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TITLE: Molecular Modulation of Inhibitors of Apoptosis as a Novel Approach for Radiosensitization of Human Prostate Cancer

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14. ABSTRACT The major goal of the project is to investigate the radiosensitization activity and mechanism of action of novel IAP-inhibitors in prostate cancer. We have investigated the in vitro radiosensitization activity of our lead IAP-inhibitors, SH-130 and Embelin, in human prostate cancer cell lines. IAP-inhibitors potently enhanced TRAIL-/radiation-induced apoptosis and growth inhibition. Using NMR and Crystal Structure Analysis, we conclusively show that these IAP inhibitors bind to the pocket in the XIAP BIR3 domain where Smac binds. Biotin-labeled SH-130 pull-down assay further confirm that BIR3 domain in XIAP and cIAP-1 is indeed the molecular target of the IAP inhibitors in apoptosis-potential. Based on our exciting data obtained from this PCR project, together with data from other collaborators, we are working with FDA for IND filing aiming for Phase I clinical trial with SH-130 as radiosensitizer for prostate cancer.						
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Table of Contents

Cover	1
SF 298	2
Introduction	4
Body	4
Key Research Accomplishments	4
Reportable Outcomes	11
Conclusions	11
References	11
Appendices	None

I. Introduction:

In this project, we will investigate in vitro and in vivo radiosensitization activity and the mechanism of action of IAP-inhibitors in human prostate cancer with IAPs overexpression. Our basic hypothesis to be tested is that (1) The IAPs play a critical role in radiation resistance of human prostate cancer overexpressing IAPs; (2) Inhibition of the anti-apoptotic function of IAPs by small molecule IAP inhibitors will overcome radioresistance rendered by the overexpressed IAPs, that in turn will enhance tumor response and restore sensitivity of prostate cancer cells to ionizing irradiation. Our goal is to investigate and validate that IAPs are promising novel targets for radiosensitization of human prostate cancer with IAP-overexpression, with the ultimate goal to establish the molecular modulation of IAPs by potent small molecule IAP inhibitors as a novel approach for overcoming radiation resistance of human prostate cancer with high levels of IAPs.

II. Research progress and key research accomplishments:

This is the first year of the project. We have finished the tasks proposed in the Statement of Work for the corresponding period of time. Specifically, we have finished the following tasks:

A. Task 1: To Investigate the role of IAP family proteins in radiation resistance of prostate cancer cells

A.1. Evaluation of radiation response of human prostate cancer cell lines and their relationship with the levels of IAP proteins.

Rapidly growing evidence has now indicated that IAP family of proteins, especially XIAP, are a highly attractive molecular target for overcoming apoptosis-resistance and improving treatment efficacy of prostate cancer. We have examined IAPs expression in human prostate cancer cell lines and found that the widely studied prostate cancer cell lines PC-3, LnCaP, CL-1 and DU-145 have high levels of IAPs expression (**Figure 1**), consistent with previous reports. XIAP blocks apoptosis induced by taxol in LnCaP prostate cancer cells through the decrease of caspase-3 activity and inhibition of the processing of pro-caspase-3. XIAP potently inhibits apoptosis induced by TRAIL in prostate cancer CL-1 cells (an androgen-independent clone from LnCaP).

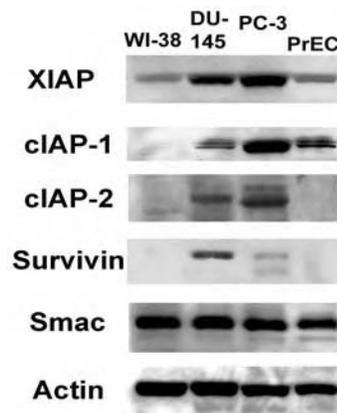


Figure 1. Western blot analysis of the expression of IAP in human prostate cancer cells and normal cells.

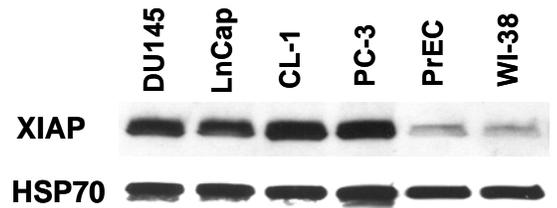
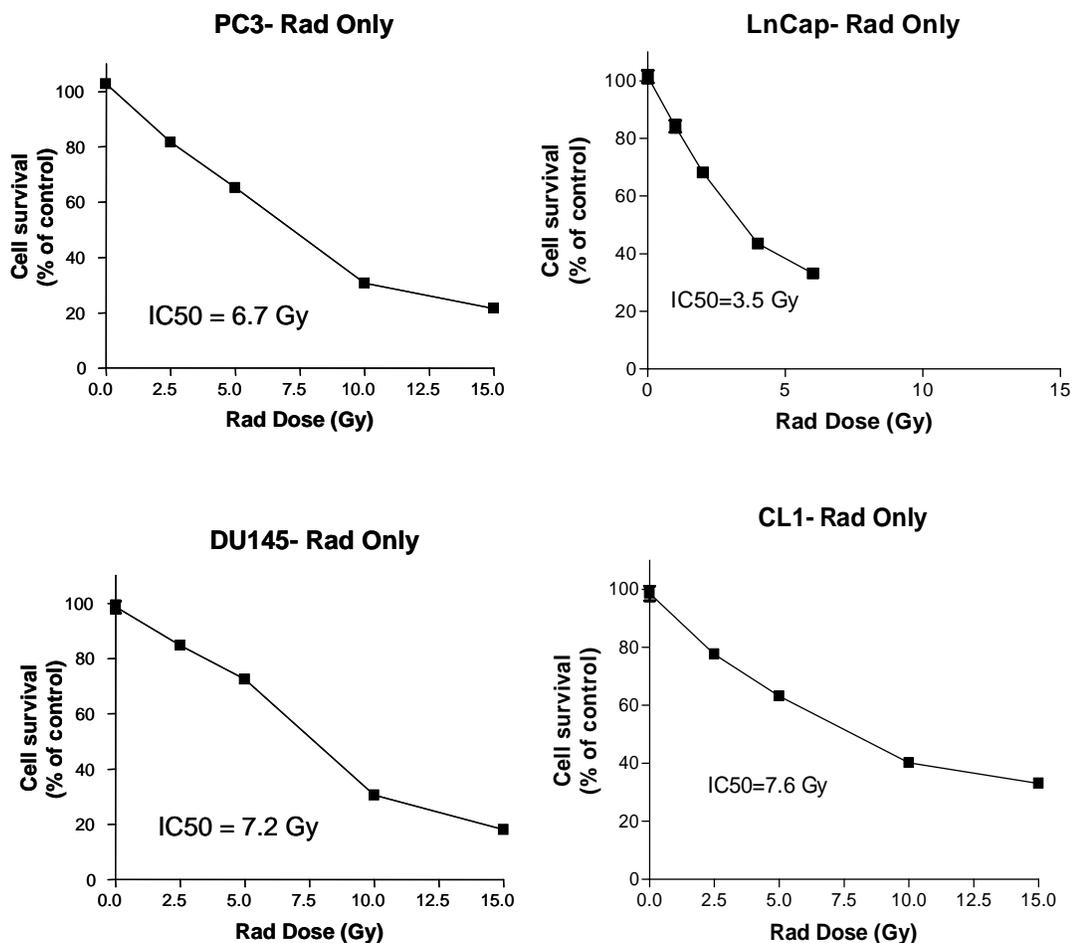


Figure 2. Western blotting analysis of the expression of XIAP in human prostate cancer cells and normal cells. PrEC: normal human prostate epithelial cells. WI-38: normal human fibroblast cell line. HSP70: heat shock protein 70kDa

CL-1 is an androgen-independent clone selected from LnCap cells cultured long term in androgen-depleted condition. CL-1 becomes highly aggressive and metastatic, resistant to a variety of chemotherapeutic agents as compared with parental LnCap cells. **Figure 2** shows CL-1 has 3-4-fold more XIAP expression than LnCap.

Figure 3 shows the radiation response of prostate cancer cell lines in a quick MTT-based cytotoxicity assay. CL-1 is much more radioresistant than parental LnCap cell, consistent with its elevated levels of IAPs. The data suggest that the cells with more IAPs are more resistant to radiation-induced cell death, supporting our hypothesis that IAPs might play a role in prostate cancer radioresistance.



Cell Line	PC3	DU145	LnCap	CL1
IC50 (Gy)	6.7	7.2	3.5	7.6

Figure 3. MTT-based cell assay for the response of prostate cancer cell lines to X-ray radiation. The WST-1 cell growth assay was performed on Day 4. The data are presented as % of control.

A.2. Effect of IAPs-overexpression on radiosensitivity of prostate cancer cells

We have constructed expression vectors for IAP family proteins, including XIAP, cIAP-1. We also constructed expression vectors for XIAP mutants, XIAP-148 (Bir-2 mutation), XIAP-310 (Bir-3 mutation), and XIAP-148/310 (Bir-2/3 double mutation). Currently, we are selecting stable clones of transfected prostate cancer or PrEC cells for radiosensitivity studies.

A.3. Effect of IAPs-down-regulation by siRNAs on radiosensitivity of prostate cancer cells

We propose to use small interfering RNA (siRNA) specific to XIAP, Survivin, c-IAP-1 or c-IAP-2 to down-regulate IAP family of proteins, to determine if down-regulation of IAPs can restore radiation response in

these cancer cell lines resistant to ionizing irradiation. **Figure 4** shows our cIAP-1 siRNA can effectively down-regulate cIAP-1 expression in DU-145 cells. The cancer cell lines will be transfected with siRNAs targeting individual IAPs, and then their radiation response will be assessed in clonogenic and apoptosis assays. We are currently screening stable clones for radiosensitivity studies.

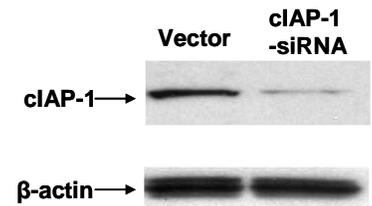


Figure 4. cIAP-1 knock-down by siRNA. DU-145 cells were transfected with siRNA on cIAP-1 for 48 hours, the cell lysates were analyzed by Western blot.

B. Task 2: To investigate in vitro radiosensitization activity of potent small molecule inhibitors of IAPs in human prostate cancer with IAP overexpression.

B.1. Small-molecule Smac-mimetic IAP-inhibitors potently sensitized PC-3 and DU-145 cells to X-ray irradiation in both cell growth assay and clonogenic assay

Overexpression of XIAP and/or other IAP proteins in cancer cells have been shown to inhibit apoptosis induced by not only chemotherapeutic agents but also radiation. Therefore, we hypothesize that treatment of cancer cells with a potent small molecule Smac-mimetic may sensitize the cancer cells to X-ray radiation by overcoming the protective effect of IAP proteins.

To test this hypothesis, we treated human prostate cancer DU-145 and PC-3 cells with our potent small-molecule Smac-mimetic IAP-inhibitors in combination of X-ray irradiation, and evaluated their effects on cell growth. As shown in **Figure 5**, Small-molecule IAP-inhibitors **SH-122** and **SH-130** potently enhanced the radiation-induced cell growth inhibition in DU-145 cells, while an inactive analog **SH-123** showed no effect on radiation response. It is worth noting that while both SH-122 and SH-130 have similar binding affinity (at nM level) to IAPs, SH-122 appears to have better cell growth inhibitory activity than SH-130 as a single therapy. When used in combination with radiation, both SH-122 and SH-130 showed similar radiosensitizing activity with up to 10-fold sensitization.

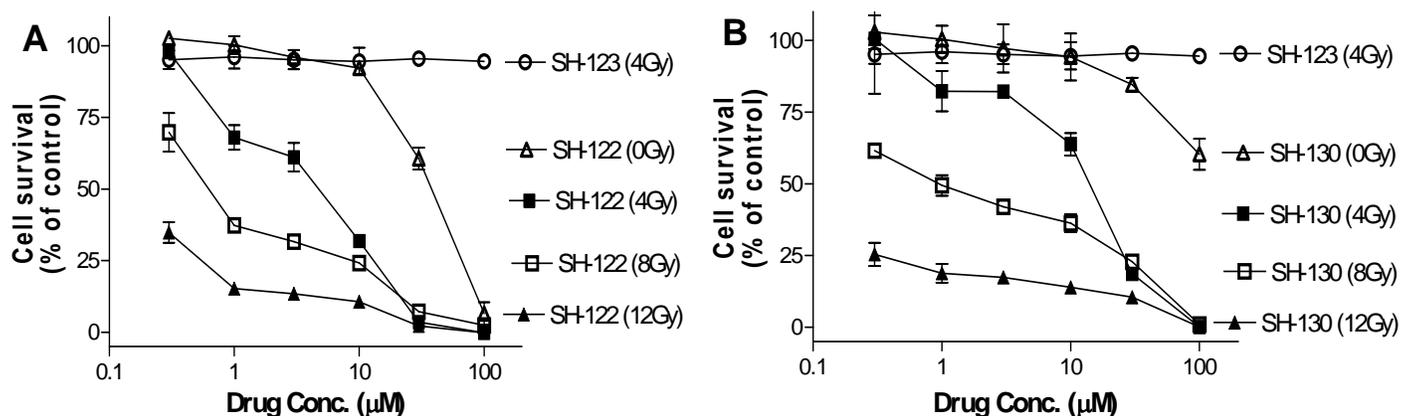


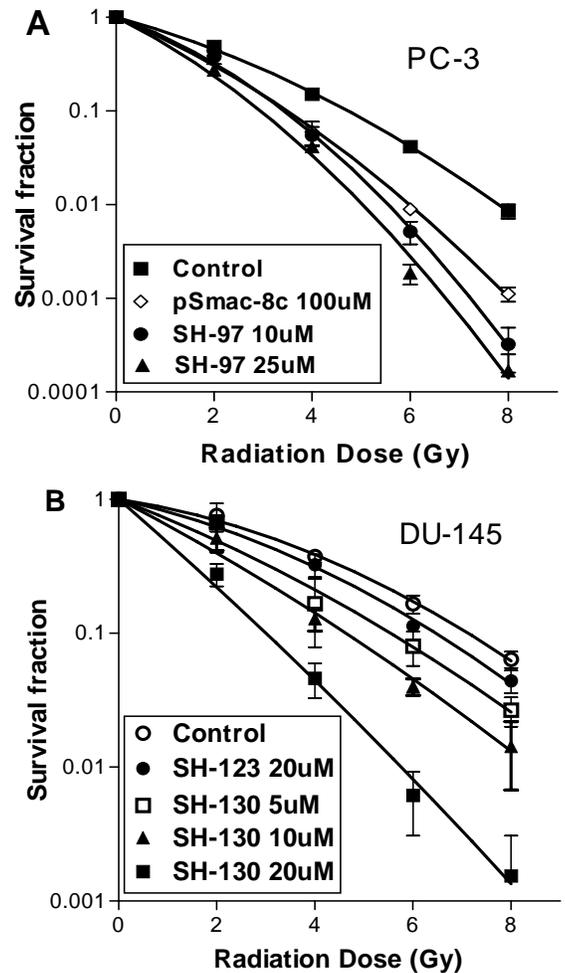
Figure 5. Small-molecule IAP-inhibitors **SH-122** (A) and **SH-130** (B) enhanced radiation-induced cell growth inhibition in DU-145 cells. The cells in 96-well plate were treated with compounds in triplicates, then subjected to various doses of X-ray irradiation within one hour. The WST-1 cell growth assay was performed on Day 4. The data are presented as % of solvent control. **SH-123**: inactive analog.

The clonogenic assay is considered to be the gold standard assay to assess radiation response of cells. We carried out the clonogenic assay to evaluate the effect of our novel IAP-inhibitors on radiation response in cancer cells. Human prostate cancer PC-3 and DU-145 cells were treated with our lead IAP-inhibitors and irradiated with X-ray within one hour. After 10-14-day's culture, the culture plates were stained with crystal violet, and the colonies with over 50 cells were counted with a ColCount colony counter. The cell-permeable Smac peptide, pSMAC-8c, was used as the positive control in this experiment. The cell survival curves were plotted with linear-quadratic curve fitting.

As shown in **Figure 6A**, pSmac-8c significantly sensitized PC-3 cells to X-ray radiation, indicating that IAP is a valid target for overcoming radiation resistance in the prostate cancer cells. Treatment of PC-3 cells with 10 and 25 μ M of SH-97 significantly increased the anti-tumor activity of radiation. At the 6 Gy dose of radiation, 10 and 25 μ M of SH-97 resulted in more than 10-fold reduction of clonogenic cell survival as compared to radiation alone. At 8 Gy, 10 and 25 μ M of SH-97 resulted in 40- and 50-fold reductions of cell survival as compared to radiation alone. 10 μ M of SH-97 appears to be more effective than 100 μ M of pSmac-8c, in terms of radiosensitizing potential, at both 6 and 8 Gy (**Figure 6A**), suggesting that SH-97 is at least 10-fold more potent than pSmac-8c in radiosensitization of PC-3 cells. In DU145 cells, IAP-inhibitor SH-130 showed dose-dependant radiosensitizing potential, while the inactive analog SH-123 showed no activity (**Figure 6B**). SH-102 and SH-122 showed similar radiosensitizing activities (data not shown).

These data provide strong support that small molecule inhibitors targeting IAPs have a great therapeutic potential to improve radiation response of human prostate cancer cells, suggesting that targeting IAPs may be a promising novel approach for overcoming radiation resistance of human prostate cancer with IAP overexpression.

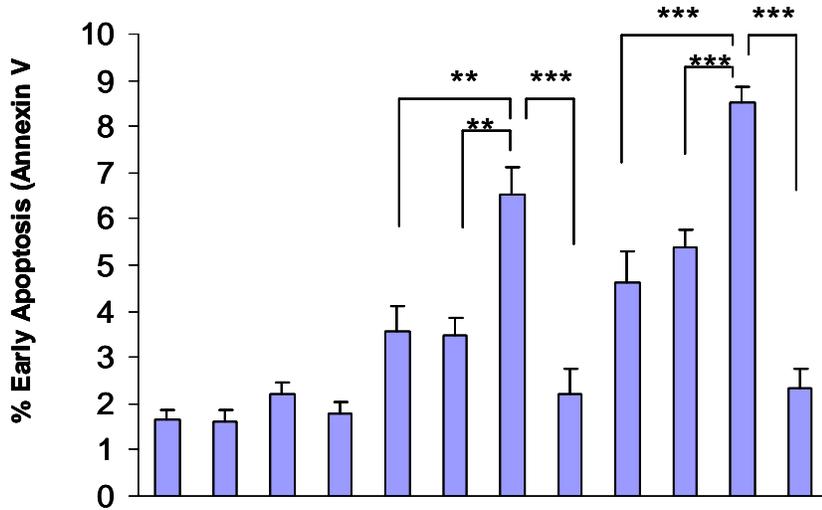
Figure 6: IAP-inhibitors-mediated radiosensitization of human prostate cancer PC-3 (A) and DU-145 (B) cells. (Clonogenic assay survival curves)



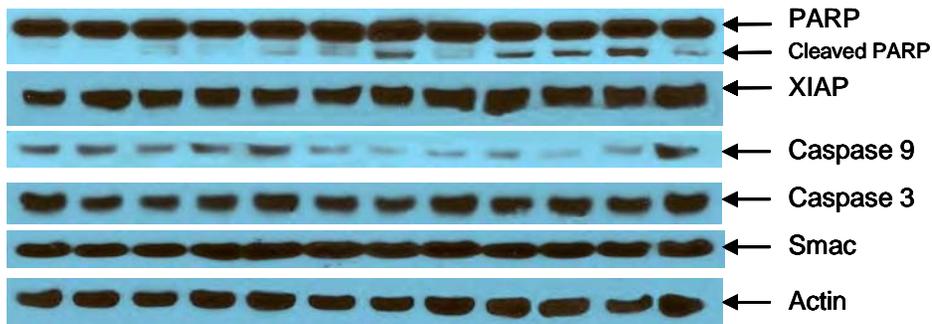
B.3. IAP-inhibitor mediated potentiation of radiation-induced apoptosis in human prostate cancer cell lines in vitro.

We have also carried out apoptosis assays to investigate whether IAP-inhibitor can potentiate radiation-induced apoptosis in prostate cancer cells with high IAPs. **Figure 7A** shows that treatment of DU145 cells with SH-130 in combination with radiation significantly increased apoptosis induction, as compared with either treatment alone. The apoptosis induction can be blocked by pan-caspase inhibitor, zVAD, indicating that caspases are involved in apoptosis induced by SH-130 + Radiation. This increased apoptosis is accompanied with increases of Caspase 9/3 activation and PARP cleavage (**Figure 7A**), in a time- and dose-dependent manner.

A



B



DMSO	+	-	-	-	+	-	-	-	+	-	-	-
SH123	-	+	-	-	-	+	-	-	-	+	-	-
SH130	-	-	+	-	-	-	+	-	-	-	+	-
zVAD	-	-	+	+	-	-	+	+	-	-	+	+

Rad. Dose
(Gy)

0

20

30

Figure 7. SH-130 enhances radiation-induced apoptosis. DU145 were seeded into 6 well plate at the concentration of 2×10^5 /ml, then exposed by SH130 (10 μ M) with or without the pan-caspase inhibitor zVAD (2.5 μ M) (Biovision), together with the irradiation at the dose of 0, 20 and 30 Gy, respectively. SH123 was used as the negative control. Twenty-four hours after incubation, cells were collected and processed to further detection. (A) Early apoptotic cell populations after treatment. Collected DU145 cells were stained by Annexin V-FITC (Trevigen), followed by flow cytometry analysis. Each sample were tested in triplicate. Data represented the mean of three measurements \pm SD, and were analyzed by unpaired Student's t test for statistical significance. ** $p < 0.01$, *** $p < 0.001$. (B), Western blot analysis of apoptosis related proteins in the corresponding samples. Whole-cell lysates were prepared, and 10 μ g of the cell lysates were subjected to SDS-PAGE (4-12% gradient gel, Invitrogen), electrotransferred to nitrocellulose membrane, and probed with antibodies against PARP, Caspase 3, Caspase 9, XIAP and Smac. Actin was shown as loading control.

C. Task 3. To investigate in vivo radiosensitization activity of small molecule inhibitors of IAP in human prostate cancer animal models with IAP overexpression.

Not planned in the first year.

D. Task 4. To investigate the mechanism of action of small molecule inhibitors of IAP in overcoming radiation resistance of prostate cancer.

D.1. Investigation of the ability of IAP inhibitors to directly activate caspase-9 and -3 in response to radiation.

We carried out plate-based caspase-3/9 assay in prostate cancer cells treated with SH-130 and radiation. DU-145 cell were treated as described in Figure 7. Cells were lysed by the lysis buffer (Biovision) as indicated. Total extracted proteins were determined and normalized, and then reacted with fluorogenic substrates (Biovision, DEVD-AFC and LEHD-AFC for Caspase 3 and 9, respectively). After 2 hours incubation at 37°C, proteolytic release of AFC was monitored at a λ_{ex} = 405 nm and λ_{em} = 500 nm using the POLARstar OPTIMA microplate reader (BMG LABTECH). Signal was expressed as relative fluorescent units (RFU). Determinations were made in triplicate, and data represented the mean of three measurements \pm SD.

As shown in **Figure 8**, SH-130 enhanced radiation-induced activation of Caspase-3 and -9, in a dose-dependent manner. Please note that this study is still ongoing, the Caspase-9 assay still has a high background. We are still working on fine-tuning the experimental conditions.

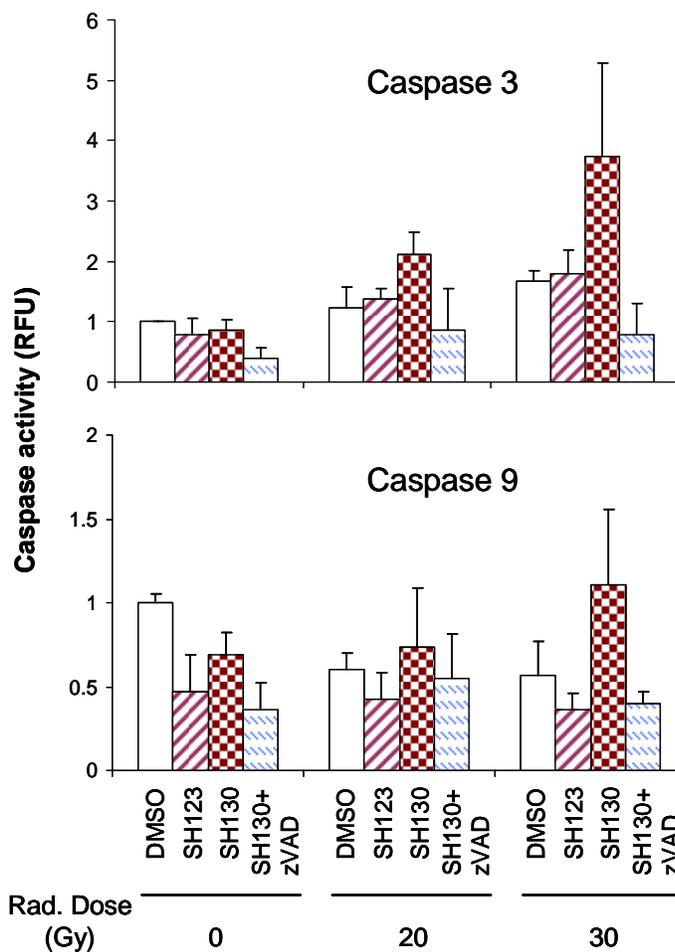
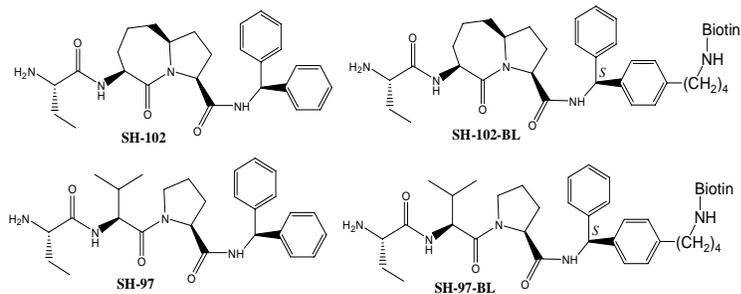


Figure 8. Analysis of Caspase 3 and 9 activation after treatment with SH-130 and radiation.

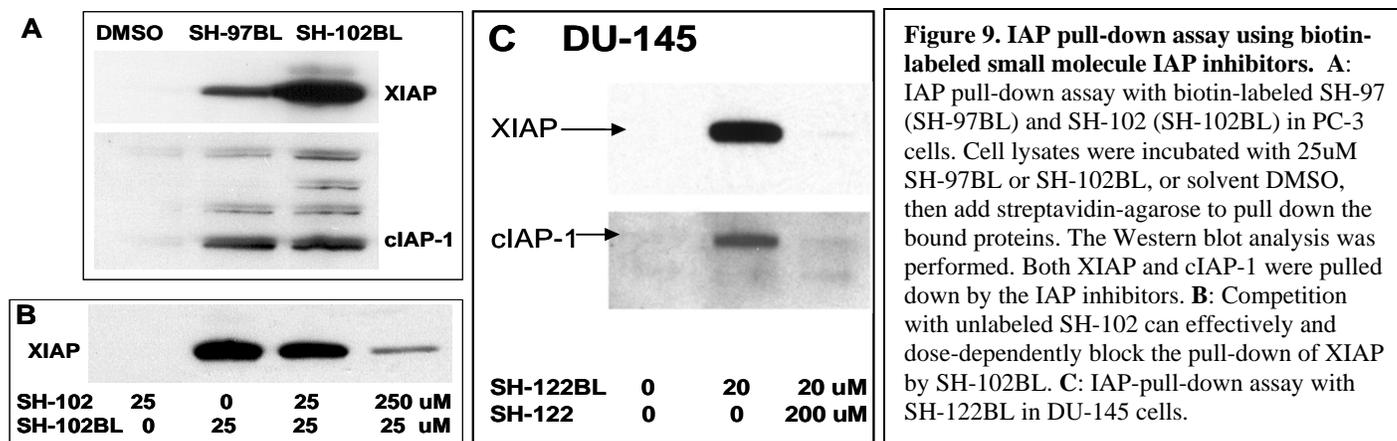
D.2. Molecular targets validation by protein pull-down assay using biotin-labeled IAP-inhibitors

To further determine if our potent small molecule IAP-inhibitors can bind to the IAP family of proteins in cells, we carried out IAP pull-down assay using biotin-labeled IAP inhibitors. SH-97 and SH-102 were labeled with biotin via chemical conjugation (**Chart I**). FP-based binding assay

Chart I. Chemical structures of biotin-labeled IAP-inhibitors.



confirmed that biotin-labeled compounds SH-97BL, SH-102BL and SH-122BL have the same binding affinity to XIAP as unlabeled compounds. Cancer cell lysates were treated with 25uM SH-97BL or SH-102BL or solvent DMSO for 1 hour on ice. Then streptavidin-agarose (Roche) was added and mixed for 2 hr at 4 °C. The agarose beads were washed 3 times and heated to 95 °C for 5 min in sample buffer. The released proteins were subject to Western blot analysis probed with antibody against XIAP or cIAP-1. As shown in **Figure 9**, biotin-labeled IAP-inhibitors successfully pulled down both XIAP and cIAP-1. To exclude the possibility of non-specific binding, we also performed competition studies using unlabeled compounds. **Figure 9B** shows that unlabeled SH-102 can effectively and dose-dependently block, or compete off, the XIAP pulled down by SH-102BL. **Figure 9C** shows the similar results with SH-122 in DU-145 cells. The data provide strong support that the IAP inhibitors can indeed target IAPs in tumor cells, suggesting that XIAP and cIAP-1 may very likely be the molecular targets of our IAP inhibitors in these cells.



The IAP family of proteins are multi-functional proteins that bind to various binding proteins or adaptor proteins. These so-called binding proteins or adaptor proteins of IAPs play critical roles in multiple functions of IAPs in regulating cell division, cell cycle, and cell signaling, besides Caspase inhibition. Therefore, it is important to explore proteins other than IAPs that can also be pulled down by the biotin-labeled IAP inhibitors, either directly or indirectly. In collaboration with the Proteomics Core in University of Michigan Comprehensive Cancer Center, we employed proteomics technology to analyze the proteins pulled down by the biotin-labeled IAP inhibitors. **Figure 10** shows the 2-D gel pictures of the pull-down assay samples. The circled spots that are outstanding over that in the control gel (**Figure 10B**) are picked for LC-MS/MS analysis and identification. Our ongoing proteomics analysis indicated that several members of IAPs were pulled down by the biotin-labeled IAP inhibitors, consistent with the Western blot data shown in **Figure 9**. More importantly, our proteomics study identified several unknown proteins and a few known proteins that have not

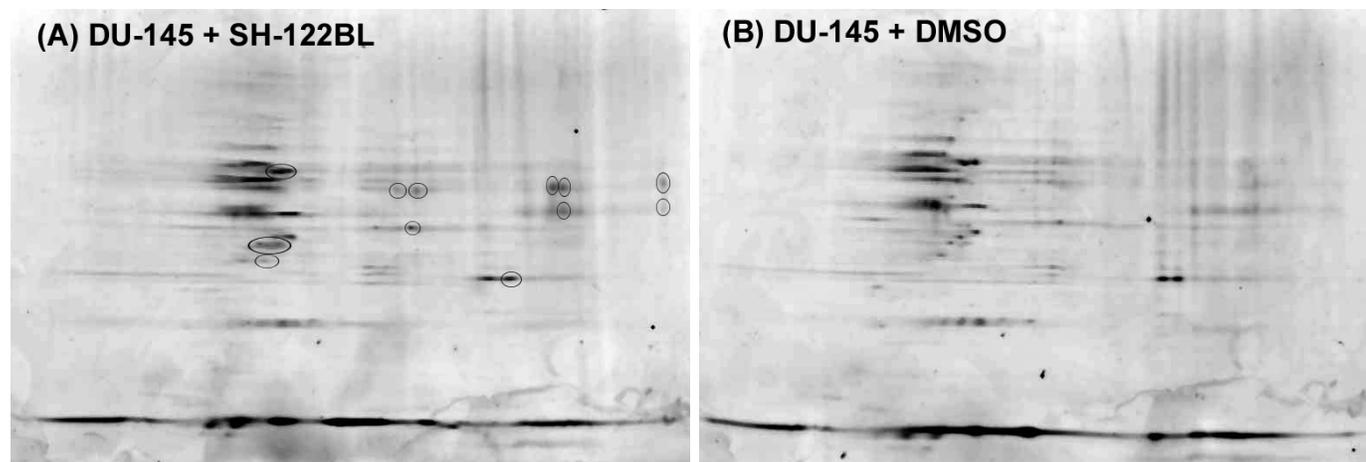


Figure 10. 2-D gel analysis of pull-down assay samples. DU-145 cell lysates were treated with SH-122BL (A) or DMSO (B), and the pull-down assay was performed as described in Figure 10. The samples were separated in the first dimension using 7cm pH 3-10 IPG gels. Second dimension gels were run on 4-20% Tris/Glycine gels. The gels were stained with SYPRO Ruby. Gel overlays were generated using Z3 gel analysis software. The circled spots were picked for LC-MS/MS analysis.

