Supplementary Data

SM-164: A novel, bivalent Smac mimetic induces apoptosis and tumor regression by concurrent removal of the blockade of cIAP-1/2 and XIAP

Jianfeng Lu[1,2], Longchuan Bai[1,2], Haiying Sun[1,2], Zaneta Nikolovska-Coleska[1,2], Donna McEachern[1,2], Su Qiu[1,2], Rebecca S. Miller[1,2], Han Yi[1,2], Sanjeev Shangary,[1,2] Yi Sun[1,5], Jennifer L. Meagher, Jeanne A. Stuckey[6,7], and Shaomeng Wang[1,2,3,4*]

[1]Comprehensive Cancer Center and Departments of [2]Internal Medicine, [3]Pharmacology, [4]Medicinal Chemistry, [5]Radiation Oncology, [6]Biological Chemistry, [7]Life Sciences Institute, University of Michigan, 1500 E. Medical Center Drive, Ann Arbor, MI 48109, USA

[*]**Corresponding author:**
Tel. 734-615-0362;
Fax 734-647-9647;
email: shaomeng@umich.edu

Supplementary Methods

Cell Lines

MDA-MB-435 melanoma cell line, DU-145 prostate cancer cell line, and WI-38 primary normal human lung fibroblasts, CCD-18Co primary normal human colon fibroblasts were purchased from the American Type Culture Collection (ATCC). Primary normal human prostate epithelial cells (PrEC) were purchased from Cambrex; human lung microvascular endothelial cells (HMVEC) and human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Walkersville, MD).

Protein expression and purification

Different constructs of human XIAP proteins, including linker-BIR2-BIR3 domain (residues 120-356), and BIR3-only (residues 241-356), were cloned into pET28 vector (Novagen) with an N-terminal 6xHis tag. Proteins were produced in *E. coli* BL21(DE3) cells, grown as previously described (1). cIAP1 BIR2-BIR3 (residues 177-363), cIAP-1 BIR3-only (residues 253-363) and cIAP2 BIR3-only (residues 238-349) were cloned into pHis-TEV vector, produced and purified using the same method as for XIAP proteins.

Fluorescence-polarization-based binding assays for XIAP, cIAP-1 and cIAP-2 proteins

A set of sensitive and quantitative fluorescence polarization (FP)-based assays were developed to determine the binding affinities of our designed Smac mimetics to XIAP BIR3, XIAP containing both BIR2 and BIR3 domains, cIAP-1 BIR3, cIAP-1 containing both BIR2 and BIR3 domains, and cIAP-2 protein.

The FP-based assay for XIAP BIR3 protein was described in detail previously (2). Briefly, 5-carboxyfluorescein was coupled to the lysine side chain of a mutated Smac peptide with the sequence (AbuRPFK-Fam) and this fluorescently tagged peptide (named SM5F) was used as the fluorescent tracer in FP-based binding assay to XIAP BIR3. The K_d value of this fluorescent tracer was determined to be 17.9 nM to XIAP BIR3.

In competitive binding experiments, a tested compound was incubated with 30 nM of XIAP BIR3 protein and 5 nM of SM5F in the assay buffer (100 mM potassium phosphate, pH 7.5; 100 μg/ml bovine gamma globulin; 0.02 % sodium azide, Invitrogen).

The K_d value of SM5F to cIAP-1 BIR3 protein was determined to be 4.1 nM (Supplementary Fig. 2). In competitive binding experiments, 10 nM of cIAP-1 BIR3 protein and 2 nM of SM5F tracer were used. The K_d value of SM5F to cIAP-2 BIR3 protein was determined to be 6.6 nM (SI Fig. S8). In competitive binding experiments, 25 nM of cIAP-2 BIR3 protein and 2 nM of SM5F tracer were used.

To determine the binding affinities of Smac mimetics to XIAP containing both BIR2 and BIR3 domains, an FP-based competitive binding assay was established using a bivalent fluorescently tagged tracer, named Smac-1F (3). The K_d value of the bivalent tagged tracer to XIAP containing BIR2 and BIR3 domains was determined to be 2.3 nM (Supplementary Fig. 1). In competitive binding experiments, a tested compound was incubated with 3 nM of XIAP protein containing both BIR2 and BIR3 domain (residues 120-356) and 1 nM of in the same assay buffer.

The K_d value of Smac-1F to cIAP-1 containing both BIR2 and BIR3 domains (residues 177-363) was determined to be 0.7 nM (Supplementary Fig. 1). In competitive binding

experiments, a tested compound was incubated with 3 nM of cIAP-1 protein containing both BIR2 and BIR3 domains and 1 nM of Smac-1F in the same assay buffer.

For each competitive binding experiment, polarization values were measured after 2-3 hours incubation using an Ultra plate reader. The IC_{50} value, the inhibitor concentration at which 50 % of bound tracer was displaced, was determined from the plot using nonlinear least-squares analysis. For each assay, Smac AVPI peptide was used as the control. Curve fitting was performed using the PRISM software (GraphPad Software, Inc., San Diego, CA). K_i value for each compound was calculated based upon the IC_{50} value using a previously reported algorithm and its associated computer program (2).

Cell-free XIAP functional assay

Dose-dependent inhibition of caspase-3/-7 activity and functional antagonism against XIAP were performed as described previously (1). XIAP containing linker-BIR2-BIR3 (residues 120-356) was used.

TUNEL Staining in Xenograft Tumor and Normal Mouse Tissues

Apoptosis was examined by TUNEL staining using the *In situ* Cell Death Detection Kit (Roche Applied Science). To determine apoptosis in xenograft tumor and normal mouse tissues, severe combined immunodeficient (SCID) mice bearing established tumor were treated as indicated or as described in figure legends. Tissues were harvested and TUNEL staining was performed on formaldehyde-fixed, paraffin-embedded tissues. Paraffin-embedded tissue sections, mounted on slides, were deparaffinized and rehydrated by washing in a descending series of alcohol solutions (100%, 95%, 70% and 50%) and PBS. Antigen retrieval was performed by the

microwave oven method using a commercially available antigen retrieval buffer (Covance Research Products, Inc.). Tissue sections were blocked with 3% BSA and 20% normal bovine serum in PBS for 30 min at room temperature (RT) to block unspecific binding sites. Tissue sections were incubated in 100 µl of TUNEL mixture [TUNEL mixture = 10 µl of Enzyme solution (Vial 1) + 90 µl of Label solution (Vial 2)] for 60 min at RT. Tissue section incubated in 90 µl of Label solution (Vial 2), instead of TUNEL mixture was used as negative control. Tissue section preincubated in DNase I recombination in 3000 U/ml of PBS, 1 mg/ml BSA for 10 min RT, followed by incubation with TUNEL mixture was used as positive control. Tissues slides were rinsed with PBS 5 min ×3, and stained with Propidium iodide and mounted and observed under a fluorescent microscope. All animal experiments were performed under the guidelines of the University of Michigan Committee for Use and Care of Animals.

Histopathological Analyses of Xenograft Tumor and Normal Mouse Tissues

To determine the effect of SM-164 on xenograft tumor tissue and normal tissues, SCID mice (two per group), treated with a single *iv* dose of SM-164 at 5 mg/kg for 24 h, was euthanized. Tumor tissues and five different organ tissues were excised, fixed in formaldehyde, paraffin embedded and tissue sections were stained with hematoxylin and eosin (H&E). To examine tissue damage, H&E stained tissue samples from mice receiving vehicle control (VEH) were compared with tissues from mice receiving SM-164. Histopathology analyses were performed by an experienced pathologist.

References

1. 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.; 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.
2. Nikolovska-Coleska, Z., Wang, R., Fang, X., Pan, H., Tomita, Y., Li, P., Roller, P. R., Krajewski, K., Saito, N. A., Stuckey, J., Wang, S. (2004) Development and Optimization of a Binding Assay for the XIAP BIR3 Domain Using Fluorescence Polarization. *Anal Biochem. 332*, 261-73.
3. Nikolovska-Coleska, Z., Meagher, J.L., Jiang, S., Kawamoto, S.A., Gao, W., Yi, H., Qin, D., Roller, P.R., Stuckey, J., Wang, S. (2008) Design and characterization of bivalent Smac-based peptides as antagonists of XIAP and development and validation of a fluorescence polarization assay for XIAP containing both BIR2 and BIR3 domains. *Anal Biochem. 374*, 87-98.

Supplementary Figure Legends

Supplementary Figure S1. Saturation curves of a bivalent fluorescently tagged tracer (Smac-1F) to XIAP (A) and cIAP-1 (B) proteins containing both BIR2 and BIR3 domains.

Supplementary Figure S2. Saturation curves of a monovalent fluorescently tagged tracer (SM5F) to cIAP-1 (A) and cIAP-2 (B) containing only BIR3 domain.

Supplementary Figure S3. Competitive binding curves of Smac mimetics to cIAP-1 BIR3 protein as determined using a fluorescence-polarization based assay.

Supplementary Figure S4. SM-164 induces processing of caspase-8, -9 and -3 and cleavage of PARP in MDA-MB-231 breast cancer line in a time-dependent manner. MDA-MB-231 cell line was treated with 100 nM of SM-164 as indicated time point and whole cell lysates were analyzed for processing of casapases and cleavage of PARP by Western blotting.

Supplementary Figure S5. Monovalent and bivalent Smac mimetics induce apoptosis in the SK-OV-3 cancer cell line in a caspases-3 and -8 dependent manner. SK-OV-3 cells were treated as indicated. (A) Apoptosis was analyzed by PI/Annexin V double staining using flow cytometry; (B) Cell viability was determined by trypan blue dye exclusion assay; and (C) Cleavage of caspases and PARP was analyzed by Western blotting; (D) Cells was transfected with siRNA against caspases-3, -8, -9 for 48 h, followed by treatment with SM-164 (middle panel) or SM-122 (right panel) for 48 h. Knock-down efficacy was examined by Western blotting and cell

growth inhibitory activity by a WST assay. (E) Cells were treated with Z-DEVD-FMK (25 μM), or Z-IETD-FMK (25 μM) for 1 h, followed by treatment with a Smac mimetic for 48 h. Cell viability was determined using a trypan blue dye exclusion assay. Data were representative of mean ± standard deviation of triplicates.

Supplementary Figure S6. Monovalent and bivalent Smac mimetics induce apoptosis in the MALME-3M cell line. MALME-3M cells were treated as indicated. (A) Apoptosis was analyzed by PI/Annexin V double staining using flow cytometry; (B) Cell viability was determined by trypan blue dye exclusion assay; and (C) Cleavage of caspases and PARP was analyzed by Western blotting.

Supplementary Figure S7. Smac mimetics induce TNFα-dependent apoptosis in cancer cell lines. (*A*) Cancer cell lines were cultured in 12-well plates, the conditioned cell culture media were collected at the indicated time points for the determination of TNFα levels using the Quantikine HS Human TNFα ELISA kit (R&D Systems). (*B*) Cancer cell lines were treated with SM-122 (3 μM) or SM-164 (1 nM) for 24 h, and the conditioned cell culture media were collected for determination of TNFα. No detectable levels of TNFα were detected in resistant cancer cell lines (DU145, MDA-MB-435 and MDA-MB-453) before and after treatment with either SM-122 or SM-164. (*C*) MALME-3M and SK-OV-3 cell lines were treated with or without TNFα or TRAIL blocking antibody for 1 h, followed with treatment of 100 nM of SM-164 or 3 μM of SM-122 for 24 h. Cell viability was determined using a trypan blue exclusion assay. (*D*) MDA-MB-231 cancer cells were treated with TRAIL alone, or in combination with TRAIL blocking antibody for 48 h and cell viability was determined by trypan blue exclusion

assay. (*E*) HCT116 colon cancer and MDA-MB-453 breast cancer cell lines were treated with a Smac mimetic alone, TNFα alone, or the combination for 48 h. Cell growth inhibition was determined using a WST-8 assay.

Supplementary Figure S8. Smac mimetics induce cIAP-1 degradation in sensitive cancer cell lines. (*A*) SK-OV-3 and MALME-3M cell lines were treated with Smac mimetics as indicated for 60 min. The levels of XIAP and cIAP-1 were examined by Western blotting. (*B*) SK-OV-3 cell line was treated with 100 nM of SM-164 at indicated time. The levels of XIAP and cIAP-1 were examined by Western blotting. (*C*) SK-OV-3 cell line was treated with 20 μM of MG-132 for 30 min, followed by treatment with Smac mimetics for 10 min. The levels of cIAP-1 were examined by Western blotting.

Supplementary Figure S9. Smac mimetics induce cIAP-1 degradation in resistant cancer cell lines. (*A*) Isogenic HCT116 XIAP wild type ($XIAP^{+/+}$) and XIAP knockout ($XIAP^{-/-}$) cancer cell lines were treated with Smac mimetics for 48 h and cell growth inhibition was examined with WST assay (top panel); HCT116 cancer cell lines were treated with Smac mimetics for 60 min, the levels of cIAP-1 and XIAP were examined by Western blotting (bottom panel). (*B*) Jurkat cell lines, stably transfected with vector control (VEC-JK) or XIAP plasmid (XIAP-JK) were treated with Smac mimetics for 48 h and cell growth inhibition was determined using a WST assay (top panel); VEC-JK and XIAP-JK cell lines were treated with Smac mimetics for 60 min, the levels of cIAP-1 were examined by Western blotting (bottom panel).

Supplementary Figure S10. Removal of cIAP-1 or cIAP-2 alone is not sufficient to induce robust TNFa-dependent apoptosis in SK-OV-3 cancer cell line. SK-OV-3 cell line was transfected with cIAP-1, cIAP-2, or control siRNA for 48 h. The levels of cIAP-1/2 was examined by Western blotting. The transfected cells were treated with TNFα for 24 h and cell viability was determined with trypan blue dye exclusion.

Supplementary Figure S11. XIAP knock-down sensitizes cancer cells to TNFα-dependent apoptosis induction by Smac mimetics. (*A*) MDA-MB-231, MALME-3M and SK-OV-3 cell lines were transfected with XIAP siRNA for 48 h, followed by treatment with 1 μM of SM-122, 10 nM of SM-164 or Mock control for 12 h. TNFα production was examined using the Quantikine HS Human TNFα ELISA kit. (*B*) MALME-3M and (*C*) SK-OV-3 cell lines were transfected with XIAP siRNA for 48 h, followed by treatment with SM-122 or SM-164 in combination with TNFα for 48 h. Cell viability was determined with trypan blue dye exclusion.

Supplementary Figure 12. SM-164 shows no or little toxicity to normal mice tissues. (*A-B*) SCID mice (two mice per group), bearing established MDA-MB-231 xenograft tumors, were treated with a single *i.v.* dose of SM-164 at 5 mg/kg, or VEH for 24 h. (*A*) Caspase processing and PARP cleavage in xenograft tumor tissue and mouse normal tissues were analyzed by Western blotting. (*B*) Mouse tissues treated with VEH or SM-164 from stomach and liver were examined by H&E staining.

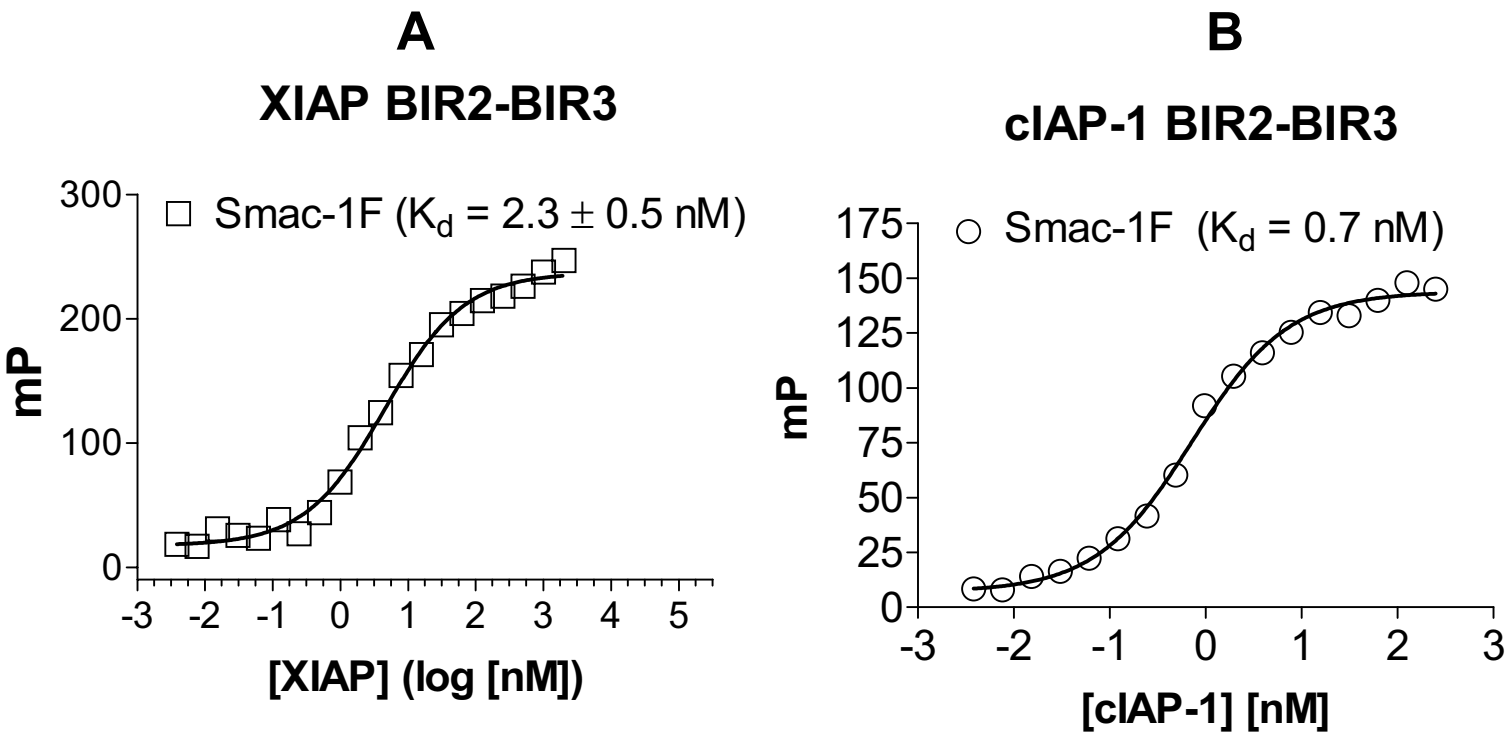
Supplementary Figure 13. SM-164 has minimal effect on mouse body weight. SCID mice (8-10 per group), bearing established MDA-MB-231 xenograft tumors, were treated with SM-164 at

5 mg/kg daily, 5 days a week for 2 weeks or VEH as indicated. Mouse body weight was measured three times a week. Data are represented as mean tumor volumes ± SEM.

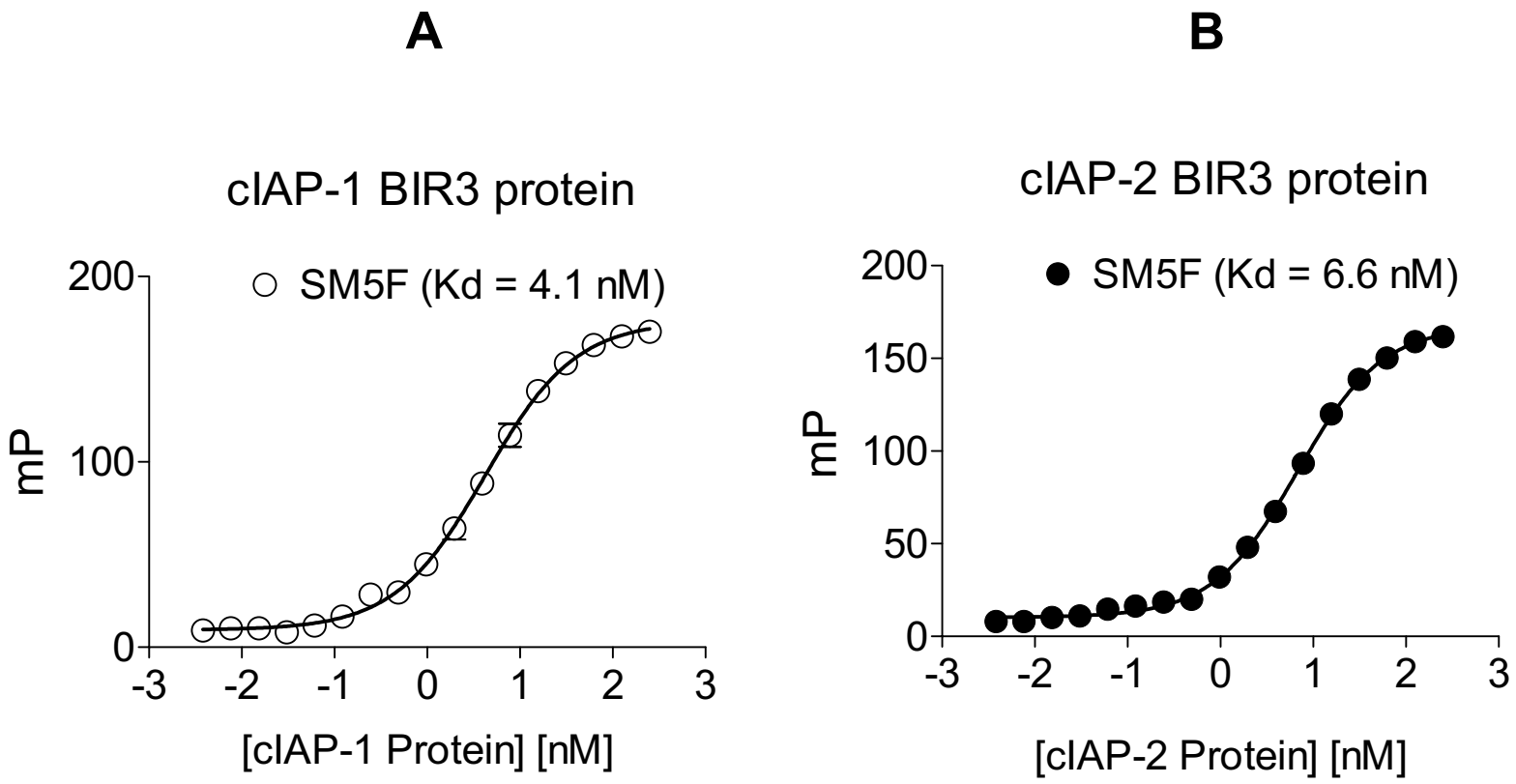
Supplementary Figure 14. Removal of cIAP-1/2 alone is not sufficient to induce robust TNFa-dependent apoptosis in resistant cancer cell lines. (*A*) MDA-MB-453 breast cancer cell line was treated with 100 nM of SM-122 alone, 100 ng/ml of TNFα alone, or the combination for 24 h. The levels of cIAP-1/2 were examined by Western blotting using a pan antibody against cIAP-1/2 (left panel, Wang L, Du F, Wang X. (2008) TNF-alpha induces two distinct caspase-8 activation pathways. Cell 133:693-703.) and cell viability was determined with trypan blue dye exclusion (right panel). (*B*) HCT116 colon cancer cell line was treated with 100 nM of SM-122, 100 ng/ml of TNFα, or the combination for 24 h. The levels of cIAP-1/2 were examined by Western blotting using a pan antibody against cIAP-1/2 (left panel) and cell viability was determined with trypan blue dye exclusion (right panel).

Supplementary Figure 15. SM-164, either alone or in combination with TNFα, shows little toxicity to normal human cells. (*A-B*). Normal cells, including WI-38 primary normal human lung fibroblasts, CCD-18Co primary normal human colon fibroblasts, PrEC primary normal human prostate epithelial cells, HMVEC human lung microvascular endothelial cells and HUVEC human umbilical vein endothelial cells were treated with 100 nM of SM-164 for 10 min and cIAP-1 degradation was examined by Western blotting. The untreated HC116, MDA-MB-231, MALME-3M and MDA-MB-436 cells were used as controls. (*C*) Normal cells were treated with 100 nM of SM-164 alone, TNFα alone, or the combination for 24 h. Cell viability was determined with trypan blue dye exclusion.

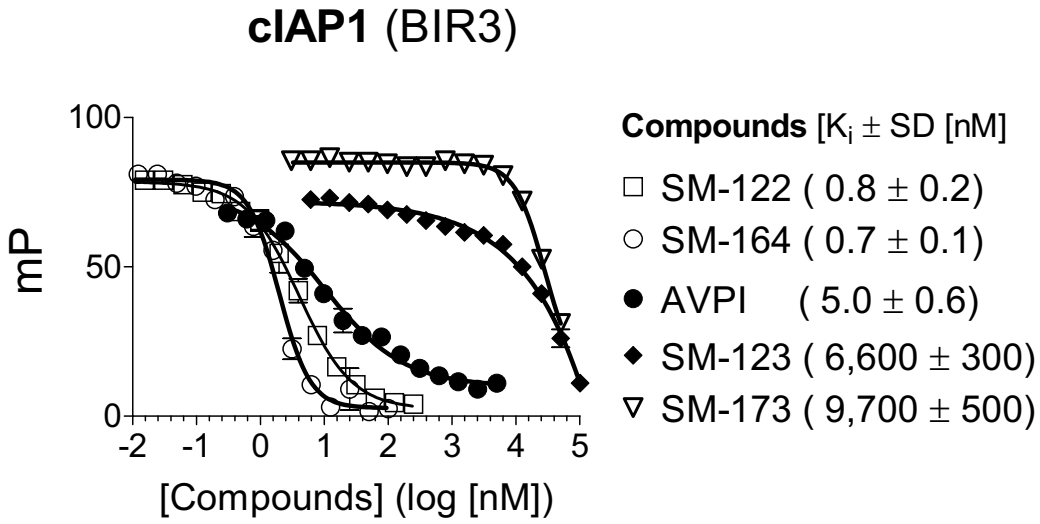
SI Fig. S1. Saturation curves of a bivalent fluorescently tagged tracer (Smac-1F) to XIAP and cIAP-1 proteins containing both BIR2 and BIR3 domains.



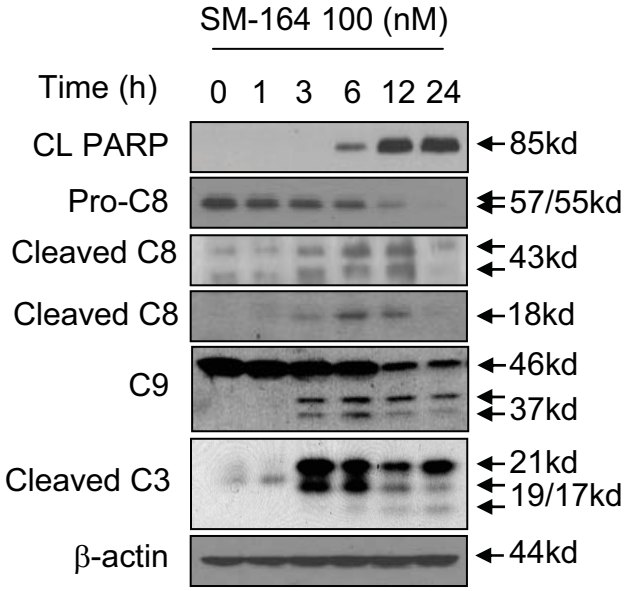
SI Fig. S2. Saturation curves of a monovalent fluorescently tagged tracer (SM5F) to cIAP-1 and cIAP-2 containing only BIR3 domain.



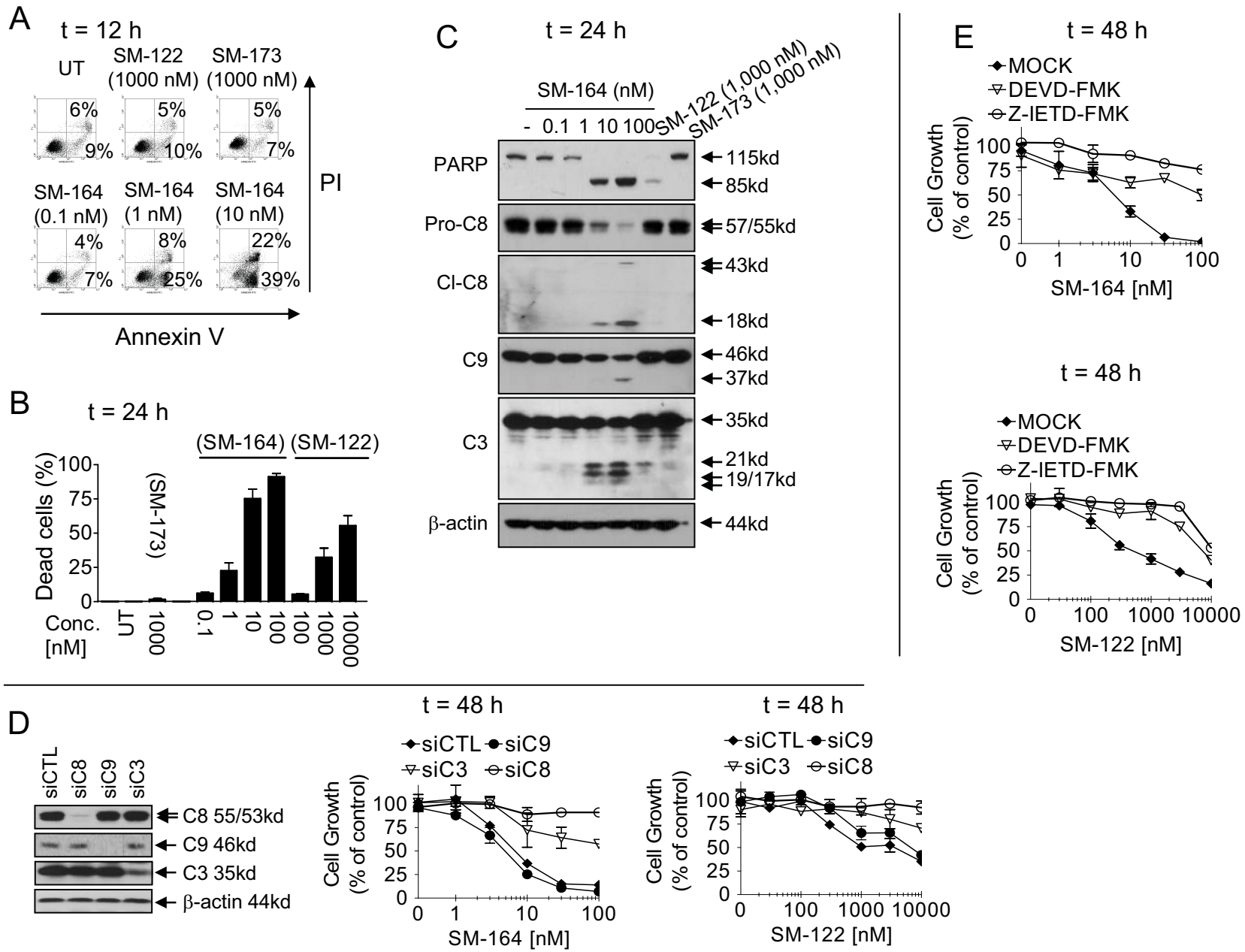
SI Fig. S3. Competitive binding curves of Smac mimetics to cIAP-1 BIR3 and cIAP-2 BIR3 proteins as determined using fluorescence-polarization based assays.



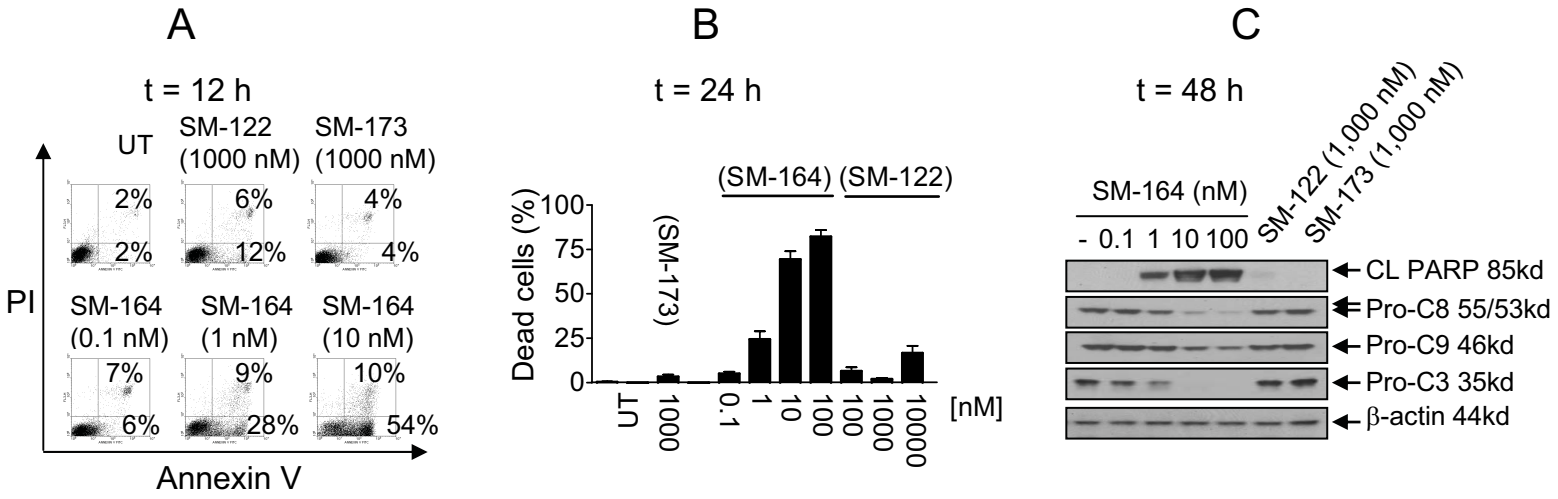
SI Fig. S4. SM-164 induces processing of caspase-8, -9, -3 and cleavage of PARP in a time-dependent manner in the MDA-MB-231 breast cancer line.



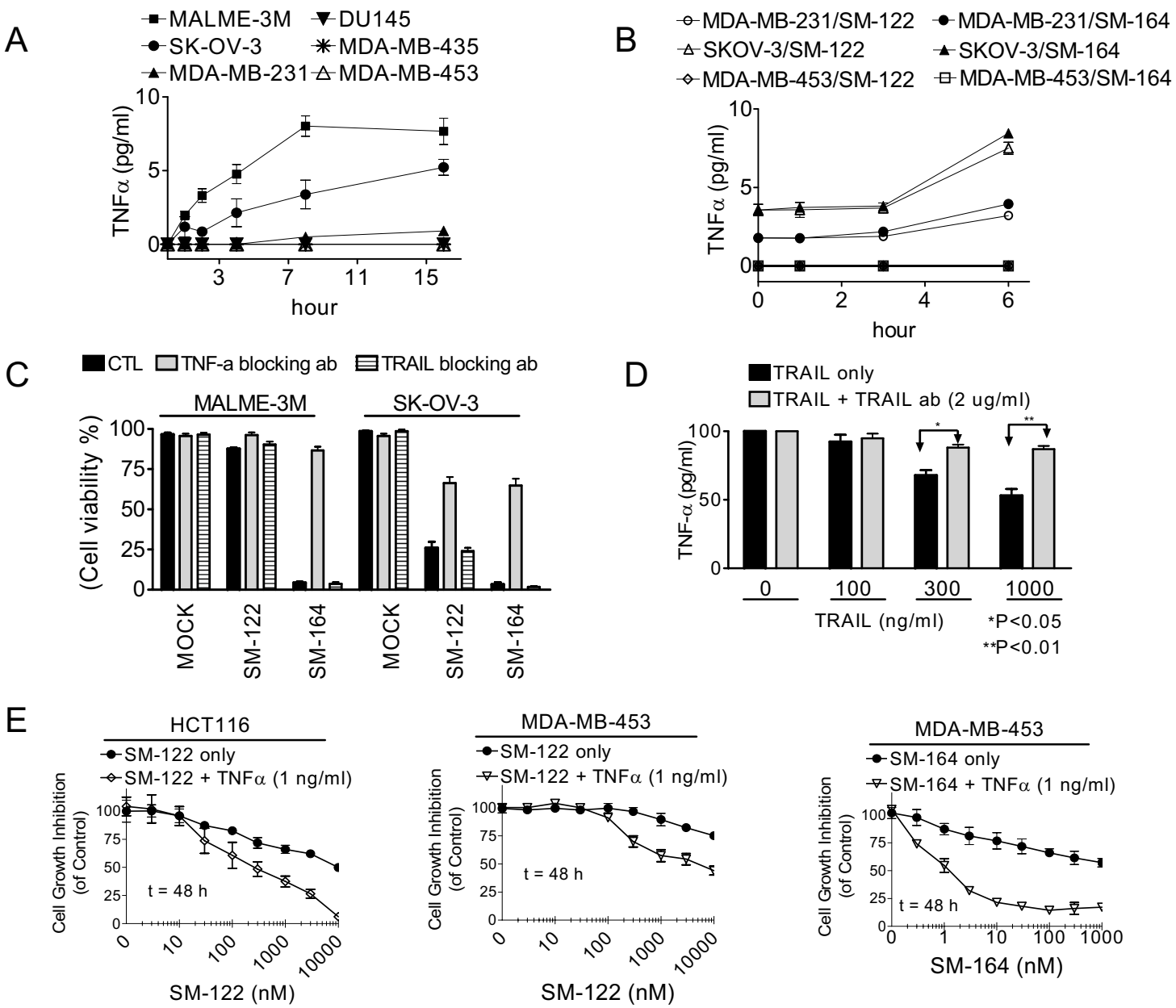
SI Fig. S5. Smac mimetics induce apoptosis in SK-OV-3 human ovarian cancer cell line in a caspases-3 and -8 dependent manner



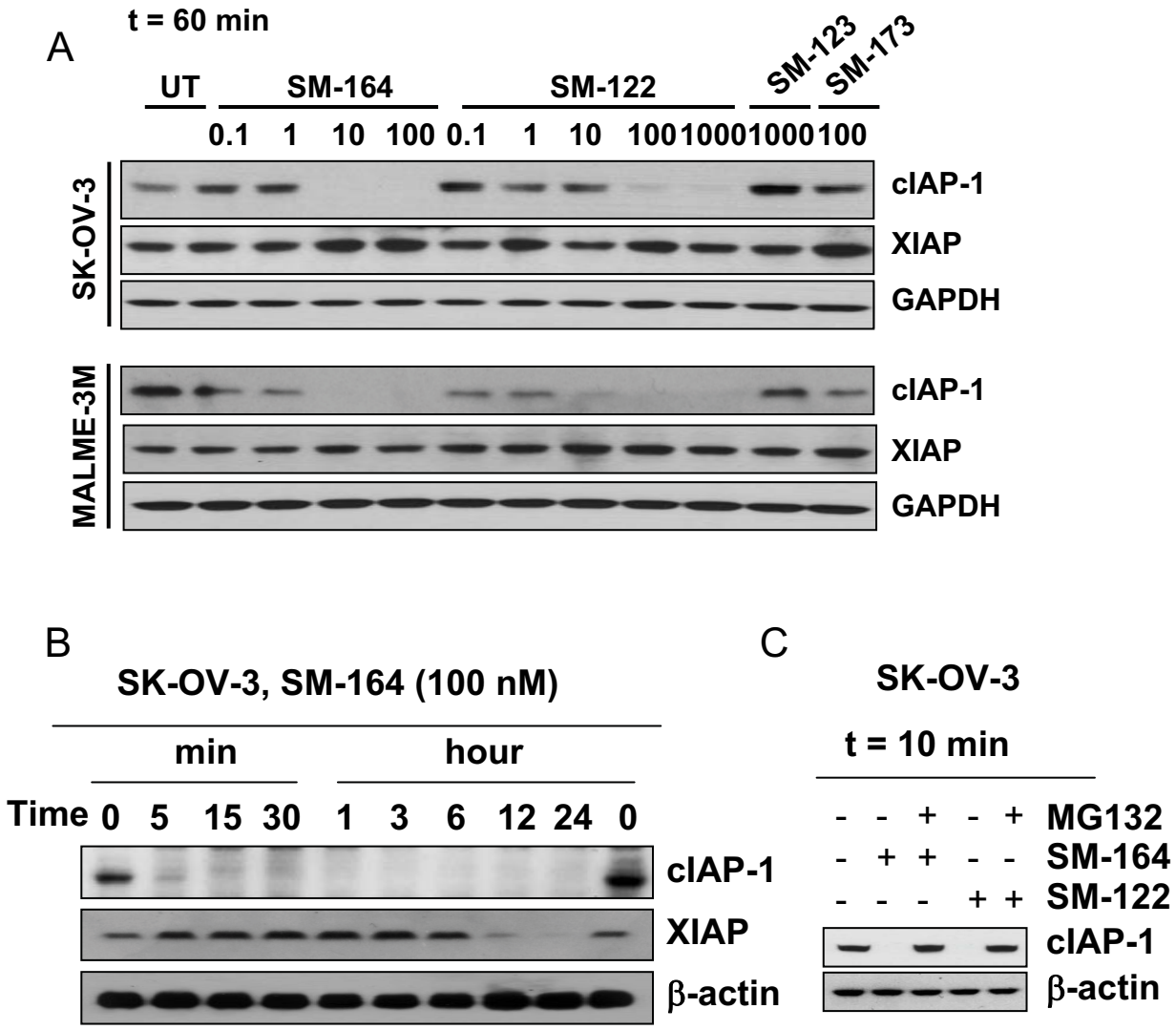
SI Fig. S6. Smac mimetics induce apoptosis in MALME-3M melanoma cell line



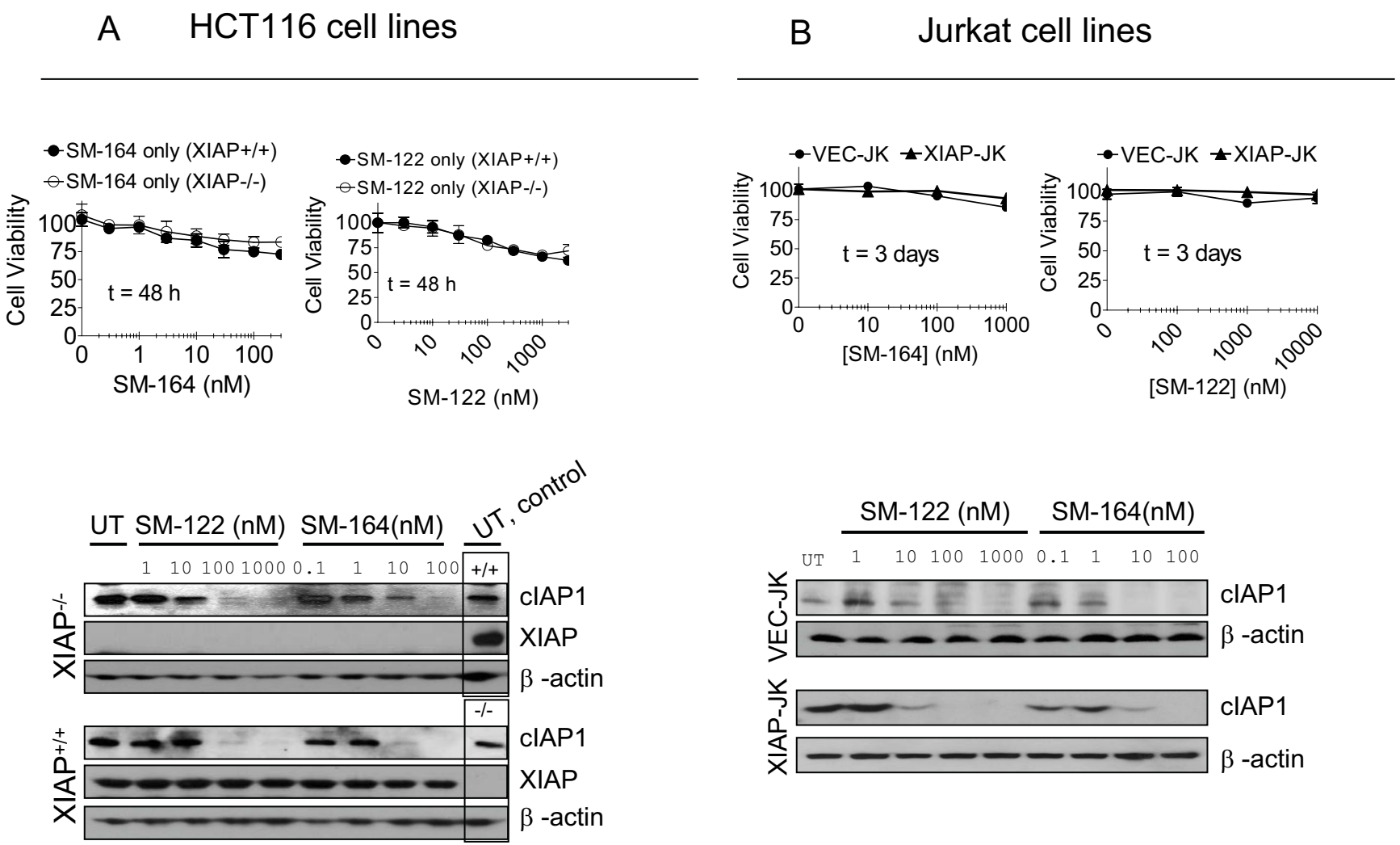
SI Fig. S7. Smac mimetics induce TNFα-dependent apoptosis in cancer cell lines.



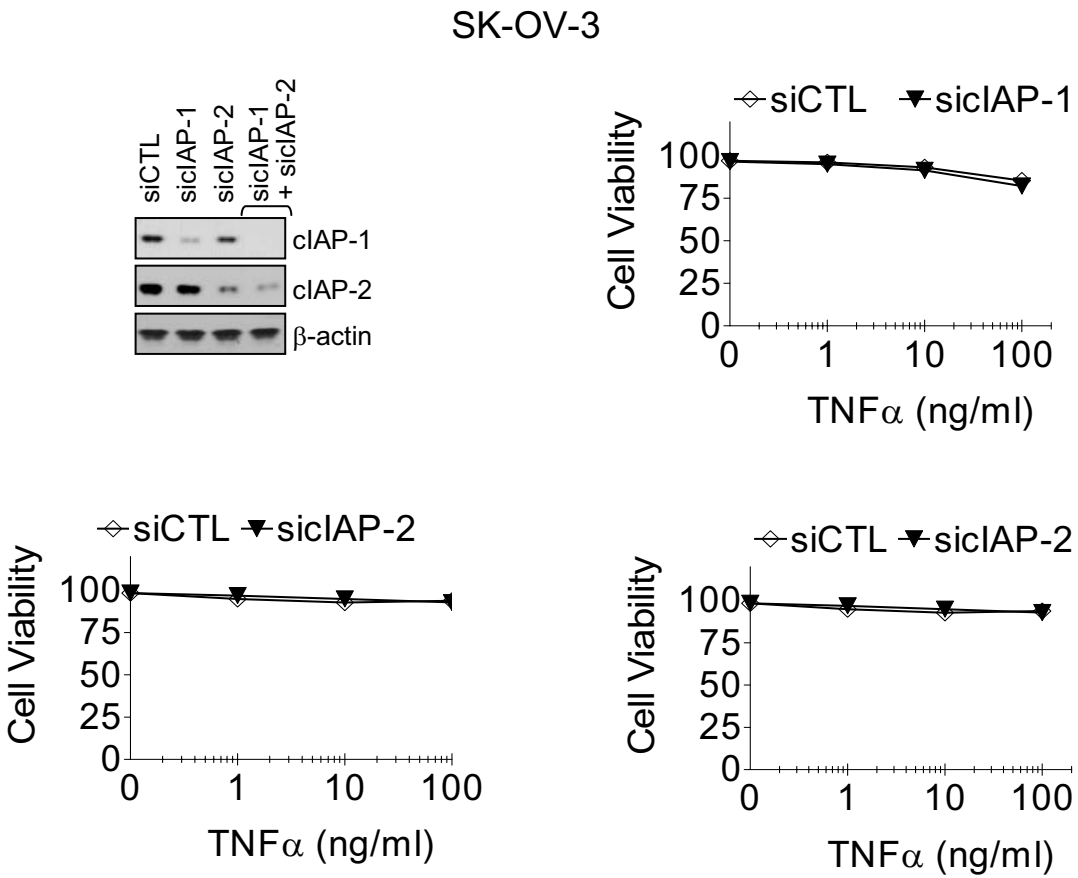
SI Fig. S8. Smac mimetics induce cIAP-1 degradation in sensitive cancer cell lines



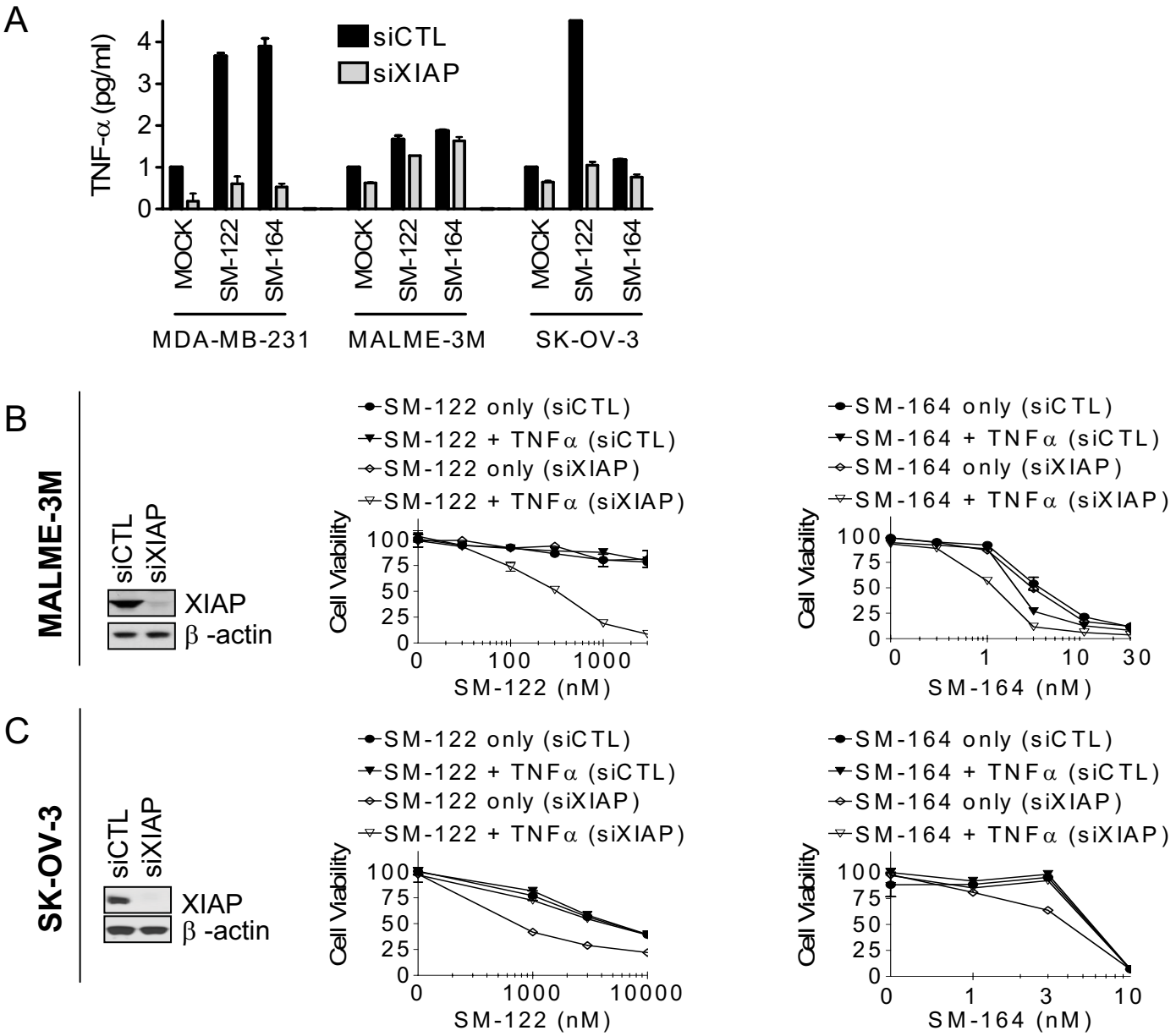
SI Fig. S9. Smac mimetics induce cIAP-1 degradation in resistant cancer cell lines.



SI Fig. S10. Removal of cIAP-1 or cIAP-2 alone is not sufficient to induce robust TNFα-dependent apoptosis in SK-OV-3 cancer cell line.

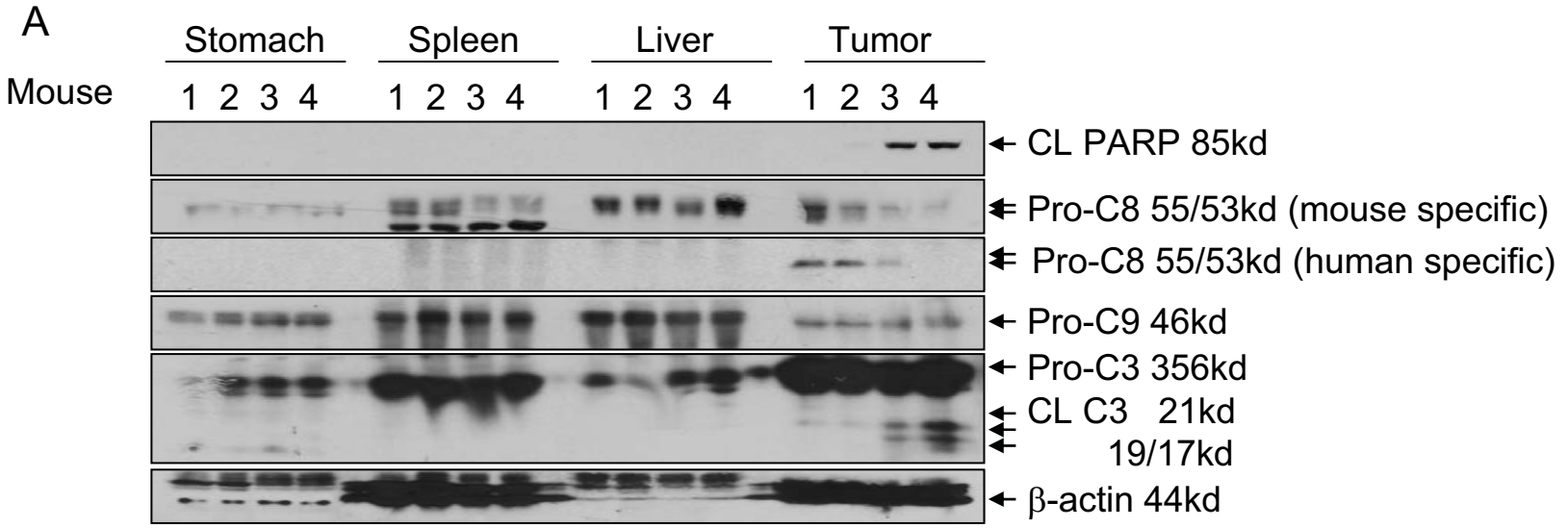


SI Fig. S11. XIAP knock-down sensitizes cancer cells to TNFα-dependent apoptosis induction by Smac mimetics.



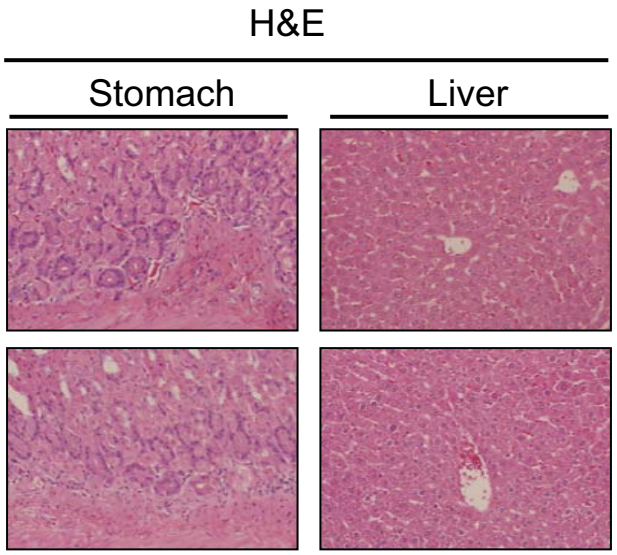
SI Fig. S12. SM-164 shows no or little toxicity to normal mice tissues.

A

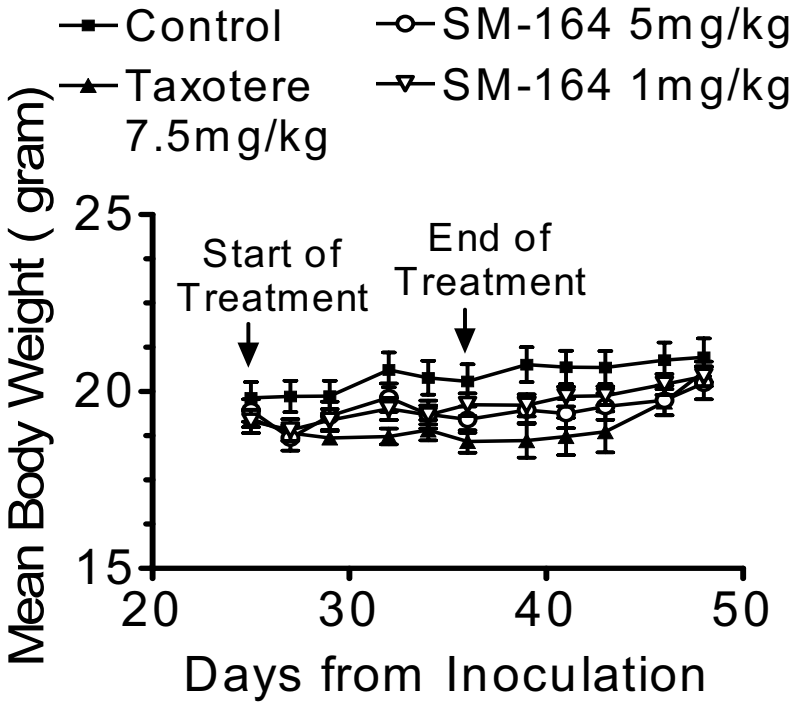


Mouse #1 and #2 in each group are treated vehicle control,
mouse #3 and #4 are treated with SM-164 for 24 hours

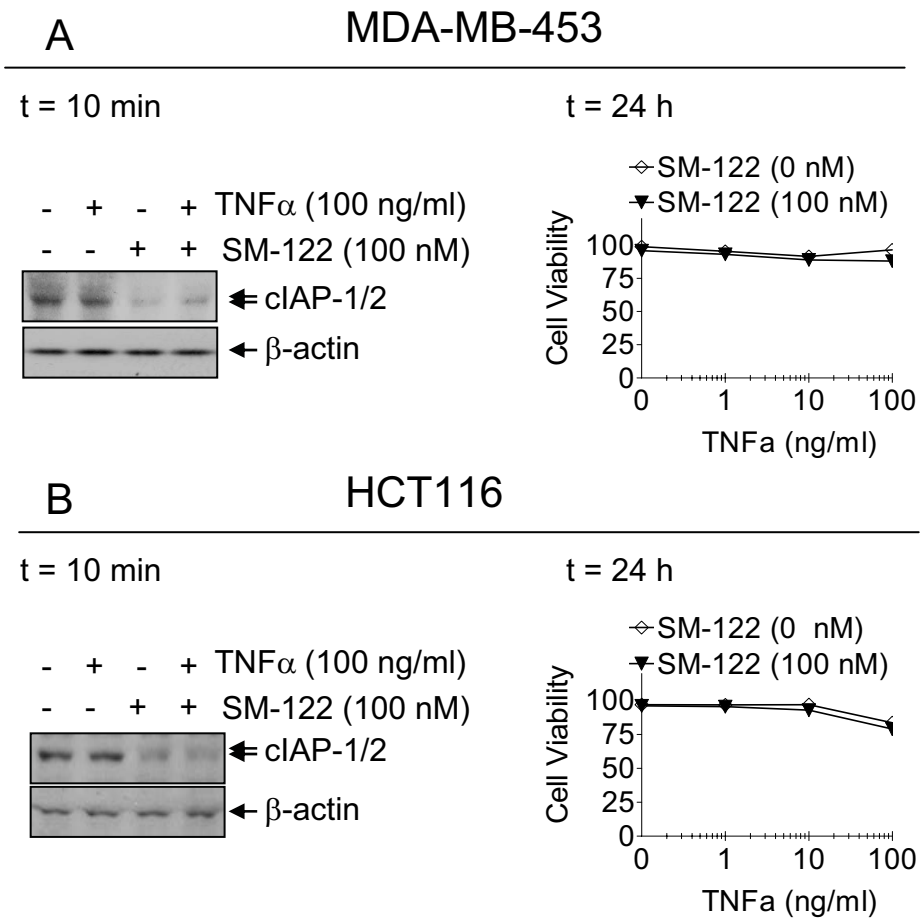
B



SI Fig. S13. SM-164 has minimal effect on mice body weight.



SI Fig. S14. Removal of cIAP-1/2 alone is not sufficient to induce robust TNFα-dependent apoptosis in resistant cancer cell lines.



SI Fig. S15. SM-164, either alone or in combination with TNFα, shows little toxicity to normal human cells.