ACS chemical biology

Articles

pubs.acs.org/acschemicalbiology

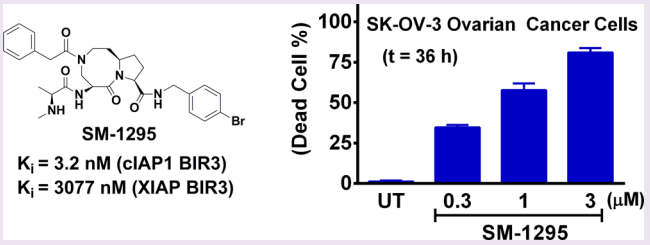
Potent and Selective Small-Molecule Inhibitors of cIAP1/2 Proteins Reveal That the Binding of Smac Mimetics to XIAP BIR3 Is Not Required for Their Effective Induction of Cell Death in Tumor Cells

Haiying Sun,[†] Jianfeng Lu,[†] Liu Liu, Chao-Yie Yang, and Shaomeng Wang*

Comprehensive Cancer Center and Departments of Internal Medicine, Pharmacology, and Medicinal Chemistry, University of Michigan, 1500 E. Medical Center Drive, Ann Arbor, Michigan 48109, United States

Supporting Information

ABSTRACT: Cellular inhibitor of apoptosis protein 1 and 2 (cIAP1/2) and X-linked inhibitor of apoptosis protein (XIAP) are key apoptosis regulators and promising new cancer therapeutic targets. This study describes a set of non-peptide, small-molecule Smac (second mitochondria-derived activator of caspases) mimetics that are selective inhibitors of cIAP1/2 over XIAP. The most potent and most selective compounds bind to cIAP1/2 with affinities in the low nanomolar range and show >1,000-fold selectivity for cIAP1 over XIAP. These selective cIAP inhibitors effectively induce degradation of the cIAP1 protein in cancer cells at low nanomolar concentrations and do not antagonize XIAP in a cell-free functional assay. They potently inhibit cell growth and effectively induce apoptosis at low nanomolar concentrations in cancer cells with a mechanism of action similar to that of other known Smac mimetics. Our study shows that binding of Smac mimetics to XIAP BIR3 is not required for effective induction of apoptosis in tumor cells by Smac mimetics. These potent and highly selective cIAP1/2 inhibitors are powerful tools in the investigation of the role of these IAP proteins in the regulation of apoptosis and other cellular processes.



Inhibitors of apoptotic proteins (IAPs) are a class of key regulators of apoptosis, characterized by the presence of one to three baculovirus IAP repeat (BIR) domains.[1,2] Among the eight IAP members that have been identified in mammalian cells, cIAP1 and cIAP2 interact with tumor necrosis factor receptor-associated factor 2 (TRAF2), blocking the formation of the caspase-8 activation complex and thereby inhibiting TNF receptor-mediated apoptosis.[3−6] The X-linked IAP (XIAP), on the other hand, binds to and antagonizes three caspases, including two effectors, caspase-3 and -7, and an initiator, caspase-9, thus blocking both death receptor-mediated and mitochondria-mediated apoptosis.[7] While the third BIR domain (BIR3) of XIAP binds to and inhibits caspase-9, the second BIR domain (BIR2), together with the linker preceding it, binds to and inhibits both caspase-3 and caspase-7.[7] These IAPs are overexpressed in many tumor cell lines and human tumor tissues and play important roles in the resistance of cancer cells to various anticancer treatments.[8−11] Accordingly, targeting these IAPs has been pursued as a new cancer therapeutic strategy.[12−16]

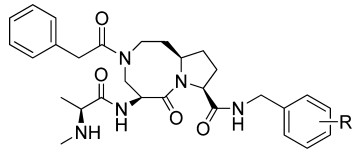
Smac, the second mitochondria-derived activator of caspases, is an endogenous antagonist of cIAP1/2 and XIAP.[17−19] After being released from mitochondria into the cytosol, the first 55 N-terminal residues in Smac are removed by a protease to expose an Ala-Val-Pro-Ile (AVPI) tetrapeptide, the so-called IAP binding motif. The interaction of Smac with XIAP, cIAP1, and cIAP2 is mediated by the AVPI binding motif in Smac and a surface binding groove in the BIR domain(s) in these IAPs. In cytosol, Smac forms a homodimer and binds concurrently to both the BIR2 and BIR3 domains of XIAP. Binding of Smac with XIAP effectively blocks the inhibition of XIAP of both caspase-9 and caspase-3/7.[20−22] In cIAP1 and cIAP2, on the other hand, only their BIR3 domain is involved in the interaction with Smac.[4]

Using the AVPI tetrapeptide as a lead compound, a number of laboratories have reported the design of both peptidic and non-peptidic, small-molecule Smac mimetics.[23−44] Smac mimetics can induce rapid degradation of cIAP1 and cIAP2 in cells and antagonize the functions of XIAP in functional assays. Smac mimetics can effectively induce apoptosis as single agents in a subset of human cancer cell lines in a TNFα-dependent manner and are capable of inhibiting tumor growth in xenograft models of human cancer.[5,6,26,28] To date, several small molecular Smac mimetics have been advanced into clinical trials.[3,23,25,26,39]

While most of reported Smac mimetics bind to cIAP1, cIAP2, and XIAP BIR3 proteins with high affinities,[23−41] one study has reported a highly selective cIAP inhibitor over XIAP BIR3 protein.[43] Because XIAP and cIAP1/2 regulate apoptosis by different mechanisms, selective IAP inhibitors can be very valuable tools to further dissect the role of these IAP proteins in the regulation of apoptosis and in human diseases. In this

Received: December 2, 2013
Accepted: February 3, 2014
Published: February 12, 2014

Table 1. Binding Affinities of Smac Mimetics to XIAP BIR3, cIAP1 BIR3, and cIAP2 BIR3 Proteins and Cell Growth Inhibition of Smac Mimetics in the MDA-MB-231 Cell Line[a]



compounds	R	binding affinity (K_i, nM) XIAP BIR3	cIAP1 BIR3	cIAP2 BIR3	selectivity for cIAP1 over XIAP	MDA-MB-231 (IC_{50}, nM)
SM-337 (**1**)		156 ± 7	2.5 ± 0.5	4.5 ± 1.5	62	52 ± 11
2	H	323 ± 31	4.7 ± 0.6	10.3 ± 1.5	69	49 ± 12
3	*p*-F	393 ± 68	1.8 ± 0.4	4.9 ± 0.8	218	17 ± 6.2
4	*p*-Cl	870 ± 81	1.1 ± 0.3	3.0 ± 0.7	791	21 ± 8
SM-1295 (**5**)	*p*-Br	3080 ± 240	3.2 ± 0.9	9.5 ± 1.8	962	46 ± 19
6	*p*-CH_3	2450 ± 290	4.0 ± 0.6	11.6 ± 2.2	613	17 ± 9
SM-1280 (**7**)	*p*-CF_3	8210 ± 1110	8.8 ± 2.1	24.6 ± 2.2	933	307 ± 44
8	*p*-Et	2320 ± 290	14.9 ± 3.3	22.0 ± 2.8	155	164 ± 29
9	*p*-iPr	38400 ± 3900	86.5 ± 25.7	157 ± 27	444	705 ± 36
10	*p*-tBu	185000 ± 25000	236 ± 41	330 ± 81	782	1320 ± 90
11	*p*-Ph	15300 ± 1100	167 ± 33	172 ± 26	92	1026 ± 177
12	*p*-OMe	16300 ± 400	18.0 ± 4.0	47.3 ± 8.0	904	121 ± 8
13	*p*-Et_2N	42500 ± 7400	148 ± 7.1	255 ± 52	287	675 ± 173
14	*p*-NO_2	11600 ± 2800	10.1 ± 2.4	31.4 ± 6.3	1149	292 ± 31
15	*p*-$CONH_2$	65500 ± 18500	274 ± 95	539 ± 119	239	>10000
16	*o*-F	532 ± 96	9.5 ± 0.9	25.7 ± 5.1	56	221 ± 123
17	*m*-F	1000 ± 176	19.0 ± 3.2	33.9 ± 7.9	53	197 ± 72
18	*o*-Cl	1457 ± 376	20.8 ± 3.0	60.9 ± 11.9	70	545 ± 255
19	*m*-Cl	1019 ± 255	15.0 ± 2.4	39.7 ± 9.9	68	294 ± 183
20	*o*-Br	1487 ± 331	22.9 ± 3.5	58.1 ± 5.8	65	667 ± 268
21	*m*-Br	1053 ± 219	22.2 ± 2.9	43.7 ± 11.7	47	327 ± 158
22	*o*-CF_3	4238 ± 372	24.2 ± 5.5	55.4 ± 7.1	175	118 ± 17
23	*m*-CF_3	3748 ± 454	32.0 ± 7.3	89.5 ± 16.5	117	224 ± 30

[a]Cells were treated with Smac mimetics for 4 days.

paper, we report the discovery of a number of highly selective cIAP1/2 inhibitors, which bind to cIAP1/2 with low nanomolar affinities and display selectivity of 3 orders of magnitude over XIAP.

RESULTS AND DISCUSSION

The starting point in our design was SM-337 (**1**), a potent and cell-permeable small-molecule Smac mimetic previously identified in this laboratory.[30] Compound **1** binds to XIAP, cIAP1, and cIAP2 BIR3 proteins with nanomolar affinities and is effective in inhibition of cell growth and induction of apoptosis in various cancer cell lines.[30] Further optimization of compound **1** has yielded SM-406 (AT-406), which is currently in clinical trials as a new anticancer drug.[23]

We reoptimized the binding assay conditions for XIAP, cIAP1, and cIAP2 BIR3 proteins[23] and have retested **1** using these assays for a direct comparison with our newly designed compounds reported in this study. In the optimized assays, compound **1** has K_i values of 156, 2.5, and 4.5 nM to XIAP BIR3, cIAP1 BIR3, and cIAP2 BIR3 proteins, respectively (Table 1), and hence has a high affinity against XIAP, cIAP1, and cIAP2 BIR3 domain proteins. Compound **1** displays a selectivity of 63-fold for cIAP1 over XIAP and is therefore a good lead compound for our design of selective cIAP inhibitors.

To assist our design of selective cIAP inhibitors, we modeled the binding modes of compound **1** complexed with XIAP BIR3 and cIAP1 BIR3 proteins (Figure 1). Our models showed that while other structural portions of compound **1** have essentially the same interactions with XIAP and cIAP1, there are some subtle differences on how the (pro-*R*)-phenyl group in **1** interacts with these two proteins. In both cases, this group in **1** interacts with a well-defined, surface binding pocket in the protein. In XIAP, the surface pocket is formed by the side chain of L292 and T308 and the hydrophobic portions of the side chains of K297 and K299. In comparison, in cIAP1, the pocket is formed by the side chain of V298 and the hydrophobic portion of the side chains of K305 and R314 and is slightly deeper than that in XIAP. As a consequence, the (pro-*R*)-phenyl group of **1** appears to interact with cIAP1 more optimally than with XIAP. We hypothesized that this difference may account for the 63-times higher binding affinity of **1** to cIAP1 BIR3 than to XIAP BIR3 and may be further exploited for the design of highly selective cIAP inhibitors. On the basis of this analysis, we decided to perform modifications of the (pro-*R*)-phenyl group for the design of selective IAP inhibitors.

Selective introduction of a substituted group to the (pro-*R*)-phenyl group in **1** proved to be synthetically challenging. Since the (pro-*S*)-phenyl group in **1** is exposed to solvent in our predicted binding models for these IAP proteins (Figure 1), removal of this phenyl group may not have a major detrimental effect for binding to these IAP proteins but can facilitate subsequent modifications of the remaining phenyl group. Accordingly, we have synthesized compound **2** (Figure 1), in which a benzyl group was used to replace the diphenylmethyl

dx.doi.org/10.1021/cb400889a | ACS Chem. Biol. 2014, 9, 994−1002

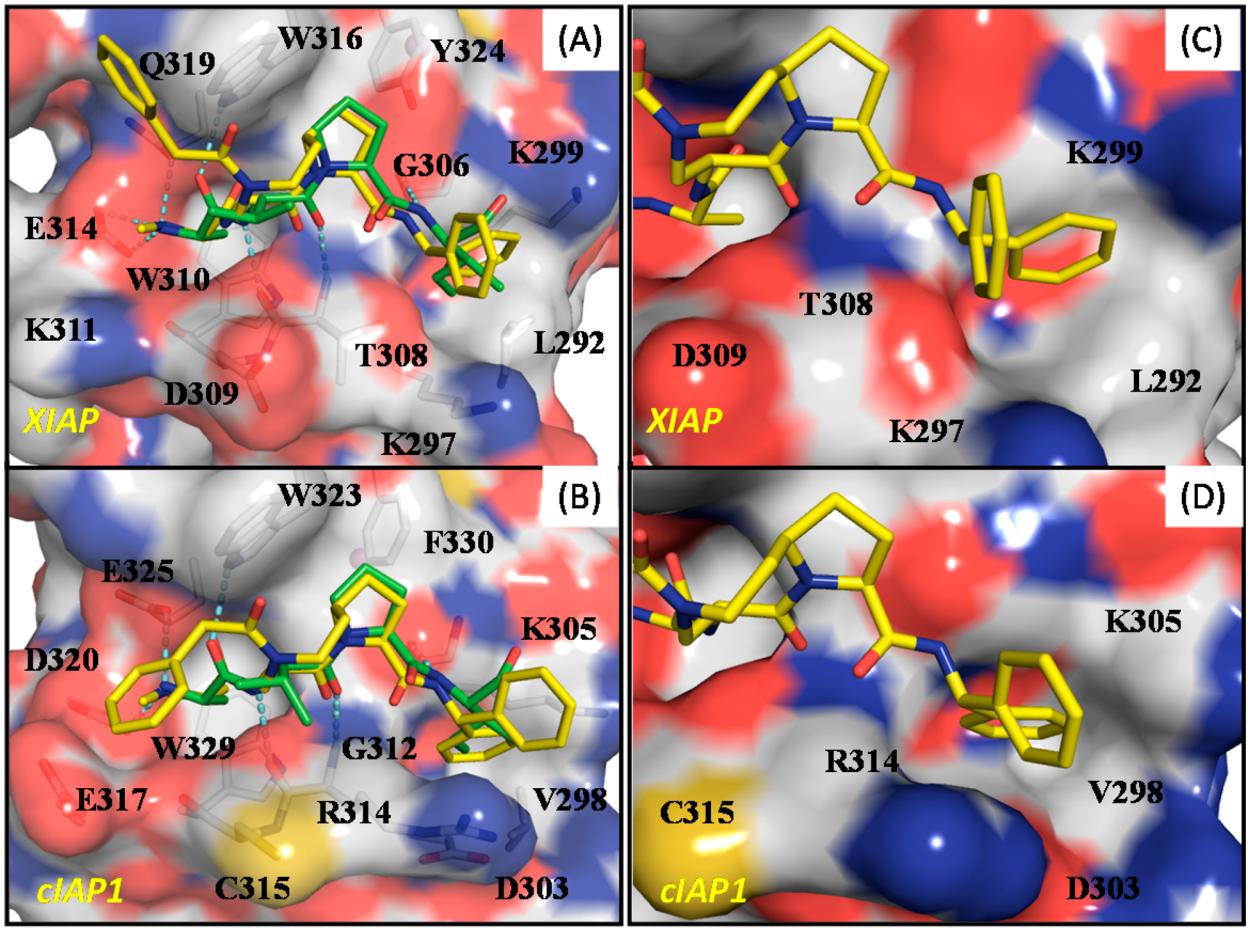


Figure 1. Computational predicting of the binding models of SM-337 complexed with XIAP BIR3 (A and C) and with cIAP1 (B and D) and design of selective cIAP inhibitors (E). The bound conformation of AVPI peptide from the Smac-XIAP BIR3 cocrystal[20] is shown in panels A and C as a reference and colored in green. Residues differing at the Ile4 (from AVPI) interacting pocket between XIAP-BIR3 and cIAP1-BIR3 are labeled in panels C and D.

group in **1**. In our FP-based binding assays, compound **2** has K_i values of 323, 4.7, and 10.3 nM to XIAP, cIAP1, and cIAP2, respectively (Table 1). Hence, it is only 2 times less potent than compound **1** in binding to these three IAP proteins. Since it was straightforward to introduce substituents on the phenyl group in **2**, this compound was used as the template for further modifications to probe the differences between cIAP1/2 and XIAP.

We have synthesized a series of new analogues by introduction of a substituted group of varying size, hydrophobicity, and polarity to the *ortho*-, *meta*-, or *para*-position of the phenyl group in compound **2**. These new compounds were tested for their binding affinities to XIAP, cIAP1, and cIAP2 BIR3 proteins, and the results are summarized in Table 1.

These binding data showed that introduction of a *para*-substituent to the phenyl group in **2** has a major effect on binding affinity and selectivity. Compounds **3**, **4**, and **5**, with a *p*-F, *p*-Cl, and *p*-Br substituent on the phenyl group in **2**, respectively, have K_i values of 1.8, 1.1, and 3.2 nM to cIAP1, respectively. These three compounds also have low nanomolar binding affinities to cIAP2 with K_i values of 4.9, 3.0, and 9.5 nM, respectively. All three compounds are less potent than **2** in binding to XIAP. Compounds **3**, **4**, and **5** display a selectivity of 218, 791, and 962 times for cIAP1 over XIAP, respectively.

Encouraged by the improved selectivity of **3**, **4**, and **5** for cIAP1 over XIAP, we synthesized and evaluated 10 new analogues with a *para*-substituent on the phenyl group. Compound **6** with a *p*-CH_3 substituent binds to cIAP1 and cIAP2 with K_i values of 4.0 and 11.6 nM, respectively, similar to

dx.doi.org/10.1021/cb400889a | *ACS Chem. Biol.* 2014, 9, 994−1002

those of compound **2** with no substituent on the phenyl ring. However, **6** has a much weaker binding affinity to XIAP and displays a selectivity of 613 times for cIAP1 over XIAP. Compound **7** with a *p*-CF_3 substituent binds to all three IAP proteins with a weaker affinity than **2** but shows a selectivity of 933 times for cIAP1 over XIAP. Compounds **8**−**10** with an ethyl, isopropyl, or *tert*-butyl substituent at the *para*-position of the phenyl ring bind to cIAP1 and cIAP2 with weaker affinities than **6** with a *p*-methyl substituent. These compounds bind to XIAP, however, with much weaker affinities. For example, compound **10** with a *p*-*tert*-butyl substituent has K_i values of 236 nM to cIAP1 and >18 μM to XIAP, a selectivity of >700 times for cIAP1 over XIAP. Compound **11** with a *p*-phenyl substituent on the phenyl ring has a decreased binding affinity to these three IAP proteins as compared to compound **2** and has a selectivity of 92 times for cIAP1 over XIAP.

To explore further different *para*-substituents on the phenyl ring, we synthesized compounds **12**−**15** with a polar substituent (OCH_3, $N(C_2H_5)_2$, NO_2, and $CONH_2$) at this position. Although compound **12** with a *p*-OCH_3 substituent is 4 times less potent than **2** in binding to both cIAP1 and cIAP2, it has a high selectivity of 904 for cIAP1 over XIAP. Compounds **13** and **14** are much less potent than compound **2** in binding to all of these three IAP proteins and have a selectivity of ∼250 for cIAP1 over XIAP. Compound **15** with a *p*-NO_2 substituent binds to cIAP1/2 proteins 2−3 times more weakly than compound **2** but has a much weaker binding affinity to XIAP, displaying a selectivity of >1000 for cIAP1/2 over XIAP. Our data thus show that compounds with Cl, Br, CF_3, and NO_2 substituents at the *para*-position of the phenyl ring have high affinities to cIAP1/2 and excellent selectivity (>500 times) for cIAP1 over XIAP.

To investigate the effect of different substitution positions, we synthesized compounds **16**−**23**, analogues with F, Cl, Br, or CF_3 at either the *ortho*- or *meta*-position of the phenyl ring. These compounds bind to both cIAP1 and cIAP2 with affinity 2−6 times weaker than compound **2**. They are 2−12 times weaker than **2** in their binding to XIAP and display a selectivity of 47−175 times for cIAP1 over XIAP. We concluded that substituents of F, Cl, Br, and CF_3 at either the *ortho*- or *meta*-position on the phenyl ring have only a relatively moderate effect on their binding affinities to these three IAP proteins as compared to the same substituents at the *para*-position.

XIAP BIR3 protein binds to caspase-9 and inhibits its activity. Accordingly, we evaluated several representative compounds (**2**, **4**, **5**, **7**, and **9**), which have different affinities to XIAP BIR3, for their ability to antagonize XIAP BIR3 protein in a cell-free caspase-9 functional assay. The results are shown in Figure 2. In this assay, the XIAP BIR3 protein dose-dependently inhibits the activity of caspase-9 and, at 500 nM concentrations, achieves 80% inhibition. Consistent with their binding data to XIAP BIR3, the rank order for these compounds in antagonizing XIAP BIR3 to recover the caspase-9 activity is **2** > **4** > **5** > **7** > **9**. While compound **2**, which has a K_i value of 323 nM to XIAP BIR3, restores 50% of caspase-9 activity at a concentration of 5 μM, compound **9**, which has a K_i of 38 μM to XIAP BIR3, restores only 23% of caspase-9 activity at 50 μM.

A previous study has shown that Smac mimetics that bind with high affinities to both cIAP1 and XIAP BIR3 are much more potent inhibitors of cell growth in cancer cell lines sensitive to Smac mimetics as single agents than those that bind selectively to cIAP1.[43] The same study suggested that

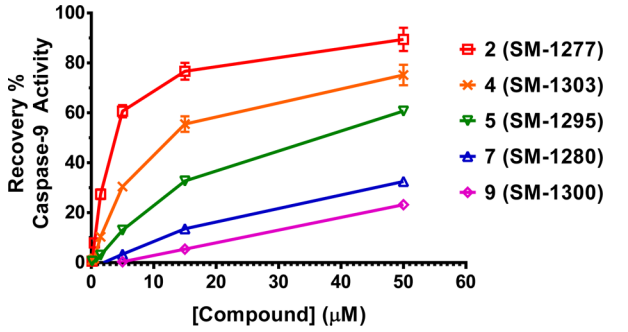


Figure 2. Smac mimetics antagonize XIAP BIR3 in a cell-free caspase-9 functional assay. XIAP BIR3 protein at 500 nM achieves 80% inhibition of caspase-9 activity in a Caspase-Glo 9 assay kit, and Smac mimetics dose-dependently restore the activity of caspase-9. Caspase-9 activity was measured after incubation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

concurrent targeting of both XIAP and cIAP1 BIR3 by Smac mimetics may be required for effective inhibition of cell growth and induction of apoptosis in cancer cells.[43] We tested our newly synthesized compounds for their ability to inhibit cell growth in the MDA-MB-231 cell line, which has been extensively used to evaluate Smac mimetics. The results are summarized in Table 1.

Interestingly, compounds that bind to cIAP1/2 BIR3 proteins with high affinities but have a weak affinity to XIAP BIR3 protein are capable of potently inhibiting cell growth in the MDA-MB-231 cell line, and a number of them achieve IC_{50} values in the low nanomolar range. For example, compounds **5** and **6**, which bind to cIAP1/2 with high affinities (K_i = 3.2−11.6 nM) and have weak affinities (K_i = 2−3 μM) for XIAP, have IC_{50} values of 46 and 17 nM, respectively, in inhibition of cell growth in the MDA-MB-231 cell line. The IC_{50} values for compounds **5** and **6** and a number of other highly selective cIAP1 inhibitors in cell growth inhibition in the MDA-MB-231 cell line are 10−100 times lower than their binding affinities (K_i values) to XIAP BIR3.

Next we selected compounds **5** and **7** for detailed evaluation of their cellular activity and mechanism of action. These two compounds bind to cIAP1 and cIAP2 with high affinities, have a weak affinity for XIAP (K_i > 3 μM), and display selectivity of >900 times for cIAP1 over XIAP. We tested their ability to induce apoptosis by Annexin-V/Propidium Iodide (P.I.) double staining and flow cytometry analysis in the MDA-MB-231 breast cancer and SK-OV-3 ovarian cancer cell lines, both of which are sensitive for apoptosis induction by Smac mimetics.[45] With a 24-h treatment, compounds **5** and **7** effectively induce apoptosis in a dose-dependent manner in both cancer cell lines (Figure 3). While compound **5** induces significant apoptosis in both cell lines starting from 100 to 300 nM (Figure 4A), compound **7** has a strong effect on apoptosis induction in both cell lines starting from 300 to 1000 nM (Figure 4B). Western blotting analysis showed that compound **5** induces cIAP1 degradation at concentrations over 30 nM in both cell lines and that compound **7** is very effective on cIAP1 degradation above 100 nM in both cell lines (Figure 4). Compound **5** induces robust cleavage of caspase-8, caspase-3, and PARP, starting from 0.3 μM with 24 h treatment in both cancer cell lines, whereas compound **7** has a significant effect on cleavage of caspase-8, caspase-3, and PARP from 1.0 μM in the same experiment (Figure 4). Interestingly, both compounds also

dx.doi.org/10.1021/cb400889a | ACS Chem. Biol. 2014, 9, 994−1002

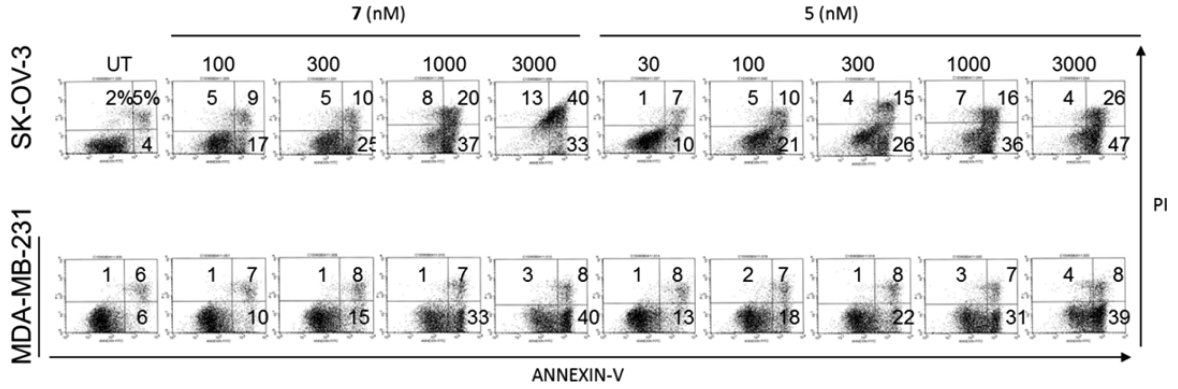


Figure 3. Inhibition of apoptosis by **5** and **7** in MDA-MB-231 and SK-OV-3 cell lines.

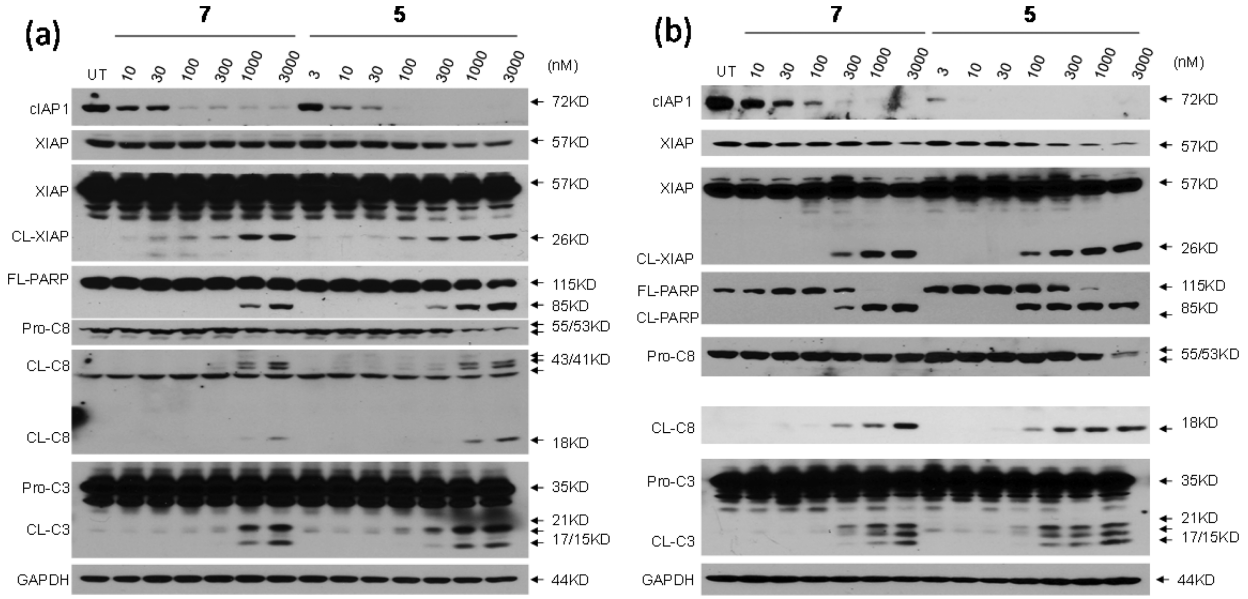


Figure 4. Selective cIAP1/2 inhibitors **5** and **7** induce cIAP1/2 degradation, XIAP cleavage, activation of caspase-3 and -8, and cleavage of PARP in (a) human breast cancer MDA-MB-231 cell line and (b) human ovarian cancer SK-OV-3 cell line. Cells were treated for 24 h.

induce cleavage of XIAP at the same concentrations at which cleavage of PARP, caspase-8, and caspase-3 take place in both cancer cell lines.

To further understand the role of caspase-8, -9, and -3 in apoptosis induction by these selective cIAP inhibitors, we knocked down each of these caspases in both MDA-MB-231 and SK-OV-3 cell lines with siRNA and tested the effect on the activity of compound **5** (Figure 5). Efficient knock-down of caspase-3 or caspase-8 has no effect on cIAP1 degradation by compound **5** but greatly attenuates the ability of **5** to induce cleavage of PARP and cell death in both cancer cell lines. Knock-down of either caspase-8 or caspase-3 greatly reduces the cleavage of the other caspase, revealing the interplay between these two caspases. Interestingly, knock-down of caspase-8 or caspase-3 also essentially blocks the cleavage of XIAP induced by **5**, suggesting that the cleavage of XIAP is dependent upon the activity of caspase-8 and caspase-3. In contrast to the profound effect observed for caspase-8 and caspase-3, efficient knocking-down of caspase-9 has no significant effect on cell-death, cleavage of PARP, caspase-3 and -8, and XIAP. These data show that caspase-8 and caspase-3 are essential for apoptosis induction by compound **5**, while caspase-9 plays no or a minimal role.

A number of studies have firmly established that Smac mimetics that bind to XIAP and cIAP1/2 with high affinities kill tumor cells in a TNFα-dependent manner.[5,6,45−47] We investigated if selective cIAP1/2 inhibitors **5** and **7** also kill tumor cells in a TNFα-dependent manner. In both MDA-MB-231 and SK-OV-3 cancer cell lines, compounds **5** and **7** at 1 μM induce robust cell death (Figure 6). The cell death induction by both compounds is effectively blocked by TNFα blocking antibody, but not by blocking antibodies against TRAIL or FasL (Figure 6). These data show that selective cIAP inhibitors **5** and **7** kill tumor cells in a TNFα-dependent manner.

Collectively, our data using two selective cIAP inhibitors, **5** and **7**, show that they effectively induce apoptosis in tumor cells that are sensitive to nonselective IAP inhibitors with the same TNFα-dependent mechanism of action. Since their potency in inhibition of cell growth and in induction of apoptosis in tumor cells is far more potent than their binding affinities to XIAP BIR3, we conclude that the binding of Smac mimetics to XIAP BIR3 is not required for their TNFα-dependent cell-death induction in tumor cells. Our conclusion thus appears to be in direct contradiction to that made from a previous study.[43] However, in the previous study, while the selective cIAP

dx.doi.org/10.1021/cb400889a | ACS Chem. Biol. 2014, 9, 994−1002

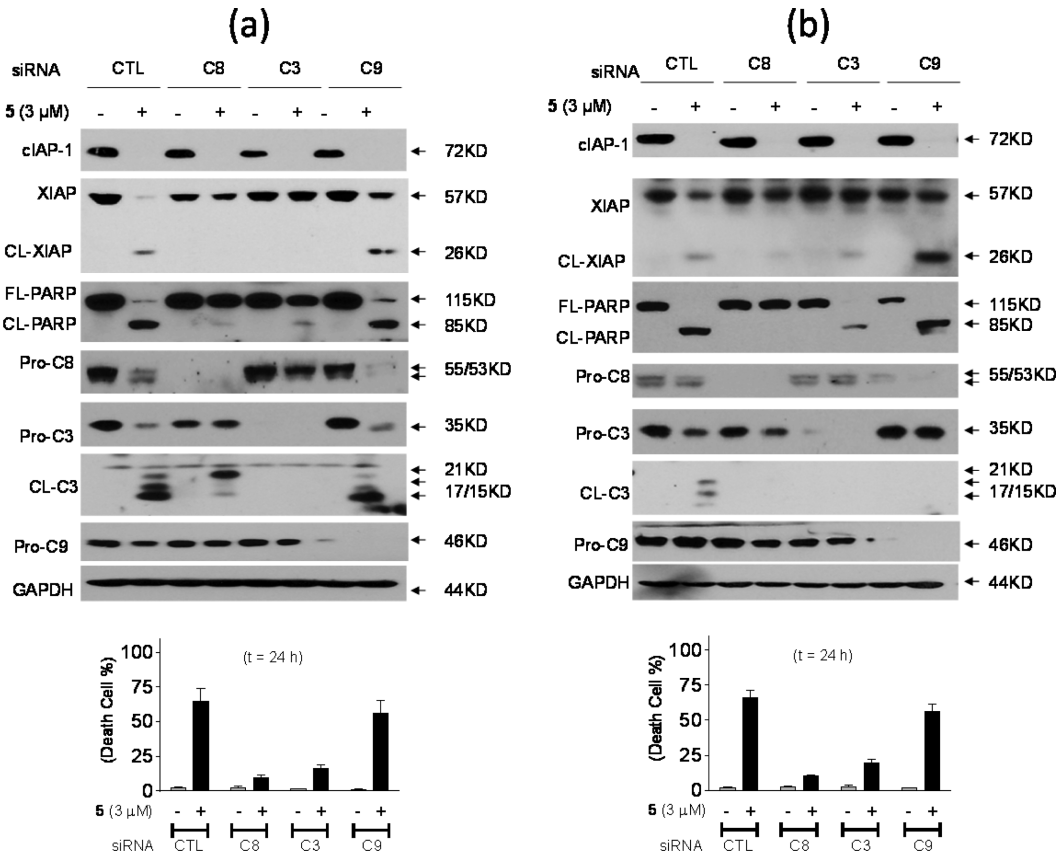


Figure 5. Investigation of the role of caspase-3, -8, and -9 in induction of cell death by compound **5** in (a) human breast cancer MDA-MB-231 cell line and (b) human ovarian cancer SK-OV-3 cell line. Cells were treated with 3 μM compound **5** for 24 h.

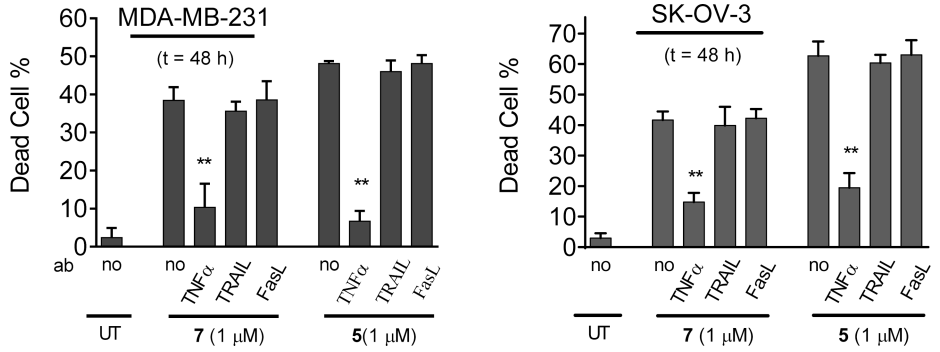


Figure 6. Compounds **5** and **7** induce TNFα-dependent cell death in both the MDA-MB-231 and SK-OV-3 cancer cell lines. Cells were treated with either **5** or **7** at 1 μM for 48 h with or without blocking antibody against TNFα, TRAIL, or FasL. Cells were harvested and stained with Trypan blue, and dead cells were counted with a Countess automated cell counter (Invitrogen).

inhibitor (CS3) is less potent than the pan IAP inhibitor (PS1), the IC_{50} values for CS3 in two different cancer cell lines in inhibition of cell growth are still <500 nM, which is much more potent than its binding affinity to XIAP (K_i > 30 μM), consistent with our data. Furthermore, although the ability for CS3 and PS1 to induce cIAP1 degradation was examined, a single concentration of 1 μM was used for both compounds in the experiment and it is therefore unclear whether PS1 is also more potent than CS3 in induction of cIAP1 degradation in tumor cells. Hence, the weaker cellular potency for the selective cIAP inhibitor CS3 compared with the pan IAP inhibitor PS1 reported in the previous study[43] could be explained by other reason(s) such as decreased cell permeability for CS3 as compared to PS1, instead of the selectivity of CS3 for cIAP over XIAP.

In summary, we have designed and evaluated a series of new Smac mimetics. Exploiting the differences between cIAP1/2 and XIAP in one particular binding pocket led to a set of highly potent cIAP1/2 inhibitors with excellent selectivity over XIAP. For example, compound **5** (SM-1295) binds to both cIAP1 and cIAP2 proteins with K_i values of <10 nM and displays a selectivity of >900-fold for cIAP1 over XIAP. Compound **5** potently inhibits cell growth in the MDA-MB-231 and SK-OV-3 cancer cell lines and induces apoptosis at low nanomolar concentrations in these cell lines. Consistent with its high binding affinities to cIAP1, **5** efficiently induces degradation of cIAP1 protein in cancer cells, as well as cleavage of PARP,

dx.doi.org/10.1021/cb400889a | ACS Chem. Biol. 2014, 9, 994−1002

caspase-8, and caspase-3. Our mechanistic studies showed that the apoptosis induction by **5** in tumor cells is dependent upon caspase-8 and caspase-3 but not on caspase-9. Interestingly, while **5** binds to XIAP with a very weak affinity, it can effectively induce cleavage of XIAP in a caspase-3- and caspase-8-dependent manner, which may provide a positive feedback for its ability to induce apoptosis in tumor cells. Compound **5** induces cell death in a TNFα-dependent manner in both MDA-MB-231 and SK-OV-3 cancer cell lines, the same as those previously reported Smac mimetics, which bind to cIAP1, cIAP2, and XIAP with high affinities. Based upon our data and also data published previously,[43] we conclude that binding of Smac mimetics to XIAP BIR3 protein is not required for effective cell-death induction in tumor cells. Our study has yielded a set of potent cIAP1/2 inhibitors that are highly selective over XIAP. Selective cIAP inhibitors, such as **5** and **7**, can be used as powerful pharmacological tools to investigate the role of these IAP proteins in the regulation of apoptosis, as well as in other biological and pathological processes in which IAP proteins may play a role.

METHODS

Chemical Synthesis and Compound Characterization. The synthesis of compounds **3**−**23** is shown in Supporting Information (Scheme S1). Detailed compound characterization is also provided in Supporting Information.

Computational Docking Studies. The crystal structure of XIAP BIR3 in a complex with the Smac protein (PDB id: 1G73) was used to predict the binding models of XIAP BIR3 bound to different compounds. For cIAP1-BIR3 domain, the complex structure between the Smac peptide and cIAP1-BIR3 (PDB id: 3D9U) was used. The binding pose of compounds with the BIR3 domain was predicted with the GOLD docking program (version 3.1.1).[48,49] Parameters used in the docking simulation have been reported previously.[30] The docking protocol was validated using a series of Smac mimetics. GoldScore, implemented in Gold, was used as the fitness function to evaluate the docked conformations of the ligands with the protein. The predicted binding pose of compound **1** shown in Figure 1 is the highest ranked conformation from the docking simulations.

Fluorescence Polarization Based Assays for XIAP, cIAP1, and cIAP2 BIR3 Proteins. Sensitive and quantitative fluorescence polarization (FP) based assays, the same as published previously,[23] were used to determine the binding affinities of designed Smac mimetics to XIAP BIR3, cIAP1 BIR3, and cIAP2 BIR3 proteins.

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, the same as described previously.[32]

Cell Growth Inhibition, Apoptosis, and Cell-Death Assays. MDA-MB-231 breast cancer and SK-OV-3 ovarian cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cell growth inhibition, cell viability and apoptosis assays were performed using the same procedures as described previously.[45]

Western Blot Analysis. Western blotting was performed using the same procedure published previously.[45] The following primary antibodies were used in the study: anti-cleaved-caspase 8, anti-XIAP, and anti-PARP (Cell Signaling Technology, Beverly, MA), anti-cIAP1 and anti-cIAP2 (R&D), anti-caspase-3, anti-caspase-9, and anti-procaspase-8 (Stressgen Biotechnologies, Victoria, Canada).

Assay To Analyze TNFα-Dependent Cell Killing. Cells were seeded in 24-well flat-bottom cell culture plates at a density of 1−2 × 10^5 cells/well. After incubation overnight, cells were pretreatment (1 h) of neutralizing antibodies (2 μg/mL) against TNFα (Biolegend, San Diego CA), TRAIL (Biolegend), or FasL/CD95 (Biolegend) prior to treatments with Smac mimetics. Cell viability was determined as previously described. Each graphical representation indicates the mean ± SD of at least three independent testing conditions.

RNA Interference. RNA interference was done as described previously.[45] Briefly, siRNA for caspase-8, -9, and -3 was purchased from Dhamacon Research, Inc. Nontargeting control siRNA was purchased from Ambion. Transfections were performed using Lipofectamine RNAiMAX (Invitrogen) in the reverse manner according to the manufacturer's instructions. Between 5 and 10 pmol of siRNA and 5 μL of Lipofectamine RNAiMAX were mixed in each well of 6-well plates for 20 min, followed by culturing 3 × 10^5 cells in the siRNA mix for 24−48 h; knockdown efficacy was assessed by Western blotting.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: shaomeng@umich.edu.

Author Contributions

†These authors contribute equally to this work.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Funding from the National Institutes of Health (R01CA109025 and R01CA127551) is greatly appreciated. We thank G. W. A. Milne for critical reading of the manuscript.

REFERENCES

(1) Salvesen, G. S., and Duckett, C. S. (2010) IAP proteins: blocking the road to death's door. *Nat. Rev. Mol. Cell Biol. 3*, 401−410.

(2) Deveraux, Q. L., and Reed, J. C. (1999) IAP family proteins-suppressors of apoptosis. *Genes Dev. 13*, 239−252.

(3) Gyrd-Hansen, M., and Meier, P. (2010) IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. *Nat. Rev. Cancer 10*, 561−574.

(4) Samuel, T., Welsh, K., Lober, T., Togo, S. H., Zapata, J. M., and Reed, J. C. (2006) Distinct BIR domains of cIAP1 mediate binding to and ubiquitination of tumor necrosis factor receptor-associated factor 2 and second mitochondrial activator of caspases. *J. Biol. Chem. 281*, 1080−1090.

(5) Varfolomeev, E., Blankenship, J. W., Wayson, S. M., Fedorova, A. V., Kayagaki, N., Garg, P., Zobel, K., Dynek, J. N., Elliott, L. O., Wallweber, H. J., Flygare, J. A., Fairbrother, W. J., Deshayes, K., Dixit, V. M., and Vucic, D. (2007) IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. *Cell 131*, 669−681.

(6) Vince, J. E., Wong, W. W., Khan, N., Feltham, R., Chau, D., Ahmed, A. U., Benetatos, C. A., Chunduru, S. K., Condon, S. M., McKinlay, M., Brink, R., Leverkus, M., Tergaonkar, V., Schneider, P., Callus, B. A., Koentgen, F., Vaux, D. L., and Silke, J. (2007) IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. *Cell 131*, 682−693.

(7) Holcik, M., Gibson, H., and Korneluk, R. G. (2001) XIAP: apoptotic brake and promising therapeutic target. *Apoptosis 6*, 253−261.

(8) Mizutani, Y., Nakanishi, H., Li, Y. N., Matsubara, H., Yamamoto, K., Sato, N., Shiraishi, T., Nakamura, T., Mikami, K., Okihara, K., Takaha, N., Ukimura, O., Kawauchi, A., Nonomura, N., Bonavida, B., and Miki, T. (2007) Overexpression of XIAP expression in renal cell carcinoma predicts a worse prognosis. *Int. J. Oncol. 30*, 919−925.

(9) Lopes, R. B., Gangeswaran, R., McNeish, I. A., Wang, Y., and Lemoine, N. R. (2007) Expression of the IAP protein family is

dysregulated in pancreatic cancer cells and is important for resistance to chemotherapy. *Int. J. Cancer 120*, 2344−2352.
(10) Nakagawa, Y., Abe, S., Kurata, M., Hasegawa, M., Yamamoto, K., Inoue, M., Takemura, T., Suzuki, K., and Kitagawa, M. (2006) IAP family protein expression correlates with poor outcome of multiple myeloma patients in association with chemotherapy-induced overexpression of multidrug resistance genes. *Am. J. Hematol. 81*, 824−831.
(11) Tamm, I., Kornblau, S. M., Segall, H., Krajewski, S., Welsh, K., Kitada, S., Scudiero, D. A., Tudor, G., Qui, Y. H., Monks, A., Andreeff, M., and Reed, J. C. (2000) Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. *Clin. Cancer Res. 6*, 1796−1803.
(12) LaCasse, E. C., Mahoney, D. J., Cheung, H. H., Plenchette, S., Baird, S., and Korneluk, R. G. (2008) IAP-targeted therapies for cancer. *Oncogene 27*, 6252−6275.
(13) Vucic, D., and Fairbrother, W. J. (2007) The inhibitor of apoptosis proteins as therapeutic targets in cancer. *Clin. Cancer Res. 13*, 5995−6000.
(14) Fulda, S. (2007) Inhibitor of apoptosis proteins as targets for anticancer therapy. *Expert Rev. Anticancer Ther. 7*, 1255−1264.
(15) Fulda, S., and Vucic, D. (2012) Targeting IAP proteins for therapeutic intervention in cancer. *Nat. Rev. Drug Discovery 11*, 109−124.
(16) Mannhold, R., Fulda, S., and Carosati, E. (2010) IAP antagonists: promising candidates for cancer therapy. *Drug Discovery Today 15*, 210−219.
(17) Shiozaki, E. N., and Shi, Y. (2004) Caspases, IAPs and Smac/DIABLO: mechanisms from structural biology. *Trends Biochem. Sci. 29*, 486−494.
(18) Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell 102*, 33−42.
(19) Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell 102*, 43−53.
(20) Liu, Z., Sun, C., Olejniczak, E. T., Meadows, R. P., Betz, S. F., Oost, T., Herrmann, J., Wu, J. C., and Fesik, S. W. (2000) Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. *Nature 408*, 1004−1008.
(21) Wu, G., Chai, J., Suber, T. L., Wu, J. W., Du, C., Wang, X., and Shi, Y. (2000) Structural basis of IAP recognition by Smac/DIABLO. *Nature 408*, 1008−1012.
(22) Huang, Y., Rich, R. L., Myszka, D. G., and Wu, H. (2003) 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. 278*, 49517−49522.
(23) Cai, Q., Sun, H., Peng, Y., Lu, J., Nikolovska-Coleska, Z., McEachern, D., Liu, L., Qiu, S., Yang, C. Y., Miller, R., Yi, H., Zhang, T., Sun, D., Kang, S., Guo, M., Leopold, L., Yang, D., and Wang, S. (2011) A potent and orally active antagonist (SM-406/AT-406) of multiple inhibitor of apoptosis proteins (IAPs) in clinical development for cancer treatment. *J. Med. Chem. 54*, 2714−2726.
(24) Cohen, F., Alicke, B., Elliott, L. O., Flygare, J. A., Goncharov, T., Keteltas, S. F., Franklin, M. C., Frankovitz, S., Stephan, J. P., Tsui, V., Vucic, D., Wong, H., and Fairbrother, W. J. (2009) Orally bioavailable antagonists of inhibitor of apoptosis proteins based on an azabicyclooctane scaffold. *J. Med. Chem. 52*, 1723−1730.
(25) Flygare, J. A., Beresini, M., Budha, N., Chan, H., Chan, I. T., Cheeti, S., Cohen, F., Deshayes, K., Doerner, K., Eckhardt, S. G., Elliott, L. O., Feng, B., Franklin, M. C., Reisner, S. F., Gazzard, L., Halladay, J., Hymowitz, S. G., La, H., LoRusso, P., Maurer, B., Murray, L., Plise, E., Quan, C., Stephan, J. P., Young, S. G., Tom, J., Tsui, V., Um, J., Varfolomeev, E., Vucic, D., Wagner, A. J., Wallweber, H. J., Wang, L., Ware, J., Wen, Z., Wong, H., Wong, J. M., Wong, M., Wong, S., Yu, R., Zobel, K., and Fairbrother, W. J. (2012) Discovery of a potent small-molecule antagonist of inhibitor of apoptosis (IAP) proteins and clinical candidate for the treatment of cancer (GDC-0152). *J. Med. Chem. 55*, 4101−4113.
(26) Gaither, A., Porter, D., Yao, Y., Borawski, J., Yang, G., Donovan, J., Sage, D., Slisz, J., Tran, M., Straub, C., Ramsey, T., Iourgenko, V., Huang, A., Chen, Y., Schlegel, R., Labow, M., Fawell, S., Sellers, W. R., and Zawel, L. (2007) A Smac mimetic rescue screen reveals roles for inhibitor of apoptosis proteins in tumor necrosis factor-alpha signaling. *Cancer Res. 67*, 11493−11498.
(27) Li, L., Thomas, R. M., Suzuki, H., De Brabander, J. K., Wang, X., and Harran, P. G. (2004) A small molecule Smac mimic potentiates TRAIL- and TNFalpha-mediated cell death. *Science 305*, 1471−1474.
(28) Sun, H., Nikolovska-Coleska, Z., Yang, C. Y., Qian, D., Lu, J., Qiu, S., Bai, L., Peng, Y., Cai, Q., and Wang, S. (2008) Design of small-molecule peptidic and nonpeptidic Smac mimetics. *Acc. Chem. Res. 41*, 1264−1277.
(29) Oost, T. K., Sun, C., Armstrong, R. C., Al-Assaad, A. S., Betz, S. F., Deckwerth, T. L., Ding, H., Elmore, S. W., Meadows, R. P., Olejniczak, E. T., Oleksijew, A., Oltersdorf, T., Rosenberg, S. H., Shoemaker, A. R., Tomaselli, K. J., Zou, H., and Fesik, S. W. (2004) Discovery of potent antagonists of the antiapoptotic protein XIAP for the treatment of cancer. *J. Med. Chem. 47*, 4417−4426.
(30) Peng, Y., Sun, H., Nikolovska-Coleska, Z., Qiu, S., Yang, C. Y., Lu, J., Cai, Q., Yi, H., Kang, S., Yang, D., and Wang, S. (2008) Potent, orally bioavailable diazabicyclic small-molecule mimetics of second mitochondria-derived activator of caspases. *J. Med. Chem. 51*, 8158−8162.
(31) Sheng, R., Sun, H., Liu, L., Lu, J., McEachern, D., Wang, G., Wen, J., Min, P., Du, Z., Lu, H., Kang, S., Guo, M., Yang, D., and Wang, S. (2013) A potent bivalent Smac mimetic (SM-1200) achieving rapid, complete, and durable tumor regression in mice. *J. Med. Chem. 56*, 3969−3979.
(32) Sun, H., Lu, J., Liu, L., Yi, H., Qiu, S., Yang, C. Y., Deschamps, J. R., and Wang, S. (2010) Nonpeptidic and potent small-molecule inhibitors of cIAP-1/2 and XIAP proteins. *J. Med. Chem. 53*, 6361−6367.
(33) Sun, H., Nikolovska-Coleska, Z., Lu, J., Meagher, J. L., Yang, C. Y., Qiu, S., Tomita, Y., Ueda, Y., Jiang, S., Krajewski, K., Roller, P. P., Stuckey, J. A., and Wang, S. (2007) 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. 129*, 15279−15294.
(34) Sun, H., Nikolovska-Coleska, Z., Lu, J., Qiu, S., Yang, C. Y., Gao, W., Meagher, J., Stuckey, J., and Wang, S. (2006) Design, synthesis, and evaluation of a potent, cell-permeable, conformationally constrained second mitochondria derived activator of caspase (Smac) mimetic. *J. Med. Chem. 49*, 7916−7920.
(35) Sun, H., Nikolovska-Coleska, Z., Yang, C. Y., Xu, L., Liu, M., Tomita, Y., Pan, H., Yoshioka, Y., Krajewski, K., Roller, P. P., and Wang, S. (2004) Structure-based design of potent, conformationally constrained Smac mimetics. *J. Am. Chem. Soc. 126*, 16686−16687.
(36) Sun, H., Nikolovska-Coleska, Z., Yang, C. Y., Xu, L., Tomita, Y., Krajewski, K., Roller, P. P., and Wang, S. (2004) 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. *J. Med. Chem. 47*, 4147−4150.
(37) Sun, H., Stuckey, J. A., Nikolovska-Coleska, Z., Qin, D., Meagher, J. L., Qiu, S., Lu, J., Yang, C. Y., Saito, N. G., and Wang, S. (2008) Structure-based design, synthesis, evaluation, and crystallographic studies of conformationally constrained Smac mimetics as inhibitors of the X-linked inhibitor of apoptosis protein (XIAP). *J. Med. Chem. 51*, 7169−7180.
(38) Sun, W., Nikolovska-Coleska, Z., Qin, D., Sun, H., Yang, C. Y., Bai, L., Qiu, S., Wang, Y., Ma, D., and Wang, S. (2009) Design, synthesis, and evaluation of potent, nonpeptidic mimetics of second mitochondria-derived activator of caspases. *J. Med. Chem. 52*, 593−596.
(39) Wang, S. (2011) Design of small-molecule Smac mimetics as IAP antagonists. *Curr. Top. Microbiol. Immunol. 348*, 89−113.

(40) Zhang, B., Nikolovska-Coleska, Z., Zhang, Y., Bai, L., Qiu, S., Yang, C. Y., Sun, H., Wang, S., and Wu, Y. (2008) Design, synthesis, and evaluation of tricyclic, conformationally constrained small-molecule mimetics of second mitochondria-derived activator of caspases. *J. Med. Chem. 51*, 7352−7355.
(41) Zobel, K., Wang, L., Varfolomeev, E., Franklin, M. C., Elliott, L. O., Wallweber, H. J., Okawa, D. C., Flygare, J. A., Vucic, D., Fairbrother, W. J., and Deshayes, K. (2006) Design, synthesis, and biological activity of a potent Smac mimetic that sensitizes cancer cells to apoptosis by antagonizing IAPs. *ACS Chem. Biol. 1*, 525−533.
(42) Hashimoto, K., Saito, B., Miyamoto, N., Oguro, Y., Tomita, D., Shiokawa, Z., Asano, M., Kakei, H., Taya, N., Kawasaki, M., Sumi, H., Yabuki, M., Iwai, K., Yoshida, S., Yoshimatsu, M., Aoyama, K., Kosugi, Y., Kojima, T., Morishita, N., Dougan, D. R., Snell, G. P., Imamura, S., and Ishikawa, T. (2013) Design and synthesis of potent inhibitor of apoptosis (IAP) proteins antagonists bearing an octahydropyrrolo[1,2-a]pyrazine scaffold as a novel proline mimetic. *J. Med. Chem. 56*, 1228−1246.
(43) Ndubaku, C., Varfolomeev, E., Wang, L., Zobel, K., Lau, K., Elliott, L. O., Maurer, B., Fedorova, A. V., Dynek, J. N., Koehler, M., Hymowitz, S. G., Tsui, V., Deshayes, K., Fairbrother, W. J., Flygare, J. A., and Vucic, D. (2009) Antagonism of c-IAP and XIAP proteins is required for efficient induction of cell death by small-molecule IAP antagonists. *ACS Chem. Biol. 4*, 557−566.
(44) Hennessy, E. J., Saeh, J. C., Sha, L., MacIntyre, T., Wang, H., Larsen, N. A., Aquila, B. M., Ferguson, A. D., Laing, N. M., and Omer, C. A. (2012) Discovery of aminopiperidine-based Smac mimetics as IAP antagonists. *Bioorg. Med. Chem. Lett. 22*, 1690−1694.
(45) Lu, J., Bai, L., Sun, H., Nikolovska-Coleska, Z., McEachern, D., Qiu, S., Miller, R. S., Yi, H., Shangary, S., Sun, Y., Meagher, J. L., Stuckey, J. A., and Wang, S. (2008) SM-164: a novel, bivalent Smac mimetic that induces apoptosis and tumor regression by concurrent removal of the blockade of cIAP-1/2 and XIAP. *Cancer Res. 68*, 9384−9393.
(46) Petersen, S. L., Wang, L., Yalcin-Chin, A., Li, L., Peyton, M., Minna, J., Harran, P., and Wang, X. (2007) Autocrine TNFalpha signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis. *Cancer Cell 12*, 445−456.
(47) Gaither, A., Porter, D., Yao, Y., Borawski, J., Yang, G., Donovan, J., Sage, D., Slisz, J., Tran, M., Straub, C., Ramsey, T., Iourgenko, V., Huang, A., Chen, Y., Schlegel, R., Labow, M., Fawell, S., Sellers, W. R., and Zawel, L. (2007) A Smac mimetic rescue screen reveals roles for inhibitor of apoptosis proteins in tumor necrosis factor-alpha signaling. *Cancer Res. 67*, 11493−11498.
(48) Jones, G., Willett, P., Glen, R. C., Leach, A. R., and Taylor, R. (1997) Development and validation of a genetic algorithm for flexible docking. *J. Mol. Biol. 267*, 727−748.
(49) Verdonk, M. L., Cole, J. C., Hartshorn, M. J., Murray, C. W., and Taylor, R. D. (2003) Improved protein-ligand docking using GOLD. *Proteins 52*, 609−623.