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A Potent Bivalent Smac Mimetic (SM-1200) Achieving Rapid, Complete and Durable Tumor Regression in Mice

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Abstract

We have designed, synthesized and evaluated a series of new compounds based upon our previously reported bivalent Smac mimetics. This led to the identification of compound **12** (SM-1200), which binds to XIAP, cIAP1 and cIAP2 with K_i values of 0.5 nM, 3.7 nM and 5.4 nM, respectively, inhibits cell growth in the MDA-MB-231 breast cancer and SK-OV-3 ovarian cancer cell lines with IC_{50} values of 11.0 nM and 28.2 nM, respectively. Compound **12** has a much improved pharmacokinetic profile over our previously reported bivalent Smac mimetics and is highly effective in induction of rapid and durable tumor regression in the MDA-MB-231 xenograft model. These data indicate that compound **12** is a promising Smac mimetic and warrants extensive evaluation as a potential candidate for clinical development.

Introduction

X-linked inhibitors of apoptosis protein (XIAP) and cellular IAP1 (cIAP1) and cIAP2 are critical regulators of apoptosis and attractive new cancer therapeutic targets.^{1–8} The second mitochondria-derived activator of caspases (Smac) also known as the direct IAP binding protein with low pI (DIABLO) is an endogenous antagonist of IAP proteins.^{9,10} Crystal structures^{11–13} reveal that the interaction between Smac and the IAP proteins XIAP, cIAP1 and cIAP2 is mediated by the AVPI tetrapeptide binding motif in Smac protein and a well-defined surface binding groove in these IAP proteins. In the last few years, various laboratories, including ours, have intensely pursued the design of small-molecules (Smac mimetics), which can mimic the interaction between Smac and these IAP proteins, as new cancer therapeutic agents.^{14–36}

Two types of Smac mimetics, monovalent and bivalent Smac mimetics, have been designed.^{14–17} Monovalent Smac mimetics are designed to mimic a single AVPI binding motif, while bivalent Smac mimetics contain two small-molecule mimics of the AVPI binding motif, tethered through a linker.^{14–17} While monovalent Smac mimetics can effectively and potently antagonize the inhibition by XIAP BIR3 protein of caspase-9

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activity, they are less effective in antagonizing the inhibition of caspase-3 by XIAP protein containing both BIR2 and BIR3 domains.^{34–36} In comparison, bivalent Smac mimetics are much more potent than the corresponding monovalent Smac mimetics in binding to XIAP containing both BIR2 and BIR3 domains. As a consequence, bivalent Smac mimetics are much more effective than monovalent Smac mimetics in antagonizing XIAP containing both BIR2 and BIR3 domains in cell-free functional assays.^{34–36} In addition to their ability to antagonize XIAP, both monovalent and bivalent Smac mimetics potently bind to cIAP1 and cIAP2 and induce rapid degradation of cIAP1/2 in cells.^{15,16} While both types of Smac mimetics effectively kill tumor cells in some human cancer cell lines, bivalent Smac mimetics can be >100-times more potent than their corresponding monovalent Smac mimetic analogues.^{30–36}

One major advantage for monovalent Smac mimetics as therapeutic agents is their favorable pharmaceutical properties stemming from their low molecular weights.^{14–17} Indeed, well designed monovalent Smac mimetics, including those in Figure 1, can achieve oral bioavailability.^{14–17} In comparison, bivalent Smac mimetics such as those shown in Figure 2, have molecular weights at least twice that of monovalent Smac mimetics and are not orally bioavailable.

To date, four monovalent and two bivalent Smac mimetics have been advanced into clinical development for the treatment of human cancers.^{14,15} For example, the orally active monovalent Smac mimetic, SM-406/AT-406 designed in this laboratory, is in Phase I/II clinical trials as a new anticancer drug.¹⁸

Because bivalent Smac mimetics are much more effective than monovalent Smac mimetics in antagonizing XIAP and inducing apoptosis against tumor cells, we have sought to develop bivalent Smac mimetics for cancer treatment and have designed, synthesized and evaluated a series of bivalent Smac mimetics.^{30–34} Although a number of highly potent Smac mimetics were obtained from our previous efforts, none of them was found to be suitable for advanced preclinical development. For example, while **8** (SM-164) effectively inhibited tumor growth and in fact induced partial tumor regression at its maximum tolerated dose (MTD) in the MDA-MB-231 xenograft model in mice, it failed to achieve complete tumor regression at its MTD.³⁴ Our pharmacokinetic (PK) study of compound **8** with intravenous administration in rats also revealed that it has a very low AUC (area-under-curve) in the plasma, indicating a poor PK profile (Table 1). These data indicated that further improvements are clearly needed toward identification of a suitable bivalent Smac mimetic for clinical development.

Toward our goal of identifying a suitable bivalent Smac mimetic for clinical development, we report herein the design, synthesis and evaluation of a series of new bivalent Smac mimetics. This study has led to the identification of a promising new Smac mimetic (**12**, SM-1200). Compound **12** has a much improved PK profile over compound **8** and is capable of achieving rapid, complete and durable tumor regression at a well-tolerated dose-schedule in mice.

Chemistry

The synthesis of the newly designed compounds **12–20** is shown in Scheme 1.

The key intermediate **23** can be synthesized according to our previously reported method.³⁶ Reaction of **23** with bis-isocyanates furnished a series of ureas, and removal of the Boc protecting groups from these ureas provided our designed bivalent Smac mimetics **12–20**.

The synthesis of the newly designed compounds **21** and **22** is shown in Scheme 2.

Condensation of **23** with 1,4-benzenediacetic acid or 1,4-phenylenedipropionic acid yielded the corresponding bisamides. Removal of the Boc protecting group in these amides gave compounds **21** and **22**

Results and Discussion

We previously reported the design of a class of bivalent Smac mimetics based upon our monovalent Smac mimetic **2**, as exemplified by compound **10**³⁶ (Figure 2). Compound **10** binds to XIAP, cIAP1 and cIAP2 with high affinities, ($K_i = 2.0$ nM, 2.9 nM and 14 nM, respectively), and potently inhibits cell growth in the MDA-MB-231 breast cancer cell line and other cancer cell lines sensitive to Smac mimetics. Pharmacokinetic evaluation of compound **10** showed that it has an improved PK profile over compound **8** (Table 2), suggesting that further modifications of compound **10** may yield a promising bivalent Smac mimetic for advanced preclinical development and clinical trials.

High flexibility of their linkers is a characteristic common to compounds **8** and **10**, and this may affect their pharmacokinetics in animals. We have therefore synthesized compound **11**³⁶ (Figure 3), which has a rigid linker. This compound binds to XIAP, cIAP1 and cIAP2 with $K_i = 5$ nM, 3.6 nM and 11 nM, respectively (Table 1). Although compound **11** has K_i values similar to those of compound **10**, **11** has IC_{50} values of 51 nM and 139 nM in inhibition of cell growth in the MDA-MB-231 breast cell line under the same assay conditions (Table 1), being >10-times less potent than **10**. Therefore, the cellular activity of compound **11** needed to be improved. Since our previous study showed that the linker in bivalent Smac mimetics plays a key role in cellular activity by modulating the cell permeability,³⁵ we have focused our modifications on the linker in compound **11** in an attempt to improve its cellular potency. The binding affinities of our newly synthesized compounds to XIAP, cIAP1 and cIAP2 were determined using our optimized competitive fluorescence-polarization (FP) assays.^{35–37} Their cell growth inhibitory activity was assessed against the MDA-MB-231 breast cancer and SK-OV-3 cell lines, which are sensitive to Smac mimetics.^{35,36} The data obtained from binding and cell growth experiments are provided in Table 1.

In a first step, both of the amide groups in the linker of compound **11** were replaced with a urea group, which resulted in compound **12**. Compound **12** binds to XIAP, cIAP1 and cIAP2 with K_i values of <1 nM, 3.7 nM and 5.4 nM, respectively, and is thus more potent than **11**. Compound **12** has IC_{50} values of 11 nM and 28 nM, respectively, against the MDA-MB-231 breast cancer and SK-OV-3 ovarian cancer cell lines and is 5–6 times more potent than **11** in these cancer two cell lines.

Based upon these promising data for compound **12**, we next made additional modifications on the linker to further explore the structure-activity relationship in both binding affinities and cellular activity (Figure 3 and Table 1). Moving the linker from 1,4 positions on the phenyl ring in **12** to 1,3 positions led to compound **13**, which binds to XIAP, cIAP1 and cIAP2 proteins with high affinities but is only half as potent as compound **12** and 5–6 times less potent than compound **12** in inhibition of cell growth in the MDA-MB-231 and SK-OV-3 cancer cell lines. Extension of the linker in compound **13** by 2 extra carbon atoms resulted in compound **14**, which is as potent as compound **12** in binding to XIAP, cIAP1 and cIAP2, as well as in inhibition of cell growth in the MDA-MB-231 and SK-OV-3 cancer cell lines. Replacement of the phenyl group in compound **12** with 4,4'-oxydibenzyl yielded compound **15**, which binds to these IAP proteins with the same affinities as compound **12**. This compound (**15**) is 4-times more potent than **12** in inhibition of cell growth in the MDA-MB-231 cell line but both **15** and **12** are essentially equipotent in the SK-OV-3 cell line. Replacement of the phenyl group in compound **12** with 4,4'-1,2-diphenylethane resulted in

compound **16**, which has a higher binding affinity to XIAP than compounds **12** and **15** based upon their IC₅₀ values but has the same high binding affinities to cIAP1 and cIAP2, as compared to **12** and **15**. Compound **16** has an IC₅₀ value of 0.9 nM in inhibition of cell growth in the MDA-MB-231 cell line and is thus >10-times more potent than **12** but is only 2-times more potent than **12** in the SK-OV-3 cell line.

Changing the phenyl group in compound **12** into a 1,4-*trans*-cyclohexyl group yielded compound **17**. Compound **17** is approximately 2-fold less potent than compound **12** in binding to XIAP, cIAP1 and cIAP2 and is also 2-times less potent than **12** in inhibition of cell growth in both the MDA-MB-231 and SK-OV-3 cancer cell lines. Compound **18** with a shorter linker than that in **12**, is 5-times less potent than **12** in binding to XIAP and both **12** and **18** have similar binding affinities to cIAP1 and cIAP2. Compound **18** is >20-times less potent than **12** in inhibition of cell growth in both the MDA-MB-231 and SK-OV-3 cancer cell lines.

To further investigate if the conformational flexibility of the linker plays a role in binding and/or cellular activity, we designed and synthesized compound **19**, in which a linear and conformationally flexible butyl group was used to replace the 1,4-disubstituted rings in **11–13**. Compounds **19** and **12** have the same binding affinities to XIAP and cIAP1/2 proteins and essentially the same IC₅₀ values in inhibition of cell growth in the MDA-MB-231 and SK-OV-3 cancer cell lines, suggesting that the flexible linker in compound **19** is not detrimental to binding to these IAP proteins and to the activity in cell growth inhibition. However, compounds with flexible linkers may not have good pharmacokinetics in animals.

In view of the high binding affinities to these IAP proteins and potent cellular activity for compound **19**, we next synthesized compound **20** with an *n*-octyl linker, four carbons longer than the linker in **19**. Compound **20** has the same high binding affinities as **12** and **19** to the three IAP proteins. However, compound **20** is 3- and 2-times more potent than compound **19** in inhibition of cell growth in the MDA-MB-231 and SK-OV-3 cancer cell lines, respectively. The cellular data for compounds **19** and **20** are consistent with our previous results that more hydrophobic linkers in general improve the cellular activity of bivalent Smac mimetics.³⁵

To confirm that the urea groups in the linker indeed improve the cell permeability with respect to the amide groups, we synthesized compounds **21** and **22** in which the linker in compound **11** has been extended by extra two or four carbons to make the linker more hydrophobic. Although compounds **21** and **22** bind to the three IAP proteins with high affinities and are only slightly less potent than compound **12**, they are >10-times less potent than **12** in inhibition of cell growth in both the MDA-MB-231 and SK-OV-3 cancer cell lines. These data show that compounds with the urea groups in the linker indeed have superior cell permeability to similar analogues with the amide groups.

Our previous study has shown that bivalent Smac mimetics are much more potent in antagonizing XIAP than monovalent Smac mimetics. We evaluated compounds **12** and **16** for their functional antagonism against XIAP containing linker-BIR2-BIR3 domains and included our monovalent Smac mimetic **1** and bivalent Smac mimetic **8** as the controls. Our data showed that bivalent Smac mimetics **8**, **12** and **16** all potently and effectively antagonize XIAP in both caspase-9 and caspase-3 functional assays (Figure 4). While compounds **8**, **12** and **16** have similar potencies, they are much more potent than monovalent Smac mimetic **1** in antagonizing XIAP both caspase-9 and caspase-3 functional assays, consistent with our previous data.

We investigated the cellular mechanism of action of compound **12** and **16** by Western blot analysis with compound **8** included as a positive control compound (Figure 5). Consistent with their high binding affinities to cIAP1 and potent cell growth inhibitory activity, compounds **8**, **12** and **16** each effectively induce the degradation of cIAP1 in the MDA-MB-231 cell line at very low concentrations. Compound **12** at concentrations as low as 1 nM, and compounds **8** and **16** at concentrations as low as 0.3 nM induce clear cIAP1 degradation and also dose-dependently induce cleavage of caspase-8, caspase-3 and PARP (Poly ADP ribose polymerase), indicative of robust apoptosis induction by these Smac mimetics.

Compounds **8** and **12**, **16** were evaluated for their ability to induce cell death by trypan blue assay in the MDA-MB-231 breast cancer cell line (Figure 6). All these three compounds potently and effectively induce cell death in the MDA-MB-231 cancer cells in a dose-dependent manner. In a 24-h treatment, compound **8** at 10 nM, **12** at 10 nM and **16** at 3 nM induced >75% of the MDA-MB-231 cells to undergo cell death.

Among these new compounds, compound **16** is the most potent based upon its activity in inhibition of cell growth in the MDA-MB-231 and SK-OV-3 cell lines, whereas compound **12** has a rigid linker and also potent cellular activity. We therefore selected these two compounds for evaluation of their pharmacokinetics (PK) in rats. While compound **12** has a significantly improved PK profile over compound **8** based upon their AUC values and clearance rates ($p < 0.01$), compound **16** has the worst pharmacokinetics among these four compounds (Table 2).

Based upon the encouraging PK data, we next evaluated the antitumor activity of compound **12** in the MDA-MB-231 xenograft model in immunodeficient mice and included docetaxel as a positive control agent (Figure 7). Docetaxel at 7.5 mg/kg effectively inhibited tumor growth but failed to achieve tumor regression. In contrast, compound **12** at both 3 mg/kg and 10 mg/kg weekly dosing induced rapid tumor regression. After a single dose, the tumors regressed on average by 55% with 3 mg/kg of compound **12** and by 88% with 10 mg/kg of compound **12**. After four weekly doses, the tumors regressed by 93% on average with 3 mg/kg of compound **12** and by 99% with 10 mg/kg of compound **12**. After treatment with compound **12** at 10 mg/kg weekly for 4 weeks, 6 out of 7 mice had no measurable tumor. The tumor regression was also durable. At day 76, 27 days after the last dose, 5 out of the 7 mice treated with 10 mg/kg of **12** remained tumor-free and 2 of the 7 mice treated with **12** at 3 mg/kg were tumor-free. Our statistical analysis showed that compound **12** at both doses was statistically more effective than docetaxel at 7.5 mg/kg (Table 3). Importantly, compound **12** at both doses was well tolerated and caused minimal weight loss in mice. In comparison, our previous study showed that while compound **8** effectively inhibited tumor growth in the MDA-MB-231 xenograft model in mice at 5 mg/kg (*i.v.*), 5 days a week for 2 weeks, it failed to achieve complete and durable tumor regression.³⁴ These data showed that compound **12** is highly effective in induction of rapid tumor regression at well-tolerated doses.

To elucidate the underlying *in vivo* mechanism of action for compound **12**, we performed a pharmacodynamic study in mice bearing the MDA-MB-231 xenograft tumors (Figure 8). Western blot analysis showed that a single dose of compound **12** at 5 mg/kg induced rapid, complete and sustained degradation of cIAP1 in tumor tissues. At 2 h time-point after dosing, the levels of cIAP1 protein in tumor tissues became undetectable and the effect persisted for at least 24 hr. Robust cleavage of both caspase-3 and PARP was observed starting from the 4 h time-point, indicative of strong apoptosis induction. Although the amount of cleaved PARP was less at the 24 h time-point than at the 4 and 8 h time-points, the levels of full-length PARP became very low, suggesting that strong apoptosis was still

occurring at the 24 h time-point. Interestingly, there was a clear decrease in the levels of XIAP, starting at 8 h time-point and lasting through the 24 h time-point. Since active caspase-3 cleaves XIAP and the decrease of full-length of XIAP occurs after robust activation of caspase-3,³⁸ caspase-3 is probably responsible for the decreased levels of full-length XIAP. Since XIAP is an efficient inhibitor of caspase-9 and caspase-3/7, the decreased levels of XIAP can further enhance the activation of caspases, leading to a positive feedback on apoptosis induction by compound **12**.

Summary

With the goal of identifying a potent and efficacious bivalent Smac mimetic for advanced preclinical development and clinical trials, we have performed modifications on compound **11**, a bivalent Smac mimetic we had previously reported, with a focus on the linker region. Our results showed that replacement of the two amide groups in the linker region with urea groups yielded new bivalent Smac mimetics with high binding affinities to these IAP proteins and potent cellular activity. For example, compound **16** binds to these IAP proteins with K_i values of 0.5–2.1 nM and inhibits cell growth in the MDA-MB-231 breast cancer and SK-OV-3 ovarian cancer cell lines with IC_{50} values of 0.9 nM and 11.1 nM, respectively. Compound **12** binds to XIAP, cIAP1 and cIAP2 with K_i values of 0.5, 3.7 and 5.4 nM, respectively and inhibits cell growth in the MDA-MB-231 breast cancer and SK-OV-3 ovarian cancer cell lines with IC_{50} values of 11.0 nM and 28.2 nM, respectively. Although compound **12** is less potent than compound **16** in inhibition of cell growth in the MDA-MB-231 and SK-OV-3 cancer cell lines, compound **12** has a much better PK profile than **16**, as well as compounds **8** and **10**, and was subjected to extensive evaluations in animals. Our *in vivo* testing showed that compound **12**, administered weekly is highly effective in induction of rapid and durable tumor regression in the MDA-MB-231 xenograft model. Compound **12**, with 10 mg/kg weekly dosing for 4 weeks achieved 99% of tumor regression and 6 out of 7 mice remained tumor free 27 days after the final dose. In comparison, while our previous reported bivalent Smac compound **8** was very effective in inhibition of tumor growth in the MDA-MB-231 xenograft model and in fact induced partial tumor regression with daily dosing for 2 weeks, it failed to achieve complete tumor regression. Although these two compounds have similar potencies in induction of cell death in the MDA-MB-231 cell line, compound **12** is much more efficacious than compound **8** *in vivo*. Our *in vivo* pharmacodynamic study showed that compound **12** induces rapid and persistent cIAP1 degradation, consistent with its mechanism of action as a Smac mimetic. In line with its ability to induce rapid tumor regression, robust induction of apoptosis was evident with a single dose of compound **12** at 5 mg/kg in the MDA-MB-231 tumor tissues based upon strong cleavage of PARP and caspase-3. Taken together, these data indicate that compound **12** is a promising bivalent Smac mimetic and warrants extensive evaluations as a candidate for clinical development.

Experimental Section

I. Chemistry

General Methods. 1H NMR spectra were acquired at 300 MHz and ^{13}C spectra at 75 MHz. 1H chemical shifts are reported with CHD_2OD (3.31 ppm) or H_2O (4.70 ppm) as internal standards. The final products were purified by C_{18} reverse phase semi-preparative HPLC column with solvent A (0.1% of TFA in H_2O) and solvent B (0.1% of TFA in CH_3CN) as eluents. The purity of all the final compounds was confirmed by analytical HPLC to be >95%.

General synthesis of bivalent Smac mimetics—N,N-diisopropylethyl amine (3 eq) was added to a solution of **23** (1 eq) and corresponding bis-isocyanate (0.5 eq) in CH_2Cl_2 or

DMF (15 mL per mmol of **23**). The solution was stirred at room temperature overnight then concentrated and the residue was purified by chromatography to give a urea. HCl (4N in 1,4-dioxane, 2 mL per mmol of bis-triazole) was added to a solution of this urea in MeOH (5 mL per mmol of bis-triazole). The solution was stirred at room temperature overnight and then concentrated to furnish a crude product which was purified by C18 reversed phase semipreparative HPLC to give a bivalent Smac mimetic.

(S,5S,5'S,8S,8'S,10aR,10a'R)-N3,N3'-(1,4-Phenylene)bis(N8-benzhydryl-5-((S)-2-(methylamino)propanamido)-6-oxooctahydropyrrolo[1,2-a][1,5]diazocine-3,8(4H)-dicarboxamide) (**12**). Yield 63% over two steps. Purity was determined by reverse phase analytical HPLC to be over 95%. ¹H NMR (300 MHz, CD₃OD): 7.53 (s, 4H), 7.37 (m, 20H), 6.18 (s, 2H), 4.84 (m, 2H), 4.67 (t, *J* = 8.4 Hz, 2H), 4.27 (m, 2H), 4.09 (m, 6H), 3.30-3.20 (m, 4H), 2.71 (s, 6H), 2.37 (m, 2H), 2.24-2.06 (m, 6H), 1.81-1.72 (m, 4H), 1.56 (d, *J* = 6.9 Hz, 6H); ESI MS: *m/z* 1115.9 (M+H)⁺.

(S,5S,5'S,8S,8'S,10aR,10a'R)-N3,N3'-(1,3-Phenylene)bis(N8-benzhydryl-5-((S)-2-(methylamino)propanamido)-6-oxooctahydropyrrolo[1,2-a][1,5]diazocine-3,8(4H)-dicarboxamide) (**13**, SF-36). Yield 56% over two steps. Purity was determined by reverse phase analytical HPLC to be over 95%. ¹H NMR (300 MHz, CD₃OD): 8.14 (s, 1H), 7.34-7.18 (m, 23H), 6.17 (s, 2H), 4.84 (m, 2H), 4.67 (t, *J* = 8.4 Hz, 2H), 4.22 (m, 2H), 4.07 (m, 6H), 3.24 (m, 4H), 2.73 (s, 6H), 2.34 (m, 2H), 2.14-2.04 (m, 6H), 1.77-1.66 (m, 4H), 1.57 (d, *J* = 6.9 Hz, 6H); ESI MS: *m/z* 1115.9 (M+H)⁺.

(S,5S,5'S,8S,8'S,10aR,10a'R)-N3,N3'-(1,3-Phenylenebis(methylene))bis(N8-benzhydryl-5-((S)-2-(methylamino)propanamido)-6-oxooctahydropyrrolo[1,2-a][1,5]diazocine-3,8(4H)-dicarboxamide) (**14**, SF-19). Yield 62% over two steps. Purity was determined by reverse phase analytical HPLC to be over 95%. ¹H NMR (300 MHz, CD₃OD): 7.36-7.15 (m, 24H), 6.15 (s, 2H), 4.84 (m, 2H), 4.63-4.53 (m, 4H), 4.32-4.14 (m, 4H), 3.99-3.81 (m, 6H), 3.16-3.06 (m, 4H), 2.63 (s, 6H), 2.34 (m, 2H), 2.18-2.85 (m, 6H), 1.85-1.60 (m, 4H), 1.50 (d, *J* = 7.2 Hz, 6H); ESI MS: *m/z* 1143.67 (M+H)⁺.

(S,5S,5'S,8S,8'S,10aR,10a'R)-N3,N3'-(Oxybis(4,1-phenylene))bis(N8-benzhydryl-5-((S)-2-(methylamino)propanamido)-6-oxooctahydropyrrolo[1,2-a][1,5]diazocine-3,8(4H)-dicarboxamide) (**15**, SF-37). Yield 49% over two steps. Purity was determined by reverse phase analytical HPLC to be over 95%. ¹H NMR (300 MHz, CD₃OD): 7.55 (d, *J* = 9.0 Hz, 4H), 7.36-7.24 (m, 20H), 6.91 (d, *J* = 9.0 Hz, 4H), 6.17 (m, 2H), 4.84 (m, 2H), 4.64 (t, *J* = 8.1 Hz, 2H), 4.23 (m, 2H), 4.09 (m, 6H), 3.21 (m, 4H), 2.71 (s, 6H), 2.34 (m, 2H), 2.14-2.02 (m, 6H), 1.80-1.73 (m, 4H), 1.56 (d, *J* = 6.9 Hz, 6H); ESI MS: *m/z* 1207.3 (M+H)⁺.

(S,5S,5'S,8S,8'S,10aR,10a'R)-N3,N3'-(Methylenebis(4,1-phenylene))bis(N8-benzhydryl-5-((S)-2-(methylamino)propanamido)-6-oxooctahydropyrrolo[1,2-a][1,5]diazocine-3,8(4H)-dicarboxamide) (**16**, SF-31). Yield 51% over two steps. Purity was determined by reverse phase analytical HPLC to be over 95%. ¹H NMR (300 MHz, CD₃OD): 7.48 (d, *J* = 8.1 Hz, 4H), 7.33 (m, 20H), 7.11 (d, *J* = 8.1 Hz, 4H), 6.17 (s, 2H), 4.82 (m, 2H), 4.63 (m, 2H), 4.25 (m, 2H), 4.08-4.03 (m, 6H), 3.88 (s, 2H), 3.30-3.20 (m, 4H), 2.70 (s, 6H), 2.34 (m, 2H), 2.20-2.04 (m, 6H), 1.75-1.60 (m, 4H), 1.55 (d, *J* = 6.9 Hz, 6H); ESI MS: *m/z* 1206.4 (M+H)⁺.

(S,5S,5'S,8S,8'S,10aR,10a'R)-N3,N3'-(Cyclohexane-1,4-diyl)bis(N8-benzhydryl-5-((S)-2-(methylamino)propanamido)-6-oxooctahydropyrrolo[1,2-a][1,5]diazocine-3,8(4H)-dicarboxamide) (**17**, SF-46). Yield 43% over two steps. Purity was determined by reverse phase analytical HPLC to be over 95%. ¹H NMR (300 MHz, CD₃OD): 7.34-7.23 (m, 20H), 6.14 (s, 2H), 4.92 (m, 2H), 4.70 (m, 4H), 4.08-3.86 (m, 8H), 3.59 (m, 2H), 3.16-3.05

(m, 4H), 2.70 (s, 6H), 2.36 (m, 2H), 2.10-1.92 (m, 10H), 1.79-1.71 (m, 4H), 1.53-1.45 (m, 8H); ESI MS: m/z 1121.7 (M+H)⁺.

(S,5S,5'S,8S,8'S,10aR,10a'R)-3,3'-(Piperazine-1,4-dicarbonyl)bis(N-benzhydryl-5-((S)-2-(methylamino)propanamido)-6-oxodecahydropyrrolo[1,2-a][1,5]diazocine-8-carboxamide) (**18**, SF-45). Yield 41% over two steps. Purity was determined by reverse phase analytical HPLC to be over 95%. ¹H NMR (300 MHz, CD₃OD): 7.33-7.21 (m, 20H), 6.12 (s, 2H), 5.11 (m, 2H), 4.84 (m, 2H), 4.56 (t, J = 8.4 Hz, 2H), 4.25 (m, 2H), 3.93 (m, 2H), 3.66-3.53 (m, 6H), 3.22-3.15 (m, 8H), 2.67 (s, 6H), 2.34 (m, 2H), 2.15-1.96 (m, 4H), 1.83-1.77 (m, 6H), 1.54 (d, J = 6.9 Hz, 6H); ESI MS: m/z 1093.7 (M+H)⁺.

(S,5S,5'S,8S,8'S,10aR,10a'R)-N3,N3'-(Butane-1,4-diyl)bis(N8-benzhydryl-5-((S)-2-(methylamino)propanamido)-6-oxooctahydropyrrolo[1,2-a][1,5]diazocine-3,8(4H)-dicarboxamide) (**19**, SM-1386). Yield 63% over two steps. Purity was determined by reverse phase analytical HPLC to be over 95%. ¹H NMR (300 MHz, D₂O): 7.40-7.20 (m, 10H), 5.99 (s, 2H), 4.75 (m, 2H), 4.45 (m, 2H), 4.10 (m, 2H), 3.95 (m, 2H), 3.80 (m, 2H), 3.65 (m, 2H), 3.25-3.05 (m, 8H), 2.62 (m, 6H), 2.30 (m, 2H), 2.20-1.70 (m, 12H), 1.45 (m, 2H), 1.40 (d, J = 7.2 Hz, 6H); ESI MS: m/z 1095.4 (M+H)⁺.

(S,5S,5'S,8S,8'S,10aR,10a'R)-N3,N3'-(Octane-1,8-diyl)bis(N8-benzhydryl-5-((S)-2-(methylamino)propanamido)-6-oxooctahydropyrrolo[1,2-a][1,5]diazocine-3,8(4H)-dicarboxamide) (**20**, SF-16). Yield 65% over two steps. Purity was determined by reverse phase analytical HPLC to be over 95%. ¹H NMR (300 MHz, CD₃OD): 7.37-7.24 (m, 20H), 6.16 (s, 2H), 4.72-4.60 (m, 4H), 4.10 (m, 2H), 4.00-3.85 (m, 6H), 3.25-3.04 (m, 8H), 2.69 (s, 6H), 2.34 (m, 2H), 2.14-2.03 (m, 6H), 1.77-1.48 (m, 8H), 1.54 (d, J = 6.9 Hz, 6H), 1.35 (m, 8H); ESI MS: m/z 1151.8 (M+H)⁺.

(S,5S,5'S,8S,8'S,10aR,10a'R)-3,3'-(2,2'-(1,4-Phenylene)bis(acetyl))bis(N-benzhydryl-5-((S)-2-(methylamino)propanamido)-6-oxodecahydropyrrolo[1,2-a][1,5]diazocine-8-carboxamide) (**21**, SF-28). 1,4-Benzenediacetic acid (10 mg, 0.051 mmol), EDC (25 mg, 0.13 mmol), HOBt (15 mg, 0.11 mmol) and 0.3 mL of N,N-diisopropylethylamine were added to a solution of **23** (58 mg, 0.1 mmol) in 5 mL of CH₂Cl₂. The solution was stirred at room temperature overnight and then concentrated. The residue was purified by chromatography to yield an amide. HCl solution (4N in 1,4-dioxane, 2 mL) was added to a solution of this amide in MeOH (5 mL). The solution was stirred at room temperature overnight and then concentrated to furnish a crude product which was purified by C18 reversed phase semi-preparative HPLC to give 87 mg of **21** as a salt with TFA, yield 65% over two steps. Purity was determined by reverse phase analytical HPLC to be over 95%. ¹H NMR (300 MHz, CD₃OD) 7.35-7.17 (m, 24H), 6.15 (s, 2H), 4.82 (m, 2H), 4.59 (m, 2H), 4.25 (m, 2H), 4.01-3.75 (m, 10H), 3.45 (m, 2H), 3.22 (m, 2H), 2.66 (s, 6H), 2.31 (m, 2H), 2.05-1.78 (m, 10H), 1.52 (d, J = 7.2 Hz, 6H); ESI MS: m/z 1113.7 (M+H)⁺.

(S,5S,5'S,8S,8'S,10aR,10a'R)-3,3'-(3,3'-(1,4-Phenylene)bis(propanoyl))bis(N-benzhydryl-5-((S)-2-(methylamino)propanamido)-6-oxodecahydropyrrolo[1,2-a][1,5]diazocine-8-carboxamide) (**22**, SF-29). 1,4-Phenylenedipropionic acid (11 mg, 0.05 mmol), EDC (25 mg, 0.13 mmol), HOBt (15 mg, 0.11 mmol) and 0.3 mL of N,N-diisopropylethylamine were added to a solution of **23** (58 mg, 0.1 mmol) in CH₂Cl₂ (5 mL). The solution was stirred at room temperature overnight and then concentrated. The residue was purified by chromatography to yield an amide. of HCl solution (4N in 1,4-dioxane, 2 mL) was added to a solution of this amide in MeOH (5 mL). The solution was stirred at room temperature overnight and then concentrated to furnish a crude product which was purified by C18 reversed phase semi-preparative HPLC to give 84 mg of **21** as a salt with TFA, yield 61% over two steps. Purity was determined by reverse phase analytical HPLC to be over 95%. ¹H

NMR (300 MHz, CD₃OD) 7.35-7.07 (m, 24H), 6.15 (s, 2H), 4.73 (m, 2H), 4.51 (m, 2H), 4.22 (m, 2H), 3.95-3.63 (m, 6H), 3.52 (m, 2H), 3.22 (m, 2H), 3.05-2.84 (m, 6H), 2.71-2.64 (m, 8H), 2.27 (m, 2H), 1.95-1.61 (m, 10H), 1.53 (d, $J = 7.2$ Hz, 6H); ESI MS: m/z 1141.7 (M+H)⁺

II. Fluorescence polarization based assays for XIAP, cIAP1 and cIAP2 proteins

Different sensitive and quantitative fluorescence polarization (FP)-based assays were used to determine the binding affinities of the designed Smac mimetics to XIAP (linker-BIR2-BIR3), cIAP1 (BIR3), and cIAP2 (BIR3) proteins. These assays were described in our previous publications.^{35,36}

III. Caspase-9 and Caspase-3 functional assays

Cell-free functional assays were employed to determine the functional antagonism of our designed Smac mimetics. These assays have been described previously in detail.³⁵

IV. Cell growth inhibition assay

The MDA-MB-231 and SK-OV-3 cell lines were purchased from the American Type Culture Collection. Cells were seeded in 96-well flat bottom cell culture plates at a density of $3-4 \times 10^3$ cells/well and grown overnight, then incubated with compounds at different concentrations. The rate of cell growth inhibition after treatment with different concentrations of a compound was determined by assaying with (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfohenyl)-2H-tetrazolium monosodium salt (WST-8; Dojindo Molecular Technologies Inc., Gaithersburg, Maryland) which 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. The concentrations of the compounds that inhibited cell growth by 50% (IC₅₀) were calculated by comparing absorbance in the untreated cells and the treated cells.

V. Cell death analysis

The MDA-MB-231 cells were treated with compounds at different concentrations for 24 h. Cell viability was determined using the trypan blue exclusion assay. Blue cells or morphologically unhealthy cells were scored as dead cells. At least 100 cells from each treatment, performed in triplicate, were counted. Statistical analyses were performed by two-way ANOVA and unpaired two-tailed t test, using Prism (version 4.0, GraphPad, La Jolla, CA). $P < 0.05$ was considered statistically significant.

VI. Western blot analysis

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. After blocking in 5% milk, the membranes were incubated with a specific primary antibody, washed, 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 antibody against cleaved caspase 3 was purchased from Stressgen Biotechnologies; Primary antibodies against cIAP1 and cIAP2 were purchased from R&D systems; Primary antibody against XIAP was purchased

from BD Biosciences; and Primary antibodies against PARP and β -actin from Cell Signaling Technology.

VII. *In vivo* antitumor efficacy and pharmacodynamics studies

For the efficacy experiment, female nude immunodeficient mice were injected subcutaneously with 5×10^6 MDA-MB-231 cells in 50% Matrigel per mouse, one tumor per mouse. Treatment started on day 28 after inoculation of tumor cells when the tumors reached an average volume of 100 mm^3 . Mice were treated with vehicle weekly (9 mice per group), docetaxel (TXT) at 7.5mg/kg weekly intravenously (7 mice per group), compound **12** at 3 or 10 mg/kg weekly intravenously (7 mice per group). Tumor sizes and animal weights were measured 3 times a week during the treatment and twice a week after the treatment. Data are presented as mean tumor volumes $\pm$ SEM. Statistical analyses were performed by two-way ANOVA and unpaired two-tailed t test, using Prism (version 4.0, GraphPad, La Jolla, CA). $P < 0.05$ was considered statistically significant.

For pharmacodynamic experiments, xenograft tumors (one tumor per mouse) were developed by injection of 5×10^6 MDA-MB-231 cancer cells with Matrigel, subcutaneously, on the dorsal side of the SCID mice (from Charles River). When tumors grew to approximately 100 mm^3 in volume, mice bearing tumors were given a single dose of compound **12** at 5 mg/kg or vehicle intravenously. Mice were sacrificed at different time points and tumor tissues were harvested for Western blot analysis to determine levels of cIAP1 and XIAP, as well as for the cleavage of PARP and caspase-3.

The *in vivo* experiments were performed under the guidelines of the University of Michigan Committee for Use and Care of Animals.

VIII. Pharmacokinetic studies in rats

Pharmacokinetic (PK) studies of bivalent Smac mimetics were performed in male Sprague Dawley rats (body weight: 250–270 g) by Medicilon Inc, Shanghai 201203, P. R. China. Before the pharmacokinetic studies, animals were carotid cannulated.

A stock solution of a Smac mimetic was prepared by dissolving the drug in methanol to yield a final concentration of $200 \mu\text{g/mL}$. An aliquot of this solution was diluted using methanol to get a series of working solutions of 25, 5, 2.5, 0.5, 0.25, 0.05, and $0.025 \mu\text{g/mL}$. Seven calibration standard solutions containing 5000, 1000, 500, 100, 50, 10, and 5 ng/mL were obtained by adding $20 \mu\text{L}$ working solution prepared above into seven Eppendorff tubes containing $100 \mu\text{L}$ blank plasma. QC samples were prepared by spiking $100 \mu\text{L}$ blank plasma with $20 \mu\text{L}$ diluted solutions containing 20, 4, $0.04 \mu\text{g/mL}$ of analyte to get the final concentration of 4000, 800, and 8 ng/mL .

Each compound was injected into the tail vein in a group of three male rats with intravenous bolus injection. Blood samples were collected from carotid vein at pre-dose and 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h post-dose. Plasma samples were stored at -20°C until bioanalysis. Plasma samples were processed by protein precipitation. Plasma samples (0.1 mL) were transferred to Eppendorff tube, then $20 \mu\text{L}$ methanol, $50 \mu\text{L}$ IS solution ($1 \mu\text{g/mL}$) and 1 mL acetoacetic ester were added to it. After Vortexing for 5 min and centrifuging for 3 min at 15,000 rpm, 1 mL of supernatant was transferred to another glass tube and evaporated under vacuum condition. The residue was reconstituted with $100 \mu\text{L}$ mobile phase then $5 \mu\text{L}$ for injection.

The LC system comprised an Agilent (Agilent Technologies Inc. USA) liquid chromatograph equipped with an isocratic pump (1100 series), an autosampler (1100 series) and a degasser (1100 series). Mass spectrometric analysis was performed using an API3000

(triple-quadruple) instrument from AB Inc (Canada) with an ESI interface. Data acquisition and control system were created using Analyst 1.4 software from ABI Inc. Concentrations in plasma below the limit of quantitation (LOQ = 5 ng/mL) were designated as zero. Pharmacokinetic data analysis was performed using noncompartmental analysis.

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Abbreviations

IAP	Inhibitor of apoptotic protein
XIAP	X-linked IAP
cIAP	cellular IAP
Smac	second mitochondria-derived activator of caspases
BIR	baculoviral IAP repeat
PARP	poly(ADPribose) polymerase
FP	fluorescence polarization
mP	millipolarization units

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1 (SM-122) **2 (SM-406/AT-406)** **3 (GDC-0152)** **4 (LCL161)**

Figure 1.
Chemical structures of previously reported monovalent Smac mimetics.

Figure 2.
Chemical structures of previously reported bivalent Smac mimetics.

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Figure 4.

Functional antagonism of Smac mimetics against XIAP (linker-BIR2-BIR3 domains) in cell-free, caspase-9 and caspase-3 functional assays. (a). Caspase-9 functional assay. XIAP L-BIR2-BIR3 effectively inhibited caspase-9 activity and Smac mimetics dose-dependently restored the activity of caspase-9. (b). Caspase-3 functional assay. XIAP L-BIR2-BIR3 effectively inhibited caspase-3 activity and Smac mimetics dose-dependently restored the activity of caspase-3.

Figure 5.

Induction of cIAP1 degradation, cleavage of PARP, and processing of caspase-3, caspase-9 and caspase-8 by compounds **8**, **12** and **16** in the MDA-MB-231 cell line. Cells were treated with different concentrations of compounds at the indicated concentrations for 24 h. cIAP1, cleaved PARP (CL PARP), caspase-8 (C8) and cleaved caspase-8 (CL-C8), caspase-9 (C9) and cleaved caspase-9 (CL-C9), caspase-3 (C3) and cleaved caspase-3 (CL-C3) were probed by Western blot analysis.

Figure 6.

Induction of cell death by compounds **8**, **12** and **16** in the MDA-MB-231 breast cancer cell line. MDA-MB-231 cancer cells were treated with different concentrations of compounds **8**, **12** and **16** for 24 h and cell death was analyzed by trypan blue assay. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$).

(A). Tumor growth

(B). Animal body weight

Figure 7.

Antitumor activity of compound **12** in the MDA-MB-231 breast cancer xenograft model in mice. Tumors were grown to an average size of 100 mm³ and compound **12** or docetaxel (TXT) was administered intravenously at the indicated dose and schedule. (A). Tumor growth. (B). Animal body weight.

Figure 8.

Pharmacodynamic studies of compound **12** in the MDA-MB-231 xenograft tumors in mice. SCID mice bearing MDA-MB-231 xenograft tumors were treated with compound **12** at 5 mg/kg intravenously. Mice were sacrificed at indicated time-points and tumor tissue lysates were examined by western blot analysis with specific antibodies against cIAP1, XIAP, caspase-3 (C3), PARP and cleaved PARP. GAPDH was used as the loading control.

Reagents and conditions: (a) i. bis-isocyanate, CH_2Cl_2 or DMF, *N,N*-diisopropylethyl amine; ii. 4 N HCl in 1,4-dioxane, methanol.

Scheme 1. Synthesis of bivalent Smac mimetics 12–20

Reagents and conditions: (a) i. 1,4-benzenediacetic acid or 1,4-phenylenedipropionic acid, EDC, HOBT, CH_2Cl_2 , *N,N*-diisopropylethyl amine; ii. 4 N HCl in 1,4-dioxane, methanol.

Scheme 2. Synthesis of bivalent Smac mimetics 21 and 22

Table 1

Binding affinities to XIAP, cIAP1 and cIAP2 and cell growth inhibition in the MDA-MB-231 and SK-OV-3 cell lines of our designed Smac mimetics. Standard deviation (SD) was obtained from 3–5 independent experiments. Data for compounds **10** and **11** were taken from reference 36.

	Binding Affinities						Cell Growth Inhibition	
	XIAP (Linker-BIR2-BIR3)		cIAP1 (BIR3)		cIAP2 (BIR3)		IC ₅₀ (nM) (MDA-MB-231)	IC ₅₀ (nM) (SK-OV-3)
	IC ₅₀ (nM)	K _i (nM)	IC ₅₀ (nM)	K _i (nM)	IC ₅₀ (nM)	K _i (nM)		
10	11.5±3.2	2±0.6	17.4±1.9	2.9±0.4	52±6	14±2	3.4±0.6	9.6
11	24±13	5±2	12.2±1.7	3.6±0.3	43±5	11±1	50.8±8.5	180
12	4.2±1.9	<1	21.7±2.4	3.7±0.6	23.3±3.7	5.4±1.0	11±2	28±4
13	9.6±1.9	1.7±0.5	41.7±5.8	7.4±1.1	48.9±2.0	12.6±0.5	55±7	176±8
14	4.1±1.3	<1	27.4±3.0	4.7±0.6	31.4±4.0	7.7±1.1	12±4	65±11
15	5.4±0.8	0.7±0.2	28.4±1.6	4.9±0.3	39.5±1.6	9.9±0.4	3±1	35±11
16	1.1±0.1	<1	10.1±0.9	1.5±0.2	11.1±0.6	2.1±0.2	0.9±0.2	11±3
17	9.5±0.8	1.7±0.2	30.6±1.3	5.4±0.3	39.8±0.4	10.0±0.1	25±4	73±15
18	19.7±4.4	4.5±1.3	32.7±4.3	5.8±0.8	43.4±1.7	11.0±0.4	460±90	670±90
19	4.6±0.4	<1	22.9±3.8	3.9±0.7	30.1±1.4	7.4±0.4	18±4	51±9
20	4.1±1.5	<1	48.6±8.8	8.7±1.7	51.7±2.0	13.3±0.6	5±1	18±2
21	7.3±1.6	1.3±0.4	28.5±2.5	5.0±0.4	32.2±1.0	7.9±0.2	380±113	759±179
22	8.5±1.4	1.5±0.3	74.6±2.1	13.7±0.4	94.8±1.4	25.3±.4	222±50	406±11

Table 2

Pharmacokinetics of compounds **8**, **10**, **12** and **16** in rats with intravenously administration. AUC, Area-under-curve; $T_{1/2}$, half elimination time; V_z , Apparent volume of distribution during terminal phase; CL, Apparent total body clearance of the drug from plasma.

Compound	Dosing (i.v.)	AUC (hr* μ g/L)	$T_{1/2}$ (hr)	V_z (L/kg)	CL (L/hr/kg)
8	1 mg/kg	221 $\pm$ 58	0.14 $\pm$ 0.05	1.0 $\pm$ 0.6	4.7 $\pm$ 1.3
10	1 mg/kg	885 $\pm$ 275	0.85 $\pm$ 0.14	1.5 $\pm$ 0.7	1.2 $\pm$ 0.5
12	2.5 mg/kg	3771 $\pm$ 216	4.9 $\pm$ 3.9	0.5 $\pm$ 0.3	0.6 $\pm$ 0.1
16	2.5 mg/kg	457 $\pm$ 19	1.4 $\pm$ 0.1	2.0 $\pm$ 0.2	5.4 $\pm$ 0.2

Table 3

Statistical analysis of the efficacy data shown in Figure 7.

	P value (t test)	P value (t-test)
	Median tumor volume in the control group reached 750mm ³ (Day 44)	Median tumor volume in the TXT treated group reached 750mm ³ (Day 76)
Control vs 12 (3mg/kg)	0.0004	
Control vs 12 (10mg/kg)	0.0001	
Control vs TXT	0.0005	
12 (3mg/kg) vs 12 (10mg/kg)	0.1719	0.1247
12 (3mg/kg) vs TXT	0.0667	0.0107
12 (10mg/kg) vs TXT	0.018	0.003