Arginine methyltransferase inhibitor-1 inhibits sarcoma viability *in vitro* and *in vivo*

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Received July 19, 2017; Accepted April 16, 2018

DOI: 10.3892/ol.2018.8929

Abstract. Protein arginine methyltransferases (PRMTs) are a class of epigenetic modified enzymes that are overexpressed in a various types of cancer and serve pivotal functions in malignant transformation. Arginine methyltransferase inhibitor-1 (AMI-1) is a symmetrical sulfonated urea that inhibits the activity of type I PRMT *in vitro*. However, previous studies demonstrated that AMI-1 may also inhibit the activity of type II PRMT5 *in vitro*. To the best of our knowledge, the present study provides the first evidence that AMI-1 may significantly inhibit the viability of mouse sarcoma 180 (S180) and human osteosarcoma U2OS cells. Additionally, the results demonstrated that AMI-1 downregulated the activities of PRMT5, the symmetric dimethylation of histone 4 and histone 3 (a PRMT5-specific epigenetic mark) in a mouse xenograft model of S180 and induced apoptosis in S180 cells. Taken together, the results suggest that AMI-1 may exhibit antitumor effects against sarcoma cells by targeting PRMT5.

Introduction

Sarcoma is a rare type of cancer and is usually categorized into two types: Sarcomas that develop in soft tissues (including muscle, tendons, fat, blood vessels, lymph vessels, nerves and tissue around joints) and bone sarcomas. Sarcomas account for ~1% of malignancies in adults and 15% of malignancies in children (1). Chemotherapy using anthracyclines with or without ifosfamide has been widely used as the standard treatment for soft tissue sarcoma (2-6). However, these agents often fail to treat patients with soft tissue sarcoma and cause adverse side effects (7). Therefore, the available treatment options for patients with sarcoma are poor and the development of novel drugs is required.

The protein arginine methyltransferase 5 (PRMT5) is a type II arginine methyltransferase that catalyzes the symmetric dimethylation of arginine residues in histones 4 (H4R3me2s) and 3 (H3R8me2s) (8). PRMT5 is upregulated in various types of cancer (9-15) and small molecule inhibitors of PRMT5 may be attractive targets for the treatment of sarcoma (16-18). Arginine methyltransferase inhibitor-1 (AMI-1), also known as 7,7'-carbonylbis(azanediyl)bis(4-hydroxynaphthalene-2-sulfonic acid), was the first inhibitor of PRMTs to be identified (19). AMI-1 may also inhibit the activity of type I PRMT *in vitro* (20). A recent study demonstrated that AMI-1 significantly inhibited the activity of type II PRMT5 *in vitro* (15). The aim of the present study was to examine the effects of the AMI-1 in sarcoma *in vitro* and *in vivo* and investigate the underlying molecular mechanisms.

Materials and methods

Cell culture and reagent. Mouse sarcoma 180 (S180) and human osteosarcoma U2OS cells were obtained from the Chinese Academy of Science (Shanghai, China) and were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin sodium and 100 mg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2. AMI-1 was synthesized in house, according to the method of Peng *et al* (21) and Ragno *et al* (22).

Animals. A total of 22 male Kunming mice (age, 6-7 weeks old; body weight, 18-22 g) were purchased from Lanzhou University (Gansu, China). The mice were acclimated to laboratory conditions (25˚C, 12/12 h light/dark, 50% humidity and *ad libitum* access to food and water) for 3 days prior to experimentation. The present study was approved by the Institutional Animal Care and Treatment Committee of Lanzhou University (Gansu, China). On day 7, mice were

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Key words: arginine methyltransferase inhibitor 1, sarcoma, protein arginine methyltransferase 5, histone methylation, symmetric dimethylation of histone 4, symmetric dimethylation of histone 3

euthanized prior to cervical dislocation with an intraperitoneal injection of 50 mg/kg pentobarbital sodium.

In vitro cytotoxicity assay. Briefly, S180 or U2OS cells were seeded at $2x10^3$ cells/well in 96-well plates. Following 24 h of culture, cells were treated with various concentrations of AMI-1 (0.6, 1.2 and 2.4 mM) and the control group was treated with the vehicle control (PBS). Cytotoxicity was evaluated using the Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol. S180 cells were seeded at $7.5x10^4$ cells/well in 24-well plates were incubated at 37˚C at indicated timepoints (48, 72 or 96 h). The cell morphology and numbers were observed under a light inverted microscope (Olympus CK40; Olympus Corporation, Tokyo, Japan; magnification, x100). Cytotoxicity was determined by measuring the absorbance at a wavelength of 450 nm using a plate reader. IC_{50} values were evaluated using CurveExpert 1.3 software (Hyams Development, Mississippi, MS, USA).

Colony formation assay. U2OS cells were seeded at a density of 300 cells in 60 mm dishes and incubated for 24 h. S180 cells were not used due to them being suspended cells and not suitable for colony formation assay. RPMI medium with FBS was replaced with 5 ml fresh medium, containing AMI-1 (0.3 or 0.6 mM) or PBS (control) and incubated at 37˚C for 18 days. Colonies were fixed with a 7:1 ratio of methanol to glacial acetic acid for 25 min at 25˚C and then stained with 0.1% crystal violet (in 20% methanol and PBS) for 25 min at 25˚C.

Flow cytometric analysis of apoptosis. S180 cells were seeded at a density of $1.2x10^5$ cells/well in 6-well plates and treated with AMI-1 (1.2 and 2.4 mM) or vehicle (PBS) for 48 and 72 h. The cells were harvested, washed twice and resuspended in 1X binding buffer. A total of 500 μl S180 cells ($1x10^6$ cells/ml) were incubated with 5 μl annexin V-fluorescein isothiocyanate and 5 μl propidium iodide for 15 min at room temperature in dark. The samples were then analyzed using a flow cytometer equipped with FCSDiva 6.2 software (LSR Fortessa™; BD Biosciences, Franklin Lakes, NJ, USA).

Tumor implantation and treatment. A total of $2x10^6$ S180 cells (in 0.2 ml 0.9% NaCl in PBS) were subcutaneously inoculated into the right axillary region of Kunming mice. Following 3 days of implantation with S180 cells, mice were divided into two groups (11 animals/group): AMI-1-treated (0.5 mg in 200 μl 0.9% NaCl) or vehicle treated (200 μl 0.9% NaCl). The treatments were administered intratumorally (200 μl per mouse, once daily for a total of 7 days). The weight of the mice was determined daily. On day 7, mice were sacrificed by cervical dislocation and tumors were removed and weighed. The inhibition rate of tumor viability (IR) was calculated as: (1-the average tumor weight of treated group/tumor weight of vehicle group) x100%. The dose of AMI-1 was chosen *in vivo* experiments based on our preliminary experiments and previous literatures (23-26).

Western blot analysis. Western blot analysis was performed as described previously (15). Briefly, tumor tissues were lysed using RIPA buffer (cat no. P0013B; Beyotime Institute of Biotechnology, Jiangsu, China) containing phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology) for 30 min at 4˚C. The extract was centrifuged at 12,000 x g for 15 min at 4˚C to clear insoluble debris. The protein concentration was assayed using Quick Start™ Bradford (cat no. 500-0205; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts of protein (40 μg per lane) were separated by SDS-PAGE (12% gel) and then transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc.). The membranes were then blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 for 1 h at 25˚C. Following blocking, membranes were incubated overnight at 4˚C with the following primary antibodies: Anti-PRMT5 (1:500; cat no. sc-376937; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-PRMT7 (1:1,000; cat no. 14762; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-H4R3me2s (1:2,000; cat no. HW027; Signalway Antibody LLC, College Park, MD, USA), anti-H3R8me2s (1:1,000; cat no. HW015; Signalway Antibody LLC), anti-p53 (1:1,000; cat no. 9282; Cell Signaling Technology, Inc.) or anti-β-actin (1:2,000, cat no. 4970; Cell Signaling Technology, Inc.). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat nos. ZB-2301 and ZB-2305; OriGene Technologies, Inc., Beijing, China) for 1.5 h at 25˚C. The protein bands were visualized by BeyoECL Plus kit (Beyotime Institute of Biotechnology). The densitometry was performed using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. The relevant data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). The difference between two groups was analyzed using Student's t-test. For comparison of multiple groups, one-way analysis of variance followed by Dunnett's post hoc test was used. $P<0.05$ was considered to indicate a statistically significant difference.

Results

AMI-1 inhibits viability of sarcoma cells in vitro. Cytotoxicity in response to AMI-1 treatment was determined using CCK-8 assay. At 72 h, IC_{50} values for S180 and U2OS cells were 0.31±0.01 and 0.75±0.02 mM, respectively (data not shown). As presented in Fig. 1A-C, AMI-1 treatment inhibited the cell viability of sarcoma in S180 and U2OS cells in a time-dependent and dose-dependent manner *in vitro*. AMI-1 treatment significantly inhibited the viability of sarcoma S180 and U2OS cells in response to treatment of AMI-1 (0.6, 1.2 and 2.4 mM) for 48, 72 and 96 h (Fig. 1A and B).

AMI-1 induces S180 cell apoptosis in vitro. To evaluate whether AMI-1 may inhibit cell viability by regulating cell apoptosis, S180 cells were treated with AMI-1 (1.2 and 2.4 mM) or vehicle for 48 and 72 h. Cellular apoptosis was evaluated using flow cytometry. The results demonstrated that AMI-1 may increase the percentages of cells undergoing apoptosis, compared with that in the vehicle group (Fig. 2).

Table I. Evaluation of body and tumor weight of mice on d 7, following AMI-1 treatment.

Group	Body weight at d 0 (g)	Body weight at d 7 (g)	Tumor weight at d 7 (g)	IR (%)
Control	24.87±1.19	31.97±2.63	1.8170±0.41	
AMI-1	24.73±1.66	31.06±2.32	1.0684±0.27[a]	41.42±6.34

AMI-1, arginine methyltransferase inhibitor-1; d, day; IR, inhibition rate of tumor viability. [a]P<0.001 vs. Control. Mean ± standard deviation; n=11.

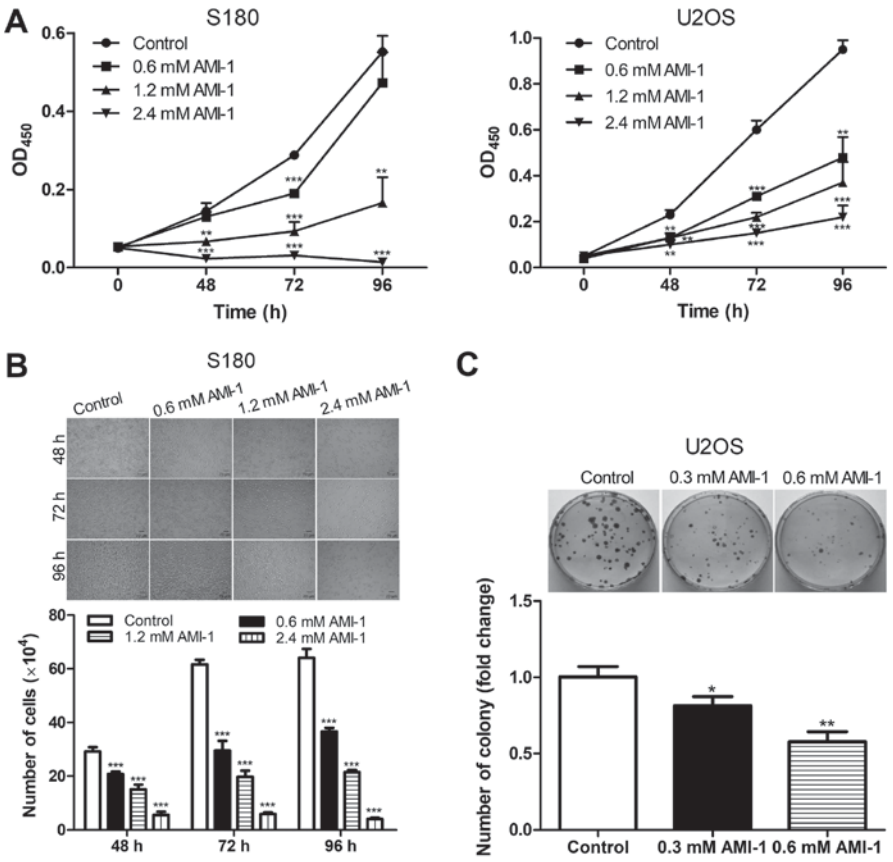

Figure 1. AMI-1 inhibits sarcoma cell viability *in vitro*. (A) S180 or U2OS cells were treated with vehicle or AMI-1 at indicated does and timepoints as indicated. The cytotoxicity of AMI-1 on sarcoma cells was assessed using a Cell Counting Kit-8. (B) Cell numbers were evaluated using a light inverted microscope (magnification, x100). (C) The effect of AMI-1 on colony formation of U2OS cells. Three individual experiments were performed. *P<0.05, **P<0.01, ***P<0.001 vs. Control; AMI-1, arginine methyltransferase inhibitor-1; OD, optical density.

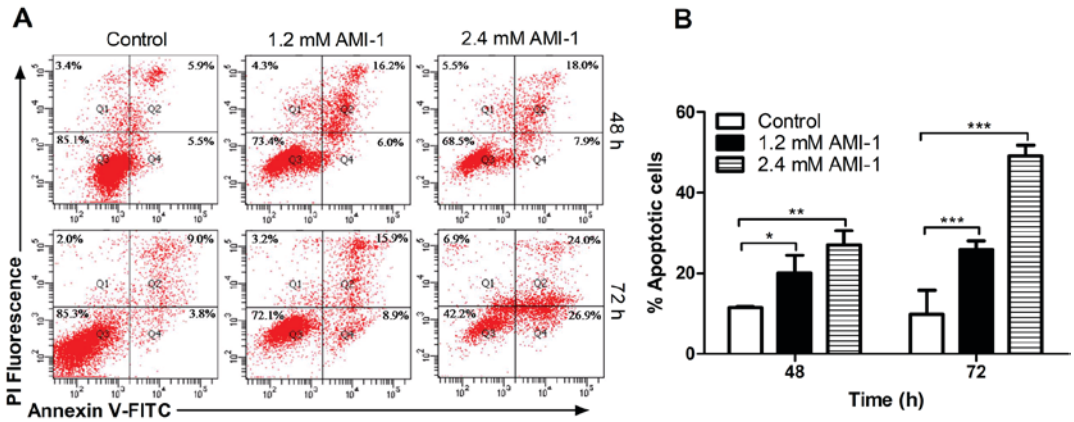

Figure 2. AMI-1 induces apoptosis in S180 cells *in vitro*. (A) S180 cells were treated with vehicle or AMI-1, and apoptosis was evaluated by flow cytometry using Annexin V-FITC/PI double staining. (B) The bar graph is a quantitative presentation of the flow cytometric data. Three individual experiments were performed. *P<0.05, **P<0.01, ***P<0.001 vs. control. AMI-1, arginine methyltransferase inhibitor-1; PI, propidium iodine; FITC, fluorescein isothiocyanate.

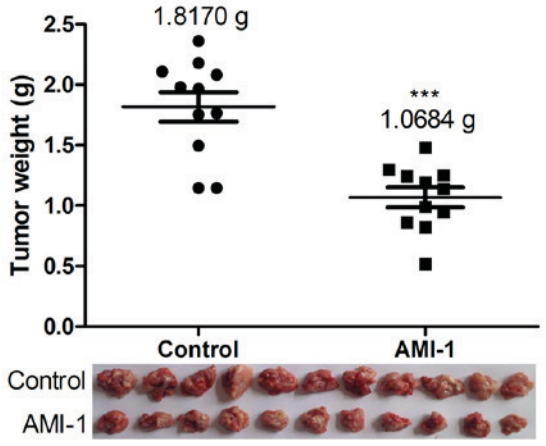

Figure 3. AMI-1 inhibits S180 viability *in vivo*. S180 cells were subcutaneously inoculated into the right axillary of mice. Mice were divided into two groups (11 animals/group): AMI-1-treated (0.5 mg in 0.9% NaCl) or vehicle-treated (0.9% NaCl). At the end of treatment tumors were dissected and weighted. ***P<0.001 vs. Control. AMI-1, arginine methyltransferase inhibitor-1.

This indicated that AMI-1 reduces S180 cell viability through the induction of cell apoptosis.

AMI-1 inhibits tumor viability of S180 cells in vivo. To assess the antitumor activity of AMI-1 *in vivo*, S180 cells were subcutaneously inoculated into the right axillae of mice. Tumor weight and body weight of control and treated groups are presented in Table I. AMI-1 treatment significantly decreased tumor weight compared with that in control-treated mice (Fig. 3). Additionally, IR of tumor viability in response to AMI-1 was 41.42±6.34% (data not shown). Furthermore, there were no significant differences in body weight of mice treated with AMI-1, compared with the control (Table I).

AMI-1 downregulates PRMT5 but does not regulate the expression of PRMT7 in a tumor xenograft model. A previous study demonstrated that AMI-1 inhibited the activity of type II arginine methyltransferase (PRMT5) (15). Therefore, in the present study, the expression PRMT5 in response to AMI-1 treatment was evaluated using a tumor xenograft model and western blot analysis. The results demonstrated that AMI-1 treatment significantly decreased the expression of PRMT5 but did not affect the expression of PRMT7 (Fig. 4). In addition, AMI-1 increased p53 protein levels, compared with control-treated tumors (Fig. 4).

AMI-1 decreases the levels of H4R3me2s and H3R8me2s in a tumor xenograft model. PRMT5 is a major type II arginine methyltransferase that catalyzes ω-NG, N'G-symmetric dimethylarginine (H4R3me2s and H3R8me2s) (8,27,28). Western blot analysis was employed to investigate the molecular mechanism by which AMI-1 may inhibit the viability of S180 cells *in vivo*. The results demonstrated that AMI-1 treatment significantly decreased the levels of H4R3me2s and H3R8me2s compared with those in the control group (Fig. 4).

Discussion

AMI-1 is a symmetrical sulfonated urea that inhibits type I PRMT activity *in vitro* (19,20). Investigation of the molecular mechanisms that lead to the inhibition of viability and induction of apoptosis of cancer cells may contribute to the design of novel therapeutic strategies and drugs (29,30). Therefore, in the present study, the possible antitumor effects of AMI-1 on S180 and U2OS cell were evaluated *in vitro*. The results demonstrated that AMI-1 significantly inhibited sarcoma cell viability using a CCK-8 assay. Additionally, the 2.4-mM dose of AMI-1 exhibited the highest antitumor activity. Next, the molecular mechanisms underlying the viability inhibitory activity of AMI-1 were investigated. Flow cytometric analysis demonstrated that treatment with AMI-1 induced apoptosis in S180 cells. Furthermore, the *in vivo* antitumor effects of AMI-1 were evaluated using S180-bearing mouse models. The

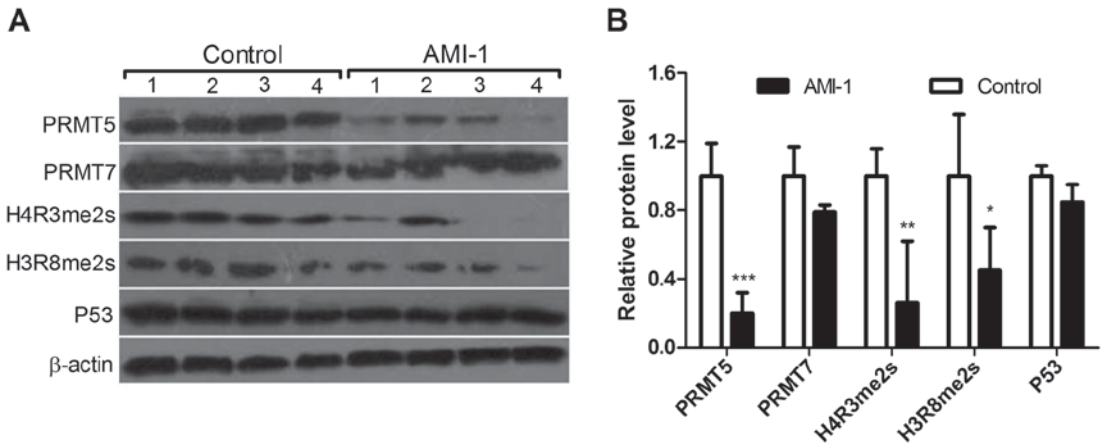

Figure 4. AMI-1 treatment decreased the expression of PRMT5 and the levels of H4R3me2s and H4R3me2s in a tumor-bearing mouse model implanted with S180 cells. (A) Western blot analysis of PRMT5, PRMT7, H4R3me2s H4R3me2s and p53 in a tumor-bearing mouse model implanted with S180 cells. Mice were divided into two groups (11 animals/group): AMI-1-treated (0.5 mg in 0.9% NaCl) or control [vehicle-treated (0.9% NaCl)]. The mice were treated for 7 days. (B) Densitometry analysis of PRMT5, PRMT7, H4R3me2s H4R3me2s and p53. β-actin was used as a loading control. *P<0.05, **P<0.01, ***P<0.001 vs. Control. AMI-1, arginine methyltransferase inhibitor-1; PRMT, protein arginine methyltransferase; H4R3me2s, symmetric dimethylation of arginine residues in histone 4; H3R8me2s, symmetric dimethylation of arginine residues in histone 3.

results demonstrated that AMI-1 significantly inhibited the viability of S180-implanted tumors *in vivo*.

PRMTs are classified into three groups of enzymes (type I, II and III) depending on their catalytic activity. Type II PRMT (PRMT5) catalyzes the transfer of methyl groups to the guanidino nitrogen atoms of arginine, resulting in ω-NG, N'G-symmetric dimethylarginine, whereas PRMT7 is the only type III PRMT catalyzing the formation of ω-NG-monomethylarginine (27,31,32). PRMT5 expression or activity is upregulated in various types of cancer and modulation of its expression regulates the viability of cancer cells. Therefore, PRMT5 may be a potential therapeutic target in cancer (33-35). Additionally, a previous study demonstrated that AMI-1 inhibited the activity of PRMT5 and suppressed the viability of colorectal cancer cells by targeting PRMT5 (15). Nevertheless, the molecular mechanism underlying the anticancer effect of AMI-1 in S180 remains unclear. In the present study, the levels of PRMT5 and PRMT7 following AMI-1 treatment were evaluated using a tumor-bearing mouse model implanted with S180 cells. The results demonstrated that AMI-1 treatment significantly decreased the expression levels of PRMT5 but did not affect the expression of PRMT7. These results suggest that AMI-1 may suppress the viability of S180 cells by downregulating the expression of PRMT5.

Similar to PRMT5, PRMT7 catalyzes the symmetrical methylation of arginine 3 of histone H4. However, PRMT7 does not catalyze the formation of H3R8me2s (a PRMT5-specific target) (36-38). In the present study, it was demonstrated that AMI-1 was able to decrease the levels of H4R3me2s and H3R8me2s in a tumor-bearing mouse model implanted with S180 cells. These results confirm that AMI-1 may inhibit the viability of S180 cells by targeting PRMT5 but not PRMT7.

The tumor suppressor p53 is an extensively studied gene in human cancer. PRMT5 is responsible for methylating p53 and PRMT5 depletion triggers p53-dependent apoptosis (39-42). Thus, in the present study, the expression of p53 expression was evaluated in a S180 tumor xenograft model treated with AMI-1. The results demonstrated that AMI-1 did not affect the expression of p53 *in vivo*. Additional studies are required to further elucidate the function of p53 in mediating the antitumor efficacy of AMI-1 in sarcoma.

In summary, the present study investigated the antitumor effects of AMI-1 on sarcoma cells *in vitro* and *in vivo*. To the best of our knowledge, the present study provides the first evidence that the effective antitumor activity of AMI-1 in S180-bearing mice was mainly due to the inhibition of the activity of PRMT5. Therefore, AMI-1 may be a potential therapeutic target for patients with sarcomas.

Acknowledgements

Not applicable.

Funding

This research work was supported in part by Fundamental Research Funds of the Central Universities (grant no. lzujbky-2013-169).

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

BZ and XueC conceived and designed the experiments. CP, SZ and XuC performed the experiments, TL and WZ analyzed the data. XueC and SG contributed to the acquisition of data, writing and revision of the manuscript. All authors read and approved final manuscript.

Ethical approval and consent to participate

Animal experiments were performed in accordance with the Institutional Animal Care and Treatment Committee of Lanzhou University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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