cancers.[1–2] Targeting key apoptosis regulators with a goal to promote apoptosis in cancer cells is a new strategy for anticancer drug design.[3–4]

Inhibitor of apoptosis proteins (IAPs)[a] are a class of key apoptosis regulators.[5,6] Among them, cellular IAP-1 (cIAP-1) and cIAP-2 play a critical role in regulation of tumor necrosis factor (TNF) receptor-mediated apoptosis,[7] and X-linked IAP (XIAP) is a central regulator of both death-receptor-mediated and mitochondria-mediated apoptosis pathways.[8] XIAP inhibits apoptosis through direct binding to and inhibition of three cysteine proteases, an initiator caspase-9 and the two effectors caspase-3 and -7.[5,6] XIAP contains three Baculoviral IAP Repeats (BIR) domains. While the third BIR domain (BIR3) of XIAP selectively targets caspase-9, the BIR2 domain, together with the immediate preceding linker, inhibits both caspase-3 and caspase-7.[6,8] Since these caspases play a critical role in the execution of apoptosis, XIAP functions as an efficient inhibitor of apoptosis.[6,8] Accordingly, these IAP proteins, especially XIAP, are considered as promising cancer therapeutic targets.[8–9]

Smac (Second Mitochondria-derived Activator of Caspases) is a potent pro-apoptotic protein and an endogenous antagonist of IAP proteins.[10,11] Previous studies have firmly established that Smac interacts with XIAP and cIAP-1/2 proteins *via* its AVPI tetra-peptide motif.[6,12–

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Supporting Information Available: An experimental section including the information on the chemical data for compounds **8**–**10** and intermediates, structural assignments of **14**, **16** and **16i** by two-dimensional NOE data, FP competitive binding assays for XIAP and cIAP-1/-2 BIR3 proteins, cell-free functional assay of XIAP BIR3, cell growth assay, Western blotting analysis and molecular modeling is available free of charge *via* the Internet at http://pubs.acs.org.

[a]Abbreviations: IAP, inhibitor of apoptosis protein; XIAP, X-linked IAP; cIAP-1/-2, cellular IAP 1/2; Smac, second mitochondria-derived activator of caspases; BIR, baculoviral IAP repeats (BIR) domain; BIR2/BIR3, the second or third BIR domain; TNF, tumor necrosis factor; FP, fluorescence polarization; NOE, Nuclear Overhauser Effect.

[15] Smac, in its dimeric form, interacts with both BIR2 and BIR3 domains in XIAP and effectively removes the inhibition of XIAP to not only caspase-9 but also caspase-3/-7.

In the last few years, intense research efforts have been devoted to the design and development of small molecules to mimic the AVPI binding motif as antagonists of IAP proteins and as a new class of anticancer drugs.[4,16–23] These small molecules are called Smac mimetics. To date, two different types of Smac mimetics have been reported, namely monovalent and bivalent Smac mimetics (Figure 1). [4,16–23] Monovalent Smac mimetics such as **1–4** are designed to mimic a single AVPI binding motif,[16–19] whereas the bivalent compounds, such as **5** and **6**, contain mimetics of two AVPI binding motifs tethered together through a linker. [4,20–23] Bivalent Smac mimetics can achieve much higher affinities to XIAP and can be much more potent than the corresponding monovalent Smac mimetics in induction of apoptosis in tumor cells.[20] However, because of their ideal low molecular weight (~500), monovalent Smac mimetics may possess major advantages for drug development as compared to bivalent Smac mimetics.

Recent studies established that Smac mimetics induce rapid cIAP-1 degradation and cIAP-1 is a key cellular target for Smac mimetics.[21–23] Furthermore, our recent study clearly showed that in order to most efficiently induce apoptosis in cancer cells by Smac mimetics, the apoptotic blockade by both cIAP-1 and XIAP needs to be concurrently removed.[24] Thus, both cIAP-1 and XIAP are important cellular targets for Smac mimetics.

Our laboratory has previously reported the structure-based design, synthesis and initial evaluation of a series of conformationally constrained monovalent Smac mimetics.[16–18,20] Among them, compound **7** (SM-122, Figure 2) binds to the XIAP BIR3 protein with a K_i value of 26 nM.[20] Our subsequent binding studies showed that **7** also binds to cIAP-1, as well as to cIAP-2 proteins with high affinities (Table 1). Compound **7** potently inhibits cancer cell growth and effectively induces apoptosis in cancer cells.[20] These data indicated that **7** is a promising lead compound for further optimization. We report herein the design, synthesis and evaluation of new analogues of **7**. These efforts led to the discovery of highly potent monovalent Smac mimetics and yielded new insights into their structure-activity relationship.

The predicted binding model of **7** in complex with XIAP BIR3 showed that while the pro-(*R*) phenyl group in **7** inserts into a hydrophobic pocket in XIAP, the pro(*S*) phenyl group does not have specific interactions with the protein (Figure 3). We have thus investigated if replacement of the diphenylmethyl group by a conformationally constrained 1-tetrahydronaphthalenyl group, which was previously used for the design of Smac peptidomimetics,[17] could further improve the binding to XIAP. Modeling predicted that the compound with the (*R*)-configuration is preferred for binding to XIAP BIR3 (Figure 3). Compounds **8** and **9** with either the (*R*)- or the (*S*)- configuration were synthesized and evaluated. Compound **8** binds to XIAP, cIAP-1 and cIAP-2 BIR3 proteins with K_i values of 14 nM, 0.68 nM, and 1.0 nM, respectively, in competitive FP-based binding assays. In comparison, **9** binds to XIAP, cIAP-1 and cIAP-2 proteins with K_i values of 209, 29, and 132 nM, respectively, substantially less potent than **8**.

Consistent with their binding affinities, **8** is 4-times more potent than **7** in inhibition of cell growth in the MDA-MB-231 cancer cell line and has an IC_{50} value of 73 nM (Figure 4). In comparison, **9** is less potent than **7** and **8** and has an IC_{50} value of 1800 nM (Figure 4).

Our predicted binding model showed that there is a hydrophobic pocket on the surface of XIAP BIR3, between W323 and Y324 (Figure 3). We next designed and synthesized compound **10**, in which a benzyl group is appended to the 5-membered ring, to test if targeting this hydrophobic pocket can further improve the binding affinities to IAP proteins and cellular activity. Modeling predicted that the pro-(*S*) configuration is preferred. Accordingly, we have developed a stereo-selective synthetic method for **10** (Scheme I). To test if the conformation

of the 8-membered ring is critical for binding to IAP proteins, we have also synthesized compound **11**, which has a double bond in its 8-membered ring.

Compound **10** binds to XIAP, cIAP-1 and cIAP-2 with very high affinities and has K_i values of 3.9 nM, 0.37 nM and 0.25 nM to these three proteins, respectively (Table 1). In comparison, **11** has K_i values of 31 nM, 1.27 nM and 2.09 nM to XIAP, cIAP-1 and cIAP-2 proteins, respectively, significantly less potent than **10**.

Consistent with its high affinities to IAP proteins, **10** potently inhibits cell growth in the MDA-MB-231 cancer cell line and has an IC_{50} value of 8.9 nM and is >25-times more potent than the initial lead **7** (Figure 4).

These Smac mimetics were evaluated for their ability to antagonize XIAP in a cell-free functional assay (Figure 5 and Supporting Information). While the XIAP BIR3 protein effectively inhibits the activity of caspase-3/-7, these Smac mimetics dose-dependently antagonize the inhibition of XIAP to caspase activity. Consistently with their binding affinities to XIAP BIR3, compounds **8** and **10** are the most potent antagonists of XIAP in this cell-free functional assay, while **9** is the least potent antagonist against XIAP BIR3.

Recent studies showed that Smac mimetics induce cIAP-1 degradation in cells, which is crucial for apoptosis induction by Smac mimetics.[21–23] Indeed, Western blotting analysis showed that compounds **7**, **8** and **10** all effectively and dose-dependently induce the degradation of cIAP-1 (Figure 6). Compounds **8** and **10** are capable of inducing marked cIAP-1 degradation at concentrations as low as 10 nM, consistent with their high binding affinities to cIAP-1. These compounds have minimal effect on the levels of XIAP protein.

We further examined the processing of caspase-8 and -3 and cleavage of poly(ADP-ribose) polymerase (PARP), several biochemical markers of apoptosis (Figure 6). Compounds **7**, **8** and **10** effectively induce processing of caspase-8 and -3 and cleavage of PARP in a dose-dependent manner (Figure 6). Compounds **8** and **10** are capable of inducing processing of caspase-3 and -8 and cleavage of PARP at concentrations as low as 10 nM and are more effective than **7**. These data indicated that these Smac mimetics not only inhibit cell growth but effectively and dose-dependently induce apoptosis in the MDA-MB-231 cancer cell line.

The synthesis of compounds **8** and **9** are similar to that for **7**[20] and is provided in Supporting Information.

The synthesis of compounds **10** and **11** is shown in Scheme I. Briefly, compound **13** was prepared from the commercially available pyroglutamate ester **12**.[25] The Boc protective group in **13** was replaced by a Cbz to obtain **14** for achieving better stereo-selectivity in subsequent steps. The stereochemistry of the benzyl group in **13** and **14** was confirmed by two-dimensional (2D) Nuclear Overhauser Effect (NOE) data of **14** (Supporting Information) and was found to be same as that in the previous report.[25] The carbonyl group in **14** was selectively reduced, followed by acetylation and introduction of a *syn*-allyl group at the C-5 position *via* the *N*-acyl iminium ion chemistry to afford compound **15**.[26] The Cbz protective group in **15** was selectively removed to generate **16**, which was coupled with (*S*)-*N*-Boc-allylglycine, followed by cyclization using the olefin metathesis method to yield the key intermediate **17**. The *syn* stereochemistry between the allyl and the benzyl groups of **16** between was confirmed by 2D NOE data of **16** and its stereoisomer **16i** (Supporting Information). Compound **17** was hydrolyzed and then coupled with (*R*)-1,2,3,4-tetrahydronaphthalen-1-amine to give amide **18**. Removal of the Boc protecting group in **18** yielded an ammonium salt, which was condensed with *N*-Boc-methyl-*L*-alanine to generate **19**. Removal of the Boc protecting group in **19** produced the designed compound **11**. Hydrogenation of the C=C double bond in **19**

catalyzed by 10% Pd-C, followed by removal of the Boc protecting group, afforded compound **10**.

In summary, we have designed and synthesized a number of novel Smac mimetics based upon lead compound **7** by modifications of two different regions. Our efforts led to the discovery of arguably the most potent, non-peptidic, monovalent Smac mimetic and insights into their structure-activity relationship in binding, functional and cellular levels. The most potent compound **10** (DM-28) binds to cIAP-1, cIAP-2 and XIAP with K_i values in subnanomolar and low nanomolar affinities, potently antagonizes XIAP in a cell-free functional assay and effectively induces cIAP-1 degradation, processing of caspase-8 and -3 and cleavage of PARP in the MDA-MB-231 cancer cell line at concentrations as low as 10 nM. Compound **10** potently inhibits cell growth in the MDA-MB-231 cell line with an IC_{50} value of 8.9 nM and is >25-times more potent than **7**. Extensive *in vitro* and *in vivo* studies are under way to further elucidate mechanism of action and therapeutic potential of **10** as a new anticancer agent and the results will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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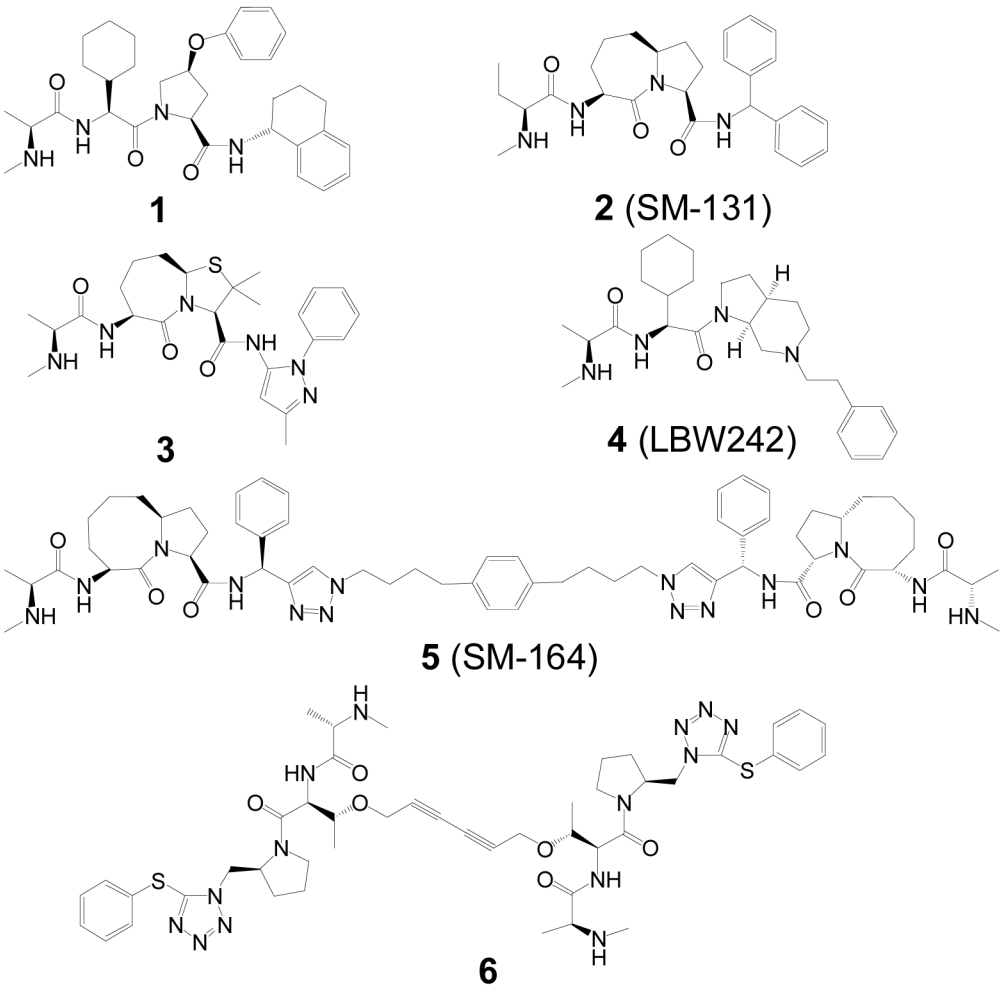


Figure 1.
Examples of monovalent (**1–4**) and bivalent (**5–6**) Smac mimetics.

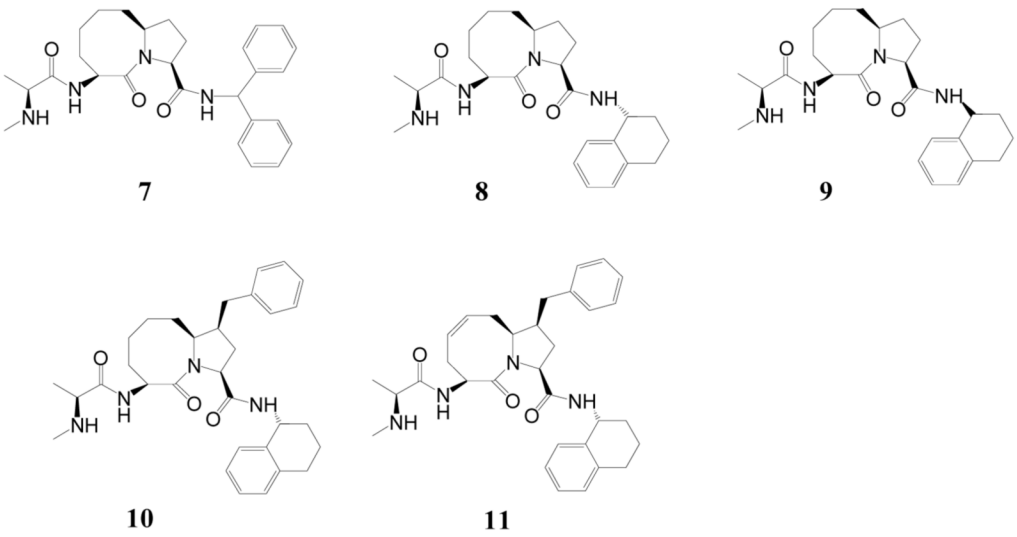


Figure 2.
Chemical structures of designed Smac mimetics.

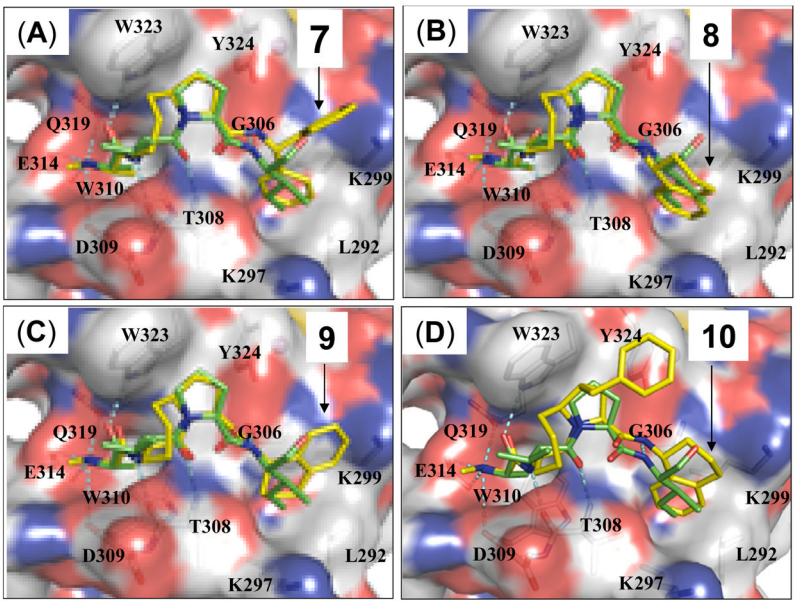


Figure 3.

Predicted binding models of compounds **7**, **8**, **9** and **10** in complex with XIAP BIR3 protein. Predicted binding models are superimposed on the crystal structure of Smac in complex with XIAP BIR3. Protein is shown in surface with key binding residues shown and labeled. Compounds **7**, **8**, **9**, **10** and AVPI peptide are shown in stick. Caron atoms in AVPI are shown in yellow and carbon atoms in **7**, **8**, **9**, **10** are shown in green; oxygen and nitrogen atoms are shown in red and blue, respectively.

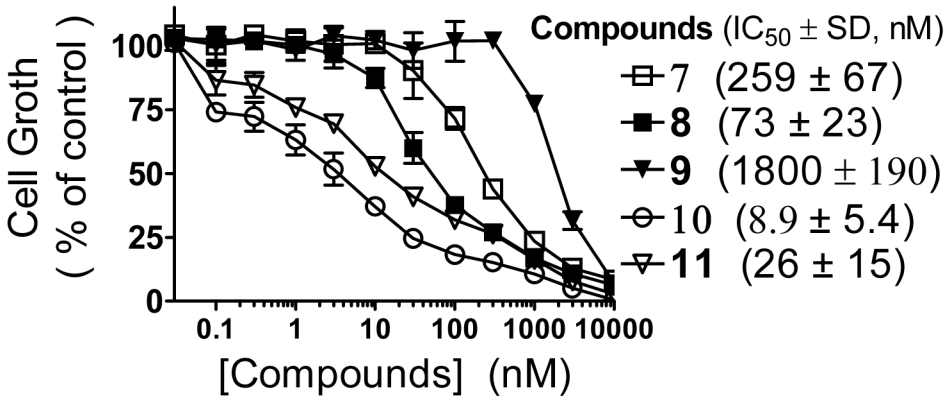


Figure 4.
Cell growth inhibition by Smac mimetics in the MDA-MB-231 cancer cell line. Cells were treated for 4 days and cell growth was determined by a WST-8 assay.

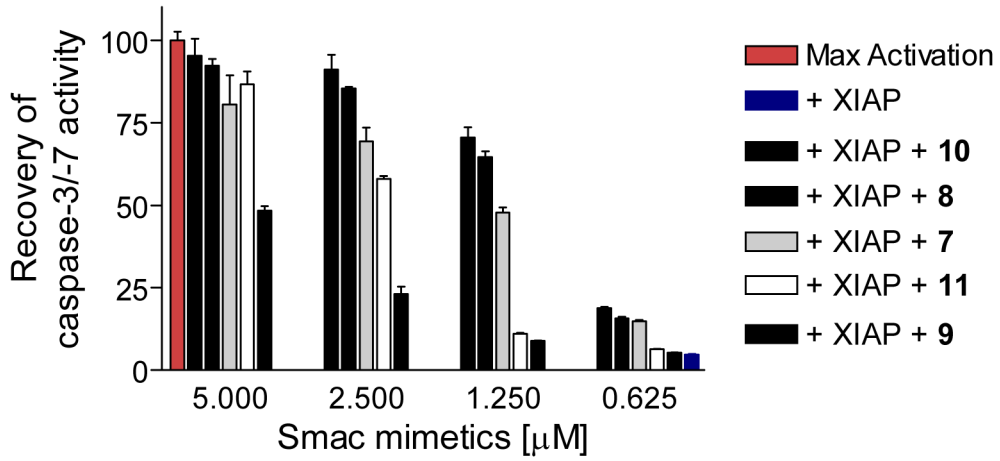


Figure 5.

Functional antagonism of Smac mimetics against XIAP BIR3 in a cell-free functional assay. dATP and cyctochrome c were added into cell lystates to activate caspase-3/7 and recombinant XIAP BIR3 protein at 500 nM effectively inhibited casapse activity. Smac mimetics dose-dependently recovered the activity of casapse-3/-7. The caspase activity data at 60 min was used (Supporting Information).

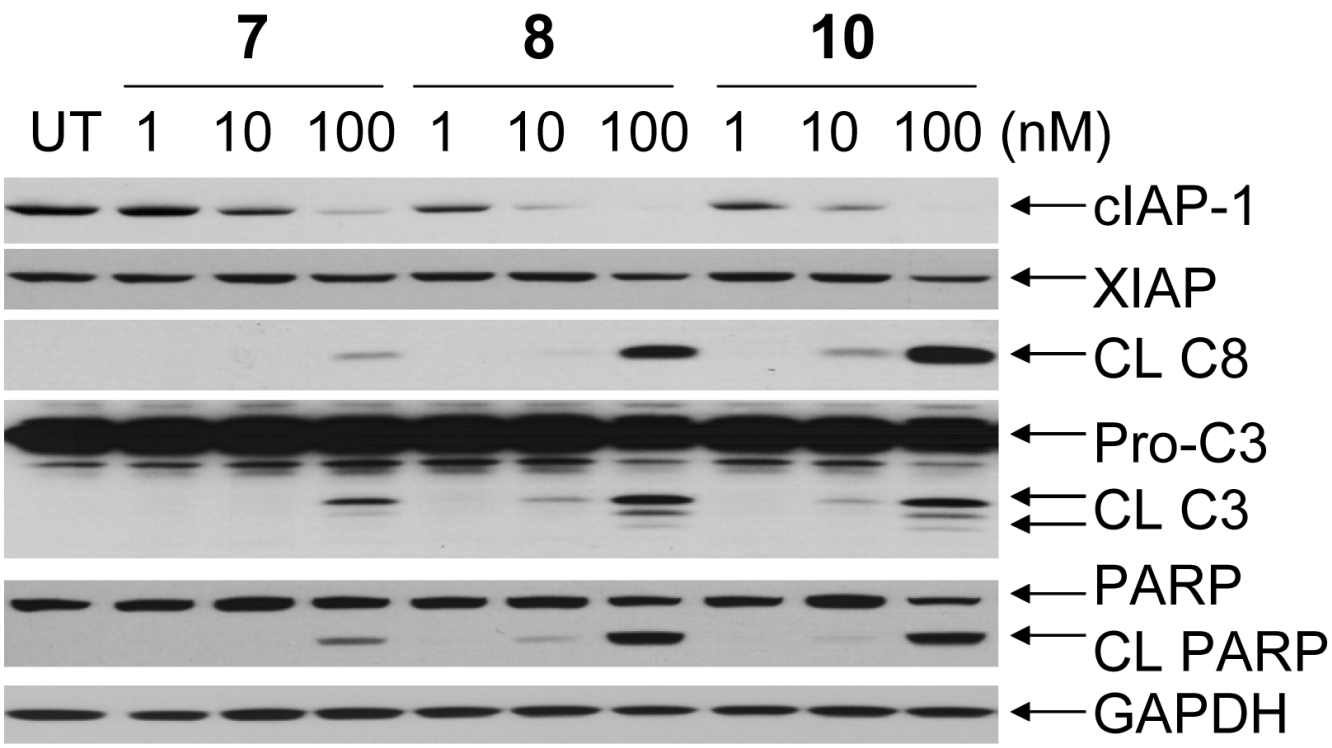
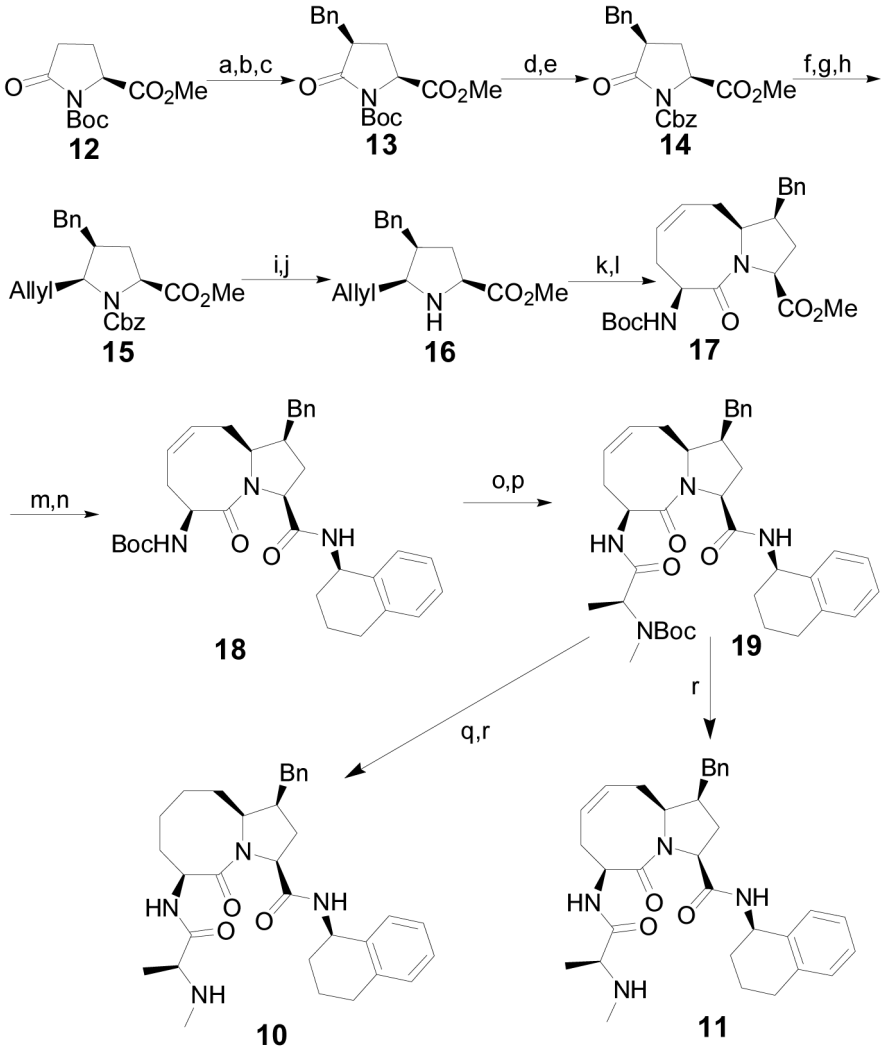


Figure 6.
Western blot analysis of the levels of cIAP-1, XIAP, cleaved caspase-8 (CL C8), pro- and cleaved caspase-3 (Pro-C3 and CL C3), cleaved PARP (CL PARP). MDA-MB-231 breast cancer cells were treated with Smac mimetics for 24 hours and proteins were probed with specific antibodies. GAPDH was used as the loading control.



Scheme I. Synthesis of compounds 10 and 11

Reagents and conditions: (a) LiHMDS, Benzaldehyde, $BF_3{\cdot}OEt_2$, THF, −78 °C; (b) MsCl, TEA, DCM, r.t.; (c) H_2, Pd/C, MeOH, r.t., 75% (3 steps); (d) TFA, DCM, 0 °C; (e) LiHMDS, CbzCl, THF, −78 °C to r.t., 80% (2 steps); (f) Super-Hydrid, THF, −78 °C; (g) Ac_2O, TEA, DMAP, DCM, r.t.; (h) $Bu_3SnAllyl$, $BF_3{\cdot}OEt_2$, toluene, −78 °C, 4, 5-syn:anti 5.6:1, 55% (3 steps); (i) $BF_3{\cdot}OEt_2$, Me_2S, DCM, 0 °C; (j) aq. sat. $NaHCO_3$, 88% (2 steps); (k) (*S*)-*N*-Boc-Allylglycin, $ClCOO^iBu$, NMM, THF, −20 °C; (l) Grubbs catalyst (1st generation), DCM, reflux, 67% (2 steps); (m) $LiOH{\cdot}H_2O$, $THF/H_2O/MeOH$, 0 °C; (n) $(R)^{-1}$,2,3,4-tetrahydronaphthalen^{-1}-amine, EDCI, HOBt, DIPEA, DCM, r.t., 95% (2 steps); (o) HCl/MeOH, 0 °C; (p) Boc-N-Me-Ala-OH, EDCI, HOBt, DIPEA, DCM, r.t.; (q) H_2, Pd/C, MeOH, r.t.; (r) HCl/MeOH, 0 °C, 85% (4 steps) for compound **10** and 91% (3 steps) for compound **11**.

Table 1

Binding affinities of Smac mimetics to XIAP, cIAP-1 and cIAP-2 BIR3 proteins. K_i and standard deviation (SD) values were calculated based upon 3–5 independent experiments.

Compounds	(K_i ± SD, nM)		
	XIAP BIR3	**cIAP-1 BIR3**	**cIAP-2 BIR3**
7	26 ± 5	0.80 ± 0.19	1.84 ± 0.62
8	14 ± 5	0.68 ± 0.15	1.00 ± 0.04
9	209 ± 73	29 ± 6	132 ± 17
10	3.9 ± 1.8	0.37 ± 0.25	0.25 ± 0.07
11	31 ± 15	1.27 ± 1.05	2.09 ± 0.78