

Effects of Calcitriol, Seocalcitol, and Medium-chain Triglyceride on a Canine Transitional Cell Carcinoma Cell Line

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Abstract. *Background:* Transitional cell carcinoma (TCC) in dogs is associated with high morbidity and mortality. Calcitriol and its analog seocalcitol, combined with medium-chain triglyceride (MCT), have potential for the treatment of this disease. *Materials and Methods:* TCC cells were treated with calcitriol or seocalcitol, alone or combined with MCT. Cell growth, cell cycle kinetics, vitamin D receptor (VDR) localization and expression, and Bcl-2 expression were measured. *Results:* Canine TCC expresses high levels of nuclear VDR. Furthermore, calcitriol and seocalcitol significantly inhibited cell growth and calcitriol caused G0/G1 cell cycle arrest. Bcl-2 expression was slightly decreased in cells treated with these compounds, although no significant changes in VDR expression were observed. MCT enhanced the growth inhibitory effect of both compounds. *Conclusion:* Calcitriol and seocalcitol inhibited TCC cell growth via induction of cell cycle arrest and MCT enhanced this effect. Therefore, calcitriol and seocalcitol with MCT may have therapeutic potential for canine bladder cancer.

The hormone 1 α , 25-dihydroxyvitamin D₃ (calcitriol), the metabolically active form of vitamin D₃, is known for its classic role in calcium regulation and bone metabolism (1).

Abbreviations: Calcitriol, 1 α , 25-dihydroxyvitamin D₃; Seocalcitol, 22, 24-diene-24a,26a,27a-trihomo-1 α , 25-dihydroxyvitamin D₃; MCT, medium-chain triglyceride; TCC cell line, canine transitional cell carcinoma cell line.

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Calcitriol mediates gene expression *via* binding to the nuclear vitamin D receptor (VDR) in target tissue, causing up-regulation of gene transcription and translation of specific proteins (1, 2). Calcitriol and its analogs have been shown to exert antiproliferative effects in a variety of human cancers, including breast, colon and prostate cancers, through interaction with the VDR (3, 4). Recently, it was demonstrated that calcitriol also has significant antiproliferative effects on human urinary bladder tumor cells *in vitro* and *in vivo* (5). We previously showed that calcitriol and its analogs inhibited the growth of primary canine prostate epithelial cells and a canine squamous carcinoma cell line (SCC 2/88) *in vitro* (6, 7).

In order to achieve the inhibitory effects of calcitriol on cell proliferation, supraphysiological doses and prolonged treatment with this active vitamin D metabolite are required. However, calcitriol has the potential to induce hypercalcemic side-effects, which limits its use in cancer patients. Therefore, during the past decade, many vitamin D analogs have been synthesized to retain the potent antiproliferative activity without the undesired hypercalcemic side-effects. Seocalcitol, or EB1089 (22, 24-diene-24a,26a,27a-trihomo-1 α , 25-dihydroxyvitamin D₃), has been reported to have much greater potency than calcitriol in inhibiting the cell growth of various human cancers *in vitro*, *in vivo* and in clinical trials, with minimal effects on the blood calcium level (8, 9).

Transitional cell carcinoma (TCC) is the most common neoplasm affecting the urinary bladder of dogs. The majority of TCCs in dogs are invasive, highly malignant tumors that often are diagnosed late during the course of disease progression (10). Current treatments for TCC in dogs are ineffective and the tumors are associated with high morbidity and mortality. Results from the use of platinum analogs, such as cisplatin and carboplatin, to treat TCC in dogs have been disappointing (11, 12). Piroxicam, a nonsteroidal anti-inflammatory drug, is the most commonly

used drug for the medical management of TCC in dogs (13). Although most dogs treated with piroxicam have improved clinical signs (e.g., stranguria, hematuria, pollakiuria), significant objective tumor regression is uncommon. Our preliminary data demonstrated that the VDR is expressed in TCC of the canine urinary bladder, suggesting a role for calcitriol and its analog on canine TCC.

Several studies have shown that the antiproliferative effects of calcitriol and its analogs are the result of cell cycle arrest (14, 15) and induction of apoptosis (16, 17). Calcitriol treatment of a squamous cell carcinoma cell line caused G0/G1 arrest by inducing the expression of the cyclin-dependent protein kinase (CDK) inhibitors p21 and p27 (14). Furthermore, treatment of the LNCaP prostate cancer cell line with calcitriol led to up-regulation of p21 protein expression, which is required for G1 to S cell cycle transition (18). Bcl-2 is a member of a large family of proteins that regulate apoptosis induction in many cell lines including urinary bladder cancer (19, 20). Treatment of primary human prostate cancer stromal cells with calcitriol led to down-regulation of Bcl-2 expression in those cultures (17). In addition, Bcl-2 over-expression prevented calcitriol-induced apoptosis of LNCaP cells (16). These findings suggest that the induction of apoptosis by calcitriol involves the Bcl-2 regulatory pathway.

Medium-chain triglyceride (MCT) is a mixture of triglycerides mainly containing caprylic and capric acids. It is used widely in foods, drugs and cosmetic formulas (21) and also is used in patients requiring parenteral nutritional supplementation (22). It has been shown that calcitriol dissolved in MCT provides greater inhibition of liver tumor cell growth *in vitro* than calcitriol alone, by acting as a sustained release drug depot (23). Moreover, MCT is retained in liver tumors when administered *via* the intra-hepatic arterial route to laboratory animals (24). Due to the fat soluble nature of calcitriol and seocalcitol, MCT is a suitable solvent for these compounds. Because of the limited effectiveness of existing therapies for advanced bladder cancer in dogs and people and the demonstrated antiproliferative effects of calcitriol and its analogs in other tumors, we designed a study to investigate: a) the effect of calcitriol and seocalcitol on TCC cell growth; b) the effect of both compounds on VDR and Bcl-2 expression; c) the effect of calcitriol on TCC cell cycle distribution; and d) the effect of the addition of MCT to calcitriol and seocalcitol on growth inhibition of TCC cells.

Materials and Methods

Vitamin D and its analog. Calcitriol and seocalcitol were generous gifts from Dr. L. Binderup (LEO Pharma, Ballerup, Denmark).

MCT mixture preparation. Calcitriol, seocalcitol and vehicle (ethanol) were mixed with MCT before adding to the cell culture

media to give final concentrations of 10^{-9} M, 10^{-7} M and 10^{-6} M. The media mixture was then sonicated in an ultrasonic bath sonicator for 15-20 minutes to emulsify the MCT and calcitriol or seocalcitol mixture with the culture media.

Cell culture. A TCC cell line was provided by Dr. D. W. Knapp (25). Cells were grown in DMEM medium supplemented with 5% FBS, 5% newborn calf serum and penicillin-streptomycin (GIBCO BRL, Grand Island, NY, USA), at 37°C in a humidified atmosphere of 5% CO_2 . Cells were plated at 50,000 cells/ml in 6-well culture plates (Corning Incorporated Life Sciences, Acton, MA, USA) and incubated for 24 hours before drug treatment. Media containing calcitriol, seocalcitol, or vehicle (ethanol) with or without MCT, as described above, were added. The final concentrations of MCT and ethanol were 1% and less than 0.1% of media, respectively. For Western blot analysis, cells were grown in 10-mm culture plates and treated with calcitriol, seocalcitol or ethanol without MCT. The cells were treated for 48 and 72 hours unless otherwise stated. At the end of each experiment, the cells were harvested and subjected to analyses accordingly. Each experiment was run in triplicate.

Fluorescence DNA concentration analysis. DNA was isolated from cells using 4 M guanidine isothiocyanate containing 0.5% sodium lauryl sarcosine and 25 mM sodium citrate (7). The DNA content was measured on a fluorescence plate reader (SPECTRAMax[®] GEMINI XS, Molecular Devices Corporation, Sunnyvale, CA, USA) using Hoechst 33258 dye. Calf thymus DNA (100 $\mu\text{g}/\text{ml}$) served as a calibration control.

Cell cycle analysis. Cell cycle analysis was performed on TCC cells treated with calcitriol at 10^{-7} M or vehicle (ethanol), as described above. At 24 and 48 hours, the cells were harvested, washed twice with cold PBS, and fixed in cold 70% ethanol for 30 minutes at 4°C . The cells then were treated with 1 unit of RNase before staining with 0.5 mg/ml propidium iodide. The DNA content was measured using a flow cytometer (Beckman Coulter[®] EPICS XL[™], Fullerton, CA, USA). The results were analyzed using Cylchred software (University of Wales College of Medicine, Cardiff, UK).

Immunohistochemistry. Normal canine urothelium (n=4), biopsy tissue from canine TCC of the urinary bladder (n=3) and the TCC cell line were fixed and processed by the freeze substitution technique for immunohistochemistry, as previously described (7). Sections were stained for localization of VDR according to our protocol (7). Briefly, samples were incubated with 5% normal goat serum (NGS) in phosphate-buffered saline (PBS, pH 7.1) for 30 minutes at room temperature, followed by application of the primary antibody, rat anti-chicken VDR monoclonal antibody (Chemicon International Inc., Temecula, CA, USA) 1:50 in PBS at 4°C overnight. After washing, the secondary antibody, goat anti-rat IgG (Chemicon International Inc.) diluted 1:20 in PBS was added to the slides and incubated for 30 minutes at room temperature. The slides were developed by incubating in rat peroxidase-antiperoxidase (PAP) (Chemicon International Inc.) diluted 1:100 in 1% NGS in PBS for 30 minutes followed by 0.05% DAB and 0.01% hydrogen peroxide in 0.05 M Tris buffer for 5 minutes. The slides were mounted in aqua mount and visualized by light microscopy.

Western blot analysis for VDR and Bcl-2. At the end of the experiment, the cells were harvested and cell lysates were prepared.

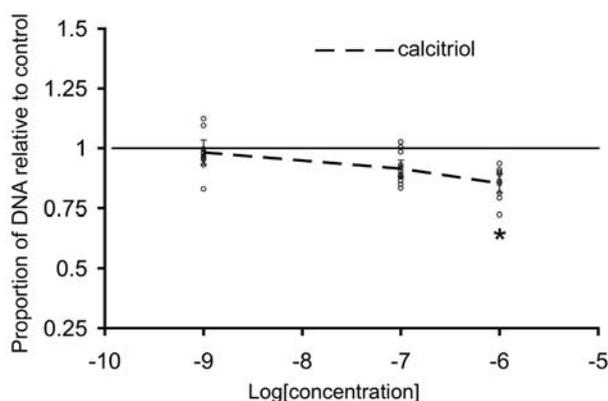


Figure 1. Antiproliferative effects of calcitriol at 72 hours: Graph demonstrates proportion of DNA remaining relative to control at 10^{-9} M, 10^{-7} M and 10^{-6} M. The DNA content of TCC cells was determined by DNA fluorometry using Hoechst 33258 dye. Calcitriol significantly inhibited TCC cell growth at 10^{-6} M. (* $p < 0.05$)

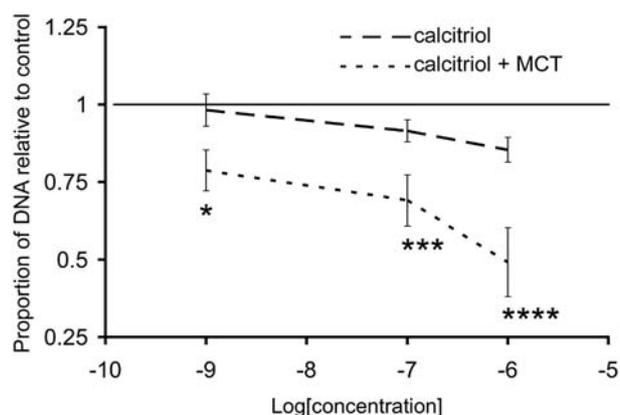


Figure 2. Antiproliferative effects of calcitriol compared to calcitriol plus 1% MCT at 72 hours. Graphs demonstrates proportion of DNA remaining relative to their respective controls at 10^{-9} M, 10^{-7} M and 10^{-6} M. MCT significantly enhanced the inhibitory effects of calcitriol at all concentration tested. (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$)

Western blot analysis was performed as previously described (7). The membranes were incubated at 4°C overnight in primary antibody, rabbit anti-human VDR monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:100, or mouse anti-human Bcl-2 monoclonal antibody (DakoCytomation California Inc., Carpinteria, CA, USA) diluted 1:200, followed by washing with deionized water for 15 minutes. Blots were incubated in goat anti-rabbit IgG-HRP for VDR blots and rabbit anti-mouse IgG-HRP for Bcl-2 blots at 1:2000 for 2 hours. Protein bands were detected using LumiGLO Chemiluminescent substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) and exposed to X-ray film for 5-10 seconds. After exposure, the blots were stripped and incubated with mouse anti- β -actin monoclonal antibody (Sigma, Saint Louis, MO, USA) to normalize protein loading. The Gel-Pro[®] Analyzer (Media Cybernetics, San Diego, CA, USA) was used to measure protein band density.

Statistical analysis. For the cell growth study, the proportion recovered was modeled using a linear regression model that treated concentration and solution as factors, and included an interaction term between concentration and solution (using PROC MIXED of SAS 9.1; Cary, NC, USA). In performing this regression analysis with PROC MIXED followed by Tukey's pairwise comparison, we considered all observations to be independent. Cell cycle analysis was analyzed using unpaired Student's *t*-test. All treatment groups were tested in triplicate.

Results

Inhibition of cell growth. We evaluated the inhibition of TCC cell growth by treatment with various concentrations (10^{-9} M, 10^{-7} M, 10^{-6} M) of calcitriol and seocalcitol with and without 1% MCT. Samples were collected at 48 and 72 hours of treatment. At 72 hours, calcitriol and seocalcitol inhibited TCC cell growth in a dose-dependent manner. Treatment of

cells with calcitriol alone caused significant inhibition only at the highest concentration of drug (10^{-6} M, $p < 0.05$) (Figure 1). However, the addition of 1% MCT to calcitriol showed a significant increase in inhibition of TCC cell growth at all concentrations tested compared to their controls ($p < 0.01$). As a result, calcitriol with 1% MCT significantly enhanced the inhibitory effects of calcitriol at all concentrations tested compared to calcitriol alone ($p < 0.05$ for 10^{-9} M; $p < 0.001$ for 10^{-7} M; $p < 0.0001$ for 10^{-6} M) (Figure 2).

Compared to the control vehicle-treated cultures, seocalcitol alone was inhibitory at 10^{-7} M ($p < 0.01$) and 10^{-6} M ($p < 0.0001$) (Figure 3). However, the addition of 1% MCT to seocalcitol was inhibitory at all concentrations tested compared to their controls ($p < 0.001$). Significant enhancement of seocalcitol activity by MCT was only at 10^{-9} M ($p < 0.05$), but not at 10^{-7} M or 10^{-6} M (Figure 4). Similar results were observed in TCC cells treated with calcitriol and seocalcitol alone or with 1% MCT at 48 hours (data not shown).

Cell cycle analysis. Cell cycle analysis was done by flow cytometry using propidium iodide staining. Calcitriol caused significant accumulation of cells in the G0/G1-phase and reduction in numbers of cell in the S-G2/M-phase of the cell cycle in TCC cells compared to vehicle-treated control after 24 hours ($p < 0.05$) and 48 hours ($p < 0.01$) treatment (Table I).

Cellular expression of VDR. Immunohistochemical staining of normal canine urinary bladder urothelium, biopsy tissue from canine TCC of the urinary bladder and TCC cell line with anti-VDR antibody demonstrated positive intranuclear staining for VDR (Figure 5).

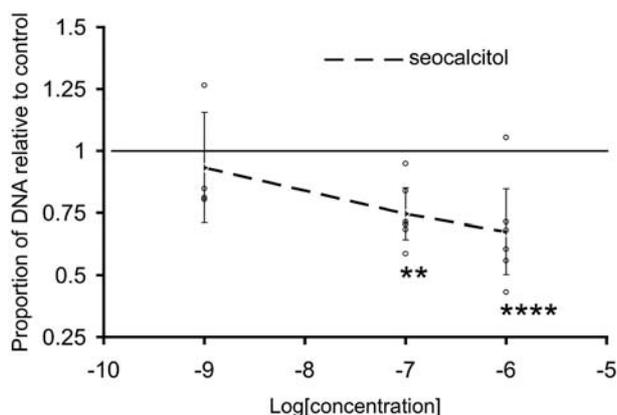


Figure 3. Antiproliferative effects of seocalcitol at 72 hours: Graph demonstrates proportion of DNA remaining relative to control at 10^{-9} M, 10^{-7} M and 10^{-6} M. The DNA content of TCC cells was determined by DNA fluorometry using Hoechst 33258 dye. Seocalcitol significantly inhibited TCC cell growth at 10^{-7} M ** and 10^{-6} M ****. (** $p < 0.01$; **** $p < 0.0001$)

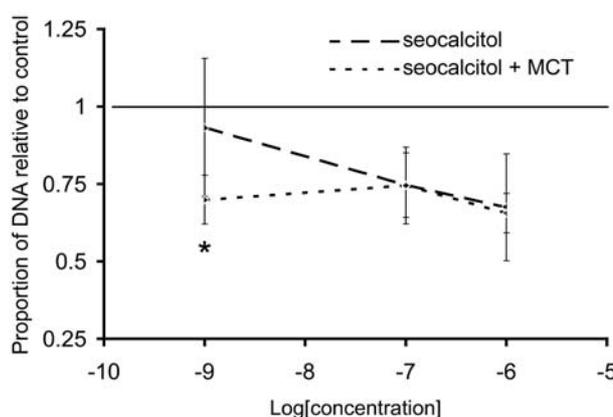


Figure 4. Antiproliferative effects of seocalcitol compared to seocalcitol plus 1% MCT at 72 hours. Graphs demonstrate the proportion of the DNA remaining relative to their respective controls at 10^{-9} M, 10^{-7} M and 10^{-6} M. MCT significantly enhanced the inhibitory effects of seocalcitol at 10^{-9} M. (* $p < 0.05$)

Footnote for Figures 1-4: The solid horizontal line represents the proportion (1.00) for the control group.

VDR protein level. Western blot analysis for VDR in normal canine urinary bladder urothelium and the TCC cell line resulted in a band with a molecular weight of approximately 62 kDa (Figure 6). Modulation of the VDR protein level was not observed in TCC cells treated with calcitriol or seocalcitol at 10^{-9} M, 10^{-7} M and 10^{-6} M compared to the vehicle-treated control after 48 and 72 hours (Figure 7).

Bcl-2 protein level. Bcl-2 was detected in TCC cells as determined by Western blot analysis. Based on quantification of the data using densitometry, Bcl-2 expression was slightly decreased in TCC cells treated with calcitriol and seocalcitol at 10^{-9} M, 10^{-7} M and 10^{-6} M compared to the vehicle-treated control after 48 and 72 hours (Figure 8).

Discussion

TCC in dogs is associated with high morbidity and mortality. Calcitriol and its analogs have been shown to exert antiproliferative effects in a variety of human cancers, including breast, colon and prostate cancers, through interaction with the VDR (3, 4). Recently, it was demonstrated that calcitriol also has significant antiproliferative effects on human urinary bladder tumor cells *in vitro* and *in vivo* (5). In order to achieve the inhibitory effects of calcitriol on cell proliferation *in vivo*, supraphysiological doses and prolonged treatment with this active vitamin D metabolite are required. However, calcitriol

Table I. Cell cycle analysis of canine TCC cells treated with calcitriol and vehicle (ethanol) at 10^{-7} M at 24 and 48 hours. DNA content (%) is presented as mean \pm SD of triplicate samples. (** $p < 0.01$; * $p < 0.05$)

| Time | %G0/G1 | | %S-G2/M | |
|------|-------------------|------------------------|-------------------|------------------------|
| | Control (ethanol) | 10^{-7} M calcitriol | Control (ethanol) | 10^{-7} M calcitriol |
| 24 h | 24.92 \pm 2.06 | 29.81 \pm 2.34* | 75.08 \pm 2.06 | 70.19 \pm 2.35* |
| 48 h | 24.67 \pm 0.79 | 38.92 \pm 2.11** | 75.34 \pm 0.80 | 61.08 \pm 2.10** |

has the potential to induce hypercalcemic side-effects, which limits its use in cancer patients. In this study, we explored the inhibition of TCC cell growth by calcitriol and its synthetic analog seocalcitol and effect of the addition of MCT as a solvent to these compounds. We have shown that normal canine urinary bladder urothelium, canine TCC of the urinary bladder and a TCC cell line express VDR by immunohistochemistry. This finding is consistent with others who reported the presence of VDR in human TCC cells (5, 26) and suggests that normal and neoplastic canine transitional epithelium are potential targets for the antiproliferative effect of calcitriol. Our findings demonstrated that calcitriol and seocalcitol significantly inhibit cell growth in TCC cells in a dose-dependent manner. These results are supported by previous studies of calcitriol-

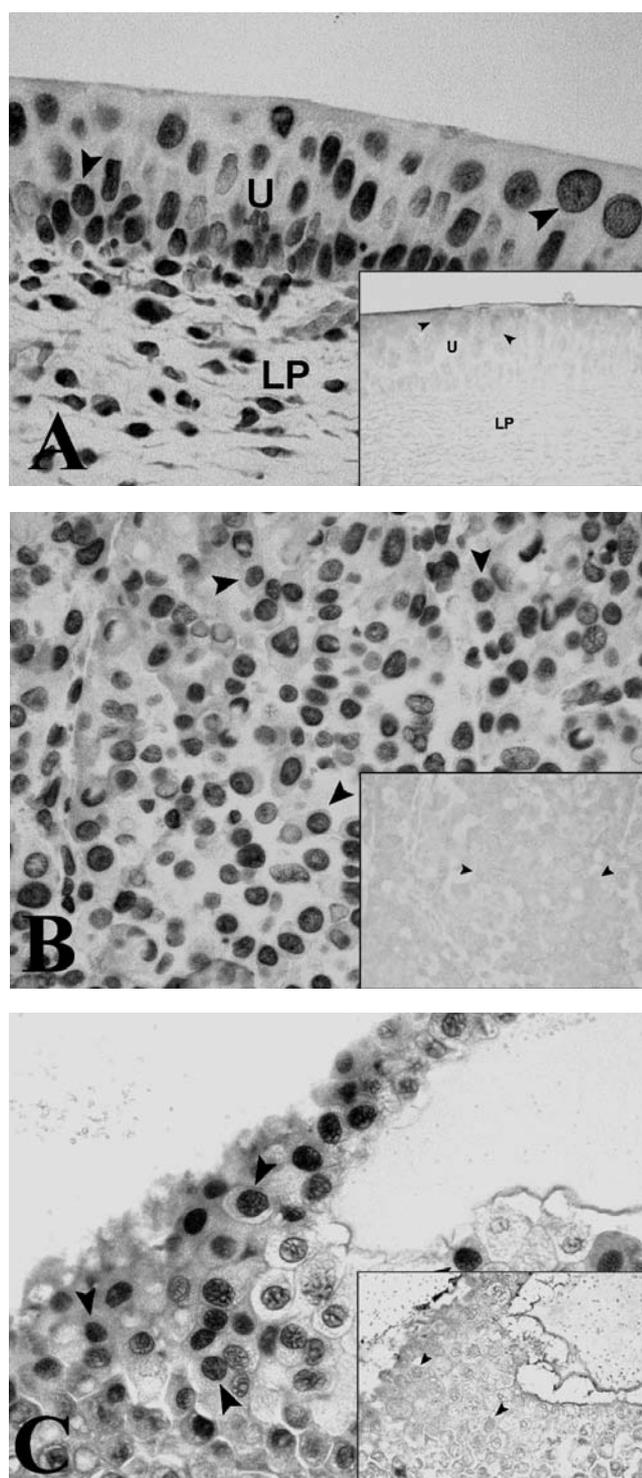


Figure 5. VDR Immunohistochemistry. (A) normal canine urinary bladder urothelium; (B) canine TCC of the urinary bladder and; (C) TCC cell line. Tissues and cells stained with rat anti-chicken VDR monoclonal antibody demonstrated positive reaction for the presence of nuclear VDR (arrowheads). Control sections (insets) were reacted with non-specific antiserum in place of a specific antibody. U: Urinary bladder urothelium; LP: Lamina propria. Magnification x 400.

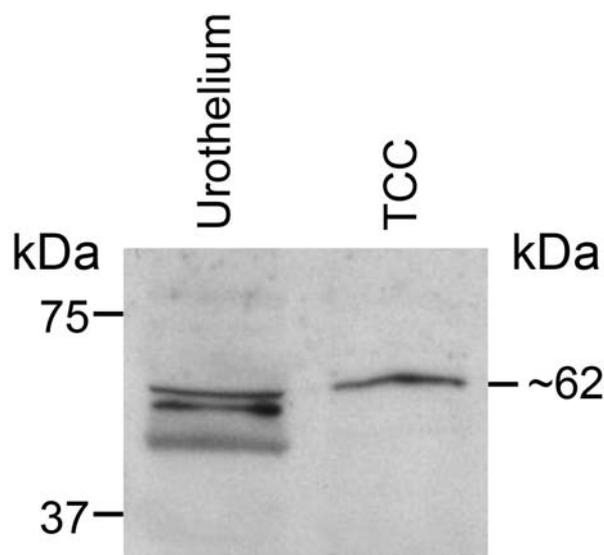


Figure 6. VDR expression in normal canine urinary bladder urothelium and TCC cell line by Western blot analysis. VDR protein bands migrated at a molecular weight of ~ 62 kDa. Blots were incubated with rabbit anti-human VDR monoclonal antibody.

mediated inhibition of cell growth of human TCC and other tumors (5, 27). However, we observed that TCC cells seem to be more resistant to the antiproliferative effects of calcitriol and seocalcitol than primary prostate epithelial cells or a squamous cell carcinoma cell line (SCC 2/88), requiring a higher dose to achieve growth inhibition. Specifically, we found that both compounds had significant inhibitory activity on TCC cell growth at tested concentration as high as 10^{-6} M, but not at 10^{-9} M. Our previous studies in primary canine prostate epithelial cells and a canine SCC 2/88 cell line showed significant growth inhibition in the 10^{-9} M to 10^{-7} M dose range (6, 7). The response of TCC cells to higher doses of calcitriol and seocalcitol compared to other cells of canine origin may explain the poor response of canine TCC of the urinary bladder to chemotherapy (10).

Importantly, the addition of 1% MCT as a solvent for calcitriol and seocalcitol significantly increased the potency of these compounds. At 10^{-9} M, neither compound alone reduced TCC cell growth after 72 hours treatment (Figures 1 and 3). The addition of 1% MCT to calcitriol significantly enhanced the growth inhibition of the compound at all concentrations from 10^{-9} M to 10^{-6} M (Figure 2). However, 1% MCT addition only enhanced the growth inhibition of seocalcitol at 10^{-9} M (Figure 4). These findings are consistent with those previously reported in HepG2 liver cells (23) and suggest that calcitriol and seocalcitol dissolved in MCT were taken up and accumulated in treated cells,

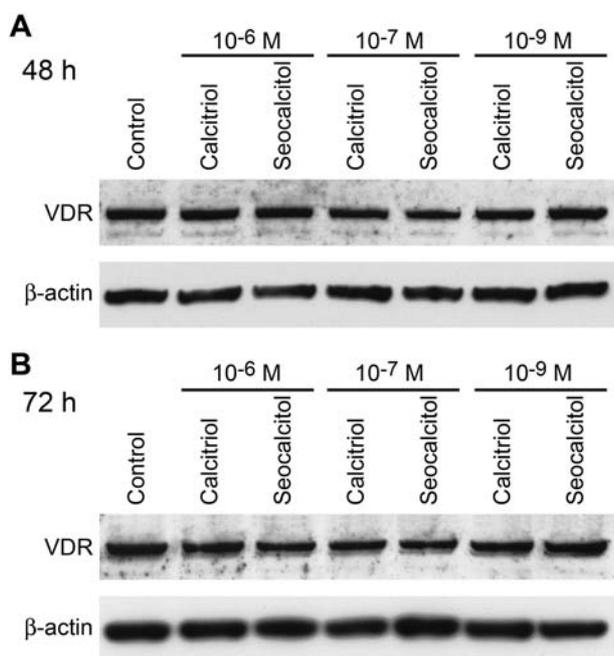
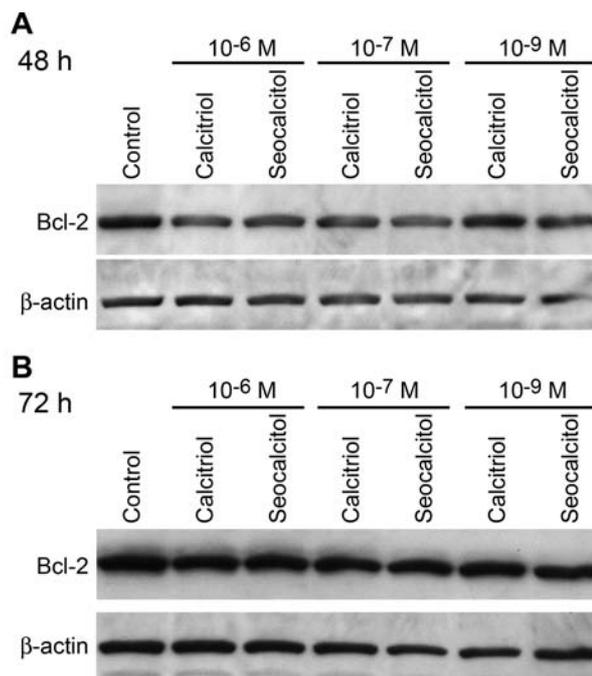


Figure 7. VDR expression in TCC cells treated with calcitriol, seocalcitol and vehicle (ethanol) was determined by Western blot analysis. Calcitriol, seocalcitol and vehicle (ethanol) at 10^{-9} M, 10^{-7} M and 10^{-6} M for 48 hours (A) and 72 hours (B). Blots were incubated with rabbit anti-human VDR monoclonal antibody.



Figures 8. Bcl-2 expression in TCC cells treated with calcitriol, seocalcitol and vehicle (ethanol) as determined by Western blot analysis. Calcitriol, seocalcitol and vehicle (ethanol) at 10^{-9} M, 10^{-7} M and 10^{-6} M for 48 hours (A) and 72 hours (B). Blots were incubated with mouse anti-human Bcl-2 monoclonal antibody.

then released in a sustained manner. It has been proposed that MCT as a solvent can prevent exposure of the compounds to cellular metabolizing enzymes, thereby prolonging degradation and resulting in greater stability of the compounds (23). The fat soluble nature of calcitriol and seocalcitol make MCT an ideal delivery reagent while maintaining the antiproliferative activity of the compounds. Altogether, the results from this study and others suggest the potential use of MCT as a solvent to enhance the efficacy of calcitriol and seocalcitol *in vivo*.

Studies in human bladder cancer cell lines and other human tumors have indicated that the growth inhibitory effects of calcitriol are associated with G₀/G₁ cell-cycle arrest (14, 28), which appears to be cell-specific (20). Similarly, our results showed that calcitriol treatment resulted in the accumulation of cells in the G₀/G₁-phase and the reduction of cells in the S-G₂/M-phase at 24 and 48 hours after treatment. Calcitriol up-regulates the CDK inhibitor p21 in LNCaP cells, suggesting that p21 is necessary for the growth inhibitory function of calcitriol by inhibiting CDK2 activity, which is required for cell cycle transition from G₁- to S-phase (18). Studies with HL-60 cells have shown that seocalcitol induces G₁ arrest by up-

regulation of p21 and p27 expression (29). p21 and p27 have been reported to be important regulators of vitamin D-mediated cell cycle arrest by inhibiting CDK activity, which leads to arrest in the G₀/G₁-phase (18, 30). The mechanism of cell cycle arrest by calcitriol in TCC cells is not known; however, our findings demonstrated that calcitriol induces G₀/G₁ arrest in TCC cells. Therefore, cell cycle arrest is, in part, involved in the growth inhibitory pathway induced by calcitriol in this cell line.

Several studies have demonstrated that the growth inhibitory effects of calcitriol are mediated by induction of apoptosis (5, 16-17). Mechanistically, treatment of some cancer cell lines with calcitriol and its analogs resulted in marked down-regulation of Bcl-2, which correlated with the pro-apoptotic actions of these compounds (17, 20). However, calcitriol and seocalcitol have been shown to induce apoptosis in the human colorectal adenoma cell line AA/C1, which does not express Bcl-2 (31). In addition, down-regulation of Bcl-2 in other colorectal carcinoma cell lines (SW260 and HT29) in response to seocalcitol was not observed. Taken together, these data indicate that the induction of apoptosis in response to calcitriol or seocalcitol is cell- and species-specific, and may or may not

occur *via* Bcl-2-dependent regulatory pathways. Our studies demonstrated that Bcl-2 expression in TCC cells treated with calcitriol and seocalcitol was only slightly decreased. Therefore, we conclude that Bcl-2 down-regulation is not a major factor of apoptosis induction in a canine TCC cell line in response to calcitriol and seocalcitol treatment.

A previous study from our laboratory reported a slight increase in VDR mRNA expression in primary cultures of canine prostate epithelial cells treated with calcitriol (6); however, a similar increase in VDR protein expression was not observed in TCC cells treated with calcitriol or seocalcitol. Previous literature reports have shown that cell lines treated with μM concentrations of calcitriol or its analogs produced antiproliferative effects *via* both VDR-dependent and VDR-independent mechanisms (15). The proposed mechanisms of the VDR-independent activity of vitamin D compounds include binding to alternative nuclear receptors, generation of toxic metabolites and byproducts, or direct interaction with components of cell signaling pathways. In this study, we conclude that the growth inhibitory mechanisms of calcitriol and seocalcitol in the TCC cell line are not dependent on the level of VDR expression. The inhibitory pathways utilized by calcitriol and its analogs in TCC cells are unknown and need further study.

Clinical uses of calcitriol for cancer treatment have been limited by the hypercalcemia and hypercalciurea induced by supraphysiological doses and long-term administration of these compounds. Our results demonstrated that MCT as a solvent enhanced the inhibitory potency of calcitriol and seocalcitol. These findings suggest the potential use of MCT as a solvent and enhancer for the increased efficacy of calcitriol and seocalcitol in cancer therapy. Because of the enhancing effect of MCT, the therapeutic potency of both compounds can be achieved with the lower dosage, therefore avoiding the unwanted hypercalcemic toxicity of calcitriol and its analogs. Moreover, the unique anatomy of urinary bladder cancer provides potential for intravesicular administration of high doses of calcitriol and its analogs dissolved in MCT, to achieve a high local drug concentration and to minimize systemic side-effects. Therefore, calcitriol and its analogs with MCT may have therapeutic potential for treating bladder cancer in animal and human patients.

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References

- 1 DeLuca HF: The vitamin D story: a collaborative effort of basic science and clinical medicine. *FASEB J* 2: 224-236, 1988.
- 2 Haussler MR, Whitfield GK, Haussler CA, Hsieh J-C, Thompson PD, Selznick SH, Dominguez CE and Jurutka PW: The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J Bone Mineral Res* 13: 325-349, 1998.
- 3 Colson K, Pirianov G and Mansi J: Non-classical actions of vitamin D: mechanisms and clinical aspects. Proc of the Eleventh Workshop on Vitamin D. Nashville, Tennessee, USA, pp. 477-484, 2000.
- 4 Trump DL, Hershberger PA, Modzelewski RA *et al*: Vitamin D endocrine system: structural, biological, genetic and clinical aspects. Proc of the Eleventh Workshop on Vitamin D. Nashville, Tennessee, USA, pp. 469-476, 2000.
- 5 Konety BR, lavelle JP, Pirtskalaishvili G, Dhir R, Meyers SA, Nguyen TT, Hershberger P, Shurin MR, Johnson CS, Trump DL, Zeidel ML and Getzenberg RH: Effects of vitamin D (calcitriol) on transitional cell carcinoma of the bladder *in vitro* and *in vivo*. *J Urol* 165: 253-258, 2001.
- 6 Kunakornsawat S, Rosol TJ, Capen CC, Leroy B and Inpanbutr N: Effects of $1\alpha, 25(\text{OH})_2\text{D}_3$, 25OHD_3 , and EB1089 on cell growth and vitamin D receptor mRNA and 1α -hydroxylase mRNA expression in primary cultures of the canine prostatic epithelial and stromal cells. *FASEB J* 16: A1098, 2002.
- 7 Kunakornsawat S, Capen CC, Rosol TJ, Middleton R, Hanah SS and Inpanbutr N: Effects of $1, 25$ -dihydroxyvitamin D_3 , EB 1089, and analog V on PTHrP production PTHrP mRNA expression, and cell growth in SCC S/88 cells. *Anticancer Res* 21: 3355-3364, 2001.
- 8 Haq M, Kremer R, Goltzman D and Rabbani SA: A vitamin D analogue (EB1089) inhibits parathyroid hormone-related peptide production and prevents the development of malignancy-associated hypercalcemia *in vivo*. *J Clin Invest* 91: 2416-2422, 1993.
- 9 Akhter J, Chen X, Bowrey P, Bolton EJ and Morris DL: Vitamin D_3 analog, EB1089, inhibits growth of subcutaneous xenografts of the human colon cancer cell line, LoVo, in a nude mouse model. *Dis Colon Rectum* 40: 317-321, 1997.
- 10 Mutsaers AJ, Widmer WR and Knapp DW: Canine transitional cell carcinoma. *J Vet Intern Med* 17: 136-144, 2003.
- 11 Chun R, Knapp DW, Widmer WR, Glickman NW, DeNicola DB and Bonney PL: Cisplatin treatment of transitional cell carcinoma of the urinary bladder in dogs: 18 cases (1983-1993). *J Am Vet Med Assoc* 209: 1588-1591, 1996.
- 12 Shapiro W, Kitchell BE, Fossum TW, Couto CG and Theilen G: Cisplatin for treatment of transitional cell carcinomas in dogs. *J Am Vet Med Assoc* 193: 1530-1533, 1988.
- 13 Knapp DW, Richardson RC, Chan TC, Bottoms GD, Widmer WR, DeNicola DB, Teclaw R, Bonney PL and Kuczek T: Piroxicam therapy in 34 dogs with transitional cell carcinoma of the urinary bladder. *J Vet Intern Med* 8: 273-278, 1994.
- 14 Hager G, Kornfehl J, Knerer B, Weigel G and Formanek M: Molecular analysis of p21 promoter activity isolated from squamous carcinoma cell lines of the head and neck under the influence of $1, 25(\text{OH})_2$ Vitamin D_3 and its analogs. *Acta Otolaryngol* 124: 90-96, 2004.

- 15 Valrance ME and Welsh JE: Breast cancer cell regulation by high-dose vitamin D compounds in the absence of nuclear vitamin D receptor. *J Steroid Biochem Mol Biol* 89-90: 221-225, 2004.
- 16 Blutt SE, McDonnell TJ, Polek TC and Weigel NL: Calcitriol-induced apoptosis in LNCaP cells is blocked by overexpression of Bcl-2. *Endocrinology* 141: 10-17, 2000.
- 17 Guzey M, Luo J and Getzenberg RH: Vitamin D₃ modulated gene expression patterns in human primary cancer prostate cells. *J Cell Biochem* 93: 271-285, 2004.
- 18 Rao A, Coan A, Welsh JE, Barclay WW, Koumenis C and Cramer SD: Vitamin D receptor and p21/WAF1 are targets of genistein and 1, 25-dihydroxyvitamin D₃ in human prostate cancer cells. *Cancer Res* 64: 2143-2147, 2004.
- 19 Schaaf A, Sagi S, Langbein S, Trojan L, Alken P and Michel MS: Cytotoxicity of cisplatin in bladder cancer is significantly enhanced by application of bcl-2 antisense oligonucleotides. *Urol Oncol* 22: 188-192, 2004.
- 20 Wagner N, Wagner KD, Schley G, Badiali L, Theres H and Scholz H: 1, 25-dihydroxyvitamin D₃-induced apoptosis of retinoblastoma cells is associated with reciprocal changes of Bcl-2 and Bax. *Exp Eye Res* 77: 1-9, 2003.
- 21 Traul KA, Driedger A, Ingle DL and Nakhasi D: Review of the toxicology of medium-chain triglycerides. *Food Chem Toxicol* 38: 79-98, 2000.
- 22 Adolph M: Lipid emulsions in parenteral nutrition. *Ann Nutr Metab* 43: 1-13, 1999.
- 23 Pourgholami MH, Akhter J and Morris DL: *In vitro* antiproliferative activity of a medium- chain triglyceride solution of 1 α , 25-dihydroxyvitamin D₃ in HepG2 cells. *Anticancer Res* 20: 4257-4260, 2000.
- 24 Yanai S, Okada H, Saito K, Kuge Y, Misaki M, Ogawa Y and Toguchi H: Anticancer effect of arterial administration of a medium-chain triglyceride solution of an angiogenesis inhibitor, TNP-470, in rabbit bearing VX-2 carcinoma. *Pharm Res* 12: 653-657, 1995.
- 25 Knapp DW, Chan TCK, Kuczek T, Reagan WJ and Park B: Evaluation of *in vitro* toxicity of nonsteroidal anti-inflammatory drugs against canine tumor cells. *Am J Vet Res* 56: 801-805, 1995.
- 26 Sahin MO, Canda AE, Yorukoglu K, Mungan MU, Sade M and Kirkali: 1, 25-dihydroxyvitamin D₃ receptor expression in superficial transitional cell carcinoma of the bladder: a possible prognostic factor? *Eur Urol* 47: 52-57, 2005.
- 27 Hershberger PA, Modzelewski RA, Shurin ZR, Rueger RM, Trump DL and Johnson CS: 1, 25-dihydroxycholecalciferol (1,25-D₃) inhibits the growth of squamous cell carcinoma and down-modulates p21 (Waf1/Cip1) *in vitro* and *in vivo*. *Cancer Res* 59: 2644-2649, 1999.
- 28 Caputo A, Pourgholami MH, Akhter J and Morris DL: 1, 25-dihydroxyvitamin D₃ induced cell cycle arrest in the human primary liver cancer cell line HepG2. *Hepatol Res* 26: 34-39, 2003.
- 29 Seol JG, Park WH, Kim ES, Jung CW, Binderup L, Koeffler HP, Kim BK and Lee YY: Effects of a novel vitamin D₃ analog, EB1089, on G1 cell cycle regulatory protein in HL-60 cells. *Int J Oncol* 16: 315-320, 2000.
- 30 Benerjee P and Chatterjee M: Antiproliferative role of vitamin D and its analogs – a brief overview. *Mol Cell Biochem* 253: 247-254, 2003.
- 31 Diaz GD, Paraskeva C, Thomas MG, Binderup L and Hague A: Apoptosis is induced by the active metabolite of vitamin D₃ and its analogue EB1089 in colorectal adenoma and carcinoma cells: possible implications for prevention and therapy. *Cancer Res* 60: 2304-2312, 2000.
- 32 Leman ES, DeMiguel F, Gao AC and Getzenberg RH: Regulation of androgen and vitamin D receptors by 1, 25-dihydroxyvitamin D₃ in human prostate epithelial and stromal cells. *J Urol* 170: 235-240, 2003.

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