Preclinical evaluation of vitamin D and ginsenoside metabolites in combination for prostate cancer therapy: Pharmacokinetic and Pharmacodynamic interactions

by

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**Preclinical evaluation of vitamin D and ginsenoside metabolites in combination for prostate cancer therapy: Pharmacokinetic and Pharmacodynamic interactions**

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ABSTRACT

The potential therapeutic roles of calcitriol as well as 20(S)-protopanaxadiol (aPPD), a naturally derived ginsenoside, have gained much attention in recent years for the prevention/treatment of prostate cancer (PCa). The clinical utility of calcitriol as an anticancer agent has been severely limited by its hypercalcemia related toxicity. The research presented here explores the pre-clinical efficacy of aPPD as a single agent and in combination with calcitriol in vitro and in the androgen insensitive castration resistant C4-2 human xenograft model prepared in mice to represent PCa in vivo. We further examined aPPD and calcitriol pharmacokinetic (PK) and pharmacodynamic based interactions.

Calcitriol sensitizes PCa cells to aPPD-mediated anticancer effects by enhancing its ability to induce apoptosis and reduce cell proliferation in vitro (IC_{50} of aPPD is reduced by almost 12- and 18-fold in LNCaP and C4-2 cells, respectively). An LC/MS assay for calcitriol was optimized and used in PK studies carried out in CD-1, non-tumor bearing, and C4-2 tumor bearing nude mice. The amount of calcitriol reaching the blood was significantly increased by co-administration of aPPD and calcitriol oral clearance was reduced. The novel findings described herein indicate aPPD potently inhibits PCa in vivo partly via inhibition of a site on the AR N-terminal domain. This manifested as cell cycle arrest and concurrent induction of apoptosis via an increase in Bax, cleaved-caspase-3, p27 and p21 expression in C4-2 tumors. Furthermore, aPPD significantly decreased Ki-67 and AR protein expression in xenograft tumors, while upregulating VDR. Adding calcitriol to aPPD treatments resulted in substantially greater inhibition of C4-2 growth than aPPD treated alone (76 % vs 53%, respectively). In the presence of calcitriol, aPPD enhanced significantly calcitriol mediated VDR activation while aPPD treatments alone did not affect the VDR activity, suggesting that aPPD may be an allosteric activator of VDR.
Our findings encourage use of lower dose calcitriol in combination with aPPD to establish therapeutic benefit while limiting side-effects such as calcitriol associated hypercalcemia. Inhibition of the AR signaling pathway alongside increased associated enhancement of VDR expression and activation with increased calcitriol serum exposure are likely to be mechanistically associated with this sensitization effect.
LAY SUMMARY

Prostate cancer is one of the most frequently diagnosed cancers among men. Unfortunately, many men still die of metastatic disease when cancer cells spread from the prostate gland to the surrounding tissue. To date, therapeutic options for castration resistant advance stage prostate cancer are limited. Novel anticancer compounds derived from natural products present an attractive alternative to synthetic compounds, based on their favorable safety and effectiveness profiles. Numerous clinical studies have shown that low blood levels of vitamin D increase the risk of developing cancers including prostate. The clinical utility of vitamin D as an anticancer agent has been severely limited because its antitumor activity is achieved at doses that cause toxicity including hypercalcemia. Ginseng is one of the most commonly used medicinal herbs in complementary and alternative medicine worldwide. The research described in this thesis suggests the potential benefits of using ginseng with vitamin D in combination as anticancer therapy in castration resistant prostate cancer patients. The combination demonstrated the synergism or sensitization of anticancer activities, which would be tremendous as lower doses of vitamin D would imply fewer side effects, leading to additional benefits in patients with cancer.
Chapter 1, Section 1.3. A version of Chapter 1.3 has been published (Ben-Eltriki M, Deb S and Guns ES. ‘Calcitriol Combinations for Prostate Cancer Treatment: Pharmacokinetic and Pharmacodynamic-based Interactions.’ Journal of Cancer, 2016). My contribution to this publication was more than 90%. I prepared this review article with a significant focus on critical thinking about presented data across literature. I covered all current preclinical and clinical studies assessing pharmacodynamic and pharmacokinetic interactions observed upon concurrent dosing of anti-cancer compounds with calcitriol. Dr. Emma Guns and Dr. Subrata Deb (Research Associate in the Guns lab) provided guidance, critical comments and reviewed the work. I drafted and prepared this manuscript for publication.

Chapter 1, Section 1.4. I was responsible for writing this chapter. Gehana Shanker (directed studies student who was under my supervision) helped me summarize the efficacy studies. This chapter will be further revised and submitted for publication. Dr. Emma Guns and Dr. Subrata Deb provided guidance, critical comments and reviewed the work.


Chapter 2. A version of Chapter 2 has been published (Ben-Eltriki M, Deb S and Guns ES. ‘Calcitriol and 20(S)-Protopanaxadiol Synergistically Inhibit Growth and Induce Apoptosis in Prostate Cancer Cell Models.’ The Journal of Steroid Biochemistry and Molecular Biology, 2016). It also has been published as published abstract at 13th European International Society for Study
Xenobiotics (ISSX June 2013). Published in Planta Medica Journal 2016. Available at: 
addition, newsletter commentary on article available at: https://redsenolhealth.com/rare- 
ginsenoside-benefit-calcitriol-and-appd-synergistically-induce-apoptosis-in-prostate-cancer- 
cells/ 

My contribution to this publication was more than 90 %. I performed the experiments (viability 
and proliferation assays, assessment of synergy and western blotting), as well as analysis and 
interpretation of the results. Hans Adomat guided and trained me on the LC-MS assays for 
calcitriol analysis. Dr. Helene Morin helped me in performing an experiment in response to the 
reviewer comment. I was responsible for writing this chapter. Dr. Emma Guns and Dr. Subrata 
Deb provided critical comments and reviewed the work. 

**Chapter 3.** A version of Chapter 3 has been submitted for publication (Ben-Eltriki M, Deb S, 
Hassona M, Meckling G, Adomat H and Guns ES. ‘Pharmacokinetic Interaction of Calcitriol with 
20(S)-Protopanaxadiol in Mice: Determined by LC/MS Analysis. My contribution to this chapter 
was more than 80 %. I designed the research question and experimental plan with guidance from 
my supervisor, Dr. Emma Guns. I developed LCMS assay for calcitriol analysis with guidance 
from Hans Adomat and used it to measure calcitriol concentrations in mice samples. I conducted 
the pharmacokinetic experiments, LCMS analysis as well as interpretation of the results and 
preparation of the manuscript. Gray Meckling, and Dr. Mohamed Hassona, a student and 
Postdoctoral fellow working with the Guns lab, helped me with sample collection. Dr. Emma Guns 
and Dr. Subrata Deb provided critical comments and reviewed the work. 

**Chapter 4.** A version of Chapter 4 has been published (Ben-Eltriki M, Deb S, Hassona M, 
ES. ’20(S)-protopanaxadiol Regio-Selectively Targets Androgen Receptor: Anticancer Effects in Castration-Resistant Prostate Tumors.’ Oncotarget Journal, 2018). My contribution to this publication was 70 %. I performed the animal work (efficacy studies), as well as analysis and interpretation of the results and preparation of the manuscript. Gray Meckling, and Dr. Mohamed Hassona helped me with animal work throughout the study. Mei-Yieng Chin helped me with end point sample collections. Dr. Ladan Fazli analyzed all immunohistochemistry staining of C4-2 tumors. Dr. Nada Lallous helped me performing AR activation assay. Dr.Takeshi Yamazaki performed all docking simulation analysis. Dr. Emma Guns and Dr. Subrata Deb provided critical comments and reviewed the work. All co-authors provided insight into the production of the manuscripts in this chapter.

**Chapter 5.** A version of Chapter 5 will be submitted for publication (Ben-Eltriki M, Deb S, Hassona M, Meckling G, Shankar G, Fazli L, Chin M, Yamazaki T and Guns ES. ‘Combination of 20(S)-protopanaxadiol and Calcitriol Targets Vitamin D Receptor: Activation of apoptosis and growth inhibition in prostate cancer. Under the guidance of Dr. Emma Guns and Dr. Subrata Deb, I was responsible for the research design. I conducted experiments, collected, analyzed, and interpreted data. I was responsible for writing this chapter. Gray Meckling, and Dr. Mohamed Hassona helped me with animal work throughout the study. Dr. Ladan Fazli analyzed all immunohistochemistry images of stained tumor sections. Dr.Takeshi Yamazaki performed all docking simulation analysis. Gehana Shanker helped me with VDR activation assay experiments. }
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LIST OF ABBREVIATIONS

aPPD Aglycone protopanaxadiol
ANOVA Analysis of variance
AR Androgen receptor
ASIR Age-standardized incidence rates
AST Aspartate aminotransferase
AUC Area under the plasma concentration versus time curve
BCRP Breast cancer resistance protein
BCL2 B cell lymphoma / leukemia gene 2
CAM Complementary and Alternative Medicine
CDK Cyclin-dependent kinase
CI Combination index
CL/F Apparent oral clearance
Cmax Peak Concentration
CNS Central nervous system
CRPC Castration resistant prostate cancer
CYP Cytochrome P450
CVS Cardiovascular system
DBD DNA-binding domain
DHT Dihydrotestosterone
DMEM Dulbecco's modified eagle's medium
DRI Dose Reduction Index
EGF Epidermal growth factor
FBS Fetal bovine serum
FDA Food and Drug Administration
GSP Ginseng polysaccharides
IC50 The half maximal inhibitory concentration
IGF Insulin-like growth factor
IOM Institute of Medicine
KGF Keratinocyte growth factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>LBD</td>
<td>Ligand-binding domain</td>
</tr>
<tr>
<td>LC/MS</td>
<td>High performance liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LDLr</td>
<td>Low density lipoprotein-receptor</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of Quantitation</td>
</tr>
<tr>
<td>MDR1</td>
<td>Multidrug resistance 1 (gene)</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum Tolerated Dose</td>
</tr>
<tr>
<td>NTD</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>PCa</td>
<td>Prostate Cancer</td>
</tr>
<tr>
<td>PD</td>
<td>Pharmacodynamic</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetic</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate-specific antigen</td>
</tr>
<tr>
<td>QD</td>
<td>Once daily</td>
</tr>
<tr>
<td>S.E.M</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>t1/2</td>
<td>Half-life</td>
</tr>
<tr>
<td>Tmax</td>
<td>Time to peak concentration</td>
</tr>
<tr>
<td>U/L</td>
<td>Units per litre</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VDBP</td>
<td>Vitamin D–binding globulin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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DEDICATION

This work is dedicated to

All affected by prostate cancer

To my Loved Ones

To: my great parents: Ahmed and Lutfia

To my loving wife Enas, my two lovely daughters (Maryaim and Maymona), and my son Adam

Thank you
1. CHAPTER 1: BACKGROUND AND LITERATURE REVIEW

1.1 Thesis overview

The research presented here explores the pre-clinical efficacy of aPPD as a single agent and in combination with calcitriol \textit{in vitro} and in the androgen insensitive castration resistant C4-2 human xenograft model for prostate cancer (CRPC) \textit{in vivo}. We further examined aPPD and calcitriol pharmacokinetic (PK) and pharmacodynamic (PD) based interactions.

The first chapter of this thesis provides background information on prostate cancer, combination therapy, ginsenosides (Rh2 and aPPD) and vitamin D (calcitriol) pharmacokinetics, pharmacodynamic and its drug interactions. The background information is organized in four main sections. Namely, the first section provides a clear understanding of prostate cancer, its epidemiology and risk factors, treatments options with respect to androgen dependence or independence, and CRPC development and treatment challenges. In the second section, discussions on advantages of using drug combination, methods for assessing therapeutic drug-drug interactions, the pharmacokinetic and pharmacodynamic outcomes (modulation of drug metabolism and transport processes involved in drug-drug interactions and their toxicological implications, and overall benefits of drug combination in cancer treatment). In the third section, calcitriol preclinical and clinical studies that describe the activity of calcitriol, either alone or as part of a combination therapy approach to treat PCa, were delineated. To our knowledge this is the first report highlighting the pharmacodynamic and pharmacokinetic interactions observed upon concurrent dosing of anti-cancer compounds with calcitriol. We discussed important considerations for calcitriol use in combination therapy with respect to safety and clinical outcomes. We highlighted the importance of cytochrome P450 (CYP) enzymes, particularly CYP3A4, in calcitriol disposition as well as the importance of the cross talk between androgen
receptor and the vitamin D receptor in prostate cancer. The final and fourth section depict the anticancer effects of Rh2 and aPPD ginsenosides. The Rh2 and aPPD pharmacokinetics, their efficacy and potential interactions with drugs in preclinical and clinical studies were summarized.

In Chapter 2, initially we measured the IC$_{50}$ of aPPD and calcitriol against two prostate cancer cell lines: LNCaP and C4-2. The cell viability and proliferation rate in vitro, then in combination with aPPD, at their IC$_{50}$ ratios, were assessed. The combination and drug reduction indices (as indicators of synergism, antagonism, or additivity) were subsequently determined. The potential synergistic mechanisms were assessed by measuring the expression of proteins related to prostate cancer regulation (e.g. prostate cancer specific antigen, androgen receptor, and vitamin D receptors), cell growth and programmed cell death (apoptosis).

Chapter 3, prior to initiating in vivo combination efficacy studies, a high performance liquid chromatography tandem mass spectrometry (LC/MS) method was optimized for quantitation of calcitriol serum levels in CD-1 mice. This assay was used to determine the pharmacokinetics of calcitriol following oral and IP administration to CD-1 mice, and to study the effect of aPPD on calcitriol PK with respect to its chronic serum levels in non-tumor bearing and C4-2 tumor bearing nude mice, respectively.

Chapter 4 and 5, the efficacy of aPPD treatments alone and in combination with calcitriol was subsequently assessed in the C4-2 CRPC model in vivo. The doses used for these efficacy studies were determined to be well tolerated and effectively inhibited the growth of established C4-2 tumors over a 46 day period. Cell cycle markers p21, p27, apoptotic index alongside levels of the proliferation marker Ki-67 in excised tumor specimens were examined. We further investigated the effect of treatments on the expression of proteins related to prostate cancer
regulation, AR and VDR, in C4-2 tumors. Blood chemistry, CBC and organ toxicity markers were quantitated following treatments. To further characterize the preventive and therapeutic potential of aPPD in PCa. The involvement of Androgen receptor (AR) and Vitamin D receptor (VDR) signaling in aPPD-mediated growth inhibition was studied. *In silico* docking studies for aPPD binding to different domains on the AR as well as *in vitro* assays to determine the ability of aPPD to inhibit AR transactivation were carried out. In addition, *in silico* docking studies, to determine aPPD binding to different VDR domains alongside VDR transactivation bioassays, were carried out in the presence or absence of VDR ligand calcitriol.
1.2 Prostate cancer

Prostate cancer (PCa) is the most commonly diagnosed cancer among men worldwide and the third leading cause of cancer-related deaths in Canada after lung and colorectal cancers\textsuperscript{1-5}. The age-standardized incidence rate is shown in Figure 1.1. The incidence of PCa is significantly proportional to increasing age (Figure 1.1)\textsuperscript{1-6}. Genetic predisposition like family history, lifestyle factors such as high fat diet (e.g. western diet), consumption of dairy products, eating red or processed meat, being overweight or obese, and inflammation of the prostate are the common predictive risk factors for PCa onset\textsuperscript{1-6}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig1.png}
\caption{Age-standardized incidence rates (ASIR) for selected cancers. (Males, Canada, 1988-2017). Adapted from Canadian Cancer Society.}
\end{figure}
PCa is curable disease and early detection and treatment can increase the chances of successful treatment. PCa symptoms are similar to normal prostatitis or urinary tract infection (burning, pain, difficulties upon urination, more frequent urination, and hematuria), so recognizing the symptoms are critical for early detection. PCa is often categorized into 3 stages: androgen dependent, androgen deprivation (ADT) associated regression and castration-resistant (Figure 1.2).

![Schematic of clinical progression to androgen independence prostate cancer.](image)

**Figure 1.2.** Schematic of clinical progression to androgen independence prostate cancer.

To effectively manage the disease and eventually develop novel cancer therapeutics that improve the treatment outcomes of metastatic PCa, better understanding of the different molecular mechanisms that occur during PCa initiation and progression is necessary. Prostate-specific antigen (PSA), which serves as a biomarker for PCa, has made it possible to detect the disease at the early stages and render better prediction of its behavior. However, there has been considerable debate about the benefits of screening patients using PSA due to limitations in its clinical use in
predicting the outcome or response to therapy\textsuperscript{7,8}. Combining results of the PSA test and a digital rectal examination (DRE) by a physician can help predict but not confirm the PCa. A biopsy is carried out to confirm and categorize the grade of a suspected cancer. PSA lacks specificity for PCa because as it is unable to distinguish well between benign prostatic hyperplasia and malignancy. This perhaps could lead to either over-treatment or unnecessary tissue biopsies\textsuperscript{7,8}. There has been a great deal of effort invested in searching for novel biomarkers in serum, urine and tissue but few have presented with the ability to replace PSA\textsuperscript{8}.

Initiation and progression of PCa are characterized by alterations in regulatory pathways of apoptosis, Androgen receptor (AR) signaling, cell cycle regulation, signal transduction, cell adhesion and cohesion, and angiogenesis, which may occur within the prostate cells and/or the surrounding tissues. Disruption of these pathways leads to unregulated proliferation of the damaged cells and subsequent accumulation of mutations, resulting in malignancy. Examples of potential candidate markers of prognosis and therapeutic response involving these pathways include p53 and Bcl-2, AR, p27, p21 and CDK, Ki-67, retinoblastoma protein pRb and c-Myc, epidermal growth factor (EGF) receptor family and E-cadherin, vascular endothelial growth factor (VEGF), respectively\textsuperscript{7,9}. PCa is initially dependent on androgens (mainly testosterone or dihydrotestosterone, DHT) which activate the AR to transcriptionally regulate the expression of multiple genes including PSA. AR signaling pathways play a significant role in the early development of PCa, as well as in the advanced stages of the disease where the prostate is androgen-independent that fails to respond to hormone deprivation therapy.

Early detection and treatment of localized PCa has significantly improved\textsuperscript{10}. Androgen deprivation therapy (ADT) is usually used in locally invasive PCa, which aims to shut down gonadal production of androgens in the testis. At the early stage when localized PCa cells depend
on androgens for growth and survival, radiation and prostatectomy are the standard primary therapy. Removal of testicular testosterone by castration leads to tumor regression by reducing testosterone levels in the body (castration phase)\textsuperscript{1-6}. The surgery is usually associated with post-operative pain, sexual dysfunction, urinary incontinence and emotional distress side effects\textsuperscript{11}. Luteinizing hormone-release hormone (LHRH) agonist (Leuprolide, Goserelin, Triptorelin and Histrelin) and antagonist (Degarelix) usually used in order to shut-down pituitary axis regulated gonadal steroid production\textsuperscript{1-6,10}. The survival benefits of ADT are well-established. However, vasomotor flushing, anemia, metabolic changes, gynecomastia and bone density loss are the common associated side effects and PCa usually reoccur after approximately 18 months, so other forms of treatment are needed. Anti-androgens inhibit AR by competing with endogenous steroids for binding to the ligand binding domain (LBD) including: the first generation: Flutamide, Bicalutamide, Nitutamide used alone and in combination with ADTs when they are no longer effective alone\textsuperscript{1-6}. These drugs are relatively ineffective as AR antagonists because their binding potential are much lower than DHT itself\textsuperscript{12}. The second generation Anti-androgen (Enzalutamide), with higher androgen competition binding potential, has been introduced in 2012 to effectively treat advance stage of PCa. Recent investigations have also discovered new antiandrogens (Bristol-Myers Squibb- BMS-641988) and (Vancouver Prostate Centre VPC-13566) that have high binding affinity and target the Binding Function 3 (BF3) pocket of AR\textsuperscript{13,14}. They are currently being investigated in preclinical studies and in Phase I-II clinical trials and may provide potential strategy to treat PCa\textsuperscript{13-16}.

The majority of men with advanced PCa eventually advance to more aggressive form of PCa known as “castration-resistant” (CRPC) and become refractory after about two years with an increased PSA and/or symptomatic progression\textsuperscript{17-23}. CRPC progression is a complex process by
which PCa cells acquire the ability to survive and proliferate in the absence of circulating androgens via several mechanisms of resistance including local intra-tumoural steroidogenesis. Several mechanisms of CRPC progression have been proposed: mutations in the AR resulting in amplification of the AR and emergence of AR splice variants, changes in the activity of AR coregulators or pathways other than AR-mediated pathways being involved. Studies have also shown that CRPC development triggers a release from the arrest resulting in cell cycle progression, even in the absence of androgens. Furthermore, epidermal growth factor (EGF), insulin-like growth factor (IGF), keratinocyte growth factor (KGF) and cytokines interleukin-4 and -6 have also been demonstrated to activate AR. AR is a major driving force in the development and progression of PCa to the metastatic stage and expression of AR splice variants is one of the major mechanisms of CRPC. At this stage, AR continues to drive tumour growth and AR antagonists may once again be used to procure TAB and reinitiate ADT. Furthermore, PCa tumours have been shown to be able to grow, even when circulating androgen levels are low, due to the emergence of de novo synthesis of androgens in tumour tissues. Androgens play a critical role in disease progression and are required for the growth and survival of androgen-sensitive as well as CRPC cells. Steroidogenesis inhibitors such as Abiraterone is usually used, which has recently been shown to supress the local intra-tumoural steroidogenesis and provide benefit by reducing available steroidal AR ligands.

Although, ADT remains the most effective treatment option for patients with advanced disease, to date, therapeutic options for CRPC are limited. Unfortunately, most patients who die from PCa have hormone refractory disease and while many PCas still express AR, only about 30% of CRPC patients respond to anti-androgen therapy that aims to achieve total androgen blockade. Many men still die of metastatic disease when PCa spreads from the prostate gland to the
surrounding tissue (lymph nodes and bone)\textsuperscript{34}. Chemotherapy is usually used alone and in combination with Prednisone to treat metastatic PCa. No particular class of chemotherapeutic drugs are considered highly effective\textsuperscript{34}. Among the existing therapeutics, docetaxel is currently considered to be the gold standard as demonstrates an increase in survival of only 3 to 4 months with considerable associated morbidity\textsuperscript{35-38}. New therapeutic approaches are being explored, agents with growth inhibitory properties that work independent of the androgen pathways are of current interest.

1.3 Combination therapy

Monotherapy has limited efficacy in cancer prevention and is mostly ineffective in curing cancers because of the heterogeneous pathways which contribute to their occurrence. In recent years, new drugs and drug combinations have contributed to improvements in treatment outcomes of PCa\textsuperscript{39}. Combination regimens of chemotherapeutic drugs were developed in the 1960s and early 1970s, based on observations that single drug administration at clinically tolerable dosages failed to cure cancer as normal tissue toxicity often limits the use of a single agent. Taxanes chemotherapy (e.g., paclitaxel and docetaxel) is usually used in a combination treatment strategy to treat CRPCa. Combination therapy offers significant benefits when compared to single-agent therapy by providing: (i) maximal cell kill within the range of tolerable toxicity by allowing lower drug doses; (ii) diverse interactions between the drugs procuring pleiotropic effects which may be optimal for treating a heterogeneous tumour; (iii) the potential to prevent or slow the subsequent development of cellular drug resistance as combined drugs work by different mechanisms; (iv) lower cost incurred as lower doses and/or less frequent drug administration is required; (v) reduced side effects of individual drugs because each drug can be used optimally at a lower dose; (vi) better
clinical outcomes when the approach taken is not curative but intended to reduce symptoms and prolong life.

Optimal drug combination effects are likely to be achieved when combined drugs have different mechanisms of action and no overlapping toxicities. Individual drugs which are used in combination should be partially effective against the same tumor type. In addition, PK and pharmacodynamic (PD) interactions could occur as a result of combining drugs and should therefore also be investigated at the onset. These interactions can be beneficial by allowing a therapeutic effect to be achieved with low doses of component interventions, which often minimizes potential side effects. Alternatively, they can exist as problematic antagonistic interactions when combined pharmacological outcomes lead to unwanted toxic effects and result in lower effectiveness than expected from the individual activities of the combined drugs. Drug combinations that interact in a manner that results in additive or synergistic effects, where the combined effects exceed those anticipated based on the effects of the individual agents, are ideal and therefore of great interest for therapeutic development.
1.4 Calcitriol in combination therapy for prostate Cancer: pharmacokinetic and pharmacodynamic based interactions

1.4.1 Introduction

1.4.1.1 Vitamin D and calcitriol

Two types of vitamin D exist: vitamin D$_3$ (cholecalciferol), which is animal-derived; and the plant derived vitamin D$_2$. Vitamin D$_3$ is a steroid-like molecule, which is considered to be a prohormone and is the predominant form of vitamin D in humans$^{40}$. It can be endogenously synthesized from sterol 7-dehydrocholesterol in the skin through exposure to ultraviolet B radiation. Alternatively, vitamin D, in the form of either vitamin D$_3$ or D$_2$, can be acquired from the diet or dietary supplements. Vitamin D, either as D$_3$ or D$_2$, requires a two-step activation process to become biologically active$^{40}$. Vitamin D$_3$ is highly lipophilic that is transported in the blood stream only when bound to a specific plasma protein called vitamin D–binding globulin (VDBP). It is taken up within hours following synthesis or dietary uptake to be activated by liver and kidney$^{41}$. In the liver, where multiple cytochrome P450 (CYP) enzymes are present, mitochondrial CYP27A1, microsomal CYP2J3 and CYP2R1 are readily available and capable of hydroxylating vitamin D$_3$ at the C-25 position to form 25(OH)D$_3$, which is then released into the blood stream for further activation, primarily in the kidney$^{42,43}$. Serum 25(OH)D$_3$ is often considered an acceptable measurement of vitamin D$_3$ levels in the body$^{44}$. It has a short plasma half-life and a long systemic half-life of ~6 hours and ~ 2 months, respectively$^{41,45}$.

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When serum calcium levels are low, renal CYP27B1 converts 25(OH) D$_3$ to 1, 25 dihydroxyvitamin D$_3$ (1, 25(OH)$_2$D$_3$; calcitriol). Calcitriol is the most active form that exists as a small lipophilic molecule with superior cellular penetration$^{44}$ (Figure 1.3).

Traditionally, most of the randomised clinical trials (RCT) have focused on bone mineralisation and fracture risk that associated with serum vitamin D levels$^{46}$. Numerous epidemiological studies recently have shown that low serum levels of vitamin D$_3$ increases the risk of developing cancers of the colon, ovary, lung, breast and prostate$^{43,47-51}$. However, there have been mixed results from clinical studies with respect to their promise of cancer prevention/treatment with vitamin D and we are yet to see compelling evidence that the numerous published preclinical studies may actually be translated to humans and cancer patients$^{5,52}$. The most favorable benefits observed for vitamin D intake have been documented with respect to the inverse relationship between serum levels seen in patients with colon and breast cancer$^5$. For prostate and skin cancer, however, inverse associations between cancer risk and serum vitamin D levels in clinical trial has been difficult to demonstrate so far$^5$. Consequently, we are limited in our ability to provide guidelines for vitamin D intake, which serve as a model to define optimal levels of serum 25(OH) D$_3$ that protect against the development of cancer$^{43}$. Report from the U.S. Institute of Medicine (IOM) suggests that serum levels greater than 20 ng/mL are recommended that could offer optimal health benefits$^{43,46,50,53,54}$. It is possible that, for individuals with limited sun exposure, these levels are achievable by taking vitamin D$_3$ supplements daily in the range of 1000–4000 IU.
In human, vitamin D₃ is the predominant form of vitamin D, which is synthesized from 7-dehydrocholesterol upon sunlight exposure. Vitamin D may also be obtained from dietary sources or supplements as either vitamin D₂ or D₃. Vitamin D₃ binds to vitamin D-binding protein (DBP) in the bloodstream and then taken up within hours following synthesis or dietary uptake to be activated by liver and kidney. In the liver where it is first converted by mitochondrial cytochrome P450 enzyme CYP27A1, microsomal CYP2J3 and CYP2R1 to 25-hydroxyvitamin D. This molecule is converted by the renal enzyme 1-a hydroxylase (CYP27B1) to 1, 25 dihydroxycholecalciferol (calcitriol), which is the active form of Vitamin D. Then, calcitriol binds to intracellular vitamin D receptors (VDR) in most cells in the body working both as a paracrine and an autocrine agent.
Calcitriol plays an important role in mineral homeostasis and bone growth by promoting the export of calcium from bone, absorption of calcium from the gastrointestinal tract and the stimulation of the absorption of phosphate and magnesium ions to support mineralization. It works as both a paracrine and an autocrine agent by binding to intracellular vitamin D receptor (VDR) to form a complex with the retinoid-X receptor. The resulting heterodimer binds to DNA to function as a transcription factor to either initiate or suppress gene expression\textsuperscript{55,56}. The VDR has a higher affinity to calcitriol than any other form of vitamin D, which explains its relatively high biological potency\textsuperscript{57}. Besides calcitriol, various steroidal and non-steroidal synthetic analogs, non-vitamin D related endogenous compounds (e.g. Lithocholic and arachidonic acid) and dietary ligands including curcumin and fatty acid derivatives, are also capable of activating VDR\textsuperscript{58,59}. VDR is present in moderate levels in most cells in the body including the kidney, heart, muscle, breast, colon, prostate, brain and immune cells\textsuperscript{55}. The gastrointestinal tract and bone are the two sites where VDR is highly expressed and consequently are the primary targets of calcitriol-mediated physiological effects\textsuperscript{43}. Calcitriol has potent effects on factors involved in growth and differentiation of many types of cancer cells, as well as cell adhesion and apoptosis. This could be largely due to its role in regulating the cell cycle and number of genes that are implicated in cancer initiation\textsuperscript{43}. Calcitriol has been shown to exhibit anti-proliferative, pro-differentiating, pro-apoptotic and anti-inflammatory actions in a variety of cancer cells in vitro, including PCa cells\textsuperscript{60-62}(Figure 1. 4).

Calcitriol bioavailability in tissues is largely determined by its affinity to bind to the VDBP plasma transporter, rate of synthesis from 25(OH) D\textsubscript{3} and rate of degradation by catabolizing enzymes. When calcitriol is administered orally, it is absorbed rapidly with approximately 99.9% bound to and transported via VDBP in the blood and a serum half-life of approximately 15
Calcitriol levels in the blood depend largely on its bioavailability, ratio of binding to plasma and tissue proteins, perhaps also its binding to lipoprotein as well as the equilibrium that exists for its biosynthesis from 25(OH) \(D_3\) and catabolic degradation steps in the kidney and liver, respectively. Within tissues, its levels are reflective of both blood 25(OH) \(D_3\) and 1, 25(OH) \(D_3\) free levels as well as the balance between the local biosynthesis from 25(OH) \(D_3\) and its rate of degradation\(^{65}\) (Figure 1.3).

1.4.1.2 Calcitriol in prostate cancer

The vitamin D derivatives are currently being used in the treatment of many diseases such as psoriasis and vitiligo\(^ {66}\). However, there is a limitation in the broader use of calcitriol as a single anticancer agent clinically due to its toxicity at higher doses. Calcitriol has limited bioavailability at the tumor site which is actively confounded by local CYP-mediated deactivation\(^ {44}\). Since dose elevation constraints exist due to its hypercalcemic side effects, to achieve desired clinical outcomes in cancer patients, the pharmacological doses required often lead to an extreme elevated plasma levels of calcium and phosphorus and cause adverse hypercalcemia and hypercalcaemia effects\(^ {44}\).

It has been reported that VDR is expressed in three human prostate carcinoma cell lines; LNCaP, PC-3, and DU-145 as well as in the primary culture of stromal and epithelial cells derived from normal and malignant prostate tissues\(^ {67}\). Many preclinical studies have been quite consistent in their demonstration of exposure to high calcitriol concentrations resulting in inhibition of PCa cell growth in vitro and retardation or slow-down of tumor growth in animal models of PCa\(^ {68}\). Preclinical studies demonstrated that 10 nM (24 ng/ml) concentrations of calcitriol are associated with anticancer activity in vitro and in vivo\(^ {69,70}\). Pharmacokinetic (PK) studies in normal mice indicate that calcitriol, at a dose of 0.125 \(\mu g/mouse\), is able to suppress tumor growth and results in a peak
plasma calcitriol concentrations (C\text{max}) of 12.0 ng/ml with corresponding areas under the curve from time 0 to 24 hr of 47 ng•h/ml. Calcitriol anticancer activity is mainly dependent on its dose, and its serum concentration ranges escalate from 0.5 to 41 ng/ml, resulting in significant toxicity\textsuperscript{71,72}. There have been many approaches taken to elevate tumor calcitriol levels that have been reported in the literature, making it feasible to see anticancer benefits while limiting its toxicity. Specifically, calcitriol may be administered on an intermittent schedule, analogues with low/no calcemic activity may be substituted, and using calcitriol in combination with other agents could enhance anticancer activity and allow for lower calcitriol doses to be optimised\textsuperscript{69}.

1.4.1.3 Calcitriol in combination therapy for PCa

Monotherapy has limited efficacy in cancer prevention and is mostly ineffective in curing cancers because of the heterogeneous pathways which contribute to their occurrence. In recent years, new drugs and drug combinations have contributed to improvements in treatment outcomes of PCa\textsuperscript{39}. Combination regimens of chemotherapeutic drugs were developed in the 1960s and early 1970s, based on observations that single drug administration at clinically tolerable dosages failed to cure cancer as normal tissue toxicity often limits the use of a single agent. Combination therapy offers significant benefits when compared to single-agent therapy by providing: (i) maximal cell kill within the range of tolerable toxicity by allowing lower drug doses; (ii) diverse interactions between the drugs procuring pleiotropic effects which may be optimal for treating a heterogeneous tumour; (iii) the potential to prevent or slow the subsequent development of cellular drug resistance as combined drugs work by different mechanisms; (iv) lower cost incurred as lower doses or/and less frequent drug administration is required; (v) reduced side effects of individual drugs because each drug can be used optimally at a lower dose; (vi) better clinical outcomes when the approach take is not curative but intended to reduce symptoms and prolong life.
Inhibitors of CYP 27A1, CYP24A1 and CYP3A4 isoform can play a vital role in maintaining the active form of vitamin D3. PK interaction outcomes can lead to significant modification of calcitriol levels in both serum and tissues. Diagram also delineates the essential cell signaling pathways for prostate cancer development and crosstalk between them. PD interaction are mediated through alterations in these regulatory pathways that enhance overall anticancer effects. CYP: Cytochrome P450; PK: Pharmacokinetic; PD: pharmacodynamic; VDBP: vitamin D–binding globulin; CDK: Cyclin-dependent kinases; P21: cyclin-dependent kinase inhibitor; S179D:a molecular mimic of naturally phosphorylated human Prolactin; NSAID: Nonsteroidal anti-inflammatory drug; KTZ: Ketoconazole; DEX::Dexamethasone; IR: Ionizing radiation; P53:Tumor protein; COX-2: cyclooxygenase-2 ;TBBz :4, 5, 6, 7tetrabromobenzimidazole;MMP-2: matrix metalloproteinase-2; CEACAM: carcinoembryonic antigen-related cell adhesion molecules; Pgp: multidrug resistance protein 1;EP2: prostaglandin E receptor 2; PG: prostaglandin.
Optimal drug combination effects are likely to be achieved when combined drugs have different mechanisms of action and no overlapping toxicities. Individual drugs which are used in combination should be partially effective against the same tumor type. In addition, PK and pharmacodynamic (PD) interactions could occur as a result of combining drugs and should therefore also be investigated at the onset. These interactions can be either beneficial by allowing a therapeutic effect to be achieved with low doses of component interventions, which often minimizes potential side effects. Alternatively, they can exist as problematic antagonistic interactions when combined pharmacological outcomes lead to unwanted, toxic and result in lower effectiveness than expected from the individual activities of the combined drugs. Drug combinations that interact in a manner that results in additive or synergistic effects, where the combined effects exceed that anticipated based on the effects of the individual agents are ideal and therefore of great interest.

Concurrent administration of a lower dose of calcitriol and other anticancer drugs may be the best option that helps to overcome this obstacle and lead to achieving the desired anticancer concentrations with low toxicity. The combination approach for calcitriol used recently with docetaxel in a clinical trial was successfully implemented and achieved the desired anticancer effect with no toxicity in PCa patients\textsuperscript{73}. This study demonstrated that administration of calcitriol dose up to 45 µg/week combined with weekly i.v docetaxel led to Cmax values of 3.4 ± 0.8 nM (8.2 ± 1.9 ng/ml) and was not associated with any significant toxicity\textsuperscript{73}. Other clinical studies suggest that the maximum tolerated dose (MTD) of i.v dose calcitriol in combination with gefitinib was 74 µg/week\textsuperscript{64,74}. The Cmax at the MTD was 6.68 ± 1.42 ng/mL (16 ± 3.4 nM) and the area under the curve (AUC) was 35.65 ± 8.01 ng h/mL. However, this reported Cmax was much higher
than calcitriol concentrations considered to be required for antitumor activity as demonstrated in vitro\textsuperscript{69,70,75}.

The aim of this thesis section is to summarize, the current available pre-clinical and clinical studies with calcitriol used alone and as part of a combination therapy approach to treat PCa. Articles were searched using PubMed using the keywords: vitamin D, calcitriol, 1, 25(OH)\textsubscript{2}D\textsubscript{3} and PCa. We highlighted the major considerations for the use of calcitriol in combination therapy with respect to safety and factors that influence the PK/PD interactions and clinical outcomes.

1.4.2 Pharmacokinetic Based Interactions

When calcitriol is used in combination with other drugs, calcitriol-drug PK interactions have the potential to affect pharmacological outcome in several ways: (i) alteration of calcitriol serum and tissue concentrations, (ii) alteration of calcitriol hepatic metabolism due to induction or inhibition of hepatic enzymes, (iii) alteration of extrahepatic enzymes by induction or inhibition, and (iv) induction or inhibition of drug transporters that could interfere with calcitriol cellular uptake. Alterations in the synthesis (CYP27B1 mediated) and metabolism (mediated by CYP3A4 and CYP 24A1) of calcitriol is modulated as part of the growth regulation of tumors; thus, compromising calcitriol potency and sensitivity. Overall, PK interaction outcomes can lead to the significant modification of calcitriol levels in both serum and tissues.

1.4.2.1 Calcitriol and CYP enzymes

CYP enzymes catalyze the synthesis and metabolism of a large number of endogenous substrates, including steroids, vitamins, fatty acids, prostaglandins and leukotrienes, as well as the detoxification of exogenous compounds, including drugs, environmental chemicals and pollutants,
and natural plant products. CYP monooxygenases are a major class of phase I metabolizing enzymes that acts either by adding or removing functional groups to/from substrates for further metabolism, thereby facilitating excretion of xenobiotics.

There are four CYP isoforms that participate in the synthesis and breakdown of calcitriol. CYP27A1 and CYP27B1, located in the mitochondria, are involved in the first steps of calcitriol synthesis from vitamin D3 and 25(OH) D3 is located in kidney and liver. Mechanisms of vitamin D metabolism within the prostate remain poorly understood. Historically, mitochondrial CYP24A1 has been reported to be the main enzymes responsible for 25(OH) D3 and a calcitriol metabolism. However, recently our lab and others have shown that CYP3A4, which is located in the endoplasmic reticulum, is a contributor to calcitriol degradation in vivo and in vitro. As shown in Figure 1.4, two metabolic pathways exist in the prostate; the first involves the 24-hydroxylase as the first-step in catabolism of calcitriol. There is strong data supporting 24-hydroxylase activity in the kidney; this enzyme is also present in many target tissues, such as the intestine, which possess vitamin D receptor. As depicted in Figure 1.4, the end product of this pathway is a side chain cleaved metabolite, calcitroic acid. The second pathway involves the conversion of calcitriol via the stepwise hydroxylation of carbon-26 and carbon-23, and cyclization to ultimately yield 1α, 25R (OH)2-26, 23S-lactone D3 (Figure 1.4). In some instance mutations in certain CYP genes, have been suggested to play a role in PCa development and progression. Specifically, mutations in CYP17 and SRD5A2 (encoding for 5α-reductase enzyme) genes have been reported to increase the risk of PCa and is found in some cases of hereditary PCa. Over-expression and mutations in CYP24A1 induce idiopathic infantile hypercalcemia which is linked to PCa resistance. Moreover, there is an association between polymorphisms in CYP3A4 and the increased risk of PCa in men with benign prostatic hyperplasia. In general,
changes in the DNA, RNA and/or protein levels, and the activities of the targets discussed above are found to significantly correlate with Gleason grade and may suggest PCa progression to an androgen–independent stage\textsuperscript{9,82}. Therefore, it is reasonable to suggest that CYP enzymes, such as CYP24A1, may be used as novel drug targets and also perhaps allow accurate prediction of tumor progression.

1.4.2.1.1 Combinations that affect calcitriol synthesis

1.4.2.1.1.1 Role of CYP27B1

Circulating serum levels of 1, 25(OH)\textsubscript{2}D\textsubscript{3} are tightly regulated by the renal enzyme, 25-hydroxyvitamin D-1α-hydroxylase, CYP27B1, which synthesizes calcitriol from the prohormone, 25(OH)\textsubscript{2}D\textsubscript{3}. The renal production of calcitriol is tightly regulated by plasma parathyroid hormone (PTH) levels and serum calcium and phosphorus levels\textsuperscript{87}. In response to low calcium levels, PTH levels rise up and regulate the expression of CYP27B1 leading to the synthesis of calcitriol, which is then released into plasma to serve its endocrine function to maintain calcium homeostasis and bone metabolism.

Many cell types, including prostate cells, immune cells, cells of the gastrointestinal tract, brain cells, and importantly skin cells are capable of calcitriol production. Thus, they can provide hormone for local signaling which could also explain the wide distribution of the and VDR in these organs\textsuperscript{88}. In prostate cells, since they contain VDR, an additional role of calcitriol as an autocrine/paracrine regulator of cell functions could be expected\textsuperscript{44}. It has been reported that primary cultures of normal prostate epithelial cells, as well as several PCa cell lines, express CYP27B1 and can synthesize the active hormone calcitriol intracellularly\textsuperscript{67,88}. In addition, the CYP27B1 activity declines in BPH and PCa cells compared to the normal prostate cells\textsuperscript{89}. In vitro studies using high performance liquid chromatography (HPLC) to measure calcitriol in the media have demonstrated
that two PCa cell lines (DU145 and PC3, but not LNCaP) and normal prostate cells were able to produce calcitriol after cells were exposed to 25(OH) D$_3$. In addition, in the presence of clotrimazole the ability of PCa cell lines, except LNCaP, to produce calcitriol was inhibited. These results correlated with an inhibition of CYP27B1 mRNA expression whereas no CYP27B1 mRNA or protein activity was detected in LNCaP cells, which may contribute to the resistance$^{67,88}$.

It has been reported that the control of calcitriol synthesis in tissues like bone and prostate is distinct from that in the potentially be 25(OH) D$_3$ causing calcitriol levels to fall earlier in these tissues than in the plasma$^{55}$. In vitro studies suggest that the prostate CYP27B1 enzyme is not regulated by serum levels of PTH and calcium compared to the renal enzyme. This supports the hypothesis that extrarenal administration of vitamin D may be useful as a chemopreventive agent, and that calcitriol should be synthesized from 25(OH) D$_3$ locally within prostate cells$^{90,91}$. However, a recent study in mouse prostate xenograft model has demonstrated that dietary vitamin D$_3$ administration caused an increase in serum calcitriol levels without causing any alteration in kidney CYP27B1 mRNA$^{92}$. In addition, both dietary vitamin D$_3$ and calcitriol imparted equivalent reductions in tumor volume in this xenograft model. This hypothesis was also tested clinically and calcitriol prostate levels were shown to increase significantly after oral administration of vitamin D supplements$^{65}$. These results suggest that extra renal sources of vitamin D and local synthesis within prostate do, in fact, contribute to the elevated circulating calcitriol. No studies have been reported to date which examine the effect of drugs combined with calcitriol and their impact on CYP27A1 expression or how it affects calcitriol levels within the prostate.
1.4.2.1.2 Combinations that inhibit calcitriol metabolism

1.4.2.1.2.1 Role of CYP24A1

CYP24A1 recognizes 25(OH) D₃ as a substrate and can mediate its hydroxylation to the active metabolite, calcitriol. This enzyme also recognizes other vitamin D metabolites and analogs and actively converts them to their hydroxylated products. CYP24A1 is also responsible for calcitriol metabolism, mainly in the kidney but also in a variety of other vitamin D target cells. It converts it to water-soluble calcitroic acid which is then conjugated and excreted in bile. The cyp24a knockout mice studies have confirmed the physiological role of CYP24A1 in 25(OH) D₃ and calcitriol homeostasis as a build-up of vitamin D₃ was observed in the knockout mouse phenotype.

CYP24A1 is highly expressed in the kidney but is also present in other normal tissues such as the prostate. Calcitriol can also induce CYP24A1 and its own metabolism, thus has the capacity to limit its physiological functions. The levels of CYP24A1 expression may reflect the endocrine and autocrine/paracrine effects of vitamin D₃ in the human body. CYP24A1 overexpression is a common feature of several solid tumors and was detected in a wide range of cancers such as breast, prostate, skin, esophagus, and gastrointestinal tract cancers. It can contribute to the pathology of diseases that otherwise would respond to endogenous or supplemented vitamin D sources and it is associated with poor prognosis due to rapid degradation of both 25(OH) D₃ and calcitriol, limiting their levels in the tumor cells and thus abrogating local anti-cancer effects of calcitriol.

Administration of calcitriol in combination with CYP24A1 inhibitors slows its catabolism, thereby enhancing its antitumor activity and antiproliferative effect (Table 1). Following treatment of DU145 PCa cells for 4 days with 1 µM liarozole, which is an imidazole derivative known to
inhibit CYP enzymes, in combination with 10 nM calcitriol, the inhibitory effect on cell viability was further enhanced almost by 65 % compared with no significant effect seen with either treatment alone. It is likely that this effect was mediated by inhibiting CYP24A1 activity, which resulted in a significant increase in calcitriol half-life from 11 to 31 h, thus enhancing the calcitriol anticancer activity. The combination treatments also led to greater VDR up-regulation than cells treated with either drug alone\textsuperscript{99}. A recent study reported that protein kinase CK2 positively regulates CYP24A1 expression and mediates the regulation of its expression. In keeping with this, inhibition of CK2 activity has been shown to cause a reduction in PCa cell proliferation and enhance calcitriol -mediated antitumor effect\textsuperscript{100}.

In vitro treatment of PC3 cells with calcitriol in combination with ketoconazole or RC2204 (a selective inhibitor of CYP24A1) acted synergistically by potentiating calcitriol-mediated antiproliferative effects, promoting the activation of caspase-independent apoptosis pathways. However, ketoconazole is a well-known non-selective inhibitor of CYP enzymes and dexamethasone (potent anti-inflammatory agent used routinely in the chemotherapeutic regimen) was added that has the ability to induce CYP and up-regulate VDR protein expression and activities in vitro\textsuperscript{101,102}. Thus, conclusions deduced following this observation are limited as they are confounded by the contribution of other mechanisms. The authors proposed that potentially ketoconazole inhibit the CYP24A1-mediated oxidative metabolism of calcitriol leading to increase in half-life and systemic exposure previously observed in PC3 cells and C3H/HeJ mouse kidney tissues. These observations were confirmed in vivo when the calcitriol and ketoconazole-dexamethasone combination therapy suppressed the clonogenic survival and enhanced growth inhibition observed with calcitriol treatment alone in the PC3 human PCa xenograft mouse model\textsuperscript{70}. 
1.4.2.1.2 Role of CYP3A4

CYP3A4 is a major hepatic phase I oxidative drug-metabolizing enzyme. It has broad substrate specificity and is inducible after exposure to therapeutic, dietary, and environmental agents. Variability in CYP3A4 activity accounts for large interindividual differences in the disposition if endogenous, xenobiotic and therapeutic drugs. CYP3A4 polymorphisms could contribute specifically to potential inter-individual and interethnic variation in steroid metabolism as it is characteristically involved in the oxidative metabolism of testosterone, thus mediating prostate cell growth. Consequently, CYP3A4 function may play a role in androgen-mediated prostate carcinogenesis if the bioavailability of testosterone is affected. CYP3A4 is expressed in many organs including prostate, breast, gut, colon, and small intestine; however, its expression is the most abundant in the human liver, accounting for 30 percent of the total hepatic CYP protein content. CYP3A4 is also highly expressed in intestine but with remarkably different composition and abundance than the liver. The intestinal CYP system is mainly comprised of CYP3A enzymes (82%) but only approximately 1% of that in liver. It exhibits a broad substrate specificity and is responsible for oxidation of many therapeutic drugs and a variety of structurally unrelated compounds, including steroids, fatty acids, and xenobiotics. It can also be inhibited by a variety compounds including drugs, nutrients, and carcinogens, resulting in low CYP3A4 activity in all of the tissues in which it is expressed, including the prostate.

It has previously been shown that in human liver and intestine CYP3A4 is responsible for the oxidative metabolism of calcitriol. Specifically, CYP3A4 mediates 24- and 25-hydroxylation of calcitriol, but not vitamin D₃, in human liver microsomes. Using specific CYP chemical inhibitors we have reported that CYP3A isoforms are responsible for the microsomal
biotransformation of calcitriol in liver and intestine in vitro. Co-incubation of calcitriol with commonly used CYP3A4 substrates/drugs (e.g. ketoconazole, tamoxifen, ritonavir or clarithromycin) led to approximately 60-100% inhibition of CYP3A4-mediated inactivation of calcitriol in human liver and intestine microsomes. In addition, recently we have shown that ginsenoside metabolites from ginseng herbs, mainly 20(S)-protopanaxadiol and 20(S)-protopanaxatriol, strongly inhibited CYP3A4 activity in vitro and could potentially therefore provide additional benefits to patients with cancer, neurodegenerative and metabolic diseases when used in combination with calcitriol. Similarly, we have also reported that abiraterone, a CYP17A1-mediated steroidogenesis inhibitor used in late stage PCa treatment, inhibits the CYP3A4-mediated inactivation of calcitriol in human liver and intestine, potentially providing additional anti-cancer benefits to PCa patients. In the context of PCa, the inhibition of CYP3A4 may lead to reduced ability of the enzyme to oxidize calcitriol in the liver and thus sparing calcitriol catabolism to yield higher intracellular levels. Therefore, the metabolism of calcitriol in the intestine and liver could contribute more to calcitriol bioavailability and tissue levels than CYP24A1. The combination of calcitriol with CYP3A4 inhibitors, therefore, could allow for the use of lower doses of calcitriol and still achieve significant anticancer effects.

1.4.3 Pharmacodynamic Based Interactions

1.4.3.1 VDR and AR cross talk

VDR is a nuclear receptor that binds calcitriol and regulates the transcription of target genes in the nucleus. VDR is universally expressed in both androgen-dependent and independent PCa cell lines which have differential sensitivity to calcitriol treatment. Six PCa cell lines were reported to contain VDR mRNA and had high-affinity saturable binding sites for calcitriol. Many factors regulate the amount of VDR in target cells that could potentially alter the magnitude of
response to calcitriol exposure. Specific to the prostate, calcitriol response depends not only on absorption, transport into the circulation, plasma levels, ultimate prostate tissue levels and metabolism but also on intact VDR for signaling. The absence of intact VDR or loss of its function would decrease or eliminate the pharmacological responses to calcitriol in the prostate. A recent report that examined the expression of VDR protein in 841 PCa patients concluded that a high expression of VDR in prostate tumors is associated with a reduced risk of lethal cancer\textsuperscript{110}. In addition, genetic predisposition is seen as a risk factor to prostate cancer, there are several studies suggesting the association between genetic variants in VDR and the development of PCa or likelihood of progression to an advanced stage\textsuperscript{111-113}. Significant associations with five common genes (Fok1, Bsm1, Taq1 and Apal), with high Gleason score have been reported with strongest evidence for Fok1 and Bsm1\textsuperscript{112,114}. The expression of VDR could, therefore, be used to help improve prognostic prediction of PCa progression and guide treatment decisions for men. In addition, VDR polymorphisms should also be integrated as a prediagnostic indicator of vitamin D status\textsuperscript{115}.

PCa is known as an androgen-dependent malignancy and the fact that other hormones such as calcitriol are now recognized as modulators of PCa growth and differentiation, suggest a role for pharmacological agents, which might make use of this endocrine axis. There is now considerable evidence to suggest that cross-talk exists between VDR and AR that may vary between different PCa cell lines\textsuperscript{116}. In addition, the phenomenon of sharing the same co-regulators which results in interdependence of AR and VDR signaling may contribute to the therapeutic activity of vitamin D in PCa\textsuperscript{116,117}. The AR belongs to the superfamily of steroid nuclear receptors. AR coregulators, which were originally identified as AR-associated proteins, can modulate many other steroid receptors in this super-family. Co-regulators can act to enhance (co-activate) or
decrease (co-repress) the AR and changes in its expression have been shown to correlate with poor prognosis in PCa patients\textsuperscript{24}. It has been reported that a large number of coregulators are overexpressed in PCa\textsuperscript{118}. Several AR regulators, including ARA54, ARA70, gelsolin, and supervillin, have been reported to promote VDR\textsuperscript{116}. Thus, by targeting this cross talk new therapeutic strategies designed to treat PCa could be developed.

1.4.4 Summary of preclinical studies

Several studies carried out in cell culture and animal models, as well as clinical trials, have shown that calcitriol pre-treatment or combination with other therapeutic agents provides anticancer benefits which are superior to treatment with either agent alone\textsuperscript{119}. Calcitriol apparently sensitizes cancer cells, enhancing their antitumor activity, and can act synergistically with other therapeutic agents. Targeting multiple pathways or the same pathway through a different mechanism maximizes the likelihood of a therapeutic effect while limiting tissue toxicity (Table 1 and 2).

1.4.4.1 Calcitriol combinations which sensitize PCa cells to anticancer drugs

Calcitriol has been shown to sensitize PCa to low temperature-induced, non-ice rupture-related cell death in vitro and in vivo\textsuperscript{36}. When calcitriol used with cryotherapy treatment, it significantly increased cell death and reducing cell viability via activation of apoptosis\textsuperscript{36}. Calcitriol enhanced antitumor effect of cryoablation by increasing necrosis and apoptosis and reduced cell proliferation. Thus, calcitriol could potentially be an applicable reagent as a freeze sensitizer to cryoablation\textsuperscript{40}. Radiotherapy, which is often a curative treatment option for PCa, however, also presents with a high risk of side effects at effective doses of ionizing radiation (IR). So when human androgen-insensitive DU145 PCa cells were pre-treated with a combination of 100 nM calcitriol and 1 mM sodium valproate, a well-tolerated histone deacetylase, led to sensitization of
the PCa cells to IR\textsuperscript{120}. This calcitriol pre-treatment with IR efficiently suppressed PCa cell proliferation and increased DNA double-strand breaks compared with non-pretreated cells. Combined pre-treatment of calcitriol with IR also resulted in 16 % enhancement in IR-induced activation of DNA damage CHEK2 compared to untreated cells. These molecular changes led to DNA replication blockade, S-phase cell cycle arrest and enhanced apoptosis. This combination approach could, therefore, be useful and allow for doses of radiation administered to cancer patients to be attenuated such that side effects are reduced\textsuperscript{60,121}.

Calcitriol enhanced the anti-proliferative and the cytotoxic effects of taxanes chemotherapy (e.g., paclitaxel and docetaxel) in vitro when used in a combination treatment to treat PC3 cell lines\textsuperscript{122}. This is thought to be due to its ability to reduce multidrug resistance-associated protein-1 expression and, therefore, inhibit transporter mediated efflux of docetaxel from cells\textsuperscript{123}. Calcitriol also sensitizes PCa cells by inducing apoptosis and slowing cell cycle and cell proliferation. The growth of PCa cell lines (LNCaP and DU145) were inhibited when cells were treated with a platinum compound (cis- or carboplatin), and its synthetic analogue, Ro 25-6760. However, the growth inhibition was further enhanced by calcitriol. The enhancement of inhibitory effect was greater when lower concentrations of these compounds were used in combination with higher concentrations of calcitriol. At IC\textsubscript{20} doses, calcitriol and a platinum compound acted in a synergistic manner to inhibit the growth of PC cells\textsuperscript{124}.

1.4.4.2 Calcitriol Combinations which act additively or synergistically with other anticancer agents on PCa cells

Historically, mitoxantrone combined with glucocorticoids was widely used to treat androgen-independent PCa\textsuperscript{125}. Calcitriol synergistically increased mitoxantrone/dexamethasone mediated growth inhibition of PC-3 cells in vitro\textsuperscript{126}. The combination also caused significantly
greater tumor regression in the PC-3 xenograft model system compared with treatment with mitoxantrone/dexamethasone or untreated controls\textsuperscript{126}.

There is increasing evidence that the isolation of a single compound from complex foods may not be effective in preventing cancer even when administered at toxic doses, whereas combination therapy using lower doses with no or lower toxicity might work. Genistein is a dietary-derived isoflavonoid found in high concentrations in serum after ingestion of soy-rich meals\textsuperscript{127}. Studies reported that low concentrations of 0.5µM genistein tested in vitro in combination with 0.1 or 0.5 nM calcitriol was synergistic in the inhibition of primary human prostatic epithelial and LNCaP PCa cell viability. However, treatment of cells with genistein or calcitriol alone had no significant effect on cell viability. In primary cells, the combination acted synergistically as both resulted in G (2) M and G (1/0) cell-cycle arrest, whereas in LNCaP cells the combination had similar effects compared to treatment alone\textsuperscript{128}. When concentrations higher than 5µM genistein were used in combination with 10 nM calcitriol, up-regulation of cell cycle inhibitor p21 levels were seen compared to treatment alone and genistein potentiated the effect of calcitriol regulation of VDR expression.

Calcitriol and 20% dietary soy protein were also examined in vivo as a combination diet in a mouse xenograft model of PCa\textsuperscript{129}. The combination diet resulted in a more substantial inhibition of tumor growth than treatment with either agent alone. Upon examination of the tumor tissue, it was deduced that potential mechanisms affected by combination treatment included up-regulation of several genes involved in prostate cell regulation such as anti-proliferative (p21, IGFBP-3) and pro-apoptotic (Bax) genes, down-regulating the anti-apoptotic (Bcl-2), compared to treatment with either calcitriol or 20% dietary soy alone\textsuperscript{129}. A significant enhancement was also observed in the combination treated group in the up-regulation of the expression of the prostaglandin G-degrading
enzyme 15-PGDH. However, the combination, on the other hand, had the potential to increase the risk of hypercalcemia as indicated by elevated expression of intestinal calcium absorption genes (TRPV6, calbindin-9k)\textsuperscript{129}. Laboratory studies have also indicated that calcitriol and dietary omega 3-polyunsaturated fatty acids act synergistically to inhibit the growth of the high passage androgen-independent PCa cell line, LNCaP-c115 at the level of the G(1)/S-phase transition and cell division\textsuperscript{69}. The combination of vitamin A and vitamin D synergistically reduced cell viability, expression of cyclin D1 and induced apoptosis by enhancing Bax protein expression\textsuperscript{130}.

The combination of cetuximab, which is an anti-epidermal growth-factor receptor antibody, with calcitriol efficiently, suppressed hormone-resistant DU145 PCa cell growth. The combination inhibited DU145 cell proliferation, caused considerable cell-cycle arrest in the Go/Gal-phase and enhanced apoptosis\textsuperscript{68}. The combined effect of calcitriol (10 nM) with ibuprofen, a well-known non-steroidal anti-inflammatory drug (NSAID) on LNCaP PCa cells was enhanced in vitro. This effect was found only to be additive. However, significant synergistic cell growth inhibition was achieved by combined treatment of calcitriol and ibuprofen in DHT-stimulated LNCaP cells. This combined treatment was effective in decreasing the cell transition from G1- to S-phase and enhanced apoptosis compared with the effect of single drugs\textsuperscript{120}. Neither calcitriol (1-10 nM) nor liarozole (1-10 mM) had any effect on DU145 cell viability when used alone. However, 10 nM calcitriol plus 1 mM liarozole acted synergistically and significantly reduced cell viability. Liarozole induced considerable increase in calcitriol half-life from 11 to 31 h. It also enhanced calcitriol activity by potentiating its ability to up-regulate VDR protein compared with the effects of either drug alone. The mechanism of interaction is due to the ability of liarozole to inhibit CYP24A1 activity, thus increasing calcitriol half-life and effect\textsuperscript{99}. 
Sodium butyrate and trichostatin A are inhibitors of histone deacetylases activities, which acted synergistically with calcitriol in DU-145 PCa cells by enhancing apoptosis\textsuperscript{131}. Moreover, in vitro calcitriol acted synergistically with IR to inhibit the growth of the LNCaP human PCa cell line by potentiating IR-induced apoptosis. At radiobiologically relevant doses of IR, calcitriol also showed synergistic inhibition of growth of LNCaP cells. However, at higher doses of IR, the combination resulted in moderate antagonism. The results suggested that the synergistic effect could permit a reduction in the dose of radiation given clinically and thus potentially reduce treatment-related morbidity\textsuperscript{132}.

In general, these combinations acted on common pathways as well as on independent pathways, thus increasing overall, anticancer effect. The molecular mechanisms of this PD interaction are mediated through alterations in regulatory pathways within the prostate that change and enhance overall, anticancer effects such as the increase in cellular apoptosis demonstrated when calcitriol combined with IR therapy, paclitaxel, docetaxel, vitamin A, genistein, cetuximab, ibuprofen, sodium butyrate and trichostatin A. Moreover, the interaction was mediated via the inhibition of calcitriol metabolism, which increases the bioavailability of endogenous and exogenously administered calcitriol, thereby synergistically enhancing its anticancer effects. Up-regulation of VDR by genistein and liarozole was observed as a result of higher levels of calcitriol reaching the tumor site following increased half-life of calcitriol (Table 1.1).

1.4.4.3 Combinations which sensitize PCa cells to anticancer effects of calcitriol

Some compounds have been reported to enhance the antitumor activity of calcitriol. The combination of S179D, which is a molecular mimic of naturally phosphorylated human prolactin and calcitriol, was shown to inhibit the growth PC3 and DU145 PCa cell lines in vitro and vivo\textsuperscript{62}. In addition, S179D sensitized the cells to calcitriol at concentrations below those that typically
result in hypercalcemia. This effect was demonstrated to be in part mediated via the induction of cell death, increased protein expression of VDR and p21\textsuperscript{62}. Androgen-independent PCa cells DU-145 and PC-3 are relatively insensitive to the anti-proliferative action of calcitriol. This is thought to be due to an increase in the calcitriol metabolism, as a result of CYP24 enzyme induction, which in turn leads to decreased anti-proliferative efficacy\textsuperscript{133}. In vitro studies supporting this hypothesis include a rat kidney mitochondria assay which incorporates CYP24A1 inhibition using 2-(4-hydroxybenzyl)-6-methoxy-3, 4-dihydro-2H-naphthalen-1-one and calcitriol that led to produce a greater inhibition of proliferation in DU-145 cells compared to single agent treatments\textsuperscript{133}.

Examination of the regulation of VDR target gene mRNA in DU-1455 cells revealed that co-treatment of calcitriol plus inhibitor of CYP enzymes co-ordinately up-regulated CYP24, p21 and GADD45alpha\textsuperscript{133}. When calcitriol was combined with a CK2 inhibitor, it enhanced calcitriol-mediated antitumor effects\textsuperscript{100}. The inhibition of CK2 by 5, 6, 7-tetramobenzimidazole, a protein kinase CK2 selective inhibitor, was also shown to inhibit CYP24A1 promoter activity induced by calcitriol in PC3 cells. Furthermore, the ability of calcitriol to induced CYP24A1 mRNA expression was reduced by using CK2 siRNA knockdown and this significantly enhanced calcitriol-mediated antiproliferative effects in vitro and vivo in a xenograft model. These observations suggested that protein kinase CK2 contributes to calcitriol mediated target gene expression and is involved in the regulation of CYP24A1 expression. To summarize, combining selected drugs with calcitriol sensitizes prostate cells to the actions of calcitriol by i) inhibiting its metabolism and ii) up-regulating VDR and its target genes, thus enhancing overall calcitriol antitumor activity.
<table>
<thead>
<tr>
<th>Drug/Agent combined</th>
<th>Calcitriol dose</th>
<th>Model used</th>
<th>Mechanisms PK/PD Based interaction</th>
<th>Pharmacological Outcomes &amp; Comments</th>
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<td>PC3</td>
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<td>↓ Bcl-2, ↑ Bax, MRP mRNA and protein levels</td>
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<td>DU145</td>
<td>↑ p21 mRNA</td>
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<td>↑ Calcitriol t½</td>
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<td>DU145</td>
<td>Bax mRNA and protein</td>
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<td>PC3</td>
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<td>Synergistically ↓ % Cell viability</td>
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<td></td>
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<td>↑ apoptosis</td>
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<td>↑ Caspase 8 activation</td>
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<td>Synergistically ↓ Tumor growth</td>
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<td>↓ PC3 tumor growth</td>
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<td>↓ Ki-67</td>
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<td>↑ Apoptosis ↑, caspase-3</td>
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<td>Transfected PC3 cells with siRNA-CK2</td>
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<td>Cryoablation</td>
<td>4.0 µg/kg of calcitriol (intratumoral injection)</td>
<td>Murine prostate tumors (RM-9) in male C57BL/6J mice</td>
<td>↓Ki-67 ↑apoptosis ↓Procaspase-9 ↑Procaspase-9 cleavage ↑caspase-3 cleavage ↓AKT</td>
<td>Sensitization ↑Necrosis ↑Apoptosis ↓Proliferation ↓Tumor volume</td>
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<td>Sodium valproate</td>
<td>Cotreatment 10 nM calcitriol + Radiotherapy</td>
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<td>↑DNA damage ↓DNA replication S-phase cell-cycle arrest ↑apoptosis</td>
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<td>S179D</td>
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<td>PC3 DU145</td>
<td>↑VDR ↓P21</td>
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<td>▼CYP24A1 activity ▲Calcitriol ( t_{1/2} ) (11 to 31 hr)</td>
<td>DU 145</td>
<td>Synergistically ▼cell viability</td>
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<td>Ginsenoside aPPD, aPPT</td>
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<td>▼CYP3A4 activity ▲VDR activity</td>
<td>Human microsomal protein</td>
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PK: Pharmacokinetic; PD: pharmacodynamic; CYP: Cytochrome P450; IC\(_{50}\): The half maximal inhibitory concentration; \( t_{1/2} \): half-life; VDR: vitamin D receptor; HPEC: Primary human prostate epithelial cells; CK2: Casein kinase 2; ki67: proliferation marker; BAX: pro-apoptotic protein; Bcl2: anti-apoptotic protein; VDBP: vitamin D-binding globulin; CDK: Cyclin-dependent kinases; P21: cyclin-dependent kinase inhibitor. TBBz: 4, 5, 6, 7-tetramethylbenzimidazole; S179D is a molecular mimic of naturally phosphorylated human Prolactin; aPPT: 20(S)-protopanaxatriol; aPPD: 20(S)-protopanaxadiol.
1.4.5 Summary of Clinical Studies

1.4.5.1 Combining calcitriol with cytotoxic agents

Although all preclinical data published so far strongly support the significance of calcitriol as an anticancer agent for prevention and/or treatment of PCa, its use as an anticancer agent alone or in combination has not yet been fully established clinically (Table 1. 2). A phase I clinical trial has shown that weekly dosing allows substantial dose-escalation of calcitriol, and in follow-up a phase II trial suggested that adding weekly high-dose calcitriol may enhance the activity of weekly docetaxel in patients with advance stage disease\textsuperscript{138}. Adding DN-101, a high-dose oral formulation of calcitriol designed for cancer therapy, to docetaxel treatment significantly enhanced the reduction in serum PSA levels in response to docetaxel. The use of high oral calcitriol combined weekly with docetaxel for the treatment of metastatic androgen-independent PCa (AIPCa) patients was recognised as safe and generally well tolerated compared with the toxicity related complications experienced with single-agent docetaxel treatment\textsuperscript{73,138-145}. Metastatic AIPCa patients were treated with 60 mg calcitriol orally in a combination with estramustine and docetaxel every 21 days for up to 12 cycles. High dose calcitriol (60 mg daily) appeared to be safe when added to this chemotherapeutic regimen and was well tolerated\textsuperscript{146}. When CRPC patients were given high weekly doses of calcitriol (0.5 µg/kg orally), in combination with docetaxel and zoledronic acid, half of the patients had a PSA response and this regimen was also well tolerated\textsuperscript{140}. In addition, the pharmacokinetics of either calcitriol or docetaxel was not affected by the presence of its companion drug. In an exploratory substudy, PSA and measurable disease response rates as well as time to progression and survival were also promising when compared with phase II studies reported in the literature for single-agent docetaxel for the same patient population\textsuperscript{143}. High dose oral calcitriol (0.5 µg/kg) used in combination with intravenous carboplatin in patients with
metastatic AIPCa was not associated with an increase in the response rate compared with the reported activity for carboplatin alone\textsuperscript{145}. The outcomes were similar to that expected with single-agent carboplatin with only one of seventeen patients in the study achieving a confirmed PSA declined response and no patient achieved the palliative response end point (2-point reduction or normalization of pain on the present pain intensity scale without increased analgesic consumption\textsuperscript{72} (Table 2).

1.4.5.2 Combining calcitriol with corticosteroids, NSAID

One year of dosing with weekly calcitriol and daily naproxen treatment to 21 patients with PCa relapse was well tolerated by most patients and effective in delaying PCa growth and progression according to a reduction in PSA doubling time was which was achieved in 75\% of patients. High-dose intermittent calcitriol plus dexamethasone appears to be safe, feasible, and also demonstrated antitumor activity\textsuperscript{64,147,148}. The combination effect of the daily calcitriol, dexamethasone and carboplatin in thirty-four patients with HRPCa also produced a PSA response in 13 of 34 patients. PSA was decreased in 13 treated patients, and the median overall survival was 97.7 weeks. However, significant side effects were reported\textsuperscript{144}. The combination of intermittent high doses (8, 10 and 12 mg) calcitriol plus dexamethasone in 43 patients with AIPCa caused slight PSA decline with minimum side effects. In this case, there was no clear evidence that combination treatment was better that dexamethasone treatment alone\textsuperscript{144}. Calcitriol, dosed in a pulsatile manner, was safe and tolerated when its toxicity was examined with zoledronate and the addition of dexamethasone at the time of disease progression\textsuperscript{149}. In patients with progressive PCa, adding dexamethasone to calcitriol at escalating doses from 4 µg up to 30 µg per day was well tolerated. In addition, when administered three times per week in combination with intravenous zoledronate (4 mg monthly) with or without dexamethasone. Patients tolerated therapy
well, even in those patients who received higher calcitriol doses of 30 µg treatment group; a maximum tolerated dose was therefore not definable. Cmax observed in the 24 µg and 30 µg cohorts ranged from 391 to 968 pg/mL with minimal antitumor effects observed. Cmax in the 24 µg and 30 µg cohorts were greater than the preclinical levels associated with antitumor effects. Conversely, adding calcitriol to mitoxantrone and prednisone in AIPCa patient did not cause any apparent enhancement of mitoxantrone toxicity (Table 1.2)
<table>
<thead>
<tr>
<th>Drug combined</th>
<th>Patient Type &amp; size</th>
<th>Calcitriol Dose or Formulation</th>
<th>Clinical Endpoint</th>
<th>Clinical Outcomes</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Docetaxel</td>
<td>AIPCa 37</td>
<td>calcitriol (0.5 µg/kg) weekly (Rocaltrol, Roche Pharmaceuticals, Basel, Switzerland)</td>
<td>PSA, Safety &amp; Efficacy</td>
<td>PSA ↓ n=30</td>
<td>No different in toxicity compared to docetaxel single treatment</td>
<td>No PK interaction</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>AIPCa 250</td>
<td>45 µg DN-101 weekly</td>
<td>PSA</td>
<td>PSA ↓ n=145</td>
<td>Survival time ↑</td>
<td></td>
</tr>
<tr>
<td>Docetaxel</td>
<td>Metastatic AIPCa 250</td>
<td>45 µg DN-101 weekly</td>
<td>PSA</td>
<td>PSA ↓ n=113</td>
<td>PSA ↔ n=113</td>
<td>Progress n=24</td>
</tr>
<tr>
<td>Docetaxel + Zoledronic acid</td>
<td>CRPC 30</td>
<td>Calcitriol (0.5 µg/kg orally in 4 divided doses over 4 h)</td>
<td>Safely &amp; efficacy</td>
<td>PSA ↓ n=23</td>
<td>Survival time ↑</td>
<td></td>
</tr>
<tr>
<td>Estramustine + Docetaxel</td>
<td>metastatic AIPCa 24</td>
<td>High dose calcitriol 60 µg orally</td>
<td>Safety &amp; Efficacy</td>
<td>Asymptomatic hypercalcemia n=4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naproxen open label</td>
<td>Relapse 21</td>
<td>High dose calcitriol (DN101, Novacea) 45 µg /week</td>
<td>Safely &amp; efficacy</td>
<td>PSA doubling time ↓ n=4</td>
<td>PSA doubling time ↑ n=14</td>
<td></td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>CRPC 18</td>
<td>i.v 74 µg weekly</td>
<td>PSA</td>
<td>PSA doubling time ↓ n=4</td>
<td>No patient had response Progress n=14 Toxicity n=7</td>
<td></td>
</tr>
<tr>
<td>Drug Combination</td>
<td>Disease Type</td>
<td>Dose</td>
<td>Response</td>
<td>Toxicity</td>
<td>Notes</td>
<td></td>
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<td></td>
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<tr>
<td>Dexamethasone + Carboplatin</td>
<td>HRPC 34</td>
<td>0.5 µg daily</td>
<td>PSA</td>
<td>PSA ↓ n=13</td>
<td>Significant adverse events. This regimen has shown a good response with an acceptable side effect profile.</td>
<td></td>
</tr>
<tr>
<td>Dexamethasone 43</td>
<td>AIPCa</td>
<td>Intermittent high different doses of 8, 10 and 12 µg 3 times a week for a month (Rocaltrol, Roche Pharmaceuticals, Indianapolis, IN)</td>
<td>Toxicity</td>
<td>Partial PSA↓ n=8</td>
<td>Toxicity was low. No clear different and superior to dexamethasone treatment alone. Calcitriol at high dose was safe, feasible, and had antitumor effects.</td>
<td></td>
</tr>
<tr>
<td>Dexamethasone + Prednisolone + Docetaxel</td>
<td>Metastatic CRPC 953</td>
<td>45 µg DN-101</td>
<td>Overall survival (OS), assessed By the Kaplan-Meier method.</td>
<td>Shorter survival than the control.</td>
<td>Clinical trial response failed. This failure might be due to either docetaxel regime or calcitriol dose.</td>
<td></td>
</tr>
<tr>
<td>Carboplatin</td>
<td>metastatic AIPCa patients 17</td>
<td>(0.5 µg/kg) repeated dose every 4 weeks</td>
<td>PSA Palliative response</td>
<td>PSA ↓ n=1 Partially PSA ↓ n=4 Pain reduction n=3</td>
<td>No different compare to single agent. No dose related toxicity. This might be due to low dose of calcitriol given.</td>
<td></td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>19 metastatic AIPCa patient</td>
<td>Calcitriol ((DN-101) 180 µg orally Every 3 weeks</td>
<td>PSA decline to half PSA↓ n=5</td>
<td>PSA↓ n=5 No different in Toxicity physical functioning↓ fatigue, insomnia, and diarrhea↑</td>
<td>This might be due to low dose of calcitriol given</td>
<td></td>
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</table>

RCT: Randomized control trial; HRPC: Hormone-refractory prostate cancer; CRPS: AIPCa: Androgen independent prostate cancer; RECIST: Response Evaluation Criteria in Solid Tumors; PSA: Prostate cancer antigen; IV: Intravenous; PK: Pharmacokinetics; Ref: References; CRPC: Castration-resistant prostate cancer; DN-101: a high-dose (15 µg) formulation of calcitriol.
1.5 PPD ginsenoside, its pharmacokinetic, anticancer effects in prostate cancer

Novel anticancer compounds derived from natural products present an attractive alternative to synthetic compounds, based on a long history of traditional clinical use, their favorable safety, patient tolerance and effectiveness profiles. Approximately 70 of FDA approved drugs are directly or indirectly comes from natural products. Plants are an important source of novel pharmacologically active compounds. There are many examples of commercially available drugs that are naturally derived from herbal traditional medicines such as (morphine, codeine, digoxin, quinine, pilocarpine and anticancer drugs like artemisinin and paclitaxel). As numerous side effects are associated with chemotherapy used in cancer patients, more patients are turning to Complementary and Alternative Medicine (CAM), such as ginseng, for relief of their symptoms or to supplement their current prescribed regimen. Traditional medicines can inhibit tumor growth and metastasis, prolong patients’ life span, and improve patients’ life quality.

Ginseng is a traditional Chinese medicine is used for its healing effects of various ailments. Ginseng consumption can be dated back more than a 1000 years in Asia. Ginseng is mainly cultivated and distributed in South Korea, China, United States of America and Canada and is consumed as a dietary supplement in various forms. Ginseng contains polysaccharides, phenolics, flavanoids and saponins (ginsenosides). Ginseng root extracts significantly reduce blood glucose levels. Studies have shown that ginseng polysaccharides stimulate the immune system. Ginseng also has antineoplastic, anti-stress and antioxidant activities. Phenolics and flavanoids in ginseng act as antioxidants. Ginsenosides are reported to be responsible for...

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2 A version of this chapter will be will be further revised and submitted for publication.
the majority of ginseng’s pharmacological activities on the CNS, CVS, endocrine and immune system\textsuperscript{160,164-166,168-174}.

Ginseng intake is correlated with decreased total cancer incidences\textsuperscript{175-178}. Several clinical trials have shown that ginseng extract alone and in combination therapy with chemotherapy significantly improved cancer related fatigues in cancer patients\textsuperscript{175-178}. The combination of ginseng polysaccharides (GSP) and dendritic cells has a greater effect on immune function than DCs alone in non-small cell lung cancer patients\textsuperscript{179}. Xie et al. reported on the combined effect of GSP and radiotherapy therapy (RT) on nasopharyngeal carcinoma (NPC) growth and patient immune function in total of 121 NPC patients. The results showed that RT-GSP dramatically improved immune function in NPC patients\textsuperscript{180}. Daily doses of COLD-fX (a ginseng extract that is commercially available in Canada) for up to 12 weeks was shown to reduce the severity of flu symptoms and significantly improved patients’ quality of life\textsuperscript{181,182}. Ginsenosides have been shown to have anticancer effects\textsuperscript{183-185}, which is elaborated in the following sections.

1.5.1 Ginsenosides

Ginsenoside are the active constituent of ginseng that are responsible for the anticancer, anti-oxidant, anti-depressant, anti-inflammatory, neuroprotective and anti-metabolic syndrome effects of ginseng\textsuperscript{186-188}. Ginsenosides are mostly concentrated in the root of ginseng\textsuperscript{189}. Ginsenosides composition varies between ginseng species, age, plant parts, cultivation strategies, and time of harvesting and preservation method\textsuperscript{186,190-192}. Standardized ginseng extracts or pure form of ginsenoside are the solution to reduce variability in preparation methods\textsuperscript{193}.

They are categorized into two major groups 20(s)-protopanaxatriols (PPT) and 20(s)-protopanaxadiols (PPD) distinguished by the carboxyl group on C6\textsuperscript{194} (Figure 1.5). Ginsenosides have steroid like structure consisting of four rings and sugar moieties attached to C3, C6 and C20
positions. Side chains of different ginsenosides contain different sugar moieties at different sites that create variation among their structure and pharmacological effects\textsuperscript{195,196}. The amphipathic and lipid soluble nature of many ginsenosides allows it to be integrated into the plasma membrane easily and interact with several membrane receptors and activate cellular responses\textsuperscript{197}. However, many ginsenosides have poor permeability across cell membranes due to their bulky sugar moieties. They need first to undergo transformation to become biologically available metabolites. The binding site and number of hydroxyl groups can determine the way ginsenosides insert into the plasma membrane, biological activity and potency of pharmacological effect\textsuperscript{198}.

![Chemical structures of 20(S)-Protopanaxadiol (aPPD) and Rh2](image)

**Figure 1.5.** Chemical structures of 20(S)-Protopanaxadiol (aPPD) and Rh2

1.5.2 Rh2 and aPPD

Following oral administration of ginseng, the bacteria and stomach acid in gastrointestinal tract degrades Rh2 (the naturally occurring ginsenoside) through deglycosylation to aPPD, the aglycone of Rh2\textsuperscript{199} (Figure 1.6). The chemical structure of Rh2 consists of a glucose sugar moiety attached to steroid nucleus (Figure 1.5). Our research group has studied ginsenosides Rh2 and aPPD for over a decade\textsuperscript{47-53}. We demonstrated its pharmacokinetics, anticancer effects alone and
in combination with other anticancer agents in numerous preclinical PCa models\textsuperscript{193,200-206}. Rh2 and aPPD pharmacokinetics and anti-prostate cancer effects will be discussed in following sections. In addition, its potential PK/PD interaction with other drugs.

Figure 1. 6. Conversion of Rh2 to 20(S)-Protopanaxadiol (aPPD) in small intestine by microbiota.

1.5.3 Rh2 and aPPD Pharmacokinetics

1.5.3.1 Absorption and distribution

The parent ginsenosides usually have low bioavailability due to their large molecular weight and poor membrane permeability\textsuperscript{207,208}. Rh2 has very poor transmembrane permeability and when Rh2 was given orally, more potent anticancer, bioavailable aglycon PPD was formed\textsuperscript{209}. Carrier-mediated transport, especially efflux ABC transporters is evident in the transportation of Rh2 in the intestine\textsuperscript{210}. Rh2 intracellular accumulation increased when p-glycoprotein were inhibited\textsuperscript{211}. Xie et al. showed Rh2 and aPPD uptake increased markedly when coadminstrated with CYP3A4 and P-glycoprotein (P-gp) inhibitors (verapamil or cyclosporine) compared to control in the human epithelial Caco-2 cell line \textsuperscript{212}. Cyclosporine and verapamil are known to inhibit both hepatic and intestinal CYPs and efflux transporter\textsuperscript{213-215}. 
A novel oral dosage formulation of Rh2 and aPPD was designed by our laboratory. When 120 mg/kg Rh2 was administered orally to nude mice in -propylene glycol-ethanol – water (7:2:1 ratio) solvent. After oral administration to PC-3 xenograft nude mice, Rh2 was found to be structurally intact when isolated from prostate tissue. Emulsification of ginsenosides into lipid based formulations and suppression of p-glycoprotein efflux system resulted in bioavailability increased. Voruganti et al. showed the encapsulation of 25-OCH3-PPD (GS25) in nanoparticles (GS25NP) enhanced its oral bioavailability, increased its half-life to 7 hrs from 2.1 hrs, tumor uptake of GS25NP was 8-fold higher compared to GS25 in CD-1 mice models. as a consequences better anticancer efficacy was seen in human PCa models in vitro and in vivo.

1.5.3.2 Metabolism

Multiple metabolic enzymes such as CY3A4, the sulfotransferases, UDP-glucuronosyltransferases and the glutathione S-transferases reside in the Caco-2 cell monolayer. An inhibition of Rh2 and aPPD’s metabolism can resulted in a high bioavailability. In addition, extracellular pH influences Rh2 and aPPD cellular uptake. While testing pH range of 5.0- 8.0, Rh2 and aPPD uptake were maximum at pH 7.0 and pH 8.0, respectively

Rh2 aPPD and aPPT are a substrate for CYP3A4 and can undergo oxidative metabolism and dehydrogenation. Li et al. reported CYP3A4 as the major enzyme involved in metabolising Rh2 with less than 5% production mediated by CYP3A5. Ketoconazole, (CYP3A inhibitor) inhibits CYP3A mediated Rh2 oxidative metabolism. We have also shown previously that CYP3A4 and CYP3A5 isoforms are the predominant enzymes responsible for oxygenation of aPPD in Human Intestine Microsomes (HIM) and Human Liver Microsomes (HLM) 220. After the incubation of aPPD, three major monoxygenated metabolites and five minor dioxygenated
metabolites were identified using LC-MS\textsuperscript{220}. We conducted also enzyme inhibition studies using KTZ and SKF, the formation of aPPD monooxygenated metabolites inhibited by 85-95\% and 91-97\% respectively. Chiu et al. also evaluated aPPD biotransformation using 12 human recombinant P450 enzymes and found that CYP3A4 and CYP3A5 were the major enzymes involved in aPPD transformation to monooxygenated metabolites\textsuperscript{220}.

1.5.3.3 Excretion

After oral administration, aPPD was found only in trace amounts in the urine, which suggests that PPDs are most likely excreted in the feces\textsuperscript{221}. Metabolites of aPPD were found in feces and urine\textsuperscript{222,223}. However, Hu et al. reported that aPPD and aPPT only were found in the bloodstream and not in the urine. Qian and Cai\textsuperscript{224} conducted \textit{in vivo} PK studies in rats, a dose of 100 mg/kg Rh2 to rats and rat feces were sampled between 0 to 48 hours. The deglycosylated and oxygenated metabolites were detected in the feces determined by LC-MS-MS analysis. Human subjects were given an oral dose of Sanqi extract, the dried root of Panax notoginseng, then plasma and urine samples were analyzed for ginseng metabolites. From the urine analysis except Rh2 and Rg3 other ginsenosides including aPPT and aPPD were excreted in the urine\textsuperscript{219}.

1.5.4 aPPD Pharmacodynamic

The amphipathic and lipid soluble nature of Rh2 and aPPD allow them to be integrated into the plasma membrane easily with membrane lipids, change the membrane composition and fluidity, interact with receptors and activating cellular responses via cell signalling cascades\textsuperscript{197}. Rh2 and aPPD can bind to multiple targets, performing a variety of biological functions and elicit different cellular responses, which can explain their complex pharmacological effects\textsuperscript{186,225}. They are potential ligands for steroid receptors such as glucocorticoid receptors (GR), AR and estrogen receptors (ER)\textsuperscript{164,193,226}. Rh2 and aPPD can bind to effector molecules within the nucleus in order
to produce transcriptional changes and consequently, changes in protein synthesis. This property is responsible for their antineoplastic effects where the cell cycle is arrested due to transcriptional changes of interactions certain cell cycle regulators\(^{227}\). aPPD binds and inhibit AR signaling \textit{in vivo} in different models of PCa\(^{200,228-230}\). As reported previously\(^{200,206,231,232}\), aPPD can lead to increased levels of p21 and p27, leading to decreased cyclin dependent kinase activity and subsequent arrest cell cycle in G1 phase. aPPD-induced apoptosis via activation of the Bax/caspase-3 pathway in PCa models \textit{in vitro}\(^{206,233,234}\). aPPD has multiple anticancer activities which have both anti-proliferative and pro-apoptotic mechanisms. In addition, its metabolite 25-OH-PPD significantly induced apoptosis by upregulating Bax, causing an increase in cleaved caspase-3 via binding and downregulation of MDM2 oncprotein in PC-3 xenograft tumors (AR negative)\(^{204}\). MDM2 is a potent negative regulator of p53 via enhancement of P53 protein degradation\(^{235}\). MDM2 also has p53-independent functions in cellular differentiation processes and signaling and is known to interact with AR protein\(^{236}\). Tovar et al\(^{237}\) have shown that MDM2 antagonist (nutlin-3a) in combination with androgen depletion \textit{in vitro} and \textit{in vivo} additively increased apoptosis and further downregulated AR expression in AR positive LNCaP and 22Rv1 (androgen-independent) cell lines. This was secondary to p53 activation\(^{237}\). MDM2 antagonism also led to a greater tumor regression and dramatically increased survival in LNCaP-bearing nude mice (p53 wild type PCa)\(^{237}\).

\subsection*{1.5.5 Summary of preclinical studies}

Several studies have shown that aPPD provides anticancer benefits via multiple pathways (Table 1.1). aPPD inhibited the cell growth and induced apoptosis in different tumor-cell lines such as B16 melanoma mouse cells, human ovarian cancer cells, human prostate cells, and human breast-cancer cells MCF7 and MDA435/LCC6\(^{206,238}\). Li et al. compared the efficacies of naturally
occuring ginsenosides (NOGs) and intestinal bacterial metabolites (IBMs) on PC3 cells and found that IBMs were more potent than NOGs (aPPD IC_{50} 22.5±2.9 µM)\textsuperscript{239}. aPPD (25µM) and PPT (50µM) treated for 48 hrs showed a greater apoptosis index with 3 and 4 fold increases respectively. aPPD and aPPT treatment resulted in a decrease in proliferation index of 63.0% and 36.1% respectively compared with controls. In addition, 25µM PPD and 50µM PPT arrested cells at S-phase by 32.9% and 27.7% respectively compared to control cells (18.5%). Western blot analysis also revealed that cyclin D1 and cyclin A expression were reduced after treatment with PPD and PPT. Overall, IBMs reduced survival rate, inhibited proliferation, induced apoptosis and arrested cell cycle\textsuperscript{239}.

In previous studies carried out in our laboratory, treatment with Rh2 (aPPD parent ginsenoside, Figure 1.5) at a dose of 120 mg/kg alone for 40 days suppressed the growth of the LNCaP and PC3 growth\textsuperscript{200,201,203}. Liu et al. also reported that 20(S)-Rh2 inhibited LNCaP, PC-3 and DU145 cell growth by 70%, 40% and 20% respectively\textsuperscript{240}. Rh2 inhibited the cell growth via G1 phase-specific cell cycle arrest which resulted in the suppression of cdk2 and cyclin dependent histone kinase activities as a result of p21 up regulated, which binds and inactivates cdk2 \textsuperscript{206,238}. Recently, Wang \textit{et al.}\textsuperscript{206,231} also investigated possible mechanisms responsible for the antiproliferative, pro-apoptotic, and cell cycle arrest effects of Rh2 against LNCaP androgen-sensitive and PC-3 androgen-insensitive PCa cells. \textsuperscript{197,222} Rh2 increased the expression of the cdk inhibitor p21 and tumor suppressor protein p53, while decreased protein levels associated with cell proliferation such as cdk2, 4, and 6 and up regulated pro-apoptotic proteins (cleaved PARP, cleaved caspase-3, -8, and -9). Kim et al. tested the effects of ginsenoside Rh2 on LNCaP and PC-3 prostate cancer cells and showed that Rh2 at doses of 20 to 200 µM had significant DNA
synthesis inhibition in LNCaP and PC-3 cells\textsuperscript{241}. In LNCaP cells, Rh2 exhibited IC\textsubscript{50} values of 4.4 µM, while 5.5 µM in PC-3 cells\textsuperscript{241}.

Cao et al. reported the effect of aPPD on the expression of AR and its target genes\textsuperscript{229}. aPPD downregulated full length (AR-FL) and AR splice variants (AR-Vs) in an androgen deprived condition in 22Rv1 and CWR-R1 cells. After 6 hrs and 12 hrs treatment \textit{in vitro}, aPPD inhibited AR activity by 36\% and 66\% respectively in a time-dependent manner. After \textit{in vivo} treatment of aPPD dosed orally at 40mg/Kg for 6 days per week, aPPD significantly reduced tumor size by \textasciitilde 67\% in castration resistance LNCaP cell models. aPPD also was shown to reduce PSA levels by 50\% as dropped from 173 ng/ml/g in the control group to 97 ng/ml/g in the aPPD group. Additionally, aPPD group did not cause acute toxicity in the mice as measured as no change in body weight. IHC analysis of the AR protein expression was significantly downregulated with aPPD treatments. Interestingly, aPPD inhibited tumour growth by approx. 35\% compared to enzalutamide (which did not inhibit tumor growth in either doses used 10 and 30 mg/kg) when aPPD efficacy was compared to enzalutamide in castration resistant 22Rv1 xenograft models in which treatment with 40mg/Kg aPPD compared to 10 or 30mg/Kg enzalutamide, were given orally for 6 days per week. The results of this study showed that aPPD is effective in reducing tumor growth, suppressing PSA levels and exhibited strong suppression of AR and its splice variants in castration resistant tumors\textsuperscript{229}. AR is a major driving force in the development and progression of PCa and expression of AR splice variants is one of the major mechanisms of CRPC development\textsuperscript{29,242}. This highlights the potential of aPPD in PCa prevention and/or therapy\textsuperscript{228,229}.

Wang et al. studied the effects of 25-OCH3-PPD (aPPD metabolites) on \textit{in vitro} and \textit{vivo} cancer models of LNCaP and PC-3 cells\textsuperscript{231}. Comparing 25-OCH3-PPD with aPPD, Rh2 and Rg3 in both models, they found that 25-OCH3-PPD had the lowest IC\textsubscript{50} value of 12.0µM and 5.6 µM
in LNCaP and PC-3 cell lines respectively compared to PPD’s 44.8 µM and 29.3 µM, Rh2’s 46.7µM and 35.7µM, and Rg3’s 302.1µM and 266.5µM in LNCaP and PC-3 cell lines respectively. At the concentration of 25 µM, 25-OCH3-PPD inhibited LNCaP cell proliferation and PC-3 proliferation by 40% and 80% respectively, rendering PC-3 more sensitive to 25-OCH3-PPD. 25-OCH3-PPD arrested cell cycle at G1 phase in both LNCaP and PC-3 cells at the lowest concentration of 25µM and induced apoptosis by 6-fold increase in LNCaP cell apoptosis and 10-fold increase in PC-3 cell apoptosis at 50 µM concentration. The researcher claimed 25-OCH3-PPD had the most potent effects compared to other ginsenosides native to ginseng. 25-OCH3-PPD, PPD and Rh2 had similar effects on down-regulation of the expression of proliferation related proteins MDM2, E2F1, cyclin D1 and cdk5,4 and 6, and up-regulated p21 and p27, whereas Rg3 failed to change expression levels except for a slight increase in p21 expression. Apoptosis related proteins such as cleaved PARP and cleaved caspases were up-regulated and caspases- 8 and 9 were activated by treatments in LNCaP cells after 72 hrs. 25-OCH3-PPD was very effective in reducing PSA secretion in a concentration-dependent manner. 25-OCH3-PPD arrested cells at G2/M stage in a dose-dependent manner in MDA-MB435 and C4-2B cell lines (IC50 7.54 to 25.91µM, but did not affect the normal cells).

Wang et al. studied the anti-cancer effects of 25-OH-PPD on LNCaP, PC-3 cells in vitro and in vivo in mouse PC-3 xenograft models. 25-OH-PPD reduced survival of LNCaP and PC-3 cells in the concentration dependent manner. LNCaP cells were more sensitive to the effects of 25-OH-PPD compared to PC-3 cells. 25-OH-PPD at 50µM concentrations inhibited the proliferation of PC-3 cells almost fully and LNCaP cells by about 40% at 100µM 25-OH-PPD concentration. Apoptosis related proteins such as Bax and PARP expression significantly increased and Bcl expression was decreased after 25-OH-PPD treatments. As a result, the activities...
of caspases 3 and 9 suggest increased apoptotic response. A dose induction in apoptotic index was seen in LNCaP cells with a 3-and 10-fold increase respectively after 50 and 100μM 25-OH-PPD treatments. In addition, 100μM 25-OH-PPD had 18-fold increase in apoptosis in PC-3 cells compared to control. 25-OH-PPD arrested LNCaP cells and PC-3 cells at G1 phase in a dose-dependent manner. 25-OH-PPD upregulated p21 and p27 expressions and significantly downregulated the expression of cdks 2, 4 and 6, MDM2, E2F1 and cyclin D1. However, p53 expression did not change. In vivo administration of 5, 10 and 20 mg/Kg/day for 3 days per week in PC-3 xenograft mice, showed significant decrease in tumor growth by 66% at the 20 mg/kg dose. In another study when 25-OH-PPD was administered (10mg/kg/day) for 5 days/week, 50% growth inhibition was observed. Additionally, these doses did not cause any significant weight loss in mice which indicated minimal, if any, acute toxicity.

![Figure 1.7. Mechanism of action of aPPD as an anticancer agent (Focus on prostate Cancer)](image-url)
1.5.6 aPPD Drug Interactions

1.5.6.1 Pharmacokinetic Interactions

Most immunocompromised patients including cancer patients take natural products with their regular medications for different purposes. As mentioned above, ginseng has been used historically as part of TCM to enhance the immunity and improve fatigues associated with chemotherapy use in cancer patients. There are examples of CYP and transporter substrates/drugs that are commonly used in the treatment regimens of patients with cancer including ketoconazole, tamoxifen, taxanes, as well as in immunocompromised patients to treat AIDS (e.g., ritonavir, clarithromycin), as well as inflammatory disorders (e.g., dexamethasone, prednisone).

Studies have reported that CYP are overexpressed in tumor tissues compared with normal tissues\(^{85,97,243}\). Tumors expressing high levels of the CYP3A4 isoform specifically are likely to have a poor treatment response to drugs\(^{85,97,243}\). Recently we have shown that aPPD and aPPT, strongly inhibited CYP3A4 activity in vitro and could potentially therefore cause a pharmacokinetic interaction which has the potential to yield additional benefits to patients with cancer when used in combination\(^{81}\). Liu et al.\(^{244}\) also showed that Rh2 exhibits inhibitory activity against CYP3A4 with an IC\(_{50}\) of 94.1 +/- 7.9 \(\mu\)M. Based on this enzyme inhibition kinetics experiment reported by Liu et al.\(^{244}\) it was determined that aPPD strongly inhibits 6-beta hydroxylation of testosterone in HLM (mediated by CYP3A4 in human liver microsomes) as well as cDNA expression of CYP3A4\(^{244}\). They found that there was no time-dependent or NADPH-dependent inactivation of CYP3A4 by either Rh2 or aPPD\(^{244}\). aPPD was shown to inhibit CYP3A4-mediated testosterone 6-beta hydroxylation activity with an IC\(_{50}\) value of 14.1 +/- 2.3 \(\mu\)M. aPPD was also shown to inhibit CYP2C9 with an IC\(_{50}\) of 42.7 +/- 2.2 \(\mu\)M when evaluated in human liver microsomes. However, aPPD also weakly inhibited CYP1A2, CYP2A6 and CYP2D6...
with an IC$_{50}$ value of $>$100 $\mu$M. Preliminary results in our laboratory determined that aPPD has the ability to inhibit CYP17A1 and CYP11A1, which are the vital enzymes for androgen synthesis from cholesterol in CRPC$^{245}$. As a consequence aPPD may also inhibit de novo synthesis of androgens, a known treatment resistance phenomenon observed in advanced CRPC patients. As described earlier, this CYP17A1-mediated steroidogenesis inhibition can be used in late stage PCa treatment potentially providing additional anti-cancer benefits to PCa patients$^{80}$. Studies also reported that Rh2 can inhibit CYP3A4 activities$^{218,246-249}$. CYP3A4 impacts drug bioavailability and inhibitors of CYP3A4 such as Rh2 and aPPD might be able to increase bioavailability of coadministered drugs. Theoretically, inhibition of CYP enzymes could result in higher drugs levels in the target tumor tissues, thus enhancing their anticancer effect. Although, the potential for the development of related side effects and toxicity is also possible. Kim et al. reported that aPPD inhibits the enzymatic activity of several Uridine 5'-diphospho-glucuronosyltransferase (UGT), major players in the human phase II drug metabolism.$^{250}$ Other studies showed that structure-dependent inhibition is related to the inhibition of ginsenosides with UGT isoforms$^{251}$. UGT enzymes express in high levels in the liver and also intestine which is known to have an impact on the first-pass and enterohepatic cycling of drugs. These processes may be impacted by UGT substrates when concomitantly administered with aPPD. Whether aPPD interferes with other phase II enzymes remains to be evaluated.

aPPD has been reported to inhibit P-gp transporter and CYP3A4 activity in human subjects$^{252,253}$. Rh2 and aPPD mediated inhibition of Pgp and breast cancer resistance protein (BCRP) increases the oral availability of coadministered drugs, thus enhance its anticancer effects by modulating its pharmacokinetics and reverse the multidrug resistant characteristics of cancer cell$^{252,253}$. In addition, Jin et al. examined the effect of aPPD and PPT containing ginsenosides on
BCRP, additional member of the ABC transporter family. Similar to Pgp and multidrug resistance-associated protein (MRP), BCRP overexpression is evident in cancer cells and BCRP has an important role clinically in multidrug resistance (e.g. mitoxantrone, methotrexate, topotecan and flavopiridol). aPPD was the most potent followed by Rh2 and PPT as inhibitors of BCRP. Methotrexate efflux and cytotoxicity to human breast carcinoma MCF-7/MX cells was significantly enhanced by Rh2 and aPPD co-treatment. Thus, inhibition of BCRP could potentially increase the oral bioavailability, slow its elimination from the body and enhance chemotherapeutic effects in cancer patients by modulating its pharmacokinetics. The combination of P-gp and CYP3A4 inhibition procured by Rh2 and aPPD could result in significant increases in bioavailability for co-administered drugs when given in combination. We also cannot exclude the possibility of changes in function or expression of other transporters by Rh2 or aPPD, as they are involved in the absorption and clearance of some anticancer agents. Jiang et al. reported that aPPD is a potent inhibitor of human OATP1B3.

There is insufficient clinical pharmacokinetic data available for aPPD to conclude human levels. Our lab and Li et al. have shown after oral administration of aPPD (50 to 70 mg/kg) to mice, an average aPPD plasma concentrations was 20 µM. Due to inter-individual variability in gut microbiota, CYP levels and co-administered drugs, aPPD exhibits different pharmacokinetics depending on its commercial preparation and duration of intake. The use of aPPD may result in inhibition of CYP3A4, eliciting a so-called ‘drug–herb interaction’. On the other hand, Malati et al. evaluated the pharmacokinetic effects of administered ginseng in healthy volunteers taking midazolam (CYP3A4 substrate) and 120 mg fexofenadine (Pgp substrate). A single dose pharmacokinetic study was conducted with 12 health participants, 500 mg of ginseng extracts was taken twice daily for 28 days, then a single oral dose of 8 mg midazolam or 120 mg
fexofenadine was given. Only midazolam pharmacokinetics was decreased by co-administration of ginseng. American ginseng did not affect fexofenadine pharmacokinetics. Based on these studies, Panax ginseng appeared to inhibit CYP3A activity and patients taking Panax ginseng in combination with CYP3A substrates should be monitored closely for side effects, perhaps potential additive or synergism therapeutic responses can occur. This is particularly important if ginseng was given with a drug has a narrow therapeutic index.

1.5.6.2 Pharmacodynamics Interactions

Studies carried out in our laboratory have shown Rh2 and aPPD demonstrated additive or synergistic anticancer effects when combined with other anticancer drugs such as docetaxel or paclitaxel, and tamoxifen to reduce tumor growth in human PCa and breast cancer models, and with tamoxifen to exert an anti-proliferative effect on MCF-7 human breast cancer cells. We have shown that Rh2 and paclitaxel acted synergistically in cultured LNCaP cells. In the same study, the oral treatment of LNCaP tumors by Rh2 produced a significant decrease in tumor growth which was accompanied by a significant decrease in serum prostate specific antigen (PSA) when combined with intravenously administered paclitaxel.

Xie et al. have shown that when Rh2 was given in combination with paclitaxel and mitoxantrone, Rh2 significantly synergistically enhanced their cytotoxicity on multidrug-resistant breast cancer cell lines. Rh2 action was mediated via inhibiting paclitaxel and mitoxantrone efflux and enhanced its uptake. Jin et al. have reported that Rh2 and PPT are inhibitors of BCRP, Methotrexate cytotoxicity to human breast carcinoma MCF-7/MX cells was significantly enhanced when combined with Rh2 and aPPD. Mitoxantrone, methotrexate are both BCRP substrate and paclitaxel is Pgp substrate. We reported previously that Rh2 acted either additively or synergistically when combined with docetaxel in three different prostate cancer cell
lines (PC-3, DU145 and C4-2) *in vitro* \(^{201}\). In addition, Rh2 and docetaxel combination *in vivo* caused established PC-3 tumors to regress by 15\% compared to their initial tumor size, and cell proliferation rate (measured by Ki-67 positive cells) was significantly lower for combination treatment \(^{201}\). Daily oral administration of Rh2 to nude mice bearing human ovarian cancer xenograft caused an inhibitory effect comparable to a weekly administration of 4 mg/kg of cisplatin \(^{203}\).

Wang et al. reported that combination treatments of 25-OH-PPD IP at dose of (20 mg/kg, 3 times per week for 4 weeks) along with Taxotere™ (15 mg/kg) revealed a growth inhibition percentage increase from 70\%, Taxotere™ alone, to >98\% in combination in PC3 model of PCa. 25-OH-PPD (20 mg/kg) in combination with gemcitabine (160 mg/kg) increased tumor growth inhibition from 80\%, gemcitabine alone, to >92\% in combination. In another study, 25-OH-PPD along with radiation increased growth inhibition effect from 40\%, radiation alone, to 75\% in combination\(^{206}\). Wang et al. shows that combination therapies using conventional chemotherapy drugs such as Taxotere™ and Gemcitabine with 20-OCH3-PPD increased the efficacy of tumor growth reduction from 69\% and 80\% in alone treatments to 94\% and >96\% in combination treatments. Additionally, they reported that apart from temporary weight loss, the mice models did not suffer from long term weight loss or demonstrate other observable toxic effects\(^{231}\).

Fung et al. conducted a randomized, double bind, controlled clinical trial with 25 health participants to test the anti-platelet and anti-coagulant effects of Curcuma longa, Angelica sinensis and Panax ginseng. The study tested three phases of treatment of only herbal product, only aspirin and herbal product plus aspirin. The results showed that the herbal products did not have clinically relevant anticoagulant or anti-platelet effects indicating that use of these herbal products does not include a risk of bleeding\(^{260}\).
<table>
<thead>
<tr>
<th>Agents</th>
<th>Model used</th>
<th>Mechanisms of action</th>
<th>Pharmacological outcomes</th>
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<tr>
<td>12-Chloracetyl-aPPD</td>
<td>LNCaP</td>
<td>Production of reactive oxygen species</td>
<td>↓ cell viability (concentration dependent)</td>
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<td></td>
<td>C4-2B</td>
<td>G2/M cell cycle arrest</td>
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<td></td>
<td>PC3</td>
<td>↓ MDM2 expression</td>
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<td></td>
<td>DU145</td>
<td>↑ p53 expression</td>
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<td>↑ apoptosis</td>
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<tr>
<td>aPPD</td>
<td>LNCaP</td>
<td>↓ cell viability</td>
<td>Synergistically acted with calcitriol to ↓ cell viability</td>
<td>200</td>
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<td></td>
<td>C4-2</td>
<td>↓ cell proliferation</td>
<td>and proliferation</td>
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<td></td>
<td></td>
<td>↓ CDK2</td>
<td>Induces apoptosis</td>
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<td>↓ AR expression</td>
<td>Inhibits cell cycle</td>
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<td>↑ VDR expression</td>
<td>↓ IC50</td>
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<td>↑ BAX</td>
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<td></td>
<td></td>
<td>↑ cleaved caspase-3</td>
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<td>Encapsulated 25-OCH3-PPD (GS25NP)</td>
<td>LNCaP</td>
<td>↑ Bioavailability tumor side</td>
<td>Nano-formulation of PPD improved its oral bioavailability and</td>
<td>204</td>
</tr>
<tr>
<td></td>
<td>PC3</td>
<td>↓ IC50 values</td>
<td>anticancer efficacy.</td>
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<td></td>
<td>DU145</td>
<td>↓ AR, ↓ PSA</td>
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<td></td>
<td>Nude mice bearing PC3</td>
<td>↑ PARP</td>
<td>Nano-formulation of PPD enhanced its anticancer efficacy.</td>
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<td>↑ P53, ↑ Bax</td>
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<td>↓ tumor volume</td>
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<td>↑ Bax, ↑ PARP</td>
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<td>↓ MDM2</td>
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<td>aPPD</td>
<td>LNCaP + castration-resistant 22Rv1</td>
<td>↓ AR expression and activities</td>
<td>Inhibition of progression to castration-resistant</td>
<td>228</td>
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<tr>
<td></td>
<td></td>
<td>↓ tumor growth</td>
<td>Delayed castration-resistant regression</td>
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<td>↓ full-length AR</td>
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<td>Compound</td>
<td>Cell Lines</td>
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<tr>
<td>aPPD</td>
<td>LNCaP, PC-3, DU145, C4-2</td>
<td>↓ AR splice variants, ↓ PSA, ↑ proteasome-mediated degradation AR, ↓ full-length AR, ↓ AR splice variants</td>
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<td>aPPD</td>
<td>LNCaP, PC-3, DU145, C4-2</td>
<td>↓ tumor growth, ↑ BAX, ↓ Ki67, aPPD is a stable compound which can be used for oral gavage formulations</td>
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<tr>
<td>Rh2</td>
<td>PC-3, LNCaP, DU145, C4-2</td>
<td>↓ tumor growth, ↑ BAX, ↓ Ki67, ↓ cell proliferation rate, ↑ apoptosis</td>
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<td>Rh2</td>
<td>PC-3, LNCaP, DU145, C4-2</td>
<td>↓ cell viability, 20(S)-Rh2 only showed growth inhibition effect, Stereochemistry is important</td>
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<tr>
<td>aPPD PPT</td>
<td>PC-3</td>
<td>↑ survival rate, ↓ proliferation, ↑ apoptosis, cell cycle arrest, IBMs exhibited more anticancer activities activity than NOGs</td>
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<tr>
<td>Rh2 PPT</td>
<td>PC-3</td>
<td>↑ p21, p27, ↑ BAX, ↑ PARP cleavage and activated caspases, ↓ MDM2, EFF2, Bcl2, cdk2/4/6 and cyclin D1, 25-OH-PPD &gt; 25-OH-PPT antiproliferative effects arrest of G1 phase cell cycle</td>
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<tr>
<td>Rh2 Rg3</td>
<td>PC-3</td>
<td>↓ MDM2, E2F1, cyclin D1, cdk2/4, Most potently decreases survival rate, ↓ proliferation, ↑ apoptosis</td>
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229, 203, 201, 240, 239, 206, 231
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<tr>
<td>Rh2</td>
<td>LNCaP, PC-3</td>
<td>↑ cell detachment, ↓ proliferation, ↑ MAP kinases activity</td>
<td>↓ cell growth</td>
</tr>
<tr>
<td>Rh2</td>
<td>PC-3, Xenograft tumors</td>
<td>↓ tumor growth, ↓ Ki67</td>
<td>The novel formulation of Rh2 is a stable and bioavailable after oral gavage</td>
</tr>
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<td>aPPD, aPPT, Rh2 + calcitriol</td>
<td>Human, liver and intestine</td>
<td>Strongly attenuated hydroxylation of the dihydroxy metabolite of Vitamin D3</td>
<td>Inhibits CYP3A4-mediated catabolism of active Vitamin D3</td>
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<tr>
<td>Rh2+ docetaxel</td>
<td>PC-3, LNCaP, DU145, C4-2</td>
<td>↓ tumor growth, ↓ Ki67</td>
<td>Synergistic effect. ↓ tumor cell proliferation rate</td>
</tr>
<tr>
<td>Rh2+ Paclitaxel</td>
<td>LNCaP cells</td>
<td>Apparent but not significant effect of proliferation markers on LNCaP cells</td>
<td>Decreases tumour growth that is more effective than individual components</td>
</tr>
</tbody>
</table>

Mouse double minute 2 (MDM2), mitogen-activated protein (MAP), natural occurring ginsenosides (NOGs), intestinal bacterial metabolites (IBMs)
1.6 Rationale

Due to the hypercalcemic side effects of vitamin D and its derivatives in higher plasma concentrations, the anti-cancer benefits of calcitriol have not been fully realized in PCa chemoprevention and/or treatment. From our experience in natural health product research, it is evident that tissue-specific sensitization of vitamin D actions by concomitant use of natural products is an effective way of blocking the development or progression of PCa. We have examined a class of naturally derived ginsenoside molecules that not only inhibit the cytochrome P450-mediated catabolism and deactivation of calcitriol, but also target key cell signaling pathways involving AR and steroidogenesis known to be dysregulated in PCa while enhancing VDR expression. VDR has been shown to have an anti-cancer role and so enhancing its expression/activation may be particularly relevant in the treatment of PCa. By virtue of their multi-targeting potential, it is not surprising that ginsenosides have highly pleiotropic therapeutic activities and are of current clinical relevance. A series of ginsenoside analogs, structurally based on 20(s)-protopanaxadiol (aPPD) (known as drug entity S111 in China) that has already been used as an antidepressant in humans in China, have been synthesized through a combinatorial chemistry approach developed by the Shanghai Innovative Research Centre of Traditional Chinese Medicine (SIRC). Combining calcitriol with aPPD (S111) is logical for many reasons: low vitamin D levels are known to be a contributing cause of depression, immune disorders, endocrine-related cancers as well as increasing the risk of developing cancers of the colon, ovary, lung, breast and prostate. The fact that aPPD exhibited good efficacy in inhibiting AR and its splice variants, major factors driving PCa growth and progression, highlights the potential of aPPD in PCa prevention and/or therapy. Importantly, preliminary results in our laboratory determined that aPPD has the ability to inhibit CYP17A1 and CYP11A1, which are
the vital enzymes for androgen synthesis from cholesterol in CRPC. As a consequence aPPD may also inhibit *de novo* synthesis of androgens, a known treatment resistance phenomenon observed in advanced CRPC patients. In addition, there is now considerable evidence to suggest that crosstalk exists between VDR and AR. This may vary between different PCa cell lines and could be mediated via sharing of coregulators required for receptor activation. Several AR coregulators including ARA54, ARA70, gelsolin, and supervillin, have been reported to modulate other steroid receptors and promote VDR function\textsuperscript{116}. In addition, we have also shown that aPPD treatment led to inhibition of enzyme-mediated inactivation of calcitriol in human liver microsomes *in vitro*, potentially providing additional vitamin D-related benefits to patients with cancer, neurodegenerative and metabolic diseases\textsuperscript{81}. Preclinical pharmacokinetic studies conducted in our laboratory have convincingly demonstrated that ginsenosides can reach the prostate tumor site in xenograft models\textsuperscript{203} and can potentially alter the prostate levels of calcitriol. This phenomenon is contributes to the synergistic anti-cancer mechanism of action of this combination. Furthermore, it has been shown that both ginsenosides and calcitriol can induce apoptosis and cause cell cycle arrest independently. Therefore, this combination is promising as a therapeutic strategy since it can offers a contemporary approach to maximize the multifaceted biological and therapeutic actions of vitamin D at a lower dose when combined with ginsenoside metabolites in the context of PCa. The chemosensitization of calcitriol activity by aPPD is a highly promising approach for PCa chemoprevention as well as treatment and is achievable at clinically relevant concentrations.

1.7 Hypothesis

Combining calcitriol with ginsenosides for the treatment of PCa significantly sensitizes their respective anti-cancer activities and limits toxicity.
1.8 Research Objectives

1. To determine whether combined use of aPPD and calcitriol can enhance the anticancer efficacy based prostate cancer therapy.

Specific aims:

- The anticancer activities of aPPD ginsenoside alone and in combination with clinically relevant concentrations of calcitriol on the growth of two well characterized human prostate cancer cell lines (LNCaP and C4-2) were evaluated and assessed:
  
  I. LNCaP and C4-2 cell viability
  
  II. LNCaP and C4-2 cell proliferation rate
  
  III. Determine Combination Indices (CI) and Dose Reduction Indices (DRI)

- The potential mechanisms of pharmacodynamic interactions were investigated by measuring the expression of proteins related to prostate cancer regulations as following:
  
  I. Cell death (apoptosis) markers (Bcl-2, Bax, and caspase-3)
  
  II. Cell cycle markers (cyclin D1 and cdk2)
  
  III. AR, PSA and VDR proteins

2. To investigate the impact of aPPD ginsenosides on the calcitriol pharmacokinetic in vivo.

Specific aims:

- To optimize the LCMS method for determining calcitriol pharmacokinetics and study the effect of aPPD on calcitriol pharmacokinetics in mice
I. To enhance the sensitivity of the current (LC-MS) method

II. Determine calcitriol PK *in vivo* after oral and IP dosing in CD-1 mice

III. To explore whether aPPD changes calcitriol PK *in vivo* in non-tumor-bearing nude mice. (Cmax, Tmax, AUC 0-24h and oral clearance F/CL were calculated)

IV. To investigate the impact of chronic administration aPPD on the calcitriol levels in C4-2 nude mice

V. Considerations for calcitriol use in combination therapy with respect to safety and clinical application will also be examined.

3. To determine the effect of aPPD on CRPC prostate cancer growth and examine the involvement of AR signaling in aPPD anticancer effect.

Specific aims:

- The changes in tumor growth and markers were examined in C4-2 tumors alongside signs of acute toxicity
  
  I. C4-2 tumor volume
  
  II. Apoptosis markers (Bax, cleaved-caspase 3)
  
  III. Proliferation markers (ki67)
  
  IV. Cell cycle markers (p21 and p27)
  
  V. Toxicity (CBC, chemistry, liver, kidney function test)

- To determine the involvement of AR signaling in aPPD-mediated growth inhibition, and to further investigate the effect of aPPD on AR expression, binding and activities.
I. In silico analyses of aPPD-AR binding to different AR domains

II. Effect of aPPD on AR protein expression in tumors

III. Determine the ability of aPPD to inhibit AR transactivation.

4. To assess the efficacy of aPPD in combination with calcitriol in the C4-2 mouse xenograft model and further study the nature of aPPD-VDR interaction

Specific aim:

- The changes in tumor growth and markers were examined in C4-2 tumors alongside signs of acute toxicity
  I. Determine if aPPD in combination with calcitriol can inhibit further C4-2 tumors.
  II. Apoptosis markers (Bax, cleaved-caspase 3)
  III. Proliferation markers (ki67)
  IV. Cell cycle markers (p21 and p27)
- To determine the involvement of VDR signaling in aPPD-mediated growth inhibition, and examine the nature of aPPD-VDR interaction
  I. In silico analyses of aPPD-VDR binding to different VDR domains
  II. Investigating the effect of aPPD on VDR protein expression in tumors
  III. Determine the effect of aPPD on VDR transactivation in presence or absence of VDR ligand calcitriol.
2. CHAPTER 2: CALCITRIOL AND 20(S)-PROTOPANAXADIOL SYNERGISTICALLY INHIBIT GROWTH AND INDUCE APOPTOSIS IN HUMAN PROSTATE CANCER CELLS

2.1 Introduction

Novel anticancer compounds derived from natural products present an attractive alternative to synthetic compounds due to their relatively favorable safety profiles. As a result, the potential role of vitamin D and ginsenoside metabolites in PCa prevention/treatment has gained much attention in recent years. The desired anticancer actions of pharmacological doses of calcitriol have been constrained by its hypercalcemic side effects and degradation by endogenous cytochrome P450 (CYP24A1 and CYP3A4 activity), the key enzymes responsible for calcitriol metabolism. Thus, one possible approach could limit calcitriol toxicity by using lower concentrations in combination with other anticancer agents that have independent and distinct activities perhaps that could enhance overall treatment efficacy.

Calcitriol apparently sensitizes cancer cells, enhancing their antitumor activity, and can act synergistically with other therapeutic agents. Most of these benefits are mediated via pharmacodynamic interaction by targeting multiple pathways or the same pathway through a different mechanism, consequently maximizing the therapeutic potential of calcitriol while limiting its toxicity. The combination approach for calcitriol used recently with docetaxel and gefitinib in a clinical trial was successfully implemented and achieved the desired anticancer effect.

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with no toxicity in PCa patients with peak plasma calcitriol concentrations of of 3.4 ± 0.8 nM (1.42 ± 1.9 ng/ml) and 16 ± 3.4 nM (6.68 ± 1.42 ng/mL), respectively. In addition, pharmacokinetic interactions via inhibition of CYP drug metabolizing enzymes could lead to significant increase in both plasma and tissue levels of calcitriol, thus delivering more bioavailable calcitriol inside the tumor.

We have also shown that aPPD treatment led to inhibition of enzyme-mediated inactivation of calcitriol in human liver microsomes in vitro, potentially providing additional vitamin D-related benefits to patients with cancer, neurodegenerative and metabolic diseases. In addition, it has been shown that both ginsenosides and calcitriol can induce apoptosis and cause cell cycle arrest independently and based on the synergistic features of ginsenosides, the combination of aPPD and calcitriol could show synergistic anticancer efficacy.

The study described in this chapter was designed to evaluate the effects of aPPD alone and in combination with clinically relevant concentrations of calcitriol on two well characterized human PCa cell lines (androgen-dependent LNCaP and androgen-independent C4-2) in vitro. We identified the pathways that potentially contribute to the combinatorial anticancer effects of these agents as well as the nature of their interaction, and postulated plausible mechanistic explanations for the effects observed.

2.2 Materials and Methods

2.2.1 Chemicals

Ginsenoside aPPD (MW 460.73 g/mol, with a purity of ~98.9%, which was confirmed in our lab by using LCMS/MS), was provided as a gift by the Shanghai Innovative Research Center of Traditional Chinese Medicine (Shanghai, China). aPPD stock solutions were prepared in 100% ethanol. Calcitriol (MW 416.63 g/mol, purity >99%), was obtained from Sigma-Aldrich Canada.
Ltd. (Oakville, ON, Canada). The stock solution was prepared by dissolving calcitriol in 100% ethanol. The stock was stored at -20°C protected from light. Drugs were added into the culture media at a final ethanol concentration of <0.1%. High-performance liquid chromatography grade chemicals and solvents were purchased from Fisher Scientific (Ottawa, ON, Canada). All other chemicals were obtained from commercial sources.

2.2.2 Cell culture

The androgen-sensitive LNCaP human prostate cancer cell line (from passage 41 to 53) and the LNCaP derived androgen-independent cell C4-2 (from passage 20 to 26) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) containing L-glutamine and supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Both cell types were maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C in an incubator, and the media was changed every 48 h. When cultures grew to 70% confluence in 15 cm (diameter) plates, the media was aspirated and the cells were washed with phosphate buffer solution (PBS), and then used for further culturing and treatments.

2.2.3 Cell Viability Assay

Cell number was determined using tetrazolium [3-(4,5-dimethylthiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium,inner salt (MTS; Promega Corporation, Fitchburg, WI). The assay measures the reduction of the yellow MTS solution into insoluble blue coloured formazan crystals by the action of mitochondrial succinate dehydrogenase enzyme present in the viable cells. Briefly, the cells were initially cultured in 96-well plates (Corning Inc., Corning, NY) at a density, which is 3000 cells per well in 100 µL of media (RPMI 1640 with 5% FBS), designed to reach 70–80% confluency at the time of assay. The cells were
allowed to attach for 24 hr, and then treated with varying concentrations of calcitriol (0, 2.5, 5, 10, 15, 25, 50, 75 and 100 nM) prepared in media. In a separate experiment, cells were treated with different concentrations of aPPD (0, 2.5, 5, 10, 15, 25, 50 and 100 µM), either alone or in combination with 1 and 10 nM calcitriol. After 24, 48, or 72 hr of treatment, 10 µl of the MTS solution (5 mg/ml; Sigma-Aldrich, St. Louis, MO) were added into each well. The plates were incubated for 2–4 hr at 37°C inside the incubator. Absorbance, which served as an estimation of the intact functional mitochondria and cell viability in the wells, was measured spectrophotometrically at 490 nm using an OptiMax microplate reader (Molecular Devices, Sunnyvale, CA). The number of viable cells was derived from the mean value of three independent experiments after normalizing to the number of untreated cells.

2.2.4 Cell Proliferation Assay

The effects of aPPD and calcitriol on cell proliferation were determined using the 5-bromo-2′-deoxyuridine (BrdU) incorporation assay (Roche, Jolla, CA). Cells were seeded in 96-well plates (3000 cells per well) and were incubated with varying concentrations of aPPD (ranging from 0 to 100 µM) or calcitriol (ranging from 0 to 100 nM) for 72 hr. The culture medium containing agents was left for the full 72 hours undisturbed. BrdU reagent was added to the culture media 10 hr before termination of the experiment. The extent of BrdU incorporation into cells was determined with an anti- BrdU antibody, and absorbance was measured at dual wavelengths of 450/540 nm with an OptiMax microplate reader (Molecular Devices, Sunnyvale, CA). Each experiment was repeated three independent times in duplicates.

2.2.5 Preparation of Whole Cell Lysates and Protein Assay

Following treatment with aPPD and/or calcitriol for 72 h, cells were rinsed with Dulbecco’s phosphate buffered saline (Invitrogen) and lysed with a buffer consisting of 50 mM Tris–HCl, pH
7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM Na3VO4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 10 g/mL aprotinin, and 10 g/mL leupeptin. Subsequently, the cells were harvested using a plastic scraper and transferred into Eppendorf micro centrifuge tubes and vortexed. The suspension was incubated for 30 min on ice. The samples were centrifuged at 14000 rpm for 15 min and the supernatant was collected. Protein concentration was quantified using bicinchoninic acid (Thermo Scientific Pierce) protein assay using bovine serum albumin as the protein standard.

2.2.6 Gel electrophoresis and Immunoblotting Assays

Thirty to Fifty micrograms of protein were loaded per lane into 12% SDS-acrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membrane in 48 mM Tris, 39 mM glycine, 0.1% SDS and 20% methanol (pH 8.3). The membranes were then blocked using Odyssey blocking buffer (Li-COR) containing 5% non-fat milk in wash buffer (Dulbecco’s phosphate-buffered saline with 0.1% Tween 20) for 2 h and incubated overnight at 4°C with primary antibodies, followed by at room temperature for 3 h. Subsequently, membranes were washed and incubated in Odyssey secondary antibody for 30-45 minutes according to manufacturer’s instructions. Blots were imaged using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Quantification was performed on single channels with the analysis software provided and normalized to β-actin for loading and transfer. Antibody dilutions, duration of second antibody incubation and film exposure were optimized to produce bands linearly related to the amount of protein. The following antibodies and dilutions were used to develop the immunoblots: mouse monoclonal antibody for β-actin as loading control (at a dilution of 1:5000; Sigma-Aldrich), mouse monoclonal antibody for AR (1:200; Santa Cruz Biotechnology Inc.), and goat polyclonal antibody for PSA (1:100; Santa Cruz Biotechnology Inc.). Rabbit polyclonal antibodies for VDR
(1:200), Cdk2 (1:100) and cyclin D (1:100) were obtained from Santa Cruz Biotechnology Inc. Mouse monoclonal antibodies for caspase-3 and cleaved caspase-3 (1:500), (1:300), Bcl2 (1:100) and Bax (1:100) used in the present study were also procured from Santa Cruz Biotechnology Inc. Conjugated secondary antibodies (Goat anti rabbit IRDye 680 at a dilution of 1:20,000 and Goat anti-mouse IRDye 800 at a dilution of 1:5000) were obtained from Cedarlane Laboratories (Burlington, ON, Canada).

2.2.7 Data Analysis

Data were analysed using the SigmaPlot Enzyme Kinetics Module (version 1.3, Systat Software Inc., Richmond, CA). IC₅₀ values (the concentration required to achieve a 50% reduction in MTS labeling or cell proliferation by BrdU assay compared to controls), were determined by nonlinear regression analysis using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). The IC₅₀ values are expressed as means ± SEM.

2.2.8 Combination Index (CI) and Dose Reduction Index (DRI)

Data from the proliferation assay was analyzed using the Calcusyn software package to assess drug–drug interactions based on the median effect principle (MEP), which can be used to define the combination index (CI) function. A CI<1 indicates synergy, a CI>1 indicates antagonistic interactions and a CI=1 indicates additive effects. The MEP was also used to obtain the DRI.

2.2.9 Statistical analysis

Parametric tests were used when the data passed the normality and equal variance test. Nonparametric tests were used when the data failed to pass the normality test and equal variance test (Sigma Statistical Software, Version 3.1). Differences between the mean values of treatment groups were analyzed using one way ANOVA test followed by a Tukey Test. The values of controls and aPPD or calcitriol treated samples alone and in a combination were compared and the
results were expressed as mean ± SEMs in the figures. All the statistical analyses were performed using the Sigma Statistical Software program Version 3.1. The level of significance was set a prior at p<0.05. All the studies were carried out as three independent experiments in duplicate except the experiments for protein expressions of the apoptotic and proliferation markers, which was conducted twice.

2.3 Results

2.3.1 aPPD Effects on Cell Viability was Enhanced by Calcitriol

At clinically relevant concentrations (1 and 10 nM) calcitriol had no effect on cell viability in both the cell lines determined after incubation for 72 hr (Figure 2.1). In contrast, calcitriol at concentrations >25 nM and >75 nM showed a concentration-dependent reduction in cell viability in C4-2 and LNCaP cells, respectively. aPPD treatment alone inhibited LNCaP and C4-2 cell growth with an IC$_{50}$ of 41 and 53 µM, respectively (Figure 2.2). However, adding 1 nM calcitriol to aPPD significantly lowered the aPPD IC$_{50}$ values to 27 and 28 µM in LNCaP and C4-2 cells, respectively. A greater degree of inhibition was seen when 10 nM calcitriol was added with a significant reduction of the aPPD IC$_{50}$ to 23 and 13 µM in LNCaP and C4-2 cells, respectively (Figure 2.2). The dotted line on the plots represents the cell number at the beginning of the incubation. The viable cell number falls below this line at concentrations above 50 µM in both cell lines; this indicates cell death is occurring in addition to inhibition of cell proliferation. The calculated IC$_{50}$ values have been summarized in Table 1. The androgen-independent cells (C4-2) were more sensitive to calcitriol, while the LNCaP cells were most sensitive to aPPD. The stability of the agents were measured using high performance liquid chromatography tandem mass spectroscopy (LCMS-MS) as previously described by our laboratory, with minor modification using vitamin D derivatization. LC/MS-MS analysis indicated that the drug
concentration did not change over the 3 day time period. There was very little or no spontaneous degradation of the treatment agents in the cell-free control medium, both calcitriol and aPPD were stable with 98% and 94% remained after 72 hours, respectively. There was no nonspecific binding of the drugs to the well.

**Figure 2.1.** Effect of calcitriol on LNCaP and C4-2 prostate cancer cell viability.
Cells were exposed to various concentrations of calcitriol for 72 hr followed by measurement of cell viability by MTS assay. Data were obtained from three independent experiments performed in duplicate and expressed as Mean ±SEM. The dotted line indicates the number of cells at the beginning of treatments. *Significantly different (p < 0.05) from the control (vehicle-treated group).

Cells were exposed to various concentrations of aPPD alone and in combination with 1 and 10 nM calcitriol for 72 hr followed by measurement of cell viability by MTS assay. Data were obtained from three independent experiments performed in duplicate and expressed as Mean ±SEM. The dotted line indicates the number of cells at the beginning of treatments. *Significantly different (p < 0.05) from the control (vehicle-treated group) and # significantly different (p < 0.05) from the aPPD alone treated group.
**Figure 2.2.** Effect of aPPD alone and in combinations on LNCaP and C4-2 prostate cancer cell viability.

**Table 2.1.** Inhibitory values of aPPD treatment alone and in combination with calcitriol on prostate cancer cell viability determined by MTS assay following treatment for 72 hr. Mean values ± SEM, n=3.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Inhibitory Concentrations</th>
<th>aPPD µM</th>
<th>aPPD µM + 1 nM Calcitriol</th>
<th>aPPD µM + 10 nM Calcitriol</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>IC_{50}</td>
<td>41.6 ± 6.5</td>
<td>27.9 ± 0.7*</td>
<td>23.6 ± 3.1*</td>
</tr>
<tr>
<td>C4-2</td>
<td>IC_{50}</td>
<td>53.3 ± 1.6</td>
<td>28.7 ± 0.4*</td>
<td>13.2 ± 2.9*</td>
</tr>
</tbody>
</table>

*P<0.05 versus aPPD only treated group.

2.3.2 **aPPD and Calcitriol Synergistically Inhibit PCa Cell Proliferation**

To test whether antiproliferative effects of aPPD are synergistic with calcitriol treatment of LNCaP and C4-2 PCa cells, we first examined the effect of aPPD and calcitriol alone on LNCaP and C4-2 proliferation rate. For both cell lines, antiproliferative effects of calcitriol and aPPD were evident (Figure 2.3). C4-2 cells were generally more sensitive to treatments than LNCaP cells. Following single agent treatment the IC_{50} values of aPPD range between 22 µM and 12.5 µM in LNCaP and
C4-2 cells, respectively, while for calcitriol the IC$_{50}$ values vary between 24 nM and 8.5 nM in LNCaP and C4-2 cells, respectively (Table 2.1). Assessment of synergy was done by calculating the combination indices determined at doses required to produce 50%, 75%, and 90% decreases (relative to controls) in BrdU labeling. The data demonstrated statistically significant synergy between calcitriol and aPPD, which has been summarized in Figure 2.4. The dose reduction indices have been summarized in Figures 2.5. When using drug combinations that result in synergistic interactions, significantly lower aPPD doses (compared to those used when aPPD added alone) are required, to achieve a specific effect level. In the presence of calcitriol, the amount of aPPD required to achieve a 50% decrease (relative to controls) is reduced by almost 12- and 18-fold in LNCaP and C4-2 cells, respectively (Figure 2.5).

**Table 2.2.** Growth inhibitory values of aPPD and calcitriol on proliferation rate for prostate cancer cells determined by BrdU assay following treatment for 72 hr. Mean values ± SEM, n=3.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Inhibitory Concentrations</th>
<th>aPPD µM Mean ±SEM</th>
<th>Calcitriol nM Mean ±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>IC$_{50}$</td>
<td>22 ± 3.3</td>
<td>24 ± 1.3</td>
</tr>
<tr>
<td>C4-2</td>
<td>IC$_{50}$</td>
<td>12.5 ± 1.2</td>
<td>8.5 ± 1.6</td>
</tr>
</tbody>
</table>
Figure 2.3. Antiproliferative effect of calcitriol (A, B) and aPPD (C, D) on LNCaP and C4-2 prostate cancer cell lines.

Cells were exposed to various concentrations of calcitriol or aPPD for 72 hr followed by measurement of cell proliferation by BrdU assay. Data were obtained from three independent experiments performed in duplicate and expressed as Mean ±SEM.*Significantly different (P<0.05) from the corresponding control (vehicle-treated) group.
**Figure 2.4.** In vitro drug combination studies analyzed using the combination index (CI) method in two prostate cancer cell lines: LNCaP and C4-2.

aPPD and calcitriol were combined according to the constant ratio experimental design. Dotted lines at CI=1 indicates an additive effect. CI<1 and CI>1 indicate synergism and antagonism, respectively. Mean values ± SEM, n=3.

**Figure 2.5.** Dose reduction index (DRI) expressed as % reduction in IC50 for aPPD in combination with calcitriol, compared to their individual IC50. Mean values ± SEM, n=3.
2.3.3 Combined Effects of aPPD and Calcitriol on AR, VDR and PSA Protein Levels

To determine whether observed synergy between aPPD and calcitriol is mediated by modulating the expression of proteins involved in the regulation of PCa, the effect of low nontoxic concentrations of each agent, either alone or in combination, was analyzed after 72 hr. aPPD alone at concentrations of 2.5 and 5 µM, has no effect on cell viability but caused a significant reduction in AR protein levels in both the cell lines (Figure 2. 6A and B). The 10 nM calcitriol slightly increased the AR levels in LNCaP cell lines. In addition, we observed that calcitriol also significantly increased the AR levels in C4-2 cells. These results are consistent with the effects of aPPD and calcitriol on PSA levels in LNCaP cells. However, neither aPPD nor calcitriol showed any appreciable effect on PSA levels in C4-2 cells.

For the first time we have shown that aPPD substantially increases in VDR protein levels in C4-2 cells, and a minor increase in VDR expression was also observed in LNCaP cells also (Figure 2. 6C and D). In addition, calcitriol treatment alone caused upregulation of VDR protein levels in LNCaP cells. Surprisingly, calcitriol caused a significant reduction in VDR levels in C4-2. These observations are consistent with the idea that aPPD affects AR signalling at the AR protein level and that it has an inverse impact on VDR levels, leading to increased cellular calcitriol effects. Adding calcitriol further enhanced the inhibitory effect of aPPD on AR and PSA expression in LNCaP cells. However, the combination increased the cellular content of the AR in C4-2 cells with no associated change in PSA compared with aPPD treatment alone. A similar trend was observed with VDR expression where addition of calcitriol to aPPD induced the VDR expression in C4-2 and significantly upregulated expression in LNCaP cell lines.
<table>
<thead>
<tr>
<th></th>
<th>LNCaP</th>
<th>C4-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcitriol</td>
<td>- + - - + +</td>
<td>- + - - + +</td>
</tr>
<tr>
<td>2.5 μM PPD</td>
<td>- + - + - -</td>
<td>- + - + - -</td>
</tr>
<tr>
<td>5 μM PPD</td>
<td>- + - + - +</td>
<td>- + - + - +</td>
</tr>
</tbody>
</table>

![AR B-actin](image)

(A) * (B) #
Figure 2.6. Western blot analysis of proteins involved in the regulation of prostate cancer cells. Androgen receptors (A, B), vitamin D receptor (C, D) and prostate specific antigen (E, F) protein levels were measured in response to various treatment concentrations of aPPD alone and in the combinations with 10 nM calcitriol in LNCaP and C4-2 prostate cancer cell lines. All experiments were performed in three independent experiments in duplicate expressed as Mean ±SEM. *Significantly different ($p < 0.05$) from the protein level of control (vehicle-treated group). #Significantly different ($p < 0.05$) from the protein level of the corresponding aPPD alone treated group.
2.3.4 Combined Effects of aPPD and Calcitriol on Apoptosis and Proliferation Markers

We further examined whether synergy occurred between aPPD and calcitriol mediated via affecting the cellular expression of proteins known to control cell cycle progression (cyclin D1 and cyclin dependent kinase-2 inhibitor (cdk2) and regulate apoptosis (Bcl-2, Bax, and caspase-3). Effects of low nontoxic concentrations of each agent alone and in combination were analyzed after treating the cells for 72 h. The data indicate that aPPD (2.5 and 5 µM) or calcitriol alone significantly upregulates Bax protein expression in LNCaP and C4-2 cells (Figure 2.7 and 2.8). In addition, procaspase-3 and cyclin D protein levels remain unchanged following treatment of LNCaP and C4-2 cells.

aPPD at 5 µM concentration and calcitriol alone significantly down-regulated the Cdk2 expression in both cell lines. The data at 72hr indicated that calcitriol and aPPD led to activation of caspases. The combination of aPPD and calcitriol significantly increased the expression of cleaved caspase 3 (Figure 2.9). Adding calcitriol to aPPD decreased the expression of Bcl2 in C4-2 cells while the inhibition of cdk2 levels in both cell lines were significantly enhanced (Figure 2.7 and 2.8).
Western blot analysis of proteins that are known to control apoptosis markers: Bax (A) and Bcl-2 (B), cell cycle regulation: cdk2 (C), and cell proliferation: cyclin D1 (D). LNCaP prostate cancer cells were treated with aPPD (2.5 µM or 5 µM) and calcitriol (10 nM), either alone or in combinations. The experiments were performed in duplicate as Mean ±SEM. *Significantly different ($p < 0.05$) from the protein level of control (vehicle-treated group). #Significantly different ($p < 0.05$) from the protein level of the corresponding aPPD alone treated group.

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Figure 2. Western blot analysis of proteins that are known to control apoptosis markers: Bax (A) and Bcl-2 (B), cell cycle regulation: cdk2 (C), and cell proliferation: cyclin D1 (D). C4-2 prostate cancer cells were treated with aPPD (2.5 µM or 5 µM) and calcitriol (10 nM), either alone or in combinations. The experiments were performed in duplicate expressed as Mean ±SEM. *Significantly different ($p < 0.05$) from the protein level of control (vehicle-treated group). #Significantly different ($p < 0.05$) from the protein level of the corresponding aPPD alone treated group.
Discussion

Our study was designed to investigate the combination of vitamin D and ginsenoside metabolite as a possible preventive and/or treatment strategy for PCa that would improve treatment outcomes in a manner that is not associated with overt toxicity. We determined the effect of the agents alone and in combination on the growth, protein expression of specific proteins involved in PCa regulation, apoptosis and proliferation markers in clinically relevant androgen-sensitive (non-metastatic) LNCaP and androgen-independent (metastatic) C4-2 PCa cell lines.

For the first time, our study clearly suggests that aPPD and calcitriol interact in a manner that imparts a synergistic anticancer effect. The combination acted synergistically in inhibiting...
the growth of LNCaP and C4-2 cells at nontoxic, clinically relevant calcitriol concentrations of 1 and 10 nM \(69,70,75\), and at aPPD levels achievable in vivo \(203,258\). Addition of calcitriol to aPPD resulted in synergistic enhancement of the antiproliferative effects of aPPD and more substantial inhibition of PCa cell growth than either agent alone. Our results described here focus on a constant ratio experimental design, where the drug: drug ratios are defined on the basis of similar inhibitory concentrations of the individual drugs. Calcitriol exhibits an IC\(_{50}\) that is 916- to 1470 -fold lower than the IC\(_{50}\) of aPPD. Previous studies from our laboratory have shown that aPPD and docetaxel can be combined to produce synergistic effects in C4-2 cells. However, the combinations appeared to produce antagonistic results in LNCaP cells \(201\). As demonstrated in Figure 2.5, the DRI suggests that substantially less aPPD is required to achieve a desired effect when using the combination as opposed to the agents alone. On the other hand, more therapeutic efficacy can be achieved with a combination potentially reducing systemic toxicities.

The possible mechanisms actuating the synergistic actions of aPPD and calcitriol were explored. It is known that androgen receptor regulation is important not only for the androgen-sensitive PCa cell lines but also in the androgen-independent PCa cell lines such as C4-2. Androgen signaling is vital to the development and progression of PCa. In addition, changes in PSA production, which is upregulated by AR activation, is routinely used as an indicator in evaluating the efficacy of PCa treatment. Androgens directly or indirectly impact vitamin D signaling pathways, and vice versa. Recent literature reports have shown that calcitriol antiproliferative effects on LNCaP cells appear to be mediated via calcitriol effects on AR \(265-267\). In the present study the effects of aPPD and calcitriol, either alone or in combinations, on AR and PSA expression and activity were examined in vitro. We found a significant inhibition in AR expression caused by aPPD not only in LNCaP but also in C4-2 cells. Similarly, previous studies
reported that ginsenoside aPPD suppressed AR expression in PCa models\textsuperscript{228,229,231}, and caused a reduction in serum PSA levels in castration restraint PCa in vivo. The fact that aPPD does not inhibit PSA expression in C4-2 cells suggests androgen independent regulation of PCa cells. This is supported by Cao et al.\textsuperscript{228}, who determined that aPPD supplementation leads to inhibition of AR signaling independently of androgen. In contrast, calcitriol showed an inhibition of proliferation as well as induction of AR protein in both cell lines and a significant increase in PSA protein expression in LNCaP cells. Calcitriol has been reported to increase PSA production in LNCaP cells in vitro\textsuperscript{268}. Interestingly, adding calcitriol to aPPD significantly enhanced its ability to reduce AR and PSA expression in LNCaP cells. However, a similar decrease in PSA expression was not seen in C4-2 cells treated with either single agent or combination treatment at similar concentrations. The results also suggest that a decrease in PSA does not necessarily correlate with a decrease in cell proliferation or an increase in cell death. PSA expression is therefore not a useful end point to assess treatment efficacy since it is subjective and dependent on the cell lines evaluated.

Interestingly, the fact that aPPD significantly upregulated VDR expression in LNCaP and C4-2 cells suggests a crosstalk between VDR and AR regulatory pathways. Ting et al.\textsuperscript{116} support our finding in that they suggest that VDR and AR may share the same coregulators. This was also confirmed by our western blot data which demonstrated an increase in VDR protein expression alongside an associated decrease in AR levels. However, the combination treatment showed an enhancement of AR with aPPD only in LNCaP cells. Consistent with the results of Cao and Wang et al,\textsuperscript{206,228,229,231}, we found a significant inhibition in AR expression caused by aPPD in LNCaP and C4-2 cells. AR coregulators, which were originally identified as AR-associated proteins, can modulate many other steroid receptors in the nuclear receptor super-family. Previous reports have
shown that a large number of coregulators are overexpressed in PCa\textsuperscript{118}. Coregulators can enhance (co-activate) or decrease (co-repress) the AR transcription and changes in its expression have been shown to correlate with poor prognosis in PCa patients\textsuperscript{24,269}. Several AR regulators, including ARA54, ARA70, gelsolin, and supervillin, have been reported to modulate other steroid receptors including AR and promote VDR\textsuperscript{116,117,270,271}. It is possible that aPPD or calcitriol changes the balance between AR co-activator and co-repressor expression providing additional factors for consideration in their mechanism of action other than other than the levels of AR and VDR proteins. For example, Hic-5 is an AR coactivator, which binds to nuclear receptors, required for full transactivation of the AR target genes. A recent study\textsuperscript{270} has examined the effects of Hic-5 on VDR-mediated transcriptional activation of CYP24A1 and has provides evidence of Hic-5 as a coregulator of VDR expression. Hic-5 reduced the VDR expression and the ability of calcitriol subsequent to induce transactivation of the CYP24A1 promoter, reducing CYP24A1 expression. The inhibition of AR expression by aPPD could potentially be mediated via Hic-5, leading to decrease in calcitriol-induced CYP24A1 expression and calcitriol metabolism.

Since bioavailability of calcitriol is a critical factor in the in vivo efficacy, we determined the calcitriol pharmacokinetic parameters in CD-1 and nude mice using an LCMS/MS assay as previously described by our laboratory\textsuperscript{79,202}. Following a single oral and intraperitoneal calcitriol dose, it was bioavailable at peak plasma concentrations ranging from 6 and 41 ng/ml respectively (unpublished data). From a metabolic perspective, we have shown previously that both calcitriol and aPPD are human CYP3A4 substrates\textsuperscript{79,220}. Thus, another possible mechanism that could plausibly explain the enhanced antiproliferative effect observed by combining calcitriol with aPPD is that aPPD inhibits calcitriol metabolism, increasing its half–life, thus increasing its effects. CYP3A4 is expressed predominantly in the liver but is also be found in many other organs.
including prostate, breast, gut, colon, and most markedly small intestine\textsuperscript{106}. We have previously shown that aPPD strongly inhibited CYP3A4-mediated calcitriol inactivation in human liver and intestine in vitro \textsuperscript{81}. This mechanism could also potentially contribute to a synergistic enhancement in anticancer effects if studied in vivo.

It is critical to recognize that CYP24A1 also metabolizes calcitriol and is expressed in the kidney. It is also present in other normal tissues such as the prostate and has the ability to metabolize calcitriol\textsuperscript{96}. However, CYP3A4-catalyzed metabolism of calcitriol in the intestine and liver is likely to contribute more to calcitriol bioavailability and tissue levels than the kidney since it is an organ largely involved in excretion\textsuperscript{107}. In the media containing prostate cells, the calcitriol concentration was higher in the presence of aPPD compared to the calcitriol alone treatment group. Similar observations were reported previously with genistein and liarozole on calcitriol half-life in DU145 PCa cells\textsuperscript{99,272}. Both the compounds downregulated the protein expression and activity of CYP24A1 enzyme which is known to efficiently catalyze the metabolism of calcitriol. Calcitriol is known to induce its own metabolism by upregulating CYP24A1 and limits VDR activity. Catabolic activity of CYP24A1 renders calcitriol inactive and acts as a negative feedback on VDR activation\textsuperscript{270}. LNCaP cells express CYP24A1. Thus, enhanced antiproliferative effects of calcitriol and aPPD combination could be due to inhibition of CYP24A1-mediated calcitriol metabolism by aPPD, leading to longer half-life of calcitriol, VDR activity and potentiated effects.

Further, we investigated the role of apoptosis and cell cycle pathways in the enhanced antiproliferative effects observed with aPPD dosed alongside calcitriol. In agreement with the cell viability and proliferation assays, combined treatment resulted in a significant decrease in the expression of anti-apoptotic and cell cycle proteins and increased expression of pro-apoptotic proteins. Previous reports suggest that aPPD and calcitriol induce apoptosis in PCa models by
regulation of Bcl-2 and Bax and inhibit cell cycle by inhibiting cdk2 and cyclin D. Our results indicate that calcitriol further enhanced aPPD's ability to induce apoptosis by up-regulating protein levels of pro-apoptotic Bax and enhanced inhibition of anti-apoptotic, Bcl-2 and cell cycle regulating protein, cdk2. Consistent with their effects on apoptosis, the combined treatment increased the expression of the cleaved caspase 3 in both cell lines, indicating both compounds induces apoptosis via the intrinsic pathway. C4-2 cells showed a greater magnitude of induction compared with LNCaP cells. Further investigations are needed to identify the precise molecular mechanisms.

In summary, this is the first study to demonstrate synergy between aPPD and calcitriol for anticancer activity in LNCaP and C4-2 PCa cell lines. Further studies may demonstrate that the combination of aPPD with calcitriol can similarly sensitize efficacy in vivo. In addition, it is necessary to investigate whether a functional AR is important to calcitriol response, activation of VDR and its associated cofactors, or downregulation of CYP3A4 and/or CYP24A1 by aPPD could contribute to calcitriol action. The potential benefit of the synergism or sensitization of anticancer activity to PCa patients would be tremendous as lower doses of calcitriol would imply fewer side effects including calcitriol associated hypercalcemia.
3. CHAPTER 3: PHARMACOKINETIC INTERACTION OF CALCITRIOL WITH 20(S)-PROTOPANAXADIOL IN MICE: DETERMINED BY LC/MS ANALYSIS

3.1 Introduction

The purpose of this study was to develop a sensitive liquid chromatography/mass spectrometry (LC/MS) assay for calcitriol quantification with which to carry out in vivo PK studies and design the appropriate dosing regimens for calcitriol. By determining a feasible route of administration we were able to evaluate safety while also establishing PK parameters when combined with aPPD. We used CD-1 mice and calcitriol was administered intraperitoneal (IP) and by oral gavage alone. We undertook in vivo PK studies in non-tumor-bearing or tumor-bearing (C4-2) nude mice to determine aPPD effects on calcitriol serum PK before performing efficacy studies in vivo. We used nude mice because they are extensively and routinely used to establish human tumor xenografts for cancer research. In this study, for the first time we report a pharmacokinetic herb-nutrient interaction between vitamin D and the ginsenoside metabolite aPPD that resulted in increased systemic exposure to calcitriol in nude mice. This interaction could affect vitamin D homeostasis, potentially providing additional benefits with respect to the anticancer effects of calcitriol in vivo.

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Figure 3.1. Chemical structures of 20(S)-Protopanaxadiol (aPPD), 20(S)-Protopanaxatriol (aPPT), 1,25-dihydroxyvitamin D (calcitriol) and Deuterated calcitriol (D6-calcitriol).
3.2 Methods

3.2.1 Test Compounds and Materials

Ginsenoside aPPD (MW 460.73 g/mol, with a purity of ~98.9%, which was confirmed in our lab by using LC-MS) was provided as a gift by the Shanghai Innovative Research Center of Traditional Chinese Medicine (Shanghai, China). Calcitriol was purchased from Vancouver General Hospital Pharmacy as a 1 µg/ml solution (Vancouver, BC, Canada). Deuterated calcitriol and 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) were obtained from Cedarlane (Burlington, ON, Canada) and Sigma-Aldrich (Oakville, ON, Canada), respectively. High-performance liquid chromatography grade chemicals and all other chemicals were obtained from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada) and Fisher Scientific (Ottawa, ON, Canada).

3.2.2 Oral gavage formulation

Calcitriol solution was dosed orally alone at a dose of 4 µg/kg (100–128 µl) solubilized in ethanol: propylene glycol: water (2:7:1, v/v/v ratio) as a treatment control and in combination with aPPD (aPPD 70mg/kg; calcitriol 4 µg/kg). aPPD ginsenoside was formulated just prior to oral administration as previously described by our laboratory. Briefly, aPPD solubilized in ethanol: propylene glycol: water (2:7:1, v/v/v ratio) was prepared prior to the administration by oral gavage at a dose of 70mg/ kg (highest achievable dose), limited due to gavage volume ethical considerations (150 µl) imposed by the institutional animal care committee. Dose selection for aPPD was based on previous work completed in our lab for safety, solubility and potency determined in solvents amenable to animal dosing prior to optimizing formulation for animal studies.
3.2.3 In vivo pharmacokinetic study design in non-tumor bearing mice

All animal experiments were conducted in accordance with the University of British Columbia Committee on Animal Care. First, a calcitriol dose response analysis was carried out in CD-1 mice after administration of 4 µg/kg (100–128 µl) orally or intraperitoneally and serum quantification was performed using our optimized LC/MS method. The goal was to determine calcitriol PK and select a feasible route of administration. In these PK studies, 50 µl of blood samples (collected from tail veins) was promptly collected at 0, 0.5, 1, 2, 4, 8, 12 and 24 h after calcitriol administration. Blood was centrifuged at 3000rpm for 10 min and the serum supernatant was placed into Eppendorf tubes which were stored at -20ºC pending analysis by LC/MS.

As shown in Figure 3.4, seventy two male nude mice age 6–8 week old (Harlan Sprague Dawley, Inc.) weighing 25–31 g were used in our PK study. Eight groups of three mice were dosed for 1 week by oral gavage with vehicle or aPPD at a dose of 70 mg/kg twice daily (117–150 µl) at an equivalent volume based on weight. At time 0, mice were given a single oral calcitriol dose of 4 µg/kg alone or in a combination with aPPD at a dose of 70 mg/kg. Mice were euthanized at each blood sampling point (n=3) using CO2 asphyxiation and cervical dislocation, upon which blood (obtained by cardiac puncture) was promptly collected at 0, 0.5, 1, 2, 4, 8, 12 and 24 h following calcitriol administration. Typically, 500–700 µl of blood was obtained and centrifuged and the supernatant was placed into eppendorf tubes which were stored at 20ºC pending analysis by LC/MS.

3.2.4 Measurement of Calcitriol levels in Xenograft Tumor –bearing C4-2 Mice

All animal experiments were conducted in accordance with the University of British Columbia’s Committee on Animal Care and protocol # A11-0377 held by Dr. Guns at the Vancouver Prostate Centre. Male Athymic mice age 6–8 week old (Harlan Sprague Dawley, Inc.) weighing 25–31 g
were used for the purpose of this study. The C4-2 cell line was used to establish human xenografts and 2x10^6 cells in 0.5 mL Matrigel (BD Biosciences), were subcutaneously inoculated at the posterior dorsal site, similar to previous experiments. When serum PSA levels reached more than 25 ng/ml, mice were castrated. Post-castration, animals were monitored and when PSA recovered to pre-castration levels. Tumors were allowed to establish until they reached a volume of 100 mm^3 at which point sixteen C4-2 xenograft bearing mice were castrated and then treated for a 46 day period. Eight mice were dosed orally three times a week with 4 µg/kg calcitriol alone or in a combination with aPPD at 70 mg/kg daily (117–150 µl) based on weight. Terminal blood samples were collected after 1 and 3 weeks (obtained by tail veins) and again at 6 weeks (obtained by cardiac puncture at time of euthanasia) approximately 18 h after calcitriol administration.

3.2.5 Assessment of toxicity

Animals were monitored daily for changes in body weight (g), appearance and signs of acute toxicity including death, lethargy, blindness, and disorientation. Blood samples were collected by cardiac puncture for CBC, liver (Alanine Aminotransferase, Amylase), and kidney (Creatinine) function tests, serum electrolytes, serum albumin and total blood protein levels. Calcitriol toxicity was evaluated by determining calcium and phosphorous serum levels. In addition, liver, spleen, kidney and lung tissues were collected for further histopathological analysis.

3.2.6 Serum extraction and PTAD derivatization

Deuterated calcitriol (D6-calcitriol, 10 ng/ml) was used as an internal standard (IS) in the assay. Calcitriol and IS were extracted from 50 µL of mouse serum using liquid–liquid extraction with Hexane: Ethyl acetate (75: 25 %v/v) under alkaline conditions and the extraction process was carried out twice. After thawing, the samples were vortex-mixed for 5 min and centrifuged at 12,000 rpm for 5 min at 4°C. Supernatant was transferred to a clean Eppendorf tube and dried
down using a CentriVap centrifugal evaporation system (35°C). The derivation was performed using 4-phenyl-1,2,4-triazoline-3,5-dione derivatizing agent (PTAD) (Figure 3.2). After the evaporation step, 20 µl of PTAD (PTAD was dissolved in acetonitrile to prepare 750 µg/mL concentration) was added to each sample which was then vortexed for 5 minutes and kept at room temperature for 2 hours. Thereafter, the samples were reconstituted with 20 µl of 50% methanol, and 10 µl an aliquot was injected into the LC/MS.

### 3.2.7 Quantification of calcitriol by LC/MS Method

Chromatographic separations of calcitriol and IS were performed on a Waters Acquity ultraperformance liquid chromatography (UPLC) system (Waters, Milford, MA) coupled to a Quattro Premier XE (Waters) triple quadrupole mass spectrometer (MS), using BEH C18 column (2.1 × 100 mm, 1.7 µM) at 40°C with a flow rate maintained at 0.3 ml/min and total run time of 7 min. The mobile phase consisted of solvent A (water/2 mM ammonium acetate/0.1% formic acid) and solvent B (methanol/2 mM ammonium acetate/0.1% formic acid) with the following conditions: 50% methanol (0–0.2 min), followed by a gradient of 50–100% methanol (0.2–15 min), isocratic elution with 100% methanol (12–20 min), and finally 50% methanol (17–20 min). The LC eluant was introduced into the MS, and all data were collected in electrospray ionization positive mode with a capillary voltage of 3.2 kV. Source and desolvation temperatures were 120°C and 350°C, respectively, and N₂ gas flow was 1000 l/h. MassLynx version 4.1 software (Waters) was used for data acquisition and Quanlynx analyses. The quantitative determination of calcitriol was performed by multiple reactions monitoring (mrm) of the m/z 450 > 397 transition using a cone voltage of 25 V and collision energy of 10 eV. Quanlynx (Waters) data analysis software was used with external calibration for PDA data and internal standard normalized calibration for MS data. Calibration standards ranged from 0.01 to 500 ng/mL (seven points) with R² >0.99. Standards
were prepared in a similar fashion using blank mouse serum serving as sample matrix and extracted with 50% methanol.

3.2.8 Data analysis

Data were entered into a Microsoft Excel spreadsheet using double data entry. Analyses were performed using Microsoft Excel (Version 2013; Microsoft Corp.) Non-compartmental analysis was used to calculate pharmacokinetic parameters of interest for comparison of each group. The Cmax and Tmax values were determined directly from the data. AUC 0 – 24h was determined from the concentration–time plot using the log-linear trapezoidal rule. Dose-normalized AUC 0–24h was calculated by dividing the AUC 0 – 24h by the dose per kg body weight. Differences between the mean values of two treatment groups were analysed using the Student t-test (parametric) or the Mann-Whitney test (nonparametric unpaired t-test). Two-tailed P values were considered significant below a threshold of 0.05.

3.3 Results

3.3.1 LC/MS Analysis

The reported method in the present work is liquid chromatography coupled to mass spectrometry (LC/MS). With minor modification using PTAD derivatizing agent and D6-calcitriol as the internal standard approach, we successfully enhanced the sensitivity (100 fold) and specificity of the LC/MS assay previously described by our laboratory79 (Figure 3.2). The calibration curves were linear (r² > 0.999) in the concentration ranges of 0.01-100 ng/mL (Appendices Figure D). The lowest concentration quantified was 0.01 ng/mL, which required as little as 50 µL serum. The accuracy value and the extraction recovery for calcitriol was within satisfactory range. Our method has a relatively short run time of 7 min. Liquid–liquid extraction was found to be a simple and effective method of affordable sample clean-up.
Figure 3. 2. Derivatization of calcitriol with PTAD

3.3.2 Calcitriol Pharmacokinetics in CD-1 mice

The applicability of the method was first demonstrated in its ability to quantify calcitriol after oral and IP administration in CD-1 mice. Our results show that oral administration of calcitriol, at a dose of 4 µg/kg resulted in peak plasma calcitriol concentrations (Cmax) of 4.11±0.76 ng/ml with corresponding AUC for the 0 to 24 hr range of 24±4.91 ng.h/ml (Figure 3.3). IP administration of calcitriol demonstrated a higher Cmax range of 43.09 ± 5.8 ng/ml up to 102 ± 20.5 ng/ml (Appendices Figure E). Overall, these results suggest suitability of oral administration of calcitriol. We therefore decided to administer calcitriol orally as formulated for all in vivo PK experiments as this translates well to the easier and preferred route of clinical administration.
3.3.3 Effect of aPPD on calcitriol Pharmacokinetics in nude mice

The dosing protocol used for aPPD in the PK interaction studies were developed based on our previously published PK and other solubility studies\(^7\),\(^{20}\),\(^{22}\),\(^{23}\). Mice pre-treated with aPPD did not demonstrate any change in calcitriol PK. However, co-administration of calcitriol with aPPD significantly increased calcitriol serum concentrations as observed for the duration of sampling (Figure 3.5). Enhanced systemic exposure (AUC) and delayed oral clearance (CL/F) of calcitriol were observed in aPPD co-treated mice when compared with control (calcitriol only treated) mice (Table 3.1). The systemic exposure of calcitriol was significantly enhanced by 35% with comparative AUC 0–24h values determined to be 43.92±2.46 ng.hr/mL versus 27.48±5.2
ng.hr/mL. Calcitriol Cmax values were also significantly enhanced by 41% compared with controls (10.2±1.29 versus 6.096±1.54 ng/ml), and corresponding differences in tmax were observed to be 0.5 h compared to 1 h for controls. In this study, we showed that aPPD significantly increased calcitriol serum exposure after combining calcitriol with aPPD in nude mice.

**Table 3.1:** Pharmacokinetic parameters of calcitriol after a single oral dose of calcitriol.

4 µg/kg calcitriol were administered to nude mice in the presence or absence of aPPD (70 mg/kg). Mean ±SEM, n of 3 for each time point.

<table>
<thead>
<tr>
<th>PK parameters</th>
<th>Calcitriol</th>
<th>Calcitriol + aPPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax ng/ml</td>
<td>6.096±1.54</td>
<td>10.2±1.29*</td>
</tr>
<tr>
<td>Tmax hr</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>AUC 0-24 ng.hr/mL</td>
<td>27.48±5.2</td>
<td>43.92±2.46*</td>
</tr>
<tr>
<td>Cl/F (ml/min)</td>
<td>2.1±0.33</td>
<td>1.59±0.08*</td>
</tr>
</tbody>
</table>

aPPD and calcitriol both administered p.o.

*Significantly increase in Cmax and AUC and decrease in CL/F (*P* < 0.05).

(CL/F), apparent clearance

Cmax, Peak serum concentration.

Tmax, time to Cmax
Figure 3.4. Flowchart for *in vivo* pharmacokinetic study design in nude mice.
3.3.4  Effect of aPPD on calcitriol levels in Xenograft C4-2 mice

Changes in calcitriol levels after 1, 3 and 6 weeks were measured in serum samples obtained by tail vein bleeding for calcitriol alone and aPPD combination treated C4-2 mice. Elevations in serum calcitriol levels were seen after 1 and 3 weeks in combination treated mice compared to controls. However, calcitriol levels were substantial and significantly elevated after 6 weeks in combination treated mice (Figure 3.6). After 6 weeks, calcitriol serum concentrations in the combination group was significant greater than the calcitriol alone treated group (0.61±0.19 vs 0.14 ±0.15 ng/ml, respectively, $p < 0.05$).
Figure 3.6. Serum calcitriol concentration in C4-2 mice after treated by oral gavage of calcitriol (4 µg/kg) 3 times weekly alone or in combination with aPPD (70 mg/kg for 46 days). Data are expressed as mean ± SEM. Eight mice were used for each treatment group. * Significant increase ($P < 0.05$) compared to calcitriol treated only group.

3.3.5 Effects of treatments on body weights and serum levels of calcium

There were no changes in the mean body weights between the experimental groups. Calcium levels in animals treated with calcitriol showed significant elevation (14.43 mg/dL, $p < 0.05$) compared to control (10.02 mg/dl for vehicle-treated mice and 11.01 mg/dl for aPPD-treated only groups) as described previously in C4-2 mice. However, serum calcium levels in the mice treated with the combination showed less elevation (12.83 mg/dL, $p < 0.01$).
3.4 Discussion

Vitamin D is an endogenous substance found in nanomolar quantities. Liquid chromatography tandem mass spectrometry (LC/MS/MS) is a sensitive analytical technique and is considered the gold standard for measuring calcitriol. Several LC/MS/MS assays have been reported for calcitriol quantification in serum and tissues\textsuperscript{276-279}. However, assay limitations still exist with respect to sensitivity due to low circulating calcitriol concentrations and its lipophilic nature\textsuperscript{276}. Derivatization techniques enhance the detection response of poorly ionizable compounds such as calcitriol \textsuperscript{276} (Figure 3.2). PTAD, Amplifex diene (AB SCIEX), and more recently, 2-nitrosopyridine (PyrNO) are often used to optimize MS/MS analysis by enhancing ionization\textsuperscript{276,277}. With minor modification of the LC/MS assay using PTAD derivatizing agent as previously described by our laboratory \textsuperscript{79} (Figure 3.2), we successfully improved the assay sensitivity 100 fold with an optimized LLQ of 0.01 ng /mL. With the smaller sample volume of only 50 µL serum, and the high sensitivity, this assay could provide the most efficient and reliable method for measuring calcitriol in biomedical and clinical research. Although we required derivatization procedures to enhance sensitivity for calcitriol, our method offers significant advantages in cost effectiveness and uses a small sample volume. The PTAD used is inexpensive and commercially available readily.

Our method was successfully applied in determining calcitriol serum PK parameters in CD-1 and nude mice. We used nude mice because they are extensively and routinely used as an immune compromised host to establish human xenograft tumours in cancer research. These pre-clinical models can be used to evaluate the antitumor activity of drugs on growth, invasion and metastasis that mimic, at least to some extent, the clinical situation in a human\textsuperscript{280,281}. Martignoni et al \textsuperscript{282} have shown no significant differences in drug metabolizing enzymes and drug transporter
protein expression and activities between the two strains of mice. Upon IP and oral calcitriol administration in mice, our PK results appear to be in agreement with previous reports. Muindi et al. have shown that when 6.25 µg/kg calcitriol was administered IP in normal C3H/HeJ mice, a Cmax of 8.73±1.03 ng/ml was determined alongside a calculated AUC from 0 to 24 hr of 33.6±3.5 ng.h/ml. However, when 2.1 µg/kg calcitriol was given orally, Cmax was much lower at 0.2±0.02 ng/ml with an associated AUC from 0 to 24 hr of 2.43±0.03 ng.h/ml. In addition, when calcitriol was administered IP at doses of 0.125 or 0.5 µg/mouse the resulting Cmax determined were 12.0 ng/ml and 41.6 ng/ml respectively, with corresponding AUC from time 0 to 24 hr of 47 and 128 ng.h/ml, respectively.

Calcitriol levels in the blood depend largely on its bioavailability, ratio of binding to plasma and tissue proteins, perhaps also its binding to lipoprotein as well as the equilibrium that exists for its biosynthesis from 25(OH) D3 and catabolic degradation steps in the intestine, kidney and liver. After oral administration of calcitriol, the reported recoveries ranged from 55 to 80 %, indicating the intestinal metabolism of calcitriol is an important factor for calcitriol bioavailability. CYP3A4 is the key enzyme responsible for calcitriol metabolism. We and others have demonstrated that calcitriol can be catabolized by a CYP3A4 dependent pathway. This CYP3A4 pathway may be responsible for the osteomalacia-inducing aspects of several pharmaceutical drugs [37,39]. We have previously shown that CYP3A4 is the predominant enzyme involved in the oxidative metabolism of calcitriol, indicating that first-pass extraction is an important factor in determining calcitriol bioavailability (3, 4). CYP24A1 is also responsible for calcitriol metabolism, mainly in the kidney but also in a variety of other vitamin D target cells [44,93]. The metabolism of calcitriol in the intestine and liver by CYP3A4 is likely to contribute more to calcitriol bioavailability and tissue levels than CYP24A1. Studies have also reported that CYPs overexpressed in tumor
tissues compared with normal tissues. Tumors expressing high levels of CYP3A4 are likely to have a poor treatment response to calcitriol. Therefore, inhibition of this enzyme could result in higher calcitriol levels in the serum as well as in the target tumor tissues, thus enhancing its anticancer effect potentially providing additional anti-cancer benefits to PCa patients. Previously, we have shown that aPPD strongly inhibited CYP3A4-mediated calcitriol inactivation in human liver and intestine in vitro. aPPD has been reported to inhibit P-gp transporter and CYP3A4 activity in human subjects. Because CYP3A4 impacts calcitriol absorption when given orally, inhibitors of CYP3A4 such as aPPD might be able to increase calcitriol absorption when given in combination. However, the potential for the development of hypercalcemic toxicity also exists.

For the purpose of our work, the pharmacokinetic study was conducted to determine the inhibitory effect of aPPD on calcitriol metabolism in vivo by co-administration of aPPD with calcitriol. As expected, the amount of calcitriol reaching the blood was significantly increased by co-administration of aPPD and calcitriol oral clearance CL/F was reduced (Table 3.1). Our results suggest that aPPD enhances calcitriol exposure by increasing its absorption and inhibiting its elimination as well. This is likely mediated via inhibiting mice Cyp3a isoforms, such as Cyp3a2 which has significant sequence homology variance shares some similarities to the catalytic competence to human CYP3A4. In addition, aPPD increased the pharmacological activity of calcitriol as indicated by the increased levels of serum calcium. It should be noted however that a direct cause and effect relationship between the inhibition of CYP3A4 activity and the increase in systemic calcitriol exposure was not probed in this study. The inhibition of CYP3A4 may lead to reduced ability of the enzyme to oxidize calcitriol in the liver and thus spare calcitriol catabolism to yield higher intracellular levels (Figure 3.7).
Similar serum calcitriol PK characteristics were reported in CYP24A1 knockout mouse models. The cyp24a-knockout mice studies have confirmed the physiological role of CYP24A1 in 25(OH)D3 and calcitriol hemostasis as a build-up of vitamin D3 which was observed in the knockout mouse phenotype. Administration of calcitriol in combination with CYP24A1 inhibitors slows its catabolism, thereby enhancing its antitumor activity in preclinical PCa models. Although CYP3A4 is the dominant elimination pathway of calcitriol in vivo, aPPD could also inhibit CYP24A1, thus enhance the calcitriol pharmacological effects in human subjects by increasing calcitriol exposure and decreasing its clearance and needs further investigation (Figure 3.7).

In humans, studies have shown that calcitriol undergo undergo enterohepatic circulation and can be excreted as glucuronides metabolites in the bile. UGT enzymes express in high levels in the liver and also intestine which is known to have an impact on the first-pass and enterohepatic circulation of drugs. Hashizume et al. have shown that three monoglucuronide isomeric metabolites of calcitriol conjugate at the the 25-hydroxy position in human liver. The glucuronidation of calcitriol in liver plays an important role in calcitriol bioavailability as well as its elimination. aPPD inhibits the enzymatic activity of several UDP-glucuronosyltransferase (UGT) isoenzymes, major players in the human phase II drug metabolism. Other studies showed that structure-dependent inhibition existed for the inhibition of ginsenosides towards UGT isoforms. This may be a potential for herb-drug interactions between UGT substrates such as calcitriol when concomitantly administered with aPPD. In this study, calcitriol oral clearance was decreased by aPPD, this could be mediated via inhibition of human hepatic glucuronidation by aPPD. This perhaps could resulted in decreased metabolism of calcitriol by CYP3A4 in the intestine and its subsequent elimination from the blood.
There have been many approaches taken to elevate tumor calcitriol levels that have been reported in the literature\(^{243}\), making it feasible to see anticancer benefits while limiting its toxicity. The tolerability and PK of single-dose administration of calcitriol at different doses of 15, 30, 60, 75, 90, 105, 135, and 165 µg in advanced solid tumors patients was tested. Thirty eight patients were enrolled in the study, there was a dose-proportional increase in Cmax and AUC seen across the full range of DN-101 doses tested. At the 165 µg dose, Cmax was 6.21 +/- 1.99 ng/mL, AUC 0-24 was 41.3 +/- 9.77 ng h/mL and t1/2 was 16.2 hours\(^{287}\). The pharmacokinetics of a 4 µg dose of calcitriol following oral and i.v. administration in 6 healthy volunteers and 12 uraemic patients were measured. The oral bioavailability of calcitriol was 71 and 73 % in healthy volunteers and uraemic patients, respectively\(^{288}\). When calcitriol was given orally 3 consecutive days each week, and paclitaxel (80 mg/m2) was given intravenously weekly in 36 patients at maximum dose of 38 µg calcitriol plasma concentrations of 600 to 1440 pg/mL were achieved with no toxicity\(^{289}\).

Hypercalcemia is an expected side effect of high dose vitamin D therapy alone and also the most frequently reported side effect that occurred in combination with other drugs in cancer patients\(^ {145,146,243}\). Thus, the anti-cancer benefits of calcitriol have not been fully realized in cancer chemoprevention and/or treatment. To achieve desired clinical outcomes in cancer patients, combining aPPD with calcitriol lowers required calcitriol doses at which hypercalcemic effects are no longer a concern. aPPD may sensitize cancer cells to calcitriol concentration that below the level that typically results in hypercalcemia. This combination potentially could provide additional benefits when it used in patients with cancer, neurodegenerative and metabolic diseases.

This is the first report to show the \textit{in vivo} pharmacokinetic interaction and the enhancement of the calcitriol absorption in nude mice.
Figure 3.7. Schematic representation of proposed mechanisms of 20(S)-protopanaxadiol (aPPD) blockade of 1, 25(OH) 2D3 inactivation in human liver and intestine. aPPD and other CYP3A4 inhibitory ginsenosides can play a vital role in maintaining the active form of vitamin D3.
4. CHAPTER 4: 20(S)-PROTOPANAXADIOL REGIO-SELECTIVELY TARGETS ANDROGEN RECEPTOR: ANTICANCER EFFECTS IN CASTRATION-RESISTANT PROSTATE TUMORS

4.1 Introduction

To date, therapeutic options for advanced stage PCa are limited. Since the androgen receptor (AR) continues to drive tumour growth, the current treatments include AR antagonists. These drugs are often used in combination with luteinizing hormone-releasing hormone/gonadotropin-releasing hormone agonists and antagonists aiming to shut-down pituitary axis regulated gonadal steroid production. More recently the use of steroidogenesis inhibitors has been designed to combat local intra-tumoural suppression of steroidogenesis and typically they are introduced for the treatment when the disease has progressed to castration resistant PCa (CRPC). Targeted therapies and agents with growth inhibitory properties that work independent of the androgen pathways are of current interest. We have identified a class of naturally derived ginsenoside molecules (aPPD) that target key cell signaling pathways involving the AR and steroidogenesis (known to be dysregulated in PCa) while enhancing vitamin D receptor expression.

AR is a major driving force in the development and progression of PCa to the metastatic stage and expression of AR splice variants is one of the major mechanisms of CRPC. Androgens binding to AR induces receptor dimerization, which is an absolute requirement for AR signaling. After dimerization, the AR interacts with the DNA-binding domain facilitating DNA binding.

\[\text{\textsuperscript{200}}\]

\[\text{\textsuperscript{242}}\]

\[\text{\textsuperscript{29}}\]

\[\text{\textsuperscript{29}}\]

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and the recruitment of cofactors and transcriptional machinery to regulate expression of target genes\(^{242}\). AR interaction also exists between an amino terminal domain and ligand-binding domain known as the N-terminal/C-terminal interaction, and ligand-binding domain dimerization. This N/C interaction is an essential factor in regulation of AR activity\(^{242}\). Since aPPD exhibited good efficacy in inhibiting AR and its splice variants, this highlights the potential of aPPD in PCa prevention and/or therapy\(^{229}\). The aPPD bears structural similarity to androgens that are bound in the AR androgen binding site (ABS) (Figure 4.1). Previously we have shown that the binding affinity of aPPD to AR is ~10,000-40,000-fold less than dihydrotestosterone (DHT), and it is unlikely that aPPD competes with DHT\(^{229}\).

The present study is designed to determine if aPPD can inhibit AR-positive castration-resistant C4-2 xenograft prostate tumors. We have also examined and validated potential mechanisms of aPPD-mediated anticancer effects by investigating AR protein expression in tumors, and carried out in silico analyses to determine aPPD binding to different domains on the AR as well as in vitro assays to determine the ability of aPPD to inhibit AR transactivation. In addition, the effect of aPPD on apoptosis markers (Bax, cleaved-caspase 3), and proliferation markers (ki67) expressions were examined.
**Figure 4.1.** Chemical structure of 20(S)-protopanaxadiol (PPD) (A). Dihydrotestosterone (DHT) (B) and enzalutamide (C).

**Materials and Methods**

4.1.1 Test compound and reagents

Ginsenoside aPPD (MW 460.73 g/mol, with a purity of ~98.9%, which was confirmed in our lab by using LC-MS), was provided as a gift by the Shanghai Innovative Research Center of Traditional Chinese Medicine (Shanghai, China). High-performance liquid chromatography grade chemicals and all other chemicals were obtained from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada) and Fisher Scientific (Ottawa, ON, Canada).
4.1.2 In vivo studies

4.1.2.1 Xenograft preparation and treatment

All animal experiments were conducted in accordance with the University of British Columbia’s Committee on Animal Care and protocol # A11-0377 held by Dr. Guns at the Vancouver Prostate Centre. Male athymic mice age 6–8 week old (Harlan Sprague Dawley, Inc.) weighing 25–31 g were used in our study. Two million C4-2 cells in 0.5 mL (Matrige, BD Biosciences), were subcutaneously inoculated at the posterior dorsal site, similar to previous experiments (14). When serum PSA levels reached more than 25 ng/ml, mice were castrated. Post-castration, animals were monitored and when PSA recovered to pre-castration levels, 20 mice were randomized and distributed into two treatment groups: Treatments began once the total tumor size exceeded 100 mm$^3$ with either aPPD at 70 mg/kg once daily (117–150 µl) or the vehicle control at an equivalent volume based on weight, with a total of 8 mice per group.

4.1.2.2 Oral gavage formulation

The ginsenoside aPPD was formulated just prior to oral administration as previously described by our laboratory$^{203}$. Briefly, aPPD solubilized in ethanol: propylene glycol: water (2:7:1, v/v/v ratio) was prepared prior to the administration by oral gavage at a dose of 70 mg/kg (highest achievable dose, limited due to gavage volume limitations (150 µl) implemented by the institutional animal care committee). Dose selection was based on previous work completed with aPPD in our lab for safety, solubility and potency determined in solvents amenable to animal dosing prior to optimizing formulation for animal studies.

4.1.2.3 Assessment of tumor growth and PSA

Tumor size (mm$^3$) was measured and monitored twice weekly. Calipers were used to measure the three perpendicular axes of each tumor to calculate the tumor volume using the formula: $\pi/6 \times$
Length x Width x Height (mm). PSA levels were measured by tail vein sera samples weekly using the Cobas automated enzyme immunoassay (Montreal, PQ).

4.1.2.4 Assessment of toxicity

During treatment, aPPD toxicity was determined. Animals were monitored daily for changes in body weight (g), appearance and signs of acute toxicity including death, lethargy, blindness, and disorientation. Mice were sacrificed when tumor volume exceeded 1,500 mm$^3$ or loss of > 20% body weight. All xenograft tumors were harvested after 46 days of the treatment approximately 24 hours after their last treatment dose. Blood samples were collected for CBC, liver and kidney function tests, serum electrolytes, glucose, serum albumin and total blood protein levels. In addition, liver, spleen, kidney, lung and brain tissues were collected for further toxicological and histopathological analysis.

4.1.2.5 Tumor collection and homogenization

At the end of the treatment period (46 days after treatments) mice were sacrificed and tumors were harvested and divided into two fractions: either frozen in liquid nitrogen and stored at -80 ºC for protein analysis or preserved in 10% formalin buffer and tissue sections embedded in paraffin blocks for histopathological analysis. Preparation of paraffin-embedded tissue sections and immunohistochemical analyses were carried out as previously described.$^{290,291}$

4.1.2.6 Western blot analysis

Excised C4-2 tumor tissue was homogenized using the Precellys™ tissue homogenizer system (Bertin Technologies, France) as per the manufacturer’s protocol. Proteins were extracted using RIPA buffer and Western blot was performed as previously described. Briefly, tumor tissue (100 mg) was homogenized in RIPA buffer with 1X protease inhibitor at a 1:4 (tissue: buffer) ratio using Precellys™ Tissue Homogenizing CKMix (Cat. # 3961-1-009) at 6000 rpm for two cycles
of 20 s each with a 15 s break. Thirty micrograms of protein were loaded per lane into 12% SDS-acrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membrane in 48 mM Tris, 39 mM glycine, 0.1% SDS and 20% methanol (pH 8.3). The membranes were then blocked using Odyssey blocking buffer (Li-COR) containing 5% non-fat milk in wash buffer (Dulbecco’s phosphate-buffered saline with 0.1% Tween 20) for 2 h and incubated overnight at 4 ºC with primary antibodies, followed by at room temperature for 3 h. Subsequently, membranes were washed and incubated in Odyssey secondary antibody for 30–45 min according to manufacturer’s instructions. Blots were imaged using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Quantification was performed on single channels with the analysis software provided and normalized to beta actin for loading and transfer. The fold induction or reduction of AR proteins was compared to that of the vehicle control group. Antibody dilutions, duration of second antibody incubation and film exposure were optimized to produce bands linearly related to the amount of protein. The following antibodies and dilutions were used to develop the immunobLOTS: mouse monoclonal antibody for beta actin as loading control (1:5000; Sigma–Aldrich), rabbit polyclonal anti-p27 (1:250; Santa Cruz Biotechnology Inc.), mouse monoclonal antibody for AR (1:200; Santa Cruz Biotechnology Inc.), and rabbit monoclonal anti-Bax (1:1000;Abcam). Conjugated secondary antibodies (anti-mouse IRDye 800 at a dilution of 1:5000 and anti-rabbit IRDye 680 at a dilution of 1:20,000) were obtained from Cedarlane Laboratories (Burlington, ON, Canada).

4.1.2.7 Immunohistochemistry

C4-2 tumors were isolated from mice at the end of the study described above and were prepared for immunohistochemical assessment of apoptosis and Ki-67. C4-2 tumors were sectioned and stained with hematoxylin and eosin (HE) and the desired areas marked along with their
corresponding paraffin blocks. The rabbit anti-human anti-Cleaved Caspase 3 (Asp175) (5A1E) (1:50; Cell Signaling Technology, Danvers, MA), rabbit anti-human anti-Ki 67 proliferating markers (1:50; Cell Signaling Technology, Danvers, MA) and rabbit polyclonal anti-p21 (1:150; Santa Cruz Biotechnology Inc) were used for immunohistochemical staining. All sections used for immunohistochemistry were lightly counterstained with 5% (w/v) Harris hematoxylin. Five fields of each slide were randomly chosen and images taken (400), using an AxioCam HR CCD mounted on an Axioplan 2 microscope and Axiovision 3.1 software (Carl Zeiss, Canada). Positively stained cells and whole cells in each image were counted and the percentage of positive cells was calculated. The TMAs were manually constructed (Beecher Instruments, MD) by punching quadruplicate cores of 1 mm for each sample giving a total of 144 cores. All scoring was done blinded with respect to treatment by LF and based on relative immunoreactive intensity on a four-point scale.

4.1.3 In silico docking between aPPD and AR ABS

To gain insight into why aPPD cannot compete with DHT for ABS in spite of its structural similarity, we performed in silico docking study for DHT and aPPD against AR ABS to seek the possible molecular mechanism. The X-ray crystal structure of AR LBD complexed with DHT was obtained from the Protein Data Bank (PDB ID: 2AMA), and AutoDock Vina [C2] was employed for the in silico docking 292. The protein model was prepared with Molecular Operating Environment (MOE) 2015.1001 [C3] by adding the missing residues and the side chains to the protein coordinate in the X-ray structure 293. The center of the binding pocket was defined based on the coordinate of DHT ligand in the X-ray structure, and the box dimension of 24 Å × 24 Å × 24 Å was used for the grid search which is large enough to accommodate the ligand molecule.
4.1.4 In vitro aPPD-AR binding and inhibition assays

PC-3 cells lacking the AR and authenticated by IDEXX Laboratories (Maine, USA) were maintained in RPMI 1640 media (Life Technologies) and 5% FBS (HyClone Thermo Fisher Scientific) at 37 °C and 5% CO2. Cultures were routinely monitored for mycoplasma contamination. Cells were seeded in 96-well plates (5,000 cells/well) in RPMI 1640 medium with 5% charcoal-stripped serum (CSS) (HyClone). After 24 h, cells were co-transfected with both NTD and pG5luc (10 ng each) or NTD-DBD and pARR3-tk-luciferase (5 ng each) using TransIT20/20 transfection reagent (3 µL/µg of DNA) (Mirus Bio LLC, Madison, WI, USA) in Optimem serum-free media (Life Technologies) for 24 h according to the manufacturer’s suggested protocol. At 24 h after transfection, cells were treated with either 0.1% DMSO (solvent control) or serial dilutions of increasing concentrations of aPPD. Enzalutamide (C-terminus inhibitors) and EPI-001 (N-terminus inhibitor), were used as positive controls. At 24 h after treatment, the medium was aspirated off and the cells were lysed by adding 60 µL of 1× passive lysis buffer (Promega) followed by shaking at room temperature for 15 min and two freeze/thaw cycles at -80 °C. Twenty microliters of lysate from each well were transferred onto a 96-well white flat bottom plate (Corning) and the luminescence signal was measured after adding 50 µL of luciferase assay reagent (Promega). The chemical oxidation of luciferin into oxyluciferin by the luciferase is accompanied by light production that can be quantified as luminescence by a TECAN M200Pro instrument. Each concentration was assayed in replicates n = 6, with a biological replicate of n = 3. The toxicity of aPPD (6.25-50 µM) was assessed in the same experimental conditions on non-transfected PC-3 cells using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay.
4.1.5 Statistical analysis

For each studied variable, mean and standard error of the mean (SEM) were calculated. Differences between the mean values of two treatment groups were analyzed using the Student t-test (parametric). The level of significance was set prior at a $P$ value of $< 0.05$.

4.2 Results

4.2.1 aPPD inhibits growth of castration-resistant C4-2 tumors in nude mice

The anti-cancer efficacy of aPPD was elucidated using nude mice bearing human C4-2 prostate tumor xenografts developed following subcutaneous injection of C4-2 human prostate cancer cells. The control group received only the vehicle formulation (ethanol: propylene glycol: water in 2:7:1 v/v/v ratio). During this study, aPPD produced significant inhibition of the C4-2 tumor growth rate starting on day 7 and onwards for up to 46 days compared to the control group ($p <0.05$) (Figure 4. 2A). The maximum inhibition of tumor growth was seen after 7 days of treatment and a sustained tumor suppressive effect was observed until 46 days of aPPD treatment (euthanasia point) with 53% inhibition compared to the control group (Figure 4. 2A).

The average tumor volume for control treated animals was approximately 6-7 times the size of the average tumor volume determined when treatment was initiated. For animals treated with aPPD, the tumor volumes were 3 to 4 times greater than the treatment initiation time point and the tumors at this time were significantly smaller than those tumors from animals treated with the formulation vehicle. In addition, aPPD had significantly different serum PSA levels after 7 and 14 days (Figure 4. 2B). PSA levels decreased at week 1, 2 and week 6 with ~46% and 34% inhibition, respectively, compared to control, followed by no difference with the control group between week 2 and week 5. Interestingly, the PSA levels demonstrated a significant 27% decrease again at week 6 following treatment initiation.
4.2.2 Lack of toxicity from aPPD treatment

There was no difference in animal body weight between vehicle- and aPPD-treated mice during the study period, indicating that the selected dose is safe and well tolerated (Figure 4. 2C), which is in agreement with our previous observations in other PCa xenografts models.\textsuperscript{201-203} Histopathological evaluations of the lung, liver, kidney and spleen from control or aPPD-treated mice show no signs of abnormal findings (Appendices Figure A). In addition, liver and kidney function tests do not reveal any organ toxicity following treatment (Appendices Table A). Interestingly, serum amylase (AMY) levels following aPPD administration were significantly lower than the control group. We therefore measured the levels of lipase enzyme, LIP, another pancreatitis marker, for which there were no significant differences between the two groups. Overall, both AMY and LIP levels were within the normal reported range in the literature.\textsuperscript{294-297} There was no statistical difference in serum albumin (Alb), alkaline phosphatase (ALP), alanine aminotransferase (ALT) and alanine transaminase (ALT) values which were within the expected range for normal mice. Serum creatinine levels were determined as a measure of kidney toxicity and the results suggest that there was no significant difference in serum creatinine levels between the aPPD and the control (Appendices Table A). Overall, there were no significant differences in the histological findings between the control and aPPD treatment group in any of the tissues examined (liver, lungs, kidneys, and spleen). Therefore, indicating that aPPD treatment was safe at the therapeutic doses used in the present study.
The *in vivo* effect of aPPD on the tumor volume (A), and serum PSA (B).

Change in tumor volume was followed over time for mice treated orally with either control (ethanol: propylene glycol: water (2:7:1)) or aPPD (70 mg/kg once daily) formulations. Average tumor volumes are expressed as a percentage of the average initial tumor volume of each week, post C4-2 cells inoculation and castration. *In vivo* toxicity as assessed by change in mean body weight of C4-2 mice xenograft expressed as % of control (C). No animals showed any signs of toxicity or weight loss. Data are presented as Mean value ± SEM, n of 8 in each group. A *p* value < 0.05 was considered significant (*), A *p* value < 0.01 was considered very significant (**) and a *p* value < 0.001 was considered extremely significant (***) change compared with control.
4.2.3 aPPD inhibits proliferation and induces apoptosis in C4-2 xenograft tumors

The C4-2 tumors were harvested from mice following 46 days of treatment (once daily) and were subjected to immunohistochemical analyses for proliferation (Ki-67 labeling), cell cycle regulator (p21), and apoptosis (Bax and cleaved caspase-3). As shown in Figure 4.3A, aPPD significantly inhibited cell proliferation as measured by Ki-67 labeling and the extent of suppression was approximately 25% lower than that observed in tumors isolated from animals treated with vehicle alone. Tumors from mice treated with aPPD experienced a 40% increase (p<0.001) in the number of apoptotic cells compared to control mice (Figure 4.3B). Bax is a pro-apoptotic protein, which inhibits caspase-3 activity, and therefore attenuates apoptosis. In the present study, consistent with previous *in vitro* results, an increase in Bax and cleaved caspase 3 levels were detected in the C4-2 tumors treated with aPPD. This was confirmed upon immunostaining for cleaved caspase-3 in the tumor sections from aPPD-treated and vehicle-treated groups. Thus aPPD anticancer activity appears to be mediated through mechanisms that cause a decrease in cell proliferation as well as an increase in apoptosis.

4.2.4 aPPD downregulates AR protein levels in C4-2 xenograft tumors

To elucidate the mechanistic aspect of aPPD-mediated C4-2 tumor suppression, AR protein levels were determined using Western Blot analyses. Strong downregulation of AR protein expression was seen in aPPD treated C4-2 xenograft tumors. Relative quantification of AR protein to beta actin shows that aPPD blocks AR expression by 84% compared to the control mice in C4-2 tumors (Figure 4.4).

4.2.5 aPPD causes cell cycle arrest

A significant upregulation of p27 and p21 protein was observed in tumors derived from aPPD treated mice. Results show that aPPD induces cyclin dependent kinase inhibitors (CKI) p27 and
p21, leading to decreased cyclin dependent kinase activity and cell cycle arrest in G1 phase. An increase in p21 expression and enhanced p27 accumulation correlates with the sensitivity of C4-2 tumors to aPPD treatments as shown in Figure 4.3.

![Image](image.png)

**Figure 4.3.** IHC staining of tumors derived from C4-2 xenografts. Effects of aPPD on C4-2 tumor cell proliferation(A). Effects of aPPD on apoptosis marker cleaved caspase-3 in the tumors (B). Effects of aPPD on cell cycle inhibitor p21 in the tumors
(C). C4-2 cell xenograft tumors were excised after 46 days of treatments with aPPD or control. Data are presented as Mean ± SEM, n of 4. A p value < 0.05 was considered significant (*), A p value < 0.01 was considered very significant (**) and a p value < 0.001 was considered extremely significant (***) change compared with control.

![Immunoblots of AR, p27, Bax, and B-Actin](image)

**Figure 4.** Representative Immunoblots and quantitative analyses of protein levels in C4-2 xenograft tumor as determined by Western blot. AR protein (A) is downregulated in four aPPD-treated tumors. p27 and Bax proteins (B and C) are upregulated in four aPPD-treated tumors.). The experiments were performed in duplicate and expressed as Mean ±SEM. A p value < 0.05 was considered significant (*), A p value < 0.01 was considered very significant (**) and a p value < 0.001 was considered extremely significant (***) change compared with control (vehicle-treated group).
4.2.6 In silico analysis of aPPD binding to AR ABS

Figure 4.5 presents the predicted docking poses of DHT (orange) and aPPD (green) in the AR ABS, along with DHT (light blue) in the 2AMA X-ray structure. It was found that the docking pose of DHT of the 2AMA X-ray structure and the docking pose of DHT predicted by Vina are almost identical (the root mean square deviation between them is 0.26 Å), demonstrating the Vina’s capability to predict the correct binding pose. It was also determined that aPPD can be docked into ABS without serious steric hindrance, and the four rings of aPPD occupy the similar space that is occupied by the four rings of DHT, but with a slightly different orientation. The docking score of DHT was calculated to be -11.1 kcal/mol, while that of aPPD was predicted to be -7.1 kcal/mol. A closer look at the binding orientations, delineate that the 17β hydroxyl group of DHT forms hydrogen bonds with both ASN705 and THR877, as has been observed in a previous X-ray study. The distance between the oxygen atom (O17) of the 17β hydroxyl group of DHT and the oxygen δ1 of ASN705 is 2.7 Å, and the distance between O17 and the oxygen γ1 of THR877 is 2.8 Å. On the other hand, aPPD can form the hydrogen bond with THR877, but not with ASN705. The distance between the oxygen atom of the hydroxyl group of aPPD and the oxygen γ1 of THR877 is 2.7 Å, but the distance between the oxygen atom of aPPD and the oxygen δ1 of ASN705 is now 3.9 Å. The elongation of the oxygen-oxygen distance between aPPD and ASN705 occurred mainly due to the existence of two methyl groups attached to the carbon atom adjacent to the hydroxyl group (DHT has only one methyl group attached to the corresponding carbon atom), and also the fact that aPPD needs to accommodate its methylheptyl tail into the ABS. Considering that two hydrogen bonds that 17β hydroxyl group forms with ASN705 and THR877 are conserved among the testosterone, DHT, and tetrahydrogestrinone, and also R1881, both hydrogen bonds can be the key interactions that ABS needs to accommodate its
ligands. We suggest that aPPD is a weaker binder for ABS because aPPD does not form one of the two key hydrogen bonds.

**Figure 4.5.** Predicted docking poses of dihydrotestosterone (DHT) (orange) and aPPD (green) in AR ABS, along with the DHT (light blue) in the 2AMA X-ray structure. The superimposed binding poses of aPPD around AR LBD with binding strength of -7.1 kcal/mol (in the native LBD pocket) and -11.1 kcal/mol for DHT.

**4.2.7 aPPD suppresses AR transactivation**

We have shown that aPPD (70 mg/kg daily 5 times every week for 4 weeks) was highly effective in inhibiting PC-3 tumor growth in vivo. In this study, the toxicity and effect of aPPD on AR activity was assessed in non-transfected PC-3 cells treated with increasing concentrations of this inhibitor, using a cell viability MTS assay. Up to a concentration of 12.5 µM of aPPD, there was no effect on the cell viability of PC-3 lacking the androgen receptor (Figure 4.6C). However,
aPPD at 25 μM and 50 μM demonstrated significant cellular toxicity. PC-3 cells lacking the AR activity were co-transfected with either NTD or combined NTD-DBD, followed by treatment with an ABS inhibitor (enzalutamide) or an N-terminus inhibitor (EPI-001). Except for the control cells, all the treatment groups were treated with R1881, a synthetic androgen and potent AR activator. Similar to the N-terminus inhibitor EPI-001, 300, aPPD (6.25 μM and 12.5 μM) was able to significantly inhibit both NTD- and NTD-DBD-mediated AR activation while the C-terminus inhibitor enzalutamide did not affect the activity (Figure 4. 6A-B).

**Figure 4. 6.** Inhibition of the androgen receptor by aPPD.

Inhibition of the isolated N-terminus domain (NTD) (A) and combined NTD and the DNA binding domain (NTD-DBD) (B) of AR by aPPD. (C) The toxicity of aPPD was assessed in the same experimental conditions on non-transfected PC3 cells using an MTS cell viability assay. The enzalutamide (Enza, C-terminus inhibitor) and EPI-001 (EPI, N-terminus inhibitor), and non-stimulated (no R1881) were used as controls. The results represent the mean ± SEM of 3 independent experiments with 6 replicates each.
4.3 Discussion

The effective treatment of CRPC remains a challenge. It is well established that the role of the AR persists following androgen deprivation therapy and that this very well defined therapeutic target acquires resistance via multiple evasive mechanisms. In such a dynamic progressive disease, it is essential that we come up with targeted strategies that are pleiotropic by nature in order to thwart rapid onset of advanced stages of prostate cancer. In numerous models for prostate cancer, we have identified and reported on multiple mechanisms of action of aPPD, a naturally derived compound found in ginseng. Based on our recent findings, that aPPD may have superior anticancer activities in C4-2 cells than LNCaP PCa cells in vitro, we carried out an in vivo study to examine the aPPD influence on AR-positive human C4-2 prostate xenograft tumors in mouse. Furthermore, to better understand the molecular mechanisms of aPPD-mediated anticancer effects, we explored the potential binding of aPPD to multiple sites on the AR protein in silico to further rationalize its effect on AR binding and activation in vitro.

We demonstrated that the ginsenoside aPPD significantly suppresses C4-2 tumor growth in mice bearing prostate cancer xenografts following treatment with oral gavage for 46 days. The inhibition of tumor growth was evident after seven days of treatment and the effect was pronounced as the treatment period increased. Similarly, PSA levels also decreased following aPPD treatment. The treatment dose was selected based on a previous study in PC-3 cells. Treatment with aPPD did not cause any acute toxicity in the xenograft model as determined by bodyweight, physical appearance, behavior or food and water intake. In keeping with this, upon harvesting of blood and organs, the liver and kidney function tests performed after aPPD treatment did not show any statistically significant change as indicated by ALT, AST and ALP, and serum
creatinine levels, suggesting no organ-based toxicity. Although we observed a decrease in serum amylase in aPPD-treated mice compared to the control group, the levels were still in the normal range. The accompanying markers of chronic pancreatitis were absent which therefore suggests no pancreatic abnormality. In chronic pancreatitis, amylase (AMY) and lipase (LIP) may be normal or decreased and lipase production can drop to less than 10% of the normal level. The AMY and LIP levels were within the normal range reported in the literature and there were no significant differences in LIP levels between the two groups. In addition, glucose levels were within the normal range in both groups. Collectively, this data indicates that aPPD treatment was safe at the therapeutic doses. This is consistent with our previous work where a ternary solvent system containing ethanol, propylene glycol and water (2:7:1) was used to formulate the aPPD for oral gavage. Our previous studies have established that the ternary solvent mixture is not toxic by itself when used in limited volume. This is the first report of aPPD-mediated antitumor activity in C4-2 prostate cancer model representing castration resistant disease. In corroboration with the data presented here, Cao et al. have also shown previously that aPPD inhibits the growth of LNCaP xenograft tumors (androgen-dependent) and castration-resistant 22Rv1 xenograft tumors.

To determine the mechanism of anti-tumor activity of aPPD, the C4-2 tumors were excised after the treatment period and markers of apoptosis (Bax and cleaved caspase-3) and proliferation (Ki-67) were measured. Initiation and progression of PCa are characterized by alterations and disruption in the regulatory pathways of AR, apoptosis and cell cycle regulation. Ki-67 is a marker of proliferation and can assist in the predictions of prostate cancer outcome (survival and prostate cancer recurrence). The results from the current study confirm our previous finding that
aPPD is an inhibitor of the Ki-67 proliferation marker and a stimulator of caspase-3 function that can induce apoptosis in PCa in vivo (12). As measured by Ki-67 labeling, aPPD significantly inhibited cell proliferation and the extent of suppression was significantly lower than what was observed in tumors isolated from mice treated with vehicle alone (Figure 4.3A). We have previously shown that aPPD lowers cell proliferation in PC-3 androgen-independent prostate tumors in vivo. In addition, aPPD is a strong promoter of apoptosis in C4-2 androgen-dependent prostate cancer cells as well as in LNCaP androgen dependent cells in vitro, and in PC-3 androgen-independent prostate cancer xenografts in vivo. Bax is a pro-apoptotic protein, which can inhibit caspase-3 activity, and contribute to reduced apoptosis. In the present study, consistent with previous in vitro results, an increase in Bax expression was detected in the C4-2 tumors treated with aPPD. Thus, aPPD-induced apoptosis may be associated with activation of the Bax/caspase-3 pathway. It has been reported that aPPD significantly upregulates Bax protein expression in LNCaP and C4-2 cells increasing the expression of cleaved caspase 3 in the C4-2 cell line in vitro. Other studies have also shown that ginsenosides are significant inducers of apoptosis and inhibit proliferation in prostate cancer models in vitro. These data are consistent with those shown in Figure 4.3B, where aPPD caused a significant increase in apoptotic index relative to tumors from control animals. We conclude therefore that aPPD has multiple anticancer activities which have both anti-proliferative and pro-apoptotic mechanisms.

It is well understood that AR protein is a central driving force in prostate cancer that persist in CRPC. C4-2 is an AR-dependent cell line, the effects of aPPD on AR protein expression and activity were thoroughly examined as part of this study. In spite of the close resemblance of aPPD to testosterone/DHT, aPPD is not likely a competitive antagonist of AR. Rather, aPPD was found to influence AR protein expression levels and consequent functionalities.
Quantification of AR protein levels in C4-2 xenograft tumors suggests that aPPD has the ability to downregulate AR expression and decrease in the PSA serum levels. Differences in PSA serum levels were significant for the first 2 weeks and during the last week only in aPPD treated samples compared to control. Serum PSA does not predict tumor volume but is dependent to a significant degree on the growth rate of the tumor. A rapidly growing tumor does not always predictably lead to an increased PSA level. In addition, a decrease in PSA does not necessarily correlate with increased cell death. A wide range of rates of tumor cell death are exhibited by different types of tumors and depend on the specific agent, its concentration and the type of cell lines evaluated. It has been reported that correlations between PSA and tumor volume decrease over time, ultimately PSA correlates with prostate size but not necessarily with tumor volume. In our study, the Pearson’s correlation of mean PSA with mean tumor volume in aPPD treated group was $R^2 =0.4471$ (Appendices Figure B). By contrast, PSA in the control group was more robust and had a stronger correlation with tumor volume as indicated by a Pearson value of 0.8226, p<0.001. In accordance with this finding, Cao et al. have also shown that aPPD downregulated AR expression in LNCaP xenograft tumors and it is suggested that multiple mechanisms may be involved in the aPPD-mediated downregulation of AR expression. Induction of proteasome-mediated degradation of AR protein was the primary mechanism of AR regulation within the initial 12 hr of aPPD treatment in studies conducted by Cao et al. It is postulated that blockade of interaction of N-terminus and C-terminus of AR protein instigates the AR degradation cascade. However, aPPD was also shown to subsequently decrease the promoter activities by 80% leading to decreased AR transcription. The in vivo results in the current study are consistent with our previous in vitro observations as well as other studies reported in a variety of prostate cancer cell lines including LNCaP and 22RV1 prostate cancer cells.
It has been reported that aPPD downregulates the transcription of full length and AR variants lacking the LBD and that the suppression of the AR transcriptional activity is not affected by increasing concentrations of androgen\textsuperscript{228,229}. These data suggest that aPPD binds to either the NTD or the DBD domain of AR. In order to evaluate this hypothesis, we transfected AR negative PC-3 cells with either the isolated NTD or the combined NTD-DBD domains and evaluated the effect of aPPD on these constructs. To explore the potential direct binding site of aPPD on AR, we performed \textit{in silico} docking simulation by targeting three functional binding sites, the ABS, the activation function 2 site (AF2), and the binding function 3 site (BF3). Our docking results suggest that aPPD weakly binds to the ABS compared to DHT, because aPPD does not form one of the two key hydrogen bonds which is consistent with the observation made in the previous study\textsuperscript{229}. This is the first report of aPPD docking to the different functional domains of AR.

Typically, binding of a molecule to AR may have growth stimulatory or suppressive effects. So, to evaluate the implications of aPPD binding with AR protein, we elucidated the effect of aPPD on transactivation of AR, either through NTD or combined NTD-DBD components. Interestingly, aPPD at 6.25 µM or 12.5 µM concentration inhibited AR activation in the presence of the potent AR agonist, R1881. These results suggest that the interaction of aPPD with AR occurs at multiple binding sites, leads to inhibition of AR activation and ultimately AR-mediated tumor growth suppression. The aPPD-mediated AR suppression observed in this study is likely to influence the CRPCs that are functional AR-dependent. Additionally, inherent multiple anticancer mechanisms of aPPD\textsuperscript{169,205,228,229,238,308-310} facilitate effective inhibition of both C4-2 androgen-dependent xenograft growth and androgen-independent PC-3 cells\textsuperscript{200,203}. It is worth noting that in the present study aPPD outperforms enzalutamide in that its activity goes beyond LBD binding to AR and it inhibits NTD or combined NTD-DBD transactivation. This corroborates the work of
Cao and Rennie et al. (2013) who previously demonstrated that AR and its splice variants may be inhibited by aPPD.

Consistent with previously reported studies, aPPD treatment leads to increased levels of p21 and p27 and an accumulation of cells in G1 phase of the cell cycle. A decrease in p21 expression and enhanced accumulation of p27 correlates with the observed sensitivity of C4-2 tumors to aPPD treatments as shown in Figure 4.2. A recent study has also shown that a aPPD metabolite (25-OH-PPD) significantly induced apoptosis by upregulating Bax, causing an increase in cleaved caspase-3 via binding and downregulation of MDM2 oncoprotein in PC-3 xenograft tumors (AR negative). MDM2 is a potent negative regulator of p53 that works via enhancement of P53 protein degradation. MDM2 also has p53-independent functions in cellular differentiation processes and signaling and is known to interact with AR protein. Tovar et al. have shown that MDM2 antagonist (nutlin-3a) in combination with androgen depletion in vitro and in vivo additively increased apoptosis and further downregulated AR expression in AR positive LNCaP (androgen-dependent) and 22Rv1 (androgen-independent) cell lines. This was secondary to p53 activation. MDM2 antagonism also led to a greater tumor regression and dramatically increased survival in LNCaP-bearing nude mice (p53 wild type PCa). Here, we examined the effect of aPPD in C4-2 xenograft tumors which are androgen independent - albeit AR positive. We therefore speculate that AR downregulation was secondary to knockdown of MDM2 in aPPD treated mice. Overall, aPPD led to elevated levels of p27 and p21, and enhanced cell cycle arrest and apoptosis through p53-dependent and -independent mechanisms (Figure 4.7).

In summary, the ginseng derived ginsenoside aPPD inhibited C4-2 tumor growth by 53% compared to control treatment and, in accordance with this, serum PSA was decreased by 25%. Further, the IHC and Western blot analysis of excised tumors showed that tumor cell proliferation
rate (measured by Ki-67 positive cells) was significantly lower for aPPD, and that was associated with elevated levels of Bax and cleaved caspase-3 expression (apoptotic markers), compared to the mice treated with vehicle alone. In addition, aPPD led to a significant increase in p21 and p27 (cell cycle inhibitors) protein levels. Furthermore, our finding that aPPD downregulated AR expression in vivo taken in combination with in silico and in vitro studies suggest that aPPD binds and significantly inhibits the NTD or the DBD domain of AR. The novel findings described by this study include aPPD potently inhibits PCa in vivo via inhibition of a site on AR N-terminal domain and concurrently induces apoptosis. These preclinical results support testing of aPPD in a clinical setting in advanced human PCa patients. Further research will be needed to determine whether aPPD treatment can target AR for the treatment of CRPC patients.

Figure 4.7. Proposed aPPD anti-prostate cancer mechanism in C4-2 model of castration-resistant prostate cancer.
5. CHAPTER 5: COMBINATION OF 20(S)-PROTOPANAXADIOL AND CALCITRIOL TARGETS VITAMIN D RECEPTOR: ACTIVATION OF APOPTOSIS AND GROWTH INHIBITION IN CASTRATION RESISTANT PROSTATE CANCER

5.1 Introduction

This chapter was designed to determine the involvement of VDR signaling in aPPD-mediated growth inhibition, and to further investigate the effect of aPPD on VDR protein expression in tumors. The in silico analyses were carried out to determine potential for aPPD binding to different domains on the VDR as well as in vitro assays to determine the effect of aPPD on VDR transactivation in the presence or absence of the VDR ligand calcitriol. We show that aPPD induces VDR expression which could contribute to their cooperative therapeutic effects previously reported.

5.2 Materials and Methods

5.2.1 Test compound and reagents

Ginsenoside aPPD (MW 460.73 g/mol, with a purity of ~98.9%, which was confirmed in our lab by using LC-MS), was provided as a gift by the Shanghai Innovative Research Center of Traditional Chinese Medicine (Shanghai, China). Calcitriol was purchased from Vancouver General Hospital Pharmacy as a 1 μg/ml solution (Vancouver, BC, Canada). (Oakville, ON, Canada) and Fisher Scientific (Ottawa, ON, Canada). Human VDR Kit was purchased from Indigo Biosciences Inc, PA, USA. Deuterated calcitriol and 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD)

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5A version of Chapter 5 will be further revised and submitted for publication (Ben-Eltriki M, Deb S, Hassona M, Meckling G, Shankar G, Fazli L, Chin M, Yamazaki T and Guns ES. Combination of 20(S)-protopanaxadiol and Calcitriol Targets Vitamin D Receptor: Activation of apoptosis and growth inhibition in prostate cancer).
were obtained from Cedarlane (Burlington, ON, Canada). High-performance liquid chromatography grade chemicals and all other chemicals were obtained from Sigma–Aldrich (Oakville, ON, Canada).

5.2.2  Xenograft preparation and animal treatment

All animal experiments were conducted in accordance with the University of British Columbia’s Committee on Animal Care and protocol # A11-0377 held by Dr. Guns at the Vancouver Prostate Centre. Male athymic mice age 6–8 week old (Harlan Sprague Dawley, Inc.) weighing 25–31 g were used in our study. Two million C4-2 cells in 0.5 mL (Matrige, BD Biosciences), were subcutaneously inoculated at the posterior dorsal site, similar to previous experiments. When serum PSA levels reached more than 25 ng/ml, mice were castrated. Post-castration, treatments began with a total of 8 mice per group once the total tumor size exceeded 100 mm$^3$ for aPPD at a dose of 70 mg/kg once daily (117–150 µl) alone or in a combination with calcitriol dosed at 4 µg/kg (100–128 µl) 3 times weekly, or the vehicle control at an equivalent volume based on weight. Mice were monitored daily for signs of acute toxicity including death, lethargy, blindness, and disorientation. aPPD was formulated just prior to oral administration as previously described by our laboratory. Briefly, aPPD solubilized in ethanol: propylene glycol: water (2:7:1, v/v/v ratio) was prepared prior to the administration by oral gavage at a dose of 70 mg/kg (highest achievable dose, limited due to gavage volume limitations (150 µl) imposed by the institutional animal care committee). Calcitriol solution dosed orally at a dose of 4 µg/kg (100–128 µl) solubilized in ethanol: propylene glycol: water (2:7:1, v/v/v ratio) in combination with aPPD (aPPD—70mg/kg; calcitriol 4 µg/kg). Dose selection was based on previous work completed with aPPD in our lab for safety, solubility and potency determined in solvents amenable to animal dosing prior to optimizing formulation for animal studies.
5.2.3 **Assessment of tumor growth, serum PSA and toxicity**

Triaxial tumor dimensions (mm$^3$) were measured twice weekly. Calipers were used to measure the three perpendicular axes of each tumor and the equation: volume = $\frac{1}{4}$ length x width x weight x 0.5326 was used to calculate the tumor volume. PSA levels were measured by tail vein blood sampling and sera samples analysed weekly using the Cobas automated enzyme immunoassay (Montreal, PQ). During treatment, animals were monitored daily for changes in body weight (g), appearance and signs of acute toxicity including death, lethargy, blindness, and disorientation. Mice were sacrificed when tumor volume exceeded 1,500 mm$^3$ or or body weight loss was > 20%. Otherwise mice were euthanized after 46 days of the treatment approximately 18 hours after their last treatment dose, using CO$_2$ alongside cervical dislocation. Blood samples were immediately collected (obtained by cardiac puncture) for CBC, liver and kidney function tests, serum electrolytes, glucose, serum albumin and total blood protein levels. In addition, liver, spleen, kidney, lung and brain tissues were collected for further toxicological and histopathological analysis.

5.2.4 **Tumor collection and homogenization and Immunohistochemistry**

Tumors and tissue sections were excised and either preserved in 10% formalin buffer for embedding in paraffin blocks for histopathological analysis or flash frozen in liquid N$_2$. Preparation of paraffin-embedded tissue sections and immunohistochemical analyses were carried out as previously described $^{290,291}$. C4-2 tumors were isolated from mice were prepared for immunohistochemical assessment of cell cycle, apoptosis and proliferation markers. Specifically, C4-2 tumors were sectioned and stained with hematoxylin and eosin (HE) and the desired areas marked along with their corresponding paraffin blocks. The antibodies were used for immunohistochemical staining: the anti-Cleaved Caspase 3 (Asp175) (5A1E) (#9664, 1:50, rabbit
anti-human) was procured from Cell Signaling Technology, Danvers, MA. Rabbit polyclonal anti-p21 (1:150, sc-397) was procured from Santa Cruz Biotechnology Inc. All sections used for immunohistochemistry were lightly counterstained with 5% (w/v) Harris hematoxylin. Five fields of each slide were randomly chosen and images taken (400), using an AxioCam HR CCD mounted on an Axioplan 2 microscope and Axiovision 3.1 software (Carl Zeiss, Canada). Positively stained cells and whole cells in each image were counted and the percentage of positive cells was calculated. The TMAs were manually constructed (Beecher Instruments, MD) by punching quadruplicate cores of 1 mm for each sample giving a total of 144 cores. All scoring was done blind with respect to treatment by LF and based on relative immunoreactive intensity on a four-point scale.

5.2.5 In silico docking between aPPD and VDR

The X-ray crystal structure of VDR LBD complexed with calcitriol was obtained from the Protein Data Bank (PDB ID: 1IE9), and AutoDock 4 was employed for the in silico docking. The center of the binding pocket was defined based on the coordinate of calcitriol ligand in the X-ray structure, and the box dimension of 24 Å × 24 Å × 24 Å was used for the grid search which is large enough to accommodate the ligand molecule. In order to explore the potential alternative binding places of aPPD on the VDR LBD surface, the box dimension of 96 Å × 96 Å × 96 Å was prepared for the grid search which is large enough to accommodate the entire VDR LBD surface, and in silico docking simulation was performed.

5.2.6 Western blotting for VDR

Excised C4-2 tumor tissue was homogenized using the Precellys™ tissue homogenizer system (Bertin Technologies, France) as per the manufacturer’s protocol. Proteins were extracted using RIPA buffer and Western blot was performed as previously described. Briefly, tumor tissue
(100 mg) was homogenized in RIPA buffer with 1X protease inhibitor at a 1:4 (tissue: buffer) ratio using Precellys™ Tissue Homogenizing CKMix (Cat. # 3961-1-009) at 6000 rpm for two cycles of 20 s each with a 15 s break. Forty micrograms of protein were loaded per lane into 12% SDS-acrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membrane in 48 mM Tris, 39 mM glycine, 0.1% SDS and 20% methanol (pH 8.3). The membranes were then blocked using Odyssey blocking buffer (Li-COR) containing 5% non-fat milk in wash buffer (Dulbecco’s phosphate-buffered saline with 0.1% Tween 20) for 2 h and incubated overnight at 4 °C with primary antibodies, followed by at room temperature for 3 h. Subsequently, membranes were washed and incubated in Odyssey secondary antibody for 30–45 min according to manufacturer’s instructions. Blots were imaged using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Quantification was performed on single channels with the analysis software provided and normalized to beta actin for loading and transfer. The fold induction or reduction of VDR proteins was compared to that of the vehicle control group. Antibody dilutions, duration of second antibody incubation and film exposure were optimized to produce bands linearly related to the amount of protein. The following antibodies and dilutions were used to develop the immunoblots: mouse monoclonal antibody for beta actin as loading control (1:5000; Sigma–Aldrich), and rabbit polyclonal antibodies for VDR (1:200; Santa Cruz Biotechnology Inc). Conjugated secondary antibodies (anti-mouse IRDye 800 at a dilution of 1:5000 and anti-rabbit IRDye 680 at a dilution of 1:20,000) were obtained from Cedarlane Laboratories (Burlington, ON, Canada).
5.2.7 VDR transactivation assay.

The assays were performed according to the manufacturer’s suggested protocol (Indigo Biosciences Inc, PA, USA). The effect of low nontoxic concentrations of aPPD alone or in presence of calcitriol on VDR activity, was analyzed after 24 hr. Briefly, reporter cells were prepared in Cell Recover Media (CRM) as a suspension and 100 ml was dispensed into the wells of white, cell culture treated, 96-well assay plates. We performed both agonist and antagonist’s assays, calcitriol (reference compound-VDR agonist- was provided in the kit and was used as positive control) and aPPD were diluted using compound screening medium and added into the reporter cells. Cells were treated with either 0.1 % DMSO (solvent control) or serial dilutions of increasing concentrations of aPPD alone and in combination with 0.5 nM calcitriol. Assay plates were incubated at 37°C for 24 hour. At 24 h after treatment, the medium was aspirated off and 100 µl/well of Luciferase Detection Reagent was added. The chemical oxidation of luciferin into oxyluciferin by the luciferase is accompanied by light production that can be quantified as luminescence by a TECAN M200Pro instrument to determine VDR activity. Each concentration was assayed in duplicate, with a biological replicate of n = 3. The toxicity of aPPD and calcitriol on VDR reporter cells was assessed in the same experimental conditions using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay.

5.2.8 Statistical analysis

For each studied variable, mean and standard deviation (SD) were calculated. Differences between the mean values of treatment groups were analyzed using one way ANOVA test followed by Duncan’s Multiple Range Test. The level of significance was set prior at a P value of < 0.05.
5.3 Results

5.3.1 Calcitriol sensitize PCa cells to aPPD anticancer effects

Combination-treated mice show significant inhibition of C4-2 tumor growth from 3 weeks after treatment was initiated onwards. Adding calcitriol to aPPD further inhibited tumor growth compared to aPPD alone treated group and controls (Figure 5.1A). At the end of treatment, adding calcitriol to aPPD treatments resulted in substantially greater inhibition of tumor growth than aPPD treated alone (76 % vs 53%, respectively, p < 0.01). There was no significant difference in animal body weight among treatment groups (Figure 5.1B). In combination treated mice, serum calcium levels were elevated but less than in mice treated with calcitriol alone compared with controls.

![Graph A](image1)

![Graph B](image2)

**Figure 5.1.** The *in vivo* effect of aPPD alone and in combination with calcitriol on the tumor volume of C4-2 mice xenograft (A) and serum PSA (B). Data are presented as Mean ± SEM. A p value < 0.05 was considered significant (* or #), A p value < 0.01 was considered very significant (** or # #) and a p value < 0.001 was considered extremely significant (***) or # # #) change compared with vehicle control (*) or to aPPD treated group (#). (B): *in vivo* toxicity as assessed by decrease in mean body weight of C4-2 mice xenograft. No animals showed any signs of toxicity or weight loss.
The combination of aPPD with calcitriol markedly promoted apoptosis as total apoptosis cleaved-caspase 3 was significantly higher ($p<0.001$), indicating adding calcitriol sensitizes and promotes aPPD mediated apoptosis in C4-2 cells (Figure 5.2).

![Figure 5.2.](image)

**Figure 5.2.** IHC staining of tumors derived from C4-2 xenografts. Apoptosis marker cleaved caspase-3 in the tumors. C4-2 cell xenograft tumors were excised after 46 days of treatments with aPPD, aPPD and calcitriol combination or control. Data are presented as Mean ± SEM, n of 4. A p value < 0.05 was considered significant (* or #), A p value < 0.01 was considered very significant (** or # #) and a p value < 0.001 was considered extremely significant (*** or # # #) change compared with vehicle control (*) or to aPPD treated group (#).
5.3.2 aPPD allosterically binds and activates VDR signaling

Strong upregulation of VDR protein expression was seen in C4-2 xenograft tumors of aPPD treated mice. Consistent with our previous observations in vitro, aPPD substantially also increases VDR protein levels (2 folds) in C4-2 tumors (p<0.001). A slightly increased in VDR expression was also observed when calcitriol was added to aPPD (p<0.05). (Figure 5. 3A).

![Immunoblots and quantitative analysis of VDR levels in C4-2 xenograft tumor](image)

**Figure 5. 3.** Representative Immunoblots and quantitative analysis of VDR levels in C4-2 xenograft tumor as determined by Western blot. The experiments were performed in duplicate and expressed as Mean ±SEM. A p value < 0.05 was considered significant (* or #), A p value < 0.01 was considered very significant (** or ##) and a p value < 0.001 was considered extremely significant (***) or (# # #) change compared with vehicle control (*) or to aPPD treated group (#).
To explore the potential direct binding site of aPPD on VDR, we performed in silico docking simulation by targeting the LBD binding site. Figure 5.4 (A) presents the predicted docking poses of aPPD (green) in the VDR LBD in the 1IE9 X-ray structure. *In silico* docking predicted that the binding strength of calcitriol is -13.51 kcal/mol while that of aPPD is here shown to be 12.77 kcal/mol (Figure 5.4, A), suggesting that aPPD is a slightly weaker binder to the native LBD pocket compared to calcitriol.

Further, we examined the effect of aPPD on transactivation of VDR in absence or presence of calcitriol. First, reporter cells were treated with increasing concentrations of aPPD in presence or absence of VDR ligand calcitriol and toxicity was determined. Calcitriol at 0.5 nM concentration (caused 25% activation of VDR) had no effect on cell viability determined after incubation for 24 hr (Figure 5.4-D). Up to a concentration of 4 µM of PPD with 0.5 nM calcitriol, there was no effect on the cell viability of reporter cells (Figure 5.5D). However, aPPD at 20 µM and above demonstrated significant cellular toxicity. Figure 5.5 A shows a concentration-dependent activation of VDR. At clinical relevant concentrations (0.5 nM) calcitriol had no effect on cell viability, calcitriol provokes 25% response to activate VDR. aPPD treatment alone at any concentrations has no effect on VDR activity, indicates that aPPD binds to LBD but does not activate VDR via LBD. Interestingly, in presence of calcitriol, potent VDR activator, aPPD was able to significantly enhanced calcitriol mediated VDR activation while aPPD treatments alone did not affect the VDR activity (Figure 5.5B-C).
**Figure 5.4:** Predicted docking poses of aPPD (green) in VDR in the 1IE9 X-ray structure. LBD (A), the superimposed binding poses of aPPD around VDR LBD with binding strengths between -12.77 kcal/mol (in the native LBD pocket) and -7.5 kcal/mol (B), each aPPD is colored based on its binding strength.
Figure 5.5. Calcitriol dose response analysis of Human VDR (A). No effect of aPPD on VDR activity (B). Enhancement of luminescence emission by aPPD in dose dependent manner (C). The toxicity of aPPD in combination with 0.5 nM calcitriol was assessed in the same experimental conditions on VDR reporter cells using an MTS cell viability assay (D). The Calcitriol (VDR agonist) were used as controls. The results represent the mean ± SEM of 3 independent experiments. A p value < 0.05 was considered significant (*), A p value < 0.01 was considered very significant (**) and a p value < 0.001 was considered extremely significant (***) or # # #) change compared with calcitriol treated only group (*) or to media (#)

5.4 Discussion

Our results show the potential benefits of using aPPD with calcitriol in combination as demonstrated here in AR-positive prostate castration resistant tumors in vivo. Consistent with our
in vitro results\textsuperscript{200}, calcitriol sensitizes C4-2 tumors to aPPD anticancer effects and maximize the growth inhibition actions and enhanced the ability of aPPD to induce apoptosis.

In vivo, aPPD as single agent, significantly increased VDR levels twofold and decreased AR expression (\% 80 decrease) in C4-2 tumors when compared to controls. Recently, we reported that aPPD caused a clear decline in the AR protein level and AR activities. Our findings suggest aPPD is a potent inhibitor of AR and potential activator of VDR signaling in CRPC model. aPPD also decreased p21 expression accumulation which were correlates with the sensitivity of C4-2 tumors to aPPD treatments. In addition, aPPD induced apoptosis in this xenograft model\textsuperscript{200,204-206}, as demonstrated by elevated Bax and cleaved caspase 3 levels determined in the C4-2 tumors treated with aPPD.

In the present study, adding calcitriol to aPPD caused further an increase in VDR and cleaved-caspase 3 expression. Overall, this suggests that aPPD with calcitriol treatments significantly inhibited tumor growth via mechanism-based activation of apoptosis and over expression of VDR (Figure 5.1-A). We report for the first time virtual aPPD docking to VDR LBD. We found that aPPD prefers to bind to the native LBD pocket in addition to other potential binding places. If any of these binding surfaces are associated with VDR activation (for example, in the Figure 5.4, we can see that aPPD binds to the area around Helix 2 that is associated with the A-Pocket), the proposed alternative pocket described in other studies\textsuperscript{311,312}, it is possible that aPPD activates the VDR synergistically with calcitriol. This VDR activation study indicates that aPPD is a potential activator of VDR. Therefore we postulate that there may be an effect of aPPD in enhancing calcitriol mediated activation of VDR. These results suggest that aPPD could be an allosteric activator and perhaps binds to VDR at multiple binding sites, leading to activation of
VDR activation and ultimately VDR-mediated tumor growth suppression. The hypothesis as to whether aPPD can bind to other pockets on VDR such as pocket A as proposed previously \(^{311-313}\), and activate VDR signaling, still needs further investigation. Other possible mechanisms can that could plausibly explain the enhanced tumour inhibition in vivo could be aPPD’s ability to inhibit calcitriol metabolism, increasing its half–life and thus enhancing its exposure to VDR and downstream anticancer effects. We have previously shown that aPPD inhibits CYP3A4-mediated calcitriol inactivation in human liver and intestine in vitro and in vivo \(^{81}\). This mechanism could potentially contribute to the enhancement in calcitriol mediated VDR activation in these reporter cell lines, which translates potentially to activation of the VDR pathway in vivo.

Clinically, there are no literature reports of pharmacokinetic studies of aPPD in humans, therefore no data is available on what aPPD dose that is required to achieve therapeutic levels in cancer patients. The aPPD levels determined in our study were high enough level to induce tumor reduction, perhaps via activation of the VDR signaling pathway. Furthermore, as indicated previously, aPPD and calcitriol treatment had no effect on the body weight of the animals while being sufficient to additively induce apoptosis and tumour growth suppression.

In summary, this study suggest that apoptosis is synergistically activated by aPPD and calcitriol combination treatment in C4-2 PCa models. Results reported here indicated that calcitriol further contributes to enhancement of aPPD’s anticancer effects in CRPC via VDR activation. Using aPPD in combination with calcitriol in prostate cancer patients may provide a new approach to prevent and/or treat later stage disease with minimal toxicity.
6. CHAPTER 6: CONCLUDING REMARKS

6.1 Summary of major findings and conclusions

The following conclusions can be drawn from the experimental studies described in this thesis:

Available data from preclinical studies suggest that calcitriol, administered either alone or in combination, have potential as an anticancer therapeutics. The existing clinical data, however, is insufficient to demonstrate calcitriol benefits since evidence is hampered due to the fact that most randomized controlled trials reported are too underpowered to detect positive effects (Chapter 1.3). In addition, most clinical trials have taken place in patients with recurrence or castration-resistant disease; studies should be carried out in PCa patients on active surveillance and/or receiving adjuvant therapy (after surgery and/or chemotherapy). Calcitriol biosynthesis and metabolism pathways are catalyzed by multiple CYP enzymes. This provides the opportunity to the researchers and clinicians to either stimulate calcitriol biosynthesis or inhibit its metabolism in an organ-specific manner, leading to higher exposure to endogenous or exogenously administered calcitriol. Targeting different biological pathways with calcitriol and currently available drugs capitalizes on pharmacodynamics interactions, where the pharmacological actions of the combination are synergized or potentiated. Overall, combinations of calcitriol with selected drugs are promising and offer a contemporary approach to maximizing the multifaceted biological and therapeutic actions of vitamin D in the context of PCa. To our knowledge this is the first report which focuses on assessing pharmacodynamic and pharmacokinetic interactions observed upon concurrent dosing of anti-cancer compounds with calcitriol (Chapter 1.3).

As shown in Chapter 2, the sensitivity of LNCaP and C4-2 cells to the anticancer effect of aPPD is increased by co-treatment with calcitriol and that the interaction is synergistic in nature.
Calcitriol enhanced aPPD ability to induce apoptosis and reduce cell proliferation, and this synergism may limit calcitriol toxicity by facilitating the use of lower calcitriol doses. The associated increase in VDR expression and calcitriol half-life may be mechanistically associated with this sensitization effect. For the first time, the results clearly indicate that this combination is promising as a therapeutic strategy since it offers a contemporary approach to maximize the multifaceted biological and therapeutic actions of vitamin D at a lower dose when combined with ginsenoside metabolites in the context of PCa. Dietary supplements with calcitriol and ginsenosides could be a potential strategy in prevention/treatment of cancers.

As described in Chapter 3, we successfully enhanced the sensitivity (～100 fold) of our previously published calcitriol LC/MS assay and subsequently defined a pharmacokinetic herb-nutrient interaction between aPPD and calcitriol that resulted in aPPD-mediated increase in serum calcitriol levels in nude mice that could affect vitamin D homeostasis. We speculate, based on our recent associated published work^{81}, that the PK interaction might be CYP-mediated, indicating that oral co-administration of calcitriol with aPPD could provide additional anticancer benefits to vitamin D. However, it may also increase the risk of hypercalcemia. This is the first report to show the in vivo pharmacokinetic interaction and the enhancement of the calcitriol absorption in nude mice. The important question here is whether a similar finding could occur in humans considering the limitations obtained here in murine models^{314}.

Chapter 4 reports for the first time that aPPD inhibited C4-2 tumor growth by 53% compared to control treatment and, in accordance with this, serum PSA was decreased by 25%. Further, the IHC and Western blot analysis of excised tumors showed that tumor cell proliferation rate (measured by Ki-67 positive cells) was significantly lower for aPPD, and that was associated
with elevated levels of Bax and cleaved caspase-3 expression (apoptotic markers), compared to the mice treated with vehicle alone. In addition, aPPD led to a significant increase in p21 and p27 (cell cycle inhibitors) protein levels. Furthermore, our finding that aPPD downregulated AR expression in vivo, considered in association with in silico and in vitro studies, suggest that aPPD binds and significantly inhibits the NTD or the DBD domain of AR. The novel findings described by this study therefore suggest that aPPD potently inhibits PCa in vivo via inhibition of a site on AR N-terminal domain and concurrently induces apoptosis. These preclinical results support testing of aPPD in a clinical setting in CRPC patients.

As shown in Chapter 5, data suggest that combining calcitriol with aPPD potentially could be synergistic or sensitize anticancer activity mediated via VDR activation in PCa patients. Adding calcitriol to aPPD treatments resulted in substantially greater inhibition of tumor growth than aPPD treatment alone (76 % vs 53%, respectively). IHC analysis demonstrated that combination therapy caused a significant increase in total apoptosis marker cleaved caspase-3 and Bax compared to controls. As determined in cell-based luciferase reporter gene assays, in the presence of the potent VDR ligand calcitriol, aPPD activated human VDR (Chapter 5). Human VDR was also activated by aPPD at low concentration with maximal effects seen at 4 µM. In addition, aPPD was shown to increase VDR activity at concentrations ≥ 0.16 µM. The mechanism through which aPPD enhanced activation of VDR with calcitriol does not appear to involve competitive direct or indirect binding to the receptor LBD. As shown in Chapter 4, aPPD also induced expression of VDR target genes (p21 and p27) in C4-2 tumors. Overall, results suggest therefore that aPPD are allosteric agonists or indirect activator of VDR via inhibiting calcitriol metabolism, increasing its half–life, thus increasing its VDR effects.
We aim to contribute to existing knowledge regarding the anticancer activities of both vitamin D and ginseng derived ginsenosides by reporting on the combinatorial study described by this thesis research. Our findings suggest, that aPPD is a potent inhibitor of AR and a potential activator of VDR signaling in prostate cancer. We believe that development of combinatorial strategies to treat or prevent cancer have significant potential in the context of a variety of cancers including PCa. This study suggest that apoptosis pathways are synergistically activated as a consequence of aPPD and calcitriol treatments in the CRPCa model studied. Using aPPD in combination with calcitriol for PCa may provide a new approach to prevent and/or treat prostate cancer. Combining treatment in this way would allow the use of lower doses of calcitriol in order to encounter fewer side effects, including calcitriol associated hypercalcemia.

Figure 6. 1. Overall schematic diagram of aPPD anticancer activities and mechanisms studied/proposed in this thesis (Focus on Prostate Cancer)
6.2 **Strengths and limitations of the present work**

6.2.1 **Strengths**

1. Our conclusions *in vitro* were drawn based on a constant ratio experimental design, where the drug:drug ratios are defined on the basis of similar inhibitory concentrations of the individual drugs. The dose reduction index analysis were calculated, suggests that substantially less drug is required to achieve a desired effect when using in combination as opposed to the agents alone, potentially reducing systemic related toxicities.

2. The LCMS assay could provide the most efficient and reliable method for measuring calcitriol in biomedical and clinical research (Chapter 3). It offers significant advantages in cost effectiveness and uses a small sample volume only 50 µL serum, and the high sensitivity.

3. The novel findings described herein indicate aPPD potently inhibits PCa *in vivo* partly via inhibition of a site on the AR N-terminal domain (Chapter 4).

4. In the various *in vitro* and cell-based assays performed in the present study, aPPD concentrations (up to 20 µM) inhibited NTD and NTD-DBD of AR (Chapter 4, Figure 4.6) and activated VDR (Chapter 5, Figure 5.5 C). Similarly, calcitriol concentrations (up to 10 nM). These concentrations are comparable to plasma concentrations achieved following oral administration *in vivo* 70,75,202,315 and in human for calcitriol 63,71,74,289.

5. Our LCMS method was successfully applied in determining calcitriol serum PK parameters in CD-1 and nude mice (Chapter 3). Nude mice have been extensively and routinely used to establish human tumor xenografts for cancer research 280,281. Martignoni et al 282 have shown no significant differences in drug metabolizing enzymes and drug transporter protein expression and activities between CD-1 and nude mice.
6. In presence of calcitriol, aPPD enhanced significantly calcitriol mediated VDR activation suggesting that aPPD may be an allosteric activator of VDR (Chapter 5).

7. The conclusions in this thesis were drawn based on multiple in vitro and in vivo studies, cell viability assay, proliferation rate and markers, apoptosis and cell cycle markers, tumor growth rate, toxicity data, LCMS and PK studies and analysis, mechanistic studies obtained from drug interactions with prostate regulatory receptors: AR and VDR signaling pathway, docking simulation competitive ligand binding for AR and VDR domains assay and target gene expression assays, cell-based luciferase reporter gene assays, immunohistochemistry and immunoblot analysis.

6.2.2 Limitations

1. In the present study, it is not known whether the concentration of aPPD used to investigate the effects of aPPD on AR (Chapter 4) and VDR (Chapter 5) is physiologically relevant. We have chosen these concentrations based on our in vitro toxicity results and our in vivo PK data in mice. However, no data are available for aPPD PK in human.

2. The PSA data were inconsistent and does not match with the changes that seen in tumor volume. Differences in PSA serum levels were significant for the first 2 weeks and during the last week only in aPPD treated samples compared to control (Chapter 4, Figure 4.1). Serum PSA does not always predict tumor volume but is dependent to a significant degree on the growth rate of the tumor. A rapidly growing tumor does not predictably lead to an increased PSA level. In addition, a decrease in PSA does not necessarily correlate with increased cell death. A wide range of rates of tumor cell death are exhibited by different types of tumors and depend on the specific agent, its concentration and the type of cell lines
evaluated \(^{307}\). It has been reported that correlations between PSA and tumor volume decrease over time, ultimately PSA correlates with prostate size but not necessarily with tumor volume \(^{307}\). In our study, the Pearson’s correlation of mean PSA with mean tumor volume in aPPD treated group was \(R^2 = 0.4471\). By contrast, PSA in the control group was more robust and had a stronger correlation with tumor volume as indicated by a Pearson value of 0.8226, \(p<0.001\). Details were included in the revised manuscript in the discussion section on page 13, line 287 to 297. The PSA and tumor volume correlation data have been included in the appendices (Appendices Figure B).

3. The fact that calcitriol can induce AR and CYP3A4 expression, this increases aPPD metabolism and perhaps can antagonist aPPD effect on AR signaling. However, aPPD metabolites such as 25 (OH)PPD have shown similar anticancer activities to aPPD (Chapter 1, section 4)

### 6.3 Future studies

In future studies, further characterizations need to be carried out to fully elucidate the potential of aPPD and calcitriol in prostate cancer intervention.

1. Coadministration of calcitriol with aPPD treatment may have been as a result of a decrease in CYP3A-mediated calcitriol deactivation as calcitriol’s absorption enhanced. Although CYP3A4 is the dominant elimination pathway of calcitriol \(in vivo\), aPPD could also inhibit CYP24A1 \(^{47,70,95,100,243}\) (Figure 3.7) and several other CYPs such as UGT isoenzymes \(^{250,251,284-286}\), and/or interfering with calcitriol cellular uptake. This perhaps could result in decreased metabolism of calcitriol in the intestine and its subsequent elimination from the blood. Given that CYP3A4 and UGT enzymes are a key enzymes involved in drug disposition, which also
responsible for metabolism of various drugs, it is possible aPPD modulate PK parameter of other common administered drugs if taken with ginseng. Whether aPPD affects expression of other intestinal and liver enzymes and transporters and whether that leads to enhanced efficacy of drug therapy remain to be investigated. This may be a potential for aPPD-drug interactions when concomitantly administered. This hypothesis requires further investigation.

2. Although combination treatments significantly inhibit C4-2 tumor growth compared with aPPD treated only group, unexpectedly calcitriol treatment alone increases tumor growth in C4-2 tumors in this study (as shown in appendix data, Figure C). There were discrepancies in calcitriol effects data on tumor volume among the 8 mice. The lack of growth inhibition by calcitriol in C4-2 xenograft tumors may be real and reflect differences in model used. Ajibade et al reported that early intervention with vitamin D in TRAMP model led to reduced tumor proliferation. However, prolonged calcitriol treatment resulted in development of a resistant and significantly more aggressive disease associated with increased distant organ metastasis, which is in agreement with our observation in this study. Ajibade et al also reported that castration resistant PCa was unresponsive to vitamin D intervention, which may be due to the already aggressive nature of the castration resistant phenotype. On other hand, previous in vivo studies showed that calcitriol can significantly inhibit the proliferation of PC3 and LNCaP xenograft tumors. The calcitriol effect in C4-2 could be model specific and perhaps aPPD attenuated calcitriol-mediated promoting effects on tumor growth. This requires further investigation to confirm our finding in similar model and other models. Specifically, aPPD may have attenuated calcitriol-mediated effects via its inhibitory effects on AR which may dominate over VDR when the two agents were combined in C4-2 model. This hypothesis requires further investigation.
3. A recent study has shown that a aPPD metabolite (25-OH-PPD) significantly induced apoptosis via binding and downregulation of MDM2 oncoprotein in PC-3 xenograft tumors (AR negative)\textsuperscript{204}. Here, we examined the effect of aPPD in C4-2 xenograft tumors which are androgen independent - albeit AR positive. We therefore speculate that AR downregulation was secondary to knockdown of MDM2 in aPPD treated mice. Overall, aPPD led to elevated levels of p27 and p21 (Chapter 4), and enhanced cell cycle arrest and apoptosis through p53-dependent and -independent mechanisms (Chapter 4, Figure 4.7). It could be one of the potential aPPD metabolites responsible for its action. It still remains to be verified whether aPPD metabolites can have similar or even potent anticancer activities. It is also not known whether aPPD metabolites exert a similar effect on AR and VDR signaling pathways.

4. aPPD activated VDR by a mechanism that did not involve binding to the LBD of VDR (Chapter 5, Fig. 5.5). Activation of Pocket A on VDR is proposed mechanism in this study and has been reported by others\textsuperscript{311,312} as an indirect mechanism and important factor for activation of human VDR (Chapter 5). Similarly, an increase in calcitriol cellular uptake and/or inhibition of its metabolism is proposed to be another indirect VDR activation mechanism (Chapter 5). Whether these mechanisms contribute to activation of human VDR by aPPD warrants further investigation.

5. Although AR expression significantly increased in C4-2 tumors in combination treated mice. Overall treatments significantly inhibit tumor growth, the mechanism-based activation of apoptosis and over expression of VDR dominates over AR upregulation. There are several avenues of aPPD and calcitriol PK/PD interaction research that could be proposed for future studies. The aPPD and calcitriol PD interaction \textit{in vivo} is complex and perhaps other potential
mechanisms can be investigated further. Factors responsible for growth inhibition after combination therapy can be determined. MDM2 could be one of the potential negative factor.

6. In this study, we have shown that aPPD suppress AR signaling, upregulates VDR and induces apoptosis, others have shown aPPD metabolites binds to MDM2, thus inhibits AR singling and also induce apoptosis. As shown in Chapter 2 and 4, in aPPD treated groups, downregulation of AR expression was associated with upregulation expression and function of VDR in C4-2 cells in vitro and in vivo. From a pre-clinical perspective, a better understanding of cross talk between AR and VDR and their role in PCa growth and progression will open new avenues to explore regarding therapeutic aPPD-calcitriol combination regimens.

7. Lysine-specific demethylase 1A (LSD1) is an important regulator of steroid receptors (AR, VDR and estrogen receptor) mediated endocrine pathways. Studies have shown that LSD1 levels correlated with tumor aggressiveness. An overexpression of LSD1 promotes AIPC development thought AR activation and suppression apoptosis pathways, thus influencing apoptosis and cell cycle progression. LSD1 acts as corepressor and coactivator for VDR and is also a known transcriptional regulator of p53. The correlation between LSD and AR, VDR expression in human biopsy/samples from early, late stage PCa, alongside effect of aPPD and its metabolites on LSD1 expression and activates is worth evaluating. It would be interesting to see whether there is functional cross-talk between AR and VDR and transcription regulators such as LSD1. In addition, whether aPPD shifts the balance between AR-VDR perhaps mediated via its effect on LSD1.

8. Lipoprotein and protein receptors, such as LDL and LDLr, play an important role in drug disposition specifically in highly lipophilic drugs. aPPD is high lipophilic compound that have lots of structure similarity to steroids and cholesterol, perhaps can bind and competes with
cholesterol on LDL. PCa have been shown to use cholesterol and is required for local steroids synthesis and AR activation\textsuperscript{12,322}. Given that aPPD directly inhibits AR and growth of PCa cells and tumors (Chapter 2 and 4), the effects of aPPD may also be indirect or additive via mechanism that interfere with cholesterol supply to PCa cells, thus inhibiting local steroidogenesis, thereby blocking AR activation. This is indirect mechanism and whether this aPPD modulates expression or function of CYPs that are involve in cholesterol synthesis and metabolism remains to be investigated.
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Figure A. Histopathological evaluation of the organs of male nude mice treated with aPPD for 6 weeks. Histological analysis of the organs (the lungs, liver, kidneys, and spleen) from the control group (A) and from the mice treated with aPPD (70 mg/kg) (B). No abnormal histopathological findings were observed. Samples were stained with hematoxylin and eosin (H&E. Digital image were obtained by using the Leica SCN400 scanning system with the SL801 autoloader (Leica Microsystems; Concord, Ontario, Canada) at magnification equivalent to 10x.
Figure B. Correlation between prostate-specific antigen (PSA) levels with tumor volumes for control and aPPD treated groups. Panel A, scatter plot with raw data (n=8 in each group). Panel B, Pearson’s correlation coefficients between mean PSA level with mean tumor volume (n=8).
**Figure C.** The in vivo effect of calcitriol on the tumor volume of C4-2 mice xenograft.

Data are presented as Mean ± SEM, n of 8 in each group. A p value < 0.05 was considered significant (*), A p value < 0.01 was considered very significant (**) and a p value < 0.001 was considered extremely significant (***) change compared with control.
Figure D. Calcitriol standard calibration curve. Linearity from 0.01 to 50 ng/ml in serum.
**Figure E.** Mean serum concentration-time curves of calcitriol following oral and ip administration at 4 µg/kg in CD-1 mice.
Table A: Measures of toxicity in C4-2 nude mice serum. Data represents as mean ± SEM (n = 3).

<table>
<thead>
<tr>
<th>Parameters/Units</th>
<th>Control</th>
<th>aPPD</th>
<th>Calcitriol</th>
<th>aPPD + Calcitriol</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALB (g/L)</td>
<td>44.5 ± 0.71</td>
<td>47.33 ± 1.53</td>
<td>48.67 ± 1.15</td>
<td>46 ± 1.01</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>39 ± 11.31</td>
<td>29 ± 7.55</td>
<td>36 ± 9.53</td>
<td>27 ± 5.56</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>48 ± 8.48</td>
<td>43.5 ± 10.6</td>
<td>50.6 ± 14.74</td>
<td>43.5 ± 3.29</td>
</tr>
<tr>
<td>AMY (U/L)</td>
<td>952±54.02</td>
<td>860.5±14.85*</td>
<td>1298.3 ± 114.6</td>
<td>1332 ± 15.17</td>
</tr>
<tr>
<td>LIP (U/L)</td>
<td>99±36.01</td>
<td>74.67 ± 14.52</td>
<td>92 ± 26.23</td>
<td>82 ± 15.01</td>
</tr>
<tr>
<td>TBIL (umol/L)</td>
<td>7 ± 0.05</td>
<td>7.66 ± 3.01</td>
<td>6.33 ± 0.57</td>
<td>6 ± 1.0</td>
</tr>
<tr>
<td>BUN (mmol/L)</td>
<td>5.25 ± 0.77</td>
<td>4.73 ± 0.15</td>
<td>3.4 ± 0.49</td>
<td>4.5 ± 0.78</td>
</tr>
<tr>
<td>CA (mmol/L)</td>
<td>2.565 ± 0.05</td>
<td>2.77 ± 0.06</td>
<td>3.66 ± 0.24**</td>
<td>3.3 ± 0.25*</td>
</tr>
<tr>
<td>PHOS (mmol/L)</td>
<td>2.28 ± 0.08</td>
<td>2.37 ± 0.26</td>
<td>2.16 ± 0.42</td>
<td>3.1 ± 0.63</td>
</tr>
<tr>
<td>CRE (umol/L)</td>
<td>&lt;18</td>
<td>&lt;18</td>
<td>&lt;18</td>
<td>&lt;18</td>
</tr>
<tr>
<td>Glu (mmol/L)</td>
<td>8.7 ± 0.56</td>
<td>9.5 ± 2.58</td>
<td>9.03 ± 0.83</td>
<td>10.3 ± 3.29</td>
</tr>
<tr>
<td>NA (mmol/L)</td>
<td>154.5 ± 0.71</td>
<td>158 ± 1.73</td>
<td>160 ± 1.02</td>
<td>159.67 ± 3.21</td>
</tr>
<tr>
<td>K (mmol/L)</td>
<td>7.3 ± 0.28</td>
<td>7.7 ± 0.85</td>
<td>7.36 ± 0.85</td>
<td>7.65 ± 0.21</td>
</tr>
<tr>
<td>TP (g/L)</td>
<td>52.5 ± 0.71</td>
<td>57.33 ± 0.57</td>
<td>58.67 ± 2.08</td>
<td>56. ± 1.2</td>
</tr>
<tr>
<td>GLOB (g/L)</td>
<td>8 ± 0.65</td>
<td>10.33 ± 2.08</td>
<td>9.67 ± 1.15</td>
<td>10 ± 1.32</td>
</tr>
</tbody>
</table>

Abbreviations: Albumin (Alb), Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT), Amylase (AMY), Lipase (LIP), Blood Urea Nitrogen (BUN), Calcium (Ca), Phosphorus (PHOS), Creatinine (CRE), Glucose (Glu), Sodium (Na), Potassium (K), Total Protein (TP), Globulin (Glb). Gram (g), Litter (L), Mole (mol), Unit (U). A p value < 0.05 was considered significant (*) change compared with control (vehicle-treated group).