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Melarsomine suppresses canine osteosarcoma cell survival via inhibition of Hedgehog-GLI signaling

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ABSTRACT. The Hedgehog-GLI signaling pathway is activated in human and canine osteosarcoma (OSA) and represents a potential therapeutic target for cancers, including OSA. Arsenic trioxide represses GLI expression. Melarsomine, an arsenic compound-containing drug, has been approved for the treatment of canine heartworm disease. Hence, we hypothesized that melarsomine inhibits GLI signaling in canine OSA cell lines. The present study aimed to assess this hypothesis. Cell viability and colony formation were decreased in the canine OSA cell lines Abrams and D17 after treatment with melarsomine. Melarsomine-induced apoptotic cell death was assessed via cell cycle analysis using propidium iodide staining. Quantitative real-time reverse transcription polymerase chain reaction and western blot analyses revealed a downregulation of genes downstream of the Hedgehog signaling pathway, including *GLI1*, *GLI2*, and *PTCH*, after melarsomine treatment. The present results suggest that melarsomine exerts antitumor effects and serves as a GLI inhibitor in canine OSA cells. Additional studies are required to evaluate and confirm the anticancer effect and relevant therapeutic dose of melarsomine *in vivo*.

KEY WORDS: dog, GLI, Hedgehog signaling pathway, melarsomine, osteosarcoma

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Osteosarcoma (OSA) is the most common bone malignancy of dogs, occurring primarily in middle-aged and large or giant breeds, including Rottweiler, German Shepherd, Boxer, Doberman Pinscher, and Irish Setter [2, 30, 32]. OSA occurs at diverse locations, from the appendicular and axial skeleton, including the forelimbs, hind limbs, ribs, and skulls, to the extraskeletal muscles, including visceral organs [5, 15]. Moreover, OSA has high metastasis and recurrence rates despite surgical resection and chemotherapy [2, 30]. Although the mechanisms underlying OSA pathogenesis are currently fully defined, several human and canine studies have suggested the involvement of the Hedgehog (Hh)-GLI signaling pathway in OSA pathogenesis and progression [12, 18, 27].

In general, the Hh-GLI signaling pathway plays key roles in embryonic development and differentiation [8, 33]. Among the three Hh ligands—Sonic Hedgehog (SHH), Indian Hedgehog, and Dessert Hedgehog—SHH is the most well-described [23]. The Hh pathway comprises several proteins, including patched (PTCH) and smoothened (SMO) ligands and the GLI family of transcription factors [8, 33]. When the Hh-GLI signaling pathway is re-activated, it contributes to carcinogenesis and metastasis in several types of cancer [33]. SMO is inhibited by PTCH in the absence of the Hh ligand. However, if Hh is present, it binds to PTCH1, thereby alleviating SMO inhibition and promoting its translocation to the cytoplasm, followed by the activation of GLI proteins, including GLI1 and GLI2. The activated GLI proteins then enter the nucleus and regulate Hh target genes, including GLI itself and PTCH1 [26, 33].

Many clinical studies have investigated the regulation of the cancer-related Hh-GLI signaling pathway [33]. Arsenic trioxide

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(ATO) is an FDA-approved GLI inhibitor used to treat acute promyelocytic leukemia in humans and has been used in clinical trials for several other hematological and solid tumors [1, 16, 27, 36]. Two recent studies have reported that Hh-GLI signaling is activated in canine OSA and transitional cell carcinoma and that the GLI inhibitor GANT61 decreases cell viability [10, 28].

Melarsomine is an arsenic compound–containing drug widely used to treat canine heartworm and is known to produce various arsenic metabolite species in the liver [35]. The present study aimed to examine whether melarsomine exerts antitumor effects *in vitro* and whether melarsomine inhibits Hh-GLI signaling in canine OSA cell lines.

MATERIALS AND METHODS

Cell culture

The canine OSA cell line Abrams was kindly provided by Dr. Douglas Thamm, Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University. The canine OSA cell line D17 was purchased from American Type Culture Collection (ATCC; Manassas, VA, U.S.A.). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; PAN-Biotech, Aidenbach, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS; PAN-Biotech) and 1% penicillin-streptomycin (PAN-Biotech) in a humidified atmosphere of 5% CO_2.

Drug preparation

Melarsomine dihydrochloride (Immiticide®) was purchased from Merial (Duluth, GA, U.S.A.). The powder in the vial was aseptically reconstituted in sterile water to a final concentration of 25 mg/m*l* (50 mM). The solution was stored at 4°C and used within 24 hr. Corresponding vehicle controls were prepared using glycine USP (Sigma-Aldrich, St. Louis, MO, U.S.A.) since glycine is used as a lyophilization stabilizer in Immiticide®.

Cell viability assay

Abrams and D17 cells were seeded in triplicate into 96-well cell culture plates at a density of 3,000 cells/well. After incubation overnight, the media were replaced with 2% FBS/DMEM containing melarsomine at concentrations of 0, 20, 40, 60, 80, 100, 150, 200, 300, and 400 μM. The cells were incubated for 48 hr at 37°C and 5% CO_2. To determine cell viability, 10 μl WST-1 reagent from an Ez-Cytox Cell Viability Assay Kit (Dogenbio, Seoul, Korea) was added to each well, and the absorbance was measured at 450 nm using a Model 680 microplate reader (Bio-Rad, Hercules, CA, U.S.A.). The inhibitory concentrations of melarsomine that reduced cell survival by 50% (IC_{50}) were calculated from the assay results.

Trypan blue exclusion assay

Cells were seeded in six-well cell culture plates at a density of 5×10^4 cells/well. After 24 hr incubation, cells were cultured with 2% FBS/DMEM containing 80 μM or 120 μM melarsomine. The cells were harvested at 48 and 72 hr and stained with 0.4% trypan blue solution (Gibco, Grand Island, NY, U.S.A.). Viable cells were enumerated using a LUNA™ automated cell counter (Logos Biosystems, Annandale, VA, U.S.A.). Experiments were performed in quadruplicate.

Clonogenic assay

Cells were seeded at a density of 400 cells/well in six-well cell culture plates and were incubated until the cells attached to the plates but before they started replicating. Attached cells were cultured in 2% FBS/DMEM containing melarsomine at concentrations of 0, 40, 80, 120, and 160 μM for 48 hr. After the cells were incubated for an additional 7 days in fresh medium without the drug, the plates were washed with phosphate-buffered saline (PBS), and the colonies were stained with 2 m*l* of a mixture of 6.0% glutaraldehyde and 0.5% crystal violet for 30 min. Digital images of the stained colonies were captured, and the number of colonies per well were determined.

Cell cycle analysis

To investigate the effect of melarsomine on the cell cycle, Abrams and D17 cells were cultured with different concentrations (0, 80, and 120 μM) of melarsomine in 2% FBS/DMEM for 48 hr. Thereafter, cells were harvested, washed, fixed with 70% cold ethanol at −20°C for 2 hr, and washed twice with PBS. The cells were resuspended with propidium iodide (PI)/RNase staining buffer (BD Biosciences, San Jose, CA, U.S.A.) and incubated for 15 min at room temperature to stain DNA. The samples were analyzed by flow cytometry using a FACSVerse™ system (BD Biosciences).

Quantitative real-time reverse transcription polymerase chain reaction analysis

Total RNA from cultured cells was extracted using an Easy-BLUE Total RNA Extraction Kit (Intron Biotechnology, Seongnam, Gyeonggi, Korea) according to the manufacturer's instructions. One microgram of total RNA was used as the template for complementary DNA (cDNA) synthesis using the SuPrimeScript RT Premix (GeNet Bio, Daejeon, Korea). Expression levels of *GLI1*, *GLI2*, and *PTCH1* mRNA were quantified by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) using AMPIGENE qPCR Green Mix Lo-ROX with SYBR Green dye (Enzo Life Sciences, Farmingdale, NY, U.S.A.). The primer sequences used in this study were taken from a study by Gustafson *et al*. [10] and are listed in Table 1. Data were analyzed using the ΔΔCt method, and relative expression levels of target mRNAs were normalized to those of *HPRT1* (internal control).

Table 1. Primers used for quantitative real-time reverse transcription PCR[a)]

Target gene	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)	Size of product (bp)
GLI1	CAGCAGCTGAACCTTATGGA	GGGTGGTTCAGGATAGGAGA	116
GLI2	GCCTCAAGAAAGTGGGAAGA	TGGAGAAACAGGATTGGGTAAA	103
PTCH1	TGTCTGTAATCCTTCATGGGC	AAAGAGATGCCTTGGACCTG	139
HPRT1	TGCTCGAGATGTGATGAAGG	TCCCCTGTTGACTGGTCATT	191

a) Reverse transcription polymerase chain reaction.

Western blot analysis

Total proteins were extracted using PRO-PREP Protein Extraction Solution (Intron Biotechnology) and quantified using the Bio-Rad DC Protein Assay Kit (Hercules) according to the manufacturer's instructions. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis using an 8–12% resolving gel and electro-transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, U.S.A.). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing Tween 20 and incubated at 4°C overnight with primary antibodies. The primary antibodies included mouse anti-SHH (1:500; Santa Cruz Biotechnology, Dallas, TX, U.S.A.), rabbit anti-GLI1 (1:500; Aviva Systems Biology, San Diego, CA, U.S.A.), rabbit anti-PTCH1 (1:500; Aviva Systems Biology), and mouse anti-β-actin (1:1,000; Santa Cruz Biotechnology). The membranes were then washed and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 2 hr. Protein bands were visualized using enhanced chemiluminescence (Advansta, Menlo Park, CA, U.S.A.) and an ImageQuant LAS 4000 mini biomolecular imager (GE Healthcare Biosciences, Uppsala, Sweden).

Statistical analysis

All results are presented as mean ± standard deviation (SD) values. The IC_{50} values for melarsomine were fitted to a non-linear regression model. Statistical analyses were performed using repeated-measures ANOVA using GraphPad Prism v.6.01 software (GraphPad Inc., La Jolla, CA, U.S.A.). *P*-values less than 0.05 were considered statistically significant.

RESULTS

Inhibitory effect of melarsomine on cell viability and colony formation in Abrams and D17 cell lines

On the basis of WST-1 assays, the cell viability of both Abrams and D17 cells was significantly reduced by melarsomine treatment in a dose-dependent manner (Fig. 1A). The IC_{50} values (95% confidence interval) for Abrams and D17 cells were 111.2 μM (105.3–117.4, R^2=0.9886) and 133 μM (127.2–139.1, R^2=0.9937), respectively. There were no significant differences in cell viability among cells treated with the same doses of glycine. Enumeration of cells by trypan blue staining revealed that treatment with both 80 and 120 μM melarsomine resulted in dose-dependent and time-dependent cell death at 48 and 72 hr post treatment (Fig. 1B). Similarly, colony-forming ability was inhibited by melarsomine in both cell lines (Fig. 2A). The number of colonies was significantly lower at 80, 120, and 160 μM melarsomine compared to that for cells in the absence of the drug (0 μM) (P<0.001). The reduction was more evident in Abrams cells than that in D17 cells (Fig. 2B).

Effect of melarsomine on the cell cycle of Abrams and D17 cell lines

To determine whether melarsomine affected the cell cycle, we performed PI staining and flow cytometric analysis of the canine OSA cell lines Abrams and D17 (Fig. 3). Compared with untreated control cells, the population of Abrams and D17 cells treated with 120 μM melarsomine in the sub-G1 phase increased significantly (P<0.001). Treatment with glycine had no significant effect on the cell cycle of either cell line (Fig. S1).

Hh-GLI expression in Abrams and D17 cell lines treated with melarsomine

The effect of melarsomine on the expression of Hh-GLI mRNA in canine OSA cells was verified by qRT-PCR. In Abrams cells, both 80 μM and 120 μM melarsomine significantly downregulated *GLI1* and *GLI2* mRNA compared to untreated and glycine control cells (P<0.05) (Fig. 4A and 4B). In contrast, the expression levels of *PTCH1* mRNA remained unaffected by melarsomine treatment (Fig. 4C). Similarly, *GLI1* and *GLI2* mRNAs were downregulated in D17 cells treated with 80 μM and 120 μM melarsomine (P<0.05) (Fig. 4D and 4E). *PTCH1* mRNA levels were downregulated in D17 cells after treatment with 120 μM melarsomine (Fig. 4F).

We also examined the expression of Hh-GLI proteins in cells treated with melarsomine. Western blot analysis demonstrated that SHH and GLI1 were downregulated in Abrams and D17 cells after melarsomine treatment. PTCH1 levels were only affected by melarsomine treatment in D17 cells and not in Abrams cells (Fig. 5A). The bar graphs in Fig. 5B show SHH and PTCH1 protein levels quantified and normalized to that of β-actin.

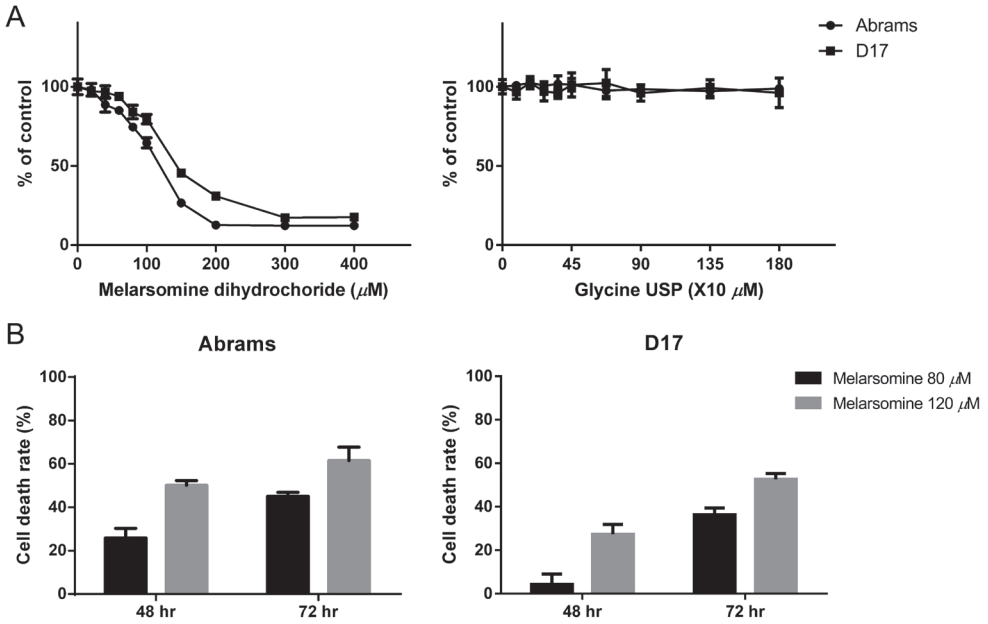

Fig. 1. Melarsomine decreased cell viability in the canine osteosarcoma cell lines Abrams and D17. (A) WST-1 assays revealed that melarsomine inhibited cell viability in a dose-dependent manner, while no significant differences were found among cells treated with the same doses of glycine. The results are expressed as the mean ± SD of triplicate samples. (B) Cell death rate after treatment with 80 and 120 μM of melarsomine was assessed by cell counting with trypan blue exclusion. Graph bars represent the mean ± SD of four independent experiments.

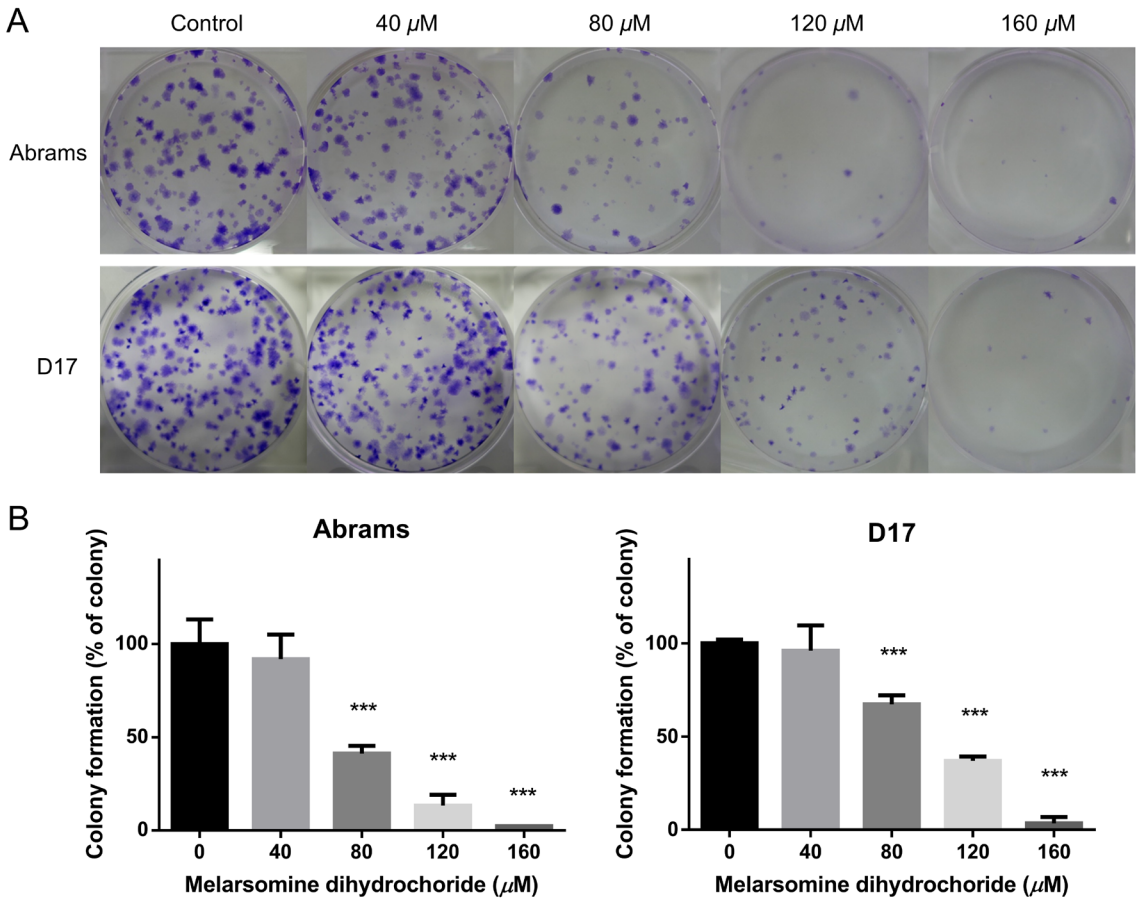

Fig. 2. Inhibitory effect of melarsomine on the colony-forming ability of the canine osteosarcoma cell lines Abrams and D17. (A) Colony formation assays were performed at the indicated concentrations. Representative cultures from two independent experiments are shown. (B) Quantification of colonies treated with melarsomine. The results are presented as the mean ± SD from duplicate observations. ***P<0.001

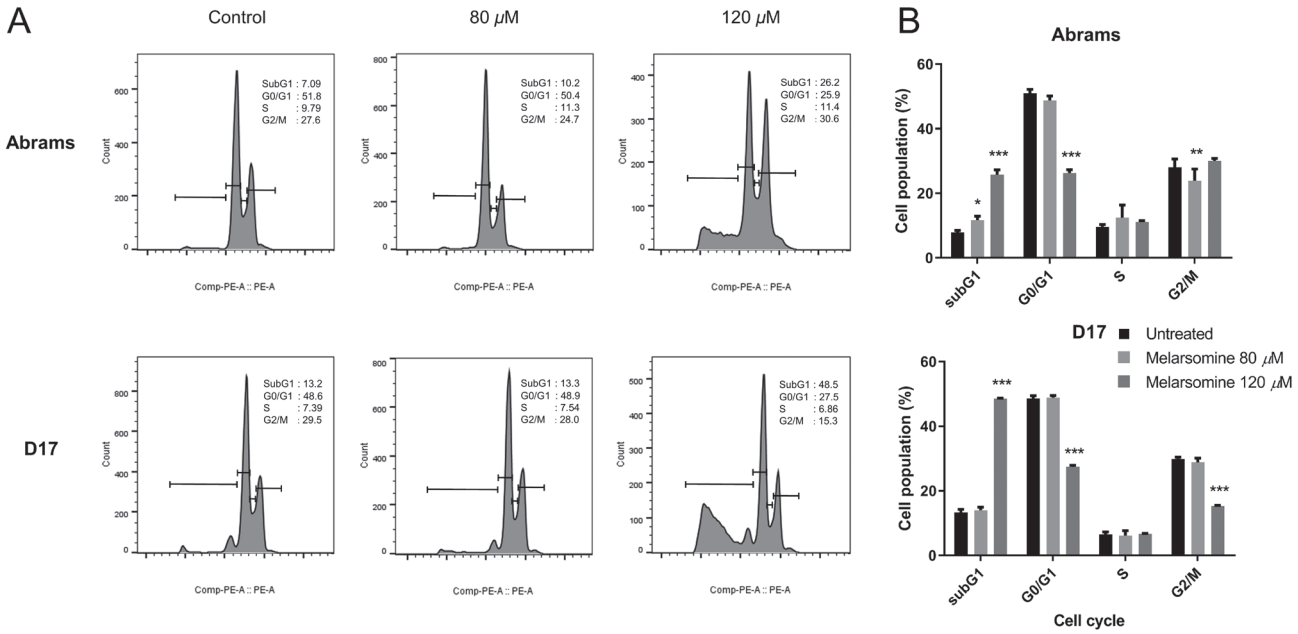

Fig. 3. Cell cycle analysis of the Abrams and D17 canine osteosarcoma cells treated with 0, 80, or 120 μM melarsomine. (A) Representative flow cytometric images exhibiting changes in cell cycle progression. (B) The histogram shows the percentages of cells in each cell cycle phase. Values represent the mean ± SD from four independent experiments. *, **, or *** indicate $P<0.05$, $P<0.01$, or $P<0.001$, respectively.

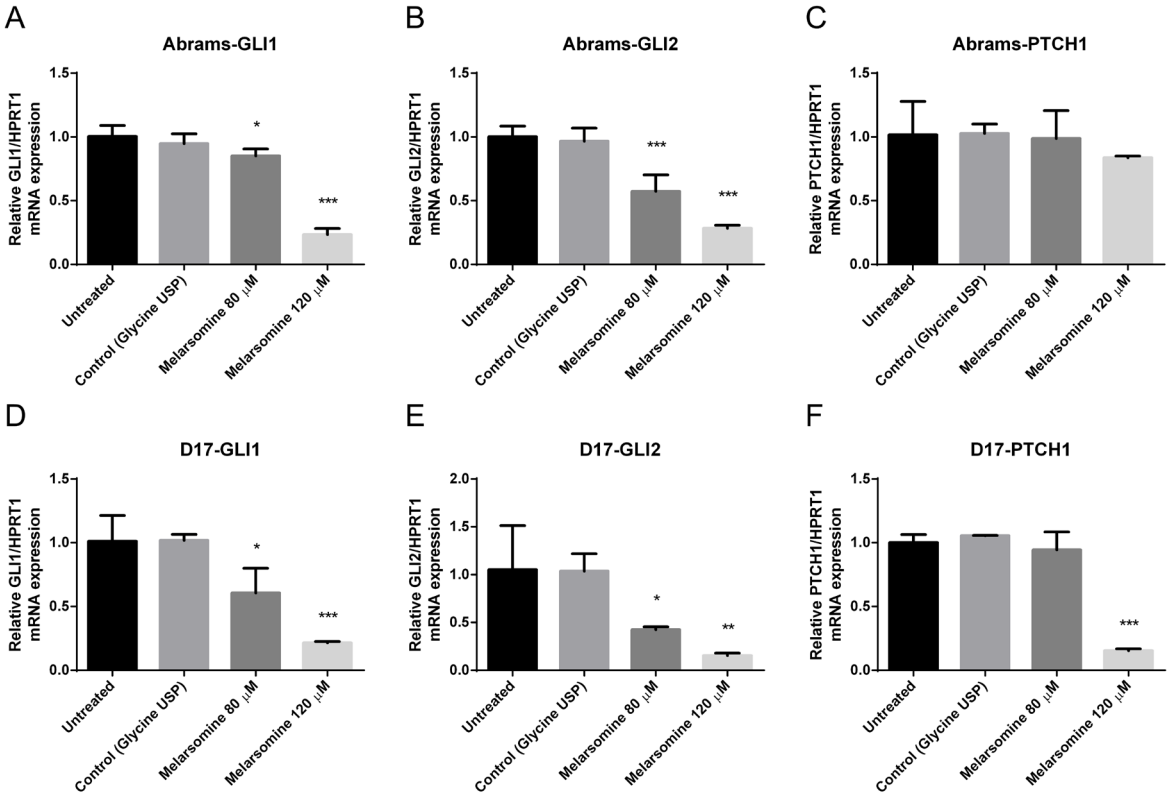

Fig. 4. Relative expression of the GLI transcription factor in Abrams and D17 canine osteosarcoma cell lines after treatment with melarsomine. Cells were cultured with 80 μM or 120 μM melarsomine for 48 hr, and the relative mRNA expression levels were determined by quantitative real-time reverse transcription polymerase chain reaction. Melarsomine treatment significantly downregulated *GLI1* and *GLI2* mRNA expression in both cell lines compared with that of untreated control cells. *PTCH1* expression in Abrams cells was not affected by melarsomine treatment, but that in D17 cells was significantly downregulated by treatment with 120 μM melarsomine. Glycine USP, a lyophilization stabilizer used in the melarsomine stocks, did not affect the expression of its target *GLI* compared with untreated control cells. The graph bars represent the means ± SD of triplicate reactions. *, **, or *** indicate $P<0.05$, $P<0.01$, or $P<0.001$, respectively.

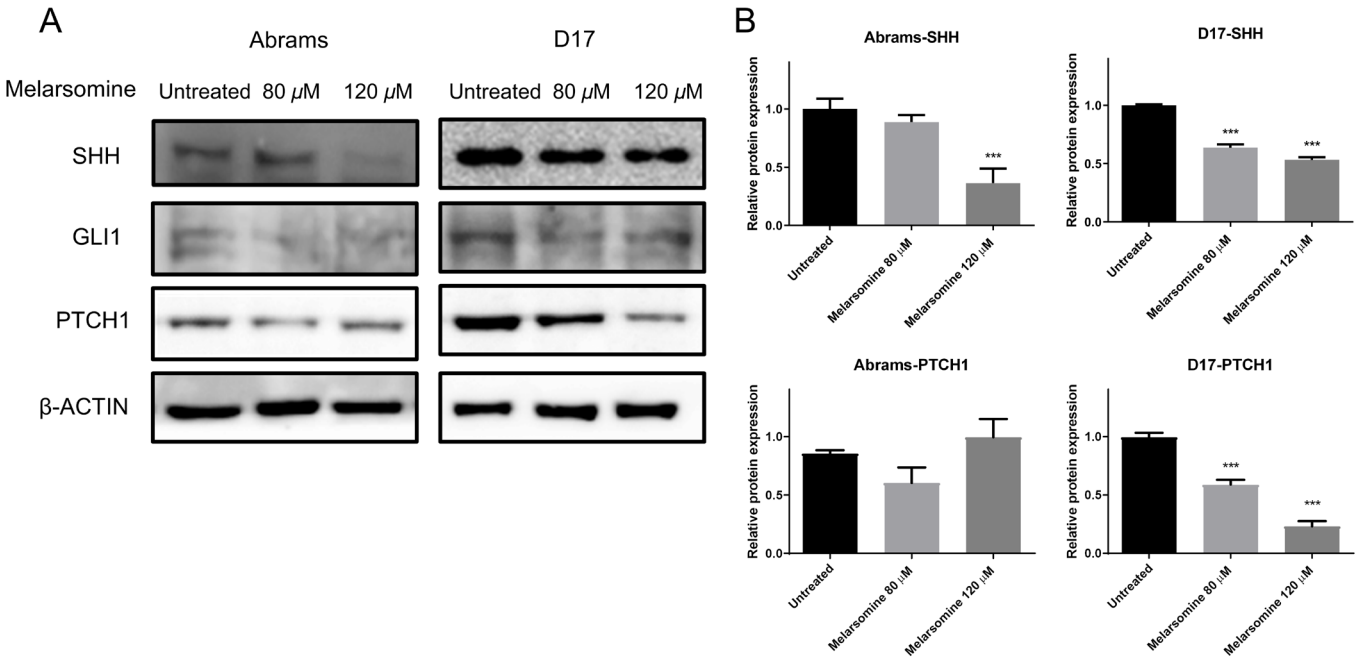

Fig. 5. The expression levels of Hedgehog pathway proteins in Abrams and D17 canine osteosarcoma cell lines treated with melarsomine were determined by western blot analysis. The Hedgehog pathway proteins SHH and PTCH1 were quantified and normalized to β-actin, and the results are shown in the graphs. GLI1 expression was suppressed in both cell lines by treatment with 80 μM or 120 μM of melarsomine. SHH expression in Abrams cells was suppressed only by treatment with 120 μM melarsomine. SHH and PTCH1 were both downregulated in D17 cells treated with either 80 μM or 120 μM melarsomine. All blots were performed in three independent experiments, and error bars represent SD. ***P<0.001 compared with the control.

DISCUSSION

To our knowledge, this is the first study to investigate the anticancer effect of melarsomine in canine cancer cell lines. Numerous clinical efforts have attempted to improve the outcome for dogs with OSA, including surgical excision, novel chemotherapeutic agents, and palliative radiation therapy [2, 3, 19, 22, 24]. Recently, diverse combinations of drugs, including pamidronate, gemcitabine, and suramin, have been administered in combination with classical OSA therapy to enhance survival rates and reinforce OSA therapeutic alternatives [31]. Previous studies have reported antitumor effects of arsenic compounds, including ATO, through the repression of GLI transcription in several cancers, including human OSA [1, 11, 21]. In the current study, we explored the potential antitumor effect of melarsomine, an arsenic-containing therapeutic agent for canine heartworm, on canine OSA cell lines.

Due to the growing interest in the role of the Hh signaling pathway in tumorigenesis, pathway components such as SMO, GLI, and PTCH have been widely studied as therapeutic targets for cancer [6, 12, 28]. The inhibition of Hh signaling by the SMO inhibitor cyclopamine, the GLI inhibitor GANT61, or the knockdown of *GLI2* prevents OSA cell proliferation [20, 28, 34]. In the present study, melarsomine treatment decreased the cell viability and colony-forming ability of Abrams and D17 cell lines. Melarsomine also affected the cell cycle, with the percentage of cells in the sub-G1 phase being significantly increased, which was consistent with an increase in apoptotic cells [9], although assessment for necrosis or caspase activation was not performed. ATO is a representative FDA-approved GLI1 and GLI2 inhibitor [16], previously reported to promote cancer cell apoptosis, reduce cell proliferation, and downregulate downstream Hh signaling genes in several cancers, including OSA, promyelocytic leukemia, malignant pleural mesothelioma, rhabdomyosarcoma, prostate cancer, and colon cancer [4, 13, 20, 36, 37]. ATO blocks Hh signaling by targeting GLI transcriptional effectors and reducing the ciliary accumulation of GLI2 [14]. In addition, ATO exposure stimulates apoptosis in OSA cells through the accumulation of DNA damage [21]. Another arsenic-containing agent, sodium arsenite, significantly suppresses SHH and GLI2 signaling in P19 mouse embryonic stem cells [17].

The two canine OSA cell lines used in this study, Abrams and D17, were selected on the basis of their previously reported relative expression levels of GLI transcription factors [28]. Among three canine OSA cell lines previously examined, D17 cells show the highest expression levels of *GLI1* and *GLI2* mRNA and are the most sensitive to the effect of GANT61 [28]. In the current study, Abrams cells were more sensitive than D17 cells to the inhibitory effect of melarsomine on cell viability and colony formation. In addition, 80 μM melarsomine increased the sub-G1 population only in Abrams cells. It seems that the antitumor effect of melarsomine was greater in the Abrams cell line than in the D17 cell line. However, the level of mRNA suppression by melarsomine was lower in Abrams cells than in D17 cells. These inconsistencies may have resulted from off-target effects or other cell line–specific characteristics.

Whether the knockdown of *GLI* affects canine OSA cell survival has not been evaluated. Several studies have reported that the transcriptional activity of *GLI2* is more important than that of *GLI1* for cell migration and metastasis in human OSA, since the knockdown of *GLI1* does not affect OSA cell proliferation and *GLI1* expression is not significantly increased in OSA biopsy samples [12, 14, 20]. In the current study, the repression of *GLI2* mRNA was more discernible than that of *GLI1*; however, this may not have been significant. The expression of GLI2 protein was not verified since we were unable to obtain an anti-GLI2 antibody that has been validated in dogs. If *GLI* knockdown inhibits OSA cell growth, it will be more clearly that the antitumor effect of arsenic compounds including melarsomine depends on the expression levels of Hh-related genes. The levels of PTCH1 decreased in D17 cells upon treatment with 120 μM melarsomine, but no significant difference was observed in Abrams cells following melarsomine treatment. A previous study reported that *PTCH1* mRNA levels were downregulated after treatment with ATO; however, the inhibition rates were lower than those seen for GLI1 and GLI2 in HsOs1 and U2OS cell lines [21]. These findings suggest that arsenic-containing GLI inhibitors would have a lesser impact on PTCH1 expression than on GLI1 and GLI2 expression.

Melarsomine, which has a narrow therapeutic margin, should be administered at a dose of 2.5 mg/kg twice, 24 hr apart, for the treatment for canine heartworm. A 3X error in dosing can result in pulmonary inflammation, edema, and death [25]. The concentration of melarsomine used in this study seemed rather high considering the PK value of melarsomine, however, there is no conversion formula from an *in vitro* dose to an *in vivo* dose. Before *in vivo* application, studies of measures to reduce clinical dosing are necessary, for example, repeated low-dose administration and change in formulation or route of administration.

In conclusion, this study showed that melarsomine decreased cell viability and colony-forming ability, induced apoptotic cell death in the canine OSA cell lines Abrams and D17, and inhibited GLI transcription factors. These results support the potential for the drug repositioning of melarsomine as a drug approved by the FDA for canine heartworm to one that might be used in treating cancers [29]. If melarsomine fulfills the requirements for clinical application as an anticancer agent in careful *in vivo* safety and efficacy analyses, its approval would save time and costs compared to the processing of a new drug, from drug discovery and development to the FDA monitoring of post-market drug safety [7]. However, further investigation is required before initiating any clinical studies to confirm the *in vivo* anticancer effects and better evaluate achievable therapeutic dosing.

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