·Basic Research·

MS-275, a histone deacetylase inhibitor, induces apoptosis and alters survivin gene expression in human myeloma cell line U266

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[Abstract] **Background and Objective:** Histone deacetylase inhibitors (HDACi) exert antitumor effects through the induction of apoptosis. This study was to investigate the effects of HDACi on survivin gene expression, cell proliferation and apoptosis in human myeloma cell line U266. **Methods:** U266 cells were exposed to different concentrations of MS-275, a histone deacetylase inhibitor, at different time courses. Cell viability was measured using the trypan blue exclusion assay. Changes in cell morphology were observed with Wright-Giemsa staining. Cell cycle was analyzed using flow cytometry. Protein expressions of poly (ADP-ribose) polymerase (PARP), caspase-3, survivin, P21 and CDK4 were detected by Western blot. **Results:** MS-275 inhibited the growth of U266 cells in a dose- and time-dependent manner. After exposure to 1.39 μmol/L MS-275 for 48 h, the cell cycle was arrested at the G_0/G_1 phase, and the cell viability was decreased by 50%. After the treatment with 2μmol/L MS-275 for 24 h and 36 h, the ratios of U266 cells at the G_0/G_1 phase were increased to 66.39% and 89.80%, respectively. Obvious changes in morphology of U266 were observed under microscopy. Cleaved PARP appeared, along with an increased protein level of P21 and a decrease of survivin and CDK4 levels in U266 cells treated with MS-275. **Conclusion:** MS-275 could suppress the proliferation, induce apoptosis and reduce the expression of survivin in human myeloma cell line U266, which may be associated with the down-regulation of survivin.

Key words: histone deacetylase inhibitors, human myeloma cell line U266, MS-275, survivin, apoptosis

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Histone acetylation/deacetylation modification is a key mechanism to mediate gene transcription and is closely correlated with occurrence of cancer.[1] Histone deacetylase inhibitors (HDACi) are a class of novel drugs with anti-tumor activity. Research found that HDACi, with wide anti-tumor activity, exerted suppression on hematological tumors and solid tumors in terms of cell cycle arrest, and induction of differentiation and apoptosis.[2] MS-275 is a strong HDACi of the benzamides, and has been demonstrated to have a strong anti-tumor effect on many human tumor cell lines with high efficiency, low toxicity, good toleration, as well as strong specificity

and selectivity for tumors.[3] The current study was to investigate the effect of MS-275 on proliferation, apoptosis and survivin expression in U266 cells, revealing the relevant mechanism of malignant proliferation of myeloma cells.

Materials and methods

Main reagents. MS-275 was obtained from Prof. Bolin Liu (University of Colorado School of Medicine, USA). Wright and Giemsa stain set was purchased from Beijing Saichi Biotechnology, Inc (Beijing, China). RNase A and PI were purchased from Sigma-Aldrich China Inc. (Shanghai, China). The primary antibodies, PARP and Acetyl-Histone H3, and secondary antibody, Anti-Mouse/Rabbit IgG (HRPlinked) were purchased from Cell Signaling Technology, Inc.(Danvers, Massachusetts, USA). P21 was purchased from Santa Cluz Biotechnology, Inc. (Santa Cluz, California, USA). BCA™ Protein Assay Kit and Chemiluminescent Substrate were purchased from Thermo Fisher Scientific Inc. (Rockford, Illinois, USA). Laemmli Sample Buffer was purchased from Bio-Rad Laboratories, Inc. (Hercules, California, USA). RPMI-1640 medium was obtained from Invitrogen Corporation (Carlsbad, CA, USA).

Cell culturing. Human myeloma cell line, U266, was provided by courtesy of Prof. Lisheng Wang (Institute of Radiation Medicine, the Academy of Military Medical Sciences). U266 cells were cultured in RPMI-1640 supplemented with 10% inactivated bovine serum, 100u/mL penicillin and 100u/mL streptomycin, and then were incubated in the incubator with 5% CO_2 at 37℃. U266 cells were in the logarithmic growth phase in experiment. Living cells accounted for 95% to 100%.

Cell viability. U266 cells in the logarithmic growth phase were selected and centrifuged at 1500 × g for 5 min. The cells were collected and their density was adjusted to 2 × 10^5/mL. Following exposure to MS-275 of different concentrations, including 0, 0.5, 1, 2, 5, and 10μ mol/L for 24 h and 48 h, living cells and dead cells were counted to calculate the cell survival rate using trypan blue exclusion assay. The cell survival rate was calculated as per the following formula: cell survival rate = (count of living cells/ (count of living cells + count of dead cells)) × 100%.

Wright-Giemsa stain method. U266 cells in the logarithmic growth phase were selected and centrifuged. Then the centrifuged cells were collected and their density was adjusted to 2 × 10^5/mL. Following exposure to 2μ mol/L MS-275 for 18h and 36h, a small quantity of cell suspension (1× 10^6/mL) were attached to the slide by cytospin and fixed with methanol for 10 min. They were stained with Giemsa for 15 min, rinsed mildly with distilled water, and stained again with Wright for 30 min. The stained cells were examined under the microscope.

Assay of cell cycle. U266 cells in the logarithmic growth phase were seeded in the 6-well plate. Following exposure to MS-275 (2μ mol/L) for 12, 24, and 36 h, they were centrifuged for 5 min at 1500 × g. After rinsed with PBS, deposited and centrifuged again, they were collected in a 1.5 mL EP tube. They were fixed with 70% ice-cold ethanol (PBS containing 3% FBS) and stored in a refrigerator at −20℃ overnight. They were centrifuged at 2 000 × g for 3 min the next day, rinsed with PBS and deposited twice. The deposited cells were re-suspended with PBS 0.1mL supplemented with RNase A (with a final concentration of 1mg/mL) and were bathed in water at 37℃ for 30 min. They were centrifuged to remove RNase. PI was added to the EP tube (with a final concentration of 100g/mL) for reaction in avoidance of light for 20 min. The stained cells were examined with the flow cytometry.

Western blotting analysis. U266 cells routinely cultured and those exposed to MS-275 (2μ mol/L)for 12, 24 and 36 h were centrifuged at 10000 × g for 5 min. They were collected and cleaved with Laemmli Sample Buffer. The cleaved cells were heated on the thermostat at 99℃ for 8 min and were bathed in ice-cold water. They were centrifuged at 10000 × g at 40℃ for 10 min. The supernatants were transferred to a sterile EP tube. Appropriate samples were obtained to evaluate protein

concentrations in the BCA kit (detailed procedures and relevant reagents were as specified in the instructions for the kit). 50 g of total protein was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) with 5% β -mercaptoethanol at 80v for 2h until trypan blue reached the bottom of the slab gels. Proteins were transferred to the polyvinylidene difluoride (PVDF) membrane and subjected to electrophoresis at 80v for 2h. The membrane was blocked for 1 h in PBS containing 5% defatted milk and washed with TBS+ Tween for 5 min for three times. It was blocked again with the primary antibody diluted with 5% defatted milk or 5% BSA and incubated overnight at 40℃. After the membrane was washed, it was incubated with the secondary antibody (diluted with 5% defatted milk) for 1 h at ambient temperature and washed for three times. ECL Chemiluminescent substrate A and B mixed of the same volume were added to the PVDF membrane and colored for 5 min. After exposure, the X-ray films were developed and fixed consecutively. β -actin was regarded as the internal reference.

Statistical analysis. Cell survival rates were expressed as mean± SD. SAS 6.0 was applied to perform one-way ANOVA for cell viability. Differences among the treatment groups with any concentrations and the control group were compared. A statistically significant difference existed if p is <0.05.

Results

Killing effect of MS-275 on U266 cells. The trypan blue exclusion assay was performed to measure cell viability (Fig 1). Results showed that MS-275 inhibited growth of U266 cells in a dose- and time-dependent manner. After exposure to MS-275 for 24 h and 48 h, cell survival rates decreased in parallel to concentrations of MS-275. There was a statistically significant difference among treatment groups exposed to different concentrations of MS-275 ($p<0.01$). In the meantime, the killing effect of MS-275 on cells was also obviously time-dependent. A statistically

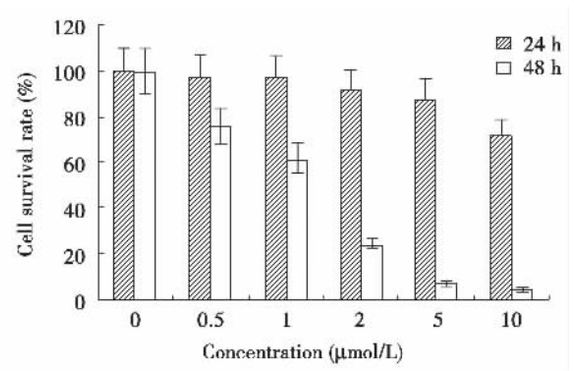

Figure 1 Cell viability of U266 treated with different concentrations of MS-275 at different time points

All results are presented as means ±SD of three independent experiments.

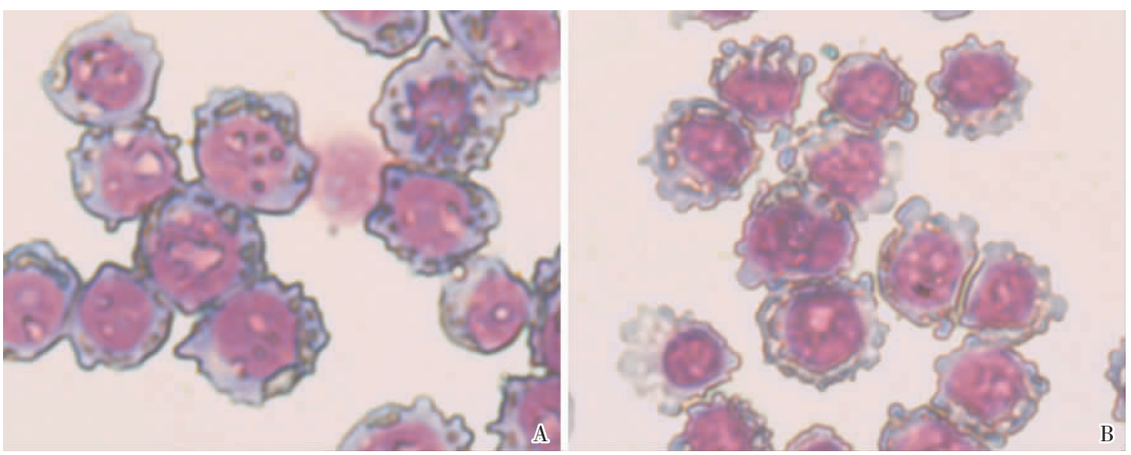

Figure 2 Morphological change in U266 cells after the treatment with MS-275 for 36 h (×400)

A: U266 cells; B: U266 cells treated with MS-275.

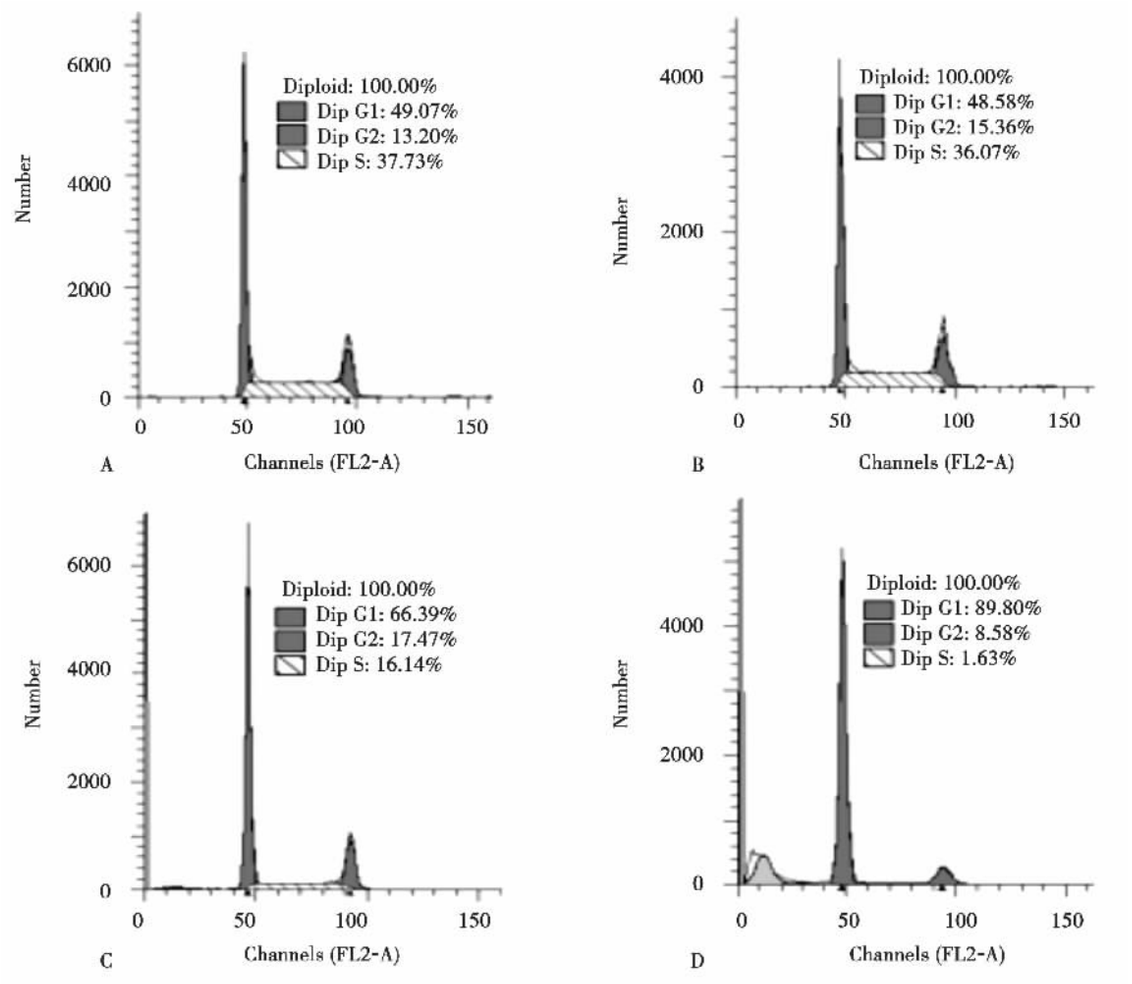

Figure 3 Cell cycle of U266 cells after the treatment with MS-275

A: U266 cells; B: U266 cells treated with MS-275 for 12 h; C: U266 cells treated with MS-275 for 24 h; D: U266 cells treated with MS-275 for 36 h.

significant difference was noted regarding the killing effect among different time points ($p<0.01$). The IC_{50} was 1.39 μ mol/L at 48 h after exposure. After exposure to 2 μ mol/L MS-275 for 24 h, the cell survival rate was 91.4%, while it was only 24.2% after exposure for 48 h. A statistically significant difference was noted in the survival rate between the two time points ($p<0.01$). So the concentration of 2 μ mol/L was adopted in sequent experiments.

Morphological change of U266 cells exposed to MS-275. After exposure to 2 μ mol/L MS-275 for 36 h, U266 cells displayed an obvious morphological change in terms of nuclear contraction and uneven alignment of complete membranes using the Wright-Giemsa stain method (see Fig 2).

Influence of MS-275 on U266 cells. After exposure to 2μ mol/L MS-275 for 12 h, 24 h and 36 h, the U266 cell cycle was arrested at the G_0/G_1 phase as shown by the flow cytometry, and the effect of cell cycle arrest became more significant when exposure period increased. After exposure for 36 h, the ratio of U266 cells at the G_0/G_1 phase increased to 89.80% from 40.07% in the control group. At the same time, the cells at the S phase decreased significantly and the ratio of cells at the S phase decreased to 1.63% from 37.73% in the control group (Fig 3).

Apoptosis of U266 cells induced by MS-275. As shown by Western blotting, MS-275 up-regulated expression of P21 and decreased expression of survivin and CDK4 (Fig 4). Following exposure to MS-275 for 12 h, 24

h and 36 h, apoptosis was induced in U266 cells, since caspase3 was activated and cleaved PARP appeared, and more obviously as time went on (Fig 5).

Acetylase effect of MS-275 on histone H3 in U266 cells. Following exposure of U266 cells to MS-275, histone H3, the substrate of HDAC1 and HDAC2 was acetylated obviously. As exposure continued, there were more acetylated histone H3 in U266 cells (Fig 6).

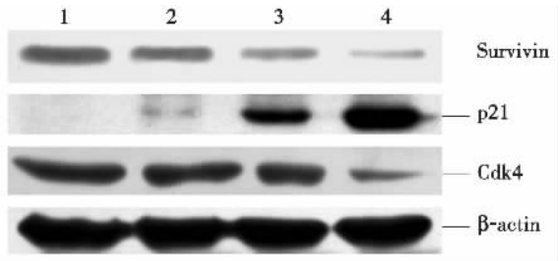

Figure 4 Protein expression of survivin, p21 and Cdk4 in U266 cells after the treatment with MS-275

1: U266 cells; 2: U266 cells treated with MS-275 for 12 h; 3: U266 cells treated with MS-275 for 24 h; 4: U266 cells treated with MS-275 for 36 h.

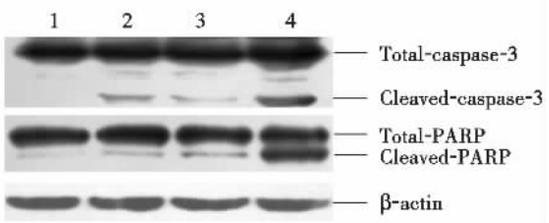

Figure 5 Protein expression of caspase-3 and cleaved-PARP in U266 cells after the treatment with MS-275

1: U266 cells; 2: U266 cells treated with MS-275 for 12 h; 3: U266 cells treated with MS-275 for 24 h; 4: U266 cells were treated with MS-275 for 36 h.

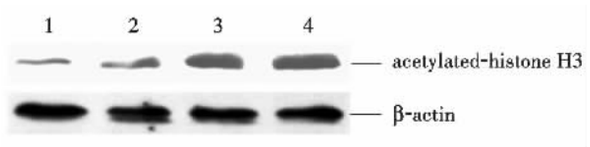

Figure 6 Protein expression of acetylated histone H3 in U266 cells after the treatment with MS-275

1: U266 cells; 2: U266 cells treated with MS-275 for 12 h; 3: U266 cells treated with MS-275 for 24 h; 4: U266 cells treated with MS-275 for 36 h.

Discussion

Histone acetyltransferase (HAT) and histone deacetylase (HDAC) is a pair of enzymes that mediate histone acetylation.[1] In normal physiological conditions, these two enzymes are in the condition of balance and check in mediating acetylation of histone. However, when cells are in the transition of cell cycle, HDAC are especially active, so that the balance is damaged. The unbalanced molecular expression thus influences cell proliferation and cell cycle, which leads to malignancies. In recent years, HDACi have been demonstrated to inhibit cancer cell proliferation, arrest cell cycle and promote cell apoptosis. HDACi have a wide anti-tumor activity. In vitro and in vivo experiments show that HDACi has a potential prospect for clinical application.[2] MS-275 is one kind of HDACi of the benzamides with a small molecular weight of 376.4. It has potent anti-tumor activity for many human tumor lines with high efficiency, low toxicity, good toleration, along with strong specificity and selectivity for tumors.[3] What deserves attention is that MS-275 can check cell cycle of normal cells and tumor cells. However, it only induces rapid apoptosis of tumor cells, not normal cells.[4]

Survivin belongs to the inhibitor apoptosis (IAP) family. In recent years, it is found to express not in normal tissues but in tumors, implying that it has an important role in apoptosis inhibition and abnormal proliferation of tumor cells. A number of reports have verified that survivin is positively correlated with proliferation of tumor cells and negatively correlated with apoptosis tumor cells.[5] High expression of survivin in cancer tissues often signals poor prognosis. The current study showed that the mechanism of apoptosis inhibition and proliferation promotion mainly correlated with inhibition of activation of caspases. caspases are regarded as an integral part of the apoptosis pathway that involves in sequential activation of caspase proteins.[6] After binding with caspases specifically, survivin inhibits activity of both Caspase 3 and Caspase 7, leading to inhibition of

cell apoptosis. Survivin also indirectly inhibits activity of Caspases through p21. Survivin binds with the cell cycle mediator, CDK4, and this surviving-CDK4 complex facilitates release of $p21^{wafl}$ from the $p21^{wafl}$-CDK4 complex, which in turn enables combination of $p21^{wafl}$ and Caspase 3, inhibiting activity Caspase 3.[5] The current study found that post exposure to MS-275 for 12h, 24h and 36h, survivin level is significantly down-regulated whereas the caspase is activated, leading to cleavage of PARP, and this two events appear to be correlated each other.

The upstream regulation mechanism regarding to survivin in the signaling pathway in tumor cells is poorly understood. The JAK-STAT signaling pathway was initially identified in interferon (IFN) signaling pathway, which is a rapid pathway from outside the cell to the inside. The signal transducers and activators of transcription (STAT) family is concentrated in the cytoplasm and can transfer into the nucleus to bind with DNA. It has a dual function of transduction and transcription. Among the STAT family, STAT3 is closely correlated with tumors and plays a role in several signaling pathways involving in various cell factors. It is found to be consistently over-activated in many tumor cells. When STAT3 is over activated, it promotes cell proliferation and malignancy transformation, and inhibits cell apoptosis, contributing to carcinogenesis.[7, 8] Some reports has showed that activation of JAK-STAT3 is closely correlated with over-expression of survivin, implying that survivin gene is a target gene for STAT3.[9, 10], and that AG490 can also inhibit activity of STAT3 binding to DNA, resulting in growth inhibition of U266 cells.[12] Yu C et al.[13] observed activity change of STAT3, change of survivin mRNA expression and breast cancer cell apoptosis prior to and post inhibition of JAK. They observed that after action of AG490, P-STAT3 expression, and survivin mRNA and protein expressions significantly reduced, while proliferation inhibition and apoptosis of tumor cells increased, implying that activation of JAK-STAT3 is correlated with expression of survivin. Therefore, intervention of steps in the JAK-STAT signaling pathway to reduce expression of survivin may inhibit malignant proliferation of tumor cells. However, the molecular mechanism at each step in the JAK-STAT signaling pathway needs to be further investigated.

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