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A THESIS FOR THE DEGREE OF MASTER

**Antitumor Activity of Rivoceranib against
Canine Mammary Gland Tumor Cell Lines**

개의 유선종양 세포주에 대한
리보세라닙의 항암 효과

2020년 2월

서울대학교 대학원
수의학과 임상수의학 (수의내과학) 전공
이 정 하

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이 논문을 수의학 석사 학위논문으로 제출함

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Abstract

Antitumor Activity of Rivoceranib against Canine Mammary Gland Tumor Cell Lines

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Canine mammary gland tumors (CMGTs) are the most common tumors in female dogs. Rivoceranib (also known as apatinib) is a novel antiangiogenic tyrosine kinase inhibitor that selectively binds to vascular endothelial growth factor receptor-2 (VEGFR2). The aim of this study was to disclose the antitumor effects of rivoceranib on CMGT cell lines. The direct effects of rivoceranib on CMGT cells in vitro were analyzed by cell proliferation and migration assays. Cell-cycle distribution and apoptotic ratio were analyzed by flow cytometry. Expression levels of phosphorylated VEGFR2 were evaluated by western blot analysis. Rivoceranib treatment significantly reduced the proliferation and migration of CMGT cells in a dose-dependent manner. Flow cytometry results revealed significant increases in

G₀/G₁ phase arrest and apoptosis proportional to the drug concentration used. Rivoceranib reduced the level of phosphorylated VEGFR2. I confirm that rivoceranib exerts antitumor effects on CMGT cells by inhibiting biological functions.

Keywords : Apatinib, Antitumor, Canine mammary gland tumor cell, Rivoceranib, VEGF, pVEGFR2

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1. Introduction

Canine mammary gland tumors (CMGTs) are the most common neoplasms in female dogs (1, 2). Over 95% of CMGTs are of epithelial origin, half of which are diagnosed as malignant (3, 4), and approximately 50% of malignant CMGTs have the potential to undergo metastasis. Adjuvant chemotherapy is recommended for patients with high risk of metastasis or recurrence (1, 2, 5). However, very few studies have demonstrated the efficacy of adjuvant chemotherapy in patients with CMGT (1), highlighting the need for an effective chemotherapy agent for these patients.

As vascular endothelial growth factor (VEGF) and its receptors are crucial in carcinogenesis (6-8), agents targeting VEGF may prove useful for metastatic neoplasms (9). The combination of anti-angiogenic drugs and conventional chemotherapy was proved to exert therapeutic effects against malignant tumors (10). VEGF family members mediate angiogenesis, and VEGF receptor-2 (VEGFR2) is a major receptor (11, 12). As VEGF-mediated angiogenesis is rare and occurs during wound healing and the female reproductive cycle, targeting VEGF is deemed to exert minimum effects on normal physiological procedures (13).

Rivoceranib (also known as apatinib; distributed by HLB Life Science Co., Ltd) is a small tyrosine kinase inhibitor targeting VEGFR2 (14). It is approved in China as a third line anti-angiogenic drug for advanced metastatic gastric cancer (15). Few phase II studies have demonstrated the effects of rivoceranib on human breast cancer (16, 17). However, there have been no reports on the direct effects of

rivoceranib on canine mammary cancer cell lines.

In the present study, I explored the direct effects of rivoceranib on the proliferation, migration, cell-cycle distribution, and apoptosis of CMGT cells. Furthermore, I measured the protein levels of cyclin D1 and phosphorylated VEGFR2 in rivoceranib-treated CMGT cells. Our findings provide experimental evidence on the application of rivoceranib as an antitumor agent in clinical practice.

2. Materials and Methods

2.1. Cell cultures

Canine malignant mammary gland tumor cell lines (CIP) were obtained from the Department of Veterinary Clinicopathology, Seoul National University (SNU). CIP cells were derived from both primary (CIPp) and metastatic tumors (CIPm). The CIPp and CIPm cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% solution of 10,000 units/ml penicillin and 100 µg/ml streptomycin (PAN Biotech, Aidenbach, Germany) at 37°C in a humidified chamber containing 5% CO₂. The media were replenished every 3 days. The cells were subcultured upon reaching 90% confluency.

2.2. Antitumor agents

Powder-type rivoceranib (known as apatinib) gifted by HLB Life Science Co. Ltd. (Seongnam, Republic of Korea) was dissolved in dimethyl sulfoxide (Duchefa Biochemie, Haarlem, the Netherlands) at a concentration of 40 mM. The solution was stored at -20°C.

2.3. Isolation of canine peripheral blood mononuclear cells

(cPBMCs)

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of SNU (protocol no. SNU-190517-2), and all protocols were in accordance with approved guidelines. Blood samples were obtained from healthy canine donors (n=3, Maltese, 3-11 years old, a female and two males) with the consent of the owner. The donors had been regularly presented for medical check-up at the Seoul National University Veterinary Medicine Teaching Hospital. The blood samples were diluted with an equal volume of Dulbecco's phosphate-buffered saline (DPBS; Pan Biotech) and layered over Ficoll-Paque solution (GE Healthcare Life Science, Chicago, IL, USA) in conical tubes. After centrifugation at $450 \times g$ for 30min with low brake, the buffy coat layer was aspirated with a pipette. RBC lysis buffer (Sigma-Aldrich) was added to the aspirated cells, which were then incubated for 5min at 20°C. The cells were washed with DPBS and centrifuged at $450 \times g$ for 10min. After removal of the supernatant, cPBMCs were resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% solution of 10,000 units/ml penicillin and 100 µg/ml streptomycin.

2.4. Cell viability assay

CIPp and CIPm cells were seeded in 96-well plates at 1,000 cells/well and incubated overnight. The cells were then treated with rivoceranib at 0, 3.125, 6.25,

12.5, 25, 50, and 100 μM or 0.5% dimethyl sulfoxide without rivoceranib for 24, 48, and 72h. Following incubation, 10 μl Cell Counting Kit (CCK) solution (DPlus™ CCK cell viability assay kit; Dong-in Biotech, Seoul, Republic of Korea) was added and the cells were incubated in the dark at 37°C for 1h. The absorbance at 450 nm wavelength was determined with a spectrophotometer (Epoch Microplate spectrophotometer; BioTek Instruments, Winooski, VT, USA).

cPBMCs were seeded in 96-well plates at a density of 1×10^5 cells/well. Two-third of cells were stimulated with 25 $\mu\text{g}/\text{ml}$ concanavalin A (Sigma-Aldrich) or 1 $\mu\text{g}/\text{ml}$ lipopolysaccharide (Sigma-Aldrich) and one-third of cells were left in an unstimulated state. All cells were subsequently treated with different concentrations of rivoceranib. After incubation for 24, 48, and 72h, the cells were treated with 10 μl CCK solution (D-Plus™ CCK Cell Viability Assay kit; Dong-in Biotech) for 1h and the absorbance at 450 nm was determined with a spectrophotometer (BioTek Instruments).

2.5. Wound-healing assay

CIPp and CIPm cells were seeded in 12-well plates at a density of 1×10^5 cells/well. After reaching 100% confluency, the cells were treated with 2 $\mu\text{g}/\text{ml}$ of mitomycin (Enzo Life Science, Farmingdale, NY, USA) for 2h. The cell monolayer was manually scratched with a 1 ml pipette tip and floating debris was removed with two washes of PBS. The adherent cells were cultured in RPMI medium supplemented with 10% fetal bovine serum and 1% solution of 10,000 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin in the presence or absence of rivoceranib

(12.5 or 25 μM) for 24h. Wound healing was estimated by measuring the width of the wound area under a microscope (ULWCD 0.30; Olympus, Tokyo, Japan) at 100 \times magnification. The width was imaged at 0, 12, and 24h using a TCapture program (Tucsen Photonics, Fuzhou, China). The width of the wound was calculated as the percentage relative migration width as follows: $[(\text{Relative width at 0 h} - \text{relative width at 12h or 24h})/\text{relative width at 0 h}] \times 100$.

2.6. Cell-cycle analysis

CIPp and CIPm cells were seeded in six-well plates at a density of 1×10^5 cells/well and incubated at 37°C overnight. The cells were treated with different concentrations of rivocecranib (0, 12.5, or 25 μM) for 48h. Following incubation, the cells were harvested, washed with PBS, and centrifuged at $300 \times g$ for 5min. The cells were fixed with pre-chilled 70% ethanol at -20°C for 2h. After fixation, the cells were washed with PBS and centrifuged at $300 \times g$ for 5min. The cells were stained with 0.5 mL of propidium iodide (PI)/RNase staining buffer (BD Pharmingen, San Diego, CA, USA) and incubated at 20°C for 15min in the dark. The cell-cycle distribution was analyzed within 1h using a flow cytometer (FACs Aria II; BD Bioscience, San Jose, CA, USA).

2.7. Apoptosis analysis

CIPp and CIPm cells were seeded in six-well plates at a density of 1×10^5

cells/well at 37°C overnight. The cells were treated with different concentrations of rivocecanib (0, 12.5, and 25 µM) for 48h. After treatment, the cells were harvested, washed twice with cold PBS, and centrifuged at 300 × g for 5min. After washing, Annexin V Apoptosis Detection Kit I (BD Pharmingen) was used according to the manufacturer's instructions. The cells were resuspended in a 1× binding buffer at a of ~1×10⁶ cells/ml, and 100 µl of the solution (~1×10⁵ cells) was transferred to a 15 ml conical tube. To stain cells, 5 µl of fluorescein isothiocyanate (FITC) annexin V and 5 µl of PI were used. The cells were incubated for 15min at 20°C in the dark and the apoptosis rate was analyzed within 1h using a flow cytometer (BD Bioscience).

2.8. Western blot assay

CIPp and CIPm cells were treated with 0, 12.5, and 25 µM of rivocecanib for 48h. Total protein was extracted from cells using PRO-PREP Protein Extraction Solution (Intron Biotechnology, Seong-nam, GG, Republic of Korea). The protein concentration was quantified using the Bio-Rad DC Protein assay kit (Bio-Rad, Hercules, CA, USA). For cyclin D1 expression analysis, 20 µg protein samples were loaded and separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For pVEGFR2 analysis, 50 µg protein samples were separated with 8% SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride membranes (0.45 µM; Merck Millipore, Burlington, MA, USA), and then blocked with 5% skim milk diluted in Tris-buffered saline containing 0.1% Tween-20 for 1h at 20°C. The membranes were probed with antibodies against cyclin D1 (1:1000; Novus Biologicals, Centennial, CO, USA), phosphorylated (p)VEGFR2

(1:250; Cell Signaling Technology, Danvers, MA, USA), and β -actin (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C overnight. Following incubation, the membranes were washed and treated with either goat anti-mouse horseradish peroxidase-labeled secondary antibody (Bethyl Laboratories, Montgomery, TX, USA) or goat anti-rabbit horseradish peroxidase-labeled secondary antibody (Enzo Lifesciences) for 1h. Immunoreactive bands were detected using ImageQuant Las4000mini (GE Healthcare Life Sciences, Chicago, IL, USA).

2.9. Statistical analysis

Each experiment was performed at least thrice. GraphPad Prism (version 5) software (GraphPad Software, San Diego, CA, USA) was used for statistical analyses. The data were analyzed using the Student's t-test and one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test. The results are presented as the mean value \pm standard deviation (SD). Differences with a value of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Rivoceranib inhibited the proliferation of CMGT cell lines

To assess the cytotoxic effects of rivoceranib, CIPp and CIPm cells were treated with different concentrations (0-100 μM) of the drug for 24, 48, and 72h. The viability of both CIPp and CIPm cells decreased in response to treatment with rivoceranib in a dose-dependent manner (Figure 1). Rivoceranib significantly reduced the viability of CIPp cells at 12.5, 25, 50 and 100 μM after 24 and 48h as compared with the control group (Figure 1A; $p < 0.001$). The viability of CIPp cells was significantly reduced at 25, 50 and 100 μM after 72h (Figure 1A; $p < 0.001$). Rivoceranib also significantly reduced the viability of CIPm cells at concentrations of 12.5 μM and above at all time points compared to the control group (Figure 1B; $p < 0.001$). However, no significant difference was observed in the viability of the cells treated with rivoceranib at concentrations below 6.25 μM for different time points. Thus, rivoceranib was found to inhibit the proliferation of CMGT cells in a dose-dependent manner.

3.2. Rivoceranib exerted no cytotoxic effects on cPBMCs

To determine the cytological effects of rivoceranib on cPBMCs, the cells were treated with different concentrations (0, 12.5, and 25 μM) of rivoceranib for 24, 48, and 72h. Cell viability was not significantly different following treatment with 0,

12.5, and 25 μ M rivoceranib for different times (Figure 2A). I used concanavalin A or lipopolysaccharide to stimulate cPBMCs before rivoceranib treatment. The viability of the stimulated cells was not significantly different at all the evaluated time points (Figure 2B and C). Thus, rivoceranib had no significant cytotoxic effects on normal immune cells.

3.3. Rivoceranib inhibited the migration of CMGT cell lines

I estimated the effects of rivoceranib on cell migration using the wound-healing assay. Rivoceranib disrupted wound closure in CIPp and CIPm cells (Figure 3A). As shown in Figure 3B, rivoceranib significantly inhibited the migration of both CIPp and CIPm cells in a dose-dependent manner ($p < 0.001$). These results confirm the ability of rivoceranib to suppress the migration of CIPp and CIPm cells.

3.4. Rivoceranib induced cell-cycle arrest in CMGT cell lines

The cell-cycle distribution of CIPp and CIPm cells after rivoceranib treatment for 48h was determined using flow cytometry. As shown in Figure 4A, naïve CIPp and CIPm cells were mostly found in the G_0/G_1 phase ($66.33\% \pm 0.21\%$ and $66.09\% \pm 0.32\%$, respectively), and similarly distributed between the S phase ($14.09\% \pm 0.16\%$ and $14.82\% \pm 1.07\%$, respectively), and G_2/M phase ($19.58\% \pm 0.09\%$ and $19.09\% \pm 0.99\%$, respectively). After treatment with 12.5 μ M rivoceranib, $72.76\% \pm 0.17\%$ and $69.11\% \pm 0.18\%$ of CIPp and CIPm cells, respectively, were

detected in the G₀/G₁ phase, 13.09%±0.74% and 12.97%±0.64% in the S phase, and 19.58%±0.58% and 17.92%±0.58% in the G₂/M phase. Treatment with 25 μM rivoceranib led to 76.96%±0.56% and 82.62%±0.29% of CIPp and CIPm cells, respectively, were found to be in the G₀/G₁ phase, 11.85%±0.84% and 6.58%±0.08% in the S phase, and 11.19%±0.75% and 10.80%±0.28% in the G₂/M phase. The ratio of the cells in the G₀/G₁ phase after rivoceranib treatment significantly increased in a dose-dependent manner (Figure 4B; p < 0.001). Thus, rivoceranib induced G₀/G₁ phase arrest in a dose-dependent manner.

3.5. Rivoceranib promoted apoptosis of CMGT cell lines

I assessed the apoptotic ratio of CIPp and CIPm cells after incubation with rivoceranib for 48h using flow cytometry (Figure 5A). The apoptotic ratio significantly increased for CIPp cells treated with 12.5 μM (p < 0.01) and 25 μM (p < 0.001) of rivoceranib, while that of CIPm cells significantly increased at 25 μM (p < 0.01; Figure 5B). These results demonstrate that rivoceranib induced apoptosis in CMGTs.

3.6. Rivoceranib down-regulated the expression of cyclin D1 and pVEGFR2 in CMGT cell lines

To investigate the underlying mechanism, I measured the effect of rivoceranib on the protein level of cyclin D1. As shown in Figure 6A, the expression level of

cyclin D1, a G₀/G₁ phase-related protein, significantly decreased in both CIPp and CIPm cells after rivoceranib treatment in a dose-dependent manner (Figure 6B; $p < 0.001$). Thus, rivoceranib induce G₀/G₁ phase arrest.

I measured the effect of rivoceranib on the protein level of pVEGFR2. As shown in Figure 7A, the expression level of pVEGFR2, an angiogenesis-related protein, was significantly reduced in CIPp and CIPm cells in a dose-dependent manner by rivoceranib (Figure 7B; $p < 0.001$). However, the intensity of pVEGFR2 was stronger in CIPp than in CIPm cells. These data indicate that rivoceranib treatment down-regulated the expression of pVEGFR2 in CMGTs.

4. Discussion

In the present study, rivoceranib was found to significantly inhibit the proliferation and migration of CMGT cells in a dose-dependent manner. However, I failed to note any significant effect on the viability of normal cPBMCs or immune cells. I found that the cytotoxic effects of rivoceranib were attributed to an increase in the apoptosis rate and G₀/G₁ phase arrest. The level of the G₀/G₁ phaserelated protein cyclin D1 was reduced in a dose-dependent manner, consistent with the result of G₀/G₁ phase arrest. Furthermore, I found that the expression of pVEGFR2, a crucial signal transducer in angiogenesis pathway (13), was significantly reduced in a rivoceranib concentration-dependent manner. In addition, CIPp cells showed more prominent down-regulation of pVEGFR2 expression than did CIPm cells. Together these findings are consistent with the clinical implications of rivoceranib, suggestive of its application as an efficient antitumor agent for the adjuvant chemotherapy of patients with CMGT.

CMGTs are the most prevalent tumors in female dogs (1, 2). Approximately 50% of these tumors are malignant, and half of the malignant forms metastasize (18, 19). Surgery alone is insufficient for the treatment for CMGTs at risk of recurrence or metastasis (5). Therefore, adjuvant treatment is necessary for patients at high risk (19, 20). Current adjuvant chemotherapy includes doxorubicin, carboplatin, 5-fluorouracil, and cyclophosphamide (1, 21). However, it is difficult to demonstrate clinical efficacy of adjuvant chemotherapy without any anti-angiogenic therapy (22).

The concept of using anti-angiogenesis as an antitumor treatment regimen was

introduced by Judah Folkman who revealed angiogenesis to be the key factor in tumor growth (23). Tumors obtain nutrients from the blood supply; hence, tumor cells secrete various pro-angiogenic growth factors in order to survive (9, 24). Among the pro-angiogenic growth factors, VEGF is a key factor involved in developmental angiogenesis (24-27). VEGF was reported to be overexpressed in approximately 30-60% of solid tumors (28) and inhibition of VEGF expression was shown to reduce tumor growth in an animal experiment (29-31). VEGF binds to two receptors, VEGFR1 and VEGFR2 (32). Of the two receptors, VEGFR2 is the key mediator of VEGF (33). All approved anti-angiogenic drugs are known to affect the binding of VEGF to VEGFR2 or to activate intracellular VEGFR2 (33).

Rivoceranib (also known as apatinib or YN968D1) is a novel selective VEGFR2 tyrosine kinase inhibitor with a 10-fold stronger binding capacity than sorafenib (14). In human gastric cancer, rivoceranib was shown to be a possible third line antitumor agent in a phase III clinical trial (15). A few studies have reported the promising therapeutic effects of rivoceranib on various human tumor types in phase II clinical trials (16, 34-37). In murine xenograft models such as of human lung, colon, stomach and nasal cancer, tumor growth was inhibited by rivoceranib alone or in combination with other conventional chemotherapy agents (14, 38, 39). As rivoceranib exerts indirect anti-angiogenesis antitumor effects, the studies on rivoceranib have mostly reported its indirect effects (14, 40, 41). However, recent studies have confirmed the direct antitumor activity of rivoceranib in various human tumor cell lines (42-46). Rivoceranib directly inhibited the proliferation and migration (44-46) and enhanced apoptosis and cell-cycle arrest of human tumor cell lines (45-47). These results are consistent with our canine data. In the present

study, I confirmed that pVEGFR2 expression was significantly down-regulated in rivocecanib-treated CMGTs.

The present study has a few limitations. As I conducted experiments without recombinant VEGF except for the small quantity of VEGF in the serum-containing media and used CMGT cells alone without endothelial cells, the protein expression of pVEGFR2 was weak. Studies have revealed higher levels of circulating VEGF in dogs with malignant tumors than in healthy dogs (48-53). Secondly, further research including *in vivo* experiments and clinical trials are warranted to confirm my findings.

5. Conclusion

In conclusion, the present study demonstrates the inhibitory effects of rivoceranib on the biological functions of CMGTs *in vitro*. To the best of my knowledge, this study revealed the direct inhibitory effects of rivoceranib on CMGT cell lines for the first time. These findings provide the basis to support the potential clinical application of rivoceranib as an adjuvant chemotherapy for patients with CMGT.

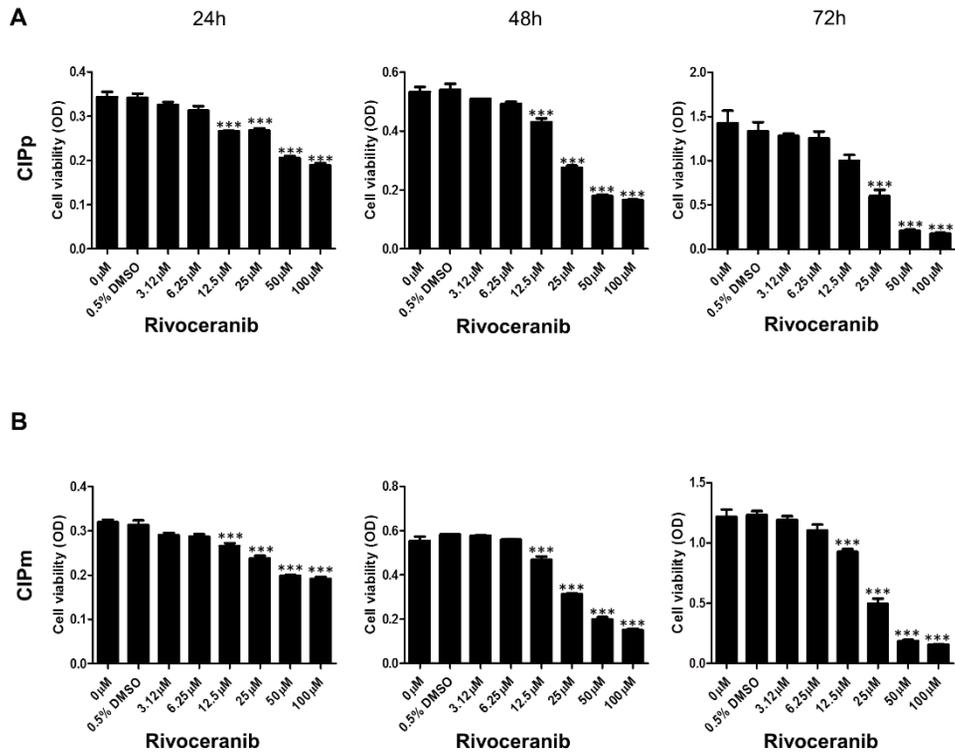


Figure 1. Rivocecanib inhibited proliferation of CIP canine mammary gland tumor cells in a dose-dependent manner. A: Viability assay of primary CIP (CIPp) cells treated with different concentrations of rivocecanib for 24, 48, and 72h. B: Viability assay of metastatic CIP (CIPm) cells treated with different concentrations of rivocecanib for 24, 48, and 72h. All experiments were performed in triplicates. The data represent mean \pm standard deviation (n=3). ***Significantly different at $p < 0.001$ versus control group (0.5% dimethyl sulfoxide).

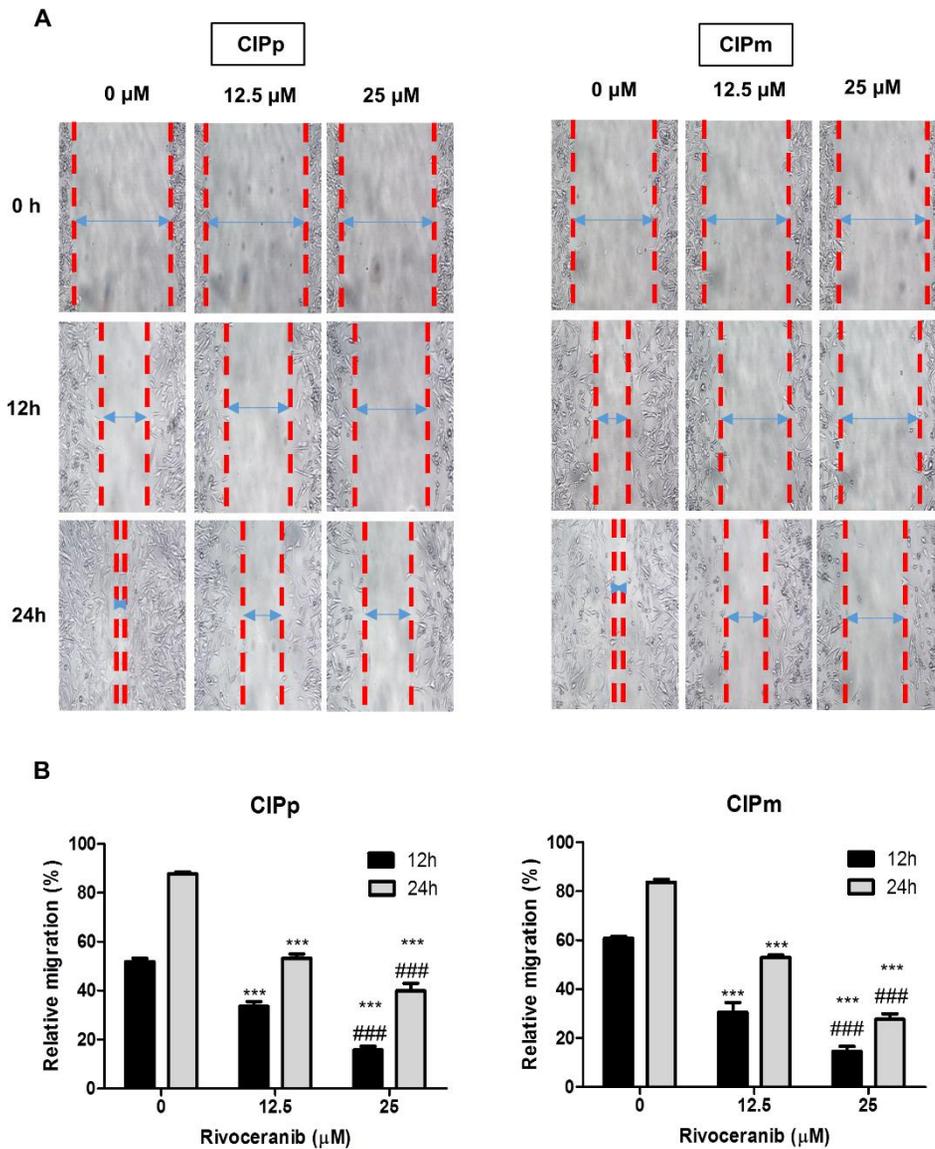


Figure 3. Rivoceranib inhibited the migration of CIP canine mammary gland tumor cells. A: The migratory capacity of primary (CIPp) and metastatic (CIPm) CIP cells was measured after treatment with rivoceranib (0, 12.5, and 25 μM) for 48h using scratch wound-healing assays. B: The relative migration is shown via histograms. All experiments were performed in triplicates. The data represent the mean \pm standard deviation ($n=3$). Significantly different at: *** $p < 0.001$ versus control group. ### $p < 0.001$ in cells treated with 25 μM versus those treated with 12.5 μM .

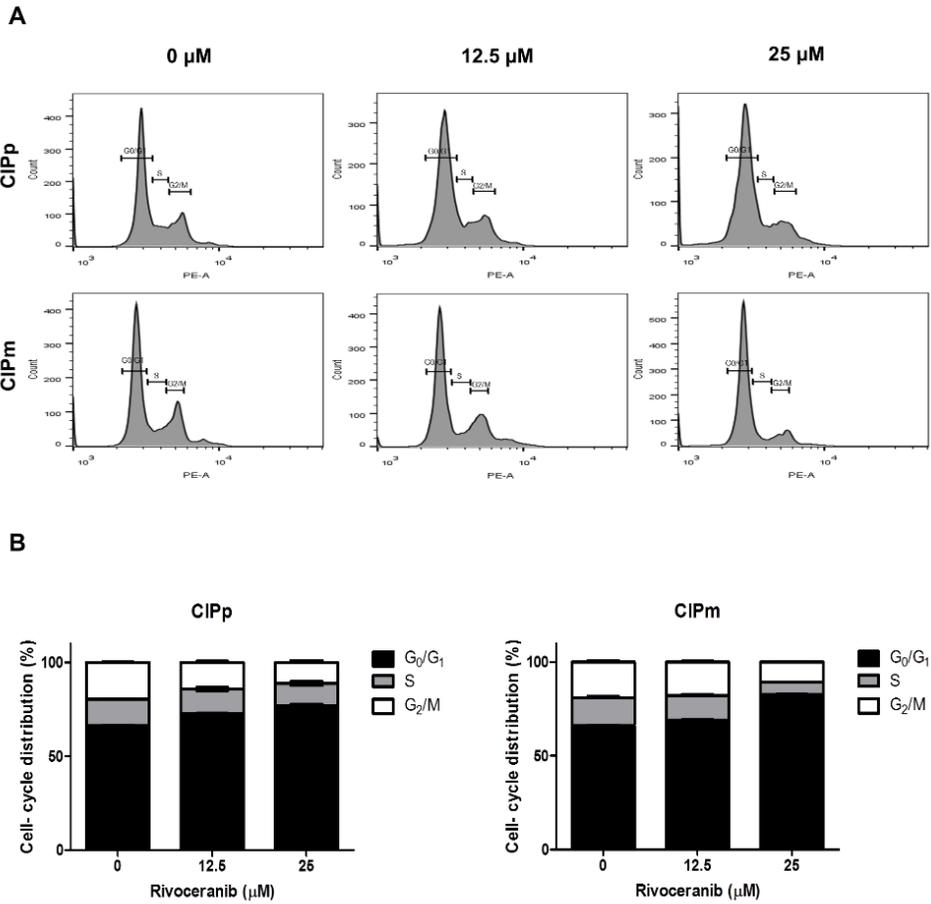


Figure 4. Rivoceranib induced G₀/G₁ phase arrest of CIP canine mammary gland tumor cells in a dose-dependent manner. A: The cell-cycle distribution of primary (CIPp) and metastatic (CIPm) CIP cells treated with rivoceranib (0, 12.5, and 25 μM) for 48h as detected using flow cytometry. B: Quantification of data shown in A. The percentages of cells in G₀/G₁, S, and G₂/M phases are shown. All experiments were performed in triplicates. The data represent the mean ± standard deviation (n=3).

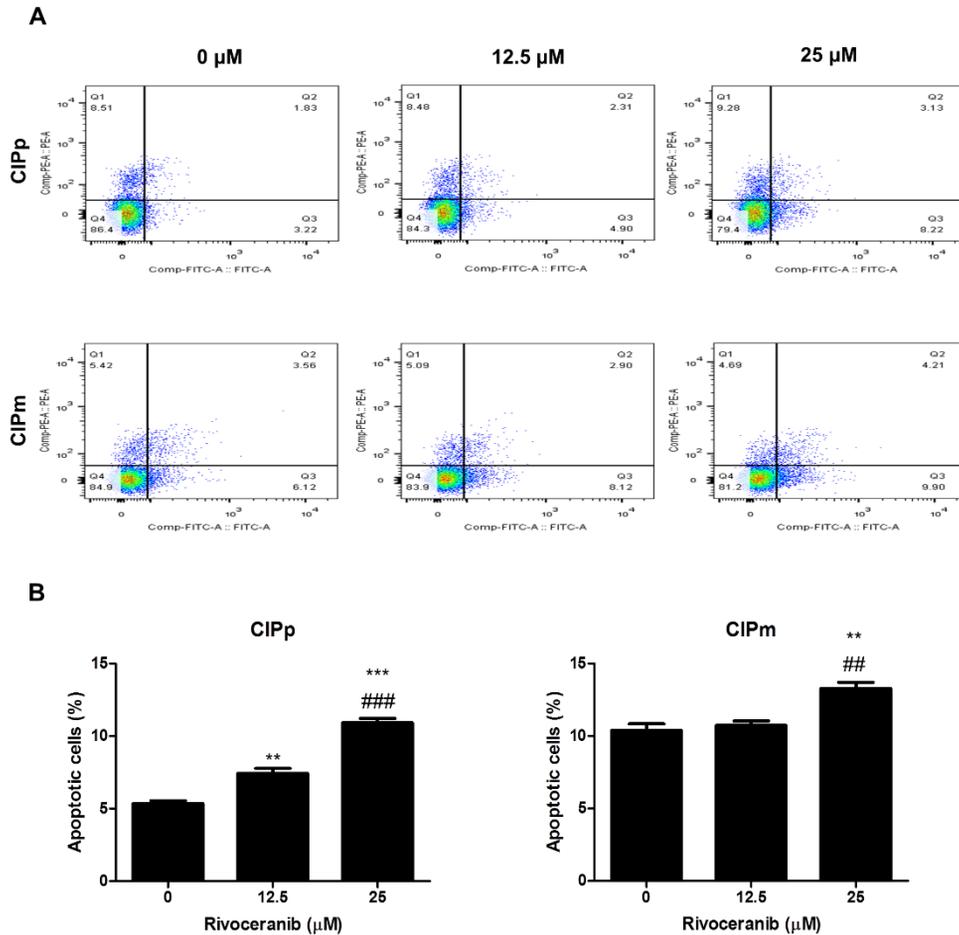


Figure 5. Rivoceranib promoted apoptosis of primary (CIPp) and metastatic (CIPm) CIP canine mammary gland tumor cells. A: The apoptotic ratio was determined using flow cytometry. CIPp and CIPm cells were treated with rivoceranib (0, 12.5, and 25 μM) for 48h. B. Quantification of data shown in A. The percentage of apoptosis was detected using annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining. Early apoptotic cells stained as annexin V⁺/PI⁻. Late apoptotic cells were stained as annexin V⁺/PI⁺. All experiments were performed in triplicates. The columns represent the mean \pm standard deviation (n=3). Significantly different at: **p < 0.01 and ***p < 0.001 versus control group; ##p < 0.01 and ###p < 0.001 in cells treated with 25 μM versus those treated with 12.5 μM .

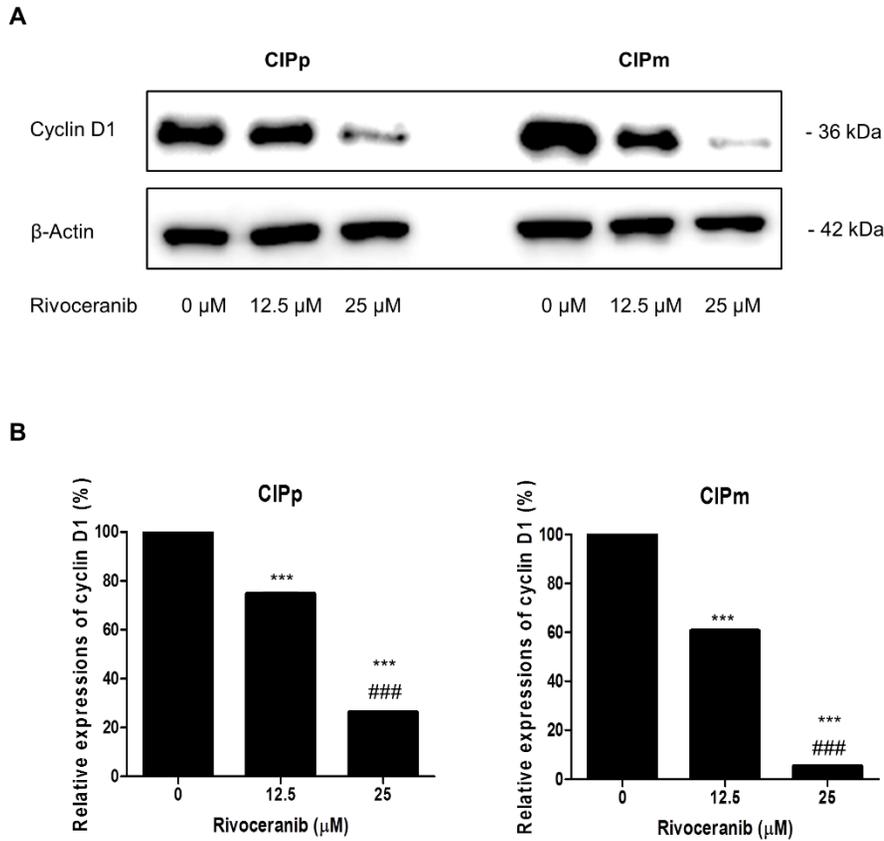


Figure 6. Rivoceranib inhibited the expression of cyclin D1 in CIP canine mammary gland tumor cells in a dose-dependent manner. A: Primary (CIPp) and metastatic (CIPm) CIP cells were treated with rivoceranib (0, 12.5, and 25 μ M) for 48h. The expression of cyclin D1 was measured with western blotting. Beta-actin was used as a loading control. B: Quantification of data shown in A. Relative expression of cyclin D1 in rivoceranib-treated and control cells is shown. All experiments were performed in triplicates. The column represents the mean \pm standard deviation (n=3). Significantly different at: ***p < 0.001 versus the control group. ###p < 0.001 in cells treated with 25 μ M versus those treated with 12.5 μ M.

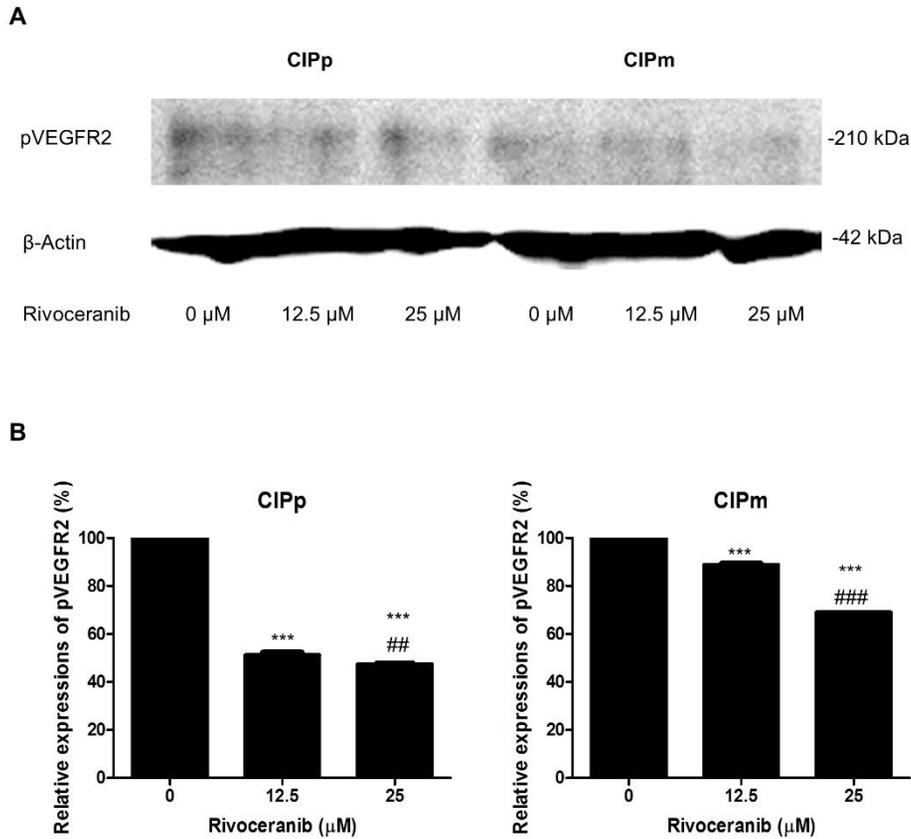


Figure 7. Rivoceranib inhibited the expression of phosphorylated vascular endothelial growth factor receptor-2 (pVEGFR-2) in CIP canine mammary gland tumor cells in a dose-dependent manner. A: Primary (CIPp) and metastatic (CIPm) CIP cells were treated with rivoceranib (0, 12.5, and 25 μ M) for 48h. The expression of pVEGFR2 was measured with western blotting. Beta-actin was used as a loading control. B: Quantification of data shown in A. Relative expression of pVEGFR2 in rivoceranib-treated and control cells is shown. All experiments were performed in triplicates. The data represent mean \pm standard deviation (n=3). Significantly different at: ***p < 0.001 versus the control group; ##p < 0.01 and ###p < 0.001 in cells treated with 25 μ M versus those treated with 12.5 μ M.

6. References

1. Withrow SJ, Vail DM and Page RL: Withrow and MacEwen's small animal clinical oncology. In: Tumors of the Mammary Gland. Karin US, Deanna RW and Michael HG (eds.). Missouri, Saunders Elsevier Inc., pp. 538-552, 2013.
2. Sleenckx N, De Rooster H, Veldhuis Kroeze E, Van Ginneken C and Van Brantegem L: Canine mammary tumours, an overview. *Reprod Domest Anim* 46(6): 1112-1131, 2011.
3. Klopfleisch R, Von Euler H, Sarli G, Pinho S, Gärtner F and Gruber A: Molecular carcinogenesis of canine mammary tumors: News from an old disease. *Vet Pathol* 48(1): 98-116, 2011.
4. Benjamin S, Lee A and Saunders W: Classification and behavior of canine mammary epithelial neoplasms based on life-span observations in beagles. *Vet Pathol* 36(5): 423-436, 1999.
5. Gilbertson S, Kurzman I, Zachrau R, Hurvitz A and Black M: Canine mammary epithelial neoplasms: Biologic implications of morphologic characteristics assessed in 232 dogs. *Vet Pathol* 20(2): 127-142, 1983.
6. Chatterjee S, Heukamp LC, Siobal M, Schöttle J, Wiczorek C, Peifer M, Frasca D, Koker M, König K and Meder L: Tumor VEGF: VEGFR2 autocrine feed-forward loop triggers angiogenesis in lung cancer. *J Clin Invest* 123(4): 1732-1740, 2013.
7. Fontanella C, Ongaro E, Bolzonello S, Guardascione M, Fasola G and Aprile G: Clinical advances in the development of novel VEGFR2 inhibitors. *Ann Transl Med* 2(12), 2014.

8. Jayson GC, Kerbel R, Ellis LM and Harris AL: Antiangiogenic therapy in oncology: Current status and future directions. *Lancet* 388(10043): 518-529, 2016.
9. Vasudev NS and Reynolds AR: Anti-angiogenic therapy for cancer: Current progress, unresolved questions and future directions. *Angiogenesis* 17(3): 471-494, 2014.
10. Gasparini G, Longo R, Fanelli M and Teicher BA: Combination of antiangiogenic therapy with other anticancer therapies: Results, challenges, and open questions. *J Clin Oncol* 23(6): 1295-1311, 2005.
11. Gasparini G, Longo R, Toi M and Ferrara N: Angiogenic inhibitors: A new therapeutic strategy in oncology. *Nat Clin Pract Oncol* 2(11): 562, 2005.
12. Scott LJ: Apatinib: A review in advanced gastric cancer and other advanced cancers. *Drugs* 78(7): 747-758, 2018.
13. Kowanzetz M and Ferrara N: Vascular endothelial growth factor signaling pathways: Therapeutic perspective. *Clin Cancer Res* 12(17): 5018-5022, 2006.
14. Tian S, Quan H, Xie C, Guo H, Lü F, Xu Y, Li J and Lou L: Yn968d1 is a novel and selective inhibitor of vascular endothelial growth factor receptor-2 tyrosine kinase with potent activity in vitro and in vivo. *Cancer Sci* 102(7): 1374-1380, 2011.
15. Aoyama T and Yoshikawa T: Targeted therapy: Apatinib—new third-line option for refractory gastric or GEJ cancer. *Nat Rev Clin Oncol* 13(5): 268, 2016.
16. Hu X, Cao J, Hu W, Wu C, Pan Y, Cai L, Tong Z, Wang S, Li J and Wang Z: Multicenter phase II study of apatinib in non-triplegenegative metastatic breast cancer. *BMC Cancer* 14(1): 820, 2014.
17. Hu X, Zhang J, Xu B, Jiang Z, Ragaz J, Tong Z, Zhang Q, Wang X, Feng J and

- Pang D: Multicenter phase II study of apatinib, a novel VEGFR inhibitor in heavily pretreated patients with metastatic triple-negative breast cancer. *Int J Cancer* 135(8):1961-1969, 2014.
18. Salas Y, Márquez A, Diaz D and Romero L: Epidemiological study of mammary tumors in female dogs diagnosed during the period 2002-2012: A growing animal health problem. *PLoS One* 10(5): e0127381, 2015.
 19. Sorenmo K: Canine mammary gland tumors. *Vet Clin North Am Small Anim Pract* 33(3): 573-596, 2003.
 20. Novosad CA: Principles of treatment for mammary gland tumors. *Clin Tech Small Anim Pract* 18(2): 107-109, 2003.
 21. Chen YC, Chang SC, Huang YH, Lee YJ, Chang CC, Liao JW and Hsu WL: Expression and the molecular forms of neutrophil gelatinase-associated lipocalin and matrix metalloproteinase 9 in canine mammary tumours. *Vet Comp Oncol* 17(3): 427-438, 2019.
 22. De Campos CB, Lavalle GE, Monteiro LN, PÊGAS GRA, Fialho SL, Balabram D and Cassali GD: Adjuvant thalidomide and metronomic chemotherapy for the treatment of canine malignant mammary gland neoplasms. *In Vivo* 32(6): 1659-1666, 2018.
 23. Folkman J: Tumor angiogenesis: Therapeutic implications. *N Engl J Med* 285(21): 1182-1186, 1971.
 24. Ferrara N: VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer* 2(10): 795, 2002.
 25. Ferrara N: Vascular endothelial growth factor as a target for anticancer therapy. *Oncologist* 9(Supplement 1): 2-10, 2004.

26. Ferrara N, Gerber H-P and LeCouter J: The biology of VEGF and its receptors. *Nat Med* 9(6): 669, 2003.
27. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ and Holash J: Vascular-specific growth factors and blood vessel formation. *Nature* 407(6801): 242, 2000.
28. Bergsland E and Dickler MN: Maximizing the potential of bevacizumab in cancer treatment. *Oncologist* 9(Suppl 1): 36-42, 2004.
29. de Oliveira RL, Hamm A and Mazzone M: Growing tumor vessels: More than one way to skin a cat—implications for angiogenesis targeted cancer therapies. *Mol Aspects Med* 32(2): 71-87, 2011.
30. Carmeliet P and Jain RK: Molecular mechanisms and clinical applications of angiogenesis. *Nature* 473(7347): 298, 2011.
31. Ellis LM and Hicklin DJ: VEGF-targeted therapy: Mechanisms of anti-tumour activity. *Nat Rev Cancer* 8(8): 579, 2008.
32. Ferrara N: Vascular endothelial growth factor: Basic science and clinical progress. *Endocr Rev* 25(4): 581-611, 2004.
33. Ferrara N and Kerbel RS: Angiogenesis as a therapeutic target. *Nature* 438(7070): 967, 2005.
34. Liao Z, Li F, Zhang C, Zhu L, Shi Y, Zhao G, Bai X, Hassan S, Liu X and Li T: Phase II trial of VEGFR2 inhibitor apatinib for metastatic sarcoma: Focus on efficacy and safety. *Exp Mol Med* 51(3): 24, 2019.
35. Li J, Qin S, Xu J, Guo W, Xiong J, Bai Y, Sun G, Yang Y, Wang L and Xu N: Apatinib for chemotherapy-refractory advanced metastatic gastric cancer: Results from a randomized, placebocontrolled, parallel-arm, phase II trial. *J*

- Clin Oncol 31(26): 3219-3225, 2013.
36. Langer CJ, Mok T and Postmus PE: Targeted agents in the third-/fourth-line treatment of patients with advanced (stage III/IV) non-small cell lung cancer (NSCLC). *Cancer Treat Rev* 39(3): 252-260, 2013.
 37. Chen X, Qiu T, Zhu Y, Sun J, Li P, Wang B, Lin P, Cai X, Han X and Zhao F: A single-arm, phase II study of apatinib in refractory metastatic colorectal cancer. *Oncologist* 24(7): 883-e407, 2019.
 38. Peng Q-X, Han Y-W, Zhang Y-L, Hu J, Fan J, Fu S-Z, Xu S and Wan Q: Apatinib inhibits VEGFR-2 And angiogenesis in an in vivo murine model of nasopharyngeal carcinoma. *Oncotarget* 8(32): 52813, 2017.
 39. Song Y-A, Ma T, Zhang X-Y, Cheng X-S, Olajuyin A-M, Sun Z-F and Zhang X-J: Apatinib preferentially inhibits PC9 gefitinib-resistant cancer cells by inducing cell-cycle arrest and inhibiting VEGFR signaling pathway. *Cancer Cell Int* 19(1):117, 2019.
 40. Fornaro L, Vasile E and Falcone A: Apatinib in advanced gastric cancer: A doubtful step forward. *J Clin Oncol* 34(31): 3822, 2016.
 41. Chan MM, Sjoquist KM and Zalcborg JR: Clinical utility of ramucirumab in advanced gastric cancer. *Biologics* 9: 93-105, 2015.
 42. Peng H, Zhang Q, Li J, Zhang N, Hua Y, Xu L, Deng Y, Lai J, Peng Z and Peng B: Apatinib inhibits VEGF signaling and promotes apoptosis in intrahepatic cholangiocarcinoma. *Oncotarget* 7(13): 17220, 2016.
 43. Lin C, Wang S, Xie W, Zheng R, Gan Y and Chang J: Apatinib inhibits cellular invasion and migration by fusion kinase KIF5BRET via suppressing RET/SRC signaling pathway. *Oncotarget* 7(37): 59236, 2016.

44. Huang M, Huang B, Li G and Zeng S: Apatinib affect vegfmediated cell proliferation, migration, invasion via blocking VEGFR2/RAF/MEK/ERK and PI3K/AKT pathways in cholangiocarcinoma cell. *BMC Gastroenterol* 18(1): 169, 2018.
45. He K, Wu L, Ding Q, Haider F, Yu H, Wang H and Xiang G: Apatinib promotes apoptosis of pancreatic cancer cells through down-regulation of hypoxia-inducible factor-1alpha and increased levels of reactive oxygen species. *Oxid Med Cell Longev* 2019: 5152072, 2019.
46. Lu W, Ke H, Qianshan D, Zhen W, Guoan X and Honggang Y: Apatinib has anti-tumor effects and induces autophagy in colon cancer cells. *Iran J Basic Med Sci* 20(9): 990, 2017.
47. Liu K, Ren T, Huang Y, Sun K, Bao X, Wang S, Zheng B and Guo W: Apatinib promotes autophagy and apoptosis through VEGFR2/STAT3/BCL-2 signaling in osteosarcoma. *Cell Death Dis* 8(8): e3015, 2017.
48. Clifford CA, Hughes D, Beal MW, Mackin AJ, Henry CJ, Shofer FS and Sorenmo KU: Plasma vascular endothelial growth factor concentrations in healthy dogs and dogs with hemangiosarcoma. *J Vet Intern Med* 15(2): 131-135, 2001.
49. Gentilini F, Calzolari C, Turba ME, Agnoli C, Fava D, Forni M and Bergamini PF: Prognostic value of serum vascular endothelial growth factor (VEGF) and plasma activity of matrix metalloproteinase (MMP) 2 and 9 in lymphoma-affected dogs. *Leuk Res* 29(11): 1263-1269, 2005.
50. Mohammed SI, Bennett PF, Craig BA, Glickman NW, Mutsaers AJ, Snyder PW, Widmer WR, DeGortari AE, Bonney PL and Knapp DW: Effects of the

cyclooxygenase inhibitor, piroxicam, on tumor response, apoptosis, and angiogenesis in a canine model of human invasive urinary bladder cancer. *Cancer Res* 62(2): 356-358, 2002.

- 51.** Troy G, Huckle W, Rossmeis J, Panciera D, Lanz O, Robertson J and Ward D: Endostatin and vascular endothelial growth factor concentrations in healthy dogs, dogs with selected neoplasia, and dogs with nonneoplastic diseases. *J Vet Intern Med* 20(1): 144-150, 2006.
- 52.** Wergin MC, Ballmer-Hofer K, Roos M, Achermann RE, Inteworn N, Akens MK, Blattmann H and Kaser-Hotz B: Preliminary study of plasma vascular endothelial growth factor (VEGF) during low-and high-dose radiation therapy of dogs with spontaneous tumors. *Vet Radiol Ultrasound* 45(3): 247-254, 2004.
- 53.** WERGIN MC and KASER-HOTZ B: Plasma vascular endothelial growth factor (VEGF) measured in seventy dogs with spontaneously occurring tumours. *In Vivo* 18(1): 15-20, 2004.

국문 초록

개의 유선종양 세포주에 대한 리보세라닙의 항암 효과

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이 정 하

개의 유선종양은 암컷 개에서의 발병률이 높은 종양이다. 리보세라닙 (또는 아파티닙으로 알려짐)은 혈관 신생을 억제하는 새로운 항암제로써 혈관 내피세포 성장인자 수용체-2 (VEGFR-2)에 선택적으로 결합하는 타이로신 키나아제 억제제이다. 그러나 아직까지 리보세라닙의 개 유선종양 세포주에 대한 연구는 보고 된 바 없다. 본 연구의 목적은 리보세라닙의 개 유선종양 세포주에 대한 항암효과를 밝히는 것이다. 리보세라닙의 개 유선종양 세포주에 대한 직접적인 효과를 세포 증식 및 이동과 관련된 세포 실험을 통해 분석하였다. 세포주기 분포와 세포 사멸율은 유세포 분석법을 통해 분석하였다. VEGFR-2의 인산화 형태

(pVEGFR-2)와 Cyclin D1의 단백질 발현 정도는 Western blot 분석으로 확인하였다. 추가적으로, 리보세라닙의 정상 개의 말초 혈액 내 단핵세포에 대한 세포독성 효과도 확인하였다. 리보세라닙 처리는 CIPp와 CIPm 세포들의 증식과 이동을 농도에 비례하여 유의적으로 감소시켰다. 유동 세포 분석법 결과, 약물 농도에 비례하여 세포 주기 중 G₀/G₁ 비율의 증가와 세포 자살율이 유의적으로 증가하였다. 리보세라닙은 pVEGFR-2와 Cyclin D1의 단백질 발현을 유의적으로 감소시켰다. 또한, 리보세라닙은 정상 개의 말초 혈액 내 단핵세포에 대한 세포독성은 없는 것으로 확인되었다. 따라서, 이러한 연구 결과는 리보세라닙이 개 유선종양 세포주의 생물학적 기능을 억제함으로써 항암 효과를 발현하는 것을 입증하였다. 본 실험 결과는 리보세라닙을 개 유선종양의 치료에 적용하기 전 근거가 될 수 있는 세포학적 실험 데이터를 제공하고 있다. 이 연구는 전 임상 단계에서 새로운 항암제 중 하나로 리보세라닙의 잠재적 적용 가능성을 제시하고 있으며 개 유선종양 세포주에서 리보세라닙의 항암 효과를 확인한 최초의 연구라는 점에서 의의가 있다.

주요어 : 개 유선종양 세포주, 리보세라닙, 아파티닙, 항암효과, 인산화 형태의 혈관 내피세포 성장인자 수용체-2, 혈관 내피세포 성장인자

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