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YM155 enhances the cytotoxic activity of etoposide against canine osteosarcoma cells

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ABSTRACT. Canine osteosarcoma (OSA) is an aggressive and highly malignant primary bone tumor. Its poor survival outcome remains problematic despite recent advances in anti-cancer therapy, therefore highlighting the need for alternative treatment options or drug repositioning. The aim of this study was to determine if YM155, a small-molecule survivin inhibitor, potentiates the chemotherapeutic efficacy of etoposide against canine OSA *in vitro* and *in vivo*. In cell culture, YM155 enhanced the cytotoxic effect of etoposide against canine OSA cell lines; however, the molecular mechanism behind this effect was heterogeneous, as only one cell line had an elevated apoptotic level. In addition, this effect was not associated with survivin suppression in two of the cell lines. These results suggest that the molecular target of YM155 is not restricted to survivin alone. When tested on a murine xenograft model, the average tumor volume of the combination treatment group (YM155, 5 mg/kg, intraperitoneally, 5 consecutive days/week; and etoposide, 20 mg/kg, intraperitoneally, every 5 days) was 66% smaller than the control group, although this difference was not statistically significant (P=0.17). Further studies to improve the treatment protocol are necessary to confirm the findings of this study.

KEY WORDS: canine osteosarcoma, etoposide, survivin, synergism, YM155

Appendicular osteosarcoma (OSA) is a locally invasive and highly metastatic bone tumor accounting for 80–90% of canine primary bone neoplasms [33]. Surgical amputation and limb sparing surgery are the standard treatment modalities for appendicular OSA and provide effective pain relief [27, 48]. However, surgical resection is only a palliative procedure because 90% of dogs with OSA have micrometastases at the time of presentation, which is the ultimate cause of death [32]. Primary bone tumors in dogs and humans exhibit strong pathophysiological and pathohistological similarities, and chemotherapy administration improves survival of both species [51]. The 5-year survival of human osteosarcoma patients undergoing surgical and adjuvant chemotherapy treatments ranges from 75–80% for good responders and 45–55% for poor responders [20]. Although adjuvant chemotherapy with doxorubicin or platinum-based drugs extends the survival of dogs treated with surgery from approximately 11–21% to 35–50% at 1 year, it is incapable of impeding the development of metastasis [4, 5–7, 9, 27, 31, 36, 41, 47, 48]. The vast difference in the treatment outcome between human and canine OSA patients indicates that there is considerable room for improvement in the efficacy of canine OSA treatment. While there have been various advances in novel anti-cancer therapy, the lack of clinical evidence that the overall prognosis for canine OSA is improving highlights the necessity for alternative treatment options or drug repositioning [26, 43, 49].

Survivin, an inhibitor of apoptosis proteins, is crucial for normal cell proliferation. Its aberrant expression enables cancer cells to overcome the apoptotic checkpoint, resulting in tumorigenesis [11, 24]. Survivin is not expressed in most normal adult tissues, but is expressed in a wide range of human and canine tumors, including canine OSA, and its expression is associated with a poor prognosis in various malignancies [1, 8, 21, 30, 42, 45]. Recent reports have implicated that suppression of survivin potentiates the efficacy of chemotherapeutic agents against cancer by inducing apoptosis [16, 37, 44, 54]. Considering that survivin plays a critical role in tumor progression and determination of sensitivity to anti-cancer agents, and is preferentially expressed in cancer cells, it has been proposed as a cancer therapeutic target. YM155 is a small-molecule that selectively suppresses survivin promoter activity and its subsequent protein expression, resulting in apoptosis in a broad array of human cancer cell lines and mouse xenograft models [3, 12, 25, 28, 34, 52]. Furthermore, it chemosensitizes both human and canine cancer cells to cytotoxic agents [19, 25, 29, 53, 55].

We have previously described the cytotoxic mechanisms of etoposide and its synergistic inhibitory effect with piroxicam,

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mediated by survivin downregulation in canine osteosarcoma cell lines [38]; however, this synergism was not evident in a murine xenograft model [40]. Given the apparent aberrant expression of survivin, particularly in tumor cells such as canine OSA, as well as the anti-tumor and chemosensitizing effects demonstrated by YM155, an investigation of the effects of YM155 in combination with etoposide on canine OSA would be compelling. Therefore, the purpose of the current study was to determine if YM155 enhances the chemotherapeutic efficacy of etoposide against canine OSA cells.

MATERIALS AND METHODS

Cell proliferation assay

The canine OSA cell lines HMPOS, POS, and HOS were maintained as previously described [38]. Etoposide (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) and YM155 (EMD Millipore, Billerica, MA, U.S.A.) were reconstituted in dimethyl sulfoxide (DMSO) at stock solutions of 20 mM and stored at −20°C in small aliquots. The drugs were diluted for each experiment with medium supplemented with 10% FBS (Gibco BRL, Grand Island, NY, U.S.A.) such that the DMSO concentration did not exceed 0.5%. The canine OSA cells were seeded into 96-well plates in quadruplicate at their optimal seeding number, 2×10^3 cells per well for HMPOS and POS cells, and 3×10^3 cells per well for HOS cells. We have previously reported that single agent etoposide treatment at 0.2 μM inhibited proliferation of HMPOS, POS, and HOS cells [38]. Therefore, the same concentration was used in the *in vitro* experiments of the present study. Etoposide (0, 0.2 μM), with or without YM155 at 50% inhibitory concentration (IC_{50}), was added after adherent growth was observed. Cell viability was determined using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) in accordance with the manufacturer's instructions. Three independent assays were performed.

Clonogenic assay

Canine OSA cells were seeded at 1.5×10^2 cells per well in 6-well plates in triplicate and incubated at 37°C for 1 hr to allow cells to adhere. Following incubation, etoposide (0, 0.2 μM) and/or YM155 at IC_{50} concentration were added to the wells, and colonies were stained with crystal violet (5 g/*l* in 6% glutaraldehyde) 7 or 8 days later. The colonies were counted, and the surviving fraction was calculated. Three independent assays were performed.

Cell cycle analysis

Seeded cells were treated with etoposide and/or YM155 when adherent growth was observed. Floating and trypsinized adherent cells were collected and subjected to cell cycle analyses using a BD FACSVerse flow cytometer (BD Biosciences, San Jose, CA, U.S.A.) as previously described [38]. For each sample, at least 10,000 events were acquired, and data were analyzed using BD FACSuite software (version 1.0 suite 1.0.3, BD Biosciences). Three independent experiments were performed.

Apoptosis assay

Treated cells were evaluated for apoptosis using an annexin fluorescein isothiocyanate (FITC)-labeled annexin V/propidium iodide (PI) double staining technique as previously described [38]. At least 10,000 events were acquired for each sample using a BD FACSVerse flow cytometer and analyzed using BD FACSuite software (version 1.0 suite 1.0.3, BD Biosciences). Three independent assays were performed.

Western blot analysis

To determine if YM155 suppressed the endogenous expression of survivin in canine OSA cells, adherent cells were exposed to YM155 at increasing concentrations (0−100 nM), and the survivin expression was visualized by western blot analysis as previously described [38]. The etoposide and/or YM155 treated cells were subjected to immunoblotting using mouse anti-poly (ADP-ribose) polymerase (PARP) (1:1,000; 611038; BD Biosciences), rabbit anti-survivin (1:1,000; NB500-201; Novus Biologicals, Littleton, CO, U.S.A.), and mouse anti-actin (1:10,000; MAB1501; Millipore, Billerica, MA, U.S.A.) antibodies.

In vivo study

This study was performed upon approval of the University of Tokyo Animal Care and Use Committee (Ref.: P16-265). BALB/c nude mice (*nu/nu*; 5-week-old females; SLC Japan, Tokyo, Japan) were maintained under specific pathogen-free conditions at 24 ± 1°C in 40–70% humidity and with a 12-hr light/dark cycle throughout all experiments. Sterilized food (CL-2; Clea Japan, Tokyo, Japan) and distilled water were provided *ad libitum*. Sub-confluent HMPOS cells were trypsinized, washed, and resuspended in phosphate buffered saline (PBS) at a density of 1×10^7 cells/m*l*, and injected subcutaneously into the right flank of all mice (1×10^6 cells per mouse). Mice were randomized into 4 treatment groups 3 days later (6 mice per group): (1) negative control (saline), (2) YM155 (5 mg/kg, intraperitoneally, 5 consecutive days/week) [3, 19], (3) etoposide (20 mg/kg, intraperitoneally, every 5 days; Nippon Kayaku, Tokyo, Japan) [40], and (4) combination of YM155 (5 mg/kg, intraperitoneally, 5 consecutive days/week) and etoposide (20 mg/kg, intraperitoneally, every 5 days). Body weights and tumor volumes were recorded every 3 days until the endpoint. Tumor volume was assessed using a caliper and calculated according to the following formula: (length × width^2)/2. All mice were humanely euthanized after 21 days of treatment. Harvested tumor samples were fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. The tumor samples were not subjected to decalcification.

Immunohistochemical studies

All tumor samples were analyzed immunohistochemically for the expression of Ki-67 and survivin protein as previously described [40]. Antibodies used for immunostaining included mouse anti-human Ki-67 (clone MIB-1; ready-to-use; IS-626; Dako, Glostrup, Denmark) and rabbit anti-survivin (1:800; NB500-201; Novus Biologicals). The proliferation index and survivin expression were quantified as the number of Ki-67- or survivin-positive nuclei (per 400 ×microscopic field) × 100 per total number of nuclei, respectively; at least 1,000 cells were counted.

Terminal deoxyribonucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay

The presence of apoptotic cells was assessed by the TUNEL assay using the DeadEnd^{TM} Colorimetric TUNEL System (Promega, Madison, WI, U.S.A.) as previously described [40]. The apoptotic index was calculated according to the following formula: number of TUNEL-positive areas (per 400 × microscopic field) × 100 per total number of nuclei; at least 1,000 cells were counted.

Statistical analyses

The IC_{50} value of YM155 for each OSA cell line was determined by dose-response analysis using R package drc. Data are presented as means ± standard deviations (SDs). Statistical analysis was performed using a one-way analysis of variance (ANOVA), and *post-hoc* comparisons were made using Dunnett's test, the Bonferroni test, or Student's *t*-test for unpaired data. Calculations were performed using SPSS statistical software (version 23, SPSS Inc., Chicago, IL, U.S.A.), and values of $P<0.05$ were considered statistically significant.

RESULTS

YM155 potentiated the inhibitory effects of etoposide on canine OSA cell lines

We confirmed that all cell lines expressed survivin, and YM155 dose-dependently suppressed their endogenous survivin expression (Fig. 1). YM155 potently inhibited growth of all canine OSA cell lines; IC_{50} values ranged from 2.2 to 29.4 nM (Table 1). Concomitant exposure to etoposide and YM155 significantly enhanced the anti-proliferative activity (Fig. 2) and reduced the number of colony forming cells (Fig. 3) when compared with etoposide alone.

The mechanism of growth inhibition of etoposide in combination with YM155 was cell line-dependent

The enhanced inhibitory effect demonstrated by co-treatment with etoposide and YM155 was not accompanied by an increase in the percentage of sub-G_1 fraction (Fig. 4), with the exception of the HOS cell line. Further analysis via the apoptosis assay showed similar results (Fig. 5). The effects of the treatments on apoptosis-regulating proteins, survivin, and PARP were assessed by western blot analysis. Excluding the HOS cell line, treatment with YM155 at IC_{50}, alone or in combination with etoposide, did not suppress endogenous survivin protein expression nor enhance PARP cleavage (Fig. 6).

Combination treatment did not significantly delay canine OSA xenograft tumor growth

All mice inoculated with HMPOS cells developed firm, non-calcified, subcutaneous tumors at the injection site. The mean ± SD tumor volume for each treatment group (total tumor volume per group/number of treated mice per group, $n=6$) at the end of the experiment was 1,634 ± 1,233 mm^3 for the control group, 1,523 ± 1,181 mm^3 for the etoposide treatment group, 1,162 ± 683 mm^3 for the YM155 treatment group, and 548 ± 710 mm^3 for the combination treatment group (Fig. 7A). Single agent treatment with either etoposide or YM155 did not significantly impede tumor progression. Although not statistically significant, the mean tumor volume of the combination treatment group was 66% smaller than that of the control group ($P=0.17$). The mean body weight of the mice in the combination treatment group was reduced on day 13 and 19 after the initiation of treatment, but otherwise was not significantly different from that of the control group (Fig. 7B). Weight loss was the only adverse sign observed in this study. At necropsy, no distant metastatic lesions were detected upon gross examination.

Combination treatment reduced cell proliferation and survivin expression

The expression of the cell proliferation marker Ki-67 in xenograft tumors of the etoposide treatment group was decreased ($P<0.05$), and the expression was further suppressed in the combination treatment group ($P<0.01$). The expression level of Ki-67

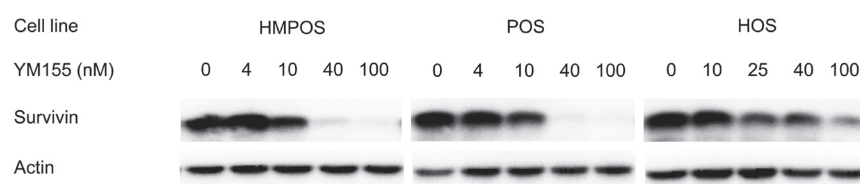
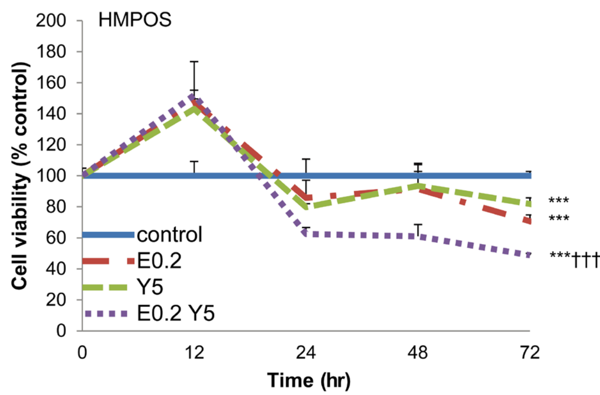


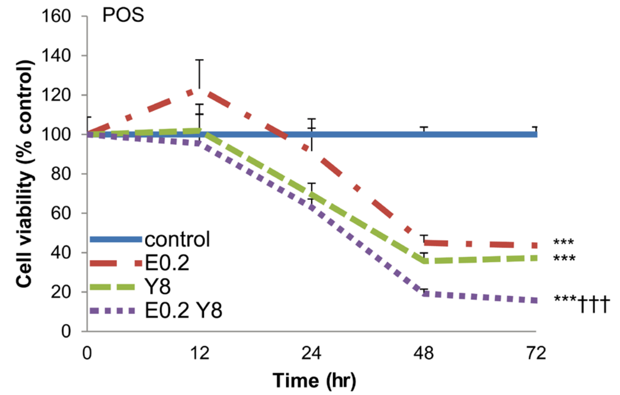
Fig. 1. Expression of survivin and actin visualized by western blot analysis. YM155 suppressed the endogenous survivin protein expression in a dose-dependent manner 24 hr after treatment. Actin was used as a loading control.

Table 1. The 50% inhibitory concentration (IC_{50}) values of canine osteosarcoma (OSA) cell lines treated with YM155

Cell line	IC_{50} (nM)
HMPOS	2.26 ± 2.07
POS	7.76 ± 0.18
HOS	29.35 ± 1.97

Data are expressed as means ± SDs.





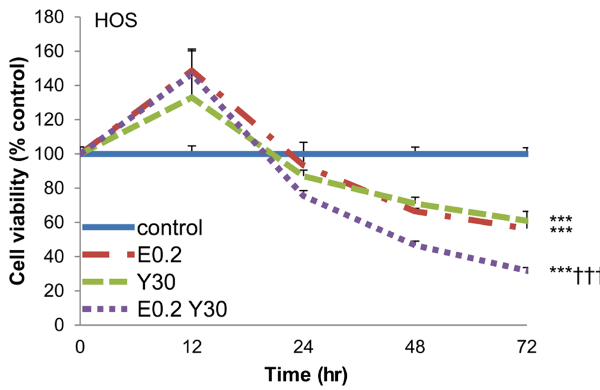


Fig. 2. Effect of etoposide and YM155 combination treatment on the viability of canine osteosarcoma cell lines, determined using the Cell Counting Kit-8 assay. Data are expressed as means ± SDs. The figures are representative of three independent experiments. Drug concentrations for etoposide and YM155 are in μM and nM, respectively. Bonferroni test; ***†††P<0.001; *compared with control; †compared with etoposide alone; E, etoposide; Y, YM155.

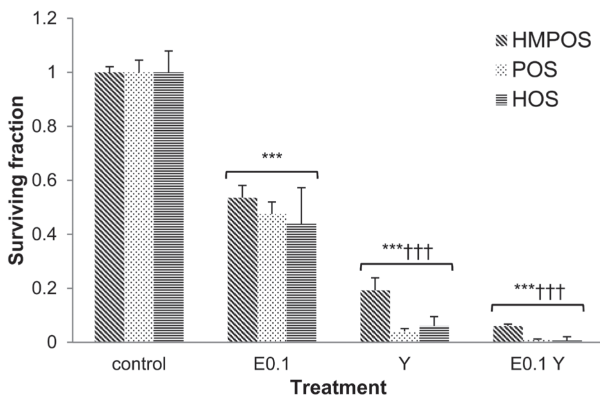


Fig. 3. Inhibitory effect of the treatments on colony formation in HMPOS, POS, and HOS cell lines. Data are expressed as means ± SDs. Data are representative of three independent experiments. Drug concentrations for etoposide and YM155 are in μM and nM, respectively. Bonferroni test; ***†††P<0.001; *compared with control; †compared with etoposide alone; E, etoposide; Y, YM155.

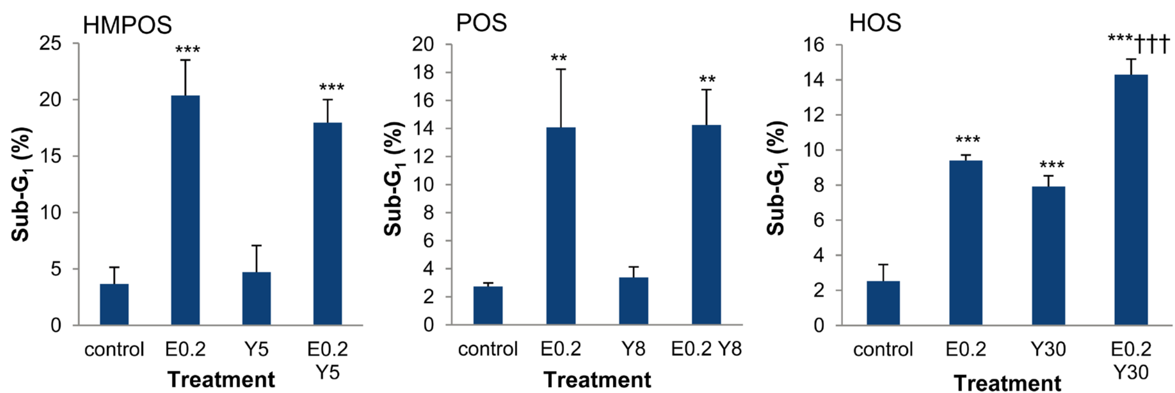


Fig. 4. Average percentages of sub-G_1 cells after treatment for 72 hr. Data are expressed as means ± SDs. Drug concentrations for etoposide and YM155 are in μM and nM, respectively. Bonferroni test; **P<0.01; ***†††P<0.001; *compared with control; †compared with etoposide alone; E, etoposide; Y, YM155.

Fig. 5. Apoptosis assay by FACS in HMPOS, POS, and HOS cells treated with etoposide, YM155, or etoposide and YM155 combined. (A) Dot plots from annexin V/PI assay are representative of three independent experiments performed on HMPOS, POS and HOS cell lines. (B) Average percentages of canine OSA cells that underwent early apoptosis at 48 hr. Data are expressed as means ± SDs. Drug concentrations for etoposide and YM155 are in μM and nM, respectively. Bonferroni test; *†P<0.01; **P<0.01; ***P<0.001; *compared with control; †compared with etoposide alone; E, etoposide; Y, YM155.

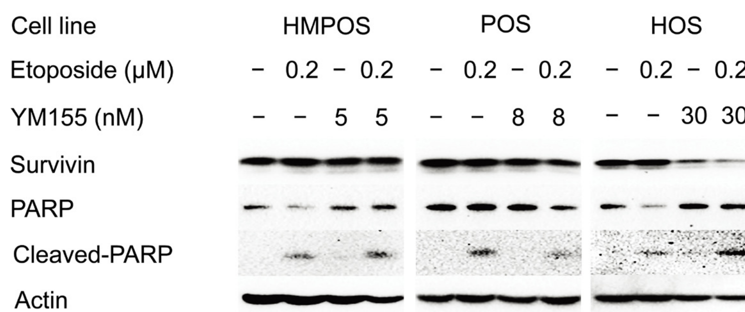


Fig. 6. Western blot analyses of canine osteosarcoma cells exposed to treatments for 24 hr (HMPOS and POS cell lines) or 72 hr (HOS cell line). Treatment with YM155 at IC_{50} either alone or in combination with etoposide did not down-regulate survivin expression of HMPOS and POS cells. Combination treatment enhanced the expression of cleaved poly (ADP-ribose) polymerase (PARP) in HOS cell line only.

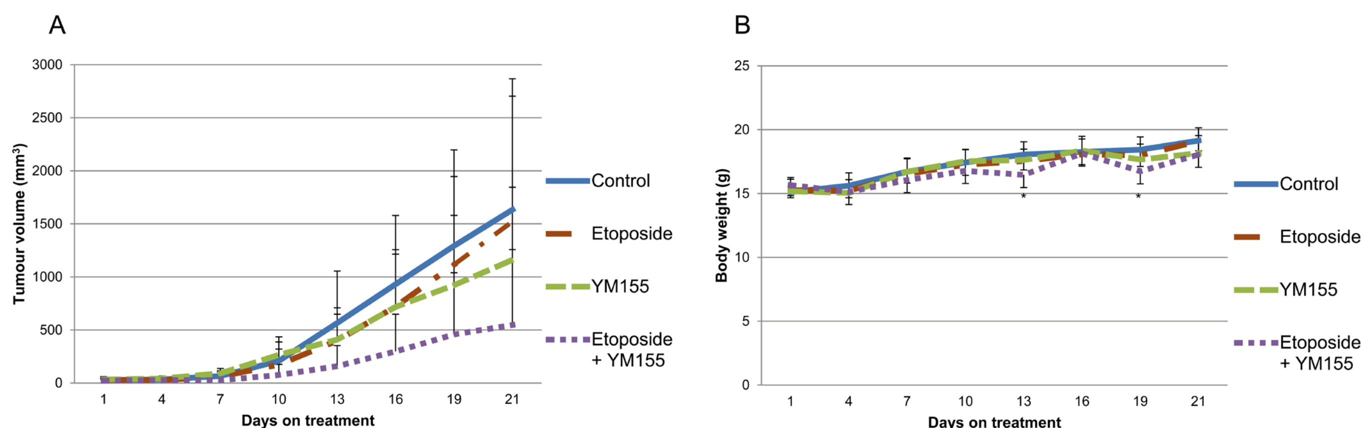


Fig. 7. Effect of treatments on xenograft tumor progression and body weight. (A) Average tumor mass of the combination treatment group was smaller than the control group but did not reach statistical significance. (B) The body weight of the combination treatment group was reduced on day 13 and 19 after initiation of treatment. Data are expressed as means ± SDs. Dunnett's test; *P<0.05 compared with control.

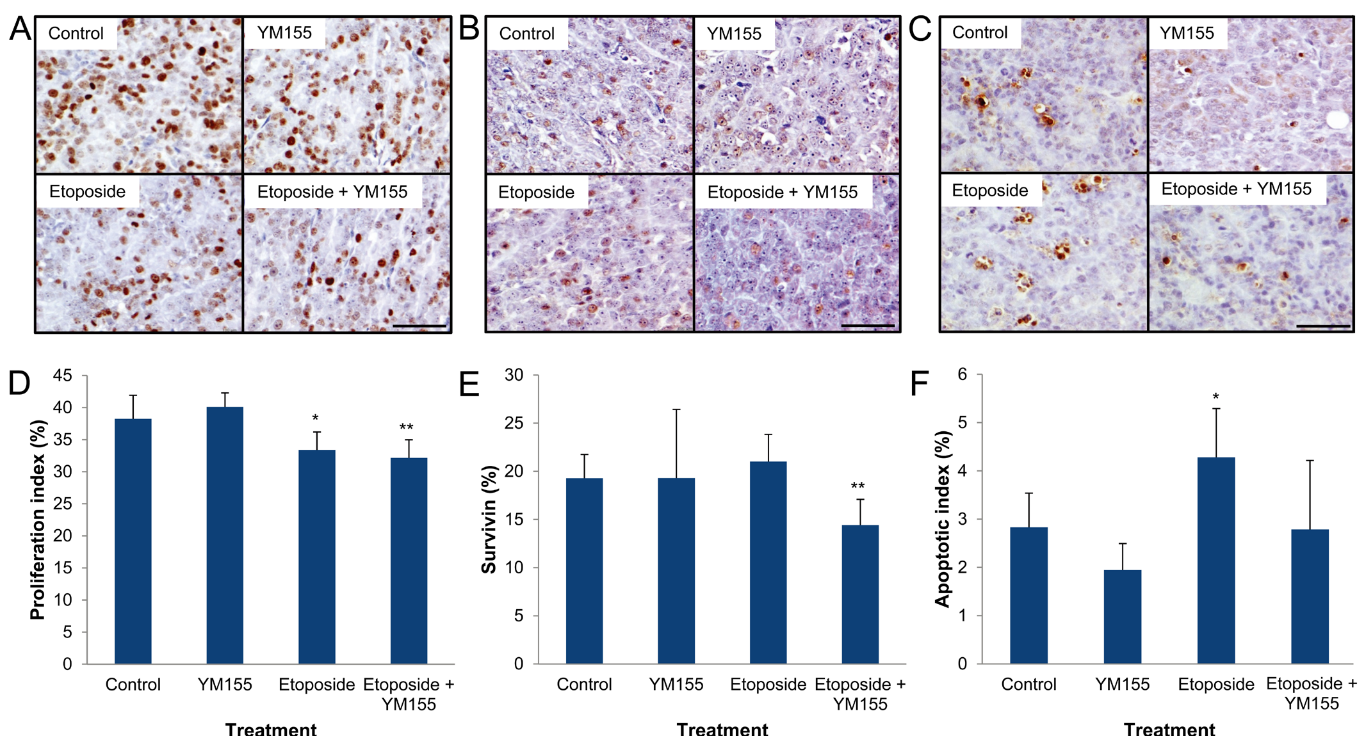


Fig. 8. Representative photomicrographs showing immunoreactivity for (A) Ki-67, (B) survivin, and (C) TUNEL analysis in the xenograft tumors. (D) Effects of the treatments on cell proliferation activity. Both etoposide single agent and combination treatment regimens reduced the proliferation index values of the xenograft tumors. (E) Effects of the treatments on the expression of survivin. Combination treatment suppressed survivin protein expression. (F) Effects of treatments on apoptosis. A slight increase in apoptosis was observed in the tumors from etoposide-treated mice. Data are expressed as means ± SDs. Student's t test for unpaired data; *P<0.05 compared with control, **P<0.01 compared with control; bar=100 μm.

was similar in the control and YM155 groups (Fig. 8A and 8D). Single treatment with either etoposide or YM155 did not alter the level of survivin expression, but combination treatment down-regulated the intra-tumoral survivin expression (Fig. 8B and 8E). Although the combination treatment reduced tumor cell proliferation and survivin expression, it was not associated with elevated apoptotic activity (Fig. 8C and 8F).

DISCUSSION

In the present study, we have demonstrated that YM155 may enhance the anti-tumor efficacy of etoposide against canine OSA.

While previous studies have shown that YM155 sensitizes cancer cells to conventional chemotherapeutic agents by inducing apoptosis [25, 29, 52, 53], our findings suggest that YM155 does not cause different canine OSA cell lines to undergo identical molecular changes that contribute to the enhanced anti-proliferative effects. Canine OSA is a heterogeneous group that can be classified into several histological subtypes [51]. In addition, each canine OSA cell line has different accumulations of genetic changes. These factors may be some of the elements associated with the heterogeneous response observed in this study. Y de Jong *et al.* [11] also reported similar observations, where YM155 treatment altered the cell cycle distribution in only two out of three human chondrosarcoma cell lines tested.

We further verified the molecular mechanism using western blot analysis. We observed that YM155 dose-dependently suppressed the endogenous survivin levels of all canine OSA cell lines. Although exposure to YM155 at the IC_{50} elicited growth inhibitory effects, it failed to suppress survivin expression in two of the cell lines, and no PARP cleavage was observed. In accordance with earlier suggestions, either survivin may not be the only target of YM155 [46], or the cellular effect is dependent on the level of survivin inhibition [3]. As survivin is also a key regulator of mitosis, canine OSA cells treated with YM155 at the IC_{50} could have reduced cell proliferation capacity instead of apoptotic cell death. The lack of apoptotic cells in the xenograft tumors also implies that YM155 may potentiate the anti-tumor effect of chemotherapeutic drugs via mechanisms that do not aggravate cancer cell apoptosis.

The anti-tumor efficacy and safety profile of YM155 have been extensively investigated in various xenograft models. YM155 has been shown to reduce survivin expression and growth of adenoid cystic carcinoma and esophageal cancer xenografts when administered intraperitoneally at 5 mg/kg for more than one week [50, 56]; however, a dose equal to or greater than 10 mg/kg may be toxic [50]. Unexpectedly, the YM155 treatment regimen used in our study (5 mg/kg, intraperitoneally, 5 consecutive days weekly) not only failed to significantly inhibit tumor progression, it did not suppress survivin expression of canine OSA xenografts. Nakahara *et al.* [35] reported that YM155 exhibited time-dependent anti-tumor activity and had superior anti-tumor effect when administered continuously compared with intravenous bolus injection. The exposure time of YM155 given intravenously by bolus injection was insufficient as the plasma half-life was 1.06 hr only. Researchers have also shown that better inhibition is obtained when YM155 is delivered continuously at a low dose [35, 46, 50]. Therefore, in the current experiment, we believe that intraperitoneal bolus administration resulted in inadequate exposure time and consequently undermined the potency of YM155. Although its efficacy was compromised, we hypothesize that YM155 enhanced the chemotherapeutic activity of etoposide against the HMPOS canine OSA cells by suppressing intra-tumoral survivin protein level that contributed to a profound decrease in Ki-67, thus leading to a smaller tumor burden, although not statistically significant. We also postulate that combination therapy with etoposide and YM155 will have a better safety profile and treatment outcome following refinement of the dosage regimen and delivery method.

Etoposide is commercially available in oral and intravenous preparations with proven efficacy for a wide range of neoplasms, including small cell lung cancer, testicular cancers, Hodgkin's and non-Hodgkin's lymphomas, and acute leukemia in humans [14, 15]. However, dogs that received intravenous administration of etoposide developed hypersensitivity reactions, which were attributable to the solvent, polysorbate 80, in the etoposide formulation [13]; therefore, the clinical applicability of etoposide in canine cancer treatment has been questioned. Considering that YM155 may potentiate the chemotherapeutic efficacy of etoposide, which is poorly tolerated at high doses, combinational therapy with YM155 would enable dosage reductions of etoposide, thus reducing etoposide-induced side effects. Furthermore, metronomic regimens of single agent etoposide, or in combination with other drugs, have exhibited favorable outcomes in human oncology [2, 10, 18, 23]. Previous evidence has also demonstrated the efficacy and safety of etoposide when administered to canine cancer patients at low doses, both orally and intravenously [13, 17, 22]. Our previous works have demonstrated the anti-tumor efficacy of single agent etoposide against canine osteosarcoma cells [39, 40]; however, the *in vivo* result of the present study was inconsistent with our previous finding. The reason for this discrepancy is unclear, but it could be attributed to several sources of variation, including the passage number of the cell culture used, differences in mouse groups, and the inherent stochasticity in biological responses to treatment. The findings reported here have to be interpreted in light of some limitations that could be addressed in future research. First, only one cell line was used to evaluate the *in vivo* anti-tumor efficacy of etoposide and YM155. Second, the treatment protocol evaluated in this study is suboptimal and further experimentation is necessary to refine the regime. Besides, to minimize errors or stochasticity, larger studies are required to confirm our findings.

The present study revealed that YM155 enhances the inhibitory effect of etoposide against canine OSA cells *in vitro*. The underlying mechanism by which YM155 improves the *in vitro* efficacy of etoposide against canine OSA is cell-line dependent; however, the precise molecular mechanism involved has yet to be elucidated. Our findings also indicate that combinational therapy using etoposide and YM155 may yield better anti-tumor effects against canine OSA. Furthermore, it would be of interest to investigate the anti-neoplastic efficacy of metronomic etoposide therapy in combination with YM155 against canine OSA.

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