Discovery of Novel Second Mitochondria-Derived Activator of Caspase Mimetics as Selective Inhibitor of Apoptosis Protein Inhibitors^[S]

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ABSTRACT

Inhibitor of apoptosis (IAP) proteins are widely considered as promising cancer drug targets, especially for drug-resistant tumors. Mimicking the IAP-binding motif of second mitochondria-derived activator of caspases (SMAC) is a rational strategy to design potential IAP inhibitors. In this report, we used the bioactive conformation of AVPI tetrapeptide in the N terminus of SMAC as a template and performed a shape-based virtual screening against a drug-like compound library to identify novel IAP inhibitors. Top hits were subsequently docked to available IAP crystal structures as a secondary screening followed by validation using *in vitro* biologic assays. Four novel hit compounds were identified to potently inhibit cell growth in two human melanoma (A375 and M14) and two human prostate (PC-3 and DU145) cancer cell lines. The best compound, UC-112

[5-((benzyloxy)methyl)-7-(pyrrolidin-1-ylmethyl)quinolin-8-ol], has IC₅₀ values ranging from 0.7 to 3.4 μ M. UC-112 also potently inhibits the growth of P-glycoprotein (P-gp)-overexpressed multidrug-resistant cancer cells, strongly activates caspase-3/7 and caspase-9 activities, and selectively downregulates survivin level at a concentration as low as 1 μ M. Coincubation of UC-112 with a known proteasome inhibitor Z-Leu-Leu-Leu-CHO (MG-132) rescued survivin inhibition, consistent with the anticipated mechanism of action for UC-112. As a single agent, UC-112 strongly inhibits tumor growth and reduces both X chromosome-linked IAP and survivin levels in an A375 human melanoma xenograft model *in vivo*. Overall, our study identified novel scaffolds, especially UC-112, as new platforms on which potent and selective IAP antagonists can be developed.

Introduction

The family of inhibitor of apoptosis proteins (IAP) bind to caspases, block the assembling of proapoptotic protein signaling complexes, and thereby prevent the activation of caspase proteolytic cleavages and the subsequent triggering of apoptosis. There are eight IAP proteins identified in human: NAIP, cIAP1, cIAP2, XIAP, ML-IAP, ILP2, survivin, and apollon (Arora et al., 2007; Wu et al., 2010; Fulda and Vucic, 2012). As overexpression of IAPs frequently occurs in cancer cells and has been linked to tumor progression, treatment failure, and poor prognosis, IAPs are considered

to be promising therapeutic targets in either directly eliciting cell death or lowering the threshold for cell death induction of current anticancer therapeutics (Tian and Lee, 2010; Wang, 2011; Fulda and Vucic, 2012).

SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP binding protein with low pI) molecules released from mitochondria antagonize the IAPs and can protect caspases from IAP inhibition (Wu et al., 2000; Tian and Lee, 2010; Wang, 2011; de Almagro and Vucic, 2012; Fulda and Vucic, 2012). SMAC interacts with IAPs mainly via its N-terminal AVPI binding motif. A proven strategy for inhibiting IAPs is to disrupt the interaction between an IAP and SMAC by developing SMAC mimetics (Wu et al., 2000). SMAC-mimicking IAP antagonists can induce apoptosis in tumor cells and effectively inhibit tumor growth in mice (Nikolovska-Coleska et al., 2004, 2008; Sun et al., 2007; Lu et al., 2008; Flygare et al., 2012; Peng et al., 2012). They can also inactivate nuclear factor κ B and produce secretion of tumor necrosis factor α (Wang, 2011; Peng et al., 2012). Several SMAC mimetics have entered late-stage preclinical development or human clinical testing as novel cancer

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ABBREVIATIONS: BIR, baculoviral IAP repeat; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; HDFa, human dermal fibroblast adult cells; IAP, inhibitor of apoptosis protein; MDR, multidrug resistance; MG-132, Z-Leu-Leu-Leu-CHO; MTD, maximal tolerable dose; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PBS, phosphate-buffered saline; P-gp, P-glycoprotein; siRNA, small interference RNA; SMAC, second mitochondria-derived activator of caspase; TGI, tumor growth inhibition; TUNEL, terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling; UC-112, 5-((benzyloxy)methyl)-7-(pyrrolidin-1-ylmethyl)quinolin-8-ol; XIAP, X chromosome-linked IAP; YM155, sepantronium bromide, C20H19BrN4O3.

therapeutics (Nikolovska-Coleska et al., 2004, 2008; Sun et al., 2007; Lu et al., 2008; Flygare et al., 2012; Peng et al., 2012). For example, Birinapant (TL32711; TetraLogic Pharmaceuticals, Malvern, PA), now in Phase II study, can effectively suppress cIAP1 and XIAP at well-tolerated doses and promises antitumor activity either as a single agent or in combination with standard-of-care chemotherapeutic drugs in adult patients with advanced solid tumors or lymphoma (Krepler et al., 2013). However, no IAP inhibitors have been approved by the U.S. Food and Drug Administration as of today, and there are limitations with many existing IAP inhibitors. For example, YM155 is a well-known survivin inhibitor that has gone through clinical trials, but it has been shown to be a substrate for the P-glycoprotein (P-gp) drug efflux pump (Iwai et al., 2011), suggesting that it could suffer from multidrug resistance (MDR) in its eventual clinical use. Thus, exploring novel scaffolds to develop potent and selective IAP antagonists is still much needed. Because all IAP proteins share the signature baculoviral IAP repeat (BIR) domain (Fulda and Vucic, 2012), which interacts with SMAC, shape-based virtual screening will be helpful in identifying potential small-molecule SMAC mimetics for regulating apoptosis in cancer cells.

In this report, we describe our efforts to identify novel small-molecule SMAC mimetics through an integrated virtual screening and biologic validation approach. Their efficiency in inhibiting IAPs, especially XIAP and survivin (BIRC5), and inducing apoptosis in cancer cells was further validated in serial biologic studies both *in vitro* and *in vivo*. These compounds represent novel scaffolds for IAP inhibition and can be further optimized to serve as a potential targeted agent for various types of cancers.

Materials and Methods

Shape-Based Virtual Screening. The University of Cincinnati's Drug Discovery Center Library (contains 362,910 compounds) was used to conduct the shape-based virtual screening. All structures were first prepared using the LigPrep module in Maestro Suite 2012 (Schrodinger, LLC, New York, NY) to generate conformers and charged states. We used the phase_shape program in Canvas (version 1.4; Schrodinger, LLC). Conformers with a shape similarity below 0.7 were filtered out, and hits with a similarity value above this threshold were selected for subsequent molecular docking process.

Molecule Docking. Crystal structures of SMAC bound to XIAP BIR3 domain [Protein Data Bank codes 1G73 (Wu et al., 2000) and 1TW6 (Vucic et al., 2005)] were processed with the Protein Preparation Wizard, and the grid of AVPI binding site was defined by Glide (version 5.7; Schrodinger, LLC). One thousand hits with top-ranked similarity value were docked into the AVPI binding site in each separate complex. The best docking complexes were subject to restricted molecular dynamics to release any strains by using the MacroModel module with OPLS-2005 force-field. The ligand and its surrounding residues within 15 Å were allowed to move freely, whereas residues outside the 15-Å radius were kept rigid.

Cell Culture and Reagents. Human melanoma A375 cell line and human prostate PC-3 and DU145 cell lines were acquired from American Type Culture Collection (Manassas, VA). Human melanoma M14 cell line was kindly provided by Dr. Robert Clarke (Georgetown University, Washington, DC). Human keratinocyte Hacat cell line was a gift from Dr. Andrzej T. Slominski (University of Tennessee Health Science Center, Memphis, TN). Human dermal fibroblast adult cells (HDFa) cells were purchased from Life Technologies (Thermo Fisher Scientific Inc., Waltham, MA). All cell

lines were authenticated prior to use for this study. Cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM; for melanoma cells) or RPMI 1640 (for prostate cancer cells) medium (Mediatech, Inc., Manassas, VA), supplemented by 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 1% antibiotic/antimycotic mixture (Sigma-Aldrich, St. Louis, MO), and 5 µg/ml bovine insulin (Sigma-Aldrich). Hacat cells were cultured in 10% FBS-supplemented DMEM. HDFa cells were maintained in supplemented Gibco Medium 106 (M-106-500, Life Technologies). Compounds were dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich) to make a stock solution of 10 mM. Compound solutions were freshly prepared by diluting stocks with cell culture medium before use (final solution contained less than 0.5% DMSO). Proteasome inhibitor MG-132 was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY).

Cell Viability Assay. The antiproliferation effect against cancer cells of all UC compounds was first screened *in vitro* by using the MTS assay as described previously (Chen et al., 2012; Wang et al., 2012; Xiao et al., 2013). Then the IC₅₀ values of selected hits were tested through the sulforhodamine B assay as reported before (Wang et al., 2012).

Small Interference RNA Silencing Survivin or XIAP Expression. Signalsilence survivin or XIAP small interference RNA (siRNA) and fluorescein conjugated control siRNA were purchased from Cell Signaling Technology, Inc. (Danvers, MA). They were transfected into preseeded A375 or PC-3 cells by mixing with Lipofectamine RNAiMAX reagent (Invitrogen and Thermo Fisher Scientific Inc.) at the final concentration of 100 nM in serum-free medium. Twenty-four hours after the transfection, cells were treated with compound solution to determine the changes of cell viability.

Caspase Functional Assay. The caspase activity of cancer cells treated by DMSO control or compound solution after 24- or 48-hour incubation was analyzed using Caspase-Glo 3/7 and Caspase-Glo 9 assay kits from Promega Corporation (Madison, WI) as per manufacturer's instructions. The readings of relative luminescence unit were normalized by the cell viability results from the same well determined by compatible CytoTox-Fluor Cytotoxicity assay kit (Promega, WI).

Western Blotting. After treatment of the indicated time, A375, PC-3, and DU145 cells were lysed to determine by Western blotting the relevant IAP family protein levels. Primary rabbit antibodies were purchased from Cell Signaling Technology, Inc.: anti-cIAP1, anti-cIAP2, anti-survivin, anti-XIAP, anti-livin, or anti-GAPDH. Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Cell Signaling) then was used to detect the target protein level. Lane intensities were quantified with ImageJ software (National Institutes of Health, Bethesda, MD).

Flow Cytometry Analysis. Flow cytometry analysis was performed as described previously (Wang et al., 2012). In brief, A375 cells ($n = 3$) were synchronized through 24-hour starvation in growth media containing only 0.1% FBS. The cells then were treated with 0, 1, or 4 µM UC-112 in full growth media (10% FBS) for another 24 hours. The cell-phase distribution then was determined on a BD LSR-II cytometer (BD Biosciences, San Jose, CA) with 10,000 cells scored, and data were processed using Modfit 2.0 program (Verity Software House, Topsham, ME).

Human Melanoma A375 Tumor Xenograft Model and Treatment. We first estimated the acute maximum tolerable dose (MTD) for compound UC-112. Progressively increasing injection doses via intraperitoneal injection route to BXD mice ($n = 3$) determined the estimated MTD to be above 200 mg/kg with 1-week continuous treatment. To ensure a large safety margin during the 3-week treatment and considering the practical doses in clinical, we scaled down the dose to 20 and 40 mg/kg in the xenograft model study.

Seven- to eight-week-old male nude mice were purchased from Charles River Laboratories International, Inc. (Wilmington, MA). Right before use, A375 cells were suspended in ice-cold phenol red-free and FBS-free DMEM and mixed with a high concentration of Matrigel (BD Biosciences) at a ratio of 1:1. One hundred microliters of

this mixture containing 3×10^6 A375 cells was injected subcutaneously to the left-side dorsal flank of each mouse. One week after the inoculation, the mice were randomized into four groups ($n = 7$), and treatments were started. UC-112 compound was suspended in sterile phosphate-buffered saline (PBS) buffer and administered through intraperitoneal injection once per day, 5 days per week, for 3 continuous weeks. Vehicle control group was intraperitoneally injected with the same volume (100 μ l) of PBS buffer at the same dosing frequency. At the end of the experiments, mice were killed, and tumor tissues were isolated and weighed separately. One small piece (~50 mg) from each tumor then was cut and stored in liquid nitrogen immediately. The rest of the tumor tissue was fixed in 10% buffered formalin phosphate solution for more than 1 week before pathology staining analysis.

Tumor volume and body weight of each mouse were evaluated three times a week. We calculated the tumor volume with the formula $a \times b^2 \times 0.5$, where a and b represented the larger and smaller tumor diameters. Data were presented as mean $\pm$ S.D. for each group and plotted as a function of time. Tumor growth inhibition (TGI) ratio (%) was calculated as $100 - 100 \times [(T - T_0)/(C - C_0)]$, where T , T_0 , C , and C_0 were the mean tumor volume for the specific group on the last day of treatment, mean tumor volume of the same group on the first day of treatment, mean tumor volume for the vehicle control group on the last day of treatment, and mean tumor volume for the vehicle control group on the first day of treatment, respectively.

Pathology Analysis. Tumor tissues fixed in formalin buffer for more than 1 week were stained with hematoxylin and eosin. All the slides were scanned to create a digital replica of the entire tissue on a glass microscopic slide by using the ScanScopeXT at 0.25 pixel/ μ m.

Terminal Deoxynucleotidyl Transferase-Mediated Digoxigenin-Deoxyuridine Nick-End Labeling Assay. To evaluate the nuclear DNA fragmentation within the A375 tumor tissue, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed using the DeadEnd Fluorometric TUNEL system (Promega) following the manufacturer's protocols. In brief, the formalin-fixed and paraffin-embedded tumor sections were deparaffinized and rehydrated before 100 μ l of 20 μ g/ml proteinase K solution was added to permeabilize the tumor sections. After pre-equilibration in room temperature for 10 minutes, tumor sections were incubated with nucleotide mix and recombinant terminal deoxynucleotidyl transferase enzyme at 37°C for 1 hour. The reaction was stopped with saline-sodium citrate buffer, and the tumor sections were washed with PBS to remove the excess reagents. These slides were mounted in Vectashield + 4,6-diamidino-2-phenylindole (H-1500; Vector Laboratories, Burlingame, CA) to stain nuclei. Finally, coverslips were added, and the slides were analyzed immediately under a fluorescence microscope (EVOS FL Cell Imaging System; Thermo Fisher Scientific Inc.).

Statistical Analysis. Data were analyzed using Prism Software 5.0 (GraphPad Software, Inc., San Diego, CA). The statistical significance (P value) was evaluated by one-way analysis of variance followed by nonparametric Dunnett's test for in vitro apoptosis detection and in vivo xenograft study. Every treated group was compared with the vehicle group separately.

Results

Identification of Initial Hits from Virtual Screening.

We started the study with shape-based virtual screening. The three-dimensional structure of SMAC N-terminal AVPI tetrapeptide [Protein Data Bank code 1G73 (Wu et al., 2000)] in its bioactive conformation was selected as the query template to screen molecules within the prepared compound library that could achieve similar conformations to that of the active AVPI peptide. In our study, the highest shape similarity score of UC library compounds reached 0.81; over 800 compounds had

Fig. 1. In silico similarity alignments of template ligand, SMAC N-terminal tetrapeptide AVPI (blue stick), with representative UC compounds (UC-222 in brown tube; UC-112 in yellow tube).

scores over 0.75. Figure 1 illustrates the alignments generated from the similarity screening where template ligand AVPI peptide overlapped well with representative hit compounds UC-222 and UC-112.

To validate the theoretical interaction between the shape-based screening hits and IAP protein, we docked the top 1000 hits into the SMAC AVPI binding pocket in two widely used crystal complexes of SMAC-XIAP (X chromosome-linked IAP) BIR3 domain [Protein Data Bank codes 1G73 (Wu et al., 2000) and 1TW6 (Vucic et al., 2005)]. Hit compounds with best docking scores in either of the crystal structures were examined for their drug-like properties. Fifty hits from each of the two crystal structures (100 hits in total; their binding poses on the AVPI pocket are shown in Supplemental Fig. 1) were selected and combined to produce 71 unique final hits for subsequent in vitro assay against a panel of cancer cells.

Validation of Virtual Screening Using In Vitro Antiproliferation Assay. We first performed a two-concentration quick screening assay for the 71 unique hits identified in the previous step. Hits that displayed more than a 10% growth inhibition at a concentration of 3 or 10 μ M on either of A375 or M14 melanoma cells after 48-hour incubation are listed in the column diagram (Supplemental Fig. 2). Compounds UC-274, UC-476, UC-112, and UC-222 (Table 1) achieved more than 50% growth inhibition in A375 or M14 cells at low micromolar concentration. We subsequently determined the IC_{50} values for these four UC compounds and embelin [a reference IAP inhibitor (Nikolovska-Coleska et al., 2004; Wu et al., 2010) serving as a positive control] on an expanded panel of human melanoma (A375 and M14) and prostate cancer (PC-3 and DU145) cell lines (Table 1). Embelin is a potent and nonpeptidic small molecular XIAP inhibitor with good in vivo antitumor and anti-inflammation activity. Among the four, UC-112 was the most active compound: its IC_{50} was as low as 0.7 μ M against PC-3 cell line. All four UC compounds had comparable or better antiproliferation activity than did embelin (Table 1).

We also investigated the cell toxicity of these UC compounds on two non-cancerous cell lines (Supplemental Table 1). Compared with its average IC_{50} value in the tested cancer cell lines, UC-112 has around 2.5-fold higher IC_{50} values on Hacat or HDFa cells. The IC_{50} values of UC-222 increased 8- to 12-fold in the tested noncancerous cells, compared with the average IC_{50} on the cancer cell lines.

TABLE 1
Growth inhibitory percentages of UC compounds
Data are shown as mean ± S.E.M. Compounds were tested at a concentration of 3 or 10 μM (MTS assay, n = 4) and IC₅₀ (μM) values of compounds were compared with embelin against cancer cell proliferation (sulforhodamine B assay, n = 4).

		UC-274	UC-476	UC-112	UC-222	Embelin
Compound ID						
Growth inhibition (%)	A375	12.8 ± 3.5	16.7 ± 2.3	50.7 ± 5.1	67.0 ± 1.6	N.D.
	M14	73.7 ± 2.4	84.0 ± 1.6	65.0 ± 3.4	94.6 ± 1.0	N.D.
	PC-3	39.9 ± 3.3	20.6 ± 2.0	81.1 ± 1.7	73.7 ± 2.0	N.D.
	Average*	70.2 ± 1.1	85.3 ± 3.8	99.6 ± 0.6	99.2 ± 0.7	N.D.
IC ₅₀ (μM)	A375	13.4 ± 0.7	10.4 ± 0.2	1.6 ± 0.1	2.7 ± 0.4	14.3 ± 3.2
	M14	12.3 ± 0.4	10.2 ± 0.2	2.5 ± 0.3	3.5 ± 0.1	20.7 ± 5.4
	PC-3	15.1 ± 0.3	12.8 ± 2.1	0.7 ± 0.1	2.3 ± 0.2	13.0 ± 1.8
	Average*	14.5 ± 1.1	11.4 ± 2.2	3.4 ± 0.8	4.5 ± 1.7	23.2 ± 3.7

N.D., not determined.
*Average IC₅₀ value was calculated using the data across all four tested cell lines of the same compound.

Hit Compounds Increased Caspase Activities Significantly Better than Embelin. All UC compounds effectively increased the caspase-3/7 (the executor caspases) and caspase-9 (the initiator caspase) levels on A375 and PC-3 cells in a time-dependent manner (Fig. 2). In addition, UC-222 at a concentration of 10 μM and UC-112 at a concentration of 4 μM (10 μM UC-112 was too high for this assay because of its higher potency) had significantly stronger ($P < 0.01$) caspase activation potency compared with embelin at 4 or 10 times higher concentration (40 μM).

Western Blotting Analyses of IAP Level Change in Cancer Cells. Having established that the four UC hit compounds can potently increase the caspase activities, we asked the question whether these compounds produced their effects by directly inhibiting IAPs, because they were screened in silico as SMAC N-terminal AVPI mimetic molecules. To answer this question, we studied the changes of IAP levels in cancer cells when they were treated by the two most potent UC compounds, UC-112 and UC-222, or the reference IAP inhibitor embelin. Although there were no significant changes of XIAP, cIAP1, cIAP2, or survivin expression at short time incubation (3 hours; data not shown), when the incubation time was increased to 24 hours, UC-112 at concentrations of 4 or 10 μM (Fig. 3A) significantly suppressed survivin level on A375, PC-3, and DU145 cell lines in a dose-dependent manner. UC-112 potently decreased survivin level (94 and 97% at 4 and 10 μM , respectively; Supplemental Fig. 3A) in A375 cells, whereas its inhibition of the other major IAPs (i.e., XIAP, cIAP1, cIAP2), which also interact with SMAC, was much weaker. At the same time point (24 hours), UC-222 at concentrations of 4 or 10 μM decreased the level of cIAP2 on

PC-3 cells and XIAP on DU145 cells but only had very mild effects on the survivin level in either cell line (Fig. 3A).

In comparison, the inhibition effects of embelin at low concentrations (4 and 10 μM) on the IAP levels were not clear in these cell lines (Fig. 3A). When we increased its concentration to 40 μM , embelin could inhibit XIAP expression in PC-3 cells with an 8-hour incubation (Fig. 3B), consistent with literature report (Nikolovska-Coleska et al., 2004). In contrast, UC-112 at 40 μM only moderately decreased the level of XIAP but substantially downregulated the level of survivin in A375 cells (Fig. 3B). UC-222 at this concentration (40 μM) was most potent in decreasing XIAP levels in A375 and DU145 cells, but PC-3 cells were more resistant to the UC-222 treatment. Thus, UC-112 demonstrated selective inhibition against survivin among the IAPs tested (Supplemental Fig. 3A).

To validate whether the toxicity of UC-112 on cancer cells results from its downregulation effects on IAP levels, we silenced the gene of survivin or XIAP by transfecting A375 and PC-3 cells with specific siRNAs. Then the antiproliferation efficacy of UC-112 on these transfected cancer cells was determined with MTS assay, and the data were shown in Fig. 3C (Supplemental Fig. 4). Compared with the results from the nontransfected (blank group) or nonspecific siRNA transfected cells, IC₅₀ values of UC-112 increased 7- to 10-fold when either XIAP or survivin expression was silenced.

Encouraged by the results that compound UC-112 is a relatively potent inhibitor of survivin, we quantified the potency of the compound by incubating A375 and PC-3 cells with serially diluted UC-112 solutions (from 4 μM to 10 nM). We also selected YM155 (Nakahara et al., 2007, 2011; Yamanaka et al., 2011; Jane et al., 2013), a highly efficient

Fig. 2. Relative in vitro caspase activity of human melanoma A375 or human prostate cancer PC-3 cells after treatment with UC compounds or embelin at different concentrations ($n = 4$). Testing concentrations (4 or 10 μM) of UC compounds were selected in reference to their in vitro antiproliferation potency. All the relative light unit (RLU) data were normalized according to the cell viability results read from the same well in a 96-well plate. (A) Caspase-3/7 in A375 cells; (B) caspase-3/7 in PC-3 cells; (C) caspase-9 in A375 cells (48-hour incubation); (D) caspase-9 in PC-3 cells (48-hour incubation). ** $P < 0.01$ compared with corresponding results of embelin group.

Fig. 3. Compounds UC-112 and UC-222 downregulate the IAP levels in vitro. (A) Western blotting analysis using cell lysates of A375, PC-3, or DU145 cells that have been treated by compounds at concentrations of 4 or 10 μM for 24 hours. (B) At concentration of 40 μM , Western blotting analysis showed that UC-112 and UC-222 can effectively induce degradation of XIAP and survivin, after 8- or 24-hour incubation. (C) Silencing survivin or XIAP gene in A375 or PC-3 cells increased the IC_{50} values of UC-112. Treatment started at 24 hours after the siRNA transfection. The cell viability after 48-hour treatment was determined using MTS assay ($n = 4$). (D) Comparison between compound UC-112 and YM155 regarding the potency of reduced survivin level in A375 or PC-3 cells. Photographs show representative data from three independent experiments. (E) MTS assay ($n = 4$) revealed that UC-112 overcomes P-gp-mediated multidrug resistance much better than does YM155.

survivin promoter inhibitor as the experiment's positive control. Results (Fig. 3D, Supplemental Fig. 3B) showed that UC-112 significantly downregulated survivin levels ($>50\%$ compared with control group) on A375 cells at a concentration of 400 nM in 24 hours, whereas a higher concentration (1 μM) or longer treatment time (48 hours) (data not shown) was needed in PC-3 cells. YM155 showed to be a more potent survivin inhibitor and strongly inhibited the expression of survivin at concentrations as low as 40 nM in A375 cells or 100 nM in PC-3 cells, consistent with data in published reports (Nakahara et al., 2007; Nakahara et al., 2011). No significant change in XIAP levels was observed in either cell line treated with either UC-112 or YM155 at any concentrations tested. This observation confirmed that compound UC-112 could selectively decrease the survivin protein level over the levels of other IAP family proteins, including the

XIAP. Although YM155 is extremely potent in binding and inhibiting the survivin promoter, its main drawback is that it is a substrate for the P-gp drug efflux pump (Lamers et al., 2012). In fact, when tested in P-gp-overexpressed M14/MDR1 melanoma cells, UC-112 has comparable IC_{50} values in the resistant M14/MDR1 and the sensitive M14 parental cells (Fig. 3E; Supplemental Table 2, small resistance index is desirable to overcome drug resistance), displaying a small resistance index value ~ 2 . In contrast, the resistance index of YM155 was larger than 2900, consistent with the finding that it is a P-gp substrate.

Proteasome Inhibitor MG-132 Rescued the Down-regulated Survivin Levels in Cancer Cells Treated by UC-112. As shown in Fig. 4A and Supplemental Fig. 5, coinubation of pan-proteasome inhibitor MG-132 (10 μM) with UC-112 for 24 hours effectively rescued the survivin levels

Fig. 4. Proteasome inhibitor MG-132 can rescue survivin inhibition by UC-112. (A) Western blotting analysis after coinubation of MG-132 (10 μ M) with UC-112 for 24 hours in A375, PC-3, and DU145 cells. (B) A375 cells were pretreated by high doses of MG-132 (20 or 50 μ M) for 6 hours and washed once by blank cell culture medium. UC-112 then was added for an additional 24-hour incubation. Photographs showed representative data from three independent experiments. (C) Cell cycle analysis of A375 cells incubated with 1 or 4 μ M UC-112 for 24 hours ($n = 3$).

in PC-3 and DU145 cells. MG-132 at 10 μ M in this study was unable to rescue survivin in A375 cells, because these cells are more sensitive to the survivin downregulation effect resulting from UC-112 treatment. However, if A375 cells were pretreated with higher concentrations of MG-132 (20 or 50 μ M) for 6 hours followed by incubation with UC-112 for another 24 hours after washing away MG-132, the survivin level could be rescued from UC-112 treatment (Fig. 4B).

UC-112 Arrested A375 Melanoma Cells in G₁ Phase. The results (Fig. 4C) of flow cytometry analysis showed that incubation with 1 μ M UC-112 for 24 hours caused A375 cells to accumulate in the S phase, with a concurrent reduction of cells in the G₂/M phase. When the concentration of UC-112 increased to 4 μ M, a large percentage of A375 cells ($76 \pm 2\%$ in treatment versus $51 \pm 1\%$ in vehicle control) accumulated in the G₀/G₁ phase with escalated sub-G₁ events, which indicated induction of cell apoptosis. The percentage of cells remaining in

the G₂/M phase was decreased ($13 \pm 1\%$ in treatment versus $21 \pm 2\%$ in vehicle control).

UC-112 Inhibited Tumor Growth in a Melanoma Xenograft Model In Vivo. After 3 weeks of continuous treatment, UC-112 was highly effective in inhibiting melanoma tumor growth in the A375 xenograft model in a dose-dependent pattern (Fig. 5A; Supplemental Fig. 6) without causing ($>10\%$) body weight loss in nude mice (Fig. 5B). Treatment with UC-112 at 20 mg/kg achieved $65.59 \pm 19.56\%$ tumor growth inhibition (TGI) compared with vehicle control group, whereas the dose at 40 mg/kg almost completely inhibited tumor growth (TGI was $95.23 \pm 3.11\%$). The average tumor weight in the UC-112 40 mg/kg treatment group was only 13.56% of that in the vehicle control group (Fig. 5C). To determine whether the potential mechanisms of action for UC-112 observed in vitro will remain in vivo, we compared the IAP protein expressions between the control and treatment

Fig. 5. UC-112 (20 and 40 mg/kg) strongly suppress melanoma tumor growth in an A375 xenograft mouse model ($n = 7$, i.p. injection 5 days per week). (A) Tumor volume growth curve. (B) Mice body weight versus time plot. (C) Tumor weight comparison bar graph. $**P < 0.01$, $***P < 0.001$ compared with the corresponded results of vehicle group. (D) Western blotting analysis of survivin and XIAP levels in fresh tumor lysates. Each lane represents tumor tissue lysate from a single mouse. (E) Representative immunohistochemistry images for H&E staining of A375 tumor tissue sections. The scale bar (yellow) in each image represents 200 μm . Photographs show representative data from three independent experiments. (F) Representative fluorescence microscope images for TUNEL assay of A375 tumor tissue sections. The scale bar (white) in each image represents 100 μm .

tumors. As shown in Fig. 5D, UC-112 significantly reduced both survivin and XIAP levels in the lysates of fresh tumors isolated at the end of the treatment, confirming the in vivo efficacy of UC-112 was at least in part through the strong inhibition of IAPs. Hematoxylin and eosin staining showed that UC-112 induced extensive tumor cell death in these A375 melanoma tumors (Fig. 5E). Furthermore, TUNEL assay, which measured nuclear DNA fragmentation (Fig. 5F), clearly

showed the dose-dependent increasing of apoptosis in tumor sections of UC-112 treatment groups.

Discussion

Identification of Initial Hits from Virtual Screening.

We used the phase shape program in Canvas to run the virtual screening. It initiates finding trial alignments based

on the principle of the distribution of atom triplets and then refines the top alignments to maximize the volume overlap (Sastry et al., 2011). The hits with high structure similarity to the AVPI template then were further screened through the docking study. We selected two widely used SAMC-XIAP complexes at this step because XIAP is the most characterized IAP and a direct inhibitor of initiator caspase-9 (Hiscutt et al., 2010; Fulda and Vucic, 2012). Apoptotic resistance was found to correlate with expression levels of XIAP in human prostate, melanoma, and non-small cell lung cancer cells (Tian and Lee, 2010). In addition, the ability of XIAP to inhibit apoptosis has been shown to allow melanoma cells to escape endoplasmic reticulum stress-mediated cell death (Hiscutt et al., 2010). Here we selected four well-established cancer cell lines (two human melanoma and two human prostate cancer) to first screen the antiproliferation potency of the hit compounds. Those cell lines had been used widely to evaluate the efficacy of IAP inhibitors both in vitro and in vivo (Nikolovska-Coleska et al., 2004; Peng et al., 2012; Jane et al., 2013).

Hit Compounds Increased Caspase Activities Significantly Better Than Embelin In Vitro. The SMAC N-terminal IAP binding motif interacts with the IAPs and protects caspase function either directly or indirectly. Because the overexpression of IAPs in cancer cells result in the suppression of caspase functions, the capability of raising caspase activities is often used to measure the potency of IAP inhibitors (Lu et al., 2008; de Almagro and Vucic, 2012; Flygare et al., 2012). Embelin was reported to increase caspase activity in PC-3 cells at a concentration of 40 μ M after 42-hour incubation (Nikolovska-Coleska et al., 2004). However, our pilot study showed that four most potent UC compounds at the concentration of 40 μ M would kill most of the cancer cells, therefore making the measurement of caspase level below the assay detection limit. For this reason, we used much lower concentrations for UC compounds and performed the caspase functional Glo-assay (Fig. 2, A–D). UC compounds showed much higher potency of activating caspase activities compared with embelin, and this result is consistent with their antiproliferation efficacy in vitro.

UC-112 Downregulated Survivin Level in Cancer Cell Lines, and the Survivin Level Can Be Rescued by Proteasome Inhibitor MG-132. As shown in Fig. 3, UC-112 demonstrated some degree of selectivity in its ability to downregulate survivin levels in cancer cells at the concentration as low as 1 μ M after 24-hour incubation. Survivin is a unique and the smallest member of IAPs. It is highly expressed in most types of cancer but has very low or undetectable expression in differentiated normal tissues (Asanuma et al., 2005; Fukuda and Pelus, 2006; Altieri, 2010; Kanwar et al., 2011; Pavlyukov et al., 2011; Du et al., 2012). Consistent with this differential expression, survivin inhibitors have been shown to have broad efficacy in many types of cancer and low toxicity (Erkanli et al., 2006; Mamori et al., 2007). Additionally, survivin expression has been well correlated with tumor progression, resistance to existing therapies, and poor patient survival (Kanwar et al., 2001; Altieri, 2010; McKenzie and Grossman, 2012; Okamoto et al., 2012). Survivin is involved in a myriad of oncogenic pathways and is considered to be a nodal protein. Recent studies have also revealed that high levels of survivin positively correlate with the overactivation of known oncogenic pathways [hypoxia-inducible factor 1 α (Kanwar et al., 2001; Mamori

et al., 2007), heat shock protein 90 (Fortugno et al., 2003; Cheung et al., 2010), phosphatidylinositol 3-kinase/protein kinase B (Asanuma et al., 2005; Peng et al., 2006), extracellular signal-regulated kinase (Carter et al., 2006), Bcl-2 (Liu et al., 2005; Wang et al., 2009), and RAS pathways (Sommer et al., 2007; Okuya et al., 2010)] but negatively correlate with tumor suppressor genes [p53, phosphatase and tensin homolog (Erkanli et al., 2006; Guha et al., 2009; Nabili et al., 2009)]. Together, these characteristics make selective inhibition of survivin an ideal target for novel cancer drug discovery.

Although the degradation mechanisms of survivin still need to be clearly delineated, XIAP-associated factor 1 was reported to activate E3 activity in XIAP and directly led to survivin degradation through ubiquitination (Arora et al., 2007). To further understand the possible mechanism leading to survivin downregulation by UC-112, we hypothesized that this compound involved the ubiquitin-related degradation process of survivin. To test this hypothesis, we determined whether the addition of a pan-proteasome inhibitor, MG-132, would counteract the ubiquitin-mediated degradation process and rescue survivin from the action of UC-112. In fact, either coincubating or pretreating cells with MG-132 successfully maintained the survivin levels in UC-112-treated cancer cells. The results suggest that UC-112 may produce its survivin inhibition effect, at least in part, via the ubiquitin-mediated degradation of survivin.

UC-112 Arrested A375 Melanoma Cells in G₁ Phase. It is well established that survivin plays complex and critical roles during cell mitosis as a chromosomal passenger protein to promote chromosome segregation and cytokinesis in the G₂/M phase of the cell cycle (Kanwar et al., 2011; Du et al., 2012; McKenzie and Grossman, 2012). Survivin as a cytoplasmic protein is dominantly expressed in the G₂/M phase, followed by a rapid degradation via the ubiquitin-proteasome pathway during G₁ (Zhao et al., 2000; Asanuma et al., 2005; Kanwar et al., 2011; McKenzie and Grossman, 2012). Therefore, the survival of cancer cells in the G₂/M phase is highly dependent on the presence of survivin, whereas cells in the G₁ phase are insensitive to survivin expressions. If UC-112 potentially degrades survivin in cancer cells, we would expect that cells in the G₂/M phase would collapse while the less survivin-dependent cells in the G₁ phase would remain, leading to an overall G₁ phase accumulation upon UC-112 treatment. To test this hypothesis, we performed cell-cycle analysis to determine how UC-112 would influence the cell-cycle distribution. In our study, UC-112 blocked the A375 cell cycle in G₁ phase in a dose-dependent manner. This observation is consistent with the proposed mechanisms of action for UC-112 as described above, providing additional evidence that UC-112 targets survivin degradation, a posttranslational modification process. In contrast, downregulation of survivin gene expression by YM155 was a cell-cycle-independent event without G₁ cell arrest in a wide panel of cancer cells (Cheng et al., 2012). Thus, unlike YM155, UC-112 downregulated the survivin levels in cancer cells more likely by promoting the ubiquitin-mediated degradation of survivin rather than by altering the expression of survivin at the transcription level.

UC-112 Inhibited Tumor Growth in a Melanoma Xenograft Model In Vivo. To further investigate the therapeutic potential of compound UC-112, we evaluated its

antitumor efficacy in an A375 xenograft mouse model. We first estimated the maximum tolerable dose (MTD) in mice. UC-112 was well tolerated in mice up to 200 mg/kg, which was the highest dose we tested. The very low toxicity displayed by UC-112 is consistent with the above evidence suggesting that UC-112 is a selective survivin inhibitor, because survivin is highly expressed in tumor cells but minimally expressed in normal tissues (Fukuda and Pelus, 2006; Mamori et al., 2007; Altieri, 2010). Considering realistic doses that are normally used clinically, we decreased the dose of UC-112 to 10% (20 mg/kg) and 20% (40 mg/kg) in subsequent in vivo efficacy studies. UC-112 significantly inhibited the tumor growth in human melanoma A375 xenograft model after 3-week continuous treatment in a dose-dependent manner without showing obvious toxicity in mouse. It also effectively decreased the survivin level and induced cell apoptosis in tumor tissues in vivo. All these results are consistent with our in vitro observations that UC-112 could efficiently inhibit tumor cell proliferation by downregulating the level of IAPs, especially survivin protein.

In conclusion, using shape-based virtual screening combined with biologic activity evaluation, we identified four SMAC mimetic hits with higher or comparable antiproliferation potency compared with embelin. The four identified hits significantly activated caspase-3/7 and caspase-9 function in human melanoma A375 and prostate cancer PC-3 cell lines. Compounds UC-112 and UC-222 strongly suppressed XIAP and survivin levels in vitro and had scaffolds suitable for modification to further improve their potency. In particular, compound UC-112 showed good selectivity in decreasing the level of survivin (BIRC5) in a dose-dependent manner, possibly through the ubiquitin-mediated degradation pathways, and it also has the potential to overcome P-gp-mediated multidrug-resistance, which is a limitation for many of the existing IAP inhibitors. More excitingly, UC-112 displayed very low toxicity, showed potent efficacy of tumor growth inhibition in an A375 melanoma xenograft model, and maintained its mechanisms of actions in vivo. This integrated chemical biology study provided promising lead compounds for further structural optimization to develop new selective IAP inhibitors as potential therapeutic agents for resistant cancers, either as a single agent or in combinations with existing drugs.

Authorship Contributions

Participated in research design: Wang and Li.
Conducted experiments: Wang and Li.
Contributed new reagents or analytic tools: Wang and Li.
Performed data analysis: Wang and Li.
Wrote or contributed to the writing of the manuscript: Wang and Li.

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