$1,25(OH)_2D_3$ increase caspase-3 activity in LNCaP cells after 2 minutes and 48h separately

Honours Thesis in Biomedicine C10, 30 ECTS

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ABSTRACT

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Cancer or malignant tumors has a high death frequency in many countries. Nowadays many research facilities are dedicated to find new substances and techniques which would lead to better cancer therapies. Seven years ago a research team from Finland made a remarkable connection between vitamin D deficiencies and an increased chance of getting prostate cancer. The research investigating this statement has lead to findings of a new non-classical effect of the calcium controlling vitamin, $1,25(OH)_2D_3$. This effect involves anti-proliferatory effects and more importantly apoptotic effects resulting in the hope of finding a new drug that can cure prostate cancer with the smallest amount of harm to the body.

In an attempt to find out if the signalling pathway of this apoptotic effect is fast or slow, an experiment designed to detect when the apoptotic protein caspase-3 is induced has been performed. Cells from the cell line LNCaP has been cultured and incubated with $1,25(OH)_2D_3$ and after 0min - 48h an assay was performed to detect the relative amounts of caspase-3 present in every sample. The optimal time period (48h) was then subjected to three different concentrations of $1,25(OH)_2D_3$ and read in the same way as the previous samples. The results showed an increase in caspase-3 expression as early as 2 min, but disappear to be seen again at 24h and are more profound in 48h samples. The caspase-3 expression was also seen to form a possible exponential curve in dose-response.

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1 Introduction

There are many forms of cancer (malignant tumors) and all are not treatable, but lately a new possibility in treating cancer has appeared in the studies of prostate cancer, namely the use of the different forms of vitamin D. In 2000 a link between vitamin D deficiency and risk of prostate cancer was proposed by Ahonen *et al.* leading to research within this area (Ahonen *et al.,* 2000). Since then many effects and pathways of the different forms of vitamin D have been discovered and verified, which in the future can lead to a new way of treating cancer both in preventive ways and as a possible new cure.

1.1 The prostate gland, hypertrophy and prostate cancer

The prostate gland is a part of the male genital positioned beneath the urinary bladder encircling the urethra as it leaves the bladder. The prostate gland produces a weak acidic fluid that is known as prostatic fluid. This fluid makes up 20-30% of the total volume of semen (Martini, 2004)

When the male individual becomes older the hormonal balance changes and an enlargement of the prostate could occur, a so called benign prostatic hypertrophy. The degree of enlargement is individual and usually not deadly but can give some symptoms, such as trouble urinating. This is easily treated with a procedure called TURP (transurethral prostatectomy), a non invasive treatment involving a tube which is inserted through the urethra and cuts away the excessive tissue of the prostate. In more severe cases the hypertrophy can result in prostate cancer, a malignant metastasizing cancer of the prostate gland. This cancer has the second highest killing rate in male cancers, but since the average age of diagnosis is 72 the patients are more likely to die of other causes. The prostate cancer normally originates from one of the secretory glands and spread mostly to glands and nearby lymph nodes. The easiest way of diagnosis is a blood sample screening for PSA (prostate-specific antigen), this usually leads to an early diagnosis and therefore an early treatment. The PSA usually circulates in low levels and an elevation can indicate cancer formation. The treatment is usually directed radiation and prostatectomy. When the cancer has started to spread few things can help the patient, although treatments involve aggressive chemotherapy, widespread irradiation, hormonal manipulation and lymph node removal. (Martini, 2004)

1.2 Vitamin D and cancer treatment

Vitamin D is mostly known for its actions and effects on bone and plasma levels of calcium, but recently non-classical effects of vitamin D has been discovered, including suppression of cell growth, regulation of apoptosis and many more; these effects could be useful in the treatment of malignant tumors that has metastasized. The suppression of cell growth is mediated through a number of mechanisms, for example the 1,25-$(OH)_2D_3$-VDR complex arrest the cell in G0/G1 through induction of the cyclin-dependent kinase inhibitor p21, the complex also induces the synthesis and stabilization of p27 arresting the cell growth. The apoptotic effects on the other hand are mediated through mechanisms which include down regulation of two antiapoptotic proteins; Bcl2

and $BclX_L$ (Dusso *et al.*, 2004). The active form of vitamin D is 1,25-$(OH)_2$ vitamin D_3 (also called calcitriol) and has an established effect on cancer cells. The other forms of vitamin D, 25$(OH)D_3$ and 24,25$(OH)_2$ D_3 has a similar effect on the cancer cells but the mechanisms are still not completely understood (Larsson *et al.*, unpublished). Although resent findings of the protein 1α-hydroxylase, which transforms 25-(OH) vitamin D_3 to calcitriol, in prostate cells indicates the importance of 25-(OH) vitamin D_3 in plasma, since the prostate on it's own can produce calcitriol inhibiting cancer growth. The effect of 24,25-$(OH)_2$ vitamin D_3 has yet to be thoroughly investigated (Chen & Holick 2003).

1.3 Prostate cell lines

The different lines of prostate cells, both cancerous and normal, LNCaP-, PC3- and PNT2-cells are the cell lines commonly used. LNCaP-cells are from a human prostate carcinoma. They were retrieved from the left supraclavicular lymph node metastasis of a 50-year-old man with prostate carcinoma in 1977 (DSMZ 1989). This cell line was later described as androgen-sensitive prostate cancer (Horoszewicz *et al.,* 1983).

1.4 Apoptosis

The preferred way of eliminating cancer cells is by inducing apoptosis since the cells then produce minimal inflammatory effect and no operation is needed. Apoptosis, programmed cell death, is necessary during an individual's entire life mostly during fetal development and growth but also during the adult's life. In the fetal development and growth, the temporary cells such as the webbed finger cells go through apoptosis, resulting in the ready fetus. In adults cells that has suffered some damage, such as DNA damage, or infection go through apoptosis to protect the body from ill effects (Kumar *et al.,* 2003).

Apoptosis can be divided into four main integrating steps; signaling, control and integration, execution and removal of dead cells. The signaling is both death and survival signals which integrate in the cell and control the different signal pathways in the cell. When a sufficient amount of death signals or the loss of survival signals appear the execution begins leading to apoptosis, cell death, and then all that remains is the removal of the dead cell (Kumar *et al.,* 2003). All these steps are under the influence of the receptor pathways, the death receptor pathway and the mitochondrial pathway. The death receptor pathway is direct, usually a death factor binds to a receptor, the complex then connect to an adaptor protein which activates initiator caspase-8 which in turn activates the effector caspase-3. The mitochondrial pathway on the other hand is more complex, it can be induced internally by for example p53 which is activated by DNA damage. The p53 then induces a pro-apoptotic Bcl-2 protein which stimulates the mitochondria to release cytochrome c. The cytochrome c activates the apoptosome that activates caspase-9 an initiator which induces caspase-3 resulting in cell death. The difference from the death receptor pathway is that the mitochondria continuously receive survival signals from for example growth factors and adhesive binding, which when bound to the receptor induces an anti-apoptotic Bcl-2 protein. The important factor in these

pathways is caspase-3 the protease which cleaves DNA repair enzymes, protein kinase C, cytoskeletal components and many more, killing the cell. Studying apoptosis by assay earlier than the caspase-3 step could give to many faults, such as only one pathway would be read or the recovery from the signaling could occur giving a false positive apoptosis reading. The optimal step is therefore to measure the caspase-3 activity since it then is confirmed that the cell is undergoing apoptosis and this regardless of the inducing signaling pathway (Rang *et al.*, 2003).

2 Aims of Thesis

The general aim of this thesis was to study the effect of $1,25(OH)_2D_3$ on apoptosis in LNCaP prostate cancer cells.
The specific aims of this project were to;

- optimize the protocol of caspase-3 detection.
- study if $1,25(OH)_2D_3$ incubation of cells induced apoptosis through the caspase-3 pathway.
- study if the effect of $1,25(OH)_2D_3$ increase with concentration.

3 Materials and methods

When starting this method a number of things had to be considered since this method was new to the laboratory. For example the cells were to be grown in vials that could hold at least one million cells since the results would be to feeble to detect otherwise. A way of washing the cells before lysation had to be defined since the protocol only gave the direction; wash the cells.

3.1 Culturing cells

The LNCaP cells on which the study was performed were ordered from Sigma-Aldrich Sweden AB, these where then cultured in a cell culture media (CCM) consisting of 10% foetal bovine serum, 2mM L-glutamine, 10mM HEPES (N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)), 1mM Na^+-pyruvat, 100 units/ml PEST (pencillin-streptomycin), everything solved in a RPMI 1640 solution. The cells were cultured and incubated with $1,25(OH)_2D_3$ at 37˚C, in 95% humidity with a 5% CO_2 air saturation, in a sterile environment to eliminate infection risks from bacteria and virus. Harvest and relocation utilized trypsin. When a sufficient number of cells were achieved the exposure to $1,25(OH)_2D_3$ could begin.

3.2 Caspase-3 Assay

During the time optimization cultured LNCaP cells was incubated with $1,25(OH)_2D_3$ in a 10^{-7}M concentration during 2, 30, 90, 240 min, 24 and 48h separately, a control containing the same amount of ethanol, that was needed to solve the $1,25(OH)_2D_3$ in, was added and incubated during the same time period. All samples were done in 4 repeats. A final control was done with no

ethanol and/or $1,25(OH)_2D_3$ added. Following $1,25(OH)_2D_3$ incubation the cells were harvested with trypsin and washed with PBS through resuspension and centrifugation. The cells were then lysed using a cell lyses buffer in the EnzCheck® Caspase-3 Assay kit #2. Following the proposed laboratory protocol (found on www.probes.com; revised 15-April-2003) the lysate was then mixed in equal parts with the Z-DEVD-R110 substrate (nonluminescent) and incubated. The caspase-3 cleaved product, rhodamin 110 (R110) was exitized and the fluorescence measured with FlouStar microplatereader (BMG labtechnologies Germany) employing the Floustar galaxy software. A standard curve was created through the use of R110 in known concentrations (0, 2.5, 5, 7.5, 10 and 15 µM) with 2 repeats. The results were then compiled and calculated in the Graph Pad Prism software (retrieved from www.graphpad.com) and graphs were created.

To confirm that the activity seen in the experiment was caspase-3 activity a separate experiment was performed. Samples were treated as described above) with an incubation time of 90min and an additional control added, two of treated and control samples were exposed to a caspase-3 specific inhibitor, giving two inhibited and two normal samples, and these were then compared with the two zero-activity samples.

The dose/response experiment was performed as described above with samples incubated in concentrations of 10^{-11}, 10^{-9} and 10^{-7}M with a control incubated in ethanol concentration which corresponds to the amount in which the $1,25(OH)_2D_3$ was solved.

3.3 Calculations

The caspase-3 activity was calculated by deciding the product R110´s concentration from the standard curves hyperbolic equation (R110 concentration=Relative fluorescence*(105024/15.59)). Goodness of fit was determined for the standard equation to R^2=0.9726.

3.4 Statistics

The time optimization results were then subjected to a paired t test to test the statistical possibility of actual difference between controls and samples, as was the confirmation experiment the normal was compare with the inhibited and the inhibited was compared with the zero-activity samples. The dose-response was subjected to a one-way ANOVA comparing all samples with the control measuring probability of differences between samples.

4 Results

Standards of all experiments were consistent and the standard curve was found to be hyperbolic (Figure 1). The resulted caspase-3 activity calculated from the standard curve is seen in figure 2. When all results of the time optimization is shown in one graph it’s clear that the induction of apoptosis occurs at 2 min but

then subsides to be shown again in the 24h sample and with further increase in the 48h sample. The p-values of 30, 90 240min and 24h are all larger than 0.05 (difference confirmed when $p<0.05$), which is no difference, however the value for 24h is only 0.1131. The p-values for 2min and 48h are 0.0109 and 0.0275 separately giving a clear difference between controls and samples.

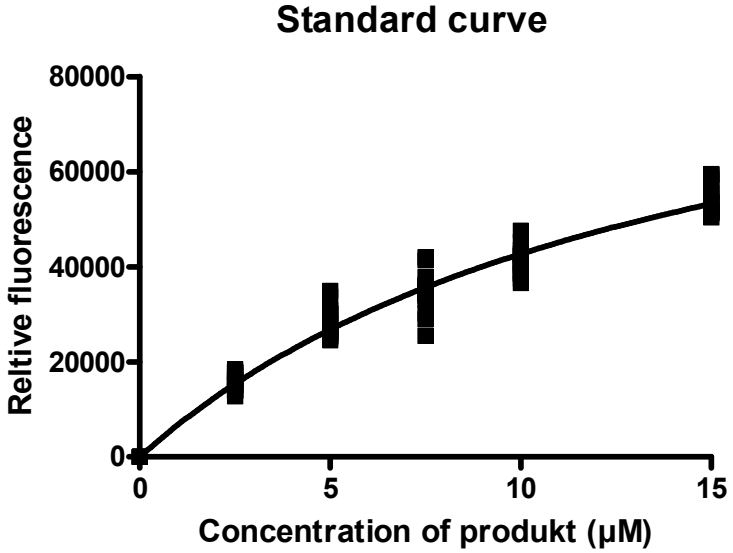

Figure 1: Compiled graph of standard values against the relative fluorescence. Samples at 0, 2.5, 5, 7.5, 10 and 15µM of Rhodamin 110 (R110)

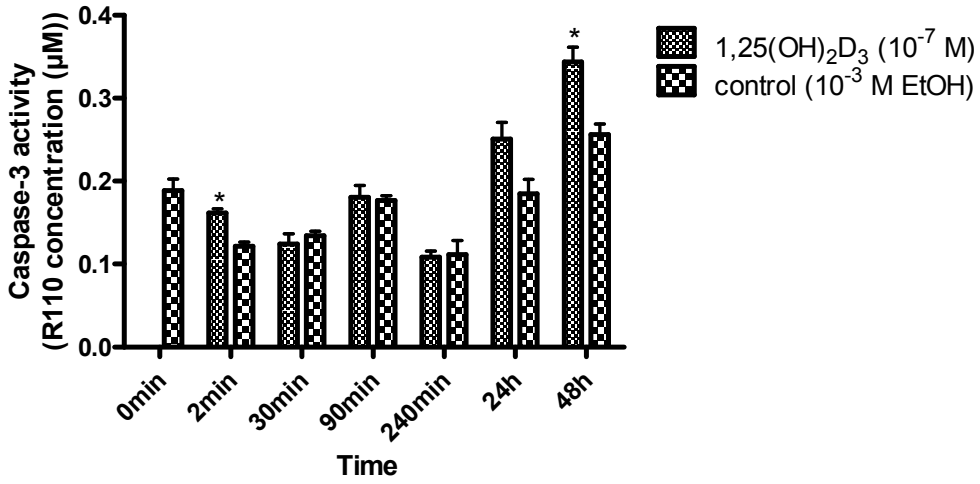

Figure 2: The resulted caspase-3 activity of samples treated with 10^{-7} M $1,25(OH)_2D_3$ for 2, 30, 90, 240min, 24 and 48h and the control samples at 0 min. The stars mark samples with statistically confirmed differences.

The inhibited samples showed a significant decrease in caspase-3 activity, with the statistical p-value 0.0103. The difference between the inhibited and zero-activity samples are nearly the same, but no statistical calibration could be done due to too few samples.

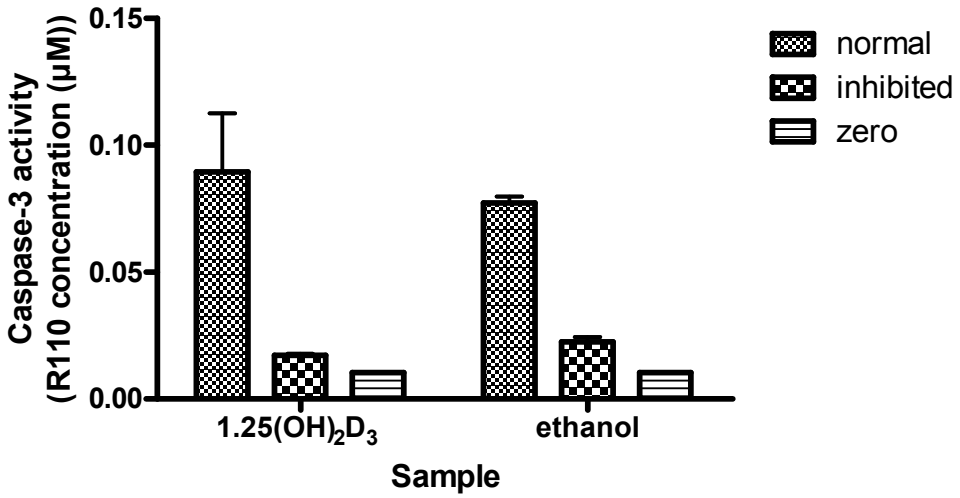

Figure 1: Samples treated with 10^{-7}M $1,25(OH)_2D_3$ and 10^{-3}M ethanol against caspase-3 activity. Normal samples are treated as earlier describe while inhibited samples has been exposed to an inhibitory substance specific for caspase-3. The zero samples are the two zero-activity samples.

When doing the dose/response experiment a possible exponential curve is shown. There is no apparent caspase-3 induction until the $1,25(OH)_2D_3$ concentration reaches 10^{-7}M (figure 3). The one-way ANOVA however firmly states that there is no relevant difference between samples and controls.

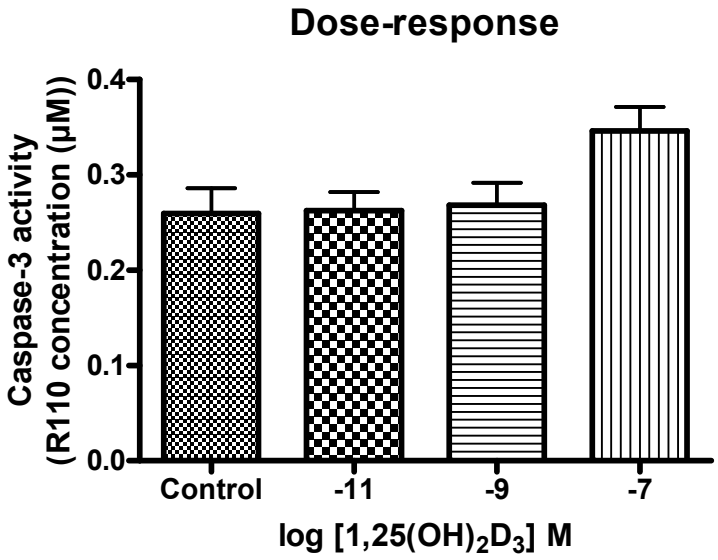

Figure 3: The dose variable samples and the control against caspase-3 activity.

5 Discussion

Previous studies confirm the effect of $1,25(OH)_2D_3$ to be apoptotic in the LNCaP cell line. Amongst other things a Bcl-2 over expression was

demonstrated to inhibit the apoptotic effect of $1,25(OH)_2D_3$ (Blutt *et al.* 2000). Further studies showed that $1,25(OH)_2D_3$ treatment decreased the expression of Bcl-2 in LNCaP cells visibly after two days (Blutt *et al.* 2000; Guzey *et al.* 2002). This is also the time of which a visible decrease of live cells is detected (Guzey *et al.* 2002). As seen in the results there indeed was a confirmed increase in caspase-3 activity at 48h, an indication of the started increase in caspase-3 activity was seen in the graph for 24h, but it was not confirmed with statistical analysis. This is all confirmed by earlier studies. The increased caspase-3 activity at 2min however indicates an early onset of apoptosis which is gone by the 30min sample. This 2min sample suggests a fast apoptotic response via a different pathway than the Bcl-2 pathway. A recent study has revealed an increased activity of p38 MAPK and JNK by $1,25(OH)_2D_3$ in muscle tissue of chicks (Buitrago *et al.* 2006). The increase was seen within a couple of minutes, as in this study. A review by Nagai *et al.* (2007) verifies that the pathway which p38 MAPK and JNK are part of is apoptotic. This could indicate that the fast response seen in the 2min samples are mediated by the p38 MAPK and JNK pathway, but no other studies has yet confirmed or rejected this theory.

The difference between inhibited and non-inhibited is confirmed statistically proving that it is caspase-3 activity being measured. The inhibited samples were not able to be compared statistically with the zero-activity samples because of lack of samples, but the resulted non-caspase-3 activity of the inhibited samples were so similar in value (see figure 1) that the non-caspase-3 activity were determined redundant. This leads to the conclusion that the activity seen in the experiments are indeed caspase-3 activity.

The dose-response experiment did not have enough samples to give a statistical certain result but the indication of the graph shows an exponential increase in caspase-3 activity. Further studies are needed to test dose dependency of $1,25(OH)_2D_3$ on caspase-3 mediated apoptosis.

When evolving this method a great deal of thought went into how to grow and incubate at least 1 million cells and then harvest and wash the cells with PBS. The solution was to plant 1 million cells in a T25 growth flask and let the cells adhere for 48h, then incubate the cells in the bottle before harvesting with trypzination. The cells were centrifuged and all trypzin and CCM were removed before the pellet was resuspended in PBS, centrifuged again and then the cell lysisbuffer was added.

Although much has been verified additional attempts should be done where the method is more adjusted to the specific goal of the experiment. Things that can be improved are 1. a possible cell count before analyses could be important to estimate the number of cells that are involved in the resulted caspase-3 activity, 2. one more wash before lysis would increase the results efficiency, 3. the CCM could be harvested at the end of the incubation period, to get a proper cell count and eliminate the risk that cells that die end up not being tested, 4. perform experiment with a higher initial number of cells, 5. the caspase-3 activity confirmation could be done on samples with proper induction of caspase-3 activity as in the 2min or 48h samples

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