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Original Article

SMAC mimetic SM-164 enhanced adriamycin induced apoptosis and cell cycle arrest in osteosarcoma cell line HOS

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Abstract: Osteosarcoma is the most common form of primary bone cancer in adolescent with a male predominance. Current treatment for osteosarcoma is still limited and ineffective. With metastasis or recurrence, the chance of long-term survival always bellows 20%, which suggested a requirement of developing novel therapy. In this study, we investigate if Second mitochondria-derived activator of caspases (SMAC) mimetic SM-164 could enhance Adriamycin induced apoptosis and cell cycle arrest in Osteosarcoma cell line. Our data suggested that SM-164 induced osteosarcoma cell proliferation inhibition in a dose depended manner. Combined treatment SM-163 and Adriamycin enhanced apoptosis induction in HOS cells. Treatment of the cell with SM-164 modestly induced protein degradation of both c-IAP1 and XIAP, which were inhibitors of apoptosis. Moreover, SM-164 enhanced Adriamycin induced cell cycle arrest in HOS cells. In conclusion, our data suggested that SM-164 may be a novel candidate as adjuvant for chemotherapy of osteosarcoma.

Keywords: Osteosarcoma, apoptosis, second mitochondria-derived activator of caspases, SM-164, adriamycin

Introduction

Osteosarcoma is the most common form of primary bone cancer and most prevalent in children and young adults with a male predominance [1-3]. Osteosarcoma is considered as aggressive malignant neoplasm which originated from cells with mesenchymal origin (and thus a sarcoma) [4]. Therefore, osteosarcoma generally exhibits osteoblastic differentiation and produces malignant osteoid [4].

Although osteosarcoma can occur in any bone, most malignancy sites are frequent found in the metaphyseal regions of the distal femur, proximal tibia and proximal humerus [3], Osteosarcoma is generally characterized by a local invasion of bone and soft tissues, function loss of the affected extremity and distant metastasis [3]. Without metastases during diagnosis, osteosarcoma patients have a five-year survival rate from 60% to 70% if aggressive surgical resection and chemotherapy were combined together for therapy [5]. However, in these cases with metastasis or recurrence, the chance of long-term survival always bellows 20% [4]. Therefore, development of novel therapy is urgently needed.

The second mitochondria-derived activator of caspases (SMAC), also named as Diablo homolog (direct IAP binding protein with low pI), a mitochondrial protein locating in the intermembrane space of mitochondrial, promotes cytochrome c- and TNF receptor-dependent activation of apoptosis (intrinsic pathway) by inhibiting the effect of inhibitor of apoptosis proteins (IAP), a class of proteins that negatively regulates apoptosis [6, 7].

The full-length of SMAC protein contains 239 amino acids residues, but the 55aa N-terminal which containing mitochondrial-targeting sequence (MTS) will be cleaved after it transportation to mitochondrial and leads to the expose of N-terminal Ala-Val-Pro-Ile sequence (AVPI) of

cleaved SMAC [8-10]. The tetrapeptide sequence AVPI is the core of binding domain to IAP, which is essential for inhibiting function of XIAP [10]. Many studies suggested that SMAC is involved in cancer due to its pro-apoptotic function and overexpression of SMAC in tumor cells leads to sensitized tumor cells for apoptosis induction [11]. As a result, small-molecule mimetics for the tetrapeptide AVPI sequence of SMAC have been developed to mimic the in the IAP binding domain of SMAC to induce cell death in tumor cells [12-15].

SM-164 is a bivalent cell-permeable Smac mimetic, which is 1,000 times more potent than SM-122 as an inducer of apoptosis in tumor cells [16, 17]. Previous reports demonstrated that combination of SM-164 with TNF-related apoptosis inducing ligand (TRAIL) represented a new therapeutic strategy for cancer and SM-164 is able to enhance Doxorubicin-mediated anticancer activity in hepatocellular carcinoma cells [17, 18]. Moreover, SM-164 is able to induce radiosensitization in breast cancer cells as well [19]. In this study, we investigate the role of SMAC mimetic SM-164 as adjuvant for chemotherapy for osteosarcoma cell line. Our data suggested that SM-164 may be a novel adjuvant for future development of more effective chemotherapy of osteosarcoma.

Materials and methods

Cell and chemicals

Osteosarcoma derived cell line HOS (ATCC® CRL-1543™) was purchased from ATCC and maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco). SM-164 was purchased from Beyotimes (Beijing, China) and solved in DMSO according to manufacturer's instruction. Doxorubicin hydrochloride (Adriamycin) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell proliferation assay (MTT)

The trypsinized HOS cells were first stained by trypan blue (Sigma) for counting of living cells. Then the single cell suspension was seeded in 96 well plates with a density of 2×10^4 cell in each well. After overnight incubation, indicated treatment were conducted, then the cell proliferation was determined at the indicated time points by using MTS Cell Proliferation Colorimetric Assay kit (Biovision, Milpitas, CA, USA) according manufacturer's instruction with VICTOR™ X5 Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA).

Flow cytometry based cell apoptosis and cell cycle assay

Cells from indicated groups were treated accordingly. The trypsinized cells were fixed with 70% ethanol and permeabilized by PBS containing 0.5% Triton X100 (Sigma-Aldrich). A totally 1×10^6 cells were stained with FITC labeled Annexin V and Propidium iodide. Then the stained cells were analyzed via flow cytometry machine (FACSCalibur, BD Biosciences, San Jose, CA, USA) for apoptosis analysis.

For cell cycle analysis, a totally 1×10^6 cells of each group were fixed with 70% ethanol and permeabilized by PBS containing 0.5% Triton X100 (Sigma) along with treating of DNase-free RNase A (Sigma). Then the fixed cells were stained by Propidium iodide for cell cycle analysis by flow cytometry machine (FACSCalibur, BD Bioscience).

SDS-PAGE and Western blotting

The SDS-PAGE was conducted as previously described [20, 21]. Briefly, SDS-PAGE separated proteins were transferred into PVDF membrane, followed by blocking with SuperBlock™ (PBS) Blocking Buffer (ThermoFisher Scientific, Waltham, Massachusetts, USA) for 15 mins. Then the membrane was probed by rabbit anti-XIAP (SC-11426, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-LC-3 (Sigma-Aldrich). Specific reactions between antibodies and corresponding proteins were detected by using goat anti-rabbit poly clonal antibodies conjugated with horseradish peroxidase (Sigma-Aldrich) and revealed by SuperSignal Chemiluminescence Substrate (ThermoFisher Scientific). The luminescence signal was digitally recorded by the ChemiDoc MP system (Bio-Rad, Hercules, CA, USA) and analyzed with the ImageLab Program (Version 6.1, Bio-Rad). The same membrane was also blotted with antibody targeting β-actin (Santa Cruz) for normalization the total protein loading during SDS-PAGE.

Statistical analysis

All quantification data and statistical analysis was conducted using Excel program of Office

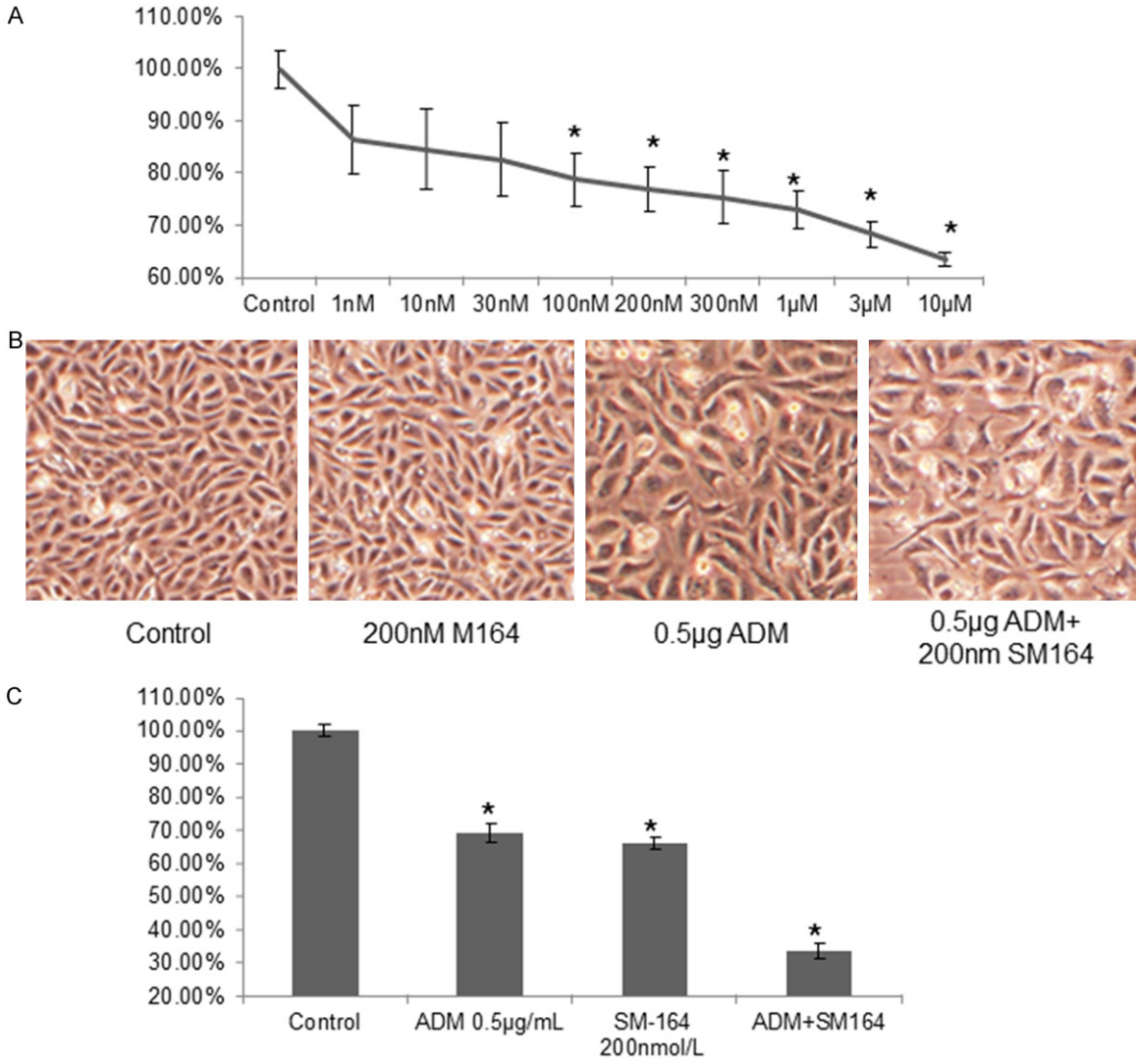

Figure 1. SM-164 combined with Adriamycin inducing osteosarcoma cell proliferation inhibition. A: Dose depended inhibition of HOS cell proliferation by SM-164. B: HOS cell was seeds in 12 well plate for 24 h, then treated with 200 nM SM164, or 0.5 μg ADM or both for 24 hours. Then cells were observed under microscope. C: MTT based cell proliferation assay for HOS cell treated with 200 nM SM164, or 0.5 μg ADM or both for 24 hours. Error bar represented SD from 3 times repeats of experiments. Significant differences between cancer tissues and normal he are shown by "*" to indicate significant difference ($P < 0.05$).

2010 software suit (Microsoft, Seattle, WA, USA). Data are presented as the Mean ± SD. Difference in indicators between samples were subjected to the Student's *t* test. A two tailed *P*-value of less than 0.05 was considered statistically significant.

Results

SM-164 induced osteosarcoma cell proliferation inhibition in a dose depended manner

To investigate the role of SM-164 in osteosarcoma derived HOS cell, we first check the proliferation of HOS cell under different concentrations of SM-164. As demonstrated in **Figure 1A**, with the adding of SM-164, proliferation of HOS cell stated to reducing as the concentration of SM-164 increased. However, increasing the SM-164 dose from 1nM to 10 μM (10000 folds) only modestly reduced the HOS proliferation rate from 90% to 64% (**Figure 1A**), implied proliferation inhibition induced by SM-164 alone was not enough. Therefore, it is interesting know if SM-164 combined with a chemodrug together could resulted a stronger inhibition since previous studies demonstrated that SM-164 is able to enhance Doxorubicin-mediated anticancer activity in hepatocellular car-

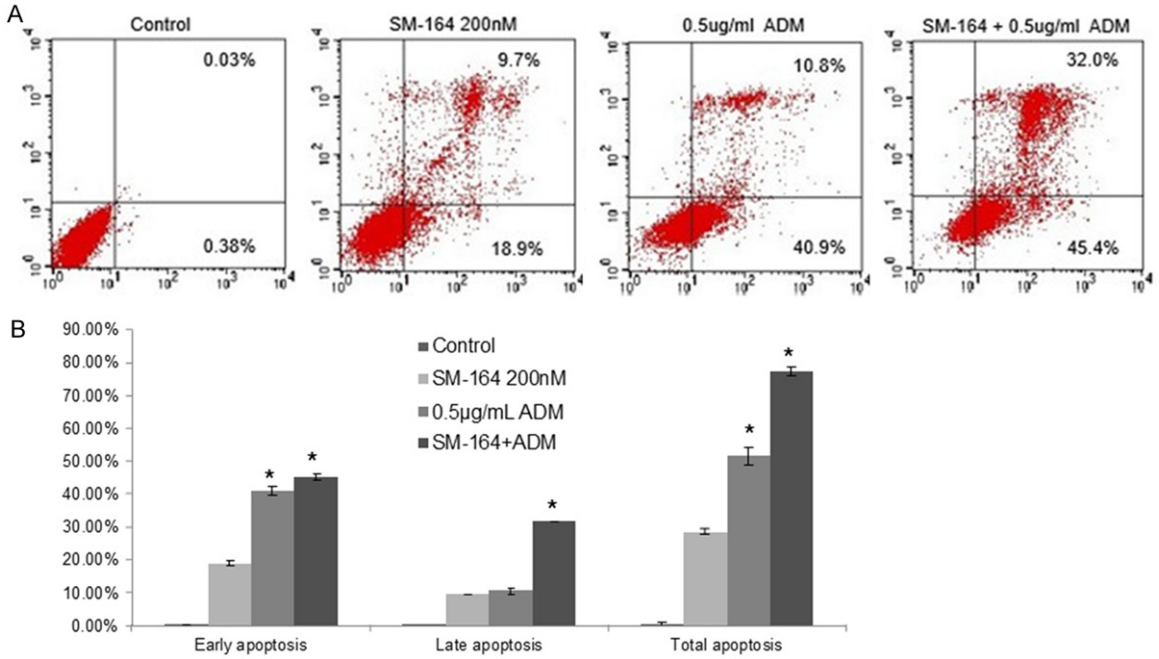

Figure 2. SM-163 enhance Adriamycin induced apoptosis in HOS cells. A: Flow cytometry based apoptosis analysis for HOS cell treated with 200 nM SM164, or 0.5 μg ADM or both for 24 hours. B: Statistical analysis for FCM based apoptosis assay, Error bar represented SD from 3 times repeats of experiments. Significant differences between cancer tissues and normal he are shown by "*" to indicate significant difference ($P < 0.05$).

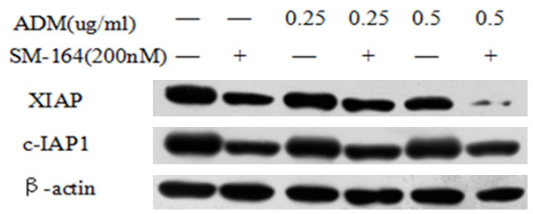

Figure 3. SM-164 treatment inhibit expression of inhibitor of apoptosis (IAPs). Western blot for protein expression level of XIAP and c-IAP1; HOS cell were treated with indicated dose of ADM and SM164, then lysed for WB analysis. Anti-β actin was included as protein loading control for normalization.

cinoma cells [17, 18]. In our study, we used Adriamycin (ADM) as the stimulator for apoptosis, since ADM has been widely used for treating primary Osteosarcoma [22-24]. Compared to the cells treating with SM-164 or ADM alone, combination of both significantly inhibited the growth of HOS cells (**Figure 1B** and **1C**).

SM-163 enhance adriamycin induced apoptosis in HOS cells

ADM induces histone eviction from transcriptionally active chromatin [25]. DNA damage response, epigenome and transcriptome deregulation lead to apoptosis in ADM-exposed cells [25]. As data above demonstrated that treatment of HOS cell with SM-164 could enhanced ADM induced cell proliferation inhibition, we further explorer examined if SM-164 enhanced ADM induced apoptosis in HOSs. Based on our flow cytometry data, under the normal condition, there were little cells experiencing early or late apoptosis (**Figure 2A**). With the treatment of 200 nM SM-164 alone, there were 18.9% and 9.7% of the total cells undergo early apoptosis and later apoptosis, respectively. Moreover, with the treatment of 0.5 μg/mL ADM, about 40.9% and 10.8% HOS cells were experiencing early and later apoptosis (**Figure 2A**). However, with the combination of both ADM and SM-164, the cells undergo early apoptosis and later apoptosis were significantly increased to 32% and 45.4%, respectively. Moreover, by repeating the experiment for 3 times, quantification analysis for FCM data also confirmed our observation (**Figure 2B**). Taken together, these data suggested the SM-163 sensitized HOS cells for apoptosis induced by ADM.

SM-164 treatment inhibit expression of inhibitor of apoptosis (IAPs)

It has been reported that SMAC proteins act as endogenous antagonist for inhibitor of apopto-

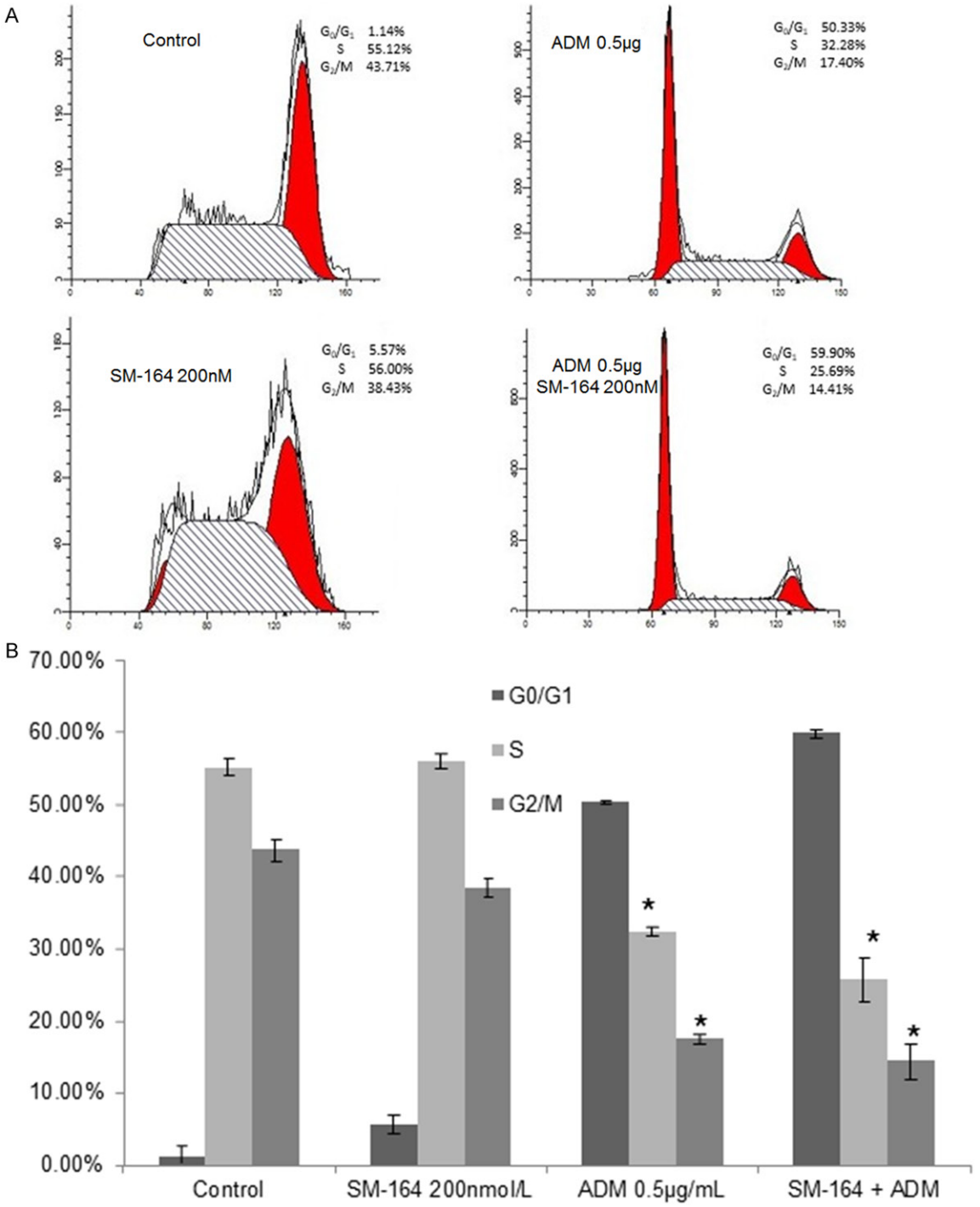

Figure 4. SM-164 enhanced ADM induced cell cycle arrest in HOS cells. A: Flow cytometry based cell cycle analysis for HOS cell treated with 200 nM SM164, or 0.5 μg ADM or both for 24 hours. B: Statistical analysis for FCM based cell cycles assay, Error bar represented SD from 3 times repeats of experiments. Significant differences between cancer tissues and normal he are shown by "*" to indicate significant difference ($P < 0.05$).

sis (IAPs) and SMAC mimetic SM-164 could induce rapid degradation of cIAP-1 in breast cancer cell line MDA-MB-231 [16]. It is interesting to know if SM-164 plays the same role in HOS cells to during the ADM mediated apoptosis induction. Therefore, we examine both cIAP-1 and XIAP protein level in treated cells. Our data demonstrated that exposed HOS cells

alone to different dose of ADM did not changed the protein level of cIAP-1 and XIAP (**Figure 3**). On the other hand, treatment of the cell with SM-164 modestly induced protein degradation of both c-IAP1 and XIAP (**Figure 3**). Notably, with increasing of the ADM dose to 0.5 μg, SM-164 induced a very strong degradation of XIAP.

SM-164 enhanced ADM induced cell cycle arrest in HOS cells

Except apoptosis induction, ADM stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby inhibiting the process of replication [26]. Therefore, we expected the SM-164 could enhance ADM induced cell cycle arrest as well. To verify this, HOS cells with indicted treatments were analyzed in FCM. It was reported that ADM mainly induced cell cycle arrest in G0\G1 phase [27]. In our study, treatment of HOS cells with ADM lead to more than half of the total cells stay in the G0\G1 phase (**Figure 4A**). On the other hand, treatment of the cells with SM-164 only did not show significant change in the cell cycle (**Figure 4A**). However, in cell with combined treatment of ADM and SM-164 significantly increase the cells in G0\G1 phase up to 60% (**Figure 4A** and **4B**). Therefore, SM-164 could enhance ADM induced cell cycle arrest in HOS cells. Taken together, our result demonstrated that SM-164 could enhanced the apoptosis induction and cell proliferation effect of ADM in osteosarcoma cell line HOS cell, which may presentation a novel method for future osteosarcoma therapy.

Discussion

With current treatment methods, long-term survival probabilities for osteosarcoma have improved dramatically during the late 20th century and approximated 68% in 2009 [1, 28]. However, unlike other cancer, osteosarcoma are mostly prevalent in children and young adults with a male predominance [1-3]. Therefore, it is necessary to improve current therapeutic methods so the young patients could survive as long as possible. As part of the standard chemo-therapy drug for osteosarcoma, Adriamycin was widely used for osteosarcoma patients after surgery removal of tumor. However, the side effect and drug-resistance is a major problem for Adriamycin application. Therefore, development of novel adjuvant which is able to enhance the anti-tumor effect of ADM is an alternative strategy.

SM-164 is a novel bivalent SMAC mimetic. Compared with its monovalent form SM-162, SM-164 is 1,000 times more potent than SM-122 as an inducer of apoptosis in tumor cells [16]. In our study, treatment of HOS cells with SM-163 could provoke apoptosis. However, the level of cells undergo apoptosis is much less than HOS cells treated with ADM, which suggested the SM-164 cannot act alone as an anti-tumor drugs. However, if combined with ADM together, the ADM induced apoptosis is enhanced by co-treatment of SM-164. Moreover, it is very interesting that our data suggesting that the degradation of cIAP-1 by SM-164 is modest sine previous data suggesting that SM-164 provokes a very rapid degradation of cIAP-1 to undetectable level in breast cancer cells [16]. This may suggest sensitivity of IAPs to SM-164 is varying in different cancer types.

On the other hand, we also observed that SM-164 could down-regulate the expression of XIAP in HOS cells, which is not reported before. It is still unclear the reduced expression of XIAP in SM-164 treated HOS cell was caused by SM-164 induced degradation in a similar manner of c-IAP1 since treatment of SM-164 to breast cancer cells did not lead to degradation of XIAP in previous report [16]. In conclusion, SM-164 could enhanced the ADM mediated proliferation inhibition of osteosarcoma cell line HOS via sensitized cancer cells for apoptosis induction and cell cycle arrest. Taken together, our data suggested that SM-164 may be a novel candidate as adjuvant for chemotherapy of osteosarcoma.

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Disclosure of conflict of interest

None.

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