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Membrane Initiated Effects of 1 α ,25-Dihydroxyvitamin D₃ in Prostate Cancer Cells: Effects on AP1 and CREB Mediated Transcription

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1. Introduction

The biologically active form of vitamin D₃, 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃], is formed through a multistep process in the liver and the kidneys, initiated in the skin by solar UVB radiation. Vitamin D compounds are transported in the body by vitamin D binding protein (DBP) to either nuclear vitamin D receptors (nVDR) or putative membrane associated vitamin D receptors (mVDR) where it exerts its biological responses in target organs by nuclear- and membrane- initiated signaling pathways (Bouillon *et al.*, 1995; Holmén *et al.*, 2009). Finally, 1 α ,25(OH)₂D₃ becomes inactivated by 24-hydroxylase which transforms it into 1,24,25(OH)₃D₃, a substance with much lower affinity for VDR (Bouillon *et al.*, 1995).

In the nuclear initiated signaling pathway, occupancy of the nuclear vitamin D receptor (nVDR) by 1 α ,25(OH)₂D₃ leads to modulation of gene transcription of hormone-sensitive genes (Krishnan & Feldman, 2010). In conformity with several other receptors of the nuclear steroid/thyroid superfamily, nVDR forms heterodimers with retinoid X receptor (RXR) (Sutton *et al.*, 2003). Subsequent interaction with the vitamin D response element (VDRE) in the promoter sequence of target genes initiates induction or repression of transcription, hence generating a biological response (Haussler *et al.*, 2011).

Vitamin D exerts multiple actions in the organism including the well-known regulation of calcium and phosphate homeostasis (Holick, 2006), but it also possesses anti-proliferative, pro-differential and pro-apoptotic actions in cancer cells as well as increasing the effect of a number of established anti-cancer drugs (Trump *et al.*, 2010).

The association of vitamin D with human cancer is well described in adenocarcinoma of the prostate gland, i.e. prostate cancer (PCa). Clear correlation between vitamin D deficiency and risk factors for PCa, such as high age and darker pigmented skin, has been observed. Thus, the amount of vitamin D decline with age, and the elevated levels of melanin in African Americans partly inhibits sun initiated vitamin D synthesis (Hsing & Chokkaligam, 2006).

Furthermore, $1\alpha,25(\text{OH})_2\text{D}_3$ has been proven to decrease the risk of PCa by controlling prostate cell proliferation (Holick, 2006).

In a previous paper, we have shown that $1\alpha,25(\text{OH})_2\text{D}_3$ regulates prostate cell differentiation, apoptosis and proliferation via multiple pathways, which involves both nuclear and membrane receptors found in the JNK/SAPK (c-Jun N-terminal kinases/stress-activated protein kinase) pathway. (Hagberg *et al.*, 2008; Larsson *et al.*, 2008; Holmén *et al.*, 2009; Karlsson *et al.*, 2010).

The JNK/SAPK pathway may be induced by several different means, such as chemical and physical stress, UV-radiation and osmotic shock, as well as pro-inflammatory cytokines, and even G-coupled receptor signaling, (Matsukawa *et al.*, 2004). Among the cytokines that triggers the JNK/SAPK pathway, TNF- α is predominant, and it is also known to regulate cellular events associated with cancer cell phenotype, such as apoptosis, cell proliferation and differentiation. Still, it has been shown that cancer cells are resistant to apoptosis induced by TNF- α (Chopra *et al.*, 2004). This resistance seems to involve certain survival signals, one of which being the transcription factor, nuclear factor-kappa B (NF- κ B). It is therefore thought that if NF- κ B is inhibited, the cancer cells would become more sensitive to TNF- α induced apoptosis. Indeed, this seemed to be the case in TNF- α resistant leukemia cells that were treated with sulforaphane, a putative anti-cancer drug that showed a non-specific inhibition of the TNF- α induced NF- κ B activation (Moon *et al.*, 2009). In addition, there were indications that this inhibition of NF- κ B lead to prolonged JNK/SAPK activation in the leukemia cells. Other studies indicate that activation of JNK also seems to regulate and can be regulated by NF- κ B (Nachmias *et al.*, 2004). A similar result to the sulforaphane inhibition of the TNF- α induced NF- κ B has been reported for breast cancer cells treated with vitamin D. Thus, Michigan cancer foundation 7 (MCF-7) breast cancer cells, were found to become sensitized to apoptosis induced by TNF- α when treated with $1\alpha,25(\text{OH})_2\text{D}_3$ (Golovko *et al.*, 2005).

Transcription factor NF- κ B can be regulated in concert with another transcription factor, activator protein 1 (AP1). AP1 is a complex consisting of homodimers and heterodimers of the jun and fos families and the activity of AP1 seems to be regulated by differential expression of the jun and fos families. The c-Jun components of AP1 can be regulated by the phosphorylating activity of active JNK (Dedieu and Lefebvre, 2006). Thus, in the two prostate carcinoma cell lines PC-3 and LNCaP, overexpression of the early growth response protein EGR-1, selectively increased the activity of both NF- κ B and AP1 and the activation of these transcription factors appeared to be essential for the induction of proliferation and anchorage independence (Parra *et al.*, 2011).

Another important mediator of cell proliferation, differentiation and apoptosis is the cyclic response element binding protein (CREB). CREB is part of the cAMP regulated pathway and is phosphorylated by protein kinase A (PKA). CREB does not have direct contact with the transcriptional machinery. 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. There are no direct evidences that vitamin D receptors are coupled to a protein complex which includes adenylate cyclase. However, several studies have demonstrated that $1\alpha,25(\text{OH})_2\text{D}_3$ evoke rapid increases in PKA activity, intracellular cAMP concentrations which have been found to be associated with G-protein-coupled signaling as well as regulation of Ca^{2+} transport through Ca^{2+} -channels (Massheimer *et al.*, 1999; Schwartz *et al.*, 2002; Dirks-Naylor & Lennon-Edwards, 2011).

Knowing how the cell cycle arrest and the anti-proliferative effects are induced on a molecular level is important when developing successful therapeutic tools against cancer. 1 α ,25(OH)₂D₃ stands out as a potential anti-cancer drug, even with its severe side effect of hypercalcemia, and treating LNCaP cells with 1 α ,25(OH)₂D₃ results in an accumulation of cells in the G1 phase, growth arrest, and to some extent apoptosis. In order to get a better understanding of how this kind of action of 1 α ,25(OH)₂D₃ is regulated we have in this study made the following investigations:

First, monitor the response on JNK/SAPK complex dependent activation of AP1 to 1 α ,25(OH)₂D₃ and TNF- α in LNCaP prostate cancer cells. Secondly, decide whether 1 α ,25(OH)₂D₃ regulates TNF- α production and release by LNCaP cells and thus have an indirect effect via the TNF- α signaling pathway on cell growth, differentiation and apoptosis, and third, evaluate the PKA dependent activation of cyclic response element binding protein (CREB) in LNCaP cells treated with 1 α ,25(OH)₂D₃.

2. Materials and methods

2.1 Cell culture

LNCaP cells were cultured in Gibco RPMI 1640 media (Invitrogen, UK). The media contained FBS (10%), PEST (1%), L-glutamine (1%), HEPES and sodium pyruvate (1%). The cells were subcultured five days after the initial culture and seeded at a density of 20 000 cells/well onto a 96-well plate (Nunc, Thermo Fischer Scientific, US) or 50 000 cells/well at a 24 well plate (Nunc, Thermo Fisher Scientific). At the point of seeding to 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 incubated at 37°C and 5% CO₂ for 48 hour prior 1 α ,25(OH)₂D₃ treatment.

2.2 Transient transfection and CREB reporter assay (cAMP/PKA)/AP1 reporter assay

SureFECT™ transfection reagent and Cignal™ CREB reporter/Cignal™ AP1 reporter kit was used according to the manufacturer's protocol (SA BioSciences, USA) to monitor cAMP/PKA pathway activity and the activity of AP1-regulated transduction pathways. The CREB 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 response to CREB 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 AP1 reporter contains a mixture of inducible AP1-responding firefly luciferase construct and a constitutively expressing *Renilla* luciferase construct. The luciferase construct codes for the firefly luciferase reporter gene which is under the control of a minimal cytomegalovirus (mCMV) promoter and tandem repeats of the TPA response element. The *Renilla* construct codes for the *Renilla* luciferase reporter gene, which acts under control of a CMV. It is used as a control for normalizing transfection efficiency and for monitoring cell viability.

Briefly, in both assays, the LNCaP cells were transfected using SureENTRY transfection reagent in a 96-well plate. The LNCaP cells were seeded at the time of transfection. The medium used for the transfection was Opti-MEM serum-free culture medium. Dilutions of

the AP1/CREB reporter and the positive and negative controls were prepared as well as dilutions for SureENTRY. The cells were then washed with PBS and trypsinised and then suspended in Opti-MEM serum-free cell culture medium. The cell pellet was then resuspended in Opti-MEM cell culture medium. A haemocytometer was used to determine cell density and 10 000 cells were then seeded into each well. The Signal reporter, negative and positive control was added to the appropriate wells and SureENTRY was added to each well and incubated for 48 hours. The cells were then treated with $1\alpha,25(\text{OH})_2\text{D}_3$ and luminescence measurements were made for selected interval.

2.3 $1\alpha,25(\text{OH})_2\text{D}_3$ and G-protein coupled PKA/CREB-dependent gene expression in LNCaP cells

Following incubation, cells transfected with the CREB reporter, were treated with $1\alpha,25(\text{OH})_2\text{D}_3$ in the concentrations 10^{-7} M with or without the G-protein inhibitor Guanosine 5'-[β -thio]diphosphate trilithium (GDP- β -S; 100 μM ; Sigma-Aldrich). Ethanol (0.001%) was used as control.

2.4 Luminescence measurements

Luminescence levels were measured at four time points, 24, 48, 72 and 96h in a luminometer (FLUOstar Galaxy, BMG Labtech, Germany) using the Dual Glo™ Luciferase Assay System (Promega, US). 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. At each measuring interval, 75 μl of Dual Glo™ was added to the examined wells to check inducible activity, according to manufacturer's recommendations and were incubated at room temperature for 12-15 minutes. Following the Firefly luciferase reading, 75 μl of Stop & Glo Reagent™ was added to check the non-inducible luciferase activity. As with Dual Glo Reagent™, the wells were incubated 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 obtained data.

2.5 Effects of $1\alpha,25(\text{OH})_2\text{D}_3$ and TNF- α on AP1-dependent gene expression in LNCaP cells

The cells were treated with $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-7} M) or TNF- α (10^{-9} M) with or without 20 μM SP600125 (JNK/SAPK inhibitor (0.001% of ethanol was used as a control)). Luminescence measurements were taken after 24, 48, 72 and 96h. The experiments were repeated in triplicates.

2.6 Effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on TNF- α production in LNCaP cells

Each well of a 24 well plate was seeded with 50 000 LNCaP cells. The cells were then treated with 10^{-7} M $1\alpha,25(\text{OH})_2\text{D}_3$ and 20 μM of the JNK/SAPK inhibitor, SP600125. As a control, 0.001% of ethanol was used. TNF- α production in the cell culture media was measured post 72 and 96 hours treatment using a commercial TNF- α specific ELISAs according to the manufacturer's instructions (Promega, USA). Each experiment was repeated three times.

2.7 Statistics

Two-way ANOVAs were performed using GraphPad Prism to evaluate the data from the assays. For the AP1 reporter assay, triplicates were used for all four time points and all treatments except for the ethanol controls, in which case duplicates were used for all time points. For the TNF- α specific ELISA four replicates were used for all the samples and duplicates for the standards, control and blank wells. The P value threshold was set to 0.05.

3. Results and discussion

Previous studies on prostate cancer cells have reported that 1 α ,25(OH)₂D₃ regulates proliferation and cell survival through membrane initiated signaling pathways (Hagberg *et al.*, 2008; Larsson *et al.*, 2008). Similar observations have been made in vitamin D responsive tissues, where membrane initiated signaling have been linked to PKA, PKC and MAPK signaling pathways (Schwartz *et al.*, 2002; Dirks-Naylor and Lennon-Edwards, 2011). This study was aimed to further elucidate membrane initiated signaling by 1 α ,25(OH)₂D₃ in LNCaP prostate cancer cells by evaluating the effects of 1 α ,25(OH)₂D₃ on AP1 and CREB-dependent gene expression as well as testing the hypothesis that 1 α ,25(OH)₂D₃ might evoke membrane initiated effects through TNF- α and TNF- α initiated signaling pathways.

3.1 Effects of 1 α ,25(OH)₂D₃ on AP1-dependent gene expression

As shown in Figure 1, 1 α ,25(OH)₂D₃ increased AP1-dependent gene expression after treatment at 48 (p<0.05), 72 (p<0.001) and 96h (p<0.0001) compared to control treated LNCaP cells (0.001% ethanol). The effect was time-dependent and at 96h, a difference in AP1-dependent gene expression was observed between the cells treated with 1 α ,25(OH)₂D₃ and cells treated with 1 α ,25(OH)₂D₃ + SP600125 (JNK/SAPK inhibitor)(p<0.05). Thus, 1 α ,25(OH)₂D₃ increase AP1-dependent gene expression through the JNK/SAPK signaling pathway in LNCaP prostate cancer cells.

Fig. 1. Effects of 1 α ,25(OH)₂D₃ (10⁻⁷ M) on AP1-dependent gene expression.

AP1-dependent gene expression was determined in LNCaP prostate cancer cells by a AP1 reporter assay (SA Biosciences) by measuring Firefly luciferase activity relative to the *Renilla* luciferase activity after treatment with 1 $\alpha,25(\text{OH})_2\text{D}_3$ (10^{-7} M) with or without SP600125 (20 μM), using ethanol as control. LNCaP cell were treated for 24, 48, 72 or 96h before measuring the AP1 activity. Data were normalised and are expressed as % of control. The level of significance was set to $p<0,05$. Data are presented as mean $\pm$ SEM

The data from this study supports previous observations in prostate cancer cells where $1\alpha,25(\text{OH})_2\text{D}_3$ decrease cell proliferation (Larsson *et al.*, 2008) and where at least a part of the decreased proliferation has been connected an increase in the phosphorylation of JNK/SAPK and c-jun (Larsson *et al.*, 2008; Hagberg *et al.*, 2008; Karlsson *et al.*, 2010). Effects on MAPK signaling pathways by $1\alpha,25(\text{OH})_2\text{D}_3$ is not limited to prostate cancer cells. In human myeloid leukemia HL-60 cells, Kim *et al.* (2007) $1\alpha,25(\text{OH})_2\text{D}_3$ induced HL-60 cell differentiation through a pathway involved with PI3-K/PKC/ERK/JNK and in human osteosarcoma SaOS-2 cells (Wu *et al.*, 2007), $1\alpha,25(\text{OH})_2\text{D}_3$ were reported to be involved in JNK/SAPK activation as well as ERK 1/2 MAPK signaling and that only sustained and not transient treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ induced AP1 activation.

3.2 Effects of TNF- α on AP1-dependent gene expression

TNF- α increased AP1-dependent gene expression after 72 ($p<0.01$) and 96h ($p<0.05$) compared to control treated LNCaP cells. The effect was not time-dependent and no differences in AP1-dependent gene expression was observed between the cells treated with TNF- α and cells treated with TNF- α + SP600125 (JNK/SAPK inhibitor) at any time-point. Thus, TNF- α increases AP1-dependent gene expression in LNCaP prostate cancer cells.

Fig. 2. Effects of TNF- α (1 nM) on AP1-dependent gene expression.

AP1-dependent gene expression was determined in LNCaP prostate cancer cells by a AP1 reporter assay (SA Biosciences) by measuring Firefly luciferase activity relative to the *Renilla* luciferase activity after treatment with TNF- α (1 nM) with or without SP600125 (20 μM), using ethanol as control. LNCaP cell were treated for 24, 48, 72 or 96h before measuring the

AP1 activity. Data were normalised and are expressed as % of control. The level of significance was set to $p < 0.05$. Data are presented as mean $\pm$ SEM

The findings in the present study are in concert with reports from MIN6N8 pancreatic β -cells (Kim *et al.*, 2005) and MCF7 breast cancer cells (Yin *et al.* 2009), where the JNK/SAPK signaling pathway was reported to increase AP1 dependent gene expression. However, Yin *et al.* (2009) showed that the AP1 transactivation activity had its peak after 3 hours but was still significantly elevated after 24h. In the present study, the response in an increased AP1 activity came after 48 hours and persisted throughout the experiment. The difference in response reported in this study and by Yin *et al.* (2009) may be because of differences between breast cancer and prostate cancer cells but could also reflect that the concentration of TNF- α in experiments performed by Yin *et al.* (2009) could have been a limiting factor.

3.3 Effects of 1 α ,25(OH)₂D₃ on TNF- α production in LNCaP cells and TNF- α concentrations in culture media

The TNF- α specific ELISA showed that 1 α ,25(OH)₂D₃ did not affect the production of TNF- α in LNCaP cells and thus, does not have an effect on TNF- α signaling pathway by increased concentrations of the growth factor in the media. This suggest that TNF- α acts independently of 1 α ,25(OH)₂D₃ in activation of the JNK/SAPK signaling pathway. These results are consistent with the findings of Golovko *et al.* (2005), who reported that under physiological conditions, 1 α ,25(OH)₂D₃ does not affect the production of TNF- α , but that TNF- α mRNA expression was up-regulated by 1 α ,25(OH)₂D₃ as well as its analogue CB1093 in LNCaP and PC3 prostate cancer cells. Chopra *et al.* (2004) studied the role of TNF- α in regulation of growth and apoptosis in three different prostate cell lines: normal prostate epithelial cells, LNCaP cells and PC3 cells and could demonstrate that normal prostate epithelial cells and PC3 cells were resistant to growth arrest and apoptosis induced by TNF- α and LNCaP cells were highly sensitive to the growth factor. Thus, from the results in the present study as well as previous studies (Golovko *et al.*, 2005; Chopra *et al.*, 2004,) we conclude that 1 α ,25(OH)₂D₃ and TNF- α acts through independent pathways ending up in an up-regulation of AP1-dependent gene expression.

3.4 Effects of 1 α ,25(OH)₂D₃ on CREB-dependent gene expression

1 α ,25(OH)₂D₃ increased CREB-dependent gene expression compared to control treated LNCaP cells (Figure 3). The effect was time- and G protein-dependent where treatment with 10⁻⁷ M 1 α ,25(OH)₂D₃ increased CREB-dependent gene expression compared to cells treated with 10⁻⁷ M 1 α ,25(OH)₂D₃ + GDP- β -s (G protein inhibitor) at 24 ($p < 0.05$), 48h ($p < 0.0001$) but were decreased compared to the G-protein inhibited cells at 72h ($p < 0.05$). Thus, 1 α ,25(OH)₂D₃ increases CREB-dependent gene expression through a G protein-dependent PKA/CREB signaling pathway in LNCaP prostate cancer cells.

PKA/CREB-dependent gene expression was determined in LNCaP prostate cancer cells by a CREB reporter assay (SA Biosciences) by measuring Firefly luciferase activity relative to the *Renilla* luciferase activity after treatment with 1 α ,25(OH)₂D₃ (10⁻⁷ M) with or without GDP- β -S (100 μ M), using ethanol as control. LNCaP cell were treated for 24, 48, 72 or 96h before measuring the CREB activity. Data were normalised and are expressed as % of control. The level of significance was set to $p < 0.05$. Data are presented as mean $\pm$ SEM

Fig. 3. Effects of $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-7}M) on PKA/CREB-dependent gene expression.

The fact that $1\alpha,25(\text{OH})_2\text{D}_3$ both activate JNK/SAPK and PKA/CREB-dependent gene expression indicate that $1\alpha,25(\text{OH})_2\text{D}_3$ exert its effects through more than one pathway and mean that there might be more than one receptor that mediates the responses of this metabolite. Alternatively, the receptor could form different complexes that upon activation start unique signal cascades. An example of a similar observation is membrane initiated signaling in skeletal muscle, where six different signaling pathways have been described for $1\alpha,25(\text{OH})_2\text{D}_3$ (Vasquez *et al.*, 1996; Capiati *et al.*, 2000; Dirks-Naylor and Lennon-Edwards, 2011). The point that there are two different vitamin D receptors (VDR, PDIA3) associated with the cell membrane (Holmen *et al.*, 2009; Karlsson *et al.*, 2010) and that PDIA3 has been suggested to form a trimer with at least three high affinity binding sites (Karlsson *et al.*, 2010) make us to postulate that depending on the docking site of $1\alpha,25(\text{OH})_2\text{D}_3$ to the receptor, the resulting change in three dimensional structure of the hormone-receptor complex, starts a subsequent signaling cascade. The response will thus be dependent on both time and space where both short-term and long-term effect will be important in regulating prostate cell biology.

3.5 Conclusions

In conclusion, our findings support previous reports and suggest that $1\alpha,25(\text{OH})_2\text{D}_3$ regulate prostate cell biology via multiple pathways and targeting of specific pathways for $1\alpha,25(\text{OH})_2\text{D}_3$ might provide more effective therapies compared to the vitamin D therapies currently clinically tested and may serve as a complementary treatment in patients with androgen independent prostate cancer.

3.6 Future directives

The nature of membrane initiated signaling as a response to $1\alpha,25(\text{OH})_2\text{D}_3$ is not yet clarified. It has been debated over the last two decades and currently there are two major candidates to be a membrane associated receptor for $1\alpha,25(\text{OH})_2\text{D}_3$ (VDR and PDIA3). Our laboratory have for several years studied membrane initiated signaling by $1\alpha,25(\text{OH})_2\text{D}_3$, *in silico* and *in vitro*, to elucidate signaling pathways and its key components with the goal to

clarify the biological role of the pathways in regulating prostate cancer. Focus on future work will be to create specific antagonists and agonists (the pharmacopore approach) to the putative membrane associated receptors to clarify their functions *in vitro* and hopefully get molecules that have a high specificity to single receptor binding site.

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Current Frontiers and Perspectives in Cell Biology

Edited by Prof. Stevo Najman

ISBN 978-953-51-0544-2

Hard cover, 556 pages

Publisher InTech

Published online 25, April, 2012

Published in print edition April, 2012

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Dennis Larsson, Adele Jonas, Niklas Bergsten, Fredrik Stahl and Sandra Karlsson (2012). Membrane Initiated Effects of 1 α ,25-Dihydroxyvitamin D3 in Prostate Cancer Cells: Effects on AP1 and CREB Mediated Transcription, Current Frontiers and Perspectives in Cell Biology, Prof. Stevo Najman (Ed.), ISBN: 978-953-51-0544-2, InTech, Available from: <http://www.intechopen.com/books/current-frontiers-and-perspectives-in-cell-biology/membrane-initiated-effects-of-1-25-dihydroxyvitamin-d3-in-prostate-cancer-cells-effects-on-ap1-and>

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