

SM-164 enhances the antitumor activity of adriamycin in human U2-OS cells via downregulation of X-linked inhibitor of apoptosis protein

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Abstract. The antitumor effects of SM-164 and adriamycin (ADM) on human osteosarcoma U2-OS cells, the underlying mechanism are yet to be investigated. In the present study, U2-OS cells were divided into control, ADM, SM-164, and ADM + SM-164 groups. In addition, cells treated with both SM-164 and ADM were further divided into three subgroups: SM-164 + ADM, SM-164 + ADM + vector and SM-164 + ADM + X-linked inhibitor of apoptosis protein (XIAP) silencing groups. XIAP expression was achieved via transfection with shRNA lentiviral vectors. Reverse transcription-quantitative polymerase chain reaction and western blotting were used to detect the expression of caspases-7, -9, and -3, poly ADP-ribose polymerase (PARP), XIAP, cellular inhibitor of apoptosis protein-1 (cIAP-1) and survivin. Cell viability and apoptosis were evaluated using MTT and flow cytometry assays, respectively. Compared with the control group, cell viability decreased, while apoptosis was increased in the ADM and SM-164-treatment group. ADM and SM-164 treatment promoted the expression of caspases-7, -9 and -3, and PARP, but reduced the expression of XIAP, survivin and cIAP-1. Compared with ADM + SM-164 group, XIAP silencing with ADM + SM-164 treatment further reduced cell viability, promoted apoptosis, increased caspase-7, -9 and -3, and PARP expression; however the expression of survivin and cIAP-1 were reduced. Combined ADM and SM-164 treatment may be considered as potential therapeutic agent in the treatment of osteosarcoma, possibly via reductions XIAP expression.

Introduction

Osteosarcoma (OS) is one of the most common types of primary malignant bone tumors, accounting for 60% of malignant bone tumors in adolescents (1). The management of OS remains challenging, particularly under conditions of metastases, and is often complicated by local recurrence following treatment. With continuing developments in adjuvant chemotherapy (2) and improvements in surgical techniques, the 5-year survival rates have risen from <20% to ~60-80% (3); however, distant metastases may occur at early stages of the condition, while recurrence is often detected without distant metastases (4). For such patients, treatment outcomes remain poor (4).

Adriamycin (ADM) is an important chemotherapeutic agent used in the treatment of OS. ADM affects the structure and function of DNA via intercalation, prevents DNA replication and RNA synthesis, and induces apoptosis in tumor cells (5). As ADM possesses a narrow therapeutic index, severe cardiotoxicity and bone marrow suppression are common side effects of treatment (6). SM-164 is a novel non-peptide, symmetric alkyne small molecule. The binding capacity of SM-164 to the baculovirus inhibitor of apoptosis protein repeat (BIR) domain is ~300-7,000 times that of monovalent Smac mimetics and of the AVPI peptide of wild Smac (7). SM-164 notably induces apoptosis in Smac-sensitive tumor cells without inflicting marked toxicity to normal cells (8). Its key mechanism of action involves the inhibition of the X-linked inhibitor of apoptosis protein (XIAP) (9).

The family of inhibitor of apoptosis (IAP) proteins contains some of the most important apoptosis inhibitors, including XIAP, cellular inhibitor of apoptosis protein (cIAP)-1 and -2, neuronal apoptosis inhibitor protein, survivin, livin/melanoma-IAP and apollon, all of which mainly inhibit caspase activity and the induction of the apoptotic pathway (10). Overexpression of IAPs has been reported in a variety of tumor cells, and is an important cause of tumor cell apoptosis and chemoresistance (11-13). At present, XIAP is the most potent known member of IAPs, while survivin has a strong inhibitory effect on apoptosis (14).

The present study investigated how combined SM-164 and ADM treatment, as well as XIAP silencing, affected U2-OS

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cells. The results may provide insight for the development of novel chemotherapeutic and genetic treatment strategies in the management of OS.

Materials and methods

Cell cultures. U2-OS cells were purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Beijing, China) and cultured in RPMI-1640 (KGM31800S-500; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). The culture was supplemented with 10% fetal bovine serum (FBS; cat. no. 04-007-1A; Biological Industries USA, Inc., Cromwell, CT, USA) and 100 U/ml penicillin-streptomycin (cat. no. P1400; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) in 5% CO₂ at 37°C. Cells that were 70% confluent were used in experiments.

Cultured cells were divided into four groups: Control, ADM, SM-164 (200 nM), and combined treatment (0.5 µg/ml ADM + 200 nM SM-164). Following the addition of ADM into cell media for 2 h at 37°C, cells were washed and then treated with SM-164 (200 nM) for 24 h at 37°C.

MTT assay. Cells (3x10³/ml) were seeded in 96-well plates. After the indicated treatments, an MTT assay was applied to evaluate cell viability as previously described (15). The optical density (OD) was determined via a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) at 570 nm and represented cell viability.

Flow cytometry. Following the indicated treatments, U2-OS cells were collected after trypsin digestion (0.25%, 2 min at 37°C) and underwent centrifugation (780 x g for 3 min at room temperature); 5 µl of Annexin V-fluorescein isothiocyanate and 5 µl propidium iodide were subsequently added to each tube for 5 min at room temperature. After light mixing, apoptosis was detected within 1 h by FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed by FlowJo version 10 (FlowJo LLC, Ashland, OR, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following the indicated treatments, U2-OS cells were collected after trypsin digestion (0.25%, 2 min at 37°C). Total RNA was extracted with a TRIzol (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Subsequently, RNA was amplified using a one-step RT-PCR kit (cat. no. 00081405; CWBIO, Taizhou, China). Primers were added into a 25-µl ULtraSYBR Mixture (cat. no. 01170; CWBIO, Beijing, China). qPCR was conducted as follows using an Applied Biosystems 7500 (Thermo Fisher Scientific, Inc.) for 6 repeats: 95°C denaturation for 10 sec, 56°C annealing for 30 sec, and 72°C extension for 30 sec (40 cycles). The primer sequences were listed in Table I. The quantification cycle value for each gene was detected and the expression levels of target genes were calculated using the 2^{-ΔΔC_q} method (16,17).

Western blot analysis. Protein was extracted from cells for western blotting using a protein isolation kit according to the manufacturer's protocols (cat. no. C1053; Applygen Technologies Inc., Beijing, China) and concentrations were

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

Genes	Primers (5'-3')
cIAP-1-F	CTCGTGGAGTGGGAAGACA
cIAP-1-R	GGAGTGATCGTGGTAAGG
XIAP-F	TGTGGGAAACAGAAATCA
XIAP-R	GGGCTTAAATGGGCATAG
GAPDH-F	GAAGGTCCGAGTCAACGGAT
GAPDH-R	CCTGGAAGATGGTGATGGG

cIAP, cellular inhibitor of apoptosis protein 1; F, forward; R, reverse; XIAP, X-linked inhibitor of apoptosis protein.

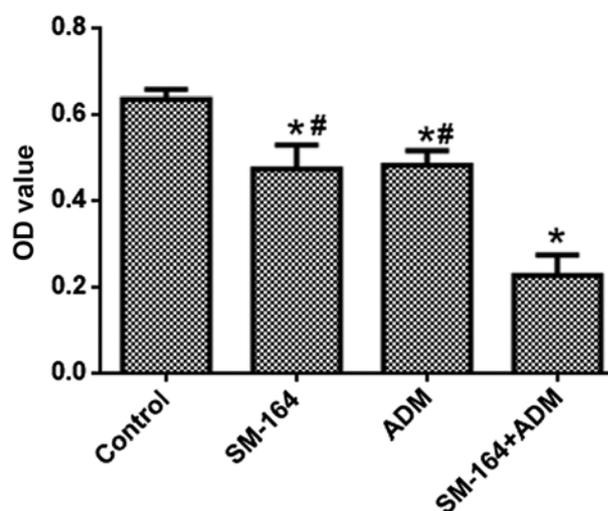


Figure 1. Combination of SM-164 with ADM inhibits the viability of U2-OS cells. The viability of U2-OS cells was significantly reduced following treatment with SM-164, ADM, and combined treatment. *P<0.05 vs. control group. #P<0.05 vs. SM-164 + ADM. ADM, Adriamycin; OD, optical density.

determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). SDS-PAGE (12%) was used to separate 25 µg of protein from each group. Samples were then transferred onto nitrocellulose membranes for western blot analysis. After a 2-h blocking in 5% bovine serum albumin (BSA) at room temperature, membranes were incubated with the following primary antibodies: Anti-GAPDH (1/2,000; TA-08; OriGene Technologies, Inc., Beijing, China); anti-caspase 7 (1/2,000; ab25900; Abcam, Cambridge, UK); anti-active caspase 9 (1/5,000; ab2324; Abcam); anti-active caspase 3 (1/10,000; ab2302; Abcam); anti-poly (ADP-ribose) polymerase (PARP) (1/5,000; ab32138; Abcam); anti-XIAP (1/500; bs-1281R; BIOS, Beijing, China); anti-cIAP1 (1/5,000; ab108361; Abcam) and anti-survivin (1/5,000; ab76424; Abcam). Following incubation with primary antibodies at 4°C overnight, nitrocellulose membranes were washed three times and incubated with a secondary antibody (horseradish peroxidase-labeled goat anti-rabbit IgG; cat. no. A16104SAMPLE; Thermo Fisher Scientific, Inc.) at 4°C for 2 h. Bands were visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific,

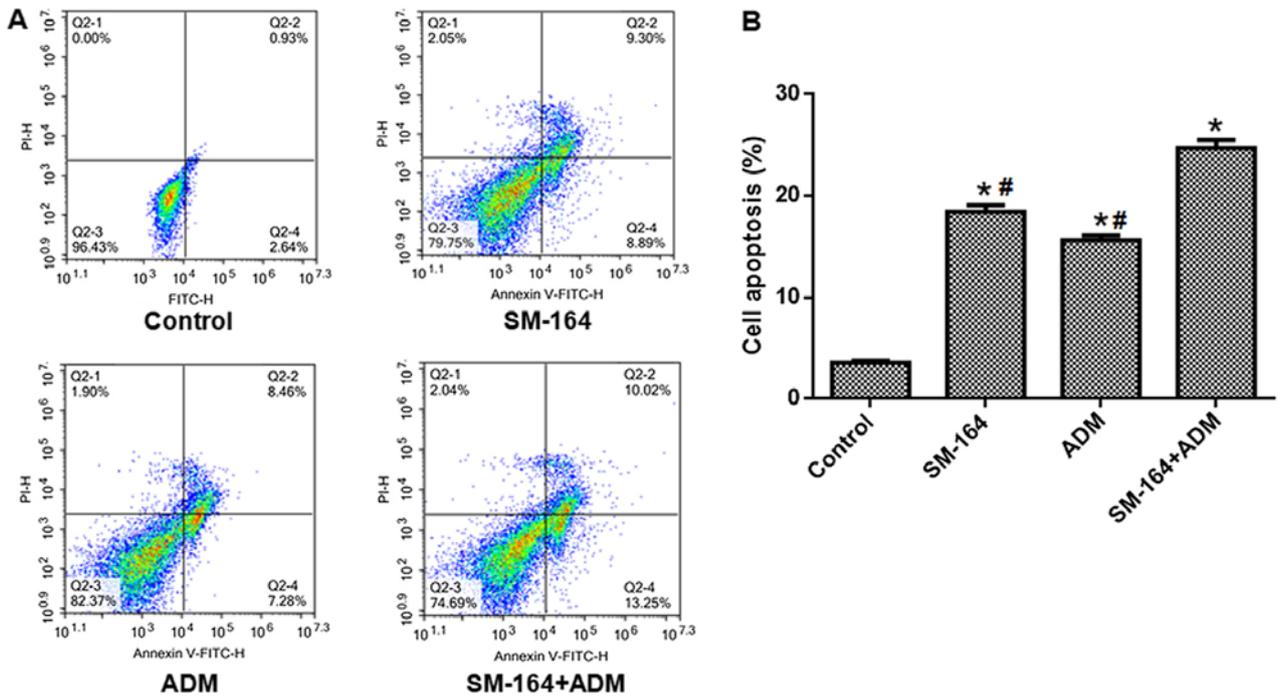


Figure 2. Combination of SM-164 with ADM promotes the apoptosis of U2-OS cells. (A) Representative flow cytometry plots. (B) Quantification data of flow cytometry analysis. *P<0.05 vs. control group. #P<0.05 vs. SM-164 + ADM. ADM, adriamycin; FITC, fluorescein isothiocyanate; PI, propidium iodide.

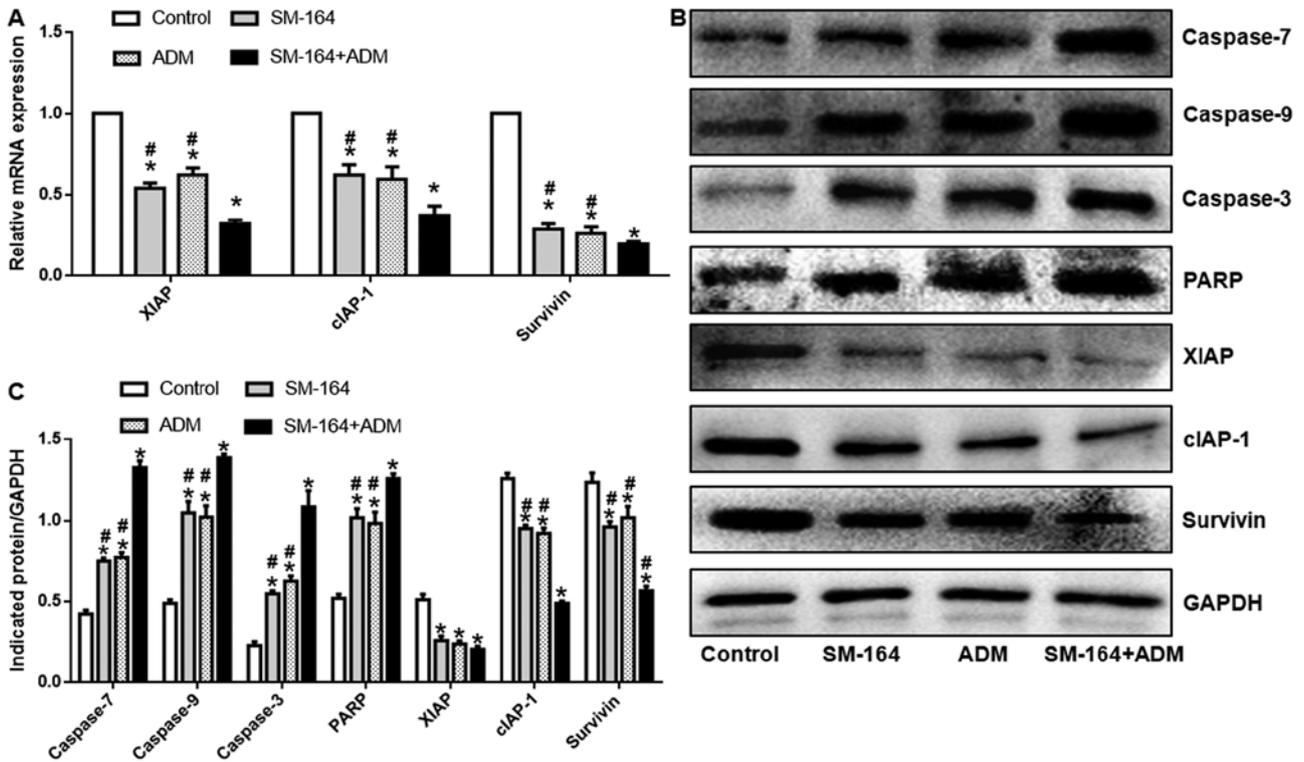


Figure 3. Combination of SM-164 with ADM promotes caspase-7, caspase-9, caspase-3 and PARP expression, but reduces that of XIAP, survivin and cIAP-1 expression. (A) mRNA expression was determined via reverse transcription-quantitative polymerase chain reaction. (B) Representative blots and (C) quantitative results of protein expression. *P<0.05 vs. control group. #P<0.05 vs. SM-164 + ADM. ADM, adriamycin; cIAP-1, cellular inhibitor of apoptosis protein 1; PARP, poly ADP-ribose polymerase; XIAP, X-linked inhibitor of apoptosis protein.

Inc.). Blot densities were quantified using Quantity One software (v4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

XIAP silencing. Short hairpin RNA (shRNA) was used to silence the expression of XIAP; the sequences were: Forward 5'-CACCCATGCAGCTGTAGATAGATGGCAATCGAA

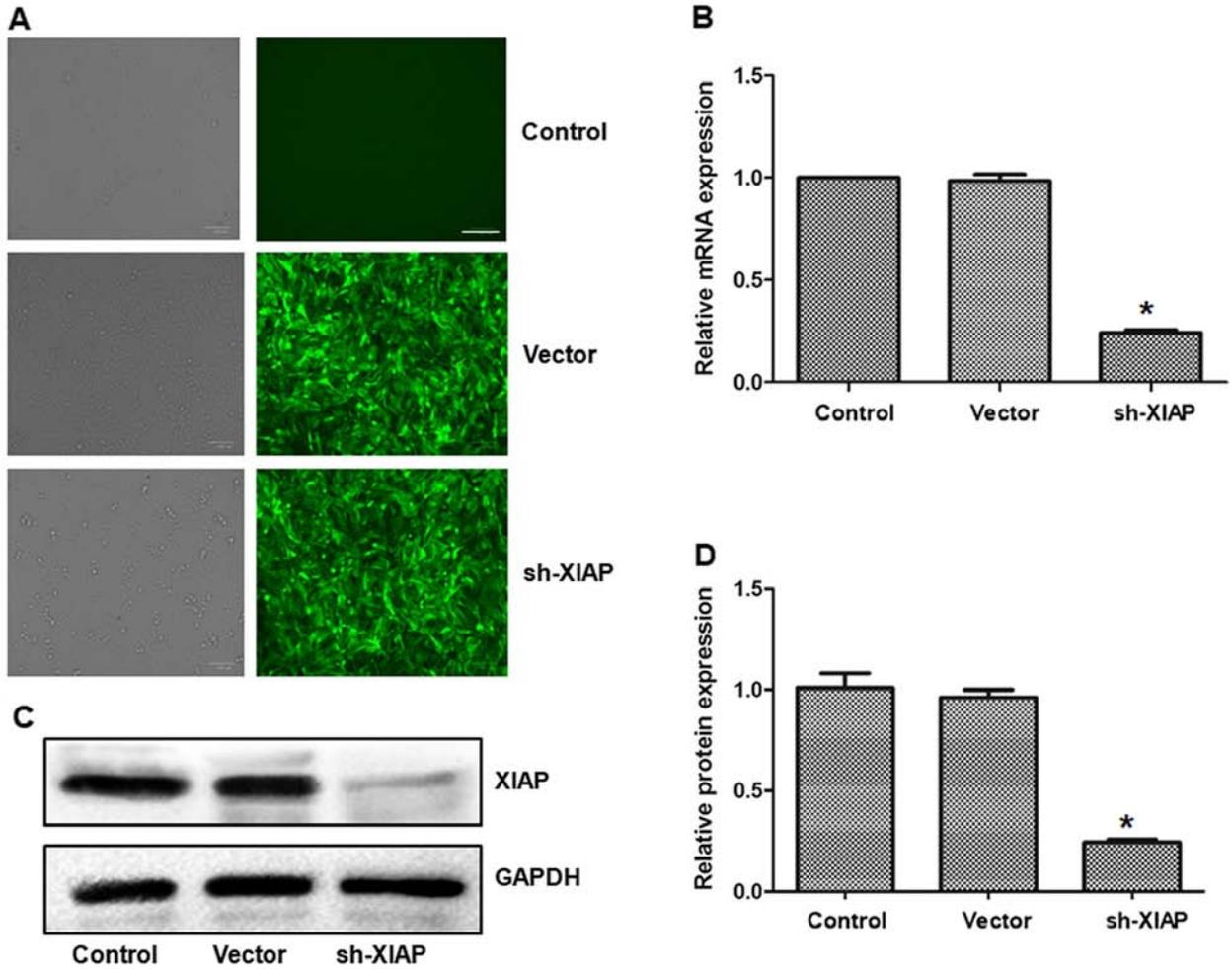


Figure 4. Silencing efficiency of XIAP silencing lentivirus. (A) Cells observed under light and fluorescence microscopes following transfection. Scale bar, 100 μ m. (B) mRNA expression of XIAP as determine via reverse transcription-quantitative polymerase chain reaction. (C) Representative western blotting of XIAP and (D) protein expression of XIAP. * $P < 0.05$ vs. control group. ADM, adriamycin; sh, short hairpin RNA; XIAP, X-linked inhibitor of apoptosis protein.

ATTGCCATCTATCTACA-3' and reverse, 5'-AAAACATGCAGCTGTAGATAGATGGCAATTTTCGATTGCCATCTATCTACA-3' (Sangon Biotech Co., Ltd., Shanghai, China). After annealing the double chain DNA at 56°C for 30 sec, the sequences were integrated into the lentiviral skeleton carrier PDS019_pL_shRNA_F (Shanghai Novobio Co., Ltd., Shanghai, China). Recombinant lentiviral plasmid liposomes (Invitrogen; Thermo Fisher Scientific, Inc.) were transfected into 293T cells (American Type Culture Collection, Manassas, VA, USA) to produce recombinant lentivirus PDS019-PL-shRNA-GFP-homo- XIAP. Viral replication was detected by fluorescence microscopy, while the titer was determined to be 1.8×10^{11} pfu/ml according to a 50% tissue culture infective dose method (18). Cells (1×10^5 /ml) were seeded in 96-well plates. The lentivirus was applied for transfection 24 h later. On the second day, 2 ml of original medium (RPMI-1640) was replaced with medium containing 6 mg/ml Polybrene. An appropriate amount of viral suspension was added. After 4 h, 2 ml of fresh medium was added to dilute the Polybrene. Fresh medium was added 2 h later.

U2-OS cells were treated with 0.5 μ g/ml ADM + 200 nM SM-164 as aforementioned. Following the addition of ADM into cellular media for 2 h, cells were washed with PBS and

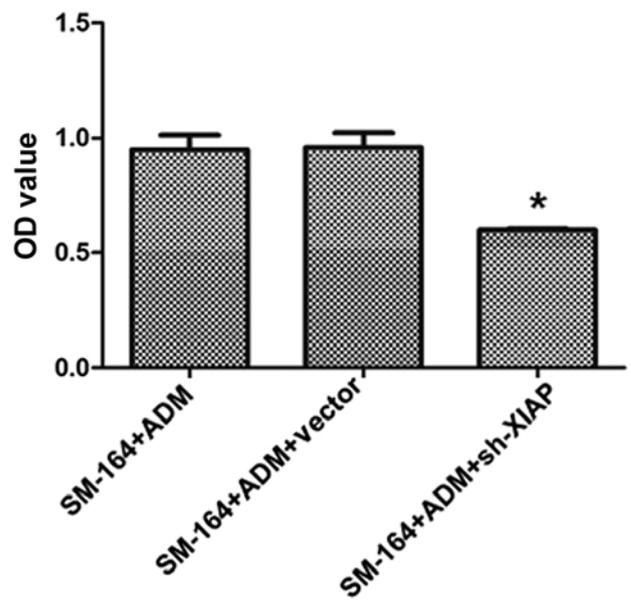


Figure 5. Effects of XIAP on U2-OS viability. Silencing of XIAP resulted in suppressed cell viability * $P < 0.05$ vs. ADM + SM-164. ADM, adriamycin; OD, optical density; sh, short hairpin RNA; XIAP, X-linked inhibitor of apoptosis protein.

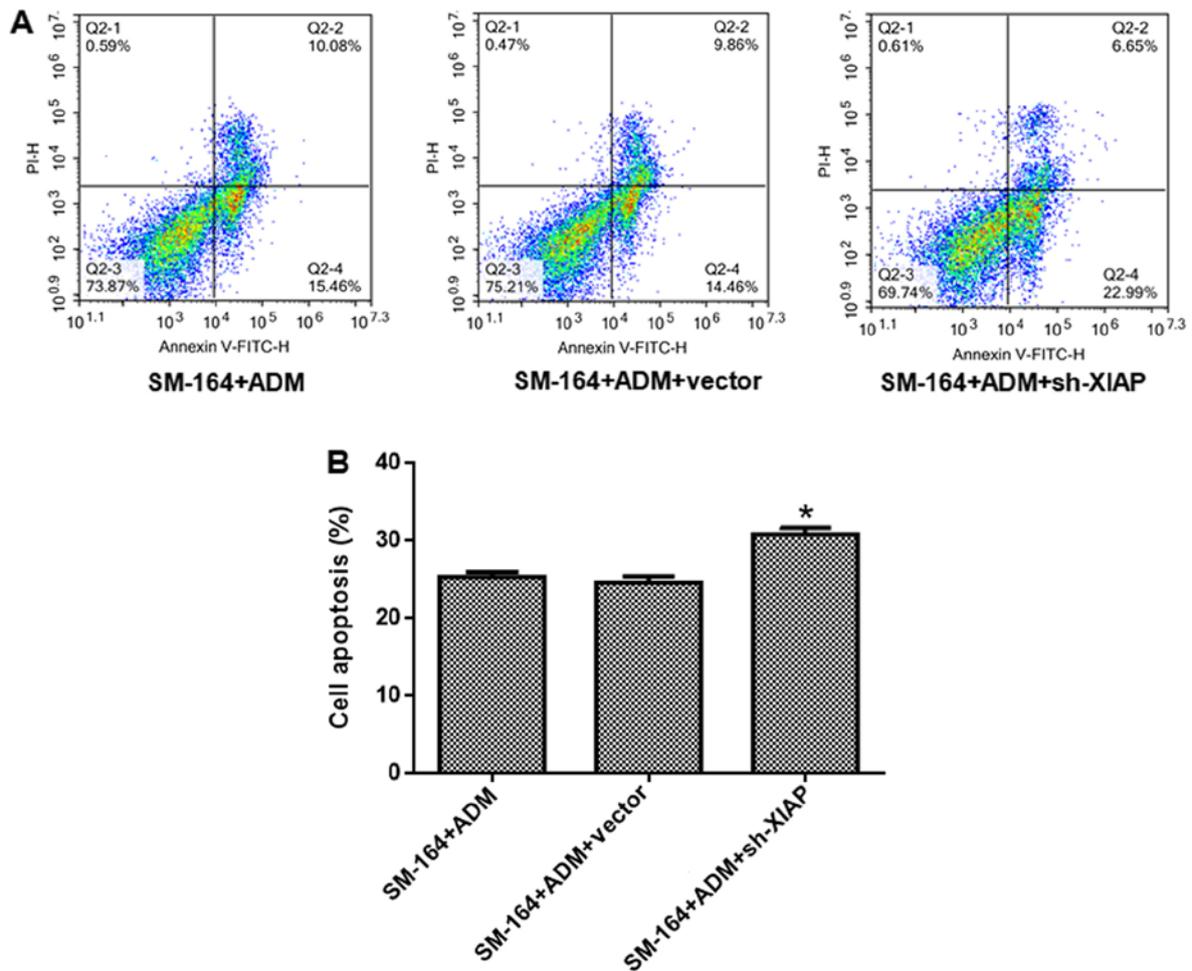


Figure 6. Effects of XIAP on apoptosis. (A) Representative flow cytometry plots. (B) Quantification of flow cytometry data. * $P < 0.05$ vs. ADM + SM-164 group. ADM, adriamycin; sh, short hairpin RNA; XIAP, X-linked inhibitor of apoptosis protein.

treated with SM-164 (200 nM). Cells were further divided into three groups after 24 h: ADM + SM-164, ADM + SM-164 + vector (empty), and ADM + SM-164 + shRNA-XIAP (sh-XIAP) groups. MTT, RT-qPCR and western blotting were performed as aforementioned 24 h after transfection.

Statistical analysis. All numerical data from six repeated experiments were expressed as the mean \pm standard deviation. Statistical analyses were performed using one-way analysis of variance followed by a Bonferroni post-hoc test. Analysis was conducted using SPSS software version 17 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Combination of SM-164 with ADM reveals enhanced inhibition of cell viability. As presented in Fig. 1, the OD values of the groups respectively treated with ADM and SM-164 were significantly decreased compared with the control group; the OD value decreased further in the combined treatment group. These data suggest that a combination of SM-164 and ADM reveals enhanced inhibition of cell viability compared with single application of SM-164 or ADM.

Combined SM-164 and ADM enhances apoptosis. As presented in Fig. 2, the apoptosis of cells treated with ADM or SM-164 alone increased significantly compared with the control group, but was significantly reduced than under conditions of combined treatment. These data suggest that combination of SM-164 with ADM reveals enhanced apoptosis compared with single application of SM-164 or ADM.

Combined SM-164 and ADM treatment exhibits enhanced effects on caspases-7, -9, -3, PARP, survivin and cIAP-1 expression. The mRNA expression levels of XIAP, cIAP-1 and survivin were significantly decreased following ADM or SM-164 treatment alone; however, expression was significantly increased compared with combined treatment (Fig. 3A). In addition, the protein expression levels of caspases-7, -9, and-3, and PARP increased significantly, while that of XIAP, survivin and cIAP-1 decreased significantly in groups treated with ADM or SM-164, compared with the control. Of note, the expression of these proteins was significantly upregulated following treatment with ADM or SM-164 compared with combined treatment (Fig. 3B and C). These data suggest that combination of SM-164 and ADM demonstrates increased effects on apoptosis-related protein expression compared with single application of SM-164 or ADM.

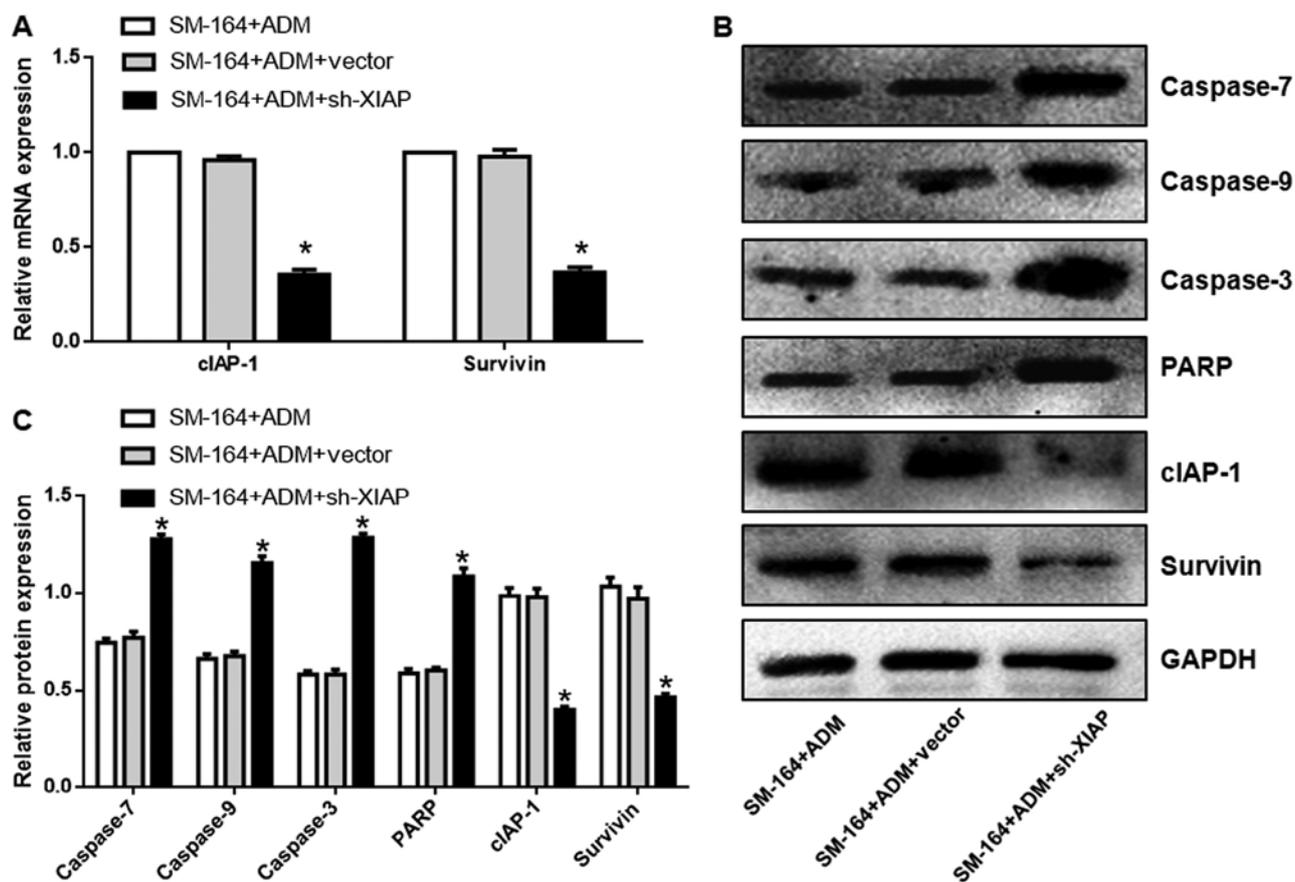


Figure 7. Effects of XIAP silencing on the expression of caspase-7, -9, -3, PARP, survivin and cIAP-1. (A) mRNA expression of cIAP-1 and survivin. (B) Representative western blot and (C) quantitative results of protein expression. * $P < 0.05$ vs. ADM + SM-164 group. ADM, adriamycin; cIAP-1, cellular inhibitor of apoptosis protein 1; PARP, poly ADP-ribose polymerase; sh, short hairpin RNA; XIAP, X-linked inhibitor of apoptosis protein.

Silencing of XIAP further suppresses viability and promotes apoptosis. As presented in Fig. 4A, cells in the vector and XIAP-silencing groups were successfully transfected. The expression of XIAP in the sh-XIAP group was significantly reduced at the mRNA and protein levels than in the ADM + SM-164 group ($P < 0.05$; (Fig. 4B-D).

As presented in Fig. 5, the OD value of the ADM + SM-164 + XIAP silencing group decreased significantly, and the difference was statistically significant compared with the ADM + SM-164 group ($P < 0.05$). As shown in Fig. 6, the number of apoptotic cells in the ADM + SM-164 + XIAP silencing group increased significantly; the difference was statistically significant compared with the ADM + SM-164 group ($P < 0.05$). These data suggest that silencing of XIAP further suppresses viability and promotes apoptosis compared with SM-164 treatment.

Effects XIAP silencing on caspases-7, -9 and -3, PARP, survivin and cIAP-1 expression. As shown in Fig. 7A, compared with the ADM + SM-164 group, the mRNA expression levels of survivin and cIAP-1 were significantly lower. In addition, the protein expression levels of caspases-7, -9, and -3, and PARP in the ADM + SM-164 + XIAP silencing group were significantly increased compared with the ADM + SM-164 group; the expression of cIAP-1 and survivin were also downregulated ($P < 0.05$; Fig. 7B and C). These data suggest that silencing of XIAP further promotes

apoptosis-related protein expression compared with SM-164 treatment.

Discussion

Neoadjuvant chemotherapy can induce primary tumor necrosis, facilitate surgical resection and eliminate micro-metastases (19). The main anticancer effect of ADM has been reported to involve the inhibition of topoisomerase II and reduced DNA stability caused by free radicals (20-22). SM-164 can combine with IAPs, promote the rapid degradation of cIAP-1, and effectively interfere with the inhibition of caspases-9, -3 and -7 mediated by XIAP. SM-164 is an effective inducer of apoptosis in tumor cells and xenotransplanted tissues (9). The results of the present study revealed that SM-164 and ADM suppressed the viability of U2-OS cells, with combined SM-164 and ADM treatment exhibiting a synergistic pharmacological effect. In addition, we reported SM-164 and ADM to promote the apoptosis of U2-OS cells, and that combined use of the two drugs further increased apoptosis.

IAPs mainly inhibit caspases, inactivate apoptosis pathways, and interfere in cell apoptosis (11). IAPs can inhibit the caspase-dependent apoptosis pathway by combining with caspases-3, -7 and -9; IAPs have become ideal target proteins for altering drug resistance of several key chemotherapeutic drugs (23). ADM can act in concert with survivin (and other

IAP family proteins) in most tumor cells via downregulation of IAP anti-apoptotic factors; thus, the mitochondrial pathway of tumor cell apoptosis may be induced (11). SM-164 is a Smac protein small molecule mimic. In a variety of tumor cells expressing IAPs, caspases-9 and -3 are activated to inhibit the apoptosis of tumor cells (24). SM-164 is able to enhance the antitumor effects of ADM and reduce toxicity to normal cells (8). SM-164 can stimulate Smac protein to initiate apoptotic signaling pathways and further alleviate the inhibitory effects of IAPs to its effectors (caspases-3 and -7, and the apoptosis-initiating factor caspase-9), which mediates a caspase cascade reaction to induce U2-OS cell apoptosis (25).

Our results revealed that treatment with ADM and SM-164 alone significantly increased the expression of apoptosis-associated factors including caspases-3, -7 and -9. The expression of anti-apoptotic factors, such as XIAP, cIAP and survivin were also reported to be decreased; combined drug treatment was observed to be the most effective. This is also consistent with the results of a recent study (24). XIAP is the most potent caspase inhibitor in the IAP family of proteins, possessing three BIR domains at its N-terminal, which can regulate the death-receptor pathway and mitochondrial pathway-dependent apoptosis (26). BIR domain 3 in XIAP can effectively inhibit the activity of caspase-9 (27). The association between BIR1 and BIR2 can selectively inhibit caspases-3 and -7 (28).

Furthermore, the results of the present study demonstrated that XIAP silencing inhibited cell viability and increased apoptosis. Our findings also revealed that silencing XIAP suppressed the expression of cIAP and survivin, yet the expression of caspases-3, -7 and -9 were upregulated. Therefore, XIAP may serve a regulatory role in tumor cell apoptosis. ADM and SM-164 were reported to inhibit cell viability and promote apoptosis, which could possibly occur via XIAP downregulation. These results suggest the potential application of this combined treatment in clinical settings; however, further investigation is required. Our future studies aim to confirm these findings using a XIAP overexpression vector. Overexpression of XIAP may provide insight into the combined effects of ADM + SM-164 as a potential treatment of OS.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JC, XuC, XiC and HS performed the experiments and analyzed the data. DY designed the study and wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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