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Improving colon cancer prevention by vitamin D: Regulation of VDR expression with Panobinostat and Bexarotene in a mouse colon cancer model

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Improving colon cancer prevention by vitamin D: 
Regulation of VDR expression with Panobinostat and Bexarotene in a mouse colon cancer model

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Abstract:

Vitamin D is generally known to protect against colon cancer; however, its beneficial effects are limited to early stages in tumorigenesis. Vitamin D binds to the Vitamin D receptor (VDR), which has been shown to mediate colon cancer risk. From previous interventional studies, it has been suggested that VDR is down-regulated in tumorigenic cells leading to the formation of cancer. Potential mechanisms by which VDR is regulated in tumors were studied. Histone deacetylase (HDAC) and Retinoid X receptors (RXR) were studied as potential factors that regulate VDR expression. APC\(^{+/\Delta14}\) mice treated with Panobinostat, a HDAC inhibitor, and Bexarotene, a RXR agonist, were measured for VDR gene expression along with other VDR target genes. The tumor and normal colon tissue were extracted, followed by a gene expression analysis using a Real-time qPCR. Mice treated with Panobinostat showed a significant increase in the expression of VDR in tumors as compared to normal tissues. This suggests that VDR expression may be regulated with HDACs. Mice treated with Bexarotene did not show a significant increase in VDR expression; RXR might not be limited by the level of the ligand. Even though vitamin D benefits are limited to the early stages in tumor growth, it may be possible to develop therapies that enhance vitamin D effects. The drug Panobinostat has been shown to up-regulate VDR expression in later stages of tumorigenesis in a mouse model.
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Introduction/Results:

Colon cancer is among the three most commonly diagnosed cancers in the world. Each year about 100,000 new cases are diagnosed and 50,000 deaths occur on average (Coble, 2013). There are many factors that contribute to the risk of developing colon cancer including diet, genetic predisposition and cellular stress. Nearly two-third of colon cancers are related to diet and lifestyle alone (Ingraham et al, 2008). The diagnosis of colon cancer is determined by the metastasis of dysplastic colon epithelium to other regions in the body. Traditionally, the main types of treatment for colon cancers have been surgery, and chemotherapy (Christakos et al, 2013). Depending on the stage of the cancer, these treatments may be combined. While the treatment for colon cancers has been the focus of many studies over the past few decades, recently studies on early detection and prevention have taken precedence. Techniques such as polyp detection and removal through colonoscopies have offered significant protection against the development of tumors; however, colon cancer related deaths are still increasing every year. Advantages of using chemopreventive mediators such as dietary agents and supplements have shown an inverse correlation with the risk of colon cancer. Moreover, these chemopreventive agents have been observed to suppress growth pathways, thereby, inhibiting cancer formation. These chemopreventive agents that are clinically recognized and still studied include folate, vitamin D and calcium to name a few (McCullough et al, 2013). The endpoint of this study is polyp recurrence and adenoma formation.

Recent studies show that cholecalciferol (a form of vitamin D) may be a chemopreventive agent that offers protection and even prevention against colon cancers. Vitamin D, is a group of fat-soluble prohormones responsible for enhancing intestinal
absorption of calcium and phosphate, and regulating mineral homeostasis. In humans, the prohormones cholecalciferol (vitamin D₃) and ergocalciferol (vitamin D₂) are obtained from diet, fortified foods and supplements; however, only cholecalciferol can be synthesized from 7-dehydrocholesterol, a type of cholesterol in skin, when exposed to ultraviolet-A light. These two vitamin D prohormones are further enhanced in the liver to their active form 1, 25-dihydroxycholecalciferol, also known as calciferol. Calciferol mediates all intercellular actions, including calcium homeostasis, regulation of cell growth and differentiation, cell adhesion, and apoptosis (Ingraham et al, 2008). Ultimately, vitamin D offers cancer preventative benefits through the transcription of target genes such as p21 and Cdh1, cell cycle regulator and tumor suppressor, respectively. Studies by Cuomo RE, Mohr SB, Gorham ED, et al. (2013) showed that higher exposure to sunlight and/or a diet high in vitamin D correlated inversely with colon cancer development. To study the protective effect of vitamin D supplementation, we used a mouse genetic model of colon cancer to analyze its response to different levels of dietary vitamin D.

Initial studies using APC+/Δ14 mice tested a trend in vitamin D protection. Higher concentrations (2500 IU compared to 250 IU) of vitamin D appear to suggest a lower tumor count (figure 1).
In order for vitamin D to induce a response, it must bind to the vitamin D receptor (VDR), a class of the nuclear receptor superfamily (Christakos et al, 2013). VDRs are found in many cell types including heart, muscle, breast, colon, prostate, brain, kidney, bone, intestine, osteoblasts, and immune cells. Studies have shown the importance of vitamin D-VDR interactions in normal and tumor colon tissues. Figure 2 shows a simplified visual of how Vitamin D and VDR interact.

**Figure 1: Comparison of colon tumor count in APC^{+/Δ14} mice cells of high (2500IU) and low (250IU) vitamin D concentrations.** Higher Vitamin D concentration show trends in lower colon tumor count. (Provided by Dr. Masako Nakanishi and Dr. Daniel Rosenberg at the UCHC).

**Figure 2: Simplified visual of vitamin D and VDR interaction.** When the vitamin D binds to the VDR, it is transcribed to produce genes that lead to regulation of cellular growth.

Recently, it has been observed that VDR expression is frequently suppressed in cancers. Early in tumorigenesis Vitamin D is effective; however, this is less clear in adenomas, which means that vitamin D is not actively binding to VDR and therefore offering its protective mechanisms (Rosa et al, 2013). For example, Hartman et al. (2005) conducted a clinical study...
called the Polyp Prevention Trial, which found that higher vitamin D intake was not significant in reducing the risk of adenomas. This ultimately means that there is a disruption in the vitamin D-VDR interaction. Furthermore, Knackstedt, Moseley, Sun & Wargovich (2013) reported lower VDR expression in tumor tissues. As a result, the concentration of vitamin D that is able to be utilized within the tumor cells is limited. Figure 3 shows an immunostaining provided by Dr. Nakanishi Masako, it was shown in a cross-section of mice tissue that VDR is absent in tumors (figure 3).

Our lab used APC^{+/Δ14} mice to confirm the initial observations of VDR down-regulation in tumors. We found that VDR is down-regulated by fivefold. Moreover, VDR target genes, p21 and Cdh1, were also down-regulated, adding to the evidence for VDR suppression in tumors (figure 4). By understanding how VDR expression is regulated in tumor cells, we may be able to gain insight into the mechanism of tumor cell progression.
HCT and HT29 colon cancer cells were used to model human colon cancer. From the cell studies it was suggested that histone deacetylase (HDAC) may be a possible regulator of VDR. HDACs ultimately decrease gene transcription by binding to the histones and removing acetyl groups, thereby allowing DNA to tightly bind to the histone. A simplified model for the actions of HDACs is shown in figure 5. HDAC expression was also measured in the APC^+/∆14 mice model, we found that tumor cells have a significantly higher expression of HDACs than normal cells (figure 6).

Figure 4: Comparison of VDR, p21, and Cdh1 expression in normal and tumor colon cancer tissues from APC^+/− mice. As compared to normal colon, tumor colon tissue have significantly lower expression of VDR, p21 and Cdh1 genes.

Figure 5: Simplified visual of HDAC and VDR interaction. HDAC will inhibit the transcription of VDR, thereby decreasing VDRs in the cell.
VDR is known to interact with the retinoid x receptor (RXR), a nuclear receptor that heterodimerizes with other nuclear receptors. Figure 7 shows a simplified view of this interaction. It was suggested that RXR-VDR interaction may be interrupted in tumor cells, thereby limiting the VDR expression. Moreover, studies by Knackstedt et al. (2013) suggested that VDR and RXR are down-regulated in a mouse model. We therefore determined the RXR expression levels in the APC$^+/\Delta14$ model. Initially, a gene expression analysis for RXRs was done in both normal and tumor tissues. It was found that RXRa, RXRb and RXRg were significantly down-regulated in tumors as compared to normal tissues (figure 8).

**Figure 6: Comparison of HDACs expression in normal and tumor colon cancer tissues from APC$^+/\Delta14$ mice.** As compared to the normal cells, tumor cells have higher expression of HDACs on average.

**Figure 7: Simplified visual of RXR and VDR interaction.** RXR will heterodimerize with VDR, thereby allowing VDR to bind to the response element on the RNA and be transcribed.
A HDAC inhibitor, Panobinostat, was used to measure the correlation between HDACs and VDR expression. Panobinostat was chosen because it is frequently used in preclinical studies on HDACs. An RXR agonist, Bexarotene, was used to measure the relationship between RXR and VDR expression. This was also chosen because it is widely used in preclinical studies.

Initially it was proposed that HDACs may be a possible mechanism by which VDR is down regulated. Figure 9 shows how this was tested. Panobinostat, the HDAC inhibitor, would inhibit the HDAC, which would not inhibit VDR. Thereby, allowing VDR to be transcribed. Vitamin D would bind to the VDR and allow for anti-tumorigenic effects. By doing an interventional study and gene expression analysis on the APC\(^{+/\Delta14}\) mice, it was discovered that the mice treated with Panobinostat showed a significantly higher VDR expression as compared to the mice that weren’t treated with Panobinostat (figure 9). Moreover, in order to assure the validity of the observed VDR expression, the relative expression of p21, a downstream target of the VDR, was measured. It was found that the relative expression of p21 was also significantly higher in mice treated with Panobinostat.

The results of the Panobinostat treated mice suggest that the HDAC up regulation in mice tumors are correlated with the VDR down regulation. This may occur because HDACs are...
binding to the DNA and decreasing the transcription of the DNA; ultimately, VDR gene is not being transcribed and the VDR proteins are not made. The lack of VDRs will limit the vitamin D utilization within the organism and lead to lower protection against tumor formation.

As for the RXR experiment, it was initially proposed that Bexarotene would allow RXR to bind to the VDR, which would subsequently bind to the DNA and transcribe specific anti-tumorigenic genes (figure 10). The results from the Bexarotene treated mice showed a slight increase in the VDR expression, which was not significant (figure 10). This means that the RXR action may not be limited by the level of the ligand but does not mean that the RXR are not factors for the down-regulation of VDR. The RXRs may play an important role in VDR regulation but it may not be direct.
Figure 9: Pathway by which HDACi, Panobinostat, acts to suppress unregulated cell growth.

Relative expression of VDR and p21 in cells of APC^+/Δ14^ mice treated with Panobinostat.

Normally, the vitamin D will bind to the VDR and regulate cell growth. However, in tumor cells, the VDR expression is low. As a result, vitamin D's health benefits will be limited and uncontrolled cellular growth may occur leading to colon cancer. Panobinostat, a HDAC inhibitor (HDACi), prevents HDACs from functioning. Thereby, allowing VDR gene expression and subsequently limiting uncontrolled cellular growth. Furthermore, an increase in p21 expression with the Panobinostat confirms the reduction in uncontrolled cellular growth.
Figure 10: Pathway by which Bexarotene acts to suppress unregulated cell growth. Relative expression of VDR in normal and tumor cells of APC^{+/Δ14} mice treated with Bexarotene. Bexarotene, a RXR agonist, will stimulate RXR binding to VDR which will transcribe the VDR gene ultimately leading to the decrease in unregulated tissue growth. The graphs show that the addition of Bexarotene does not result in significantly higher VDR expression in colon tumor cells.
**Treatment of tumor-bearing/normal mice and tissue extraction**

\( \text{APC}^{+/\Delta 14} \) mice were used to model the vitamin D-VDR expression seen in colon cancer. \( \text{APC}^{+/\Delta 14} \) mice have a heterozygous knockout for the APC gene, which gives them a tumor predisposition phenotype.

\( \text{APC}^{+/\Delta 14} \) mice were treated at the University of Connecticut Health Center (Farmington, CT). They were allowed access to laboratory rodent chow (Laboratory Rodent Diet 5001; PMI Nutrition International, Richmond IN) and water. Ten mice were fed a high vitamin D (2500 IU) diet. At five weeks of age, five of those mice were treated with Panobinostat (LBH-589) for nine weeks. The other five mice were raised normally. Concurrently, another eight mice were raised on a low vitamin D (250 IU) diet. Of those, three were injected with Panobinostat while the other five were raised normally. After the final dose, all the mice were euthanized. The tumor and adjacent normal tissues from the colons and liver were separately collected for analysis.

Another set of 18 mice were treated as above but were injected with Bexarotene instead of Panobinostat. The same collection procedure as cells treated with Panobinostat was used here as well. RNA was prepared from the samples by grinding and storing in TRIZol reagent.

**RNA extraction**

Tissues were suspended in 200uL of TRIZol reagent (Life Technologies, Carlsbad, CA) in microfuge tubes. Next, at least ten minutes were allowed in order for the TRIZol to homogenize the samples based on molecular size. Afterwards, 200uL of chloroform was added to the microfuge tubes and shaken vigorously for 15 seconds, lysing the cells and allowing the RNA to be released. The samples were centrifuged at 12,000rpm for fifteen minutes at 4°C. After the
Centrifugation was complete, the upper aqueous layer of the samples was removed and pipetted into new tubes. To the new tubes 500μl of isopropanol was added and incubated for ten minutes before they were centrifuged at 12,000rpm for ten minutes at 4°C. The centrifugation left an RNA pellet at the bottom of the tube, which was isolated by removing the supernatant from the tube. The RNA pellet was washed with 75% ethanol and centrifuged again at 12,000rpm for five minutes at 4°C. Finally, after centrifugation, the supernatant was removed, completely leaving behind the RNA pellet, which was resuspended in RNase-free water and store at 70°C.

**RNA quantification**

In order to quantify the RNA concentration in the samples, Thermo Scientific NanoDrop 8000 Spectrophotometer (cat. #13-400-413) from Fisher Scientific (Waltham, MA) was used. Initially, the NanoDrop 8000 was blanked by using RNA-free water. Then a single drop of the mice tissue RNA was pipetted into the NanoDrop apparatus and the concentration was measured. This step allowed for the confirmation that the samples actually contained RNA before proceeding to reverse transcribe them to cDNA.

**Reverse transcription**

Using the quantified concentrations of RNA, it was necessary to calculate the amount of the samples needed to make 10μl solutions in H₂O. The mixtures were added to new microfuge tubes with 10μl master mix (H₂O, 10x reverse transcription buffer, 25x dNTP mix, 10x random primers, and reverse transcriptase ) (Life Technologies, Carlsbad, CA).. The tubes were centrifuged at room temperature, then placed into the My Cycler: Bio-RAD (Conquer Scientific San Diego, CA). My Cycler is a thermal cycler that transcribes the RNA into cDNA.
Real-Time qPCR

In order to measure the expression of the specific probes on the samples, a Fast Real-Time qPCR was used. The ACTB was our control gene to which all the other probe expressions were compared. VDR is the gene for the vitamin D receptor, SAHA is a HDAC inhibitor, SNAIL1/2 are transcription repressors, and 5'-AZA is a DNMT inhibitor. Using the TaqMan gene expression assay (Life Technologies, Carlsbad, CA), it was possible to do a real-time qPCR to measure gene expression. In order to run a real-time qPCR, the probes were thawed at room temperature. Using the calculated concentrations, the master mix was prepared by mixing the TaqMan gene expression Master Mix and RNase-free water in a microfuge tube. Calculated amounts of cDNA were pipetted into new microfuge tubes. To these tubes, calculated amounts of the master mix were added. Using a 96-well microtiter reaction plate, 8ul of the cDNA/master mix were added to the appropriate wells. A repeater pipette was used to add 2ul of the specific probes of interest to each of the wells. The plate was sealed thoroughly and loaded into an Applied Biosystems 7500 Fast Real-time PCR System.

Data Analysis

The comparative C\textsubscript{T} method was used to analyze the data and quantify gene expression. Student’s t-test was used for statistical group analysis. P-values below 0.05 were considered as statistically significant.

Discussion/Literature Review
Initially, there were a few possible epigenetic mechanisms that were considered for exploration. They included SNAIL/SLUG proteins, HDACs, RXRs, and DNA methyltransferase (DMNT). However, due to time constraints and evidence from literature, only two of the mechanisms were thoroughly studied. The following review of literature will present past research studies that analyzed various means of VDR down regulation in tumor cells. Rationales for studying HDACs and RXR will be discussed.

**SNAIL/SLUG protein effects on VDR regulation**

Larriba, Bonilla & Munoz (2010) investigated a less known mechanism of VDR down-regulation, the up-regulation of transcription factors SNAIL1/SLUG. These transcription factors bind to E-box sequence and recruit HDACs, which repress the expression of the gene. Moreover, the overexpression of SNAIL1 and SLUG induces the epithelial-to-mesenchymal transition (EMT), meaning that the tissue loses its epithelial characteristics and acquires mesenchymal fibroblastic phenotype. As a result, the cells develop migratory properties favoring tumor invasion and metastasis. Using phase-contrast imaging and confocal laser immunofluorescence imaging they suggested that the tumor cells that did not express SNAIL1/SLUG were normal epithelial cells, and those that did express SNAIL1/2 had mesenchymal cell characteristics.

Normal and tumor biopsies from approximately one hundred colon cancer patients were used to test for SNAIL1, SLUG and VDR RNA expression. SNAIL1 and SNAIL2 were present in low or no quantities in normal colonic tissue, but were expressed 60-70% in colon tumor tissue. In addition, tumors that express SNAIL1 and SNAIL2 showed a strong VDR down-regulation.
Palmer et al., (2004) also looked at the significance of SNAIL proteins on human colon cancer cells. Their study examined the relationship between SNAIL and calcitriol, a form of vitamin D, suppression.

One of the beneficial effects of calcitriol is the expression of E-cadherin, a protein important in cellular adhesion within a tissue. It gives the cell membrane strength and support. If E-cadherin expression is suppressed then the cell loses some of its cellular adhesion and becomes prone to migration. Palmer et al. (2004) found that the expression of E-cadherin was reduced by SNAIL. Using chromatin-immunoprecipitation (ChIP) assays, they also showed that SNAIL is recruited on the native VDR promoter in vivo. This further adds to our understanding of SNAIL-induced suppression of VDR.

Our studies on the SNAIL/SLUG gene expression showed that they were up-regulated in tumors as compared to normal tissue by three folds (Figure 11). However, the SNAIL/SLUG expression was expressed mostly in tumor stroma and not the cells themselves (figure 12). As a result, SNAIL/SLUG was not studied in our study. It is important to note that SNAIL/SLUG recruit HDACs in order to suppress gene expression. As previously mentioned, HDACs are shown to be highly expressed in tumors; thus, they were studied.
A study done by Seuter, Heikkinen, & Carlberg (2012) looked at the Histone Deacetylase (HDAC) and its effects on VDR suppression. HDACs are a class of enzymes that remove acetyl groups from a lysine amino acid on a histone, allowing the histones to wrap the DNA more tightly thus reducing the expression of certain genes. Seuter et al. proposed a mechanism in which HDACs are up-regulated in a cell, which led to VDR down-regulation. This study further added validity to the claim that HDACs may be a regulator of VDR expression in colon cancer cells.

Figure 11 shows SNAIL/SLUG upregulated in tumors. The up regulation of SNAIL/SLUG was observed mostly in the tumor stroma and not the tumor itself.

Figure 12 shows an immunostaining of SNAIL/SLUG expression in colon tumor tissue. Areas of brown staining represent the presence of SNAIL/SLUG. Areas of blue are counterstaining for the absence of SNAIL/SLUG. The SNAIL/SLUG proteins are located in the tumor stroma, not the tumors themselves. (Provided by Dr. Nakanishi Masako)
RXR effects on VDR regulation

Knackstedt, Moseley, Sun & Warqovich (2013) studied suppression of Retinoid X receptor a (RXRa), heterodimerization with VDR and its relation to the SNAIL protein in human ulcerative colitis and colitis-associated cancer (CAC). Normally, when VDR binds to the promoter sequence it is heterodimerized with an RXRa, which allows for the VDR to bind to the correct sequence on the VDR gene. It was suggested in tumor cells that the VDR and the RXRa may be compromised. This means that the expression of both VDR and the RXRa is suppressed.

In order to explore the mechanism behind this study, Knackstedt et al. (2013) proposed a link between the VDR-RXRa suppression and the SNAIL protein’s up-regulation. Using a real-time PCR analysis they were able to show a significant up-regulation of TNFa, an upstream regulator of SNAIL, and SNAIL RNA. Moreover, with immunohistochemistry techniques they were able to show that the SNAIL proteins were evident in ulcerated areas in the colorectal cancer (CRC) models, thus, in the same locations where RXRa and VDR were down-regulated.

The study by Knackstedt et al. (2013) suggests that SNAIL may be a factor for RXR down-regulation. However, our results (figure 9) show that SNAIL is actually down-regulated in tumors. This may mean that SNAIL is not a direct reason for RXR down regulation. However, RXR down regulation in tumors was noted for further exploration.

DMNT effects on VDR regulation

Marik, Fackler, Gabrielson et al. (2010) tested to see if hypermethylation of the CpG regions, multiple repeated sequences of cytosine and guanine nucleotides linked by phosphodiester bonds, near the promoter of the VDR gene would silence the expression of VDR. First, calcitriol binds to the VDR that goes into the nucleus and binds to the Vitamin-D
response element (VDRE), a sequence on the VDR gene. In normal cells, the transcription of the VDR gene is uninhibited and promotes the transcription of VDR mRNA which ultimately leads to the production of VDR protein that allows for more calcitriol to bind.

Marik et al. (2010) aimed at studying VDR expression in breast cancer cells. Using immunohistochemistry they found that the expression of VDR was higher in differentiated cells than in proliferating cells. While many previous studies focused on the alteration of histone acetylation in the VDR promoter, Marik et al. (2010) pointed towards altered DNA methylation patterns as a proposed mechanism for VDR suppression in tumor cells. They investigated to see if methylation-induced silencing of VDR in breast cancer might account for the calcitriol insensitivity. Methylation of a nucleotide inhibits the transcription of that nucleotide; as a result, whatever a certain nucleotide sequence transcribes will be suppressed if there is methylation on it. In the case of VDR, methylation will result in decreased expression of VDR mRNA.

By means of in-silico techniques, they were able to find three CpG islands in an area spanning from -790 bp upstream to +380 bp downstream relative to the primary transcription start site. They performed a bisulfite sequencing analysis in order to look for methylation in these regions with an interest in the region closest to the promoter. They found that the CpG regions had relatively higher methylation in tumor cells as compared to normal cells (Marik et al. 2010).

Main findings of this study reported that demethylation restores the effects of calcitriol in breast cancer. They treated breast cancer cells and normal breast epithelial cells for 96 hours with calcitriol, with a demethylating agent AZA (5’ deoxy-azacytidine) and with calcitriol and
AZA. They discovered that the effects of calcitriol were amplified when induced with AZA, with a greater antiproliferative effect than with either agent alone. This meant that the overall expression of VDR increased with calcitriol and AZA combined (Marik et al. 2010). The results of this study indicate that methylation of the CpG regions may be the underlying cause for the suppression for VDR in tumor cells. However, the result of this study were limited to breast cancer cells.

HCT and HT29 colon cancer cells were used to test the effect of methylation on VDR expression. In order to see how methylation will effect VDR gene regulation, we looked at DNA methyltransferase (DMNT), a family of enzymes that catalyze the transfer of methyl groups to DNA. Using the drug 5-Azacytidine (5-AZA), an enzyme that inhibits DMNTs, VDR expression was measured via a gene expression analysis. The results showed no significant increase in VDR expression in the HCT cells and a relatively higher increase in the VDR expression in the HT29 cells (figure 11). It was determined that the results were not promising enough to study DMNTs in a mouse model, at least from the initial data.
While the goal early on in this project was to study the effect of HDACs, RXRs, SNAIL/SLUG and DMNT on VDR expression, initial studies determined that HDACs and RXRs were more effective than SNAIL/SLUG and DMNT. Therefore, in the last part of this experiment, HDACs and RXRs were studied more thoroughly.

**Future Directions**

The results from these experiments allow for future studies to focus on finding which specific HDACs lead to the significant increase in VDR expression, which will allow for a more
targeted therapy. Additionally, other intracellular proteins that affect VDR expression need to be studied. Finally, in the long run, the goal is to develop therapies for preventing the down-regulation of VDR in colon tumors. By studying the various mechanisms that control and regulate VDR, it may be possible to design therapies that will improve colon cancer prevention.

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