

# Molecular pathways mediating the anti-inflammatory effects of calcitriol: implications for prostate cancer chemoprevention and treatment

Aruna V Krishnan and David Feldman

Division of Endocrinology, Department of Medicine, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, California 94305-5103, USA

(Correspondence should be addressed to D Feldman; Email: dfeldman@stanford.edu)

## Abstract

Calcitriol, the hormonally active form of vitamin D, exerts multiple anti-proliferative and pro-differentiating actions including cell cycle arrest and induction of apoptosis in many malignant cells, and the hormone is currently being evaluated in clinical trials as an anti-cancer agent. Recent research reveals that calcitriol also exhibits multiple anti-inflammatory effects. First, calcitriol inhibits the synthesis and biological actions of pro-inflammatory prostaglandins (PGs) by three mechanisms: i) suppression of the expression of *cyclooxygenase-2*, the enzyme that synthesizes PGs; ii) up-regulation of the expression of *15-hydroxyprostaglandin dehydrogenase*, the enzyme that inactivates PGs; and iii) down-regulation of the expression of PG receptors that are essential for PG signaling. The combination of calcitriol and nonsteroidal anti-inflammatory drugs results in a synergistic inhibition of the growth of prostate cancer (PCa) cells and offers a potential therapeutic strategy for PCa. Second, calcitriol increases the expression of *mitogen-activated protein kinase phosphatase 5* in prostate cells resulting in the subsequent inhibition of p38 stress kinase signaling and the attenuation of the production of pro-inflammatory cytokines. Third, calcitriol also exerts anti-inflammatory activity in PCa through the inhibition of *nuclear factor- $\kappa$ B* signaling that results in potent anti-inflammatory and anti-angiogenic effects. Other important direct effects of calcitriol as well as the consequences of its anti-inflammatory effects include the inhibition of tumor angiogenesis, invasion, and metastasis. We hypothesize that these anti-inflammatory actions, in addition to the other known anti-cancer effects of calcitriol, play an important role in its potential use as a therapeutic agent for PCa. Calcitriol or its analogs may have utility as chemopreventive agents and should be evaluated in clinical trials in PCa patients with early or precancerous disease.

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## Introduction

Prostate cancer (PCa) is the most commonly diagnosed malignancy in men after skin cancer and is a leading cause of cancer death in men in the United States (Jemal *et al.* 2008). Surgery and radiation are common initial treatment options for localized PCa. If disease recurs, it is often treated with androgen deprivation therapy (ADT). However, many patients eventually fail ADT and develop androgen-independent PCa (AIPC), more recently described as castrate-resistant PCa (CRPC). CRPC ultimately progresses to metastasis and may be lethal. This stage of PCa is not

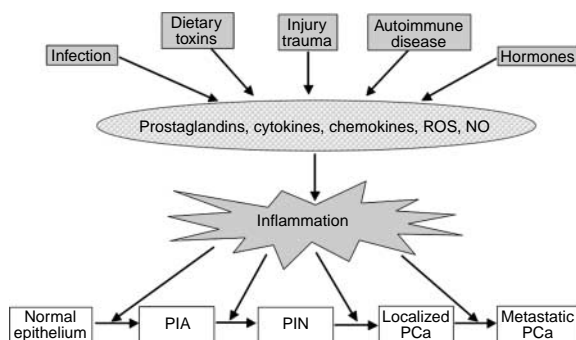
amenable to current therapy. A major goal of current research is the identification of new agents that would prevent and/or delay the development of CRPC or slow its progression.

Calcitriol (1,25-dihydroxyvitamin D<sub>3</sub>), 1,25(OH)<sub>2</sub>D<sub>3</sub>, the biologically most active metabolite of vitamin D, acts as a classical steroid hormone and regulates calcium homeostasis and bone metabolism through actions mediated by the vitamin D receptor (VDR) in intestine, bone, kidney, and the parathyroid glands (Feldman *et al.* 2007). However, calcitriol also exerts anti-proliferative and pro-differentiating effects in a number of malignant

cells and retards tumor growth in animal models of cancer raising the possibility of its use as an anti-cancer agent. Calcitriol has been shown to inhibit the development and progression of PCa (Krishnan *et al.* 2003, Stewart & Weigel 2004, Beer & Myrthue 2006, Gombart *et al.* 2006, Deeb *et al.* 2007, Fleet 2008). U.V. light is essential for the synthesis of vitamin D in the skin, and epidemiological studies suggest that mortality rates due to PCa in the US are inversely related to sunlight exposure (Hanchette & Schwartz 1992, Schwartz & Skinner 2007). Many studies suggest that vitamin D deficiency, characterized by low circulating 25-hydroxyvitamin D levels, is associated with increased PCa risk and progression (Ahonen *et al.* 2000, Giovannucci 2005, Tretli *et al.* 2009, Trump *et al.* 2009), although all studies do not support such a correlation (Platz *et al.* 2004, Ahn *et al.* 2008).

### Inflammation and PCa

Chronic inflammation has been recognized as a risk factor for the development of many cancers (Allavena *et al.* 2008, Mantovani *et al.* 2008). Inflammation can be triggered by a variety of stimuli such as injury, infection, exposure to toxins, autoimmune disease, the development of benign or malignant tumors, or other stresses and pathologies (Fig. 1). Cancer-related inflammation is characterized by the presence in tumor tissue of inflammatory cells and the overexpression of inflammatory mediators such as cytokines, chemokines, prostaglandins (PGs), and reactive oxygen and nitrogen



**Figure 1** Inflammation and PCa. Inflammation can be triggered by a variety of stresses and stimuli such as infection, injury, exposure to toxins, autoimmune disease, and changes in hormonal status. Cancer-related inflammation is characterized by the presence of inflammatory cells at the tumor sites and the overexpression of inflammatory mediators such as prostaglandins, cytokines, chemokines, reactive oxygen species (ROS), and nitric oxide (NO) in tumor tissue. These pro-inflammatory mediators promote malignant transformation and cancer progression as well as angiogenesis, invasion, and metastasis. PIA, proliferative inflammatory atrophy; PIN, prostatic intraepithelial neoplasia; PCa, prostate cancer.

species (Coussens & Werb 2002, Balkwill 2004, Lucia & Torkko 2004, Mantovani *et al.* 2008; Fig. 1). Many of these pro-inflammatory mediators activate angiogenic switches usually under the control of vascular endothelial growth factor (VEGF) and thereby promote tumor angiogenesis, metastasis, and invasion (Angelo & Kurzrock 2007, Kundu & Surh 2008).

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to reduce inflammation and prevent PCa development in a rat model of prostate carcinogenesis (Narayanan *et al.* 2009). The use of NSAIDs such as aspirin has also been shown to lower serum prostate-specific antigen (PSA) levels in men with latent PCa (Singer *et al.* 2008, Fowke *et al.* 2009). In PCa patients, a strong association between the levels of serum C-reactive protein (CRP), a nonspecific marker of inflammation, and serum PSA has been reported (Lehrer *et al.* 2005). Recent studies also show that an elevated plasma CRP level is a strong predictor of poor prognosis in patients with metastatic AIPC (McArdle *et al.* 2006, Beer *et al.* 2008). These observations suggest that inflammation might be a fundamental process in PCa. Several studies support a link between inflammation and the development of PCa (De Marzo *et al.* 1999, 2007, Nelson *et al.* 2003, 2004). De Marzo *et al.* (2004) postulate that exposure to infectious agents, hormonal alterations, and dietary carcinogens could cause injury to the prostate epithelium leading to inflammation and the formation of lesions referred to as proliferative inflammatory atrophy (PIA), which are the precursors of prostatic intraepithelial neoplasia (PIN). PIN lesions are characterized by abnormalities that are intermediate between normal prostatic epithelium and cancer, while progression to high grade PIN is the most likely precursor of prostate carcinoma (Montironi *et al.* 2004). The epithelial cells in PIA lesions have been shown to exhibit many molecular signs of stress including elevated expression of cyclooxygenase-2 (COX-2), the enzyme catalyzing the synthesis of PGs (De Marzo *et al.* 1999, Zha *et al.* 2001, Wang *et al.* 2007). Thus, mediators of inflammation appear to be involved in triggering the process of prostate carcinogenesis (Fig. 1).

### Molecular mechanisms of calcitriol actions

Many molecular pathways mediate the anti-cancer effects of calcitriol in PCa cells (Deeb *et al.* 2007). Calcitriol inhibits the proliferation of PCa cells through cell cycle arrest in the G<sub>1</sub>/G<sub>0</sub> phase (Krishnan *et al.* 2003, Yang & Burnstein 2003, Stewart & Weigel 2004) by increasing the expression of the

cyclin-dependent kinase (CDK) inhibitors *p21<sup>Waf/Cip1</sup>* and *p27<sup>Kip1</sup>* (Liu *et al.* 1996, Blutt *et al.* 1997, Campbell *et al.* 1997, Yang & Burnstein 2003), decreasing *CDK2* activity (Yang & Burnstein 2003), and causing the hypophosphorylation of the retinoblastoma protein (Jensen *et al.* 2001). Calcitriol-induced G<sub>0</sub> arrest appears to be p53-dependent (Stewart & Weigel 2004). In several PCa cell lines including the androgen-independent C4-2 cells, calcitriol reduces the expression of cellular myelocytomatosis oncogene (*c-MYC*), a transcription factor known to promote G<sub>0</sub>/G<sub>1</sub> to S-phase transition, by down-regulating *c-MYC* mRNA levels and decreasing the stability of the *c-MYC* protein (Rohan & Weigel 2009). Calcitriol also induces apoptosis in some PCa cells and suppresses the expression of anti-apoptotic genes such as *BCL-2* (Blutt *et al.* 2000). Calcitriol stimulates differentiation and modulates growth factor actions including up-regulation of the expression of *insulin-like growth factor binding protein-3 (IGFBP-3)* in PCa cells, which in turn increases the expression of *p21* causing cell cycle arrest (Boyle *et al.* 2001, Peng *et al.* 2004). Other mechanisms include the inhibition of invasion and metastasis (Sung & Feldman 2000, Krishnan *et al.* 2003, Stewart & Weigel 2004). Recent research, including observations from our laboratory, suggests that calcitriol also exerts anti-inflammatory actions that may contribute to its beneficial effects in several cancers in addition to its other known anti-proliferative actions (Krishnan *et al.* 2007a,b). In the following sections, we will discuss the role of the anti-inflammatory actions of calcitriol and its potential chemopreventive and therapeutic utility in PCa.

### Anti-inflammatory actions of calcitriol

Studies that employed cDNA microarrays to examine changes in gene expression in cancer cells treated with calcitriol or its analogs identified many novel calcitriol target genes, some of which could be important molecular mediators of its potent anti-inflammatory effects (Krishnan *et al.* 2004, Peehl *et al.* 2004, Kriebitzsch *et al.* 2009). Calcitriol regulates the expression of several genes involved in PG metabolism and signaling, thereby reducing the levels and biological activity of PGs (Moreno *et al.* 2005). PGs are pro-inflammatory molecules that promote tumorigenesis and cancer growth (Badawi 2000, Hussain *et al.* 2003, Zha *et al.* 2004). Calcitriol up-regulates the expression of *mitogen-activated protein kinase phosphatase-5 (MKP5)*; also known as *dual specificity phosphatase-10 (DUSP10)* and thereby promotes downstream anti-inflammatory

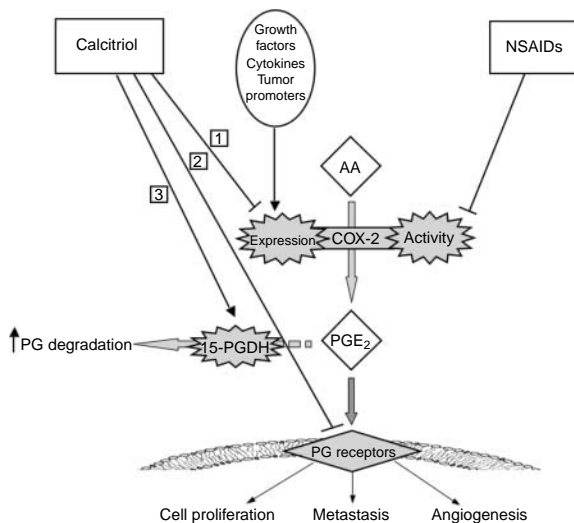
effects including a reduction in the level of expression of pro-inflammatory cytokines (Nonn *et al.* 2006). Recent research also indicates that calcitriol interferes with the activation and signaling of *nuclear factor-κB (NFκB)*, a transcription factor that regulates the expression of numerous genes involved in inflammatory and immune responses and cellular proliferation (McCarty 2004). NFκB is thought to play a key role in the process leading from inflammation to carcinogenesis (Maeda & Omata 2008). In addition, calcitriol also exhibits important anti-angiogenic effects as detailed below. In the following sections, we will discuss the importance of these molecular pathways of inflammation in the development and progression of PCa and the therapeutic significance of the inhibition of these of pro-inflammatory signals by calcitriol.

### Regulation of PG metabolism and signaling

PGs have been shown to play a role in the development and progression of many cancers, and COX-2, the enzyme that regulates PG synthesis, is an important molecular target in cancer therapy (Badawi 2000, Hussain *et al.* 2003, Wang *et al.* 2005). PGs promote carcinogenesis by multiple mechanisms such as stimulating cellular proliferation, inhibiting apoptosis, promoting angiogenesis, and activating carcinogens (Dubois *et al.* 1998, Hawk *et al.* 2002). Calcitriol has been shown to regulate the expression of several key genes involved in the PG pathway causing a decrease in PG synthesis, an increase in PG catabolism, and the inhibition of PG signaling through their receptors in normal and malignant prostate cells (Moreno *et al.* 2005; Fig. 2).

#### *Cyclooxygenase-2*

COX/PG endoperoxidase synthase is the rate-limiting enzyme that catalyzes the conversion of arachidonic acid to PGs and related eicosanoids. COX exists as two isoforms, COX-1, which is constitutively expressed in many tissues and cell types, and COX-2, which is inducible by a variety of stimuli. *COX-2* is regarded as an immediate-early response gene whose expression is rapidly induced by mitogens, cytokines, tumor promoters, and growth factors (Hussain *et al.* 2003). Evidence suggests that single nucleotide polymorphisms in the *COX-2* gene may affect PCa risk (Cheng *et al.* 2007). Genetic and clinical studies indicate that increased *COX-2* expression is one of the key steps in carcinogenesis (Markowitz 2007). Several studies suggest a causative and/or stimulatory role for *COX-2* in prostate tumorigenesis and demonstrate its overexpression in prostate adenocarcinoma (Gupta *et al.* 2000, Yoshimura *et al.* 2000). However, not all



**Figure 2** Inhibition of the PG pathway by calcitriol. Calcitriol inhibits the synthesis and biological actions of pro-inflammatory PGs by three mechanisms: (1) suppression of the expression of *COX-2*, the enzyme that synthesizes PGs; (2) down-regulation of the expression of PG receptors that are essential for PG signaling; and (3) up-regulation of the expression of *15-PGDH*, the enzyme that inactivates PGs. The combination of calcitriol and NSAIDs, which inhibit the enzymatic activity of *COX-2*, results in a synergistic inhibition of PCa cell growth. Adapted from Krishnan *et al.* (2007b).

PCa are associated with elevated *COX-2* expression (Zha *et al.* 2001, Wagner *et al.* 2005). Zha *et al.* (2001) did not find consistent overexpression of *COX-2* in established PCa. However, they detected appreciable *COX-2* expression in areas of PIA, the precursor lesions that have been implicated in prostate carcinogenesis. Silencing of *COX-2* in metastatic PCa cells arrests cell growth and causes morphological changes associated with enhanced differentiation highlighting the role of *COX-2* in prostate carcinogenesis (Narayanan *et al.* 2006). The overexpression of *COX-2* in PIA lesions also appears to be associated with elevated expression of *CCAAT/enhancer-binding protein β*, an important transcription factor involved in cellular proliferation and differentiation (Wang *et al.* 2007). *COX-2* protein expression in prostate biopsy cores and PCa surgical specimens is inversely correlated with disease-free survival (Rubio *et al.* 2005). A study analyzing archival radical prostatectomy specimens concluded that *COX-2* expression was an independent predictor of PCa recurrence (Cohen *et al.* 2006).

Local production of PGs at the tumor sites by infiltrating inflammatory cells also increases the risk of carcinogenesis and/or cancer progression (Zha *et al.* 2001, 2004, Nelson *et al.* 2004, Wang *et al.* 2005, De Marzo *et al.* 2007). In colon cancer, *COX-2*

expression is found in the carcinoma cells as well as infiltrating macrophages within the tumors (Bamba *et al.* 1999, Chapple *et al.* 2000). In other cancers, *COX-2* expression has been demonstrated in vascular endothelial cells, fibroblasts, and smooth muscle cells around the cancer (Goluboff *et al.* 1999, Mifflin *et al.* 2002). Immunohistochemical analyses of *COX-2* expression in prostate epithelium in tissue specimens from patients with PCa and benign prostatic hyperplasia (BPH) revealed high T-lymphocyte and macrophage densities in *COX-2*-positive areas raising the likelihood that pro-inflammatory cytokines released by these cells up-regulate *COX-2* expression in the adjacent tumor cells (Wang *et al.* 2004, 2005). PGs generated by *COX-2* act in an autocrine and paracrine manner to stimulate cell growth. At the cellular level, both arachidonic acid, the substrate for *COX*, and the product prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) stimulate proliferation by regulating the expression of genes that are involved in growth regulation including *c-fos* (Chen & Hughes-Fulford 2000). Studies in experimental models of cancer have shown that *COX-2* enhances tumor development and progression by promoting resistance to apoptosis and stimulating angiogenesis and tumor invasion, and it is therefore regarded as an oncogene (Zha *et al.* 2004, Kundu & Surh 2008).

#### 15-Hydroxyprostaglandin dehydrogenase

15-Hydroxyprostaglandin dehydrogenase (15-PGDH) is the enzyme that catalyzes the conversion of PGs to their corresponding 15-keto derivatives, which exhibit greatly reduced biological activity. Therefore, *15-PGDH* can be regarded as a physiological antagonist of *COX-2*. *15-PGDH* has been described as an oncogene antagonist and plays a tumor-suppressive role in colon cancer (Yan *et al.* 2004, Myung *et al.* 2006). *15-PGDH* is universally expressed in normal colon but is routinely absent or severely reduced in cancer specimens. Importantly, the stable transfection of a *15-PGDH* expression vector into colon cancer cells greatly reduces the ability of the cells to form tumors and/or slows tumor growth in nude mice demonstrating that *15-PGDH* functions as a tumor suppressor (Yan *et al.* 2004). *15-PGDH* also acts as a tumor suppressor in breast cancer (Wolf *et al.* 2006).

#### PG receptors

Prostaglandin E (PGE) and prostaglandin F (PGF) are the major PGs stimulating the proliferation of PCa cells and they act by binding to G-protein-coupled membrane receptors (prostanoid receptors). There are eight members in the prostanoid receptor subfamily,

which are distinguished by their ligand-binding profile and the signal transduction pathways that they activate, accounting for some of the diverse and often opposing effects of PGs (Breyer *et al.* 2001). PGE acts through four different receptor sub-types (EP1–EP4), while PGF acts through the FP receptor. PCa cells express both EP and FP receptors (Chen & Hughes-Fulford 2000, Moreno *et al.* 2005). PG receptors are also expressed in most endothelial cells, macrophages, and stromal cells found in the tumor microenvironment. PG interaction with its receptors can send a positive feedback signal to increase *COX-2* mRNA levels (Tjandrawinata & Hughes-Fulford 1997, Tjandrawinata *et al.* 1997, Chen & Hughes-Fulford 2000). Therefore, irrespective of the initial trigger of *COX-2* expression, PGs could mediate a wave of *COX-2* expression at the tumor sites not only in the cancer cells themselves, but also in the surrounding stromal cells and infiltrating macrophages as well as endothelial cells thereby promoting tumor progression.

#### *Calcitriol effects on the PG pathway in prostate cells*

As depicted in Fig. 2, calcitriol regulates the expression of several PG pathway genes in multiple PCa cell lines as well as cultured primary prostate epithelial cells established from surgically removed prostate tissue from PCa patients (Moreno *et al.* 2005). The levels of *COX-2* mRNA and protein are significantly decreased by calcitriol treatment of these cells (Moreno *et al.* 2005). Calcitriol increases *15-PGDH* mRNA and protein expression in various PCa cells (Moreno *et al.* 2005). Calcitriol has also been shown to regulate *COX-2* and *15-PGDH* expression in other cells such as rat macrophages and human neonatal monocytes (Pichaud *et al.* 1997, Aparna *et al.* 2008). By inhibiting *COX-2* and stimulating *15-PGDH* expression in PCa cells, calcitriol decreases the levels of biologically active PGs, thereby reducing the growth stimulation due to PGs. Further calcitriol also decreases the expression of *EP* and *FP* PG receptor mRNA in PCa cells (Moreno *et al.* 2005). The calcitriol-induced decrease in PG receptor expression results in the attenuation of PG-mediated functional responses even when exogenous PGs are added to the cell cultures. Calcitriol suppresses the induction of the immediate-early gene *c-fos* and the growth stimulation seen following the addition of exogenous PGs or the PG precursor arachidonic acid to PCa cell cultures (Moreno *et al.* 2005). The down-regulation of PG receptors by calcitriol would inhibit the positive feedback exerted by PGs on *COX-2*, thereby limiting the wave of *COX-2* expression at the tumor sites

and slowing the rate of tumor progression. Thus, calcitriol inhibits the PG pathway in PCa cells by three separate mechanisms: decreasing *COX-2* expression, increasing *15-PGDH* expression, and reducing PG receptor levels (Fig. 2). We postulate that these actions contribute to the suppression of the proliferative and angiogenic stimuli provided by PGs in PCa. The regulation of PG metabolism and biological actions constitutes an important anti-inflammatory pathway of calcitriol action.

Calcitriol analogs are being developed to find a therapeutic substitute that would have increased anti-cancer efficacy and decreased side effects of hypercalcemia (Masuda & Jones 2006). A recent study reports that the calcitriol analog elocalcitol also exhibits anti-inflammatory effects on human BPH cells (Penna *et al.* 2009). The analog causes significant decreases in *COX-2* mRNA levels and PGE<sub>2</sub> synthesis resulting in the suppression of cytokine-stimulated production of the pro-inflammatory chemokine interleukin-8 (IL-8), which is involved in BPH pathogenesis (Penna *et al.* 2009). Another study shows that a different calcitriol analog 1 $\alpha$ ,25-dihydroxy-16-ene-23-yne-vitamin D<sub>3</sub> exhibits anti-inflammatory effects causing a reduction in the expression of inflammatory markers such as *COX-2*, inducible nitric oxide synthase, and IL-2 in experimental models of inflammation (Aparna *et al.* 2008). Interestingly, this study demonstrates that calcitriol and 1 $\alpha$ ,25-dihydroxy-16-ene-23-yne-vitamin D<sub>3</sub> also directly inhibit *COX-2* enzymatic activity (Aparna *et al.* 2008).

#### *Combination of calcitriol and NSAIDs as a therapeutic approach in PCa*

NSAIDs are a class of drugs that decrease PG synthesis by inhibiting *COX-1* and *COX-2* enzymatic activities. Several NSAIDs nonselectively inhibit both the constitutively expressed *COX-1* and the inducible *COX-2*, while others have been shown to be more selective in preferentially inhibiting *COX-2* enzymatic activity. Based on our studies in PCa cells (Moreno *et al.* 2005), we hypothesized that the action of calcitriol at the genomic level to reduce *COX-2* expression, leading to decreased *COX-2* protein levels, would allow the use of lower concentrations of NSAIDs to inhibit *COX-2* enzyme activity (Fig. 2). Further, an increase in the expression of *15-PGDH* and a decrease in PG receptor levels due to calcitriol actions would lower the concentrations and biological activity of PGs thereby enhancing the NSAID effect (Fig. 2). Therefore, we hypothesized that the combination of calcitriol and NSAIDs would exhibit an

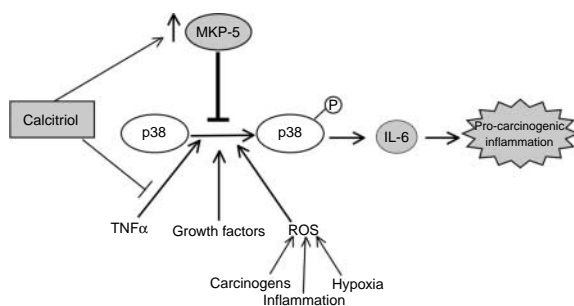
additive/synergistic activity to inhibit PCa cell growth. Combinations of calcitriol with the COX-2-selective NSAIDs, NS398 and SC-58125, and the nonselective NSAIDs, naproxen and ibuprofen, caused a synergistic enhancement of the inhibition of PCa cell proliferation, compared to the individual agents (Moreno *et al.* 2005). These results led us to further hypothesize that the combination of calcitriol and NSAIDs may have clinical utility in PCa therapy (Moreno *et al.* 2005). As discussed below (see the section on Clinical studies), we have conducted a small clinical trial examining the effects of a combination of high-dose calcitriol and the nonselective NSAID naproxen in PCa patients with far advanced disease, and the results suggest a beneficial effect of the combination to slow the course of disease progression.

### Induction of *MKP5* and inhibition of stress-activated kinase signaling

Data from cDNA microarray studies in normal human prostate epithelial cells (Peehl *et al.* 2004) revealed another novel calcitriol-responsive gene, *MKP5*, also known as *DUSP10*. *MKP5* is a member of the dual specificity MKP family of enzymes that dephosphorylate, and thereby inactivate, mitogen-activated protein kinases (MAPKs). *MKP5* specifically dephosphorylates p38 MAPK and the stress-activated protein kinase Jun-N-terminal kinase (JNK), leading to their inactivation (Fig. 3). Calcitriol up-regulates *MKP5* expression leading to downstream anti-inflammatory effects in cells derived from normal prostatic epithelium and primary, localized adenocarcinoma, supporting a role for calcitriol in the prevention and/or early treatment of PCa (Nonn *et al.* 2006). In primary cultures of normal prostatic epithelial

cells from the peripheral zone, calcitriol increases *MKP5* transcription. A putative positive vitamin D response element in the *MKP5* promoter mediates the stimulation of *MKP5* transcription by calcitriol. Interestingly, calcitriol up-regulation of *MKP5* is seen in primary cells derived from normal prostatic epithelium and primary, localized adenocarcinoma, but not in the established PCa cell lines derived from PCa metastasis. Calcitriol inhibits the phosphorylation and activation of p38 in normal primary prostate cells in a *MKP5*-dependent manner as *MKP5* siRNA completely abolished p38 inactivation by calcitriol (Nonn *et al.* 2006). A consequence of p38 stress-induced kinase activation is an increase in the production of pro-inflammatory cytokines that sustain and amplify the inflammatory response (Park *et al.* 2003). IL-6 is a p38-regulated pro-inflammatory cytokine implicated in PCa progression (Culig *et al.* 2005). Stimulation of primary prostate cells with the pro-inflammatory molecule tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) increases *IL-6* mRNA stability and concentrations of IL-6 in the conditioned media. Pre-treatment of the cells with calcitriol significantly attenuates the increase in IL-6 production following TNF $\alpha$  treatment, adding inhibition of IL-6 production to the spectrum of calcitriol-mediated actions (Nonn *et al.* 2006).

IL-6 is a major pro-inflammatory cytokine that participates in inflammation-associated carcinogenesis (Rose-John & Schooltink 2007) and has been implicated in the pathogenesis of several cancers (Schneider *et al.* 2000, Culig *et al.* 2005, Kai *et al.* 2005). Serum IL-6 levels are significantly elevated in PCa patients and in addition a positive correlation between IL-6 levels and the number of bone metastases is also seen (Tumminello *et al.* 2009). IL-6 is a growth and survival factor in human PCa cells with aggressive phenotypes and has been implicated in the progression of PCa to AIPC (Culig *et al.* 2005, Wegiel *et al.* 2008). Calcitriol reduces the production of the pro-inflammatory cytokine IL-6 by inhibiting p38 activation via *MKP5* up-regulation as well as by interfering with the signaling of pleiotropic inflammatory cytokines such as TNF $\alpha$  (Nonn *et al.* 2006; Fig. 3). These observations provide evidence for significant anti-inflammatory effects of calcitriol in prostate cells. Interestingly, established PCa cell lines derived from metastasis such as LNCaP, PC-3, and DU145 exhibit low basal levels of *MKP5*, and calcitriol is unable to induce *MKP5* expression. We therefore speculate that a loss of *MKP5* expression might occur during PCa progression, as a result of a selective pressure to eliminate the tumor suppressor activity of *MKP5* and/or calcitriol.



**Figure 3** *MKP5*-mediated inhibition of stress-activated kinase signaling by calcitriol. In prostate cells, calcitriol induces the expression of *MKP5*, which inhibits the phosphorylation and activation of the p38 stress kinase, resulting in the attenuation of the production of pro-inflammatory cytokines such as IL-6. Adapted from Nonn *et al.* (2006).

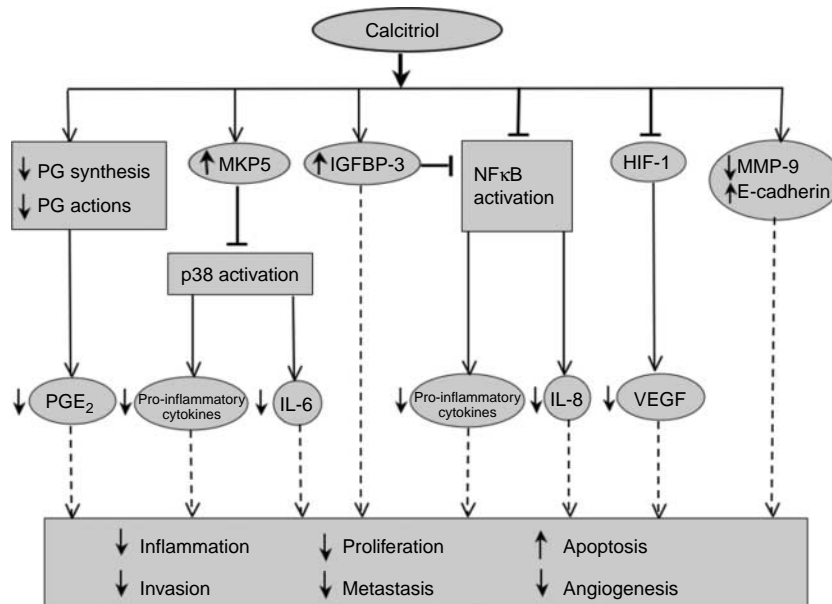
### Inhibition of NFκB activation and signaling

NFκB comprises a family of inducible transcription factors ubiquitously present in all cells that are important regulators of innate immune responses and inflammation (Karin & Lin 2002). In the basal state, most NFκB dimers are bound to specific inhibitory proteins called IκB. Many pro-inflammatory signals activate NFκB mainly through IκB kinase-dependent phosphorylation and degradation of the inhibitory IκB proteins (Maeda & Omata 2008). Free NFκB then translocates to the nucleus and activates the transcription of pro-inflammatory cytokines, chemokines, and anti-apoptotic factors (Ghosh & Karin 2002). In contrast to normal cells, many cancer cells have elevated levels of active NFκB (Sovak *et al.* 1997, Palayoor *et al.* 1999), and constitutive activation of NFκB has been observed in AIPC (Gasparian *et al.* 2002, Ismail *et al.* 2004, Suh & Rabson 2004). The NFκB protein RelB is uniquely expressed at high levels in PCa with high Gleason scores (Lessard *et al.* 2005). NFκB plays a major role in the control of immune responses and inflammation and promotes malignant behavior by increasing the transcription of the anti-apoptotic gene *BCL-2* (Catz & Johnson 2001), cell cycle progression factors such as *c-MYC* and *cyclin D1*, proteolytic enzymes such as *matrix metalloproteinase 9* (*MMP-9*) and *urokinase-type plasminogen activator*, and angiogenic factors such as *VEGF* and *IL-8* (Huang *et al.* 2001, Suh & Rabson 2004). *IL-8*, an angiogenic factor and a downstream target of NFκB, is also a potent chemotactic factor for neutrophils and is associated with the initiation of the inflammatory response (Ferrer *et al.* 1998).

Calcitriol is known to directly modulate basal and cytokine-induced NFκB activity in many cells including human lymphocytes (Yu *et al.* 1995), fibroblasts (Harant *et al.* 1998), and peripheral blood monocytes (Stio *et al.* 2007). A reduction in the levels of the NFκB-inhibitory protein IκBα has been reported in fibroblasts derived from knockout mice lacking the VDR (Sun *et al.* 2006). Calcitriol and its analogs block NFκB activation by increasing the expression of IκB in macrophages and peripheral blood mononuclear cells (Cohen-Lahav *et al.* 2006, 2007, Stio *et al.* 2007). VDR appears to play an inhibitory role in the regulation of basal NFκB signaling in colon cancer cells, and the VDR antagonist ZK191732 increases NFκB activity by decreasing IκBα (Schwab *et al.* 2007). Calcitriol and its analogs also inhibit NFκB activation in different types of immune cells (Dong *et al.* 2003, Giarratana *et al.* 2004, Penna *et al.* 2007). There is considerable evidence for the inhibition of NFκB signaling by

calcitriol in PCa cells. Calcitriol decreases the levels of the angiogenic and pro-inflammatory cytokine *IL-8* in immortalized normal human prostate epithelial cell lines (HPr-1 and RWPE-1) and established PCa cell lines (LNCaP, PC-3, and DU145; Bao *et al.* 2006a). The suppression of *IL-8* by calcitriol appears to be due to the inhibition of *NFκB* signaling. Calcitriol reduces the nuclear translocation of the NFκB subunit p65 thereby inhibiting the NFκB complex from binding to its DNA response element and consequently suppressing the NFκB stimulation of transcription of downstream targets such as *IL-8* (Bao *et al.* 2006a). Thus, calcitriol could delay the progression of PCa by suppressing the expression of angiogenic and pro-inflammatory genes such as *VEGF* and *IL-8* (Fig. 4). The calcitriol analog elocalcitol inhibits the production of *IL-8* by human BPH cells by arresting NFκB p65 nuclear translocation (Penna *et al.* 2009). NFκB also provides an adaptive response to PCa cells against cytotoxicity induced by redox-active therapeutic agents and is implicated in radiation resistance of cancers (Kimura *et al.* 1999, Criswell *et al.* 2003). Calcitriol significantly enhances the sensitivity of PCa cells to ionizing radiation by selectively suppressing radiation-mediated RelB activation (Xu *et al.* 2007). Thus, calcitriol may serve as an effective agent for sensitizing PCa cells to radiation therapy via suppression of the *NFκB* pathway.

In addition to the direct inhibition, calcitriol also indirectly inhibits NFκB signaling by up-regulating the expression of other proteins that interfere with NFκB activation such as IGFBP-3 and clusterin (CLU) as discussed below. Calcitriol up-regulates the expression of *IGFBP-3* (Boyle *et al.* 2001, Peng *et al.* 2004), which has been shown to interfere with NFκB signaling in PCa cells by suppressing p65 NFκB protein levels and the phosphorylation of IκBα (Jogie-Brahim *et al.* 2009; Fig. 4). Apart from its ability to inhibit NFκB, IGFBP-3 has multiple anti-PCa actions including inhibition of proliferation, stimulation of apoptosis, and a variety of anti-inflammatory effects (Boyle *et al.* 2001, Ali *et al.* 2003, Peng *et al.* 2004, 2008, Jogie-Brahim *et al.* 2009; Fig. 4). NFκB activity is also modulated by apolipoprotein J or CLU. CLU is a protein expressed in virtually all tissues and is regulated in inflammation, apoptosis, and cancer. Its nuclear form (nCLU) is implicated in apoptosis (Gleave & Chi 2005, Shannan *et al.* 2006). In human neuroblastoma cells and mouse embryonic fibroblasts, CLU has been shown to strongly inhibit NFκB activity through the stabilization of IκB proteins (Santilli *et al.* 2003). Calcitriol and its analogs up-regulate CLU expression with an accompanying apoptotic response



**Figure 4** Summary of molecular pathways mediating the anti-inflammatory actions of calcitriol. Several new calcitriol target genes have been identified revealing multiple molecular pathways of anti-inflammatory actions of calcitriol in prostate cells. These include (1) the inhibition of PG synthesis and biological actions; (2) the induction of *MKP5* expression and the subsequent inhibition of p38 stress kinase activation and the production of pro-inflammatory cytokines such as IL-6; (3) the inhibition of NFκB signaling resulting in the attenuation of the synthesis of pro-inflammatory cytokines such as IL-8; (4) up-regulation of the expression of IGFBP-3, which suppresses proliferation and inhibits NFκB activation; (5) inhibition of tumor angiogenesis due to suppressive effects on the expression of pro-angiogenic factors such as HIF-1, VEGF, and IL-8; and (6) the regulation of the expression of molecular mediators such as MMP-9 and E-cadherin to inhibit invasion and metastasis. Solid lines indicate direct actions of calcitriol and dotted lines indicate downstream effects of calcitriol.

in several cells including human BPH cells (Crescioli *et al.* 2004) and human breast cancer cells (Simboli-Campbell *et al.* 1997).

### Inhibition of angiogenesis

Angiogenesis is the process of formation of new blood vessels from existing vasculature and is a crucial step in continued tumor growth, progression, and metastasis (Folkman 1995). VEGF is the most potent stimulator of angiogenesis. PGs, as discussed below, are also important pro-angiogenic factors. The initiation of angiogenesis is controlled by local hypoxia, which induces the synthesis of pro-angiogenic factors that activate signaling pathways leading to the structural reorganization of endothelial cells favoring new capillary formation (Sakamoto *et al.* 2008). Stimulation of angiogenesis in response to hypoxia is mediated by hypoxia-inducible factor 1 (HIF-1), which directly increases the expression of several pro-angiogenic factors including VEGF (Giaccia *et al.* 2003, Rankin & Giaccia 2008). Early studies indicate that calcitriol is a potent inhibitor of tumor cell-induced angiogenesis in experimental models (Majewski *et al.* 1996). Calcitriol inhibits VEGF-induced endothelial cell

tube formation *in vitro* and decreases tumor vascularization *in vivo* in mice bearing xenografts of breast cancer cells overexpressing VEGF (Mantell *et al.* 2000). Calcitriol and its analogs also directly inhibit the proliferation of endothelial cells (Bernardi *et al.* 2002, Furigay & Swamy 2004, Ben-Shoshan *et al.* 2007, Chung *et al.* 2009) leading to the inhibition of angiogenesis.

At the molecular level, calcitriol exerts its anti-angiogenic effects by regulating the expression of key factors that control angiogenesis. Calcitriol reduces the expression of VEGF in several malignant cells including PCa cells through transcriptional repression of HIF-1 (Ben-Shoshan *et al.* 2007). Furthermore, as discussed above, calcitriol inhibits PCa cell-induced angiogenesis by suppressing the expression of the pro-angiogenic factor IL-8 in an NFκB-dependent manner (Bao *et al.* 2006a). Chung *et al.* (2009) established transgenic adenocarcinoma of the mouse prostate (TRAMP) -2 tumors in wild-type mice and VDR knockout mice and found enlarged vessels and increased vessel volume in TRAMP tumors in the VDR knockout mice, suggesting an inhibitory role for VDR and calcitriol in tumor angiogenesis. The study further showed increased expression of



pro-angiogenic factors such as HIF-1 $\alpha$ , VEGF, angiopoietin-1, and platelet-derived growth factor in the tumors in the VDR knockout mice (Chung *et al.* 2009).

Interestingly, another important mechanism by which COX-2 promotes tumor progression is through the stimulation of angiogenesis, and COX-2 inhibitors have been used to block angiogenesis and tumor proliferation (Sahin *et al.* 2009). The pro-angiogenic effect of COX-2-generated PGE<sub>2</sub> might be due to its action to increase HIF-1 $\alpha$  protein synthesis in cancer cells (Fukuda *et al.* 2003). In PCa cells, PGE<sub>2</sub> mediates hypoxic effects on VEGF by promoting the translocation of HIF-1 $\alpha$  protein to the nucleus (Liu *et al.* 2002). The selective COX-2 inhibitor NS398 has been shown to suppresses the growth of PC-3 xenografts *in vivo* by a combination of the direct induction of tumor cell apoptosis and the down-regulation of tumor VEGF, leading to the inhibition of angiogenesis as demonstrated by decreased microvessel density of the tumors (Liu *et al.* 2000). An immunohistochemical analysis of human PCa specimens by Wang *et al.* (2005) reveals that increased COX-2 immunostaining is associated with an increased infiltration of T-lymphocytes and macrophages and increased CD31-marked microvessel density, indicating a positive correlation between COX-2 expression and inflammation and angiogenesis. The authors postulate that pro-inflammatory cytokines released by tumor-adjacent inflammatory cells such as T-lymphocytes and macrophages may induce COX-2 in the epithelial cells in prostate atrophic lesions promoting tumor progression (Wang *et al.* 2004). Suppression of COX-2 expression by calcitriol therefore provides an important indirect mechanism by which calcitriol inhibits angiogenesis, in addition to its direct suppressive effects on pro-angiogenic factors such as HIF-1 and VEGF. It has been suggested that VEGF induction of p38 and JNK pathways is necessary for COX-2 expression in endothelial cells (Wu *et al.* 2006). As discussed above, calcitriol inactivates the p38 pathway by inducing MKP5 expression. Thus, MKP5 induction and VEGF suppression by calcitriol could further contribute to its anti-angiogenic effects through p38 inactivation.

MMPs promote angiogenesis by mediating the degradation of the basement membrane of the vascular epithelium and the extracellular matrix, thereby creating a passageway in these barriers for the formation of new capillaries (Sakamoto *et al.* 2008). In human PCa cells, calcitriol decreases the expression and activity of MMP-9 while increasing the activity of its counterpart tissue inhibitor of metalloproteinase-1 (TIMP-1), thereby decreasing the invasive potential of these cells (Bao *et al.* 2006b).

### Inhibition of invasion and metastasis

Calcitriol reduces the invasive and metastatic potential of many malignant cells including PCa cells. The mechanisms underlying this effect include the inhibition of angiogenesis (discussed above) and regulation of the expression of key molecules involved in invasion and metastasis (Osborne & Hutchinson 2002). Calcitriol decreases the tumor size and lung metastasis of the highly metastatic Mat-ly lu and R 3327-AT-2 Dunning PCa cells *in vivo* (Getzenberg *et al.* 1997). Calcitriol inhibits the invasiveness and migration potential of metastatic PCa cells such as DU145 and PC-3, due in part to decreasing the expression of  $\alpha 6$  and  $\beta 4$  integrins (Sung & Feldman 2000). In PCa cells, calcitriol and its analogs also increase the expression of E-cadherin, a tumor suppressor gene whose expression is inversely correlated to metastatic potential (Campbell *et al.* 1997). Calcitriol-mediated suppression of MMP-9 activity and increase in TIMP-1 also decrease the invasive potential of PCa cells (Bao *et al.* 2006b).

### The role of anti-inflammatory effects of calcitriol in PCa prevention and treatment

PCa generally progresses very slowly, likely over decades, before symptoms become clinically detectable and a diagnosis is made (Fang *et al.* 2001, Whittemore *et al.* 2005, Loeb *et al.* 2006, Steyerberg *et al.* 2007). Recently, inflammation in the prostate has been proposed to be an etiological factor in the development of PCa (Nelson *et al.* 2004, De Marzo *et al.* 2007). The observed latency in PCa provides a long window of opportunity for intervention by chemopreventive agents. Treatment with COX-2-selective NSAIDs such as celecoxib has been shown to suppress prostate carcinogenesis in the TRAMP model (Gupta *et al.* 2004). Our studies on the inhibitory effects of calcitriol on COX-2 expression and the PG pathway and MKP5 induction with the resultant stress kinase inactivation and inhibition of pro-inflammatory cytokine production as well as other multiple published observations of calcitriol actions to inhibit NF $\kappa$ B signaling and tumor angiogenesis suggest that calcitriol exhibits significant anti-inflammatory and anti-angiogenic effects *in vitro* and *in vivo* (Fig. 4). Therefore, calcitriol has the potential to be useful as a chemopreventive agent in PCa (Chen & Holick 2003, Banach-Petrosky *et al.* 2006, Krishnan *et al.* 2007a,b). Foster *et al.* have demonstrated that administration of high-dose calcitriol (20  $\mu$ g/kg), intermittently

3 days/week for up to 14–30 weeks, suppresses prostate tumor development in TRAMP mice (Alagbala *et al.* 2005, 2006). The efficacy of calcitriol as a chemopreventive agent has been examined in Nkx3.1; Pten mutant mice, which recapitulate stages of prostate carcinogenesis from PIN lesions to adenocarcinoma (Banach-Petrosky *et al.* 2006). The data reveal that calcitriol significantly reduces the progression of PIN from low-grade to high-grade lesions. Calcitriol is more effective when administered before, rather than subsequent to, the initial occurrence of PIN. These animal studies as well as *in vitro* observations suggest that clinical trials in PCa patients with PIN or early disease evaluating calcitriol and its analogs as agents that prevent and/or delay PCa progression are warranted.

### Clinical studies of calcitriol in PCa

Several investigators have conducted clinical trials evaluating the use of calcitriol in PCa patients. In an early clinical study in PCa patients who had undergone previous radiation or surgical treatment, a statistically significant decrease in the rate of rise of serum PSA levels was seen following the administration of modest daily doses of calcitriol (Gross *et al.* 1998), suggesting a beneficial effect of calcitriol in slowing the progression of the disease. Calcitriol is an FDA approved drug (for other indications). The side effects associated with calcitriol use are the development of hypercalcemia and renal stones and possibly increased soft tissue and vascular calcification. Some investigations have followed the approach of administering very high doses of calcitriol intermittently, such as three times a week (Trump *et al.* 2006) or once weekly (Beer *et al.* 2003), when it apparently can still elicit anti-proliferative effects but cause only transient hypercalcemia and limited toxicity (Beer *et al.* 2003, Trump *et al.* 2006). The use of nutrient vitamin D (cholecalciferol), the biochemical precursor of calcitriol, has also been evaluated in a pilot clinical study, revealing a beneficial effect of prolongation of PSA doubling time (Woo *et al.* 2005).

Many academic investigators and pharmaceutical companies have undertaken intense research to develop structural analogs of calcitriol, which exhibit increased anti-proliferative activity and reduced tendency to cause hypercalcemia (Ma *et al.* 2006, Masuda & Jones 2006). A phase II study in PCa patients with advanced hormone-refractory PCa evaluated the calcitriol analog  $1\alpha$ -OH-D<sub>2</sub> that had exhibited a reduced calcemic effect but an equal or greater anti-proliferative effect in comparison to calcitriol in preclinical studies (Liu *et al.* 2003).

PSA response was not considered a surrogate marker in this study. Although no objective responses were seen, 30% of the patients experienced stable disease for >6 months, suggesting possible cytostatic activity (Liu *et al.* 2003). In a phase I/II study of the calcitriol analog 19-nor- $1\alpha$ -25(OH)<sub>2</sub>D<sub>2</sub> (paricalcitol) in patients with AIPC, no PSA or objective responses were seen (Schwartz *et al.* 2005). However, the data suggested that paricalcitol might be beneficial in the reduction of skeletal morbidity (Schwartz *et al.* 2005). Administration of the VDR agonist elocalcitol (BXL-628) has been shown to arrest prostate growth in patients with BPH (Maggi *et al.* 2006).

Calcitriol and its analogs have also been tested in PCa patients and in other cancers in combination with various therapeutic agents. Preclinical (Torrance *et al.* 2000) and clinical studies (Meyskens *et al.* 2008) on colon and other cancers have successfully used the strategy of combining low doses of two active drugs to achieve a more effective chemoprevention and therapeutic outcome compared to the individual agents (Sporn & Hong 2008). The combination approach would also minimize the toxicities of the individual drugs by allowing them to be used at lower doses while achieving a significant therapeutic effect. Calcitriol is also being used in combination therapy with other agents that may enhance its anti-proliferative activity while reducing its hypercalcemic tendency (Trump *et al.* 2006, Beer *et al.* 2007). Trump *et al.* (2006) carried out a phase II trial in patients with AIPC using high-dose oral calcitriol (12 µg/day 3 times/week) with dexamethasone (4 mg/day 4 times/week), and the results showed a 50% reduction in PSA in 28% of the patients and no hypercalcemia. Another phase II trial tested the combination of calcitriol, dexamethasone, and carboplatin in patients with hormone-refractory PCa and found a PSA response in 13 out of 34 patients (Flaig *et al.* 2006).

Studies suggest that the addition of calcitriol to chemotherapy drugs might be beneficial in the management of advanced, hormone-refractory PCa (Berry & Eisenberger 2005, Kantoff 2005). The ASCENT I clinical trial in advanced CRPC patients who had failed other therapies studied the administration of a very high dose (45 µg) of calcitriol (DN101, Novacea, South San Francisco, CA, USA), administered once weekly along with the chemotherapy drug docetaxel (Taxotere, sanofi-aventis, Bridgewater, NJ, USA) at the usual regimen in use at the time of that trial (once weekly) compared to docetaxel alone. The results of the trial appeared to indicate that the combination caused a very significant improvement in overall survival and time to progression, suggesting that calcitriol could enhance

the efficacy of active drugs in cancer treatment (Beer *et al.* 2007, 2008). The ASCENT I trial did not meet its primary endpoint, i.e. a lowering of serum PSA. However, based on the promising survival results (16.4 months in the docetaxel arm versus 24.5 months in the docetaxel plus calcitriol arm), a larger phase III trial (ASCENT II) with survival as an endpoint was initiated. A new, improved docetaxel regimen (every 3 week dosing) was used in the control arm of the ASCENT II trial, which was compared to DN101 plus the older docetaxel dose regimen (once a week), resulting in an asymmetric study design. Unfortunately, the improved survival due to the combination demonstrated in the ASCENT I trial could not be confirmed in the ASCENT II trial (Press Release, June 04, 2008, <http://novacea.com/>). In fact, the trial was prematurely stopped by the data safety monitoring committee after 900 patients were enrolled, when an excess number of deaths were noted in the study arm (DN101 plus old docetaxel regimen) versus the control arm (new docetaxel regimen). This negative result eventually led to the company Novacea ending its DN-101 PCa program.

Another recent study has tested the combination of high-dose calcitriol (DN-101) with mitoxantrone and prednisone in patients with metastatic AIPC without previous chemotherapy, and has concluded that calcitriol did not significantly add to the activity of mitoxantrone and prednisone as assessed by the decline in serum PSA levels (Chan *et al.* 2008). In a randomized, double-blinded phase II study in a similar patient population, the addition of daily doses of doxercalciferol ( $1\alpha$ -hydroxyvitamin D<sub>2</sub>) to weekly docetaxel did not enhance the PSA response rate or survival (Attia *et al.* 2008). Interestingly, another study testing the effect of the combination of weekly high-dose calcitriol and docetaxel in PCa patients whose disease progressed after first-line chemotherapy using docetaxel alone showed that high-dose calcitriol restored the sensitivity to chemotherapy with docetaxel (Petrioli *et al.* 2007).

Preclinical observations in prostate cell cultures (Moreno *et al.* 2005) suggested that a combination of calcitriol with a NSAID would be a beneficial approach in PCa therapy. The combination strategy would allow the use of lower concentrations of NSAIDs thereby minimizing their undesirable side effects. It has become clear recently that the long-term use of COX-2-selective inhibitors such as rofecoxib (Vioxx) causes an increase in cardiovascular complications in patients (Ray *et al.* 2002, Antman *et al.* 2005, Graham *et al.* 2005, Solomon *et al.* 2006). Very recently, even the use of nonselective NSAIDs has been shown to

increase cardiovascular risk in patients with heart disease (Gislason *et al.* 2009). However, in comparison to COX-2-selective inhibitors, nonselective NSAIDs such as naproxen may be associated with fewer cardiovascular adverse effects (Bombardier *et al.* 2000, Gislason *et al.* 2009). Preclinical data showed that the combinations of calcitriol with nonselective or selective NSAIDs were equally effective in inducing synergistic growth inhibition (Moreno *et al.* 2005). Based on these data, we have recently carried out a single-arm, open-label phase II study evaluating the combination of the nonselective NSAID naproxen and calcitriol in patients with early recurrent PCa (Srinivas & Feldman 2009). Patients in this study had no evidence of metastases. All of the patients received 45 µg of calcitriol (DN-101) orally once a week and 375 mg naproxen twice a day for 1 year. The trial was prematurely stopped after 21 patients had been enrolled when the FDA put a temporary hold on DN-101 based on the preliminary data from the ASCENT II trial described above. The therapy was well tolerated by most patients in the trial. Serum PSA levels were monitored every 2 months. Bone scans were done every 3 months along with ultrasound of the kidney to assess the possibility of asymptomatic renal stones. One patient developed a small asymptomatic renal stone and was removed from the study. He required no intervention for his renal stone. Only four patients showed evidence of progression and they were removed from the study and offered other therapy. Changes in PSA doubling time (PSA-DT) post intervention were compared to baseline PSA-DT values. A prolongation of the PSA-DT was achieved in 75% of the patients suggesting a potential beneficial effect of the combination therapy (Krishnan *et al.* 2009, Srinivas & Feldman 2009).

The use of calcitriol or various vitamin D analogs in trials in men with PCa has thus far been disappointing. Based on preclinical data from cell culture and animal models showing substantial benefits, the modest efficacy thus far achieved in patients is underwhelming. The most promising study, ASCENT I, using very high doses of calcitriol intermittently (to avoid complicating side-effects) in combination with docetaxel had very encouraging findings since the data showed improved survival and not just changes in the surrogate marker PSA (Beer *et al.* 2007). However, the expanded follow-up trial, ASCENT II, not only failed to show any benefit but put a shadow over the use of calcitriol for PCa therapy when it was stopped prematurely because of increased deaths in the calcitriol arm. However, the trial was poorly designed with asymmetric placebo and treatment arms. Since the

halting of the trial, further analysis (Press Release, September 11, 2008, <http://novacea.com/>) suggested that the increased deaths in the treatment arm compared to the control arm were not due to calcitriol toxicity, but due to better survival in the control arm that had received the new and improved docetaxel regimen compared to the older docetaxel regimen. Although both ASCENT trials seemed to indicate little problem with toxicity using very high but intermittent doses, the benefits of calcitriol were decidedly unimpressive or nonexistent in the larger ASCENT II trial. Thus, data from clinical studies using calcitriol are not as hopeful as that predicted by epidemiological and preclinical studies. One obvious and major difference in the approaches is that many of the clinical studies including the ASCENT trials were carried out in men with far advanced PCa or in men who had failed all other therapies. This suggests that calcitriol may be better used in early disease and/or in chemoprevention.

However, when used in early disease, an efficacy trial would require very large numbers of subjects and long-term studies to demonstrate improved survival or prolonged time to progression. This has led many trials examining patients with early disease to rely on changes in PSA levels as a surrogate biomarker to demonstrate benefit. There is much discussion in the urology field about whether declines in absolute PSA levels, the rate of PSA rise, PSA velocity, or PSA doubling time are valid indicators of efficacy, and criteria have been established to utilize PSA change as a biomarker of efficacy (Petrylak *et al.* 2006, Scher *et al.* 2008). However, in clinical trials in men with early recurrent disease, whose prostates have been surgically removed or irradiated, changes in PSA levels remain a useful although not perfect tumor biomarker. Some studies do show a PSA benefit due to calcitriol by various criteria, at least in some men. Based on the strong rationale from preclinical data and epidemiological studies described in this review on control of inflammation and the many other actions of calcitriol that predict efficacy, it is our opinion that it would be premature to conclude from the limited number of clinical trials completed thus far that calcitriol has little or no efficacy in men with PCa. We need to determine the optimum regimen of calcitriol or its analogs to be used and the time during the course of PCa that is best to intervene. Since calcitriol has mild anti-proliferative and differentiating actions that are more cytostatic than cytolytic, we believe therapy would be most effectively used in chemoprevention or early disease, at the highest dose tolerated without side effects and probably in combination with other drugs.

The epidemiological studies indicating increased risk of cancer in vitamin D deficiency (Giovannucci 2005, Garland *et al.* 2006) and the high frequency of vitamin D deficiency in the population (Tangpricha *et al.* 2002) indicate that at a minimum, vitamin D status should be assessed and deficiency should be vigorously treated with vitamin D supplements in men at high risk for PCa or those that have already been diagnosed with cancer. The available data suggest that the likelihood of vitamin D or calcitriol having a beneficial effect in the prevention of PCa development or in treatment of early disease is better than its effects on advanced disease. Therefore, the detection and correction of vitamin D deficiency in the population at large is worthwhile for this and many other reasons. In the future, improved vitamin D analogs with increased efficacy and decreased tendency to cause hypercalcemia may be the best therapeutic option (Ma *et al.* 2006, Masuda & Jones 2006). However, in the interim until those drugs are developed and become available, treatment with high doses of dietary vitamin D seems to be a safe and worthwhile approach. The prostate expresses 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase, the enzyme that can convert circulating 25-hydroxyvitamin D to calcitriol (Schwartz *et al.* 1998, Hsu *et al.* 2001, Chen *et al.* 2003). Thus, dietary vitamin D supplements can lead to local calcitriol production within the prostate where it acts as a paracrine hormone to inhibit prostate growth and perhaps to delay or prevent the progression to cancer (Schwartz *et al.* 1998, Chen *et al.* 2003). This seems a worthwhile strategy for a safe and effective approach until new, more potent drugs become available.

## Conclusions

Recent research has identified several new calcitriol target genes revealing multiple molecular pathways of calcitriol action in prostate cells (Figs 1–4). The data suggest that calcitriol has several anti-inflammatory actions in addition to its other well-known therapeutic and cancer-preventive effects in PCa. We conclude that calcitriol or its analogs may have the best utility as chemopreventive agents in PCa and/or when used in early disease. Studies in animal models suggest that calcitriol is more effective when administered before, rather than subsequent to, the initial occurrence of PIN. We believe that calcitriol and its analogs should therefore be further evaluated in clinical trials in PCa patients with early or precancerous disease. The avoidance of vitamin D deficiency should be a goal for reducing the incidence of PCa.

Additional trials in men with minimal or early disease, treating with high doses of dietary vitamin D, new potent analogs or, combination therapy may finally demonstrate the promise of benefit of vitamin D in the prevention and treatment of PCA.

### Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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