

## **1,25(OH)<sub>2</sub>D<sub>3</sub> AND PROSTATE CANCER**

The effects on cAMP/PKA-dependent  
gene expression in LNCaP cells

Bachelor Thesis 15 hp

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## Abstract

Prostate cancer is the leading male cancer form in Sweden and maybe worldwide as well. Vitamin D is synthesized in the skin following the exposure to sunlight. Researchers have long been aware of the positive effect that vitamin D<sub>3</sub> has on prostate tumour growth. 1,25(OH)<sub>2</sub>D<sub>3</sub> have for a long time been the target of these studies and have shown good results. The steroid hormone induces cAMP accumulation and activates the cAMP dependent protein kinase A (PKA). PKA is then able to activate a transcription regulating protein. 1,25(OH)<sub>2</sub>D<sub>3</sub> is known to cause LNCaP cells to accumulate in the G<sub>1</sub> phase of the cell cycle. It has also been shown that 1,25(OH)<sub>2</sub>D<sub>3</sub> is under negative feedback control via 24-hydroxylase. In this study, PKA activity was observed by transfecting LNCaP cells with a viral vector carrying firefly and *Renilla* luciferase genes. The successfully transfected LNCaP cells would then express luciferase as a response to PKA gene expression. The LNCaP cells were then treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> and GDP-β-S (100 μM), a G-protein coupled receptor inhibitor, in order to examine if 1,25(OH)<sub>2</sub>D<sub>3</sub> regulate PKA dependent gene expression through a G-protein coupled receptor. The study could show that 1,25(OH)<sub>2</sub>D<sub>3</sub> regulate gene expression in LNCaP cells through a PKA dependent pathway. Furthermore, the PKA dependent gene expression was demonstrated to be independent of G-protein coupled receptor activation.

## Introduction

Prostate cancer is by far the most common cancer form in men, 33% of all cancers seen in males in Sweden today belong to this group. Possibly it is the most common form of male cancer in the world as well. In Sweden alone approximately 70000 men are living with or have been treated for prostate cancer. In 2008, 8870 males were diagnosed with the disease and the incidence has increased by 2.7% each year over the last two decades. It is rarely diagnosed before the age of 50 and risk of cancer development increases with age and 1 in 8 men risk developing prostate cancer before the age of 75 (Swedish institute of health and diseases, 2009).

## Vitamin D

In the search of a successful treatment for cancer, the hormone vitamin D has arisen as a likely candidate. Vitamin D is synthesized in the skin when exposed to sunlight and it is also present in various forms in dairy products and oily fishes such as salmon and mackerel. Some countries, including Sweden, fortify some food products with vitamin D to try to prevent vitamin D deficiency in the population. These forms of vitamin D are however inactive, until they reach the liver, in which vitamin D is metabolized into 25-hydroxyvitamin D<sub>3</sub>. The final hydroxylation is achieved in the kidney by the enzyme 1 $\alpha$ -hydroxylase, thus producing the active form of vitamin D<sub>3</sub>, 1,25(OH)<sub>2</sub>D<sub>3</sub>. 1,25(OH)<sub>2</sub>D<sub>3</sub> is also known as cholecalciferol or calcitriol. Parathyroid hormone stimulates the production of 1,25(OH)<sub>2</sub>D<sub>3</sub> when calcium levels are low and this in turn increase the absorption rate in the intestine. The active form participates in the regulation of calcium and phosphorous metabolism by upregulating the synthesis of calcium binding proteins. Furthermore, it controls the maturation of osteoblasts and chondrocytes, the cells involved in bone mineralization processes and it regulates the ion transport in the intestine. The metabolically active form 1,25(OH)<sub>2</sub>D<sub>3</sub> have been demonstrated to have antiproliferative effects in several types of cancerous tissue as well as prostate tissue (Johnson *et al* 2002, Holick 2006). The effect of vitamin D is depending on the presence of vitamin D receptors (VDR) which has been demonstrated to be expressed in essentially every type of tissue including skeletal, colon, breast, skin, lung and prostate. (Campbell and Farrell, 2009)

## Cyclic AMP (cAMP) and kinases

Cyclic AMP is a secondary messenger which is synthesized from ATP through catalysing effects by adenylate cyclase. An increase of cAMP intracellular stimulates the cAMP depending protein kinases, e.g. protein kinase A (PKA), which are associated with the cell being in a catabolic state. PKA then phosphorylate the cyclic response element binding protein (CREB), which connects to the cyclic response element (CRE) and activates the downstream target genes. CREB does not have any direct contact with the transcriptional machinery (RNA polymerase), as shown in figure 1. Therefore it requires

CREB binding protein (CBP) to achieve transcriptional activation. There are several steroid and thyroid hormones that act to bind CBP e.g. luteinizing hormone, glucagon and adrenaline which all exert influence over cAMP and PKA. CREB mediated transcription is involved in cell proliferation, differentiation and when the protein works to inhibit the effects of growth hormone, to slow the cell cycle and cause apoptosis. This is where the tumour suppressor genes like *TP53* come into play. The suppressor protein p53 mediates cell cycle progress in the G<sub>1</sub> phase of the cell cycle and exerts a firm control over the transcription of the *p21* gene. The suppressor protein p21 is a strong CDK inhibitor (CKI) regulates the DNA synthesis and thereby cell division. By binding to CDK, p21 effectively inhibits protein kinase activity and cell cycle arrest associated with p21 can cell differentiation. (Campbell and Farrell 2009) The role of cell cycle inhibitor that p21 exhibits, is however not induced by 1,25(OH)<sub>2</sub>D<sub>3</sub> according to experiments performed on primary cells (Peehl *et al*, 2003). P21 carries a vitamin D response element (VDRE) but no consistent data regarding the regulation by vitamin D exists. Instead it is assumed that the gene *CDKN1B* (encodes p27) is up-regulated by altering translation and extending mRNA half-life. The p27 is a CDK inhibitor protein that exerts control over cell cycle progress in the G<sub>1</sub> phase.

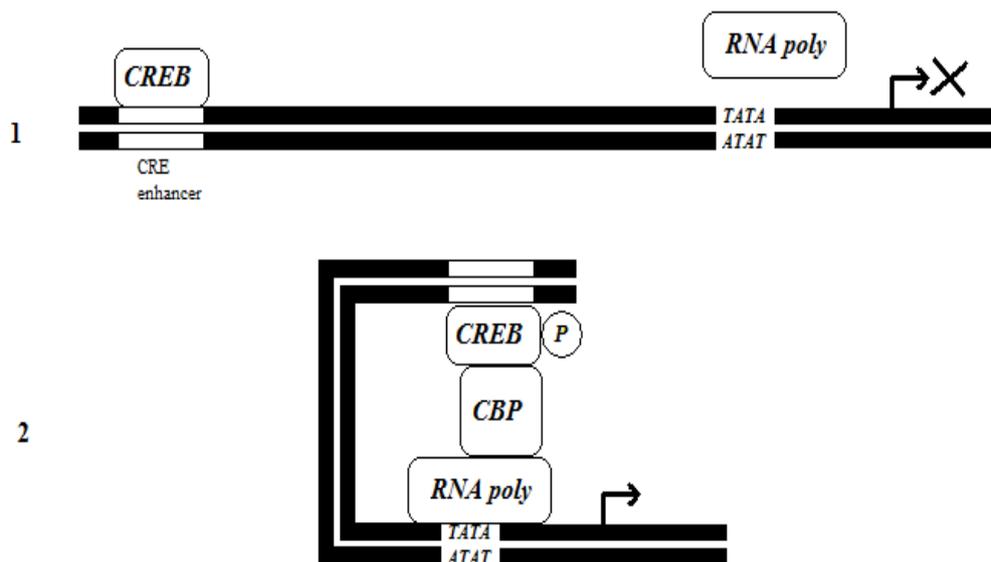


Figure 1. Regulation of transcription 1) Unphosphorylated CREB will not connect with CBP and thus will not initiate transcription. 2) CBP binds to CREB as a consequence of the phosphorylation of CREB and then forms a bridge between CREB and the transcription factor. This event will trigger the transcription. (Adapted from Biochemistry, Campbell and Farrell, 2009)

## 1,25(OH)<sub>2</sub>D<sub>3</sub> and cancer prevention

Knowing how the cell cycle arrest and the antiproliferative effects are induced on a molecular level is important when developing a successful therapeutic tool against cancer. Vitamin D stands out as a potential anti cancer tool, even with its severe side effect of hypercalcemia. It has been discovered that treating LNCaP cells with 1,25(OH)<sub>2</sub>D<sub>3</sub> results in an accumulation of cells in the G<sub>1</sub> phase, growth arrest, and to some extent apoptosis through an increase in

insulin-like growth factor binding protein-3 (IGFBP3). It has also been shown that the classical target for  $1,25(\text{OH})_2\text{D}_3$ , 24-hydroxylase, is up-regulated following vitamin D treatment (Krishnan *et al* 2003). The same is true for IGFBP-3. IGFBP-3 is known to be essential for  $1,25(\text{OH})_2\text{D}_3$  mediated growth inhibition and 24-hydroxylase transform  $1,25(\text{OH})_2\text{D}_3$  into  $24,25-(\text{OH})_2\text{D}_3$ , a form that is inactive as to any antiproliferative effects. The up-regulation of 24-hydroxylase indicates that vitamin D is inducing its own breakdown through a negative feedback system. In addition to this, Peehl *et al* (2003) concluded that primary cell lines became irreversible growth suppressed following treatment with  $1,25(\text{OH})_2\text{D}_3$  together with the absence of  $1\alpha$ -hydroxylase activity in LNCaP cell line. These cells are unable to transform  $25(\text{OH})\text{D}_3$  into the active  $1,25(\text{OH})_2\text{D}_3$ . Desiniotis *et al* (2002) could show that cAMP and PKA could perform inappropriate activation of the androgen receptor (AR) and that down-regulation of subunit 1 of the PKA enzyme decreased the signaling and severely impaired androgen expression and activity. The opposite is also true, since knock down of AR also yielded a decline in PKA activity.

## **Aim**

The aim of this study was to evaluate the PKA dependent gene expression in LNCaP cells when treated with  $1,25(\text{OH})_2\text{D}_3$ .

## **Materials and methods**

### **Cell culturing**

LNCaP cells (p4) were cultured in Gibco RPMI 1640 media (Invitrogen, UK). The media contained FBS (10%), PEST (1%), a non essential amino acid (L-glutamine, 1%), HEPES and sodium pyruvate (1% respectively). The cells were subcultured five days after the initial culture and seeded at a density of 20000cells/well onto a 96-well plate (Nunc, Thermo Fischer Scientific, US). At the point of seeding cells on the plate, the growth media was substituted for Opti-MEM (Invitrogen, UK) with 5% FBS and 1% NEAA without phenol-red-free and without antibiotics to prepare for transfection. The cells were then incubated at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  for 48 hour prior to treating them with  $1,25(\text{OH})_2\text{D}_3$ , while at the same time changing the growth media. The incubation was to allow the cells to adhere to the wells properly.

### **Transfection**

SureFECT<sup>TM</sup> transfection reagent was purchased from SABiosciences (USA) together with Cignal<sup>TM</sup> CRE reporter kit. Dilutions of SureFECT for standard transfection together with CRE reporter and controls were made according to protocols provided from manufacturer. The CRE reporter is a viral vector based on the Cytomegalo virus (CMV) that has been rendered replication incompetent and robbed of all virulence factors. It consist of inducible firefly

luciferase that respond to CRE and constitutively expressed *Renilla* constructs. The reporter is designed to monitor cAMP/PKA pathway activity and together with a Dual Glo™ Luciferase Assay System (Promega, USA) it provides an easy approach to study the activity of this pathway. The positive control consists of a mix of constitutively expressed firefly luciferase, together with *Renilla* luciferase constructs. The negative control is a mix of non-inducible firefly luciferase and constitutively expressed *Renilla* luciferase constructs.

### **1,25(OH)<sub>2</sub>D<sub>3</sub> treatment of LNCaP cells**

Following incubation, transfected cells were treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> in the concentrations 10<sup>-7</sup> M and 10<sup>-11</sup> M with or without the inhibitor Guanosine 5'-[β-thio]diphosphate trilithium (GDP-β-S). GDP-β-S was diluted from salt to final concentration of 100μM (Sigma-Aldrich). The inhibitor GDP-β-S is a non-hydrolysable, G-protein antagonist. The author chose the vitamin D concentrations based on the fact that 10<sup>-7</sup> M has shown the best antiproliferative effects and 10<sup>-11</sup> M as to compare the concentration-dependent manner of reaction. Vitamin and inhibitor were diluted in media and distilled H<sub>2</sub>O respectively. Ethanol was used as control at a final concentration of 0,01%. Media was changed in all wells at the same time as treating them and all wells were topped off with media in order to contain the same volume.

### **Luciferase Assay**

Luminescence levels were measured at four time points, 24h, 48h, 72h and 96h in a luminometer (FLUOstar Galaxy, BMG Labtech, Germany) using the Dual Glo™ Luciferase Assay System (Promega, US). The time intervals were chosen for the purpose to study transcription alterations, which may take from hours to days to develop. At the first interval, Dual Glo Reagent™ was prepared by mixing Dual Glo Substrate™ with Dual Glo Buffer™, at a ratio of 1:1. Stop & Glo Reagent™ was prepared by mixing Stop & Glo Substrate™ with Stop & Glo Buffer™, at a ratio of 1:100 at each time interval, before measuring activity. All compounds were stored according to manufacturer's recommendations. At each measuring interval, 75μl of Dual Glo™ was added to the examined wells to check inducible activity, according to manufacturer's recommendations and left to incubate at room temperature for 12-15 minutes. Following the Firefly luciferase reading, 75μl of Stop & Glo Reagent™ was added to check the non-inducible luciferase activity. As with Dual Glo Reagent™, the wells were left to incubate for 12-15 minutes. The principle is that Firefly luciferase (Dual Glo Reagent™) is inducible, while *Renilla* luciferase (Stop & Glo Reagent™) is not. This provides a reference point to compare and normalize data

### **Statistics**

Statistics and graphs were produced with GraphPad™ Prism4 (GraphPad Software Inc, USA). All experiments were performed as triplicates, except where otherwise stated. One-way ANOVA was performed and p<0,05.

## Results and discussion

Vitamin D plays a complex role in the growth control of prostate cancer cells.  $1,25(\text{OH})_2\text{D}_3$  inhibits prostate tumour growth by cell cycle arrest and induction of proliferation and differentiation. This is a complex control system that exerts its effect through a massive number of phosphorylation reactions. The androgen responsive LNCaP cells are highly responsive to vitamin D treatment. This study was conducted using a CMV-based viral vector for transfection of LNCaP cells and the subsequent luminescence measurement of these cells.

### Time dependence

The highest activity was recorded after 72 and 96 hours, both in cells treated only with  $1,25(\text{OH})_2\text{D}_3$  and cells treated with both  $1,25(\text{OH})_2\text{D}_3$  and GDP- $\beta$ -S. The change in PKA-dependent expression was significantly higher at 72 hours ( $p < 0,05$ ) and 96 hours ( $p < 0,05$ ) compared to the earlier recordings done after 24 and 48 hours. During the first 48 hours, the PKA reporter activity was similar in all wells and thus showed no dose-dependent effects. However, an increase in PKA activity was recorded after 72 hours. This increase is consistent with other findings (Nazarova *et al* 2004) and was observed after treatment with  $10^{-7}$  M and  $10^{-11}$  M of  $1,25(\text{OH})_2\text{D}_3$  and there were no effects when cells were treated with the G-protein inhibitor (figure 2). When comparing the result from the 96 hour incubations with the results from the 72 hours a significant decrease in response was observed, where the activity was decreased 2-fold ( $p < 0,05$ ). The decrease suggests that cells respond differently to the treatment once passed 72 hours, a lower affinity receptor or a negative feedback system that regulates  $1,25(\text{OH})_2\text{D}_3$  levels has exerted some effect.

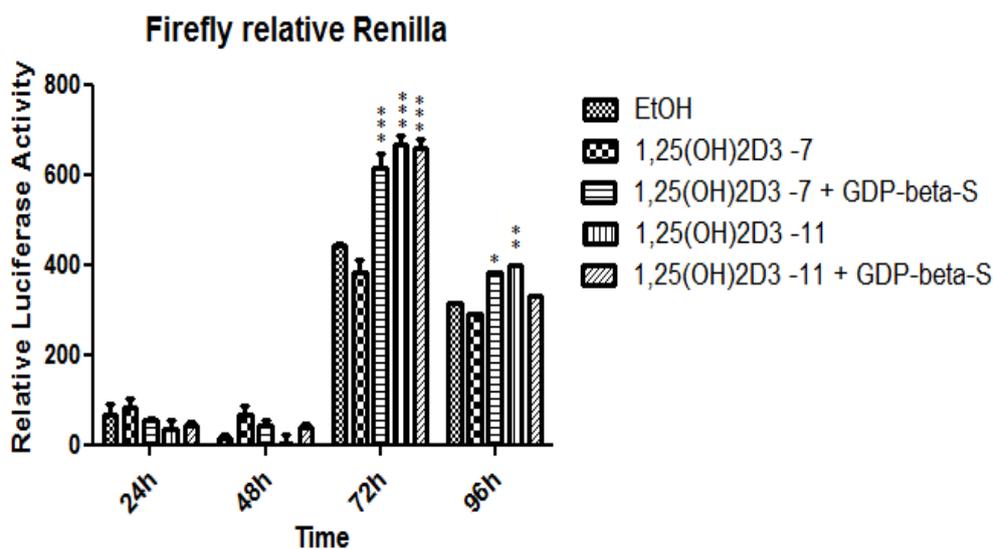


Figure 2. This graph presents the recordings of Firefly luciferase activity relative to the *Renilla* luciferase activity in LNCaP cells after treating them with  $1,25(\text{OH})_2\text{D}_3$  ( $10^{-7}$  and  $10^{-11}$ ) with or without GDP- $\beta$ -S ( $100\mu\text{M}$ ), using ethanol as control. The recorded

readings of the 72 hours treatment compared to the 96 hours treatment were significantly different. Data were analyzed by Two-way ANOVA with significance level set at  $p < 0,05$ .

The recording after 96 hours was performed in a single replicate. Because of this, those values are excluded from Two-way ANOVA.

The decrease in PKA-dependent gene expression at  $10^{-7}$  M concentration in sample treated with vitamin D<sub>3</sub> compared to the sample treated with both vitamin D<sub>3</sub> and GDP-β-S in 72 hour reading is most likely the effect of chance. The effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> are androgen dependent (Desinotis *et al* 2009) and was unaffected by the presence of the GDP analogue GDP-β-S. Findings also provide proof of LNCaP exhibiting biphasic growth as response to androgens (Zhao & Feldman 2001). This supports the theory that 1,25(OH)<sub>2</sub>D<sub>3</sub> exerts its effect through several signaling pathways. The result presented in this study suggests that perhaps a receptor with lower affinity for the treatment is activated after the treatment reaches optimum concentration. This implies that not only time affects the result but also space and different receptor populations within the same cell. The location of the receptor is just as important as the time aspect. Researchers have shown that ligand size and receptor location matters for the final hormone response (Norman 2006). The study demonstrated a smaller but more rapid response was obtained when 1,25(OH)<sub>2</sub>D<sub>3</sub> and vitamin D receptor interact directly in the nucleus.

### Dose dependence

In figure 3, the samples are displayed as separate replicates. Strikingly, 1,25(OH)<sub>2</sub>D<sub>3</sub> in  $10^{-11}$  M, both in absence and in presence of GDP-β-S showed the highest activity response. Perhaps a lower ( $10^{-11}$  M) treatment concentration would also reach optimum later than the higher ( $10^{-7}$  M) treatment concentration.

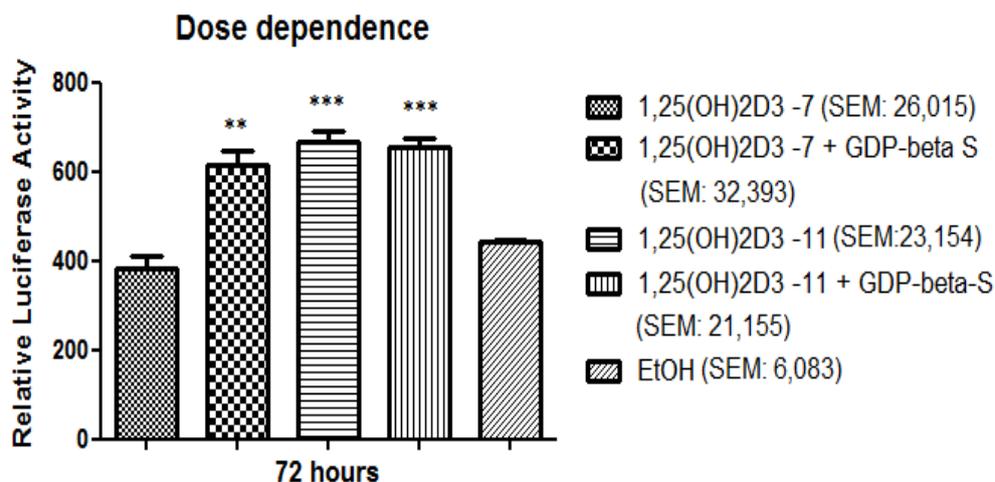


Figure 3. The Firefly luciferase activity relative to the *Renilla* luciferase activity, compared to EtOH after 72 hours treatment. PKA activity was lower in sample treated with 1,25(OH)<sub>2</sub>D<sub>3</sub>  $10^{-7}$  M concentration without inhibitor compared to the sample treated with both 1,25(OH)<sub>2</sub>D<sub>3</sub>  $10^{-7}$  M and GDP-β-S 100 μM. Cells that were treated with vitamin D  $10^{-11}$  concentration showed higher PKA activity but no difference when

comparing cells treated with GDP- $\beta$ -S to cells that were not treated with inhibitor. One-way ANOVA showed that means were significantly different with p values  $<0,05$ .

Part of the antiproliferative effect is due to  $1,25(\text{OH})_2\text{D}_3$  inducing prostate derived factor (PDF). PDF is part of the TGF- $\beta$  protein family and is associated with proapoptotic and anti cell division activities in LNCaP cells (Nazarova *et al* 2004). The highest recorded effect of  $1,25(\text{OH})_2\text{D}_3$  was after 72 hours in  $10^{-11}$  M calcitriol. This concurs with the findings of this study, showing the highest pathway activity after 72 hours. This study also utilized the. Vitamin D functions as ligand for a family of nuclear receptors that regulate transcription by binding to promoter regions. Esquenet *et al* (1996) could show that vitamin D severely, but reversibly, changed the growth pattern of LNCaP cells. They too experienced the highest effects after 72 hours and with concentrations of  $10^{-8}$  M and higher ( $10^{-7}$  M).

## Related research

GDP- $\beta$ -S inhibits  $1,25(\text{OH})_2\text{D}_3$  stimulation of calcium influx, which indicates that G-proteins are involved in the hormone effects (Vasquez *et al* 1995). Research have also uncovered that GDP- $\beta$ -S inhibits the effects of vitamin D in bird and mammalian cells. This suggest that  $1,25(\text{OH})_2\text{D}_3$  inhibits the G-protein coupled receptor subunit  $G_i$  from inhibiting adenylyl cyclase synthesis (Vasquez *et al* 1995). Further research could show that the androgen receptor (AR) can be activated by PKA in androgen sensitive LNCaP cells. They could then inhibit PKA synthesis and show that PKA is essential for appropriate activation of AR and proapoptotic events (Desinotis *et al* 2009). In this study, the findings suggest that PKA is essential, though  $1,25(\text{OH})_2\text{D}_3$  can activate PKA without G-protein coupled receptors. Inducing growth arrest in prostate cancer cells triggers expression of prostate antigen (PSA) as a differentiated function (Esquenet *et al* 1996). They hypothesized that the synergistic effect of several growth inhibitors would be more suited compared to relying on a single growth inhibitor. This further emphasizes that there is more than one way for  $1,25(\text{OH})_2\text{D}_3$  to have effect. This study could show that  $1,25(\text{OH})_2\text{D}_3$  exert full effect even when inhibitor was added. As mentioned previously, the location of the receptor is of importance. The receptor locale in combination with activation of a lower affinity receptor could be the reason for the biphasic response.

## Conclusions

From this study it is concluded that  $1,25(\text{OH})_2\text{D}_3$  regulate gene expression in LNCaP cells through a PKA-dependent pathway and that the PKA-dependent gene expression is independent of G-protein coupled receptors.

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