# Effects of $1,25(OH)_2D_3$ on Smad2 Activity in PC-3 Prostate Cancer Cells

**Project Work in Biomedicine, Advanced Level, 7.5 ECTS**

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# ABSTRACT

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The vitamin D metabolite $1,25(OH)_2D_3$ has long been known to inhibit growth of prostate cancer cells and this mainly through a VDR-mediated pathway controlling target gene expression, resulting in cell cycle arrest, apoptosis and differentiation. Another major way in which $1,25(OH)_2D_3$ inhibits cell growth in prostate cancer is via membrane-initiated steroid signalling, which triggers activation of signal cascades upon steroid binding to a receptor complex, leading to induction of genes regulating cell growth, proliferation and apoptosis. The main prostate cancer inhibiting membrane-initiated route is the TGFß signalling pathway, elicited by the protein TGFß. Another important protein downstream in this cascade is Smad2. In this study the early effects of $1,25(OH)_2D_3$ on activated Smad2 levels in PC-3 prostate cancer cells were examined. PC-3 cells were incubated for 5, 10, 30 and 60 minutes as well as 24 and 40 hours both together with $1,25(OH)_2D_3$ of the concentrations $10^{-10}$ and $10^{-7}$ M and without. An ELISA assay scanning for activated Smad2 was then performed on supernatants from both treated and untreated cells. This is a follow-up to an earlier study which examined the influence of $1,25(OH)_2D_3$ on TGFß levels using the same doses and similar time points and which found that $1,25(OH)_2D_3$ initially lowered the level of active TGFß, then increased it. The results of this study showed a statistically insignificant, time delayed $1,25(OH)_2D_3$ mediated induction of the same pattern in the levels of active Smad2.

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# 1 Introduction

## 1.1 Prostate cancer and vitamin D

Cancer of the prostate is the most common form of male cancer; each year around half a million cases are diagnosed, worldwide. The symptoms of prostate cancer mainly consist of various urination difficulties and usually do not show until the tumor has spread outside the prostate capsule. The main medical treatments of this disease are prostatectomy, radiation therapy and testosteron ablation. The side-effects of these treatments are rather severe, the most common being impotence, incontinence and hot flushes. Because of this, more effective treatments with less side-effects are required (Nystrand, 2005).

Prostate cancer is much more common in Western countries than in for instance Asia, and in the USA African Americans run a much greater risk of developing the disease than Caucasians. Increasing age is another risk factor for this type of cancer (Nystrand, 2005). It may be that differences in vitamin D levels account for the mentioned observations. Firstly, it is a fact that Japanese men consume larger amounts of fatty fish, the main dietary source of vitamin D, than do Western men (Zhao & Feldman, 2001). Secondly, light skin compared to dark contains less melanin, a compound in the skin inhibiting synthesis of vitamin D. Thirdly, as men age their serum vitamin D levels decrease as the efficiency of vitamin D synthesis decreases with age (Holick, 2005). These suggestions are also supported by research showing that vitamin D has anti-proliferative effects on prostate cancer cells (Chen *et al*, 2000; Zhao & Feldman, 2001; Holick, 2006).

The active form of vitamin D is called $1,25(OH)_2D_3$ and functions like a hormone in the body. It is, together with the parathyroid hormone, a major regulator of mineral homeostasis and bone metabolism. $1,25(OH)_2D_3$ aids intestinal calcium absorption and is important for prevention of diseases such as rickets and osteomalacia (Zhao & Feldman, 2001).

The main cellular receptor for $1,25(OH)_2D_3$ is a cytosolic/nuclear receptor called the vitamin D receptor (VDR). The genes regulated upon binding with the VDR include genes important for calcium metabolism such as osteocalcin, osteopontin, 24-hydroxylase and calbindin (Haussler *et al.*, 1998) but also genes involved in cellular proliferation and differentiation such as c-myc, c-fos, p21, p27 and Hox A10 (Freedman, 1999). Expressing VDR, the prostate, especially the tumorous prostate (Krill *et al.*, 2001), is a target organ for vitamin D and $1,25(OH)_2D_3$ has long been known to inhibit growth of prostate cancer cells. This has been ascribed to a VDR-mediated, nuclear-initiated signalling controlling target gene expression, resulting in cell cycle arrest, apoptosis and differentiation (Lou *et al.*, 2004).

However, it has been found that another major way in which $1,25(OH)_2D_3$ inhibits cell growth in prostate cancer is via membrane-initiated steroid signalling (Murthy & Weigel, 2004; Larsson *et al*, 2007). Membrane-initiated steroid signalling triggers activation of signal cascades upon steroid binding to the receptor complex, leading to induction of genes regulating cell growth, proliferation and apoptosis (Norman *et al*, 2004).

## 1.2 $1,25(OH)_2D_3$, the TGFβ signalling pathway and Smad proteins in prostate cancer

Transforming growth factor β, TGFβ, is a signalling protein widespread among mammalian tissues. In the prostate, it regulates many critical cellular functions, particularly growth arrest, differentiation and apoptosis (Danielpour, 2005). The protein is secreted from cells in

complexes composed of three proteins, the mature TGFβ dimer, the latency-associated protein (LAP) and the latent TGFβ binding protein (LTBP). TGFβ signalling is initiated by proteolytic cleavage of LTBP resulting in release of the latent TGFβ complex from the extracellular matrix. The protein is activated by dissociation of LAP from the mature TGFβ (Taipale *et al*, 1998) and then it influences the prostate cells in an autocrine and paracrine manner (Kelly & Yin, 2008).

TGFβ triggers a signalling cascade through interaction with two transmembrane serine/threonine kinase receptors, TβR1 and TβR2. The main intracellular mediators of these receptors are a family of proteins known as Smads (small mothers against decapentaplegic). The TGFβ protein first binds to TβR2, which in turn recruits TβR1 to form a ligand-receptor heteromeric complex consisting of two TβR2s and two TβR1s. A constitutively active kinase in the cytoplasmic domain of TβR2 then activates TβR1 at a juxtamembrane site. The activated TβR1, with the help of a couple of proteins named SARA and Hrs/Hgr, recruits and activates Smad2 and 3 by means of phosphorylation (Danielpour, 2005). SARA is present in an early endosome which, through clathrin-mediated endocytosis, internalizes the receptor complex (Runyan *et al*, 2004). Once activated Smad2 and 3 homodimerize, they then enter the nucleus either with or without a third Smad, Smad4. The phosphorylated complex then binds transcription promoters/cofactors and causes the transcription of DNA (Danielpour, 2005). Normal production of Smad2 and 3 proteins is essential. In studies, lack of normal Smad2 or 3 has been specifically implicated in tumor progression in both mice and humans (Dijke & Heldin, 2006; Nakao *et al*, 1996).

TGFβ has also been described to initiate other pathways such as the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathway. The mitogen-activated protein kinase (MAPK) JNK, a mediator in this cascade, has been shown to be an additional activator of Smad2 and 3 by means of phosphorylation (Mori *et al*, 2004).

In a study from 2004 it was shown that in prostate cancer cells, $1,25(OH)_2D_3$ increases both production, signalling and receptor levels of TGFβ, in turn inhibiting cell growth (Murthy & Weigel, 2004). The study was of long-term effects (1-6 days) and only referred to the cytosolic/nuclear VDR as a potential hormone receptor. However, $1,25(OH)_2D_3$ mediated elicitation of the SAPK/JNK branch of the TGFβ pathway has been detected already at the prenuclear stage; activation of JNK in the cascade has been shown as early as within 10 minutes of treatment (Larsson *et al*, 2007). Also, in the last-mentioned study the involvement of another vitamin D receptor was suggested, the protein disulfide-isomerase A3 precursor, PDIA3 (also called 1,25-MARRS).

A study in 2008 examined the early effects (3 minutesï 38 hours) of $1,25(OH)_2D_3$ on the levels of active TGFβ and found that $1,25(OH)_2D_3$ induced a statistically significant initial lowering of the active TGFβ level followed by a statistically significant successive rise of the level with time; a fall and rise which was not observed in their 0.01% EtOH treated controls (Stahel, 2008).

# 2 Aims and expectations of this Project Work

The main aim of this work was to study the early effects of $1,25(OH)_2D_3$ on activated Smad2 levels in PC-3 prostate cancer cells. The levels of phosphorylated Smad2 were expected to

show the same pattern as the levels of active TGFb after the same treatment, that is, an initial decrease followed by an increase.

PC-3 is a commonly used cell line in cancer research, which was derived in the late 1970ös from a human prostatic adenocarcinoma metastatic to bone (Kaighn *et al*, 1979).

Discovering the details of the cancer growth inhibiting mechanism behind vitamin D is important as it means progress in the search for new and less maiming treatments of prostate cancer. Included in the aim of this study was to form a lead in that search. This is a follow-up to the 2008 study investigating the early effects of $1,25(OH)_2D_3$ on activated TGFb levels in PC-3 (Stahel, 2008).

# 3 Materials and methods

## 3.1 Cell culturing and treatment with $1,25(OH)_2D_3$

Human prostate cancer cells from the cell line PC-3 (ECACC, Salisbury, UK) were used for this experiment. They were grown in monolayers on 24 well plates (TPP, Switzerland) in cell culturing medium: RPMI 1640, supplemented with 2 mM Glutamine, 10 mM Hepes, 1 mM Na-Pyruvate, 10% Fetal Bovine Serum and 100 U/ml Penicillin-Streptomycin. The culture was kept in 37°C in a humidified atmosphere with 5% $CO_2$.

The monolayers were then treated in 37° C and 5% $CO_2$ for 5, 10, 30 and 60 minutes as well as 24 and 40 hours with 0.01% EtOH or $1,25(OH)_2D_3$, $10^{-10}$ or $10^{-7}$ M, and lysates were prepared from all groups.

## 3.2 Activated Smad2 ELISA, absorbance measuring and computer analysis

### *3.2.1 The PathScan® Phospho-Smad2 Sandwich ELISA Kit, MultiSkan EX and GraphPad Prism 4*

The PathScan® Phospho-Smad2 Sandwich ELISA (Enzyme-Linked ImmunoSorbent Assay) Kit is a solid phase ELISA that detects endogenous Smad2 when phosphorylated at Ser465/467. It has microwells coated with Smad2 mouse antibodies and after incubation with cell lysates, Smad2 (phosphorylated and nonphosphorylated) is captured by the coated antibody. Following extensive washing a phospho-Smad2 detection antibody is added to detect serine phosphorylation of the captured Smad2 protein. A horseradish peroxidase (HRP) linked anti-rabbit antibody is then used to recognize the bound detection antibody. The HRP substrate TMB is added to develop color. The magnitude of the absorbance for the developed color is proportional to the quantity of Smad2 phosphorylated at Ser465/467 (Cell Signaling Technology, 2008).

In this study a microplate photometer called MultiSkan EX (Thermo Electron Corporation, MA, USA) was used for the testing. The MultiSkan measures absorbance in Arbitrary Absorbance Units, AAU.

The computer program GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis of the result figures.

### *3.3.2 Assay, measuring and analysis*

The Smad2 ELISA and microplate absorbance measuring was carried out according to the PathScan® Phospho-Smad2 Sandwich ELISA protocol (Cell Signaling Technology, 2008).

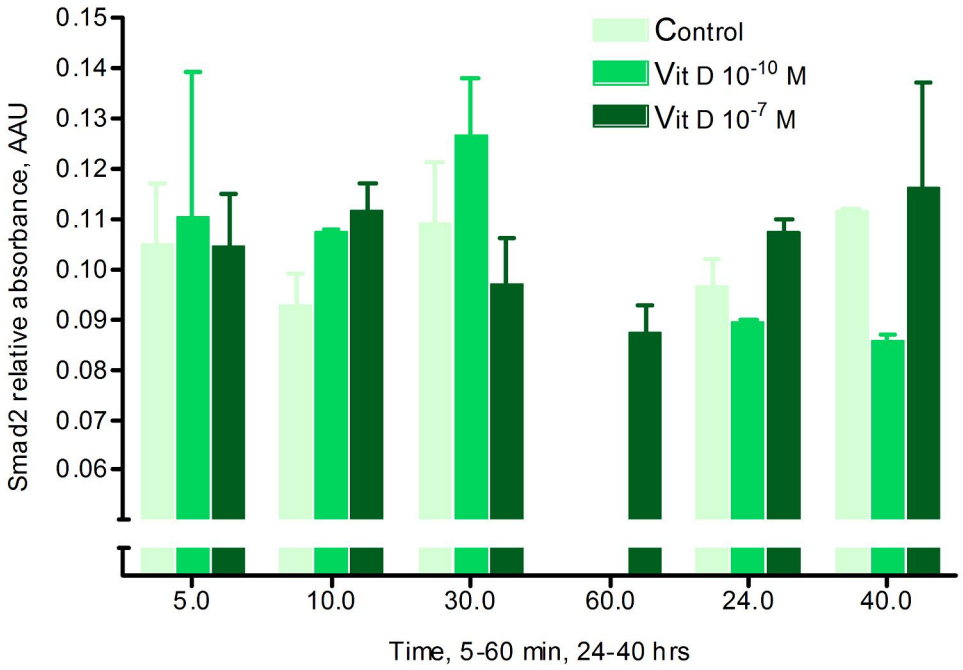
The result values from the MultiSkan were statistically analyzed in GraphPad Prism where a number of graphs were drawn on basis of the result figures. The figures used for the graphs were mean values, that is, averages were calculated for each time point for the 2 replicates of each hormone concentration as well as the EtOH controls. The analyses made were a Two-way ANOVA followed by Bonferroniõs post-hoc test and a One-way ANOVA followed by the same. The significance threshold was set to $P<0.05$.

# 4 Results

## 4.1 Early effects of $1,25(OH)_2D_3$ on activated Smad2 levels

The analysis of the result values in GraphPad Prism did not show any statistically significant effects by $1,25(OH)_2D_3$ on activated Smad2 levels in PC-3 cells. (The 60 minute control and $10^{-10}$ M values were removed due to a mistake during lysis buffer pipetting leading to incorrect figures.)

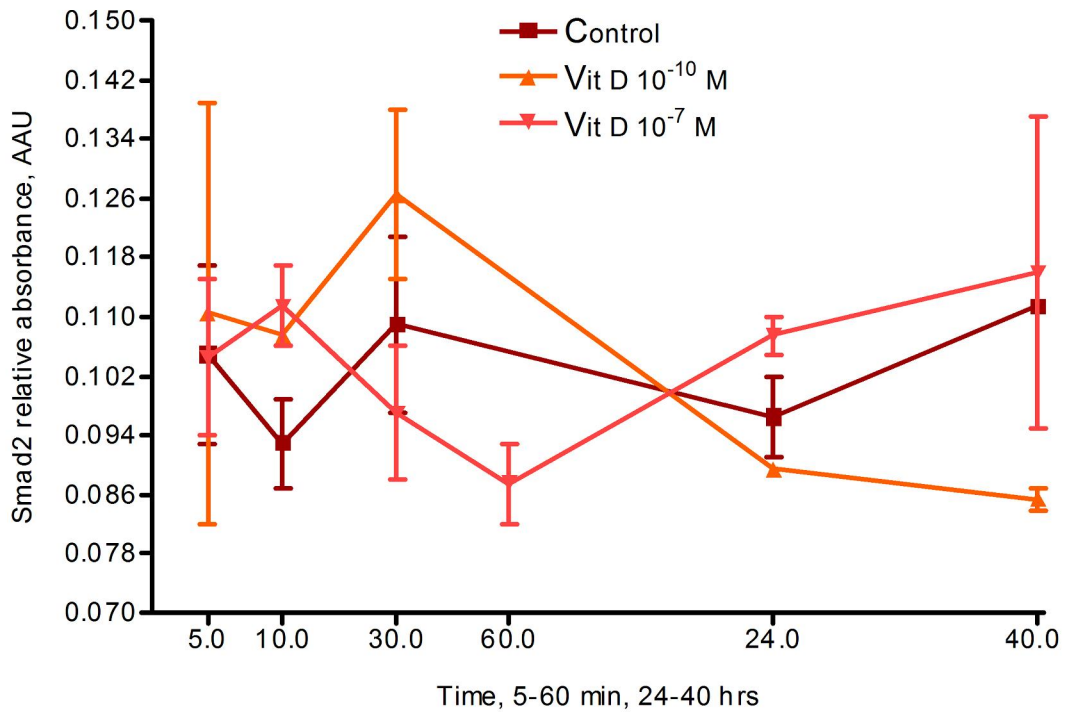
A first, vertical interleaved bar graph drawn based on the results from the GraphPad analysis is shown in Figure 1.




No statistically significant dose effect differences.

**Figure 1:** Dose- and time-dependent responses with and without $1,25(OH)_2D_3$ treatment were compared with help of a Two-way ANOVA followed by Bonferroni's post-hoc test. An absorbance-based ELISA was used where levels of active Smad2 were measured with a HRP substrate.

Analyses were also made with error bar category graphs, comparing each time-point within each separate curve. The results showed lack of any statistically significant variations of the slopes of all curves, both those for the hormone treated ($10^{-7}$ M and $10^{-10}$ M) cells and that for the untreated (0.01% EtOH, control). The results are shown in Figure 2.




No statistically significant time point differences.

**Figure 2:** Dose- and time-dependent responses with and without $1,25(OH)_2D_3$ treatment were compared with help of a One-way ANOVA followed by Bonferroniõs post-hoc test. An absorbance-based ELISA was used where levels of active Smad2 were measured with a HRP substrate.

# 5 Discussion and conclusion

The results of this study showed that treatment with $1,25(OH)_2D_3$ does not significantly alter the level of activated Smad2 in PC-3 cells initially (5 minutes ï 40 hours). However, even though the result curves from the GraphPad analyses showed lack of statistically significant variations, they still showed variations, both between doses and within each separate curve.

As can be seen in Figure 2, the curve for treatment with $1,25(OH)_2D_3$ $10^{-7}$ M first rises slightly, then lowers quite a lot, comparatively, then after an hour it starts rising again, a rise which continues through 40 hours. These changes diverge from those of the control curve, and also, they rhyme with the changes of the corresponding TGFb curve in the in the prequel of this study examining the same doses at similar time points, only in a delayed way. This makes sense since active TGFb when bound to its receptor phosphorylates Smad2; a change in active TGFb should generate a change in active Smad2 as well (Danielpour, 2005).

The mentioned TGFb curves are shown in Figure 3 for comparison.

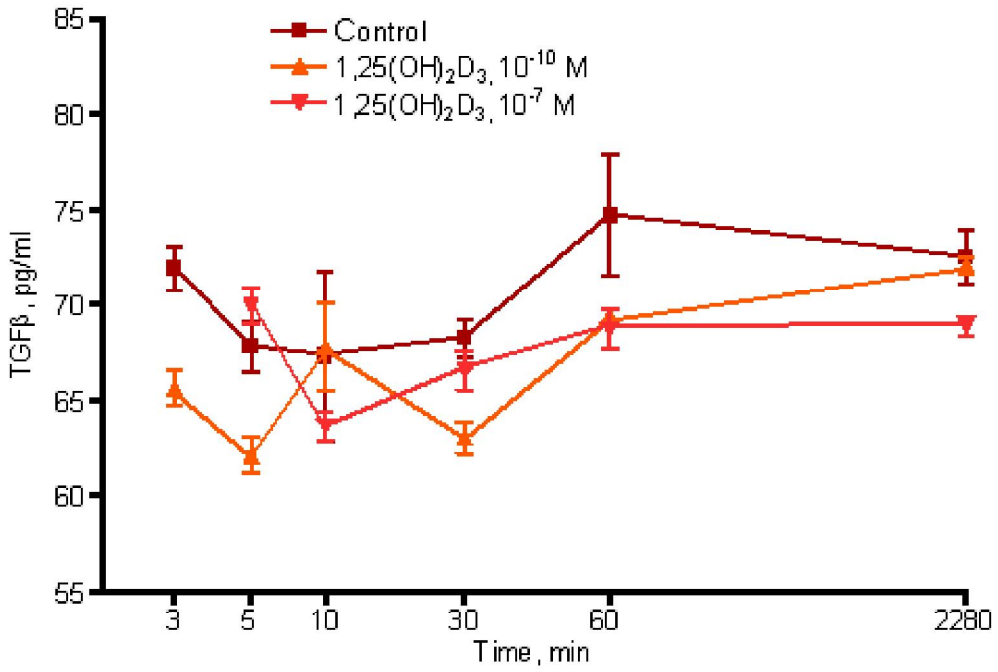



**Figure 3:** Dose- and time-dependent responses with and without $1,25(OH)_2D_3$ treatment were compared with help of a One-way ANOVA followed by Bonferroni's post-hoc test. An absorbance-based ELISA was used where levels of active TGFβ were measured with a chromogenic substrate (Stahel, 2008).

The curve for treatment with $1,25(OH)_2D_3$ $10^{-10}$ M similarly rhymes with the changes of its corresponding TGFβ curve altough in a more delayed way, and also, instead of rising upward towards the end like the curve for TGFβ, the curve for Smad2 continues downward through 40 hours. This seems contradictive considering the phosphorylation relationship between active TGFβ and active Smad2, but on the other hand it is possible that this curve for Smad2 would show an upward turn again after 40 hours should the testing be continued.

In conclusion, this study indicated the presence of a small initial influence on active Smad2 levels by treatment with $1,25(OH)_2D_3$ in PC-3 cells. It is likely that this change is an effect of the statistically significant, previously documented change in the level of active TGFβ by the hormone, especially since the pattern of the Smad2 change imitates that of TGFβ even if in a time delayed manner (indicating that it takes some time for TGFβ to exert its influence). The effect in the present study was not statistically significant, but existent, and it is possible that further, extended studies with more replicates would show significant changes or an onset of significant changes at testings beyond 40 hours.

It may or may not be that the results of this study contradict the findings by Mori *et al* in 2004 that JNK is an additional activator of Smad2 and by Larsson *et al* in 2007 that $1,25(OH)_2D_3$ activates JNK in prostate cancer cells within 10 minutes of treatment. The reason it may not is that in their studies, Mori *et al* used a normal cell line from gastric mucosa of rat while Larsson *et al* used the prostate cancer cell line LNCaP, and these cell lines both differ in several aspects from the PC-3 cell line used in the present study (Mori *et al*, 2004; Larsson *et al* 2007).

# 6 Acknowledgements

Once again I would like to thank my supervisor, Dennis Larsson, for his immense kindness, patience and trust when guiding me through my work. I would also like to warmly thank dr Thomas Strömberg at Karolinska, for the tip!

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