Abstract. The phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) signaling pathway is a therapeutic target for various types of human tumors, and dual PI3K/mTOR inhibitors demonstrate antitumor activities in both preclinical and clinical studies. However, resistance mechanisms limit their abilities. As the molecular mechanisms involved in the cellular resistance are not clear in any canine tumors, an understanding of resistance mechanisms would support the potential use of dual PI3K/mTOR inhibitors in canine tumors. The antitumor activity of gedatolisib on cell viability, protein phosphorylation, and cell cycle distribution was assessed using 12 canine tumor cell lines from 6 types of tumors. In addition, the molecular determinants involved in the cellular sensitivity to gedatolisib were explored by investigating the involvement of serum-and-glucocorticoid-induced kinase 1 (SGK1), PIK3CA, and ATP-binding cassette, subfamily B, member 1 (ABCB1). The results demonstrated that gedatolisib decreased cell viability in all cell lines, with IC50 values <1 µM in 10 of the 12 lines. Gedatolisib inhibited Akt and mTOR complex 1 substrate phosphorylation and induced G0/G1 cell cycle arrest. However, certain cell lines with higher IC50 values were more resistant to these effects. These cell lines exhibited higher ABCB1 activity and the ABCB1 inhibitor cyclosporin A enhanced the decrease of cell viability caused by gedatolisib. SGK1 overexpression did not confer resistance to gedatolisib. The mutations of E545K and H1047R in PIK3CA were not observed. The present results indicated that gedatolisib decreased cell viability in canine tumor cell lines and ABCB1 played an important role in gedatolisib resistance, supporting the potential use of gedatolisib for canine tumors.

Introduction

The phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) signaling pathway plays an important role in cellular proliferation, growth, and survival by integrating signals from growth factors, cytokines, and other environmental sources (1,2). PI3K/mTOR inhibitors are therapeutic agents developed for various types of human tumors (3). These inhibitors include PI3K inhibitors, Akt inhibitors, mTOR complex 1 (mTORC1) inhibitors, and dual PI3K/mTOR inhibitors that inhibit PI3K catalytic isoforms, mTORC1, and mTOR complex 2 (mTORC2). Dual PI3K/mTOR inhibitors are more potent because they suppress the feedback re-activation that limits the efficacy of mTORC1 inhibitors (4,5).

Studies of dual PI3K/mTOR inhibitors indicate that several molecular mechanisms affect antitumor activity. In the PI3K/mTOR signaling pathway, mutations in the PIK3CA gene, which encodes the PI3Kα isoform, and the loss of phosphatase and tensin homolog (PTEN) increase sensitivity to dual PI3K/mTOR inhibitors (6). In contrast, various biological processes, including compensatory signaling pathways (7,8), the epithelial-mesenchymal transition (9), and drug efflux with ATP binding cassette transporters (10) mediate cellular resistance to dual PI3K/mTOR inhibitors. Despite these discoveries, strategies to overcome resistance mechanisms and develop biomarkers associated with clinical outcomes are not established. Therefore, the identification of the molecular determinants that affect the antitumor activity of these inhibitors is necessary to maximize the clinical outcomes for dual PI3K/mTOR inhibitor treatments (3).

The PI3K/mTOR signaling pathway is activated in specific canine tumors, including hemangiosarcoma (11,12), mammary...
carcinoma (12,13), glioma (12), lymphoma (12), mast cell tumor (12,14), osteosarcoma (15,16), and melanoma (17-19). Dual PI3K/mTOR inhibitors decreased cell viability and induced apoptosis, primarily in melanoma and hemangiosarcoma in vitro (19,20). However, the molecular mechanisms involved in the cellular resistance are not clear in any canine tumor.

The aim of the present study was to investigate the in vitro antitumor activity of gedatolisib, a dual PI3K/mTOR inhibitor, against various canine tumors, and to explore the molecular determinants involved in the cellular sensitivity to gedatolisib. The antitumor activity of gedatolisib on cell viability, protein phosphorylation, and cell cycle distribution was assessed using 12 canine tumor cell lines from six types of tumor. In addition, the involvement of serum-and-glucocorticoid-regulated kinase 1 (SGK1), PIK3CA, and ATP-binding cassette, subfamily B, member 1 (ABCB1) was investigated in gedatolisib resistance.

Materials and methods

Cell lines and culture. Two canine osteosarcoma cell lines (POS and HMPOS) (21,22), four canine urinary bladder transitional cell carcinoma (TCC; canine equivalent of muscle-invading bladder cancer in humans) cell lines [MCTCC, LCTCC, MegTCC, and MomoTCC], which was established at our laboratory (Laboratory of Veterinary Surgery, Department of Clinical Sciences, Graduate School of Veterinary Medicine, Sapporo, Japan) and erroneously named as MonoTCC in a previous study] (23,24), two canine malignant melanoma cell lines [CMeC, which was provided by the University of Tokyo (Tokyo, Japan) and MCM-N1, which was purchased by DS Pharma Biochemical Co., Ltd.] (25,26), two canine histiocytic sarcoma cell lines (DH82, which was purchased by DS Pharma Biochemical Co., Ltd. and CHS-4, which was provided by the University of Tokyo) (27,28), a canine mast cell tumor cell line (CoMS, which was established at our laboratory) (29), a canine lung adenocarcinoma cell line [CLAC, which was provided by Azabu University, (Sagamihara, Japan)] (30), and a human embryonic kidney cell line (293T, which was provided by Laboratory of Comparative Pathology, Department of Clinical Sciences, Faculty of Veterinary Medicine, Hokkaido University) were used in the present study. Each cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) and RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences, Inc.), 100 IU/ml penicillin G (Wako Pure Chemical Industries, Ltd.) and 100 µg/ml streptomycin (Wako Pure Chemical Industries, Ltd.) at 37˚C and 5% CO₂.

Inhibitors. Two mTORC1 inhibitors, rapamycin and everolimus, were purchased from AdipoGen Life Sciences and AdooQ Bioscience, respectively. Two dual PI3K/mTOR inhibitors (gedatolisib and PF-04691502), one Akt inhibitor (MK-2206), and one PI3K inhibitor (BKM120) were purchased from AdooQ Bioscience. ABCB1 inhibitors, cyclosporin A and tariquidar, were purchased from Wako Pure Chemical Industries, Ltd. and CHS-4, which was provided by AdooQ Bioscience. Two dual PI3K/mTOR inhibitors (gedatolisib and PF-04691502), one Akt inhibitor (MK-2206), and one PI3K inhibitor (BKM120) were purchased from AdooQ Bioscience. ABCB1 inhibitors, cyclosporin A and tariquidar, were purchased from Wako Pure Chemical Industries, Ltd. and CHS-4, which was provided by AdooQ Bioscience. The primary antibodies and secondary antibody used in the present study are listed in Table SI. The proteins were visualized using the Western BLoT Ultra-Sensitive HRP Substrate (Takara Bio, Inc.) and detected using an ImageQuant LAS-4000 mini system (GE Healthcare; Cytiva).

To prepare the cell lysate, 6x10⁵ cells were seeded in 6 cm dishes and cultured for 48 h at 37˚C and 5% CO₂. For evaluation of the dose-dependent suppression of gedatolisib, the cells were treated with 0.1% DMSO or various concentrations (0.1 nM-10 µM) of gedatolisib for 24 h. For evaluation of the time-dependent suppression of gedatolisib, the cells were treated with 0.1% DMSO or gedatolisib (100 nM or 10 µM) for 0, 4, 8, 24 and 48 h. For immunoblotting analysis of p-NDRG1 (Thr346), the cells were treated with 0.1% DMSO or 100 nM gedatolisib for 4 h.

Cell cycle analysis. Cells were seeded at 1-4x10⁵ cells/10-cm dish and incubated for 72 h at 37˚C and 5% CO₂. The cells were then treated with DMSO or gedatolisib (0.1, 1, or 10 µM) for 24 h, collected using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS), and fixed with 70% ethanol overnight at -30˚C. The fixed cells were treated with RNase A (10 µg/ml in PBS; EMD Millipore) for 30 min at 37˚C and stained with 50 µg/ml propidium iodide (PI) in PBS for 20 min at 25˚C. The DNA content of the cells was measured using a FACSuite software (BD Biosciences) equipped with a 488-nm argon laser and 527/32 and 700/54 nm bandpass filters. The percentage of cells at each stage of the cell cycle was calculated by manually gating the histograms. All data were analyzed using the FACSuite software 1.0 (BD Biosciences).

Immunocytochemistry. Immunocytochemistry was performed as previously described with minor modifications (33). Cells (0.2x10⁵) were cultured in an 8-well culture slide (Iwaki®) with 400 µl of the medium containing 10% FBS. After treatment with 100 nM gedatolisib for 24 h, the cells were fixed with pre-warmed PBS containing 4% paraformaldehyde and 4% sucrose at 37˚C for 10 min. The cells were permeabilized with 0.2% Triton X-100 (ICN Biomedicals) in PBS for 10 min and blocked with 5% FBS in PBS at 25˚C for 30 min. The antibodies and probes used this assay are presented in Table SI.
Establishment of SGK1-overexpressing cell lines. The canine SGK1 sequence was identified using cDNA from the CMeC cell line and transfected into the MegTCC cell line using a lentiviral system. The primers used for cell line establishment and the specific conditions of the polymerase chain reaction (PCR) are summarized in Tables SII and SIII, respectively. Canine SGK1 was amplified using KOD-Plus-Ver. 2 (Toyobo Life Science) and the fragment was ligated to the pTAA2 vector (Toyobo Life Science). This recombinant plasmid was introduced into DH5α competent cells (Takara Bio, Inc.), purified using a NucleoSpin Plasmid EasyPure kit (Takara Bio, Inc.), and sequenced by the Kazusa DNA Research Institute (Chiba, Japan).

The recombinant plasmid was digested with XhoI and BamHI (Takara Bio, Inc.), and the digested fragment was cloned into CSII-CMV-MCS-IRES2-Bsd (developed by Dr H. Miyoshi, Keio University, Tokyo, Japan). The plasmid was provided by the Riken BioResource Research Center through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan. The plasmid (4.6 µg) and packing plasmids (2.7 µg each) were co-transfected into 293T cells using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) for 15 min at 25°C. Then, the cells were further incubated for 4 h at 37°C with 5% CO₂ and medium was replaced with fresh medium.

After co-transfection of the plasmids, the culture supernatant was collected after 24 and 48 h, and the lentiviral vector particles were concentrated using a Lenti-X™ Concentrator (Takara Bio, Inc.). MegTCC cells were infected with the lentiviral vector using polybrene (Nacalai Tesque, Inc.) and cultured in DMEM supplemented with 10% FBS for 48 h at 37°C with 5% CO₂. The cells were exposed with 10 µg/ml blasticidin (Wako Pure Chemical Industries, Ltd.) for 48 h at 37°C with 5% CO₂, and growing cells were collected and maintained in DMEM supplemented with 10% FBS with 10 µg/ml blasticidin for further passage. After at least 5 times passages, the cells were used for subsequent experimentations.

As SGK1 function is post-transcriptionally regulated (34), the mutated SGK1 (SGK1 Δ60 S422D), which encodes the constitutively active form of SGK1 (35,36), was constructed. Inverse PCR was performed using a KOD-Plus-Mutagenesis kit (Toyobo Life Science), and this construct was introduced into competent cells. The same procedure as for SGK1 was performed for transfection, virus production, and infection of the mutated SGK1.

Gene accession numbers. The sequence data were submitted to the DNA Data Bank of Japan database (http://getentry.ddbj.nih.go.jp) under the accession nos. LC424654-LC424655 and LC424656-LC424679 for PIK3CA and SGK1, respectively.

Statistical analysis. All statistical analyses were performed using GraphPad Prism software 7.04 (GraphPad Software, Inc.). For the cellular viability assay, IC50 values were calculated by fitting dose-response curves to a three-parameter variable slope sigmoidal dose-response model. For cell cycle analysis, only the G0/G1 phase was compared between groups based on light and the PI intensity, respectively. For flow cytometry analysis, only viable cells were evaluated for rhodamine-123 efflux. For the in vitro PI3K/mTOR inhibition study, the LC50 values were calculated by fitting dose-response curves to a three-parameter model, respectively. The sample number in each group was based on light and the PI intensity, respectively.

Results

Gedatolisib decreases cell viability in various types of canine tumor cell lines. Cell viability against the dual PI3K/mTOR

Figure 1. Schematic diagram of the PI3K/mTOR signaling pathway. PI3K/mTOR inhibitors used in the present study, included rapamycin, everolimus, gedatolisib, PF-04691502, MK-2206, and BKM120. PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog; TSC2, tuberous sclerosis complex 2; mTORC, mammalian target of rapamycin complex; 4E-BP1, 4E-binding protein 1; FOXO3, forkhead box O3.
inhibitor gedatolisib compared with other PI3K/mTOR signaling pathway inhibitors including rapamycin, everolimus, PF-04691502, MK-2206, and BKM120 in 12 canine tumor cell lines from 6 types of tumor were evaluated (Fig. 1). All inhibitors, except for the mTORC1 inhibitors, decreased cell viability in a dose-dependent manner (Fig. 2A). Gedatolisib exhibited a marked decrease in cell viability with lower median IC50 values than the other inhibitors and IC50 values of <1 µM in 10 of the 12 cell lines (Fig. 2B).

**PI3K/mTOR signaling pathway is activated in canine tumor cell lines.** To reveal the mode of action of gedatolisib and explore the molecular determinants affecting cellular sensitivity to this inhibitor, the basal expression and phosphorylation of PI3K/mTOR signaling pathway molecules were examined, by verifying the presence or absence of protein expression and phosphorylation rather than semi-quantitative comparison. The phosphorylation of p70S6 kinase (p70S6K) was clearly observed in all cell lines except in MegTCC, MomoTCC, MCM-N1 and CoMS cells. The phosphorylation of 4E-binding protein 1 (4E-BP1) was clearly observed in TCCs, MCM-N1, and CLAC cells. It was observed that mTORC1 and its substrates (p70S6K and/or 4E-BP1) were phosphorylated in all cell lines, which suggested that mTORC1 is active in these cell lines (Fig. 3A). The expression of PTEN and tuberous sclerosis complex 2 (TSC2) was examined because the deficiency of these suppressive regulators of the PI3K/mTOR signaling pathway has been revealed to increase cell sensitivity to PI3K/mTOR inhibitors (6,38,39). The PTEN expression was not observed in five cell lines (POS, HMPOS, MCM-N1, DH82, and CHS-4). However, there were no significant differences in the IC50 values between PTEN-positive and PTEN-negative/low cell lines (Fig. 3B). TSC2 expression was observed in all cell lines, which indicated that TSC2 expression did not sensitize the low IC50 cell lines against gedatolisib.

**Gedatolisib inhibits the phosphorylation of PI3K/mTOR signaling pathway molecules in MegTCC and LCTCC cells.** The inhibitory effect of gedatolisib on the phosphorylation of PI3K/mTOR signaling pathway molecules in
The G0/G1 phase in MegTCC and LCTCC cells, but LCTCC gedatolisib significantly increased the percentage of cells in the G0/G1 phase in canal tumor cell lines. The effects of gedatolisib on cell cycle distribution were assessed in the canine tumor cell lines. The time-course inhibition of p70S6K phosphorylation was observed in five cell lines (CLAC, CHS-4, MegTCC, MCM-N1, and HMPOS), in which the IC50 values were <100 nM. To explore the molecular determinants involved in the cellular sensitivity to gedatolisib, the canine tumor cell lines were divided into two groups, namely the low-IC50 cell lines (IC50 values <100 nM) and high-IC50 cell lines (IC50 values >100 nM). The percentage increase of cells in the G0/G1 phase in the low-IC50 cell lines was significantly higher than in the high-IC50 cell lines (Fig. 5C). The localization of p27kip1 was examined to demonstrate the mechanism of the G0/G1 cell cycle arrest. In MegTCC cells, p27kip1 expression was primarily observed in the nucleus (Fig. 5D).

Overexpression of SGK1 does not confer resistance to gedatolisib in MegTCC cells. As LCTCC cells resisted the inhibition of mTORC1 and G0/G1 cell cycle arrest, it was hypothesized that the molecular determinants were not downstream molecules from Akt, or drug efflux pumps. The compensatory signaling pathways were investigated and the involvement of SGK1, which has a similar function to that of Akt (Fig. 6A) (34) was evaluated because overexpressed SGK1 has been revealed to confer resistance to PI3Kα and Akt inhibitors (40, 41).

In CLAC and MegTCC cells, which exhibited low IC50 to gedatolisib, the phosphorylation level of N-myc down-stream-regulated 1 (NDRG1), a substrate of both SGK1 and Akt (40), was low with gedatolisib treatment (Fig. 6B), which suggested that the activities of Akt and SGK1 were low. To the best of our knowledge, this is the first study that identified the canine SGK1 sequence (Fig. S1) and established MegTCC cells expressing SGK1 to reveal the contribution of SGK1. The expression of SGK1 was observed in MegTCC cells transfected with wild-type and mutated SGK1, which was constitutively the active form of SGK1 (Fig. 6C) (35, 36). Neither the overexpression of SGK1 nor that of its mutated form was observed to confer cellular resistance to gedatolisib (Fig. 6D).

SGK1 expression was low in MegTCC cells, while it was high in LCTCC cells. To explore the contribution of PI3K, the presence of gene mutations E545K and H1047R in PIK3CA, which increase the sensitivity of tumor cells to dual PI3K/mTOR inhibitors, were examined (6). Consequently, gene mutations E545K and H1047R in PIK3CA were not observed in any of the canine tumor cell lines, which indicated that these mutations did not sensitize the low-IC50 cell lines against gedatolisib (Fig. S2).

ABC1 inhibition enhances the antitumor activity of gedatolisib in LCTCC cells. The functional integrity of ABCB1 was evaluated using rhodamine-123, a well-established ABCB1 substrate (42). It was demonstrated that the percent decrease of the mean fluorescence intensity in the high-IC50 cell lines was significantly higher than in the low-IC50 cell lines (Fig. 7A and B).

To reveal the importance of ABCB1 in gedatolisib resistance, the ABCB1 function was inhibited using cyclosporin A (43). The dose-response curve of cyclosporin A in LCTCC is presented in Fig. S3. Treatment with cyclosporin A increased the mean fluorescence intensity of...
Figure 4. Gedatolisib inhibits the phosphorylation of PI3K/mTOR signaling pathway molecules in canine tumor cell lines. (A) Dose-response effects of gedatolisib on the phosphorylation of PI3K/mTOR signaling pathway molecules in MegTCC and LCTCC cells. Cells were treated with increasing concentrations of gedatolisib for 24 h before lysis. The phosphorylation of PI3K/mTOR signaling pathway molecules was then evaluated using immunoblotting. (B) Time-course effects of gedatolisib. MegTCC and LCTCC cells were treated with 100 nM or 10 µM gedatolisib for 0, 4, 8, 24 and 48 h. PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; p-, phosphorylated; 4E-BP1, 4E-binding protein 1; p70S6K, p70S6 kinase.

Figure 5. Gedatolisib induces G0/G1 cell cycle arrest in canine tumor cell lines. (A) The cell cycle distribution (top panels) was evaluated using flow cytometry after 24 h of treatment with 100 nM, 1, 10 µM gedatolisib, or DMSO (control) in MegTCC and LCTCC cells, with the percentage of each cell cycle phase (bottom panels) analyzed. Data are presented as the mean ± SD from three independent experiments. (B) Percentage increase of cells in the G0/G1 phase after 24 h of treatment with 100 nM gedatolisib in canine tumor cell lines. Data are presented as the mean ± SD from three independent experiments. (C) The mean percent increase of cells in the G0/G1 phase in the low (IC50 values <100 nM, n=7) and high (IC50 values >100 nM, n=5) IC50 cell lines. Each point represents the individual mean from three independent experiments, with bars representing the median. (D) p27kip1 localization in MegTCC cells treated with 100 nM gedatolisib for 24 h. These cells were stained with the anti-p27kip1 antibody (green) and counterstained with DAPI (blue) and actin (gray). Scale bars, 50 µm. **P<0.01 and ***P<0.001 (for A and B, multiple t-tests; for C, Mann-Whitney U test). DMSO, dimethyl sulfoxide.
rhodamine-123, which demonstrated inhibition of ABCB1 function in LCTCC cells (Fig. 7C). Furthermore, ABCB1 inhibition enhanced the decrease in cell viability (Fig. 7D), PI3K/mTOR signaling pathway inhibitors and induced G<sub>0</sub>/G<sub>1</sub> cell cycle arrest (Fig. 7F) that resulted from gedatolisib treatment.

To demonstrate the effect of ABCB1 inhibition, tariquidar, an ABCB1 inhibitor (44), and toceranib, a receptor tyrosine kinase inhibitor with a similar structure to sunitinib (45), were evaluated. Toceranib was selected because it is frequently used to treat various canine tumors (46), and its analog sunitinib has a weaker ABCB1 inhibitory effect than did cyclosporin A (47). Consequently, tariquidar enhanced the decrease of cell viability against gedatolisib with a potent ABCB1 inhibitory effect. However, toceranib did not produce this enhancement and caused less ABCB1 inhibition than tariquidar (Fig. S3).

These results indicated that ABCB1 inhibition enhanced the antitumor activity of gedatolisib in LCTCC cells.

Discussion

In the present study, the PI3K/mTOR signaling pathway inhibitors decreased cell viability in the canine tumor cell lines derived from six types of tumors. These results were consistent with the results of previous studies that revealed that PI3K/mTOR signaling pathway inhibitors decreased cell viability in various types of tumor cells (10,12). However, the question remains whether PI3K/mTOR signaling pathway inhibitors selectively exhibit antitumor activity to tumor cells. The PI3K/mTOR signaling pathway is involved in numerous cellular functions in non-tumor and tumor cells (1). As the PI3K/mTOR signaling pathway is aberrantly activated in tumor cells, PI3K/mTOR signaling pathway inhibitors have been revealed to selectively decrease cell viability in tumor cells (3). In fact, BEZ235, which is a dual PI3K/mTOR inhibitor, exhibited anti-leukemic activities in acute myeloid leukemia cells without affecting normal hematopoiesis in vivo (48). Although the effect of PI3K/mTOR signaling pathway inhibitors in non-tumor cells remains unclear in this study, the present results suggest that PI3K/mTOR signaling pathway inhibitors decrease cell viability against various types of canine tumor cells.

Gedatolisib decreased cell viability in most of the canine tumor cell lines. The IC<sub>50</sub> values in 10 of the 12 cell lines were <1 µM, which are similar to those in human tumors (non-small cell lung cancer and breast cancer) currently in clinical trials (3,10). The two cell lines with high IC<sub>50</sub> values were from TCC and melanoma and they exhibited high ABCB1 activity. In a previous study, gedatolisib decreased cell viability of 50 diverse human tumor cell lines. There were resistant cell lines from multiple tumor types and ABCB1 inhibition enhanced the anti-proliferative activity of gedatolisib (10). The present results were consistent with these results, which suggests that canine tumors have a similar sensitivity and resistance mechanism as human tumors.

Overexpression of SGK1 did not confer resistance to gedatolisib and the gene mutations in PIK3CA were not observed in all canine tumor cell lines. Previous studies revealed that overexpressed SGK1 conferred resistance to PI3Kα and Akt inhibitors (40,41). However, the involvement of SGK1 in the cellular sensitivity to the dual PI3K/mTOR inhibitor, BEZ235, remains unclear and the results in this study could not show the involvement of SGK1 in the cellular sensitivity to gedatolisib. As for PIK3CA, several studies have reported the involvement in the cellular sensitivity to the dual PI3K/mTOR inhibitor, BEZ235, in human tumor cell lines (6). Since the gene mutation in PIK3CA was not observed in all the cell lines used in the present study, the involvement of PIK3CA remains unclear in canine tumor cell lines. To reveal its involvement, further research with gene modification is required.

In the present study, high ABCB1 activity in the resistant cell lines was observed. ABCB1 inhibition in those cells resulted in the suppression of the PI3K/mTOR signaling pathway and induction of a G<sub>0</sub>/G<sub>1</sub>-cell cycle arrest. To the best of our knowledge, this is the first study to explore the molecular mechanisms involved in the cellular resistance in canine tumor cell lines.
tumors. These results were consisted with ones in human tumor cell lines (10), suggesting that ABCB1 was involved in the resistance of gedatolisib, decreasing the suppression of the PI3K/mTOR signaling pathway in both human and canine tumor cell lines. In human tumors, however, compensatory signaling pathways are reported as resistance mechanisms; the signaling pathways cross-talk with and compensate each other (7,8,49). In the present study, the contribution of other signaling pathways in gedatolisib resistance was not clear. As ABCB1 expression is regulated by several signaling pathways and transcriptional factors (50), it may be possible that other signaling pathways are involved in the resistance mechanisms to gedatolisib via ABCB1 expression.

ABCB1 inhibition enhanced cell viability reduction due to gedatolisib in canine tumor cell lines. The same effect occurs in human tumor cell lines (10), which suggests the following two possibilities. Firstly, ABCB1 expression could be a predictive marker for the efficacy of gedatolisib. In a clinical study, stathmin, a regulatory protein for microtubule dynamics, was used as a biomarker for PI3K/mTOR signaling activation. However, stathmin levels did not correlate with the efficacy of gedatolisib (51). Evaluation of the ABCB1 expression level in tumor tissue would be beneficial to identify the predictive marker. Secondly, ABCB1 could be a therapeutic target to overcome gedatolisib resistance. In the present study, ABCB1 was inhibited using three compounds and it was discovered that the third generation ABCB1 inhibitor, tariquidar, exhibited the highest inhibition of ABCB1 (Fig. S3). Although ABCB1 inhibition by cyclosporin A and multi-kinase inhibitors has been proposed (52), the third generation ABCB1 inhibitors may be selected for further studies due to their inhibitory activity.

At least three limitations are acknowledged in the present study. Firstly, the in vivo activity of gedatolisib against canine tumors remains unclear. It is necessary to evaluate the in vivo activity and tolerability of gedatolisib using an animal model. Secondly, although 12 cell lines from six types of tumor were
used, the number of tumor types and cell lines was limited. The antitumor activity in other types of tumors is worth evaluating. Thirdly, potential mechanisms involved in cellular sensitivity to gedatolisib were explored. However, the contribution of other possible mechanisms (e.g., other signaling pathways, pH, nutrient depletion, and hypoxia) was not elucidated. Further investigation for the resistance mechanisms is warranted.

In conclusion, the dual PI3K/mTOR inhibitor gedatolisib potently inhibited the activation of the PI3K/mTOR signaling pathway, decreased cell viability, and induced a G1/S cell cycle arrest in the canine tumor cell lines. These effects were enhanced by ABCB1 inhibition. Collectively, these novel results support the potential usage of gedatolisib for canine tumors and suggest that ABCB1 plays an important role in the cellular resistance to gedatolisib.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
KH, SK and YM conceived and designed the experiments. YM and TS performed the experiments. YM analyzed the data. KH, SK, and MO supervised the experiments. YM wrote the draft manuscript. KH and MO reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

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Competing interests
The authors declare that they have no competing interests.

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