Antitumor activity of dobutamine on human osteosarcoma cells

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Abstract. Dobutamine has been widely used for the treatment of heart failure and cardiogenic shock since the 1970s. Osteosarcoma is the most commonly observed malignant bone tumor in children. Currently, there are no effective drugs for the treatment of osteosarcoma. In the present study, the potential anticancer activity of dobutamine on human osteosarcoma cells was examined. Human osteosarcoma MG-63 cells were treated with dobutamine at various concentrations and for various incubation times. The inhibition of cell growth by dobutamine was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Flow cytometry was utilized to evaluate the effect of dobutamine on cell apoptosis and the cell cycle. Furthermore, the expression levels of caspase-3 and caspase-9 were assessed by western blot analysis. The influence of dobutamine on cancer cell migration and invasion was additionally evaluated using wound-healing assay and the Boyden Chamber migration method. Dobutamine significantly inhibited the growth of MG-63 cells at a concentration of 10 μM or higher when incubated for 12 h or longer (P=0.023). Dobutamine augmented cell apoptosis and arrested the cell cycle in the G2/M phase. Western blot analysis revealed that dobutamine induces expression of caspase-3 and caspase-9. In addition, the invasiveness and migration of MG-63 cells was inhibited by dobutamine in a concentration-dependent manner. The results of the present study may lead to novel applications for dobutamine in the treatment of osteosarcoma.

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Introduction

Osteosarcoma is the most commonly observed primary malignant cancer of the bone in children, and possesses high incidence and mortality rates (1). The tumor predominantly arises from the metaphyses of the long bones with active bone growth and repairation, such as the knee joint, lower femur and upper tibia. As osteosarcoma is considered to be a radioresistant tumor, chemotherapy is the primary approach for the treatment of osteosarcoma (2). However, the currently utilized chemotherapy regimens demonstrate low efficacy for the treatment of this tumor (3). Current chemotherapeutic drugs, including ifosfamide, cisplatin and high-dose methotrexate, have a number of side-effects and their use may result in acquired drug resistance in osteosarcoma cells (4). Furthermore, the prognosis of osteosarcoma is poor and >30% of patients succumb to pulmonary metastases within 5 years of diagnosis (5). Therefore, there is an urgent requirement for the development of novel effective therapeutic drugs for the treatment of osteosarcoma.

Yes-associated protein (YAP), a transcriptional co-activator, is a key regulator of the Hippo signaling pathway (6). When YAP is recruited to the nucleus, transcription of cell proliferation-promoting and anti-apoptotic genes is continuously activated (7,8). High expression of YAP has been observed in a number of types of tumor, including osteosarcoma, hepatocellular, colorectal, ovarian, breast and lung cancer cases, as well as gastric carcinoma, and has been reported to be correlated with a poor prognosis (9-12). These findings suggest that YAP may contribute to a malignant cellular phenotype and therefore may be an important target for anticancer drugs (13).

Dobutamine is a synthetic catecholamine developed by Eli Lilly and Company in the 1970s (14). It has been widely used as an inotropic drug for hemodynamic support in the treatment of congestive heart failure, as well as cardiogenic and septic shock (15). A previous study demonstrated that dobutamine is able to attenuate YAP-dependent transcription by inhibiting its nuclear translocation (16).

In the present study, the effect of dobutamine on the proliferation, apoptosis and invasiveness of the MG-63 human osteosarcoma cell line was investigated. The results of the present study demonstrated the potential effectiveness of dobutamine for the treatment of osteosarcoma.

Table I. Cell cycle phase distribution and apoptosis of MG-63 cells.

Group	Cell cycle phase			Apoptosis, %
	G0/G1	S	G2/M	
Control	54.12±6.53	25.60±5.33	20.28±1.79	2.2±0.4
1 μM dobutamine	55.19±3.16	25.37±3.66	19.44±5.01	2.5±1.1
5 μM dobutamine	53.96±4.58	23.18±6.34	22.86±4.27	2.9±1.7
10 μM dobutamine	49.82±2.99	26.78±5.92	23.40±6.52	7.0±2.5[a]
25 μM dobutamine	50.62±5.27	21.50±4.59	27.88±4.55[b]	11.6±4.7[c]
50 μM dobutamine	51.21±6.52	18.39±2.79	30.40±7.26[d]	13.2±1.8[e]

[a]P=0.012, [b]P=0.007, [c]P=0.020, [d]P=0.003 and [e]P=0.026 vs. control group.

Materials and methods

The MG-63 human osteosarcoma cell line was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from GE Healthcare Life Sciences (Logan, UT, USA). Propidium iodide (PI) and dobutamine were obtained from Sigma-Aldrich (St. Louis, MO, USA). The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from Beckman Coulter, Inc. (Brea, CA, USA).

Cell culture. MG-63 cells were grown in medium at 37˚C in an atmosphere with 5% CO_2. Culture medium supplemented with 10% FBS, 100 U/ml penicillin (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA), 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific Inc.) and DMEM was used for MG-63 culture.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The influence of dobutamine treatment on cell viability was determined using an MTT assay. Cells were seeded into a 96-well plate (Corning, New York, NY, USA) overnight at 37˚C and incubated with various concentrations of dobutamine (1, 5, 10, 25 and 50 μM) for 12, 24, 48 and 72 h. Following the indicated treatments, the cells were incubated with MTT (0.25 mg/ml; Sigma-Aldrich) in phosphate-buffered saline (PBS; Gibco; Thermo Fisher Scientific Inc.) for 4 h at 37˚C, followed by removal of the medium and addition of 1 ml 100% dimethyl sulfoxide (Beyotime Institute of Biotechnology, Shanghai, China) to solubilize the MTT-formazan product. The absorbance at 490 nm was determined using an automatic multi-well spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The inhibitory rate of cell growth was calculated as [1-treatment group/control group)] x100. The growth curve was drawn using time as the abscissa and inhibition rate as the ordinate. Each dobutamine dose was used in triplicate, and the MTT assay was repeated at least twice.

Flow cytometric analysis. The rate of apoptosis and percentage of cells in G1, S and G2/M phases was measured by flow cytometry. Following treatment of the experimental groups in MTT for 24 h, cells were harvested, trypsinized , washed twice with PBS and resuspended in binding buffer (Beyotime Institute of Biotechnology). The cells were subsequently stained with Annexin V-FITC and PI according to the manufacturer's protocol and analyzed by flow cytometry. The cell suspension was incubated with 50 μg/ml PI solution and 50 U/ml RNase (Beyotime Institute of Biotechnology) for 30 min in order to observe the cell cycle stage. Flow cytometric analysis was performed on a BD FACSCaliber™ using CellQuest software, version 5.1 (BD Biosciences, Franklin Lakes, NJ, USA).

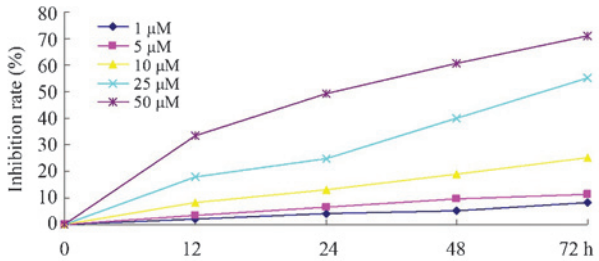

Figure 1. Effect of dobutamine treatment on the proliferation of MG-63 cells. Cells were treated with various concentrations (1, 5, 10, 25 and 30 μM) of dobutamine for 12, 24, 48 and 72 h. The inhibition rate (%) of dobutamine was calculated using the following formula: [1-treatment group/control group)]x100%.

Cell invasion analysis. The effect of treatment with dobutamine on the invasion of MG-63 cells was investigated using Transwell chambers with polycarbonate filters (pore size of 8 μm; Beyotime Institute of Biotechnology). MG-63 cells were seeded into the upper chamber at a density of $1x10^5$ cells/ml and incubated in 0.6 ml DMEM medium containing 10% FBS and various concentrations (1, 5, 10, 25 and 50 μM) of dobutamine. The lower chamber was filled with 0.6 ml DMEM medium containing 20% FBS. Following 24 h of incubation at 37˚C, cells on the upper filter that had not migrated through were removed by wiping, and the remaining cells were fixed in 4% paraformaldehyde (Beyotime Institute of Biotechnology) for a total of 1 h. Cells that had migrated through the filter were stained using hematoxylin (Beyotime Institute of Biotechnology) and visualized and counted under a microscope (Olympus IX53; Olympus, Tokyo, Japan).

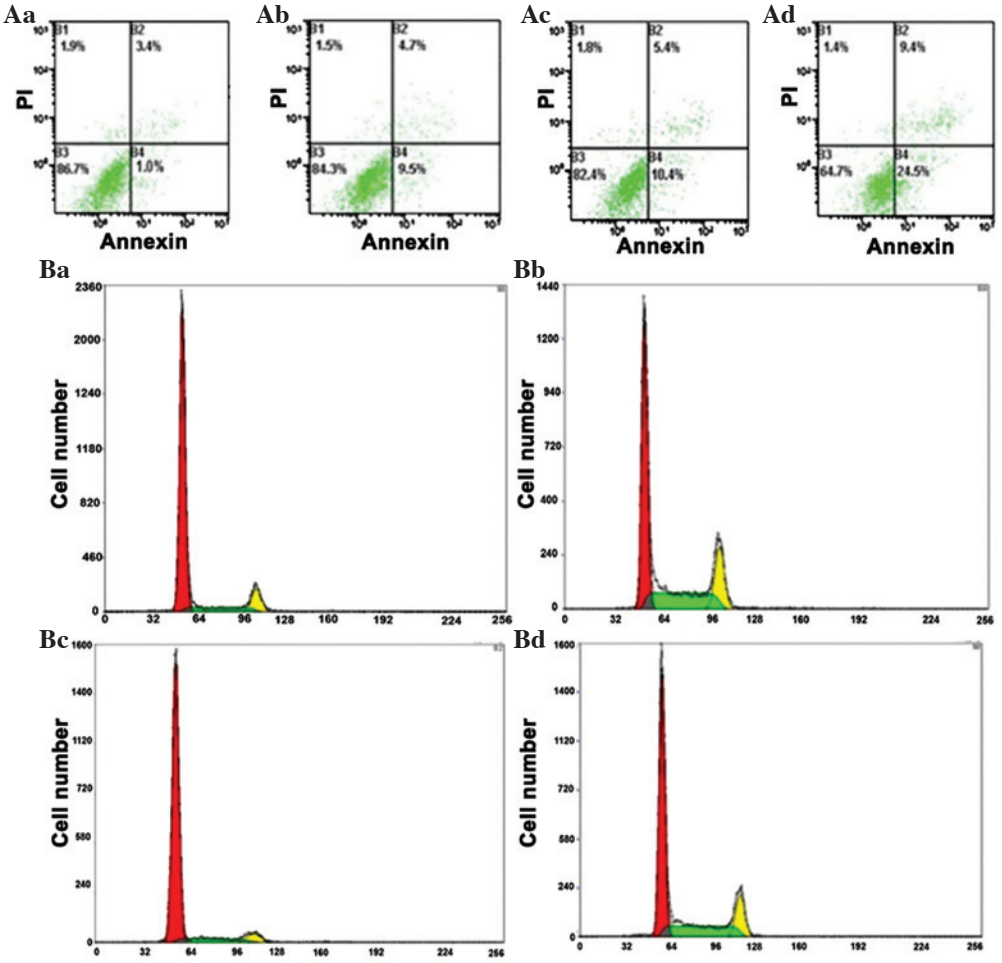

Figure 2. (A) Cell cycle and (B) apoptosis distribution of MG-63 cells in response to dobutamine treatment: (a) Control, (b) 10 μM dobutamine, (c) 25 μM dobutamine and (d) 50 μM dobutamine. Data are presented as the mean ± standard deviation (n=6). *P<0.05 compared with the control group.

Western blot analysis. The present study examined the levels of protein expression of caspase-3 and caspase-9 in MG-63 cells prior to and following treatment with dobutamine. The cells were treated with the different concentrations of dobutamine (1, 5, 10, 25 and 50 μM). The cells were then harvested in 5 ml of medium, pelleted by centrifugation (1,000 x g for 5 min at 4˚C), washed twice using ice-cold PBS and lysed in ice-cold HEPES buffer (50 mmol/l; pH7.5), 10 mmol/l NaCl, 5 mmol/l $MgCl_2$, 1 mmol/l ethylenediaminetetraacetic acid (all Beyotime Institute of Biotechnology), 110% glycerol (v/v), 1% Triton X-100 (v/v 1X complete), a cocktail of SigmaFast protease inhibitors (1X complete; Sigma-Aldrich), followed by treatment with 1 mg/l dobutamine on ice for 30 min. The cell lysates were clarified by centrifugation (15,000 x g for 10 min at 4˚C), and the supernatants were analyzed immediately or stored at -80˚C until required. Equivalent quantities of protein (50 μg) from total cell lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis using precast 12% BIS-TRIS gradient gels and transferred onto polyvinylidene difluoride membranes. Membranes were blocked overnight at 4˚C using blocking buffer [5% skimmed dried milk (v/v), 150 mmol/l NACl, 10 mmol/l Tris (pH 8.0) and 0.05% Tween 20 (v/v); Beyotime Institute of Biotechnology]. Proteins were detected by incubation in blocking buffer overnight at 4˚C with the following primary antibodies: Mouse anti-human monoclonal caspase-3 antibody (1:10,00 dilution; sc-65496), mouse anti-human monoclonal caspase-9 antibody (1:10,00 dilution; sc-56073) and mouse anti-human monoclonal GADPH antibody (1:10,00 dilution; sc-47778) (all Santa Cruz Biotechnology, Inc, Dallas, TX, USA). Unbound antibody was removed by washing with Tris-buffered saline (pH7.2) containing 0.5% Tween 20 (TBS-T; Beyotime Institute of Biotechnology). The membrane was subsequently incubated at room temperature with horseradish peroxidase-conjugated secondary antibody. Subsequent to washing with TBS-T three times, bands were visualized by enhanced chemiluminescence

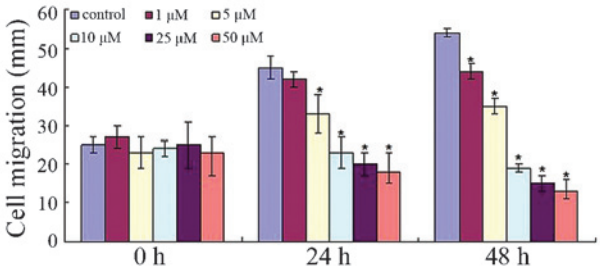

Figure 3. Effect of dobutamine treatment on the migration of MG-63 cells. Data are presented as the mean ± standard deviation (n=6).*P<0.05 compared with the control group.

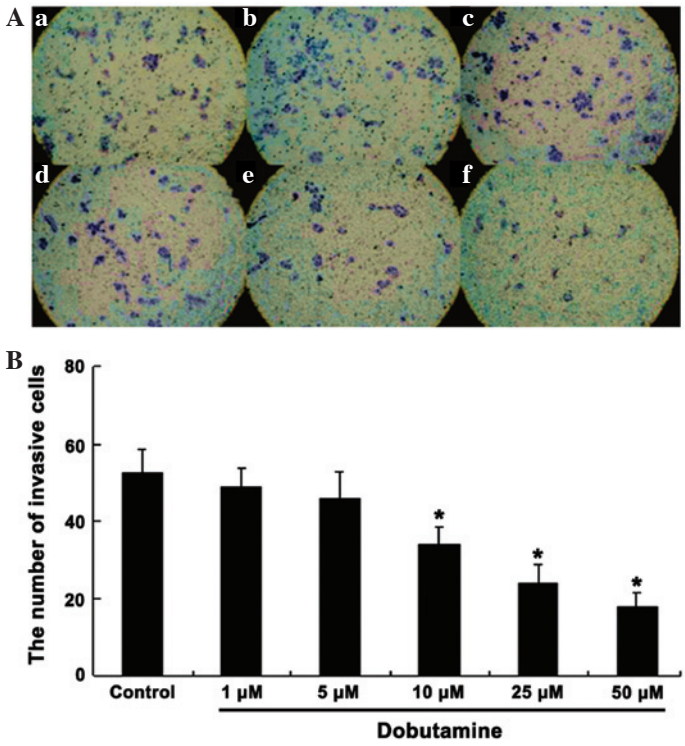

Figure 4. Effect of dobutamine treatment on MG-63 cell invasiveness. (A) Cells were treated with the following concentrations of dobutamine for 24 h: (a) Control, (b) 1 μM, (c) 5 μM, (d) 10 μM, (e) 25 μM and (f) 50 μM. (B) Number of invasive MG-63 cells following dobutamine treatment for 24 h. Data are presented as the mean ± standard deviation (n=6). *P<0.05 compared with the control group.

system (Pierce Biotechnology, Rockford, IL, USA), and the protein intensities were quantified using AlphaEaseFC 4.1.0 software (Alpha Innotech, San Leandro, CA, USA).

Statistical analysis. Statistical analyses were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). Comparisons between two samples (experimental and control group) were employed by Student's t-test. P<0.05 was considered to represent a statistically significant difference.

Results and Discussion

Dobutamine inhibits the proliferation of MG-63 osteosarcoma cells. The results of the present study revealed that dobutamine significantly inhibited cell proliferation in a time- and concentration-dependent manner compared with the control group. As demonstrated by the proliferation inhibition graph (Fig. 1), treatment with 10, 25 and 50 μM dobutamine had a significant inhibitory effect on the survival of MG-63 cells (P=0.032).

Dobutamine augments cell apoptosis and arrests the cell cycle. Annexin V/PI staining was used to measure dobutamine-induced apoptosis. Compared with the control group, dobutamine induced a significant increase in apoptotic death, following pretreatment of MG-63 cells with 5, 10, 25 and 50 μM for 24 h (P=0.028; Table I). The percentage of MG-63 cells in G2/M phases was significantly increased at dobutamine concentrations of 25 and 50 μM (P=0.007 and P=0.003, respectively), and the percentage of cells in S-phase was significantly decreased (P=0.039) compared with the control group (Fig. 2).

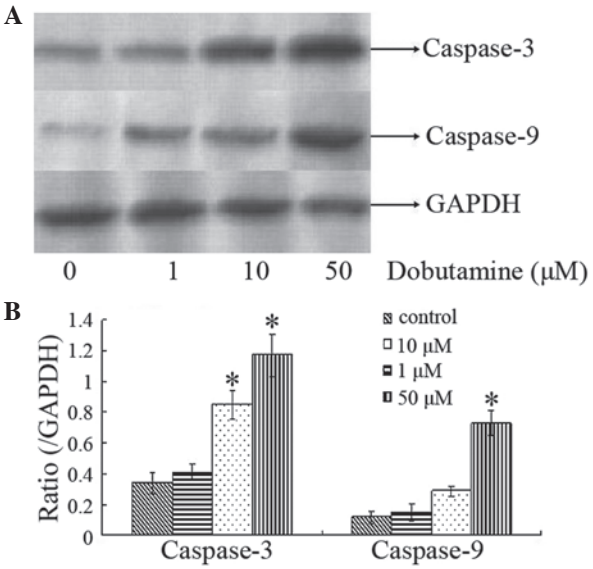

Figure 5. Effect of dobutamine treatment on protein expression of caspase-3 and caspase-9 in MG-63 cells. (A) Western blot analysis and (B) quantification of protein expression of caspase-3 and caspase-9. Cells were treated with various concentrations of dobutamine (0, 1, 10 and 50 μM) for 72 h. GAPDH was used as an internal control gene. The expression level was calculated as the ratio of caspase-3 or caspase-9:GAPDH. Data are presented as the mean ± standard deviation (n=6). *P<0.05 compared with the control group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Dobutamine reduces the migration and invasion of MG-63 cells. To investigate whether dobutamine treatment affects osteosarcoma cell movement, the migratory rate of the MG-63 cells was observed. Fig. 3 demonstrates that dobutamine

significantly decreased cell migration from the edge of the wound (P=0.041). Similarly, the cell invasion/Transwell assay showed that a large number of cells passed through the filter in the control group, whereas the cells passing through the filter were markedly reduced following dobutamine treatment. Furthermore, treatment with dobutamine reduced the number of invasive cells in a concentration-dependent manner (Fig. 4). The number of invasive cells in the dobutamine groups was significantly reduced compared with that in the control group (P=0.039 for 10 μM, P=0.015 for 25 μM and P=0.011 for 50 μM) (Fig. 4).

Dobutamine induces expression of caspase-3 and caspase-9. Caspases are a family of endoproteases that provide crucial links in cell regulatory networks that control inflammation and cell death (17). Caspase-3 and 9 are crucial mediators in apoptosis signaling pathways (18). Western blot analysis was used to investigate the expression of caspase-3 and caspase-9 in MG-63 cells following dobutamine treatment. Protein expression analysis indicated that caspase-3 levels were increased following treatment with dobutamine at the concentrations of 10 and 50 μM for 72 h (P=0.011 and P=0.013, respectively), and caspase-9 levels were increased following treatment with dobutamine at a concentration of 50 μM for 72 h (P=0.031) (Fig. 5). These findings indicated that dobutamine may induce cancer cell apoptosis and cell death.

Recent reports have demonstrated that YAP is highly expressed in human osteosarcoma MG-63 cells (19). The results of the present study indicate that the inhibitory effect of dobutamine may be associated with the inhibition of YAP translocation. Silencing of the YAP gene by RNA interference led to a similar effect to that caused by dobutamine (20). In addition, the present study found that dobutamine arrests the cell cycle at the G2/M transition stage and augments cell apoptosis. Previous studies have demonstrated that YAP activates cell apoptosis in response to DNA damage via interaction with p73 in several cancer cell lines (21). The findings of the present study may result in a novel application for dobutamine in the treatment of cancer.

In conclusion, the results of the present study demonstrated that dobutamine was able to significantly suppress osteosarcoma cell growth by inhibiting cell proliferation, inducing cell apoptosis and redistributing cell cycle phases. These findings indicate that dobutamine may become a novel therapeutic agent for the treatment of osteosarcoma. However, additional *in vivo* studies are required in order to confirm the effectiveness and safety of dobutamine in the treatment of osteosarcoma.

Acknowledgements

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