ROLES OF CALCITRIOL AND ITS ANALOG ON CANINE TRANSITIONAL CELL CARCINOMA IN VITRO AND IN VIVO, AND IN NORMAL CANINE PROSTATE TISSUE EXPLANTS

DISSERTATION
Presented in Partial Fulfillment of the Requirement for The Degree Doctor of Philosophy in the Graduate School of The Ohio State University
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ABSTRACT

Although increasing data indicates inhibitory roles of calcitriol on tumor growth in humans, little is known about its effects on canine tumors. The objectives of this study are to investigate the effects of calcitriol and its analogs on canine transitional cell carcinoma and canine prostate tissue explants. First, we investigated effects of calcitriol, seocalcitol and medium-chain triglyceride (MCT) on a canine transitional cell carcinoma cell line (TCC). The effects of calcitriol and seocalcitol on cell growth, cell cycle, vitamin D receptor (VDR) and Bcl-2 expression were determined with/without MCT. Second, we established a canine TCC mouse-xenograft model and used this model to examine effects of calcitriol, seocalcitol, and piroxicam on tumor growth. Third, the effects of calcitriol and dihydrotestosterone (DHT) were evaluated on arginine esterase (AE) activity and VDR expression in normal canine prostate tissue explants.

In summary, our results showed that the VDR is present in canine TCC tumor and in the canine prostate. Calcitriol and seocalcitol significantly inhibited cell growth and calcitriol caused cell cycle arrest. Bcl-2 expression was decreased in cells treated with these compounds, although no significant changes in VDR expression were observed. MCT enhanced the growth-inhibitory effects of both compounds.

We developed and used a canine TCC-mouse xenograft model to evaluate and compare the inhibitory effects of calcitriol, seocalcitol and piroxicam. Results showed
that only seocalcitol reduced tumor volume compared to controls. The inhibitory effect of seocalcitol on tumor growth was supported by data from a Ki-67 staining. Blood calcium was higher in both calcitriol-and seocalcitol-treated mice compared to controls. In summary, our findings suggest a potential use for calcitriol and seocalcitol for the treatment canine TCC.

We demonstrated that DHT increased AE activity and VDR expression in canine prostate tissue explants; however, there was no increase in AE activity in calcitriol-treated explants and a decreased in VDR expression. These results indicated that canine prostate tissue explants are a valuable model for the study of prostate pathobiology and pharmaceutical interventions. They also provided a basis for further investigation of roles of calcitriol as a therapeutic/preventative agent in benign prostatic hyperplasia in both dogs and humans.
Dedicated to my family,

My mother and my brother
ACKNOWLEDGMENTS

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<td>$1\alpha,25(OH)<em>{2}D</em>{3}$; calcitriol</td>
<td>$1\alpha$, 25-dihydroxyvitamin D$_3$</td>
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<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
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<td>MCT</td>
<td>medium-chain triglycerides</td>
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<td>seocalcitol</td>
<td>22-24-diene-24a,26a,27a-trihomo-$1\alpha$25-dihydroxyvitamin D$_3$ or EB1089 dihydrotestosterone</td>
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<td>DHT</td>
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<tr>
<td>TCC</td>
<td>transitional cell carcinoma</td>
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<tr>
<td>AE</td>
<td>Arginine esterase</td>
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<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
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<tr>
<td>BPH</td>
<td>Benign prostatic hyperplasia</td>
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<tr>
<td>iCa</td>
<td>Blood ionized calcium</td>
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<td>DBP</td>
<td>Vitamin D binding protein</td>
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<tr>
<td>$1\alpha$-OHase</td>
<td>$1\alpha$-hydroxylase</td>
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<tr>
<td>$24,,25(OH)<em>{2}D</em>{3}$</td>
<td>24, 25-dihydroxyvitamin D$_3$</td>
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<tr>
<td>DBD</td>
<td>DNA binding domain</td>
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<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
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<td>RXR</td>
<td>Retinoid X receptor</td>
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<td>VDRE</td>
<td>Vitamin D response element</td>
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CHAPTER 1

INTRODUCTION

1. VITAMIN D METABOLISM

Vitamin D was discovered as an essential nutrient for the prevention of rickets. Following the recognition by Sir Edward Mellanby (1919) that rickets might be a disease caused by a dietary deficiency (Mellanby, 1919); experiments in dogs were performed in an effort to identify the causative substance. The term “Vitamin D” was used when McCollum and his colleagues reported the discovery of a new lipid-soluble compound, which they named “Vitamin D” (McCollum et al., 1922). At the same time, Huldshinsky (1919) and Chick (1923), independently working on children with rickets, discovered that rickets could be cured by exposing them to sunlight. With continued study, the structure of the first vitamin D, vitamin D\textsubscript{2} or ergocalciferol, was identified by irradiation of plant sterols (Askew et al., 1931). The identification of vitamin D\textsubscript{3} (cholecalciferol), which was derived from the photolytic cleavage of the B rings of the precursor (7-dehydrocholesterol) in the skin, was later reported by Windaus and colleagues (1935).
Vitamin D plays a crucial role in calcium and phosphate homeostasis and is essential for bone development (DeLuca, 1988). Vitamin D can be obtained from the diet, or by the reaction of sunlight on the skin. Only a few natural food sources provide a significant amount of vitamin D$_2$ and D$_3$ such as fish (not only fatty fish), egg yolk, and liver (Christel, 2006). In addition, some wild mushrooms may contain significant amounts of vitamin D$_2$ (Christel, 2006). However, many foods in the United States are now fortified with vitamin D. Exposure to ultraviolet rays induces photolytic conversion of 7-dehydrocholesterol to previtamin D$_3$, followed by thermal isomerization to vitamin D$_3$ which is then transported from the skin into the blood circulation by binding to vitamin D binding protein (DBP) (Adrina et al., 2005). Both vitamins D$_2$ and D$_3$ go through the same activation process involving first, hydroxylation by 25-hydroxylase in the liver, followed by $1\alpha$-hydroxylation in the proximal tubules of the kidney, to make the biologically active forms of $1\alpha$, 25-dihydroxyvitamin D$_2$ [$1\alpha$, 25(OH)$_2$D$_2$] and $1\alpha$, 25-dihydroxyvitamin D$_3$ [$1\alpha$, 25(OH)$_2$D$_3$ or calcitriol], respectively (Jones et al., 1998). There is little information indicating the differences in the actions between these two vitamin D active forms. Only the $1\alpha$, 25(OH)$_2$D$_3$ (Figure 1.1) was used in our studies.

The 24-hydroxylation also is involved in vitamin D enzyme metabolism. The metabolic inactivation of vitamin D by 24-hydroxylation is catalyzed by 25-hydroxyvitamin D$_3$-24-hydroxylase (24-OHase). The 25-OHD$_3$ is hydroxylated in the kidney either by $1\alpha$-hydroxylase ($1\alpha$-OHase) to form $1\alpha$, 25(OH)$_2$D$_3$ (as mentioned above), or by 24-OHase to form 24, 25-dihydroxyvitamin D$_3$ [24, 25(OH)$_2$D$_3$]. Both $1\alpha$, 25(OH)$_2$D$_3$ and 24, 25(OH)$_2$D$_3$ may go through an additional hydroxylation to form 1, 24, 25-trihydroxyvitamin D$_3$. This mechanism is important in preventing hypercalcemia.
under conditions of high circulating levels of 1α, 25(OH)₂D₃. Other mechanisms involved in the inactivation of the biologically active 1α, 25(OH)₂D₃ include (i) the oxidative cleavage of the side chain which forms C-23 carboxylic acid and calcitroic acid, and (ii) enterohepatic circulation. The serum levels of 1α, 25(OH)₂D₃ remain constant in the normal physiological state and are strictly regulated in response to factors that control calcium homeostasis. The regulation of 1α, 25(OH)₂D₃ and 24, 25(OH)₂D₃ production by these factors is conducted by alternating the activities of the enzymes that hydroxylate vitamin D derivatives.

2. MECHANISMS OF VITAMIN D ACTIONS

VITAMIN D RECEPTOR

The vitamin D receptor (VDR) is a member of a superfamily of nuclear steroid receptors that regulate gene expression in a ligand-dependent manner. The VDR is a ligand-activated transcription factor (Ordonez-Moran et al., 2005) which can be divided by its function into several domains. First, the N-terminal DNA binding domain (DBD), located approximately between amino acids 20 to 90; second, the hinge region, which is located approximately between amino acids 90 to 130 lies between the DNA-binding domain and the ligand-binding domain; and lastly, the C-terminus or ligand-binding domain (LBD) which consists of the entire C-terminus of the VDR molecule.

The DBD is the most conserved region among the members of the nuclear superfamily for steroid and thyroid hormones that include the VDR. It contains two 30-residue zinc-binding motifs (zinc fingers) which are unique to the nuclear receptor
family (Peleg and Posner, 2003). The DBD is responsible for the binding of the VDR to specific DNA sequences in the promoter region of the VDR-responsive genes (Zierold et al., 1994). The DBD also is involved in the heterodimerization of the VDR with the retinoid X receptor (RXR), which is essential for the high affinity binding of the VDR to DNA (Kliewer et al., 1992). Spontaneous mutations in the zinc finger regions of the human VDR result in defective DNA binding and the most severe clinical phenotype of vitamin D resistance (Dusso et al., 2005).

The LBD domain of the VDR molecule, located in the c-terminal portion of the VDR, is responsible for the high-affinity binding of 1α, 25(OH)2D3. 25(OH)D3 and 24, 25(OH)2D3 bind nearly 100 times less avidly than 1α, 25(OH)2D3 (Mellon et al., 1979); however, the ligand binding affinity is not a predictor of the transcription activity of ligand-activated VDR (Dusso et al., 2005). Cell-specific variation in the regulation of ligand delivery to and from the VDR may modulate the ligand-VDR association rate and consequently, the half-life of the VDR molecule, which is protected from proteosomal degradation through ligand binding (Masuyama and MacDonald, 1998). After ligand binding, the cytoplasmic VDR rapidly translocates to the nucleus along the microtubules. Little is known about the nature of the nuclear localization signals within the VDR molecules (Dusso et al., 2005).

**GENOMIC ACTIONS OF VITAMIN D**

The main actions of vitamin D are mediated via the genomic pathway which involves binding of the hormone to the intracellular VDR (Feldman D et al. 1997). Most of the circulating 1α, 25(OH)2D3 is bound to DBP and albumin. The small fraction of
unbound hormone can diffuse into the cells and bind to the VDR. The ligand binding domain of the VDR molecule, located in its c-terminal portion, is responsible for the high affinity binding with 1α, 25(OH)₂D₃. The transcriptional regulation of the 1α, 25(OH)₂D₃-VDR complex involves multiple protein-protein and protein-DNA interactions that includes: (i) heterodimerization with retinoid X receptor (RXR), (ii) binding of the VDR-RXR heterodimer with the vitamin D response elements (VDRE) in the promoter region of 1α, 25(OH)₂D₃ responsive genes, (iii) additional interaction of VDR-RXR complex with various nuclear proteins (co-regulators) into the transcriptional preinitiation complex, which markedly enhance or suppress the rate of target gene transcription by the VDR. Numerous genes have been described to be sensitive to vitamin D compounds, including 26 genes in which a VDRE has been identified (Hannah and Norman, 1994; Hansen et al., 2001).

**NON-GENOMIC ACTIONS OF VITAMIN D**

In recent years, the concept of non-genomic actions by steroids that do not involve the activation of nuclear receptors has been demonstrated in number of different tissues. Evidence has been suggested that the non-genomic actions of steroids might be explained by the presence of signal-generating steroid receptors on the cell surface. It has been reported that the non-genomic pathway involves the regulation of voltage-gated calcium channels, opening of chloride channels, modulation of protein kinase C (PKC) activity, and activation of mitogen-activated protein kinases (MAP kinases) which lead to the onset of rapid biological responses (seconds to 1-2 minutes), including inhibition of cell proliferation and stimulation of cells differentiation (Hansen et al., 2001). Studies
have shown that the generation of rapid responses is mediated through putative membrane receptors with ligand binding properties different from those of the nuclear VDR (Baran et al., 1994; Kato et al., 1998). In addition, as alternative to specific membrane receptors for vitamin D, it has been suggested that the membrane-associated annexin II might serve as a receptor for vitamin D-mediated non-genomic rapid responses (MacDonald et al., 1994). However, the physiological relevance of non-genomic mechanisms for vitamin D’s actions remains unclear.

3. VITAMIN D AND CANCER

The role of vitamin D in cancer is supported by strong evidence from epidemiological studies demonstrating an association between colon, breast and prostate cancers and vitamin D deficiency (Zittermann, 2003). The receptors for vitamin D are found not only in the classic target organs (i.e., intestinal mucosa, skin, kidney and bone), but also in many other normal epithelial and mesenchymal cell types, as well as many different cancers, including colon cancer, leukemia, and breast cancer (Colston et al., 1982; Eisman, 1984). Calcitriol and its synthetic analogs have been shown to exert anti-tumor effects in many human cancer cells (Rots et al., 1999; Pourgholami et al., 2000). Several studies have reported that calcitriol induces cell differentiation and inhibits proliferation of hemopoietic, epidermal and many different cancer cell types (Suda et al., 1986; Thavarajah et al., 1991). The mechanisms by which calcitriol and its analogs contribute to the inhibitory mechanisms on tumor growth are still not well established. Many investigations have shown that multiple mechanisms are responsible
for the anticancer activities of calcitriol and its analogs, including: (i) cell cycle regulation, (ii) induction of apoptosis, (iii) induction of cell differentiation, and (iv) anti-angiogenesis and inhibition of metastasis.

(i) Cell cycle regulation

Several studies have shown that calcitriol and its analogs exert their inhibitory effects on tumor cell growth by regulating cell cycle progression. Treatment of most cell types with calcitriol and its analogs have demonstrated cell cycle arrest at the G0/G1 phase, resulting in an accumulation of cells at G0/G1 and a reduction in the number of cells that progress to S-phase of the cell cycle (Hershberger et al., 1999; Kaewsakhorn et al., 2005). Calcitriol up-regulated the cyclin dependent kinase (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-phases (Rao et al., 2004). Studies with HL-60 cells have shown that seocalcitol, a synthetic analog of calcitrol, induced G1 arrest by up-regulation of p21 and p27 expression (Seol et al., 2000). The induction of p21 and p27 in response to the treatment of vitamin D compounds is accompanied by the up-regulation of the hypophosphorylated form of retinoblastoma (Rb) protein and the down-regulation of the hyperphosphorylated form of the Rb (Simboli-Campbell et al., 1997). However, calcitriol has been shown to inhibit tumor cell growth, such as prostate cancer cell line PC-3, without altering cell cycle distribution and changes in the expression of p21 (Zhuang et al., 1998). This suggests that the regulation of the cell cycle by vitamin D compounds is cell-specific and may involve many cellular pathways.
(ii) Induction of apoptosis

Programmed cell death, or apoptosis, occurs as a part of the normal regulatory processes in the body that control and maintain a proper rate of cellular turnover. Apoptosis is closely linked to the cell cycle and is controlled, in part, by the same regulatory systems (Kasten et al., 1998). Cancer often is associated with cells that fail to undergo apoptosis. Calcitriol and its analogs have been reported to induce apoptosis in many normal and malignant cells (Peehl et al., 2004; Golovko et al., 2005). The Bcl-2 family of proteins (i.e., Bcl-2, Bcl-xl, Bax, and Bak) is important in the regulation of apoptosis in many cells. Treatment of some cancer cell lines with calcitriol and its analogs has resulted in marked down-regulation of Bcl-2, which has correlated with the pro-apoptotic actions of these compounds (Guzey et al., 2004; Wagner et al., 2003). However, in these studies apoptosis and Bcl-2 expression were not strictly correlated, since calcitriol and seocalcitol have been shown to induce apoptosis in the human colorectal adenoma cell line AA/C1, which does not express Bcl-2 (Diaz et al., 2000). Additionally, down-regulation of Bcl-2 in other colorectal adenoma 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 its analogs is cell type- and species-specific, and may or may not occur via Bcl-2-dependent pathways (Kaewsakhorn et al., 2005). In the studies of myeloma, breast, colon and prostate cancer cell lines, a down-regulation of the anti-apoptotic members of the Bcl-2 family, Bcl-2 and Bcl-xl, and up-regulation of the pro-apoptotic members, Bax and Bak proteins, have been demonstrated after the treatment with vitamin D compounds (James et al., 1998; Diaz et al., 2000; Park et al., 2000).
Calcitriol has been shown to exhibit stimulatory effects on tumor necrosis factor alpha (TNF-α) mRNA expression in prostate cancer LNCaP cell line (Goloveka et al., 2005). A study on leukemic cells transfected with the wild type p53 tumor suppressor gene demonstrated that calcitriol protected against p53-induced apoptosis and enhanced p53-induced maturation, which resulted in growth inhibition and produced cells that could either undergo apoptosis or terminal differentiation (Ehinger et al., 1996). Thus, it appears that calcitriol and its analogs can induce inhibition of cell growth by either apoptosis-dependent or apoptosis-independent mechanisms. In some cases, induction of differentiation may be more prominent than apoptosis. Overall, it is unclear what factors dictate whether cells will undergo apoptosis or differentiation in response to vitamin D compounds. Stage of the cell cycle, presence of various growth factors, and interactions between cell populations, are all likely important factors.

(iii) Induction of cell differentiation

Several studies have demonstrated that calcitriol and its analogs stimulate differentiation in a variety of normal and neoplastic cells (Kornfehl et al., 1996; Kunakornsawat et al., 2001; Zhou et al., 2006; Mosieniak et al., 2006). In human leukemia cells, the onset of differentiation is indicated by an increased expression of cell surface antigens CD14 and CD18. Superoxide production and phagocytic activity also are induced in differentiated cells. All of these markers have been shown to be up-regulated in response to the treatment of the leukemia cells with vitamin D compounds in vitro (Hansen et al., 2000; Hansen et al., 2001).
Prostate specific antigen (PSA) is a cell type-specific marker of mature human prostate epithelial cells and PSA is often used as a differentiation marker for prostate cancer. Several studies have shown that calcitriol increases the expression of PSA and the steady state level of its mRNA in the prostate cancer cell line LNCaP (Hansen et al., 2001). Serum PSA concentration has been used as a prognostic factor in benign prostatic hyperplasia (BPH) and prostate cancer in human patients (Beer et al., 2006). In one clinical study, calcitriol was shown to delay the rate of increase of serum PSA in men with early recurrent prostate cancer after primary therapy with radiation or surgery (Gross et al., 1998). Calcitriol has been reported to up-regulate apolipoprotein D, a protein associated with tumor differentiation and prognosis in women with breast cancer, in breast cancer cell lines (Rassart et al., 2000; Soiland et al., 2007). A study from our laboratory in a canine squamous cell carcinoma cell line showed that the vitamin D analog, analog V, increased the expression of involucrin, a differentiation marker protein (Kunarkornsawat et al., 2001). At present, the precise mechanisms mediating the effect of calcitriol and its analogs on cell differentiation are not conclusive.

(iv) Anti-angiogenesis and inhibition of metastasis.

Mantell et al. (2000) reported that calcitriol inhibits vascular endothelial growth factor (VEGF)-induced endothelial cell sprouting and elongation in vitro and in vivo. The anti-angiogenic effect of calcitriol was further supported by a study demonstrating a decreased formation of elongated endothelial cell networks within 3D collagen gels and a regression of sprouting endothelial cells in cell culture after treatment with calcitriol. The anti-angiogenic effects of calcitriol and its analogs may be due to the induction of
apoptosis and the inhibition of cell proliferation in sprouting endothelial cells. *In vivo* studies using different animal models provides evidence that vitamin D suppresses invasion and metastasis and exerts anti-angiogenic activity. In melanoma, lung, prostate, colon and breast cancer animal model studies, a reduction in the number and size of metastases has been observed in animals treated with calcitriol and its analogs as compared to control (Yudoh *et al.*, 1999; Lokeshwar *et al.*, 1999; El Abdaimi *et al.*, 2000). Additionally, administration of calcitriol to mice bearing different types of cancer cell xenografts and rats bearing chemically-induced colon cancers has been shown to significantly reduce tumor-induced angiogenesis (Fujioka *et al.*, 1998; Iseki *et al.*, 1999; Mantell *et al.*, 2000) These *in vivo* studies are consistent with the *in vitro* studies in that the anti-angiogenic effects of calcitriol involved the regulation of VEGF.

### 4. VITAMIN D ANALOGS

The primary aim for the synthesis and evaluation of synthetic vitamin D analogs is to identify compounds that are potent regulators of cell proliferation and cell differentiation, with reduced ability to cause hypercalcemia. The mechanisms that might be responsible for the enhanced differentiation-inducing ability and lowered hypercalcemia-inducing activity of the analogs include: (i) avidity of the binding of the analogs to vitamin D binding protein (DBP), (ii) avidity of binding to VDR, and (iii) ability to alter dimerization of the VDR, VDR-RXR, or VDR-RXR with VDREs (Uskokovic *et al.*, 1997). Some of the vitamin D analogs have been shown to bind less strongly to DBP than the parent active form, calcitriol, resulting in more free ligands
entering through the cell membrane. Due to structural modifications, the analogs may be more suitable than the parent compound to participate in the events leading to specific gene transcription activation or repression (Siu-Caldera et al., 1996). The different structural characteristics of the analogs may alter conformation of the VDR-RXR bound to VDREs, which may result in higher or lower transcriptional activity of the target genes (Cheskis et al., 1994). Moreover, the structural modification of the analogs may result in reduced ligand sensitivity and promoter selectivity (Quack et al., 1998). In the past decade, research on the development of vitamin D analogs at the cellular and molecular level has advanced greatly. More than a thousand new vitamin D analogs have been synthesized for their enhanced effects on inhibition of cell growth, immune system regulation, receptor binding and gene expression, and calcium and bone metabolism. In the future, it is thought that in-depth evaluations of the therapeutic potential of vitamin D analogs will allow the synthesis of more effectively selective analogs in the treatment of cancer.

In our studies, the synthetic analog 22-24-diene-24a,26a,27a-trihomo-1α25-dihydroxyvitamin D₃ (EB1089 or seocalcitol) was used. Seocalcitol is characterized by having two double bonds in the side chain, which makes it less susceptible to metabolic degradation (Figure1.2). Compared to calcitriol, seocalcitol was shown to be a potent inhibitor of tumor cell growth both in vitro and in vivo with reduced effects on on calcium metabolism (Colston et al., 1992; Mathiasen et al., 1993; Hensen et al., 2000). In the prostate cancer cell line LNCaP, seocalcitol was a potent inhibitor of cell growth and caused cells to undergo G0/G1 cell cycle arrest and apoptosis (Blutt et al., 2000). The ability of seocalcitol to inhibit tumor growth and induce tumor cell differentiation,
with reduced hypercalcemic effects, suggests that seocalcitol may be an effective new therapeutic agent for the treatment of cancer.

5. MEDIUM-CHAIN TRIGLYCERIDES

Medium-chain triglycerides (MCT) are a class of lipids in which three saturated fatty acids are bound to a glyceride backbone (Figure 1.3). Each fatty acid molecule consists of 6-12 carbons. MCT is a colorless to slightly yellowish oily liquid containing mostly caprylic and capric fatty acids. MCT is a component of many foods, especially coconut and palm oil. MCT also is available as a dietary supplement and is used in pharmaceutical and cosmetic formulations either as an emulsifying agent, solvent or suspending agent. MCT is used as the base for oral emulsions, solutions or suspensions for drugs that are unstable or insoluble in water, such as calciferol. MCT also have been used in parenteral formulations in the production of emulsions or solutions intended for intravenous administration. A study in the HepG2 liver cell line showed that calcitriol dissolved in MCT had a two-fold greater inhibition of cell growth than calcitriol without MCT (Pourgholami et al., 2000). In vivo, MCT has been shown to be taken up and retained by liver tumors when administrated using the intrahepatic arterial route in experimental rabbits (Yanai et al., 1995). These findings suggest that calcitriol and its analogs when dissolved in MCT results in sustained release of the compounds. Furthermore, MCT is an ideal solvent for calcitriol and its analogs due to the lipophilic nature of these compounds. We wanted to explore the enhancing effects of MCT on calcitriol and its analogs on tumor cells. Potentially, the therapeutic effects of these
compounds can be achieved at lower doses when dissolved in MCT, thus avoiding potential undesirable hypercalcemic toxicity of these compounds.

6. CANINE TRANSITIONAL CELL CARCINOMA

Canine transitional cell carcinoma (TCC) of the urinary bladder is the most common malignancy of the urinary tract in dogs. Certain breeds are at a significantly increased risk of TCC compared with dogs in other breeds (Glickman et al., 2004). An environmental risk factor for canine TCC that has been identified is exposure to lawns or gardens treated with phenoxy herbicides (Glickman et al., 2004). Currently, there is no effective treatment for this type of cancer. In contrast to humans, where TCC is commonly diagnosed early as a superficial cancer of the urinary bladder transitional cell epithelium, the majority of TCCs in dogs are infiltrative tumors of intermediate to high grade malignancy. For this reason, common therapies used in people to treat superficial tumors are not successful for treating the large and invasive tumors commonly seen in dogs. Surgical excision of canine TCC is difficult due to the trigonal location of the tumors and the high likelihood of metastases and/or urethral involvement at the time of diagnosis. While complete cystectomy accompanied by use of an external urine collection device is a viable option for treatment of human bladder cancer patients, this is not an option for canine patients. Because of the limited surgical options and the potential for metastasis, cytotoxic chemotherapy has been advocated for the treatment of TCC in the dog, as well as in human patients with advanced invasive bladder cancer. The uses of the platinum analogs, cisplatin and carboplatin (Helfand et al., 1990; Chun
et al., 1996) and, to a lesser extent, other drugs such as mitoxantrone, actinomycin, doxorubicin, and gemcitabine have been reported (Helfand et al., 1990; Henry et al., 2003). Overall, the response rates have been disappointing, ranging from 0-20%.

Currently, the use of the non-steroidal anti-inflammatory drug piroxicam has become, arguably, the standard of care for the medical management of TCC in dogs (Knapp et al., 1994). Most dogs show an improvement in clinical signs when placed on piroxicam; however, the overall objective response rate is low, similar to cytotoxic therapies. Recently, a clinical trial investigated the combined use of piroxicam and mitoxantrone in dogs. This combination resulted in an overall response rate of 35%; however, only one dog had a complete response to the treatment (Henry et al., 2003).

Calcitriol has been shown to inhibit proliferation and induce apoptosis in TCC of human origin in vitro and in a rat model of bladder cancer (Konety et al., 2001). In addition, results from our in vitro study showed that calcitriol and seocalcitol significantly inhibited cell growth and calcitriol caused G0/G1 cell cycle arrest (Kaewsakhorn et al., 2005). Due to the lack of an effective therapy to treat bladder cancer in dogs, we wanted to explore the possibility of using calcitriol and its analogs as therapeutic agents in a xenograft mice model before a canine clinical study.
VITAMIN D AND THE PROSTATE GLAND

VITAMIN D AND THE INCIDENCE OF PROSTATE CANCER

During the past decades, there have been many studies reporting a link between vitamin D and prostate cancer in humans (Barreto et al., 2000). Based on epidemiological studies, the lack of adequate vitamin D in the diet has been demonstrated as a risk factor for prostate cancer (Schwartz et al., 1990; Schwartz et al., 2000). Deficiency of vitamin D due to reduced synthesis of previtamin D_3_ is a potential common element among three major risk factors identified for prostate cancer. First, older men are generally exposed to less sunlight compared to their younger counterparts. Furthermore, the ability to synthesize previtamin D_3_ decreases with age. Second, the high melanin content of the skin of African-Americans reduces the efficiency of previtamin D_3_ synthesis. African-Americans have a higher incidence of prostate cancer than the Caucasian population. Last, the mortality rates of prostate cancer in the United States are inversely proportional to the geographically determined UV radiation exposure from the sun (Hanchette et al., 1992). This evidence supports the theory that vitamin D is involved in the pathogenesis of prostate cancer and could play an important roles in the treatment of this type of cancer (Schwartz et al., 1990; Pienta et al., 1993). Current research on the role of vitamin D in prostate cancer has included studies of vitamin D compound effects on inhibition of tumor cell growth, correlation between VDR polymorphism and prostate cancer incidence, development of treatment regimens with minimal hypercalcemic side effects, and development of vitamin D analogs as potential therapeutic and preventive agents in the treatment of prostate cancer.
PROSTATE AS A TARGET ORGAN FOR VITAMIN D

The presence of the VDR in human normal and malignant prostate cells suggested that the prostate gland is a target organ for vitamin D (Miller et al., 1992; Peehl et al., 1994). Studies in human prostate cancer cell lines have demonstrated that calcitriol and its analogs have anti-proliferative effects on prostate cancer cells (Miller et al., 1992; Schwartz et al., 1995; Muindi et al., 2007). Similar antiproliferative and pro-differentiating effects also have been reported in normal prostate cells (Peehl et al., 1994; Konety et al., 1996; Kunarkornsawat et al., 2002). These studies suggest that calcitriol can act on both normal and neoplastic prostate cells and encouraged development of clinical trials for vitamin D compounds in prostate cancer.

CANINE PROSTATE

The dog is the only known non-human species that spontaneously develops benign prostatic hyperplasia (BPH) with advancing age (Berry et al., 1986). Epidemiologic data indicates that the incidence of BPH approaches 100% of dog population by 7 to 8 years of age. Canine prostatic neoplasms generally are malignant, and benign forms have rarely been reported (Bell et al., 1991). Because of the late diagnosis and advanced status of canine prostatic neoplasia, treatment of this disease is generally unsuccessful with commonly used cytotoxic drugs. Studies in humans, in vitro, in vivo and in clinical trials have shown the antiproliferative effects of calcitriol on prostate cancer. In the canine prostate, our previous study demonstrated the antiproliferative effects of calcitriol and its analogs on canine epithelial and stromal cells.
(Kunakornsawat et al., 2002). These data suggest that the canine prostate is also a target for the action of calcitriol as in humans.

Arginine esterase (AE), the major seminal plasma protein in dogs, is a member of the kallikrein gene family (Chapdelaine et al., 1984). AE, at the amino acid level, has approximately 60% amino acid homology with human prostate specific antigen (PSA) (Dube et al., 1985). Their molecular weights are similar, 34 KDa for PSA and 29 KDa for AE. Both PSA and AE are major prostatic secretory proteins and their enzymatic activities toward their protein substrates are similar (Chapdelaine et al., 1984; Dube et al., 1985). AE, as well as PSA, are under androgenic control and AE is absent from the prostate of the castrated dogs (Chapdelaine et al., 1984; Dube et al., 1985). PSA has been used as a serum marker for the early detection of BPH and prostate cancer in men. Under normal circumstances, PSA is an excellent tumor marker that correlates well with prostate tumor growth (Osborn et al., 1995; Beer et al., 2002). Calcitriol has been shown in both clinical and in vivo studies to reduce serum PSA levels and the rate of increase in serum PSA levels (Gross et al., 1998; Beer et al., 2002).

Data from studies in human prostate and results from our previous study on primary cultures of canine prostate cells provide a strong rationale for the further study of the roles of calcitriol and/or its analogs on canine prostate. Evidence from human and canine studies strongly supports a role for vitamin D as a crucial regulator of cell growth and differentiation in the dog prostate. These effects may be important in the pathogenesis of BPH and prostate cancer.

In conclusion, the active form of vitamin D, calcitriol, and its analogs has been shown to produce growth suppressive effects in several models of human cancers.
Calcitriol is involved in the regulation of the growth, differentiation, and function of a wide range of different types of normal and malignant cells. The anti-tumor activities of calcitriol and its analogs might affect several different factors that inhibit tumor cell growth and development. These mechanisms are involved in multiple and diverse actions including: (i) cell cycle regulation: The induction of cyclin dependent kinase inhibitor p21 and p27 has been reported to be involved in the cell cycle regulation by calcitriol. However, the effects of vitamin D compounds on the cell cycle are cell-specific and may involve many cellular pathways. (ii) induction of apoptosis: The Bcl-2 family of proteins have been shown that their pathways involve in apoptosis induction. A down-regulation of the anti-apoptotic members of the Bcl-2 family, and an up-regulation of the pro-apoptotic members have been reported in response to vitamin D treatments. (iii) induction of cell differentiation, and (iv) anti-angiogenesis and inhibition of metastasis: The anti-angiogenesis effects of vitamin D compounds have been demonstrated through the inhibition of VEGF-induced endothelial cell sprouting and elongation both in vitro and in vivo. The purpose of our studies was to investigate the effects of calcitriol and its analogs on canine transitional cell carcinoma in vitro, in vivo, and on normal canine prostate tissue explants. These studies will allow a better understanding of the role of vitamin D compounds as agents to control tumor cell growth in dogs.
Figure 1.1 The chemical structure of calcitriol
Figure 1.2 The chemical structure of seocalcitol
Figure 1.3 The chemical structure of MCT
CHAPTER 2

EFFECTS OF CALCITRIOL, SEOCALCITOL, AND MEDIUM-CHAIN TRIGLYCERIDE ON A CANINE TRANSITIONAL CELL CARCINOMA CELL LINE.

2.1 INTRODUCTION

The hormone 1α, 25-dihydroxyvitamin D₃ (calcitriol), the metabolically active form of vitamin D₃, is known for its classic role in calcium regulation and bone metabolism (DeLuca, 1988). 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 (DeLuca, 1988; Haussler et al., 1998). 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 (Colson et al., 2000; Trump et al., 2000). Recently, it was demonstrated that calcitriol also has significant antiproliferative effects on human urinary bladder tumor cells in vitro and in vivo (Konety et al., 2001). We previously showed that calcitriol and its analogs inhibited the growth of primary canine prostate epithelial cells and a canine

In order to achieve the inhibitory effects of calcitriol on cell proliferation, supraphysiologic 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-1α, 25-dihydroxyvitamin D$_3$) has been reported to have much greater potency than calcitriol in inhibiting cell growth of various human cancers *in vitro, in vivo* and in clinical trials, with minimal effects on the blood calcium level (Haq *et al.*, 1993; Akhter *et al.*, 1997).

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 (Mutsaers *et al.*, 2003). 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 (Shapiro *et al.*, 1988; Chun *et al.*, 1996). Piroxicam, a nonsteroidal anti-inflammatory drug, is the most commonly used drug for the medical management of TCC in dogs (Knapp *et al.*, 1994). Although most dogs treated with piroxicam have clinical improvement (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, indicating roles of 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 (Hager et al., 2004; Valrance and Welsh, 2004) and induction of apoptosis (Blutt et al., 2000; Guzey et al., 2004). 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 (Hager et al., 2004). 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 (Rao et al., 2004). Bcl-2 is a member of a large family of proteins that regulate apoptosis induction in many cell lines including urinary bladder cancer (Schaaf et al., 2004; Wagner et al., 2003). Treatment of primary human prostate cancer stromal cells with calcitriol led to down-regulation of Bcl-2 expression in those cultures (Guzey et al., 2004). In addition, Bcl-2 over-expression prevented calcitriol-induced apoptosis of LNCaP cells (Blutt et al., 2000). 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 (Traul et al., 2000) and also is used in patients requiring parenteral nutritional supplementation (Adolph, 1999). 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 (Pourgholami et al., 2000). Moreover, MCT is retained in liver tumors when administered via the intra-hepatic arterial route to laboratory animals.
(Yanai et al., 1995). 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.

2.2 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 cell culture media to give final concentrations of $10^{-9}$ M, $10^{-7}$ M and $10^{-6}$ M. The media mixture then was 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 (Knapp et al., 1995). 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% CO₂. 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. Cells were treated for 48 and 72 hours unless otherwise stated. At the end of each experiment, cells were harvested and subjected to analyses accordingly. Each experiment was run in triplicate.

**Fluorescence DNA concentration analysis:** DNA was isolated from cells using 4M guanidine isothiocyanate containing 0.5% sodium lauryl sarcosine and 25mM sodium citrate (Kunakornsawat et al., 2001). 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 μg/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, 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. 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.
Sections were stained for localization of VDR according to our protocol (Kunakornsawat et al., 2001). Briefly, samples were incubated with 5% normal goat serum (NGS) in phosphate-buffer 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., Temecula, CA, USA) diluted 1:20 in PBS was added to the slides and incubated for 30 minutes at room temperature. Slides were developed by incubating in rat peroxidase-antiperoxidase (PAP) (Chemicon International Inc., Temecula, CA, USA) diluted 1:100 in 1% NGS in PBS for 30 minutes followed by 0.05% DAB & 0.01% hydrogen peroxide in 0.05 M Tris buffer for 5 minutes. 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, cells were harvested and cell lysates were prepared. Western blot analysis was performed as previously described (Kunakornsawat et al., 2001). The membranes were incubated at 4°C overnight in primary antibody, rabbit anti-human VDR monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) diluted 1:100, or mouse anti-human Bcl-2 monoclonal antibody (DakoCytomation California Inc., Carpinteria, CA, U.S.A.) 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, 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 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). 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.

### 2.3 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 2.1). However, the addition of 1% MCT to calcitriol showed a significant increase in inhibition of TCC cell growth at all concentration tested compared to their controls (P<0.01). As results, calcitriol with 1% MCT significantly enhanced the inhibitory effects of calcitriol at all concentration 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 2.2).

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 2.3). However, the addition of 1% MCT to seocalcitol was inhibitory at all concentration 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 2.4). 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 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 2.1).

**Cellular expression of the 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 2.5).

**VDR protein level:** Western blot analysis for VDR in normal canine urinary bladder urothelium and TCC cell line resulted in a band with a molecular weight of approximately 62 kDa (Figure 2.6). 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 2.7).
**Bcl-2 protein level:** Bcl-2 was detected in TCC cells as determined by Western blot analysis. Based on quantification of 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 2.8).

### 2.4 DISCUSSION

Transitional cell carcinoma (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 vitamin D receptor (VDR) (Colson *et al*., 2000; Trump *et al*., 2000). Recently, it was demonstrated that calcitriol also has significant antiproliferative effects on human urinary bladder tumor cells *in vitro* and *in vivo* (Konety *et al*., 2001). In order to achieve the inhibitory effects of calcitriol on cell proliferation *in vivo*, supraphysiologic 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. In this study, we explored the inhibition of TCC cell growth by calcitriol and its synthetic analog seocalcitol and effect of the addition of medium-chain triglycerides (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 that reported the presence of VDR in human TCC cells (Konety *et al*., 2001; Sahin *et al*., 2005) 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-mediated inhibition of cell growth of human TCC and other tumors (Konety et al., Hershberger et al., 1999). 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 $10^{-9}$ M to $10^{-7}$ M dose range (Kunakornsawat et al., 2001, Kunakornsawat et al., 2002). 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 (Mutsaers et al., 2003).

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 (Figure 2.1&2.3). 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 (Figures 2.2). However, 1% MCT addition only enhanced the growth inhibition of seocalcitol at $10^{-9}$M (Figures 2.4). These findings are consistent with those previously reported in HepG2 liver cells (Pourgholami et al., 2000) and suggest that calcitriol and seocalcitol dissolved in MCT were taken up and
accumulated in treated cells, 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 (Pourgholami et al., 2000). The fat soluble nature of calcitriol and seocalcitol make MCT an ideal delivery reagent while maintaining the antiproliferative activity of the compounds. Altogether, 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 (Hager et al., 2004; Caputo et al., 2003), which appears to be cell-specific (Wagner et al., 2003). Similarly, our results showed that calcitriol treatment resulted in the accumulation of cells in the G0/G1 phase and the reduction of cells in 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 (Rao et al., 2004). Studies with HL-60 cells have shown that seocalcitol induces G1 arrest by up-regulation of p21 and p27 expression (Seol et al., 2000). 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 (Rao et al., 2004; Benerjee et al., 2003). The mechanism of cell cycle arrest by calcitriol in TCC cells is not known; however, our findings demonstrate 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 (Konety et al., 2001; Blutt et al., 2000; Guzey et al., 2004). 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 (Guzey et al., 2004; Wagner et al., 2003). 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 (Diaz et al., 2000). 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 (Kunakornsawat et al., 2002); 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 (Valrance et al., 2004). Proposed mechanisms of the VDR-independent activity of vitamin D compounds include binding to alternative nuclear receptors, generation of toxic metabolites and byproducts, or directly interacting with components of cell signaling pathways. In this study, we conclude that the level of VDR expression is not affected by growth inhibitory mechanisms of calcitriol and seocalcitol in TCC cell line. 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 supraphysiologic 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, we can achieve the therapeutic potency of both compounds 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.
2.5 REFERENCES


Kunakornsawat S, Rosol TJ, Capen CC, Leroy B and Inpanbut N: Effects of 1α, 25(OH)$_2$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.


Figure 2.1 Inhibition of cell growth: Antiproliferative effects of calcitriol at 72 hours: Graph demonstrated proportion of DNA remaining relative to control at $10^{-9}$ M, $10^{-7}$ M and $10^{-6}$ M. 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). The solid horizontal line represents the proportion (1.00) for the control group.
Figure 2.2 Inhibition of cell growth: Antiproliferative effects of calcitriol compared to calcitriol plus 1% MCT at 72 hours. Graphs demonstrated 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). The solid horizontal line represents the proportion (1.00) for the control group.
Figure 2.3 Inhibition of cell growth: Antiproliferative effects of seocalcitol at 72 hours: Graph demonstrated proportion of DNA remaining relative to control at $10^{-9}$ M, $10^{-7}$ M and $10^{-6}$ M. 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). The solid horizontal line represents the proportion (1.00) for the control group.
Figure 2.4 **Inhibition of cell growth**: Antiproliferative effects of seocalcitol compared to seocalcitol plus 1% MCT at 72 hours. Graphs demonstrated 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 seocalcitol at $10^{-9}$ M (* P<0.05). The solid horizontal line represents the proportion (1.00) for the control group.
Figure 2.5 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 2.6 Western blot analysis for VDR: 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.
Figure 2.7 **Western blot analysis for VDR**: 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 2.8 Western blot analysis for Bcl-2: 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.
Table 2.1 **Cell cycle distributions**: 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 ± SD of triplicate samples. (** P<0.01; * P<0.05)

<table>
<thead>
<tr>
<th>Time</th>
<th>%G0/G1</th>
<th>%S-G2/M</th>
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<tr>
<td></td>
<td>control (ethanol)</td>
<td>$10^{-7}$ M calcitriol</td>
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<tr>
<td>24 hrs</td>
<td>24.92±2.06</td>
<td>29.81±2.34*</td>
</tr>
<tr>
<td>48 hrs</td>
<td>24.67±0.79</td>
<td>38.92±2.11**</td>
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CHAPTER 3

EFFECTS OF CALCITRIOL, SEOCALCITOL AND PIROXICAM ON TUMOR GROWTH IN A CANINE TRANSITIONAL CELL CARCINOMA XENOGRAFT MOUSE MODEL

3.1 INTRODUCTION

Transitional cell carcinoma (TCC) is the most common tumor of urinary bladder in dogs (Glickman et al., 2004). Unlike human TCC, where it is commonly diagnosed early as superficial cancer of the urinary bladder transitional epithelium (Knapp et al., 1994), the majority of canine TCCs are infiltrative tumors of intermediate or high grade malignancy. For this reason, common therapies used in people to treat superficial tumors are not successful for treating the large, exophytic, and invasive tumors that are seen in dogs. In dogs, surgical excision, even when followed by urinary tract reconstruction, generally is ineffective in controlling the primary tumor and is associated with high morbidity. Because of the limited surgical options and potential for metastases, cytotoxic chemotherapy has been the preferred form of treatment for TCC in dogs, as well as in human patients with advanced invasive bladder cancer (Helfand et al., 1994). The use of the platinum analogs, cisplatin and carboplatin (Chun et al., 1996) and to a lesser extent,
other drugs including mitoxantrone, actinomycin, doxorubicin, and gemcitabine has been reported in dogs with TCC (Helfand et al., 1994). Unfortunately, overall the response rates have been low, ranging from 0-20%.

Currently, piroxicam, a non-steroidal anti-inflammatory drug has arguably become the standard of care for the medical management of TCC in dogs (Knapp et al., 1994). Most dogs have an improvement in clinical signs when placed on piroxicam; however, the overall objective response rate is low, similar to more traditional cytotoxic therapies. A recent clinical trial in dogs with TCC combined piroxicam with mitoxantrone. This combination resulted in an overall response rate of 35%; however, only one dog had a complete response (Henry et al., 2003). Because of the lack of an effective therapy to treat bladder cancer in dogs, new therapeutic strategies need to be pursued. To this end, we developed a novel canine TCC - mouse xenograft model for the in vivo preclinical study of new systemic treatments for canine TCC.

Vitamin D is best known for its role in maintaining calcium homeostasis and regulating bone metabolism (DeLuca, 1988). The active form of vitamin D, 1, 25(OH)₂D₃ (calcitriol), acts by binding to the vitamin D receptor (VDR), a ligand-activated transcription factor (DeLuca, 1988; Haussler et al., 1998). More recent evidence shows that calcitriol and its synthetic analogs have significant antiproliferative effects on various human cancers, including bladder cancer, in vitro, and in vivo (Fujoika et al., 1998; Barreto et al., 2000; Konety et al., 2001). The antiproliferative effects of calcitriol are mediated by an increase in cell cycle arrest at the G0/G1 phase, induction of apoptosis and differentiation, and by modulation of growth factor receptors and cell signaling pathways (Sheikh et al., 1995; Muindi et al., 2004). These findings have led to
several clinical trials in humans for the treatment of cancers with calcitriol (Schwartz et al., 2000; Osborn et al., 1995). Calcitriol has been shown to inhibit proliferation and induce apoptosis in human transitional cell carcinoma in vitro (Konety et al., 2001). In addition, their in vivo study using a carcinogen-induced model of bladder cancer in rats demonstrated that intravesicular administration of calcitriol resulted in fewer tumors and reduced the tumor invasiveness (Konety et al., 2001).

In order to achieve the inhibitory effects of calcitriol on cell proliferation, supraphysiologic doses and prolonged treatment with the active vitamin D metabolite are required. However, calcitriol has the potential to induce hypercalcemic side effects, which limits its use in cancer patients. In the past decades, many vitamin D analogs have been synthesized to retain the potent antiproliferative activity without the undesired hypercalcemic side effects. Seocalcitol, or analog EB1089 (22, 24-diene-24a,26a,27a-trihomo-1α, 25-dihydroxyvitamin D₃), is one of these analogs that has been developed and reported to have 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 concentration (Sheikh et al., 1995; Schartz et al., 2000).

Our laboratory was the first to report the inhibitory effects of calcitriol and its analogs on the cell growth of normal and cancer cells of canine origin both in vitro and in vivo (Kunakornsawat et al., 2001; Kunakornsawat et al., 2002; Kunakornsawat et al., 2004; Kaewsakhorn et al., 2005). Previously, we reported that calcitriol, seocalcitol and analog V (1,25-dihydroxy-16ene-23-yne-vitamin D₃) inhibited cell growth and differentiation in the canine squamous cell carcinoma cell line (SCC 2/88) in a dose-dependent manner (Kunakornsawat et al., 2001). Studies in nude mice bearing the
canine adenocarcinoma (CAC-8) demonstrated that calcitriol, seocalcitol, and analog V reduced tumor volume in treated mice compared to vehicle-treated controls (Kunakornsawat et al., 2002). Moreover, our recent in vitro study demonstrated that calcitriol and seocalcitol, significantly inhibited cell growth and calcitriol caused cell cycle arrest at G0/G1 phase in a canine TCC cell line (Kaewsakhorn et al., 2005). Based on the observations that calcitriol and seocalcitol inhibit tumor cell growth in human cancers, including bladder cancer; calcitriol and seocalcitol inhibit cell growth of cancer cells of canine origin, including canine TCC, in vitro; and the poor tumor response rate to piroxicam treatment in canine patients with TCC, we developed a canine TCC-mouse xenograft model to evaluate the effectiveness of calcitriol, seocalcitol, and piroxicam in vivo. The first objective of this study was to develop a canine TCC - mouse xenograft model using the IVIS™ imaging system to study the study effects of new treatments on canine TCC tumor progression. Our second objective was to determine whether calcitriol and seocalcitol will reduce tumor size in a canine TCC - mouse xenograft model and whether their effects are better than those achieved using piroxicam.

3.2 MATERIALS AND METHODS

Reagents: Calcitriol was purchased from Biomol International, L.P (Plymouth Meeting, PA, USA). Seocalcitol was a generous gift from Dr. L. Binderup (LEO Pharma, Ballerup, Denmark). Piroxicam was purchased from Sigma-Aldrich (St. Louis, MO, USA).
**Animals**: Four-week-old female nude mice were purchased from Harlan Life Sciences (Indianapolis, IN, USA). The mice were fed sterilized rodent chow and housed under barrier conditions for one week prior to tumor induction.

**Cell culture**: 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% CO₂. The medium was changed every two to three days. When cells became confluent, they were washed and trypsinized with TrypLE™ (GIBCO BRL., Grand Island, NY, USA) for 10 minutes at 37°C. Trypsinized cells were centrifuged, resuspended in DMEM complete medium and replated. Cultures were passaged every five to seven days. Analysis for mycoplasma contamination was performed using MycoAlert® Mycoplasma Detection Assay (Cambrex, Baltimore, MD, USA).

**Cell transfection**: Canine TCC cells were transfected with a plasmid vector encoding a single reading frame of luc-YFP reporters driven by a hCMV promoter (W. C. Kisseberth, personal communication). Luc-YFP plasmid carries the prokaryotic selection marker, ampicillin, and a mammalian selection marker, geneticin (G418), for the selection of stable cells. The liposome formulation Lipofectamine (Invitrogen, San Diago, CA, USA) was used as a transfection reagent according to the manufacturer’s protocol. Stably transfected cells were selected, cloned in medium containing G418 (Invitrogen) and high luc-YFP expressing clones were used in in vivo studies.

**Induction of canine TCC tumors in mice**: Induction of canine TCC tumors was made in athymic nude mice. Mice were injected subcutaneously between the scapulae with luc-YFP -TCC cells (1x10⁶ cells) in DMEM medium.
Bioluminescence imaging of tumor xenograft: Bioluminescence imaging was performed once a week using an IVIS™ bioluminescence imaging system (Xenogen Corp., Alameda, CA, USA) linked to a PC running Living Image™ software (Xenogen Corp., Alameda, CA, USA). Immediately prior to imaging, animals were anesthetized with 2% isoflurane mixed with air and injected intraperitoneally with 30 mg/ml of D-luciferin (Xenogen Corp., Alameda, CA, USA) in DPBS at a dosage of 150 mg/kg body weight. During image acquisition, isoflurane anesthesia was maintained using a nose cone delivery system and animal body temperature was regulated using a digital thermostat-controlled bed integrated within the IVIS™ imaging system. An integration time of 1-20 seconds was used for luminescent image acquisition. The signal intensity was quantified as the sum of all detected photon counts within a region of interest prescribed over the mouse scapular area using the Living Image™ software package. A luciferin kinetic study was performed to determine peak signal time after luciferin administration over 40 minutes at the interval of 5 minutes. Results from luciferin kinetic study were used to estimate optimal imaging time after luciferin injection (15-20 minutes).

Treatment: Three weeks after tumor induction, mice were randomly assigned to four groups (Groups I – IV). Baseline IVIS™ signal intensity and vernier caliper measurements were recorded immediately before starting treatments. Mice were treated three times per week as follows: Group I (n=11), vehicle (vegetable oil) (0.1ml/mouse, i.p); Group II (n=16), calcitriol (0.5 ug/mouse, i.p); Group III (n=8), seocalcitol (0.1 ug/mouse, i.p); and Group IV (n=8), piroxicam (25mg/kg, oral gavage daily). Dosages of calcitriol, seocalcitol and piroxicam were established based on our previous studies and
published data (15). Body weight, IVIS™ signal intensity, and tumor volumes were
determined weekly at the time of IVIS™ imaging. Tumor volume was measured by a
vernier caliper and calculated using formula: \( \frac{1}{6} (\text{length} \times \text{width} \times \text{height}) \). Animals were
observed daily for any signs of toxicity, including hypercalcemia (e.g. lethargy, anorexia, weight loss). After 6 weeks of treatments, all mice were humanely euthanized
by CO\(_2\) asphyxiation according to the Institutional Laboratory Animal Care and Use
Committee (ILACUC) protocol of The Ohio State University.

**Blood ionized calcium:** Blood samples were collected by facial vein puncture
into heparinized microtubes at 0, 3, 6 weeks of treatment and at euthanasia. Blood
ionized calcium was determined using a calcium selective electrode (NOVA 8 Calcium
Analyzer, NOVA Biomedical, MA, USA.).

**Histopathology:** Mice were sacrificed after 6 weeks of treatments. Complete
necropsies were performed on all mice. After the grossly visible tumor tissue was
excised, bioluminescence imaging was performed on the mouse carcass in order to
detect bioluminescence of any metastases that may be hidden by the intense
bioluminescence of the primary tumor. Tumor tissues were then fixed in neutral
buffered formalin, embedded and processed for histologic and immunohistochemical
evaluations.

**Apoptosis:** Apoptosis was assessed on tissue sections using the terminal
deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) method using the
ApopTag peroxidase *in situ* apoptosis detection kit (Intergen Co., NY, USA) according
to the manufacturer’s protocol. TUNEL-positive cells were quantified in 5 randomly
selected high-power fields (x400) of each tissue sections.
**Cell proliferation:** Cell proliferation was measured on tissue sections using Ki-67 clone MIB-I (DAKO Corp., Carpintera, CA, USA) immunohistochemistry. Briefly, after deparaffinized through xylene and graded alcohols, tissue sections were heated in Dako Target Retrieval Solution (DAKO Corp., Carpintera, CA, USA) in a steamer at 95°C for 20 minutes. Endogenous peroxidase activity was blocked by immersing the slides in 3% H₂O₂ in methanol. Staining was completed by incubating the sections in Serum Free Protein Block Solution (DAKO Corp., Carpintera, CA, USA) for 20 minutes; primary antibody, mouse monoclonal anti-Ki-67 at 4°C overnight; and secondary antibody, biotinylated horse anti-mouse IgG for 30 minutes at room temperature (Vector Laboratories, Berlingame, CA, USA). The slides were then incubated with avidin-biotin-peroxidase complex for 30 minutes (VECTASTAIN® Elite ABC, Vector Laboratories, Berlingame, CA, USA). The color was developed by diaminobenzidine (DAB) (DAKO Corp., Carpintera, CA, USA) and counterstained with haematoxylin. Positive control slides consisted of sections from canine lymph nodes. Negative control slides were prepared by using universal negative control for mouse primary antibodies (DAKO Corp., Carpintera, CA, USA). The number of immunoreactive cells was quantitated using a computer-based image analyzer (Image-Pro Plus Software, Media Cybernetics, Inc., MO, USA).

**Statistical analysis:** The results were expressed as the mean ± standard error of the mean (S.E.M) and were analyzed by one-way analysis of variance using an ANOVA and Turkey’s multiple comparison test using GraphPad InStat version 3.06 for Windows® XP (GraphPad Software, San Diego, CA, USA).
3.3 RESULTS

**Selection of canine TCC cell line for the *in vivo* study:** We decided to use the TCC-2 cell line in the *in vivo* study based on its ability to rapidly produce tumors in nude mice in preliminary studies (data not shown). TCC-2 cells were transfected with luc-YFP reporters as described in Materials and Methods and stably transfected cells were used for tumor induction in nude mice (Figure 3.1A).

**Tumor induction:** Tumors developed at the site of injection in 43/43 (100%) of the mice injected. One week after subcutaneous implantation, the tumor was detectable by the IVIS™ imaging system but was not apparent by macroscopic evaluation (Figure 3.1B).

**Histopathology:** Microscopic examination of the sections from the tumor tissues showed a well differentiated transitional cell carcinoma in all groups (Group I-IV). No histopathologic differences could be observed in tumors from the control- and treated-groups. All of the tumors had large areas of necrosis surrounded by solid sheet of malignant cells (Figure 3.2). No metastases were observed either by histopathology or bioluminescence.

**Tumor volume:** Seocalcitol slightly reduced tumor volume in canine TCC-bearing nude mice compared to control (Figure 3.3) when measured by a vernier caliper. There were no differences in tumor volume in canine TCC-bearing mice treated with calcitriol or piroxicam compared to the control group. When evaluated using bioluminescence imaging, there was wide variability of tumor growth in all mice and the bioluminescence data were not conclusive.
**Cell proliferation:** Immunohistochemical staining of canine TCC mouse xenograft tumor tissue with a Ki-67-antibody produced a peroxidase reaction in the nuclei of positive cells. The number of immunoreactive cells was quantitated using a computer-based image analyzer (Image-Pro Plus Software, Media Cybernetics, Inc., MO, USA). The Ki-67-positive cell count was minimally decreased in all treatment groups compared to the control group. (Control = 231.3±14.6, Calcitriol = 214.9±10.9, Seocalcitol = 207.0±16.0, Piroxicam = 218.4±11.3); however, there was no statistically significant different in the number of Ki-67-positive cells between tumor tissue from control and treated mice (Figure 3.4).

**Apoptosis:** TUNEL assay of canine TCC mouse xenograft tumor tissue was performed to determine the apoptotic effects induced by the treatments with calcitriol, seocalcitol and piroxicam *in vivo*. Tumor tissues from all treatment groups showed slightly increased in number of apoptotic cells staining positively for TUNEL compared to control group (Control = 71.2 %, Calcitriol = 80.12%, Seocalcitol = 79.61%, Piroxicam = 86.24%).

**Body weight:** There was a significant decrease in body weight in mice treated with calcitriol (p<0.05) or seocalcitol (p<0.001) compared to controls. The piroxicam-treated mice maintained their body weight better than mice treated with either calcitriol or seocalcitol (Figure 3.5).

**Blood ionized calcium:** The blood ionized calcium concentration was higher in all mice treated with calcitriol or seocalcitol as compared to mice in the control group (p<0.001 for both). The blood ionized calcium concentration in vehicle-, or piroxicam-
treated-mice was in the normal range (whole blood ionized calcium in normal athymic nude mice is no more than 5.12 mg/dl) (Figure 3.6).

### 3.4 DISCUSSION

Recent evidence shows that calcitriol and its synthetic analogs have inhibit cell growth and promote differentiation in many different normal and malignant cell types of human origin (Barreto et al., 2000; Konety et al., 2001; Fujoika et al., 1998). Consistent with the findings in human cells, our laboratory has demonstrated the inhibitory effects of calcitriol and its analogs on cell growth of normal and cancer cells of canine origin, both *in vitro* and *in vivo* (Kunakornsawat et al., 2001; Kunakornsawat et al., 2002; Kunakornsawat et al., 2004; Kaewsakhorn et al., 2005). We also have reported that calcitriol and seocalcitol inhibited cell proliferation in a canine transitional cell carcinoma cell line *in vitro* (Kaewsakhorn et al., 2005). However, it required $10^{-6}$ M of calcitriol to demonstrated the anti-proliferative effects on the canine TCC cell line, compared to $10^{-7}$ M for other cell types such as canine SCC and prostate epithelial cell lines (Kunakornsawat et al., 2001, Kunakornsawat et al., 2002). This study demonstrated that seocalcitol at $10^{-7}$ M significantly inhibited the TCC cell line, but a higher concentration of calcitriol was requied to produce the same result. Based on data from our and other studies, we conducted a preliminary study before conducting this experiment using calcitriol at 0.05 µg/mouse and seocalcitol at 0.01 µg/mouse three times a week, to determine the general growth characteristics of the xenograft and potential toxicity of this dosage regimen. Both calcitriol and seocalcitol at these dosages
did not exhibit an inhibitory effect on tumor growth in TCC xenograft mice; however, the treatment was well tolerated (data not shown). Therefore, in this study we escalated the dosage of calcitriol to 0.5 µg/mouse and seocalcitol to 0.1 µg/mouse three times a week.

Our results demonstrated that only seocalcitol at 0.1 µg/mouse reduced tumor volume in canine TCC-bearing mice, but not calcitriol at 0.5 µg/mouse. The doses of calcitriol and seocalcitol used in this study were higher than those successfully used in most other studies treating tumors of human origin (Konety et al., 2001; VanWeelden et al., 1998). This supports the suggestion by others that the inhibitory effects of calcitriol and its analogs on tumor growth are species-specific. Our previous study using a canine adenocarcinoma mouse xenograft model demonstrated that seocalcitol and calcitriol at a ten times lower dose than used in this study, reduced tumor volume compared to controls (Kunakornsawat et al., 2002). The inhibitory effects of calcitriol and its analogs also may be tissue-specific and have a lesser effect on canine TCC. The resistance of canine TCC cell line to calcitriol in our in vitro study is consistent with findings in the in vivo xenograft mouse model. Although the basis for resistance of this canine TCC cell line to calcitriol and its analogs is unclear, these data indicate that it takes higher doses of these compounds to achieve effective growth inhibitory effects with this type of malignant neoplasm.

These findings have potential therapeutic implications for the design of treatment regimens for dogs with TCC. The poor responses to treatment seen in canine TCC patients may be due in part to the relatively higher resistance of cells of this tumor type to currently used drugs, similar to the relative resistance of TCC to calcitriol and its
analogs compared to other tumor types. Previous studies reported that canine TCC has very poor response to chemotherapy. The use of drugs such as cisplatin, carboplatin or mitoxantrone to treat TCC in the canine patients showed limited response. The few dogs that responded to the treatment had only partial remission of the tumor treated with these drugs. These findings support our hypothesis that the limited respond of canine TCC in a mouse model may be due to the resistance of these tumor cells to a variety of drugs including calcitriol and seocalcitol.

The most effective treatment regimen for canine bladder cancer is the combined use of piroxicam and mitoxantrone, which had a reported overall response rate of 35% (Henry et al., 2003). Results from our study indicated that piroxicam failed to slow tumor growth in canine TCC-bearing mice at 25mg/kg administered by oral gavage daily. The dose of piroxicam used in this study was higher than the dose that significantly delayed the progression of tumor growth in human TCC-bearing nude mice (approximately 0.8 mg/kg daily assuming that a 25 gm-mouse drinks 4 ml 0.5% piroxicam in drinking water/day) (Mohammad et al., 2006). These findings suggest that canine TCC also is resistant to piroxicam compared to human TCC.

A combination of calcitriol and non-steroidal anti-inflammatory drugs (NSAIDS) has been demonstrated to exhibit synergistic inhibition of cell growth in prostate cancer (Krishnan et al., 2007). In this study, the investigators demonstrated that calcitriol significantly suppressed mRNA and protein expression of the prostaglandin endoperoxide synthase/cyclooxygenase-2 (COX-2); up-regulated 15-prostaglandin dehydrogenase, an enzyme involves in initiating prostaglandin catabolism; and significantly increased prostate cancer cell growth inhibition when combined with
NSAIDS (known inhibitors of COX-1 and COX-2). The combination of calcitriol and NSAIDS including piroxicam might provide a useful therapeutic approach to TCC that would allow both drugs to be used at reduced dosages, leading to increased safety, and hopefully increased efficacy. In addition, this might minimize the occurrence of the hypercalcemic effects of calcitriol. Because seocalcitol is an analog of calcitriol, combining seocalcitol and NSAIDS is a logical combination treatment strategy worthy of future investigation.

Both calcitriol- and seocalcitol treatment significantly increased the blood ionized calcium concentration in TCC xenograft mice. Seocalcitol is a synthetic analog that was synthesized to maintain the antiproliferative properties of calcitriol while minimizing the hypercalcemic effects. Our results demonstrate that the escalated dose of seocalcitol did not maintain normal blood ionized calcium concentrations in TCC xenograft mice. Our previous study also reported hypercalcemia in CAC-8 bearing mice, even though the mice were treated at a lower dose of seocalcitol and calcitriol than our present study (0.01 µg/mouse, and 0.05 µg/mouse respectively). A study of daily treatments of seocalcitol in rats with nitrosomethyl urea-induced mammary tumors reported a reduction of tumor growth in rats with significantly higher serum calcium concentrations than rats in the control group. In the same study, rats treated with intermittent dosing using the same total dose of seocalcitol, had a reduction in tumor growth while maintaining a normal serum calcium concentration (Colston et al., 2003).

A study by Van Weelden et al. reported that administering seocalcitol at the same dose (60 pmol/day), but by different routes of administration, can reduce the hypercalcemic effects, but still inhibit tumor growth in xenografted MCF-7 cells (Van
Weelden et al., 1998). Mice receiving sustained release pellets of seocalcitol maintained normal blood calcium level compared to the higher calcium concentrations in the subcutaneous administration group. In addition, instead of the regular diet we used in this study, feeding mice a low calcium diet while on treatment should reduce the chance of developing hypercalcemia. Changing the dosage regimen and feeding animals a low calcium diet in future studies may demonstrate greater inhibitory effects of calcitriol and seocalcitol on canine transitional cell carcinoma with minimal hypercalcemic effects.

The measurement of bioluminescence using the IVIS imaging system has been reported as a useful technique allowing non-invasive evaluation of local (primary) tumor growth, progression, and metastasis in the living animal (Edinger et al., 1999; Edinger et al., 2003). With the sensitivity of this technique, tumor growth can be accurately detected from day one after tumor induction and used to follow the tumor progression. Our IVIS bioluminescence imaging system data showed variability after tumor size reach a detectable level at four weeks after tumor induction (data not shown). At that time, tumor volume can be reproducibly measured using vernier calipers. Consistent with our findings, a study of bone metastasis in a human pulmonary squamous cell carcinoma mouse xenograft model reported wide variability of tumor growth as indicated by the bioluminescence data (Tannehill-Gregg et al., 2006). They concluded that standard histomorphometry was more accurate than bioluminescence data in quantification of tumor burden in bone.

In order to obtain a bioluminescence signal, luc-YFP-TCC cells must actively and reproducibly express the luciferase gene. Since tumor cells must use ATP to emit bioluminescence light from luciferin, using the luciferase enzyme produced by the
plasmid construct, only viable tumor cells can emit a bioluminescence photon signal. In most cases, the larger the tumor, the more areas of necrosis that will be present. Therefore, the variable data from IVIS imaging measurements from larger tumors in our study with canine TCC may be due, in part, to the loss of expression of the luciferase gene in necrotic tumor cells. The variability of the extent of the necrotic areas from one mouse to another in all groups was supported by histopathologic examination. The advantages of using the IVIS imaging system in studying tumor growth are: 1) the ability to detect the tumor progression at very early stages, 2) detecting tumor growth in body cavities and other relatively inaccessible locations such as bone, and 3) for detecting and quantifying metastases in the living animal over time. In our experiments, the combination of using the IVIS imaging system and vernier calipers to evaluate tumor growth provided the means for a thorough evaluation of tumor progression both at the earlier and later stages of tumor development.

In summary, we established a canine TCC- xenograft mouse model to evaluate and compare the effects of calcitriol, seocalcitol and piroxicam. This model provides a tool for future studies to evaluate the efficacy of therapies for canine TCC. The results from this study showed that seocalcitol reduced tumor volume in canine TCC-bearing nude mice while calcitriol and piroxicam had no inhibition of tumor growth compared to controls. The superior inhibitory effect of seocalcitol on tumor growth was supported by data from quantitative evaluation of Ki-67 staining. Blood iCa levels were higher in both calcitriol- and seocalcitol-treated group compared to controls. This is the first description of a canine TCC-mouse xenograft model for the study of canine TCC tumor progression and treatment.
These findings suggest a potential for the therapeutic use of calcitriol analogs in the treatment of canine TCC. Given the common use of piroxicam to treat clinical canine patients with TCC, investigation of the combined effects of piroxicam and calcitriol analogs, such as seocalcitol, are warranted. Furthermore, optimal dosage regimens need to be investigated further. Recent studies suggest that intermittent pulse-dosing may be effective in minimizing the hypercalcemic effects while maintaining an antiproliferative effect of the vitamin D metabolites. These results suggest that piroxicam combined with a vitamin D analog (like seocalcitol) may be more effective than piroxicam alone in the treatment of canine TCC, especially if fed a low calcium diet to minimize effects on the blood calcium.
3.5 REFERENCES


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Figure 3.1 The transfection of the luc-YFP in canine TCC cells, tumor induction in mice: (A) and bioluminescence imaging of the stable luc-YFP transfected TCC cells in tumor bearing-mice (B). Data is expressed as photon emission per second and the color scale represents luminescent signal intensity, with blue indicating the least intense and red the most intense light originating from transfected cells.
Figure 3.2 **Histopathology:** Histologic morphology of canine TCC mouse xenograft. A well-differentiated transitional cell carcinoma. H&E X200
Figure 3.3 Tumor volumes: Effects of calcitriol, seocalcitol and piroxicam on the growth of canine TCC tumor xenograft measured by a vernier caliper. Data are presented as percent change from week 0. Each time point represents the mean value ± standard error of the mean (S.E.M).
Figure 3.4 **Cell proliferation**: Ki-67 (clone MIB-I) immunohistochemistry staining of canine TCC mouse xenograft illustrating positive labeling of nuclei in tumor cells. The inset is a negative (no primary antibody) control. X200
Figure 3.5 **Body weight**: Percent change in body weight from week 0 in mice treated with vehicle, calcitriol, seocalcitol and piroxicam. Each time point represents the mean value ± standard error of the mean (S.E.M). Significant differences in mice body weight from control mice treated with vehicle are indicated by asterisks (* p<0.05, ** p<0.01, *** p<0.001).
Figure 3.6 **Blood ionized calcium**: Blood ionized calcium levels for the treatment of canine TCC-bearing mice with vehicle, calcitriol, seocalcitol and piroxicam. Each time point represents the mean value ± standard error of the mean (S.E.M). Significant differences from control mice treated with vehicle are indicated by asterisks (*** p<0.001). Whole blood ionized calcium in normal nude mice is no more than 5.12 mg/dl.
CHAPTER 4

EFFECTS OF CALCITRIOL AND DIHYDROTESTOSTERONE (DHT) ON ARGinine ESTERase (AE) ACTIVITY AND VITAMIN D RECEPTOR (VDR) EXPRESSION IN EXPLANTS OF CANINE PROSTATE TISSUE

4.1 INTRODUCTION

Low plasma levels of calcitriol have been correlated to increased risk of human prostate cancer (Lokeshwar et al., 1999; Schwartz et al., 2000). It has been reported that calcitriol and its synthetic analogs exert anti-tumor effects on various types of human cancer in vitro, in vivo and in clinical trials. Moreover, calcitriol has been shown to suppress prostate cancer growth in human prostate cancer cell lines and in clinical trials. Several studies have demonstrated that calcitriol regulated the growth and differentiation in human prostate cancer cells through the mediation of the vitamin D receptor (VDR) (DeLuca, 1988). Data from our laboratory showed that calcitriol and its analogs inhibit proliferation and increase VDR mRNA expression in epithelial cells in primary canine prostate culture (Kunakornsawat et al., 2002).

The dog is the only known animal species that spontaneously develops benign prostatic hyperplasia (BPH) with advancing age (Berry et al., 1986). Epidemiology data
have indicated that the incidence of BPH approaches 100% of the dog population by 7 to 8 years of age. Canine prostatic neoplasms are generally malignant, and benign forms have rarely been reported (Bell et al., 1991). Because of the late diagnosis and advanced status of canine prostatic neoplasia, treatment of this disease is generally unsuccessful. There are no antitumor drugs that are effective treatments of canine prostatic carcinoma. Studies in humans, in vitro, in vivo and clinical trials have shown the antiproliferative effects of calcitriol on prostate cancer. In the canine prostate, our previous study also demonstrated the antiproliferative effects of calcitriol and its analogs on canine prostate epithelial cells (Kunakornsawat et al., 2002). This suggests that, as in human, the canine prostate is also a target for the action of calcitriol. Therefore, the study of effects of calcitriol on normal canine prostate is valuable for further evaluation of its mechanistic roles in the pathogenesis and prevention of canine BPH.

Arginine esterase (AE), the major seminal plasma protein in dogs, is a member of the kallikrein gene family (Chapdelaine et al., 1984). AE, at the amino acid level, has approximately 60% amino acid homology with human prostate specific antigen (PSA) (Dube et al., 1985). Their molecular weights are similar, 34 KDa for PSA and 29 KDa for AE. Both PSA and AE are major prostatic secretory proteins and their enzymatic activities toward protein substrates are similar (Chapdelaine et al., 1984; Dube et al., 1985). AE, as well as PSA, are under androgenic control and AE is absent from the prostate of the castrated dogs (Chapdelaine et al., 1984; Dube et al., 1985).

PSA has been used as a serum marker for the early detection of prostate cancer. Under usual circumstances, PSA is an excellent tumor marker that correlates with prostate tumor growth (Osborn et al., 1995; Beer et al., 2002). Calcitriol has been
demonstrated in both clinical and in vivo studies to reduce serum PSA levels and reduce the rate of increase in serum PSA levels (Gross et al., 1998; Beer et al., 2002). Since calcitriol can decrease PSA levels in human prostate cancer patients, and data from our laboratory has shown that calcitriol and its analogs inhibit epithelial cell proliferation and increase a VDR mRNA expression in the primary canine prostate cultures (Kunakornsawat et al., 2002); we hypothesized that calcitriol will decrease AE enzymatic activity and increase VDR expression in cultured canine prostate tissue explants. The overall objective was to develop an animal model of prostate pathobiology by using cultured canine prostate tissue explants.

4.2 MATERIALS AND METHODS

Vitamin D: Calcitriol was a generous gift from Dr. L. Binderup (LEO Pharma, Ballerup, Denmark). DHT was purchased from Sigma-Aldrich Corp., St. Louis, MO.

Tissue procurement: Canine prostate glands were collected from healthy adult male dogs (approximately 2-4 years of ages) immediately after euthanasia. The glands from younger or older dogs were also collected and processed for histological and immunohistological study for comparison purpose. The prostate glands (n=12) were brought to the laboratory in ice cold RPMI-1640 media under sterile conditions. The glands were weighed, then washed with 70% ethanol and dipped in the media to eliminate excess ethanol. The connective tissue capsule and the prostatic urethra were removed. Small pieces of tissue were fixed in formalin and saved for a freeze substitution technique for histological and immunohistochemical analysis. Tissue was
cut into approximately 1 mm³ pieces and placed on a surgical grade polyethylene mesh with 2 ml of RPMI-1640 media in 6-well plates (~15 pieces per well) and incubated at 37°C in 95% air and 5% CO₂ for 24 hours. Prostate explants (n=2) were treated with DHT at either 10⁻⁹ M or 10⁻⁷ M or vehicle (ethanol) in RPMI-1640 media for 48 hours. Prostate tissue explants from the rest of the dogs (n=10) were treated with calcitriol at 10⁻⁷ M or vehicle (ethanol) in RPMI-1640 media for 24 and 48 hours. The final concentration of ethanol was less than 0.1% of media. At the end of each experiment, tissues in each group were pooled and rapidly frozen in liquid nitrogen for the AE enzymatic activity assay and Western blot analysis.

**Immunohistochemistry:** Tissues were fixed using a freeze substitution technique (0.05% glutaraldehyde in ethanol) for 2 weeks (Kunakornsawat et al., 2001) and processed conventionally. Sections were stained for localization of VDR according to our protocol (Kaewsakhorn et al., 2005). 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 the application of the primary antibody, rat anti-chicken VDR monoclonal antibody (Chemicon International Inc., Temecula, CA) 1:50 in PBS at 4°C overnight. After washing, the secondary antibody, goat anti-rat IgG (Chemicon International Inc., Temecula, CA) diluted 1:20 in PBS was added to the slides and incubated for 30 minutes at room temperature. Slides were developed by incubating them in rat peroxidase-antiperoxidase (PAP) (Chemicon International Inc., Temecula, CA) diluted 1:100 in 1% NGS in PBS for 30 minutes followed by 0.05% 3, 3′-diaminobenzidine (DAB) with 0.01% hydrogen peroxide in 0.05 M Tris buffer for 5 minutes. Slides were mounted in aqua mount® and visualized by light microscopy. The
number of immuno reactive cells was quantitated using a computer-based image analyzer (Image-Pro Plus Software, Media Cybernetics Inc., MO).

**Western blot analysis:** At the end of the experiment, tissues in each group were pooled and a tissue homogenate was prepared. Western blot analysis was performed as previously described (Kaewsakhorn et al., 2005). The membranes were incubated at 4°C overnight in primary antibody, rabbit anti-human VDR monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:300, followed by washing with deionized water for 15 minutes. The blots were incubated in goat anti-rabbit IgG-HRP diluted 1:2000 for 2 hours. Protein bands were detected using LumiGLO Chemiluminescent substrate (Kirkegaard & Perry laboratories, Gaithersburg, MD) and exposed to X-ray film for 5-10 seconds. After exposure, the blots were stripped and incubated with rabbit polyclonal anti-histone H3 antibody (Abcam Inc., Cambridge, MA) to normalize protein loading. The Gel-Pro® Analyzer (Media Cybernetics, San Diego, CA) was used to measure protein band density.

**AE Enzymatic Activity Assay:** One gram of frozen prostate tissue was homogenized on ice (3-5 cycles, 15-20 seconds per cycle) with a tissue homogenizer (Biospec Products, Bartlesville, OK) in 5 ml cold PBS containing protease inhibitors (Roche Diagnostics Corp., Indianapolis, IN) and 0.01% Triton X-100 (Sigma-Aldrich Corp., St. Louis, MO). Homogenates were centrifuged at 20,000 x g for 45 minutes at 4°C, and the supernatants were aliquotted and stored at -80°C until assayed. Total protein concentration of the supernatants was measured with the BCA protein assay. Arginine esterase enzymatic activity of the tissues was determined by the cleavage of a 1mM solution of benzoyl arginine ethyl ester (BAEE) (Sigma-Aldrich Corp., St. Louis,
MO) in 10 mM Tris-HCl, pH 8.0, at 25°C. Cleavage of BAEE was determined by measuring the change in absorbance at 253 nm (OD$_{253}$) with a spectrophotometer (Perkin-Elmer, Fremont, CA). A positive control (fresh canine prostate homogenate) and a negative control (canine muscle homogenate) were included in each assay (Leroy et al., 2004).

4.3 RESULTS

**Vitamin D receptor expression:** Immunohistochemical analysis of canine prostate tissue demonstrated that positive peroxidase reaction of VDR mostly appears in the nuclei of epithelial cells and in a lesser amount in the stromal cells (Figure 4.1). The prostate glands collected for this study were categorized into 3 groups based on histologic morphology and number of immuno-reactive cells evaluated by a computer-based image analyzer (Image-Pro Plus Software, Media Cybernetics, Inc., MO) (Table 4.1).

**VDR protein level by Western blot analysis:** Western blot analysis for VDR in normal canine prostate tissue resulted in a protein band at a molecular weight of approximately 56 kDa. Results based on numerical data using the Gel-Pro® Analyzer (Media Cybernetics, San Diego, CA) to measure density of protein bands. DHT increased VDR protein expression compared to the ethanol-treated control groups as follows: At 24 hours treatment (control = 158.64, DHT $10^{-9}$ M = 212.00, and DHT at $10^{-7}$ M = 196.13); at 48 hours after treatment (control = 145.04, DHT $10^{-9}$ M = 210.29, and DHT at $10^{-7}$ M = 239.11) (Figure 4.2A). However, calcitriol at $10^{-7}$ M slightly decreased
VDR protein expression at both 24 and 48 hours as follows: at 24 hours, control = 356.05, calcitriol = 217.67; at 48 hours, control = 218.02, calcitriol = 201.5. Interestingly, VDR protein expression in the control group decreased after the treatment at 48 hours compared to 24 hours (Figure 4.2B).

**AE Enzyme Activity:** AE enzyme activity of canine prostate tissue explants was determined by following the change in optical density at 253 nm (OD\textsubscript{253}) upon hydrolysis of BAEE. Treatment of prostate tissue explants with DHT significantly increased AE enzyme activity at 10\textsuperscript{-7} M compared to the vehicle-treated control group at 48 hours (p<0.05) (Figure 4.3). There was no difference in AE enzyme activity observed between prostate tissue explants treated with calcitriol at 10\textsuperscript{-7} M at 24 and 48 hours compared to the vehicle-treated control (Figure 4.4 A&B).

4.4 **DISCUSSION**

In this study, we investigated the effects of DHT and calcitriol on AE enzyme activity and VDR expression in canine prostate tissue explants. AE activity increased in canine prostate tissue explants that were treated with DHT at 10\textsuperscript{-7} M. AE, a major seminal plasma protein in dogs, is under androgenic regulation. Gauthier ER et al. (1993) demonstrated that AE gene expression is regulated by androgens mainly at the transcription level. Results from our study indicate that the prostate tissue explant model was able to detect changes in AE activity associated with DHT administration. Our results confirmed a previous study which reported that AE activity in canine prostate tissue explants was significantly stimulated by 10 nM and 100 nM of DHT (Ding et al.,
Moreover, our results were consistent with the increased AE enzyme activity shown in castrated dogs treated with androgenic steroids (Juniewicz et al., 1990). However, the concentration of $10^{-7}$ M used in our experiment may not represent the maximum response to DHT of this prostate tissue explant model.

Furthermore, we showed that DHT increased VDR protein expression in canine prostate tissue explants. Our results were consistent with other studies that reported the up-regulation of VDR by DHT in human neonatal prostatic epithelial (Leman et al., 2003) and ovarian adenocarcinoma cell lines (Ahonen et al., 2000). This suggests cross-regulation between these two hormones (calcitriol and DHT) and their receptors. The possible mechanisms for the up-regulation of VDR protein level by DHT is that DHT may act by a direct interaction with the VDR gene by stimulating transcription and translation. Alternatively, DHT may induce other gene products, such as epidermal growth factor (EGF) and insulin-like growth factor (IGF-I) (Chung and Davies, 1996), which in turn increase VDR mRNA by up-regulation of transcription/translation or mRNA stabilization.

Data from our laboratory showed the presence of VDR in the nuclei of epithelial and stromal cells of canine prostate tissue which indicates that canine prostate would be a target organ for calcitriol. Calcitriol has been shown to inhibit cell proliferation in prostate cancer cell lines, reduce tumor growth in vivo, and reduce serum PSA levels in clinical studies in humans (Lukeshwar et al., 1999; Beer et al., 2002). We also demonstrated that calcitriol inhibited epithelial cell growth in primary canine prostate cultures (Kunakornsawat et al., 2002). However, results from this study showed no difference in AE activity in canine prostate tissue explants treated with calcitriol despite

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the fact that this model was able to detect changes in AE activity associated with DHT administration. This suggested that calcitriol has no direct effect on AE production in the canine prostate. The most likely explanation for this effect is that AE expression in the canine prostate involves different regulatory pathways compared to PSA expression in the human prostate. Studies with human prostate cell cultures have shown that prostatic stromal cells play an important role in affecting the growth and differentiation of prostatic epithelium in an autocrine/paracrine fashion (Chung and Davies, 1996; Krill et al., 1999). Prostatic stromal cells in humans were reported to secrete paracrine growth factors such as fibroblast growth factor (FGF), transforming growth factor (TGF), and nerve growth factor (NGF) (Chung and Davies, 1996). These paracrine factors can diffuse from the stromal compartment to the epithelial cells and subsequently influence their growth and differentiation. The prostate explant culture used in this study maintained the normal architecture of the prostate gland without disturbing the relationship between the cellular components which may be caused by enzymatic or mechanical separation, thereby allowing normal physiological interactions between canine prostate epithelial and stromal cells. Moreover, data from our laboratory showed that calcitriol stimulated stromal cell growth in the canine prostate (Kunakornswat et al., 2002). Hence, stromal cell growth factors could interact with the epithelial cells (in which cell growth is inhibited) affecting the total AE expression in prostate tissues. This may offer another explanation as to why there was no difference in AE activity in response to calcitriol in this study. Additional studies are required to determine the roles of calcitriol in the regulatory pathway of AE expression in the canine prostate.
Finally, we reported that calcitriol had no effect VDR protein expression in canine prostate tissue explants. Other studies that have demonstrated that the actions of calcitriol in prostatic epithelial and stromal cells are mediated through the VDR (Kunakornsawat et al., 2002; Tokar and Webber, 2005), which in our explant model the epithelial and stromal compartment still maintained normal architecture of the prostate gland as mentioned above. Calcitriol has been reported to mediate VDR in a variety of cell culture models by controlling VDR mRNA expression (Kunakornsawat et al., 2002). Moreover, several studies also have reported that calcitriol can stabilize VDR protein and increase protein half-life in cultured cells independently of transcriptional components (Zella et al., 2007).

CONCLUSIONS

The results from this study showed that DHT significantly increased AE enzyme activity and VDR protein expression, while calcitriol decreased VDR protein expression but did not change AE enzyme activity. We have demonstrated that cultured canine prostate tissue explants provided a valuable model for the study of prostate pathobiology and to evaluate the effects of pharmaceutical interventions. It also provided the basis for further investigation into the mechanistic roles of calcitriol and its potential uses as therapeutic/preventative agent in BPH and prostatic carcinoma in dogs and humans.


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Figure 4.1 VDR immunohistochemistry: Localization of the VDR in canine prostate tissue by immunohistochemistry. Dark staining (arrowheads) indicates the presence of VDR in the nuclei of epithelial and stromal cells (arrows) of the canine prostate tissue. Magnification x200
Figure 4.2 **Western blot analyses for VDR**: VDR expression in canine prostate tissue explants treated with calcitriol, DHT or vehicle (control) was determined by Western blot analysis. DHT and vehicle at $10^{-9}$ M and $10^{-7}$ M (A); calcitriol and vehicle at $10^{-7}$ M (B) treated for 24 and 48 hours. Blots were incubated with rabbit anti-human VDR monoclonal antibody. The Gel-Pro® Analyzer (Media Cybernetics, San Diego, CA, USA) was used to measure protein band density.
Figure 4.3 **AE enzymatic activities:** AE enzymatic activity in canine prostate tissue explants was determined by following the change in optical density at 253 nm (OD$_{253}$) upon hydrolysis of BAEE. Prostate tissue explants were treated with DHT at $10^{-9}$ M and $10^{-7}$ M for 48 hrs. DHT significantly increased AE at $10^{-7}$ M compared to vehicle treated-control. Mean OD$_{253}$ values of triplicate samples are presented. Canine muscle homogenate was used as a negative control. Data is presented in $\mu$mol/min/mg protein. (* P<0.05)
Figure 4.4 AE enzymatic activities: AE activity of canine prostate tissue explants was determined by following the change in optical density at 253 nm (OD$_{253}$) upon hydrolysis of BAEE. Prostate tissue explants treated with calcitriol at $10^{-7}$ M for 24 hrs (A) and 48 hrs (B) showed no difference in AE activity compared to the vehicle-treated control. The mean values of triplicate experiments from dogs in each group are presented. Canine muscle homogenate was used as a negative control. Data is presented in µmol/min/mg protein.
Table 4.1 Grading of canine prostate glands: The prostate glands collected in this study were categorized into 3 groups based on histologic morphology and % of VDR immuno-reactive cells. Data is presented as mean ± SD of prostate samples sample from 3-4 dogs in each group.
It has become apparent over the past decade that calcitriol, the seco-steroid hormone that is formed from the biotransformation of vitamin D in the body, plays a much wider physiological roles than was previously thought. Beyond its classical role in mineral and skeletal homeostasis, calcitriol is involved in the regulation of growth, differentiation, and other functions of a wide range of normal as well as malignant cells (Sung et al., 2000; Soiland et al., 2007). Active forms of vitamin D, calcitriol and its analogs, have been shown to produce growth suppressive effects in vitro and in vivo in a variety of human and mouse tumors. Investigation of the anti-tumor effects of calcitriol and its analogs is a very active area of ongoing research. The anti-tumor activities of calcitriol and its analogs might inhibit tumor cell growth and development in several different ways via different mechanisms of action, including: (i) cell cycle regulation (Seol et al., 2000; Rao et al., 2004), (ii) induction of apoptosis (Diaz et al., 2000; Guzey et al., 2004; Golovko et al., 2005), (iii) induction of cell differentiation (Hansen et al., 2001; Mosieniak et al., 2006), and (iv) antiangiogenesis and inhibition of metastasis (Mantell et al., 2000; Abdaimi et al., 2000). Although there is increasing data in the literature demonstating the anti-tumor effects of calcitriol and its analogs in human and
laboratory animals, little is known about the role of these compounds in the tumor cell growth in canine species. For this reason, these studies were designed to investigate the potential uses of calcitriol and its analogs as anti-tumor agents on canine tumors.

In our first study, we examined the effects of calcitriol, seocalcitol, and medium-chain triglyceride on canine transitional cell carcinoma in vitro. We hypothesized that calcitriol and seocalcitol would inhibit the growth of canine TCC in vitro and MCT will enhance the inhibitory effects of these two compounds. 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. Our results showed that canine TCC expressed high amounts 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; however, no significant changes in VDR expression were observed. MCT enhanced the growth inhibitory effect of both compounds. These findings suggest that calcitriol and seocalcitol with MCT may have therapeutic potential for canine bladder cancer.

For our next study, we investigated the effects of calcitriol and DHT on AE enzyme activity and VDR expression in canine prostate tissue explants. We hypothesized that DHT would increase AE enzyme activity and VDR expression in cultured canine prostate tissue and that calcitriol would decrease AE enzyme activity and increase VDR expression. Explants were treated with DHT, calcitriol or vehicle at $10^{-9}$ M to $10^{-7}$ M for 24 and 48 hours. After treatment, AE activity and VDR expression were measured by an enzyme activity assay and Western Blot analysis, respectively. Our
results showed that DHT significantly increased AE enzyme activity and VDR expression; however, there was no difference in AE activity observed between the calcitriol- and vehicle-treated control groups. Calcitriol slightly increased VDR expression. These findings suggested that cultured canine prostate tissue explants were an excellent model for the study of prostate pathobiology and pharmaceutical interventions, and that this model might be used as a basis for further investigation of the mechanism of calcitriol’s effects on the prostate and its potential use as therapeutic/preventative agent in BPH and prostatic carcinoma in both dogs and human patients.

Our last study was designed to investigate the use of a canine TCC-mouse xenograft model to study the effects of calcitriol, seocalcitol and piroxicam on canine transitional cell carcinoma. This study was based on the hypothesis that the administration of calcitriol, seocalcitol and piroxicam will inhibit tumor growth in TCC-tumor bearing mice compared to untreated control, and that calcitriol and seocalcitol will have greater anti-tumor effects than piroxicam. A model has been developed which allows non-invasive evaluation of tumor growth and metastasis using luciferase-expressing canine TCC cells. Light signal from luciferase-expressing cells can be detected in living animals using a bioluminescence imaging system (IVIS) following injection of luciferin substrate. In developing this model, canine TCC cells were transfected with plasmid encoding the luciferase gene. Stably transfected TCC cells were used to induce canine TCC tumors in nude mice by subcutaneous injection. Three weeks after tumor induction, mice were treated with vehicle, calcitriol, seocalcitol or piroxicam three times per week for six weeks Tumor responses were evaluated weekly by
measurement of the light signal using the IVIS™ imaging system and by caliper measurements. Body weight was determined weekly. Blood was obtained from the facial vein for the measurement of ionized calcium (iCa) every three weeks to monitor toxicity of calcitriol and seocalcitol. At the end of the experiment, mice were sacrificed and tumor tissues were collected and processed for histopathology, immunohistochemistry of Ki-67, and TUNEL assay. We showed that the canine TCC - mouse xenografts grew well in nude mice and that a light signal could be detected with the IVIS™ imaging system in established tumors; however, the correlation of light signal with tumor growth as measured with calipers was poor. Seocalcitol reduced tumor volume while calcitriol and piroxicam showed no effect; the superior inhibitory effect of seocalcitol on tumor growth was supported by data from a quantitative evaluation of Ki-67 staining cells. Blood iCa levels were higher in both calcitriol- and seocalcitol-treated mice compared to control. These findings suggest the potential use of seocalcitol for the treatment of the TCC in dogs.
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