A THESIS FOR THE DEGREE OF MASTER

Anti-tumor Effects of Oral Paclitaxel in a Mouse Model of Canine Melanoma

개 흑색종 마우스모델에서 경구용 파클리탁셀의 항암효과

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수의과대학 수의내과학 전공

양 지 인

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지도교수 윤화영

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위 원 장 채 준 석 (인)

부 위 원 장 윤 화 영 (인)

위 원 정 진 영 (인)

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Ji-In Yang

(Supervised by Prof. Hwa-Young Youn)

Department of Veterinary Internal Medicine

The Graduate School of Veterinary Medicine

Seoul National University

Abstract

Paclitaxel, a member of the taxane family, exhibits anti-tumor effects by targeting the microtubules of cancer cells. Recently, oral paclitaxel was developed to overcome the side effects of intravenous paclitaxel administration. The objective of this study was to investigate the anti-tumor effects of oral paclitaxel in vivo. To induce canine melanoma in nude mice, LMeC cells were injected subcutaneously. Three weeks after inoculation, oral paclitaxel (25 and 50 mg/kg) or saline was administered every week for three consecutive weeks. To explore the underlying mechanism, tumor

angiogenesis was examined by immunohistochemistry with anti-CD31 antibody. Apoptosis was detected by Terminal deoxynucleotide transferase dUTP nick end labelling (TUNEL) assay, and cell cycle arrest was confirmed by western blot analysis. In animal experiments, the average tumor size decreased to approximately 30% compared to that in the control. Histologically, oral paclitaxel showed anti-angiogenic effects and induced apoptosis in tumor tissues. Oral paclitaxel also downregulated the intra-tumoral expression of cyclin D1 and inhibited cell proliferation. In conclusion, oral paclitaxel demonstrated an anti-tumor effect on canine melanoma tumor. The study findings support the potential application of oral paclitaxel as a novel chemotherapeutic strategy to treat canine melanoma. This is the first study to investigate the potential of oral paclitaxel as a therapeutic strategy against canine tumors.

Key words: Chemotherapy, Dog, Melanoma, Paclitaxel, Solid tumor

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1. Introduction

Malignant melanoma is an aggressive form of skin cancer that develops from melanocytes (Gray-Schopfer *et al.*, 2007; Sulaimon *et al*., 2003). It is a life-threatening disease that accounts for 2.6% of all incidence of cancer (Parkin *et al.*, 2002), and 72% of all deaths from human skin cancer (Weinstock, 1997). Evidence suggests that global melanoma incidence and mortality rates are increasing on a yearly basis (Hall *et al.*, 1999; Guy *et al.*, 2015). Several antitumor agents have been used for treatment, but standard chemotherapy does not appear to prolong survival in human patients (Gogas *et al.*, 2007). Canine malignant melanomas are most commonly located in the oral cavity and considered to be a highly aggressive form of oral cancer (Spangler *et al.*, 2006). As in human patients, standard chemotherapy has little effect on survival, indicating the need for novel approaches to treat melanoma (Brockley *et al.*, 2013).

Paclitaxel is a microtubule-stabilizing drug that leads to cell cycle arrest (Weaver, 2014; Foa *et al.*, 1994). It is a chemotherapy drug used in human patients with broad activity against various cancers, especially ovarian cancer, breast cancer, non-small cell lung carcinoma, and malignant melanoma (Martin, 1993). Because of the non-soluble properties of paclitaxel, intravenous (IV) paclitaxel with cremophor EL as a vehicle is administered clinically (Panchagnula, 1998; Gelderblom *et al.*, 2001). However, it has been observed that cremophor EL induces hypersensitivity reactions in up to 9% of all human patients despite pretreatment (Markman *et al.*, 2000).

Novel formulations of paclitaxel have been developed in an attempt to reduce hypersensitivity (Khanna *et al.*, 2015). An oral form of paclitaxel was recently approved by the Food and Drug Administration (FDA) for the treatment of gastric cancer (Kang *et*

al., 2016). In the context of this study, we note that previous research has established the efficacy of oral paclitaxel in mice transplanted with cancer cell lines. Oral paclitaxel has been shown to induce anti-tumor effects in various cancer models, and its efficacy has been reported as similar to that of a parenteral injection of paclitaxel (Hahn *et al*., 2014; Jang *al*., 2018).

Recent developments in paclitaxel formulation have heightened the need for further investigation. Although research has been carried out on the efficacy of oral paclitaxel, no single study exists which describes its use in veterinary medicine. This study set out to investigate the efficacy of oral paclitaxel in a xenograft mouse model of canine melanoma. This is the first study to evaluate oral paclitaxel in veterinary tumors.

2. Materials and Methods

2.1. Mice

Female athymic nude mice (20–25 g) six weeks of age were purchased from Nara Biotech (Seoul, Korea) and kept under specific pathogen-free conditions. Mice were held for 1 week after arrival to adapt to the new environment. All animal studies were approved by the Institutional Animal Care and Use Committee of CHA university (permit number: IACUC 180029).

2.2. Cancer cell culture

The LMeC canine melanoma cell line was provided by Professor Nobuo Sasaki (University of Tokyo, Tokyo, Japan) (Wu SH *et al.*, 2004). LMeC cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (PAN Biotech) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

2.3. Animal model

To induce canine melanoma in mice, a suspension of 10^7 LMeC cells/100 μL in 100 μL of matrigel (Corning® Matrigel® Matrix, Corning Inc., New York, USA) (1:1 dilution) was injected subcutaneously (SC) into the right flank. Tumor volume was determined

using the following formula: $\pi/6 \times \text{width}^2 \times \text{length}$. When the tumor volume reached around 100 mm^3 (after 3 weeks), the mice were divided into control and treatment groups. The weights and tumor size of the mice were monitored every 3 days. Mice were sacrificed by cervical dislocation.

2.4. Paclitaxel treatment

Oral paclitaxel (Liporaxel®) was provided by Daehwa Pharmaceutical Co., Seoul, Korea. It was stored and refrigerated at 4°C and exists in a semisolid state. The formulation, which has a melting point of 33°C to 35°C, was melted at room temperature before administration (Hong *et al.*, 2007). After tumor cell injection, mice were divided into three groups (n = 10 per group); Group 1 mice were given 200 μL saline (control group), Group 2 mice received a 200 μL suspension of 25 mg/kg paclitaxel, and Group 3 mice received a 200 μL suspension of 50 mg/kg paclitaxel. Mice in the control group were orally administered 200 μL of saline every week. Oral paclitaxel was administered every week for three consecutive weeks. To investigate the effects of dose-dependent paclitaxel, the treated mice were divided into groups receiving 25 and 50 mg/kg of paclitaxel. Saline and paclitaxel were administered into the stomach using an oral administration blunt needle. At week 4, all mice were sacrificed and necropsied.

2.5. Blood analysis

Complete blood count (CBC) analysis was performed one week following the final

administration. Blood samples were obtained from the orbital sinus under anesthetized conditions. A CBC analysis, including white blood cell (WBC), neutrophil, red blood cell (RBC), and platelet (PLT) counts, was performed using ADVIA 2120i (Siemens Diagnostics, Tarrytown, New York) hematologic analyzer. The autopsy was performed immediately after obtaining blood samples.

2.6. Histology and Immunofluorescence

For immunofluorescence studies, 10% formaldehyde-fixed tumor sections (5 μm) were mounted on slides. Sections were deparaffinized and rehydrated. They were boiled in a microwave with sodium citrate buffer (10 mM, pH 6.0) for 10 minutes. After quenching in 3.0 % hydrogen peroxide in methanol for 15 min, the sections were incubated with 5% bovine serum albumin (BSA) for 30 min at room temperature. The sections were incubated at 4°C overnight with CD31 antibody (1:100; LSBio, Seattle, WA, USA). After rinsing, the sections were incubated for 1 h with goat-anti mouse IgG-FITC (1:100; Santa Cruz Biotechnology, Texas, USA). For nuclear labeling, 4′,6-diamidinophenylindoledihydrochloride (DAPI) staining was applied to the slides. Finally, the slides were counterstained with H&E. Stained sections were visualized using fluorescence microscopy (EVOS® FL Auto Imaging System, Thermo Fisher Scientific, MA, USA).

2.7. Western blot analysis

Protein was extracted from frozen tissue sections using Pro-Prep protein extraction solution (Intron Biotechnology, Boston, MA, USA). The protein concentration was determined by a Bio-rad DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Cellular fractions were obtained using Cell Fractionation Kit-Standard (Abcam, Cambridge, MA, USA). Protein fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to a poly-vinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were blocked with blocking buffer containing 5% skim milk in Tris-buffered saline (TBS). Cell cycle arrest was detected using anti-cyclin D1 antibodies (1:1500; Cell Signaling Technology, Beverly, MA, USA), with anti-β-actin antibody (1:1000; Santa Cruz Biotechnology, Texas, USA) as loading control. Bands were detected using chemiluminescence (Advansta, Menlo Park, CA, USA).

2.8. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Apoptosis following treatment was determined using the TUNEL assay on 10% formaldehyde-fixed frozen tumor sections. TUNEL staining was performed using In situ Apoptosis Detection Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions, and the resulting sections were examined by microscopy. For apoptotic cell quantification, TUNEL positive cells were counted in four randomly chosen fields (×400) per section.

2.9. Statistical analysis

The ten mice in the study were divided into three treatment groups. Mouse body weight, tumor size, and tumor weight were analyzed using ANOVA and statistical significance was inferred at P values less than 0.05.

3. Results

3.1. Effect of oral paclitaxel on canine melanoma tumors in vivo

To investigate the therapeutic effect of oral paclitaxel, melanoma tumors were induced by subcutaneous injection of LMeC cells. When the size of the tumor reached about 100 mm^3 at three weeks following induction, the mice were randomly divided into three groups. The tumors in the control group grew rapidly, reaching 286 ± 19 mm^3 in size after 21 days of treatment (Figures 1A and 1B). In contrast, the tumors treated with 25 mg/kg and 50 mg/kg paclitaxel had significantly smaller tumors than were seen in the control group, with mean sizes of 206 ± 12 mm^3 and 208 ± 99 mm^3 respectively ($P < 0.01$ for both groups; Figures 1A and 1B). The difference between groups treated with 25 mg/kg and 50 mg/kg paclitaxel was not significant.

Tumor weight decreased significantly in the 25 mg/kg and 50 mg/kg paclitaxel-treated mice when compared with that observed in control mice (20.5 ± 1.3 mg, 15.7 ± 1.3 mg, and 15.8 ± 0.8 mg, respectively; $P < 0.05$ for both; Figure 1C).

3.2. Toxicity of oral paclitaxel

To evaluate the toxicity of oral paclitaxel on mice, body weight and CBC analyses were performed. The body weights of control and treatment group mice remained similar and no significant differences were found during the study (Figure 2A). Similarly, there were no significant differences in white blood cell (WBC) count between control and treatment

groups (Table 1).

To examine the toxicity of orally administered paclitaxel on the gastrointestinal tract, tissue sections were stained with H&E and observed. There were no chemotherapy-related abnormalities in the stomach, small intestine, or large intestine (Figure 2B).

3.3 Anti-angiogenic activity of oral paclitaxel

The tumor sections were immuno-stained with the anti-CD 31 antibody to evaluate the effect of oral paclitaxel on blood vessels. Less blood vessels were observed in paclitaxel-treated mice (Figures 3B and 3C) relative to control mice (Figure 3A). Overall, these results indicate that oral paclitaxel has anti-angiogenic activity.

3.4 Evaluation of apoptosis induced by oral paclitaxel

To detect apoptotic cells, the tumor sections were stained with TUNEL and examined using microscopy. More apoptotic cells were observed in paclitaxel-treated mice than in control mice (Figure 4). For quantification, TUNEL-positive cells in sections were counted on blind observers. In the paclitaxel-treated group, the number of apoptotic cells was significantly increased compared to that in the control group, and 50 mg/kg paclitaxel induced significantly more apoptosis than 25 mg/kg paclitaxel ($P < 0.01$ for both comparisons; Figure 4D). These results suggest that oral paclitaxel induces apoptosis in tumors.

3.5 Effect of oral paclitaxel on the cell cycle

As cyclin D1 has been shown to be overexpressed in various cancers, we investigated the effects of oral paclitaxel on cyclin D1 expression in tumors. Western blot analysis revealed that the expression of cyclin D1 was decreased in paclitaxel-treated groups (Figure 5A). The relative band density confirmed that cyclin D1 expression was significantly decreased in the treated group compared to that in the control group, and the difference between the 25 and 50 mg/kg paclitaxel-treated groups was also significant ($P < 0.01$ for both comparisons; Figure 5B). This result suggests that oral paclitaxel downregulates cyclin D1, thereby inhibiting cell cycle progression in melanoma.

4. Discussion

Paclitaxel is a cytotoxic drug that promotes microtubule polymerization and stabilization (Weaver, 2014; Yvon *et al.*, 1999). Paclitaxel inhibits the proliferation and migration of activated endothelial cells *in vitro*; it also inhibits the angiogenic process *in vivo* (Bocci *et al.*, 2013). A number of studies have suggested that paclitaxel may have anti-tumor activity against various types of cancer (Khanna *et al.*, 2015; Foa *et al.*, 1994; Jiang *et al.*, 2010). However, clinical application of IV paclitaxel in veterinary medicine is not common owing to the high rates of adverse effects (Poirier *et al.*, 2004; Gaudy *et al.*, 1987).

Oral chemotherapy has several advantages when compared to IV administration, including convenience, reduced toxicity, and improved quality of life. In a study investigating toxicity of IV paclitaxel, it reported that allergic reactions occur in 64% of dogs despite pretreatment (Poirier *et al.*, 2004). Because of poor bioavailability of paclitaxel, however, developing an oral formulation has been difficult (Frederik *et al.*, 2013). Recently, a formulation of oral paclitaxel was approved by the FDA (Shin *et al.*, 2009) . This drug is absorbed through the gastrointestinal membrane and effectively distributed in major organs (Hong *et al.*, 2007). The mucoadhesive formulation of oral paclitaxel improves its permeability (Jang *et al.*, 2017).

The results of our study show that oral paclitaxel induces a significant decrease in tumor size and tumor weight. However, dose-dependent effects were not observed for these parameters when comparing the results of mice dosed with 25 mg/kg and 50 mg/kg of paclitaxel. Oral paclitaxel was administered at doses five times higher than conventional IV paclitaxel, because absolute bioavailability of the oral formulation was

14.6–29.0% that of Taxol® (Shin *et al.*, 2009).

The major adverse effects of paclitaxel include hypersensitivity, bone marrow suppression, and diarrhea (Poirier *et al.*, 2004). In this experiment, the body weight of tumor-bearing mice treated with oral paclitaxel did not change significantly. One week following the last administration, no blood or gastrointestinal toxicity could be observed. At doses of 25 or 50 mg/kg, no chemotherapy-related complications were observed.

To evaluate the effects of oral paclitaxel on blood vessels *in vivo*, we examined the expression of CD31 by immunohistochemical staining. When paclitaxel was administered, much less CD31 was observed in tumor sections compared to that in control sections. Paclitaxel was thus confirmed to decrease expression of CD31, implying anti-angiogenic properties. To evaluate the effect of oral paclitaxel on apoptosis *in vivo,* the TUNEL assay was used. In paclitaxel-treated mice, the number of TUNEL-positive cells was significantly decreased relative to that observed in control mice. Thus, oral paclitaxel inhibited apoptotic cell death *in vivo.* Interestingly, the number of TUNEL-positive cells was significantly decreased in 50 mg/kg paclitaxel-treated mice relative to 25 mg/kg paclitaxel-treated mice.

Cyclin D1, a member of the cyclin family, regulates progression through the G1 phase of the cell cycle (Diehl, 2002). In the cancer environment, mitogenic growth factor leads to an accumulation of cyclin D1 (Evan *et al.*, 2001). It is now well established from a variety of studies that cyclin D1 is overexpressed in multiple cancers. This study demonstrated that cyclin D1 is decreased in paclitaxel-treated groups of mice with melanoma *in vivo*. Thus, the results of this study show that oral paclitaxel induced G1 cell cycle arrest and inhibited proliferation in melanoma tumor cells.

The present study has a few limitations. Since the study used only two doses, 25 and

50 mg/kg of paclitaxel, it was not possible to find the optimal therapeutic range of oral paclitaxel in treating canine melanoma. As there was no difference in efficacy between 25 and 50 mg/kg doses, evaluation of a greater range of doses must be performed. In addition, paclitaxel is commonly administered via the IV route, and a comparison of efficacy between oral and IV paclitaxel may yield interesting insights.

In conclusion, the investigation of oral paclitaxel has shown effective chemotherapeutic properties with low toxicity in a mouse xenograft model of canine melanoma. In the animal experiments, oral paclitaxel showed therapeutic efficacy in suppressing primary melanoma tumors through anti-angiogenic, apoptotic, and cell cycle arrest activities. The results of this study indicate that oral paclitaxel may be useful as an alternative to IV paclitaxel in treating canine melanoma.

5. Conclusions

In the present study, the investigation of oral paclitaxel has shown an effective chemotherapy with less side effects in a mouse xenograft model of canine melanoma. Paclitaxel revealed both direct cytotoxic effect and anti-angiogenic effect on melanoma tumor cells. In conclusion, the findings of this research provide insights for the further investigation in the treatment of a melanoma and other malignant tumors.

Table 1. Complete blood count in tumor-bearing mice

Parameters	Control	PTX 25 mg/kg	PTX 50 mg/kg	Reference range
WBC (10^3/μL)	3.11 ± 0.89	2.76 ± 1.12	4.02 ± 0.89	2.97 – 15.83
Neutrophil (10^3/μL)	1.28 ± 0.31	1.19 ± 0.46	1.62 ± 0.72	1.02 – 5.01
RBC (10^6/μL)	9.87 ± 0.44	8.33 ± 3.32	10.01 ± 0.25	8.87 – 12.16
HCT (%)	44.09 ± 1.38	42.06 ± 1.40	45.65 ± 1.02	49.8 – 67.4
Platelet (10^3/μL)	1055.63 ± 179.77	1098.75 ± 125.20	1018.00 ± 182.84	691 – 1454

PTX, paclitaxel

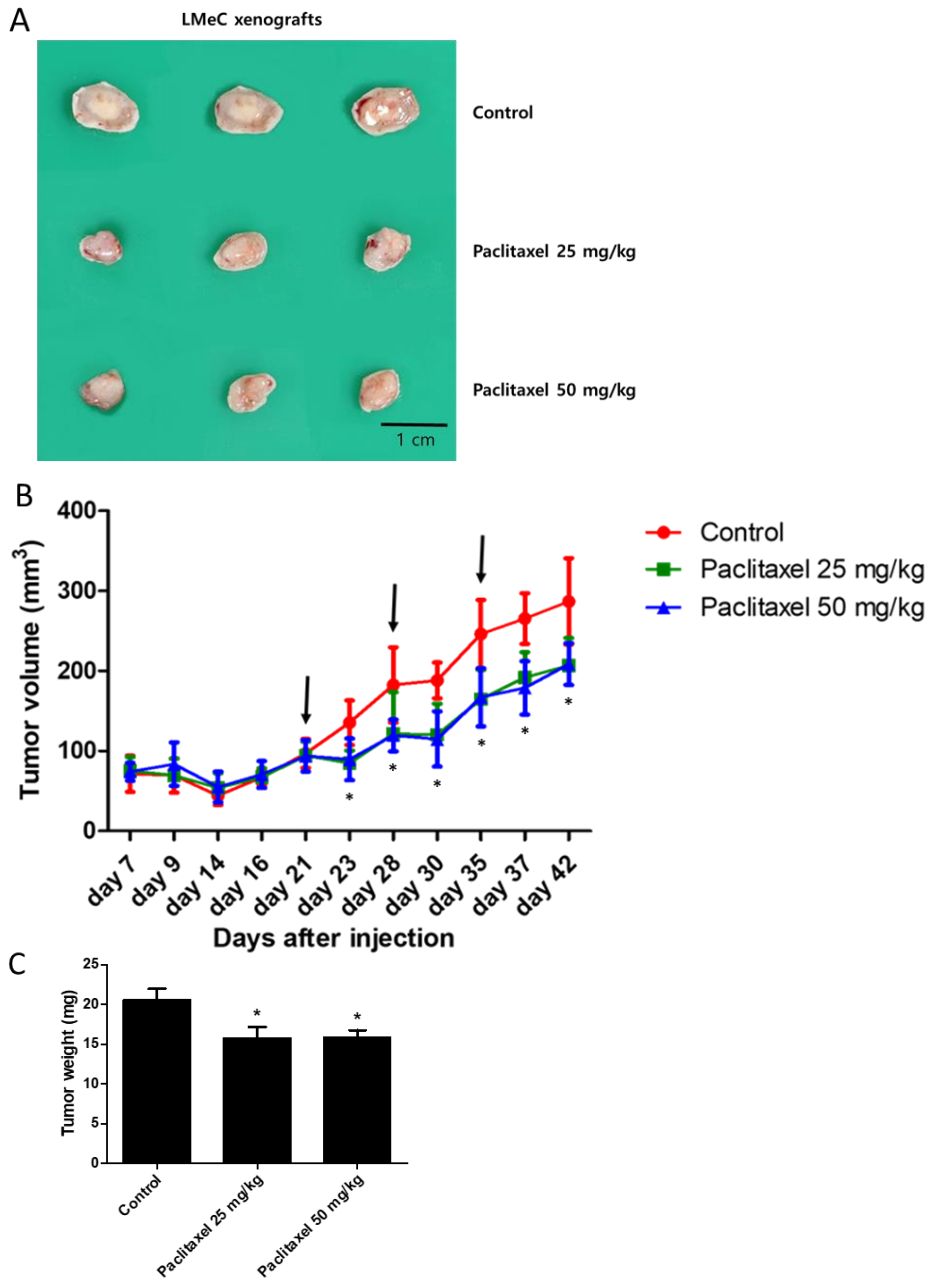

Figure 1. Oral paclitaxel inhibits tumor growth *in vivo*. (A) Representative images of canine melanoma tumors in athymic nude mice. (B) The mean tumor volumes in three groups (control, 25 mg/kg paclitaxel-treated, and 50 mg/kg paclitaxel-treated) following paclitaxel treatment. Oral administration of paclitaxel is indicated by a vertical arrow. (C) The mice were euthanized after 21 days of treatment, and the tumor weights were measured. Data are represented as means ± standard deviation. Significant difference from control is denoted by "*"; * $P < 0.05$.

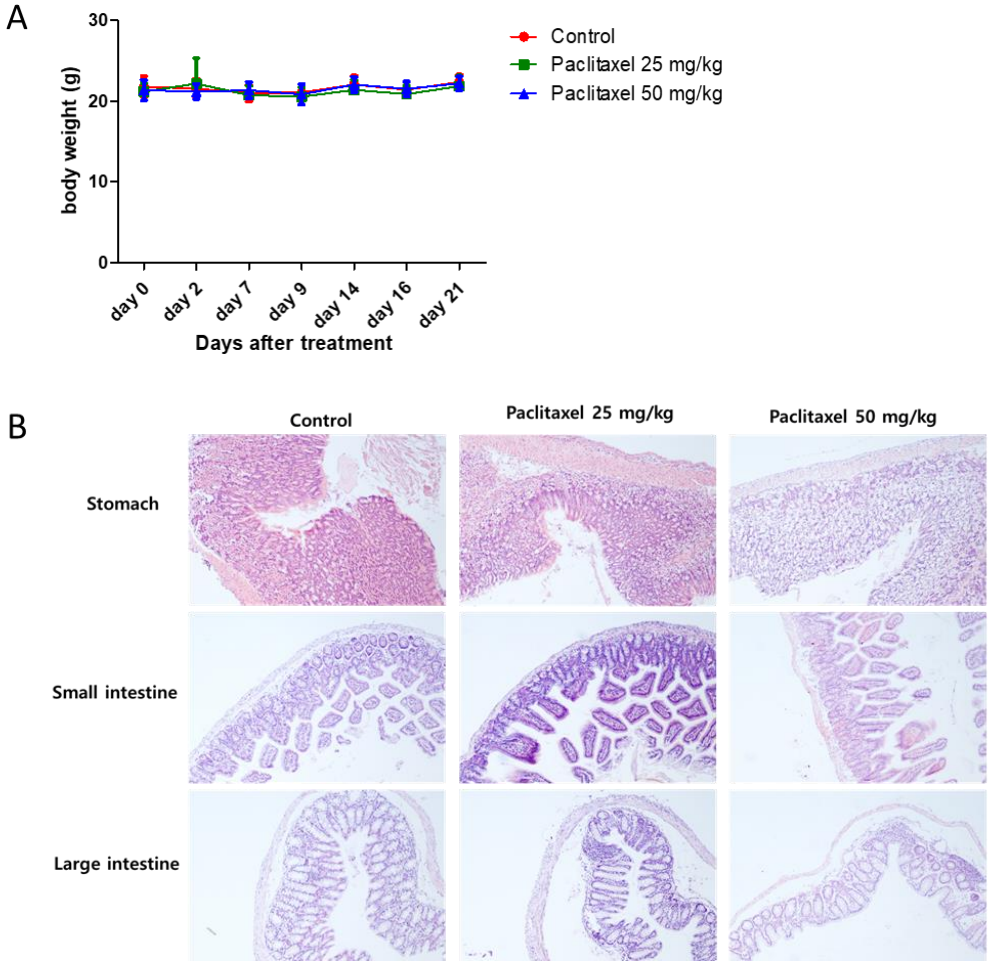

Figure 2. Toxicity of oral paclitaxel. (A) The body weights of tumor bearing mice were monitored twice a week during the course of paclitaxel treatment. (B) After 21 days of treatment, mice were euthanized, and gastrointestinal tissue samples were collected.

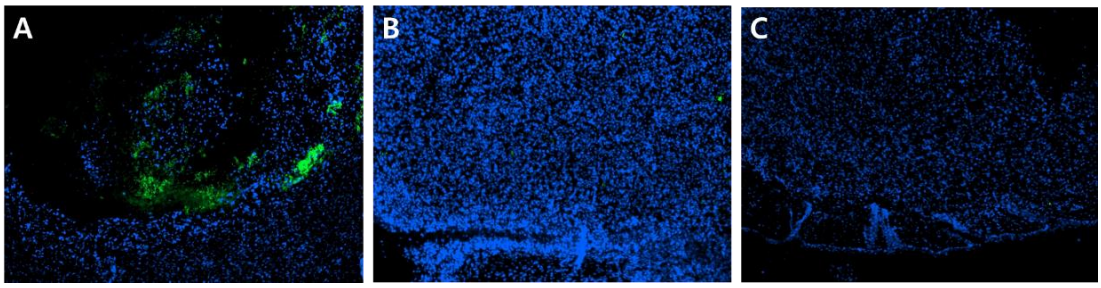

Figure 3. Oral paclitaxel has anti-angiogenic activity *in vivo*. Representative images of tumor sections immunostained with anti-CD31 antibody from the (A) control, (B) 25 mg/kg paclitaxel-treated, and (C) 50 mg/kg paclitaxel-treated groups.

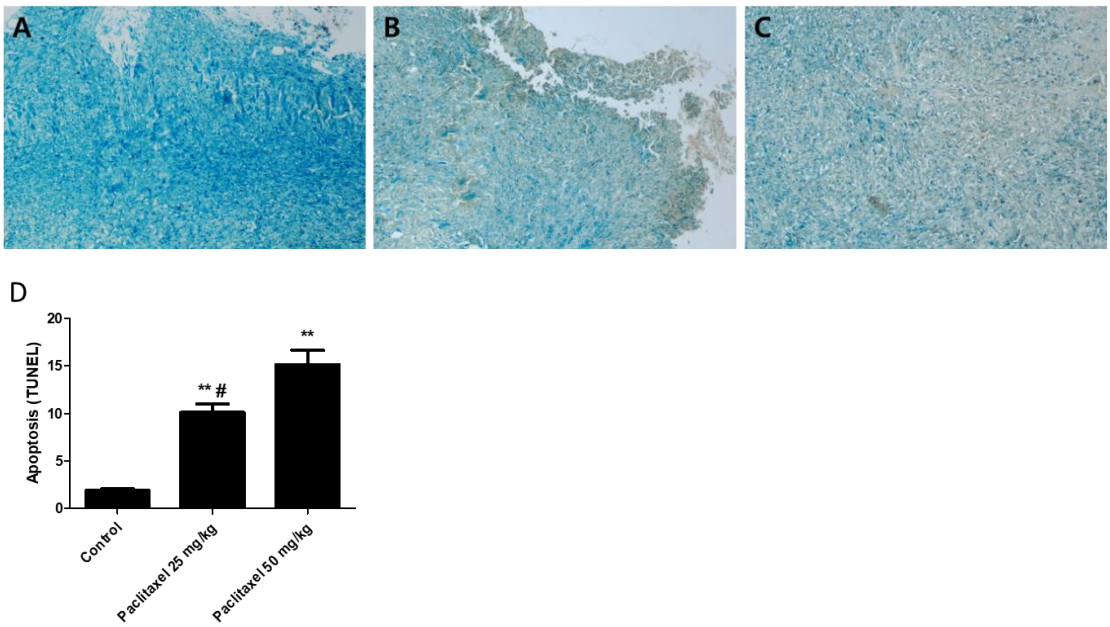

Figure 4. Oral paclitaxel induced apoptosis *in vivo*. Representative images of tumor sections subjected to TUNEL assay from the (A) control, (B) 25 mg/kg paclitaxel-treated, and (C) 50 mg/kg paclitaxel-treated groups. (D) The TUNEL-positive cells were counted in random fields. Significant difference from control is denoted by "*" and "**", and a significant difference between the 25 and 50 mg/kg paclitaxel-treated groups is denoted by "#"; * $P < 0.05$, ** $P < 0.01$; # $P < 0.01$.

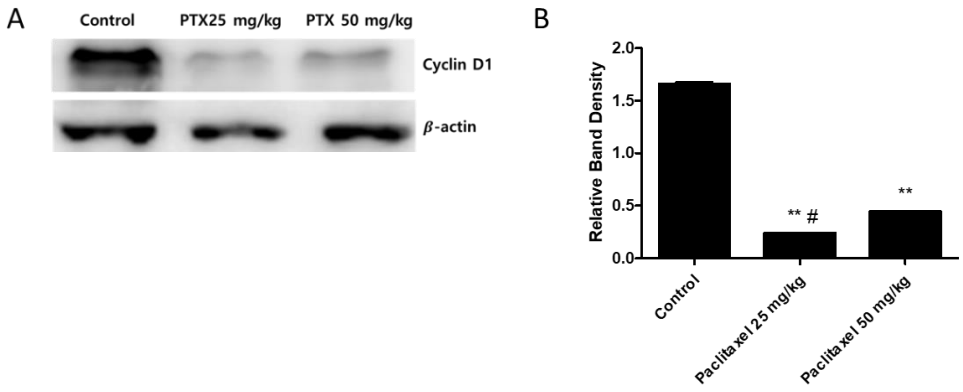

Figure 5. Oral paclitaxel induced cell cycle arrest *in vivo*. (A) Western blot was conducted on tumors from the three groups of mice (control, 25 mg/kg paclitaxel-treated, and 50 mg/kg paclitaxel-treated) using anti-cyclin D1 antibody (upper panel) and β-actin antibody (lower panel). (B) The relative densities of the bands were normalized against β-actin band densities. Significant difference from the control value is denoted by "*" or "**", and a significant difference between 25 and 50 mg/kg paclitaxel-treated group values is denoted by "#"; * $P < 0.05$, ** $P < 0.01$; # $P < 0.01$.

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국문 초록

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지도 교수: 윤 화 영

서울대학교 대학원

수의학과 수의내과학 전공

양 지 인

파클리탁셀은 탁산계 항암제로서, 암 세포의 미세소관에 작용하여 항암 효과를 나타낸다. 최근 정맥투여용 파클리탁셀의 부작용을 보완하기 위하여 경구용 파클리탁셀이 개발되었다. 본 연구는 마우스 생체내 실험에서 경구용 파클리탁셀의 항암 효과를 알아보는데 목적이 있다. 누드마우스에 개 흑색종을 유발하기 위하여, LMeC 세포주를 피하 주사 하였다. 접종 3주 후, 경구용 파클리탁셀 (25, 50 mg/kg) 혹은 생리식염수를 3주간 매주 투약하였다. 항암의 기전을 알아보기 위하여, 종양 혈관 신생은 항 CD31 항체를 이용한 면역화학검사로 알아보았고, 세포자멸사는 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) 검사로 확인하였으며, 세포 주기는 western blot으로 분석하였다. 동물 실험 결과 평균 종양의 크기는 대조군과 비교하여 약 30% 감소하였다. 조직학적으로, 경구용 파클리탁셀의 혈관신생을 억제하는 효과

와 세포자연사를 유발시키는 작용이 확인되었다. 또한 경구용 파클리탁셀이 종양내 Cylin D1 단백질을 하향조절하여 세포 증식을 막는 것이 확인되었다. 결과적으로 본 실험을 통해 경구용 파클리탁셀이 개 흑색종에 항암 효과가 있다는 것이 입증되었다. 이러한 연구 결과는 개 흑색종을 치료하는 새로운 항암 치료 방법 중 하나로 경구용 파클리탁셀의 잠재적 적용 가능성을 제시하였다. 이 연구는 개 종양에서 경구용 파클리탁셀을 적용한 첫 연구라는 점에서 임상적 의의가 있다.

주요어: 개, 고형 종양, 파클리탁셀, 항암치료, 흑색종

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