Induced expression of antimicrobial peptides in epithelia and monocytes

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90 ECTS thesis submitted in partial fulfillment of a
Magister Scientiarum degree in biology

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Abstract

In this project I studied the effects of glucocorticoid steroids (GCs) on antimicrobial peptide expression and the effects 4-phenyl butyric acid (PBA) and butyric acid have on VDR mediated *CAMP* expression.

We found that the GC dexamethasone (Dex) suppressed antimicrobial peptide expression in bronchial epithelial cell line and monocyte cell line as well as primary monocytes. This suppression could be countered by 1,25 dihydroxyvitamin D_3 (1,25D3) induction, although the induction was still tempered by Dex.

In the other part of the project I found that butyrate and PBA induction of *CAMP* and *HIF1A* do not work in the same way, as MAPK (mitogen-activated protein kinase) inhibition had different effects on these inductions. I also showed that an attempted VDR knockout cell line had impaired PBA, butyrate and vitamin D mediated *CAMP* induction. Computer docking simulations were used to look at the possibility of butyrate and PBA being direct ligands to VDR. According to these simulations butyrate and PBA are weak ligands to VDR although it is still unclear if the chemicals binding is sufficient to activate the receptor.

Útdráttur

Í þessu verkefni leit ég á áhrif glucocorticoid stera (GC) á tjáningu örverutrepandi peptíða auk þess sem ég skoðaði örvun PBA og bútyrats á tjáningu *CAMP* gensins og hvernig sú virkni fer í gegnum Vítamín D viðtakann (VDR).

Niðurstöður okkar voru á þá leið að GC minnkar tjáningu á örverudrepandi peptíðum í lungnaþekju og einkjörnunga frumulínum og einnig í einkjörnungum fengnum úr blóði. Við sáum einnig að hægt var að vinna á móti þessari minnkuðu tjáningu með D vítamíni sem örvaði tjáningu *CAMP* gensins sem tjáir fyrir LL-37 peptíðinu. Þessi örvun vann á móti minnkuðu tjáningunni en tjáningin var þó minni heldur en ef eingöngu D vítamín var notað en engir sterar.

Í hinum hluta verkefnisins skoðaði ég övun á tjáningu *CAMP* og *HIF1A* með D vítamíni, PBA og bútírati og tengsl þessarar örvunar við MAPK (mítógen virkjaður prótein kínasi). Þar fundum við að PBA og bútírat örvun er ekki eins þar sem breytingar á örvuninni við hindrun MAPK var ekki eins milli efnanna. Ég sýndi einnig að bútírat, PBA og vítamín D miðluð örvun á *CAMP* raskaðist í frumulínu þar sem reynt hafði verið að slá VDR út. Tölvulíking var notuð til þess að kanna hugsanlega bindingu bútírats og PBA í VDR til þess að sjá hvort að það gæti verið möguleiki að þessi efni væru bindlar fyrir viðtakann. Samkvæmt útreikningum forritsins eru efnin veikir bindlar en það er óljóst hvort að bindingin er nægjanlega sterk til að virkja viðtakann.

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Abbreviations

1,25D3	1,25 dihydroxyvitamin D_3
25D3	25 hydroxyvitamin D_3
AMP	Anti microbial peptide
BD	Budesonide
BSA	Bovine serum albumin
CAMP	Cathelicidin AMP
Dex	Dexamethasone
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
FP	Fluticasone Propionate
GC	Glucocorticoid
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
gRNA	Guide RNA
HAT	Histone acetyl transferase
HBD	Human beta defensin
HDAC	Histone deacetylase
HDACi	HDAC inhibition
HIF	Hypoxia-induced factor
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase 1
IL1B	Interleukin 1 beta
IP10	Interferon gamma-induced protein 10
JNK	c-Jun N-terminal kinases
MAPK	Mitogen-activated protein kinases
PBA	4-phenyl butyric acid
Poly I:C	Polyinosinic-polycytidylic acid
PPARγ	Peroxisome proliferator-activated receptor gamma
qRT-PCR	Quantitive real time PCR
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RXR	Retinoic acid receptor
SCFA	Short chain fatty acid
shRNA	Small hairpinn RNA
TSA	Trichostatin A
UBC	Ubiquitin C
VDR	Vitamin D Receptor
VDRE	Vitamin D reponse element

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1 Introduction

1.1 The immune system and its roles

The immune system is a system that the body has to fight of infections. To fulfill its duty there are four main tasks it has to be able to do. Firstly it is immunological recognition; it has to recognize the pathogens to be able to take the necessary action. This task is carried out both by the white blood cells and the innate immunity. Secondly the system has to eliminate the threat of infection. The immune effector functions are carried out by both the white blood cells, proteins in the blood for example antibodies produced by some lymphocytes. While it is important to fight off infections it is also very important to keep the immune response under control so that it does not do harm to the body. The third task of the immune system is immune regulation, the ability to regulate this response. Failure to regulate the system can cause allergies and autoimmune diseases. The fourth and last of the main tasks is the immunological memory, to be able to "memorize" pathogens and respond quickly to reoccurring infections. This task is fulfilled by the adaptive immune system. The immune system can be divided into the innate immune system and the adaptive immune system although these two "systems" are really two units of the same system (Murphy, 2012).

1.2 Innate immunity

The first lines of defense are physical and chemical barriers of epithelia like the skin for example. The innate immune system has developed to guard against infections and see that the microbes are unable to breech our barriers and multiply. The innate immune system consists of many leukocytes that secrete cytokines, ingest the pathogen or kill it directly. The innate immunity is a rapid, broad spectrum and powerful system in preventing infections. If the pathogens are however able to resist these defenses and develop into an infection the adaptive immune system comes into play with a slower but more specific line of attack through the use of lymphocytes specific for the pathogen.

The cytokines that some leukocytes secrete as a part of innate immune response are of various kinds and have different functions. There are for example numerous cytokines that affect the cells around them, thus being able to conduct the battle. Another element of the innate immune system are the antimicrobial peptides. These are peptides that are very toxic to bacteria and other microbes. They are vital in preventing pathogens from breaking through epithelial cell layers, entering the body (Murphy, 2012).

1.3 Antimicrobial peptides

Antimicrobial peptides (AMPs) are, as the name suggests, short proteins that have an important role in fighting foreign microbes. They are an integral part of the innate immune system. There are over 2700 different peptides that have been discovered in all life forms, from bacteria to plants to humans. The discovery of these peptides dates back to the 1960s when the peptide bombinin was isolated from the toad *Bombina variegate.* The first indication of these kinds of peptides had come a little earlier in bacteria and fungi. Following these discoveries the research community did not pick up on this results and remained largely unaware of the peptides until research took flight again in 1980 and since then the research on AMPs has been a growing field. In humans these peptides are secreted by diverse cell types throughout the body, both conventional immune system cells and also by some epithelial cells (Cederlund, Gudmundsson, & Agerberth, 2011).

These peptides are generally around 30 amino acid long and are characterized by cationic amphipathic properties in folded 3D structure.

The AMPs show antimicrobial properties against various types of microorganisms, including bacteria, fungi and viruses. The effects of the AMPs are non-specific and interestingly they work well against many drug resistant bacteria strains. Most AMPs directly work on the pathogens membrane, using their amphipathic nature and cationic properties to disrupt the pathogens lipid bilayer. Two of the most extensively researched categories of AMPs in mammals are cathelicidins and defensins that are split down to the subgroups of α-, β- and θ-defensins. In humans α- and β-defensins have been described along with one cathelicidin gene (Cederlund er al., 2011; Lai & Gallo, 2009; Lehrer & Lu, 2012; Leonard et al., 2012).

1.3.1 Cathelicidin

Cathelicidins have been described in mammals and other vertebrates. Their name is based on a common, highly conserved N-terminus region, known as the cathelin domain. The protein, which is characterized by two disulfide bonds, was originally thought to have the ability to inhibit the protease cathepsin-L wherefrom the name is derived. In humans only one cathelicidin gene is known (*CAMP*) but in other species many different cathelicidins have been described, often more than one in the same species. The human *CAMP* gene codes for a precursor protein of approximately 18 kDa, called hCAP18. The cathelicidins are considered a gene family because of the well-conserved domain mentioned above but the C-terminus of the proteins encoded by the genes varies greatly. However the cathelicidin peptides show little similarity between them and are only considered a group because of the comparable structure of the precursor protein, which is dominated by the cathelin domain. In humans the precursor protein is processed to release a 37 amino acid long peptide from the C terminus of the protein. The released product is the antimicrobial peptide known as LL-37. The peptide starts with two leucine amino acids, explaining the name. The hCAP18 precursor product can be stored in cells in high concentrations in the

pro-form. The processing of the protein and cleaving of the LL-37 from its precursor is important for the activation of its antimicrobial function.

LL-37 is produced both in granulocytes but also in epithelial cells all over the body, for example in the lungs, gut and skin. In neutrophils the hCAP18 pro-protein is cleaved by the serine protease, protease-3, to release the LL-37 peptide. There are examples of the pro-protein being cleaved by different serine proteases, especially in the skin, to form differently cleaved peptides. In seminal fluid a longer variant, ALL-38, has been found. These different cleave forms can have different antimicrobial properties. LL-37 has been shown to have both a direct antimicrobial function on many different pathogens while also showing other effects such as anti- and pro-inflammatory effect, angiogenesis properties and plays a role in wound healing. Overall the cathelicidin antimicrobial peptides are a highly diverse group of molecules with some similarities in their precursor (Cederlund et al., 2011; Lai & Gallo, 2009; Sørensen et al., 2001).

The expression of *CAMP* is down-regulated by certain infectious agents such as *Sigella* while the expression has been shown to be increased in other diseases like psoriasis. Known inducers of *CAMP* are amongst others vitamin D, phenyl butyric acid (PBA) and butyrate. Butyrate and PBA have been shown to counter the down-regulation by *Shigella* in rabbits. The *CAMP* gene is a direct target of the Vitamin D receptor (VDR), a VDRE element being located in the genes promoter region (Campbell et al., 2012; A. Gombart et al., 2005; A. Gombart et al., 2009; Steinmann et al., 2009; Termén et al., 2008; T. T. Wang et al., 2004).

1.4 Glucocorticosteroids

Glucocorticoids (GCs) are widely used drugs. GCs are the most effective drug in treatment of many chronic immune diseases and inflammatory disorders, including asthma, cystic fibrosis and chronic obstructive pulmonary disease. GCs act in a ligand-receptor dependent manner binding to the glucocorticoid-receptor (GR). After binding the receptor forms a homodimer and the homodimer-ligand complex then binds to the glucocorticoid response element (GRE) in the promoter regions of the GR target genes, leading to transcriptional effects. GCs influence inflammation on a broad level both by down-regulating genes encoding for pro-inflammatory cytokines and by up-regulating anti-inflammatory cytokines. Side effects that have been associated with GC treatment include secondary infections such as oral candidiasis and after long term treatment reported side effects include diabetes, osteoporosis, cataracts and pneumonia (Barnes, 2011).

In general GCs have been shown to spare or even enhance innate immune response while taking down inflammation (Schleimer, 2004; Zhang et al., 2007). Resent research on mice however has showed that GCs can have a negative effect on AMP expression. In the study allergic asthma mouse model was treated with the GC, budesonide and infected with *Pseudomonas aeruginosa*. The budesonide inhalation took down the expression of *mCRAMP* which is the cathelicidin gene found in mice, and increased the number of internalized bacteria, both *in vivo* and *in vitro* (P. Wang et al., 2013).

Some asthma patients do not show improvement following glucocorticoid treatment. These patients' $CD4^+$ T cells fail to induce IL-10 secretion upon GC treatment. It has been shown that adding a combination of vitamin D and dexamethasone (Dex) to $CD4^+$ T cells from steroid resistant patients improves IL-10 secretion to a level comparable to what is expected with Dex treatment and this suggest that co-treatment of vitamin D and GCs might be beneficial (Xystrakis et al., 2006).

1.5 Vitamin D

Vitamin D_3 can both be obtained through diet and it can also be synthesized in the skin by exposure of UV radiation.

After consumption or synthesis Vitamin D is hydroxylated by the enzyme CYP27A1 in the liver to form 25-hydroxyvitamin D (25D3) and circulates in the blood in that form. To fully activate the vitamin it is then further hydroxylated by another enzyme called CYP27B1 to form 1,25-dihydroxyvitamin D (1,25D3). This activation is primarily undergone in the primary renal tubules of the kidneys (Christakos et al., 2010; A. Gombart, 2009). However, the process is also carried out in numerous different locations of the body by cells that express CYP27B1, such as in bone, skin epithelia, lung, colon and by immune cells, especially activated macrophages (A. Gombart, 2009; Hansdottir et al., 2008; Sigmundsdottir et al., 2007).

The renal production of 1,25D3 occurs in response to decreased levels of Ca^{2+} in circulation. 1,25D3 increases the Ca^{2+} uptake by the intestine, resulting in increased level of Ca^{2+} in the circulation and lesser renal synthesis of 1,25D3. Furthermore 1,25D3 suppresses its own production through a negative feedback loop, by repressing the production of CYP27B1 and inducing the production of CYP24, which is an enzyme that catabolizes both 1,25D3 and 25D3. Thus the renal production of 1,25D3 is an important link in securing Ca^{2+} homeostasis in the blood.

The 1,25D3 production in macrophages is controlled by different means. Activation of macrophages leads to the induction of CYP27B1 and thus induces the production of 1,25D3. There is no negative feedback in macrophages to limit the production of 1,25D3. The cells do produce a different splice variant of CYP24 that is catalytically inactive and does not catabolize 1,25D3. Thus there is no limit on the macrophage production of 1,25D3 while they are activated.

1,25D3 has an overall tempering effect on adaptive immunity. 1,25D3 directly affects T-cell proliferation and production of cytokines. It decreases Th1 development and inhibits Th17 development while increasing the frequency of regulatory T-cell and Th2 production. Another key factor in the 1,25D3 driven modulation on adaptive immunity is the impact on myeloid dendritic cell phenotype and function. The dendritic cells (DC) are affected in such a way that their expression of CD40, CD80, CD86 as well as IL-12 is down-regulated while IL-10 expression is up-regulated. The phenotypic result of this overall expression

pattern is creating tolerogenic myeloid DC which leads to enhanced activity of $CD4^+$ suppressor T-cells and higher recruitment of T regulatory cells and a decrease in Th1 cell development. The overall effect of these modifications on adaptive immunity is suppressive so vitamin D is a potential factor in autoimmune disease treatment (A. Gombart, 2009). In line with this recent reports show that vitamin D decreases inflammation in virus infected lung epithelial cells via IκBα induction and suppression of its degradation, thereby inhibiting NF-κB activation (Hansdottir et al., 2010).

In contrast to the suppressive effects of vitamin D on the adaptive immune functions it has been used to treat infections, primarily tuberculosis prior to the development of modern antibiotics. This can be explained by the boost 1,25D3 gives to the innate immunity. This enhancement is mediated in part through up-regulation of antimicrobial peptide production. Vitamin D is important for regulation of both *CAMP* and *DEFB4* (A. Gombart, 2009; Liu et al., 2009).

1.6 Vitamin D receptor

The genomic effects of 1,25D3 are mediated through the Vitamin D receptor (VDR). VDR is a transcription factor of the steroid/hormone receptor family (A. Gombart, 2009).

VDR is a monomer in solution and forms a homodimer (VDR-VDR) when introduced to a specific DNA element. When stimulated by 1,25D3, its ligand, VDR forms a heterodimer with retinoid X receptor (RXR). 1,25D3 does this by significantly slowing down the conversion of monomeric VDR to a homodimer and increasing the rate of the disruption of the homodimeric structure. This together decreases the amount of DNA-bound homodimers of VDR and stabilizes DNA-bound monomers, which favors the formation of VDR-RXR heterodimer. 9-cis retinoic acid, the ligand of RXR has the opposite effect to 1,25D3 and hinders the heterodimerization (Cheskis & Freedman, 1994).

The DNA binding of VDR is carried on by two Zinc finger-like modules that have a strong affinity to so-called half-site, a DNA target site of the receptor that are organized as imperfect repeats. The favorable protein-protein contact that is necessary for the heterodimer to form is made through certain amino acid structure that is aligned by DNA-binding (Cheskis & Freedman, 1994).

1.7 Hypoxia Induced Factor 1

Hypoxia Induced Factors (HIF) are transcription factors that are as the name suggests classically linked with hypoxia. For transcription to occur the α and β subunits must form a complex with som co-factors like p300/CBP. HIF-1α is expressed widely in the body and

is expressed in essentially all innate and adaptive immune cell types. Recently HIF transcription factors have been shown to be major elements in immune cell functions. HIFs are regulated by oxygen availability through posttranslational degradation; when oxygen is abundant prolylhydroxylases mark the HIF-1α subunit for proteasome degradation. There are also different means of regulation for example hydroxylation of arginine in HIF-1α blocking protein interaction with co-activators like p300 (Palazon et al., 2014).

HIF-1α stability is however not only reached by hypoxic environment; there are many known ways of HIF activation linked to immune functions, both adaptive and innate. Bacteria dependent induction of HIF-1α has been documented in macrophages in normal oxygen conditions and what is more, some bacteria secrete iron binding agents that limit the iron supplies that are needed for the enzymes marking HIF-1α for degradation, thus stabilizing the subunit independent of oxygen availability. NF-κB is necessary for bacterial induction of HIF-1α and this induction leads to accumulation of HIF-1α in macrophages independent of stabilization(Palazon et al., 2014).

A recent report showed an interesting link between antimicrobial peptide activity and HIF-1α in infection prevention. The study showed that activation of HIF-1α with subsequent LL-37 production by commensal anaerobic bacteria is a key element in suppressing *Candida albicans* colonization and thereby preventing invasive disease (Fan et al., 2015).

1.8 HDAC inhibition

Gene expression can be influenced through different means, both by inducing or inhibiting transcription factors directly and also by manipulating chromatin by altering its epigenetic patterns for example by changing methylation or acetylation status of histones. Histones are proteins that are a unit of the nucleosome, the fundamental unit of chromatin. One nucleosome consists of approximately two turns of DNA wound around one histone octamer which consists of two of each of the histone variants; H2A, H2B, H3 and H4. Various different modifiers such as phosphorylation, methylation and acetylation occur, mainly on the histone amino-terminal tails but also on its globular domain, naturally modify the histones. The globular domain of histones comes in close contact with DNA and the effects of these modifications on that domain on gene expression depends on the nature of the affecter, its location and close environment (Fischer et al., 2016; Steliou, Boosalis, Perrine, Sangerman, & Faller, 2012).

One histone modification is acetylation. The acetylation status of histones plays a crucial part in transcription regulation. The status is determined by two opposing actions, acetylation, which is carried out by histone acetyl-transferases (HAT), and deacetylation by histone deacetylases (HDAC). Generally there is a positive correlation between histone acetylation level and transcription activity. The acetyl groups added by HAT decreases the positive charge on the histone surface which interacts with the negatively charged DNA and thereby opening up the chromatin and opening up access for transcription factors and

other proteins to start transcription. Conversely HDAC lowers the acetylation level of the histones and in turn winds the chromatin tighter around the histones and limits transcription (Fischer et al., 2016; Steliou et al., 2012).

There are a number of known HDAC inhibitors such as trichostatin A (TSA), butyric acid (butyrate), phenyl butyric acid (PBA) and many other chemicals. These inhibitors do not all work in the same way and inhibit different kinds of HDAC in diverse patterns, that is one HDAC inhibitor does not necessarily inhibit the same HDAC enzymes as another. There are 18 known HDACs in human

Although HDAC's and HAT's names don't indicate it, those enzymes have not only been linked to acetylation and deacetylation of histones but also a number of other proteins as well. Many of these proteins are transcription factors that are direct participants in transcription so HATs and HDACs influence gene expression in more than one way (Fischer et al., 2016; Steliou et al., 2012).

Many short chain fatty acids (SCFAs) have HDAC inhibitory properties. The optimal size of the acids for HDAC inhibition seems to be three- to five-carbon long chain and branches from the chain reduce the HDACi potential (Steliou et al., 2012).

1.8.1 Butyric acid

Butyrate can be gained directly through diet but is also formed by gut bacteria as a side product of the fermenting of dietary fibers the bacteria use as nutrition (Ulven 2012). This production of butyrate is an essential role of the microbiome in the gut. Butyrate is a SCFA. It is composed of a four-carbon atom long chain with a carboxyl group.

There are several known functions of this small molecule. Being a fatty acid butyrate is useful as a source of energy and is the dominant energy source of the colon epithelia. Butyrate has also been linked to wound healing and is known to reduce inflammation in the small intestine. Butyrate also affects cell proliferation and differentiation of colonocytes. Furthermore butyrate is a known ligand to G-protein coupled receptors (GPR) 40, 41, 43, 84 and 120 and has been shown to affect other receptors such as VDR and peroxisome proliferator-activated receptor γ (PPARγ) (Alex et al., 2013; Campbell et al., 2012; Ulven, 2012).

The best-known function of butyrate however is its HDAC inhibition effect. The effects of butyrate mediated HDAC inhibition on gene expression are for example an anti-inflammatory effect and anti-proliferation effect. Butyrate also induces apoptosis of T-cells, tempering the immune response in the gut. These effects are essential for the gut epithelia and butyrate is an important factor in the interaction between the microbiome of the gut and the gut itself (Zimmerman et al., 2012).

Another effect butyrate inflicts through the inhibition of HDAC is the promotion of the generation of T_{reg} cells both in vitro and in vivo in mice. Butyrate decreases the expression of pro-inflammatory cytokines of dendritic cells through acetylation of histone H3, and in

turn induces the expression of Foxp3 in $CD4^+$ T cells. These effects are inflicted through HDAC inhibition properties of butyrate (Arpaia et al., 2013; Furusawa et al., 2013).

Butyrate is known to up-regulate antimicrobial peptide expression. There are some reports of butyrate having effects on defensins expression in humans. *HBD-2* mRNA expression in colonocytes and gingival epithelial cells showed significant increase in induction of *HBD-2* in response to bacterial challenge if pretreated with sodium butyrate. Butyrate has also been shown to be a inducer of *CAMP* (Campbell et al., 2012; Raqib et al., 2006; Sarker et al., 2011). The up-regulation of *CAMP* has also been shown to work in synergy with 1,25D3 in HT-29 colon cancer cell line by Termén et al., 2008, although they did not seem to focus on this part of their results as they do not mention the synergistic effect (Termén et al., 2008).

Because of the effects mentioned above and more, the therapeutical potential of butyrate has been of interest for some time. The main problem with butyrate as a drug candidate is its poor pharmacological qualities. Butyrate has a relatively short half-life, has to be administered in high quantity to reach therapeutic concentration and tastes and smells very badly. Because of this other related chemicals have been examined, one of which is the SCFA 4-phenyl butyric acid (PBA), which is without the foul smell and odor of butyrate but still suffers the same problem of a short half-life (Steliou et al., 2012).

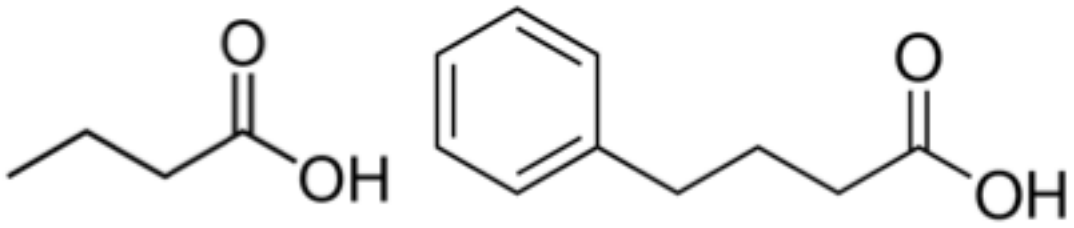

Figure 1 - Butyrate and 4-phenyl butyrate The difference between the two SCFA is the phenyl group on PBA. Both acids inhibit Histone deacetylation. Butyrate is a dietary combound and is naturally made by the normal bacterial flora in the gut as a side product of fiber digestion. PBA is a FDA approved drug for treating urea cycle disorders.

1.8.2 Phenyl Butyric Acid

4-phenyl butyric acid (PBA) is a FDA approved drug, used for treating urea cycle disorders and is also under investigation in both clinical and pre-clinical models for a treatment option for cancer, Alzheimer disease, Huntington's disease, cystic fibrosis, spinal muscular dystrophy and more motor neuron disorders (Iannitti & Palmieri, 2011).

PBA is a known HDAC inhibitor and shares a similar structure to butyric acid. PBA is also a known inducer of AMPs such as human beta defensin 2 (HBD2) and cathelicidin AMP (*CAMP*) (Steinmann et al., 2009; Steliou et al., 2012). Furthermore PBA's induction of *CAMP* gene expression works in synergy with 1,25D3 and recent reports have shown that PBA induction of *CAMP* is VDR dependent (Fig 2) (Kulkarni et al., 2014). These discoveries are the bases for the second part of my project.

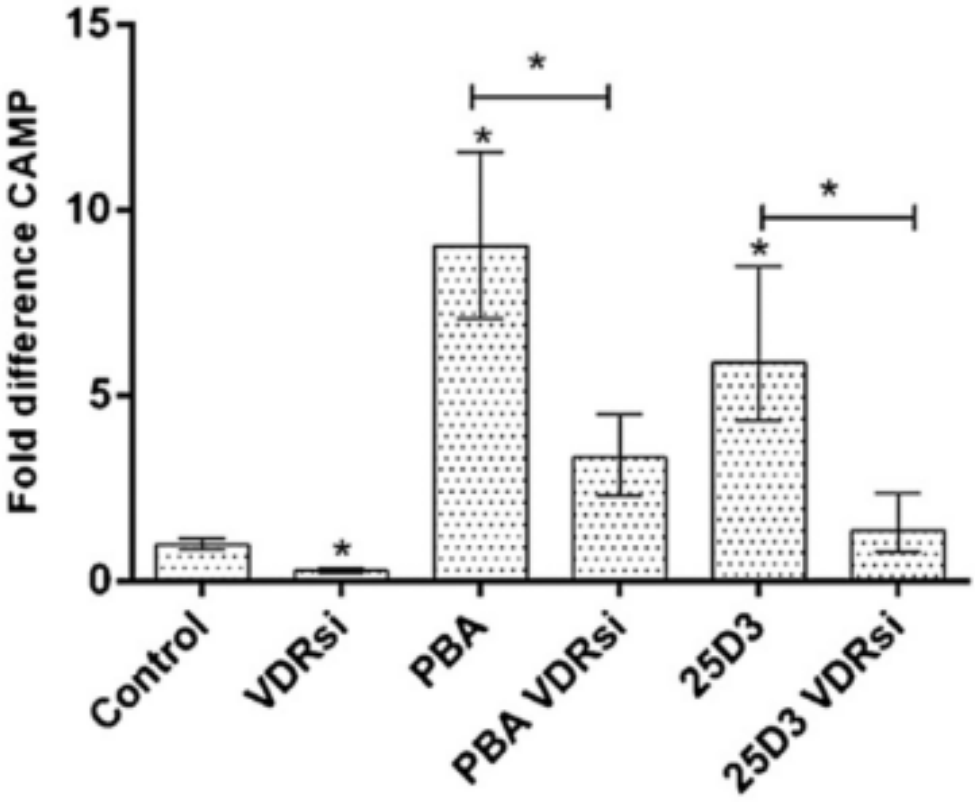

Figure 2 - siRNA knockdown of VDR effects on PBA induction of *CAMP*. VDR knockdown significantly takes down PBA mediated *CAMP* induction (*=P<0.05) (Kulkarni et al., 2014).

2 Aims of study

This study can be divided into two parts that have a common thread to them as the both revolve around the human cathelicidin expression.

In the first part I was looking into the effects of glucocorticoids on expression of antimicrobial peptides. I came into this project at a late stage and we published an article on our results in September 2015.

The primary aim of project one was to see if the glucocorticoid dexamethasone affected innate immune response, in particular if it had effects on cathelicidin expression. This was motivated by the fact that secondary infections are a known side effect of glucocorticoid expression and we sought to see if innate immune repression could be a factor in the treatment.

The second part of the study was to inspect the involvement of the vitamin D receptor in butyrate mediated *CAMP* induction. I sought to find out if VDR was a part in the induction pathway, as is the case with 4-phenyl butyric acid mediated induction. Furthermore I aimed to define how butyrate and PBA affected VDR and if RXR was a necessary part of VDR activation by those compounds as it is for activation by vitamin D.

3 Materials & methods

3.1 Cell culturing

3.1.1 Bronchial epithelial cell lines

The VA10 and BCi N.S. 1-1 bronchial epithelial cell lines were cultured in passages 14-25 on T75 plastic flasks from Falcon at 37°C in 5% CO». The medium changed every 2-3 days and the medium used was B/TEGM (Bronchial/tracheal epithelium cell growth medium from Cell Applications, 511A-500) with 5000 U/µg of PenStrep from Gibco, Life Technologies. The cells were split 4:1 when they reached about 80% confluency using Trypsin-EDTA and 10% FBS.

When seeded on plates for experiments 150.000 cells were seeded in each well after a count in a hemocytometer.

3.1.2 Colonic epithelial cell line

Human colorectal cancer cell line, HT-29 passages 58-68, was cultured in a humidified incubator at 37°C in 5% CO_2. The cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and PenStrep from Gibco, Life Technologies. The cells were split 1:10 – 1:20 when they reached 80-90% confluency using Trypsin-EDTA and 10% FBS. The presumed VDR knockout cell line was treated the same as the untreated one.

When seeded on a plate for experiments 500.000 cells were seeded per well, after a count in a hemocytometer.

3.2 Primary monocyte isolation

Buffy coat from a healthy donor was contributed from The Icelandic Blood Bank (Bb). The buffy coat diluted 1:2, then put in a 50mL tube with Ficoll layered at the bottom and finally spun down (400g) with no brake to prevent the layers from blending. After centrifuging, the mixture in the tubes was in four layers; at the bottom there was a red layer containing granulocytes, the second layer was the ficoll, on top of that was a thin layer of mononuclear cells and finally on top there was plasma.

The mononuclear layer was removed by careful pipetting and put in fresh tube. The cells were then mixed with magnetic beads covered in anti-CD14 for selection. The mixture was run through a column in a magnetic field and then washed before being taken out of

magnetic field and thereby being released to new tubes. The cells were then checked in a flow cytometer at Bb showing it to be almost exclusively monocytes.

3.3 Isolation of sample material

3.3.1 RNA isolation

After experiments cells were collected, lysed and RNA isolated by NucleoSpin RNA kit (Machery-Nagel, Germany). 350 µL RA1 buffer with 3.5 µL β-mercaptoethanol was added to each well and cells scraped of and collected. The kit was used as instructed and the concentration of the RNA in each sample was measured with Nanodrop.

3.3.2 Protein isolation

After experiments the cells were washed with cold PBS, kept on ice, and all liquid removed. 150 µL RIPA lysis buffer supplemented with 1x protease inhibitor (Complete Mini, Roche Germany) was placed on the cells. Cells were then scraped of the plates and put in a cold 1,5 mL tube and kept on ice for 30 minutes (min), vortexing them every 10 min. After the wait the samples were centrifuged at 10.000 rpm for 10 min and the supernatant, containing protein, separated from the palate and put in fresh ice cold tubes.

The protein concentration was then measured with BioRad protein assay dye (Bio-Rad laboratories USA) and a standard curve gradual BSA concentrations to determine protein concentration of samples measured in a spectrometer at 595 nm wavelength.

3.4 cDNA synthesis

For cDNA synthesis High Capacity cDNA Reverse Transcription Kit from Applied Biosystems, USA, was used. Equal amount of RNA was measured for each sample and kit instructions were followed to generate cDNA.

3.5 qRT-PCR

Power SYBR® Green PCR Master Mix (Applied Biosystems, Life Technologies, UK) was used to perform the qRT-PCR analysis. 1 µL of cDNA in each well and 9 µL of master mix containing forward and reverse primers at 300 nM concentration. The plates used for analysis were FrameStar 96 Fast Plates (4titute, UK). The two reference genes used for all analysis were ubiquitin C (UBC) and the hypoxanthine-guanine phosphoribosyl-transferase gene (HPRT1). For statistical analysis unpaired T-test was used.

Table 1 - Primers from Integrated DNA Technologies used in qRT-PCR.

Primer	Forward (5'-3')	Reverse (5'-3')
CAMP	GCACACTGTCTCCTTCACTG	CTAACCTCTACCGCCTCCT
HIF1A	CAACCCAGACATATCCACCTC	CTCTGATCATCTGACCAAAACTCA
HPRT1	GCGATGTCAATAGGACTCCAG	TTGTTGTAGGATATGCCCTTGA
IL1B	GAACAAGTCATCCTCATTGCC	CAGCCAATCTTCATTGCTCAAG
IP10	CAGTTCTAGAGAGAGGTACTCCT	GACATATTCTGAGCCTACAGCA

3.6 Western blot

Protein samples were made by measuring equal amount of protein according to measurement of concentration done earlier. To these sample buffer from NuPAGE (Novex Life Technologies, USA) and beta-mercaptoethanol as a reducing agent. The samples were then heated for 10 min at 70°C in a heat block and then cooled to room temperature. Equal amounts of samples were then loaded on a 4-12% Bis-Tris gel (Novex Life Technologies, USA) and the gel then electrophoresed in a MES running buffer. Next the proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane using a wet transfer in a transfer buffer from Novex Life Technologies, USA.

The PVDF membrane was then blocked in 3-5% bovine serum albumin (BSA) in 0.05% Tween PBS for 1 hour (h) at room temperature and primary antibodies in a 0.5% BSA, 0.05% Tween PBS were then put on and incubated over night at 4°C. The appropriate secondary antibody, tagged with HRP (horseradish peroxidase), was then put on the membrane in 0.5% BSA, 0.05% tween PBS and incubated at room temperature for 1 h. The detection reagent Pierce ECL Plus Western blotting substrate was used and added to the membrane for 5 min (Thermo Scientific, USA) and blots analyzed with Image Quant machine (GE Healthcare)

Table 2 – Antibodies from Santa Cruz Biotechnology used in protein analysis.

	Target	Species	Type	Isotype	Catalog number
Primary	VDR	Mouse	Monoclonal	IgG	sc-13133
Primary	VDR	Rabbit	Polyclonal	IgG	sc-1008
Primary	GAPDH	Mouse	Monoclonal	IgG	sc-365062
Secondary	Mouse IgG	Goat		HRP-conjugated	sc-2005
Secondary	Rabbit IgG	Goat		HRP-conjugated	sc-2004

3.7 CRISPR/Cas9 knock-out

gRNA oligo pairs for VDR were designed and bought. The oligos were then annealed and the annealed oligos were phosphorylated to have 5´phosphate. The annealed oligos were then ligated to a ready made backbone (MLM3636 cut BsmBI) and competent *E. coli* bacteria was transformed with the ligated vectors and after 45 minutes the bacteria was spread on a LB-AMP agar plate for selection and incubated overnight.

Colonies were then swiped and a colony PCR was performed to confirm that the vector was integrated. The PCR product was run on a 1% agarose gel with EtBr. Colonies that yielded good bands on the gel were selected and cultured overnight before being frozen down in 80% glycerol at -80°C. A MiniPrep was preformed with a kit from Thermo Fischer Scientific, USA, plasmid was isolated and concentration measured with Nanodrop. A sample was sent out for sequencing to Beckman Coulter Genomics.

The sequencing results were used to pick the suitable plasmids containing the correct shRNA. These were used for transfecting the BCi cells.

For the transfection 15.000 cells were seeded 2 wells each sample on a 24 well plate and then grown to 70% confluency. The plasmid and Cas9 were added in two different ratios 1:1 and 1:15 in 75 µL of antibiotic free medium and then FuGENE (Promega, USA) transfection reagent is added carefully and the mix is incubated for 15 min at room temperature. This is then added to 425 µL of antibiotic free medium and then added to the cells and incubated in 37°C 5% CO_2 overnight.

After overnight incubation the cells were trypsinated and spread on a 15 mL dish and Blasticidin selection undergone for 3 days and living colonies then picked and separated to generate single cell clones.

Table 3 - gRNA constructs used for CRISPR/Cas9 knockout of VDR.

gRNA name	Forward (5'-3')	Reverse (5'-3')
VDR-gRNA20	ACACCGCACAGATCCGGGGCACGTTCG	AAAACGAACGTGCCCCGGATCTGTGCG
VDR-gRNAgs	ACACCGAACGTGCCCCGGATCTGTGG	AAAACCACAGATCCGGGGCACGTTCG

3.8 shRNA knock-down

VDR shRNA viral particles were ordered from Santa Cruz Biotechnology, USA along with control particles. Cells were cultured to 50% confluency on a 12-well plate. Medium containing 5 µg/ml polybrene (Santa Cruz Biotechnologies, USA) was put on cells and the viral particles were thawed and mixed gently and then added to wells to infect the cells.

The next day the medium was changed to normal medium without polybrene and the day after that the cells were split 1:5 and then incubated for 48 hours in normal medium.

To select out staple clones expressiong the shRNA a puromycin selection was used. Puromycin dihydrochloride (Santa Cruz Biotechnologies, USA) was added to wells and kept on until all the cells that were not infected had died. The living cells were then cultured and split twice before moving them from the transfection room to the normal incubator.

3.9 Immune Precipitation of VDR

The cells were rinsed twice in ice cold PBS and then lysed in a non-denturing lysis buffer. The lysate was then spun down at 16.000g for 15 min. Afterwards the supernatant was put in a fresh ice cold tube. The isolated proteins were then pre-cleared by combining them with sepharose protein A/G beads and tumbling end over end for 30 min before centrifuging for 5 min at 16000g to remove the beads and in turn the proteins that might have bound to them.

For immune precipitating VDR two different antibodies from Santa Cruz Biotechnology, USA. A rabbit polyclonal antibody (sc-1008) was conjugated to sepharose protein A/G beads. The beads and antibody were mixed thoroughly and tumbled end over end at 4°C for an hour. Beads were then washed 3 times with ice cold PBS before re-suspending them in a non-denaturing lysis buffer, containing triton X-100; tris-Cl, 7.4pH; NaCl and EDTA with iodoamine, leupeptin and PMSF added right before use. Another set of beads were prepared using GAPDH antibody (sc-25778) as a negative control for VDR precipitation.

One μL of 10% BSA was then added to the antibody conjugated beads that were suspended in 0.5 mL of ice cold lysis buffer and the proteins were then added to the mix. These tubes were then tumbled end over end overnight and the centrifuged for 5 seconds at 16000g and the supernatant was removed and kept. The beads were then washed 4 times in a washing buffer, containing the same as the lyisi buffer (listed above) without adding the iodoamine, leupeptin and PMSF. The proteins were then eluted from the beads with an elution buffer containing SDS; Tris-Cl 7.4pH and DTT. The buffer was added and the samples then incubated for 5 min at room temperature and the 5 minutes at 95°C.

Then a western blot was run and the mouse monoclonal VDR antibody from Santa Cruz biotechnology was used for detection (sc-13133).

The immune precipitation yielded no results presented in this thesis. It could however prove a useful tool to analyze VDR mechanism further.

4 Results

4.1 Glucocorticoid steroids and innate immunity

In the first part of my project I contributed to the final stages of a project on GC effect on innate immunity. Our hypothesis was that GCs negatively impact the innate immune system resulting in easier access for potential pathogens leading to infections and that vitamin D might counter this negative effect.

4.1.1 Dexamethasone's effect on innate immune response in THP-1 cells

Firstly, we examined the effects of glucocorticoid dexamethasone (Dex) on THP-1 monocytes, combined with Poly I:C to mimic viral infection and with 1,25D3, a known inducer of *CAMP* and other innate immune genes. We found that Dex down-regulated basal *CAMP* expression significantly. 1,25D3 up-regulated *CAMP* expression, as expected, and reversed the Dex down-regulation although the Dex significantly took down the 1,25D3 induced effect. The Poly I:C also took down the 1,25D3 effect significantly and a combination of Poly I:C and Dex reduced the vitamin D effect even further (Fig 3 a).

We also looked at the effect on the expression of pro-inflammatory cytokines IP-10 and IL1B. We found that Poly I:C induced *IP-10* expression and worked in synergy with 1,25D3 to further up-regulate IP-10 expression. Dexamethasone significantly took this induction down, both the induction of Poly I:C alone and the synergistic induction by Poly I:C and vitamin D (Fig 3 b).

Dexamethasone down-regulated basal expression of *IL1B*. The combination of Poly I:C and 1,25D3 up-regulated the expression of *IL1B* and Dex reduced that up-regulation (Fig 3 c).

4.1.2 Dexamethasone effects on bronchial epithelial cells

The next step was to study the effects of Dex on bronchial epithelial cells. For this we used the BCi cell line. The Dex treatment took down *CAMP* expression in a doze dependent manner and also took the 1,25D3 induced expression down somewhat. The vitamin D however neutralized the down-regulation to the extent that the high doze of Dex in combination with vitamin D showed similar *CAMP* expression to untreated cells (Fig 4).

4.1.3 Effects of Dex and Vitamin D on primary monocytes

Next we analyzed the effects of Dex on primary monocytes isolated from buffy coat gotten from healthy donors. The monocytes were treated with 1,25D3 and Dex both individually

and in combination. Dex seems to down-regulate *CAMP* basal expression and possibly vitamin D induced expression as well. The up-regulation induced by 1,25D3 however is pronounced, both individually and in combination with Dex (Fig 5).

In the published research we found that the effects of dexamethasone on *CAMP* expression were mediated through the glucocorticoid receptor. Inhibition of the receptor resulted completely hindered the down-regulation of *CAMP* by Dex. Furthermore it was showed that two other GCs, Fluticasone Propionate (FP) and Budesonide (BD) had similar inhibitory effect on 1,25D3 induced *CAMP* expression that dexamethasone has. The group also showed that Dex down-regulated *DEF1B*, *SLPI* & *LYZ* basal expression. Lastly we looked at other cell type to see if the effect of Dex on *CAMP* expression with and without 1,25D3 was similar. For this we looked at BCi N.S. 1.1, a human basal bronchial epithelial cell line, immortalized with hTERT (Walters et al., 2013). We showed that there was a similar expression pattern, that Dex down-regulated *CAMP* basal and induced expression and that vitamin D induced *CAMP* to counteract the reduction. The work on glucocorticoid steroids was published in September 2015 in Immunobiology (Nikhil Nitin Kulkarni et al., 2015).

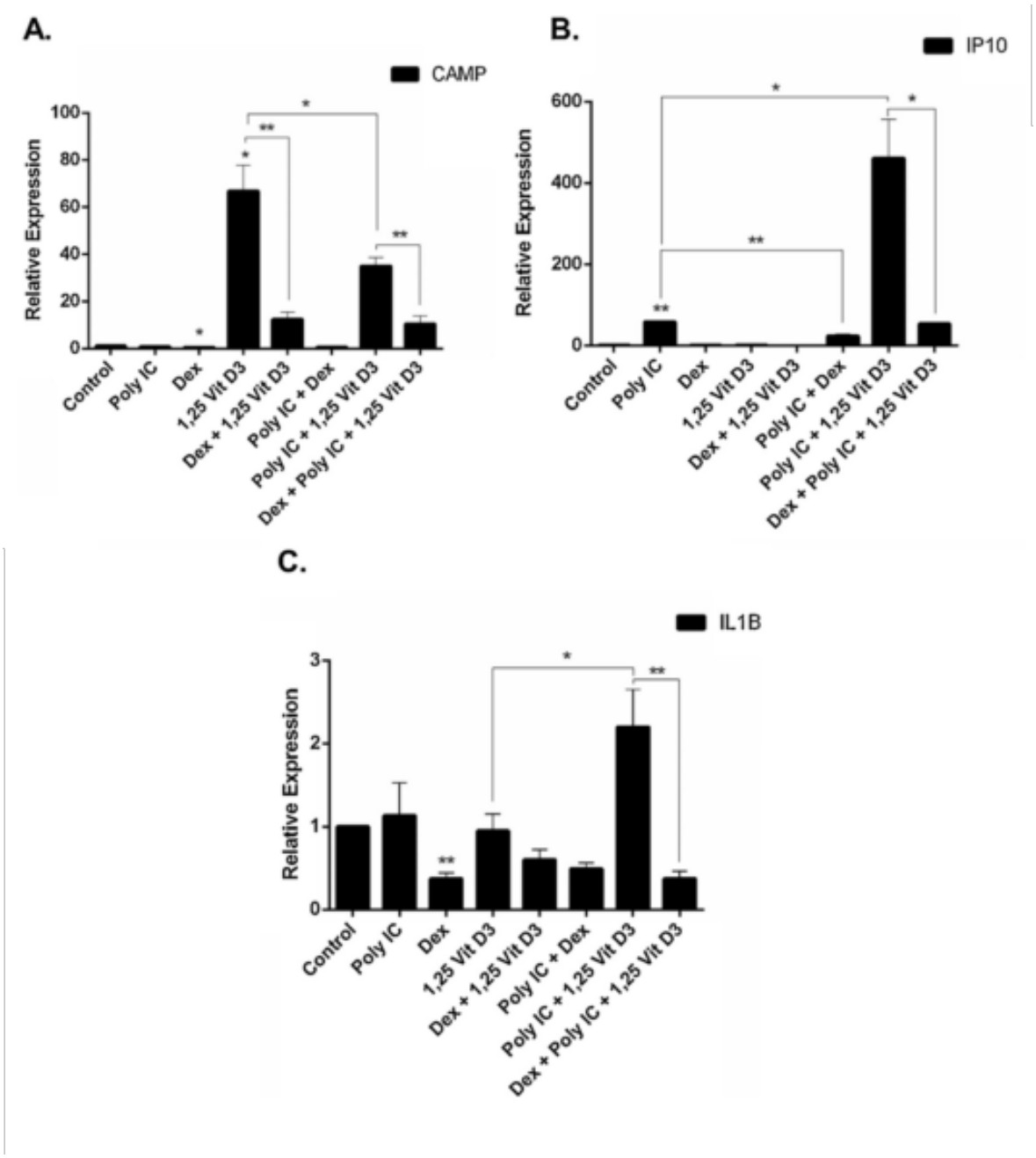

Figure 3 - Dexamethasone's effect on innate immune response in THP-1 cells. (A) Dex down-regulates *CAMP* expression, both on basal level and when the cells are challenged with 1,25D3 and Poly I:C. (B) Dex down-regulates Poly I:C induced expression of *IP10* and especially takes down the expression induced by the combination of Poly I:C and 1,25D3. (C) The expression of *IL1B* is up-regulated by the combination of Poly I:C and 1,25D3 relative to the expression when the cells are only challenged with 1,25D3. Dexamethasone takes down the up-regulation as well as basal expression of *IL1B* (*=P<0.05; **=P<0.01).

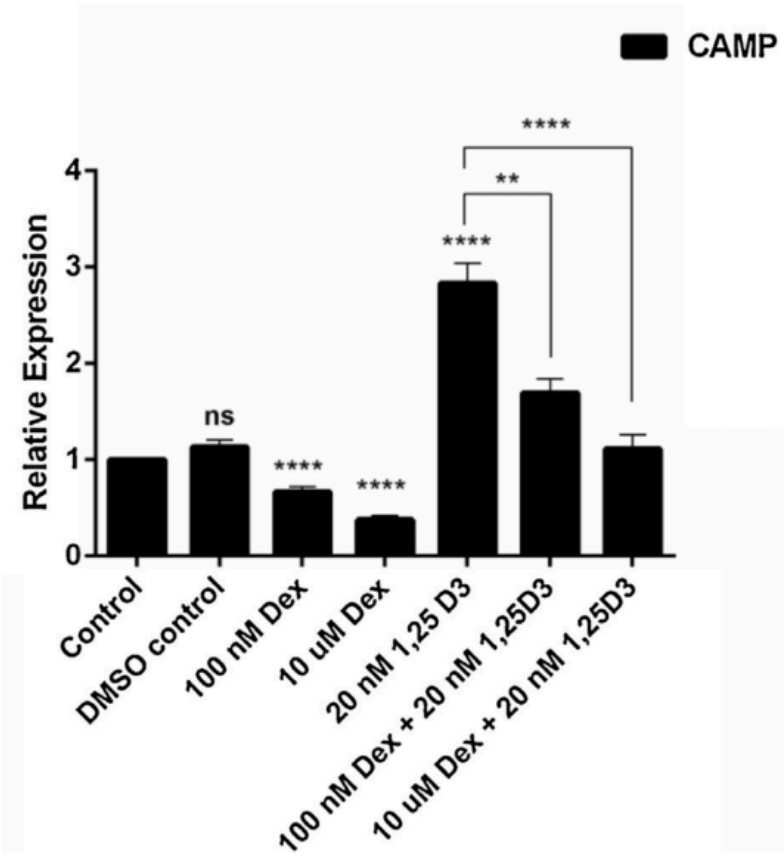

Figure 4 - Dexamethasone effects on BCi cells. Dex takes down basal expression of *CAMP*. Furthermore Dex takes down Vitamin D induced *CAMP* expression in a doze dependent manner (*=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001).

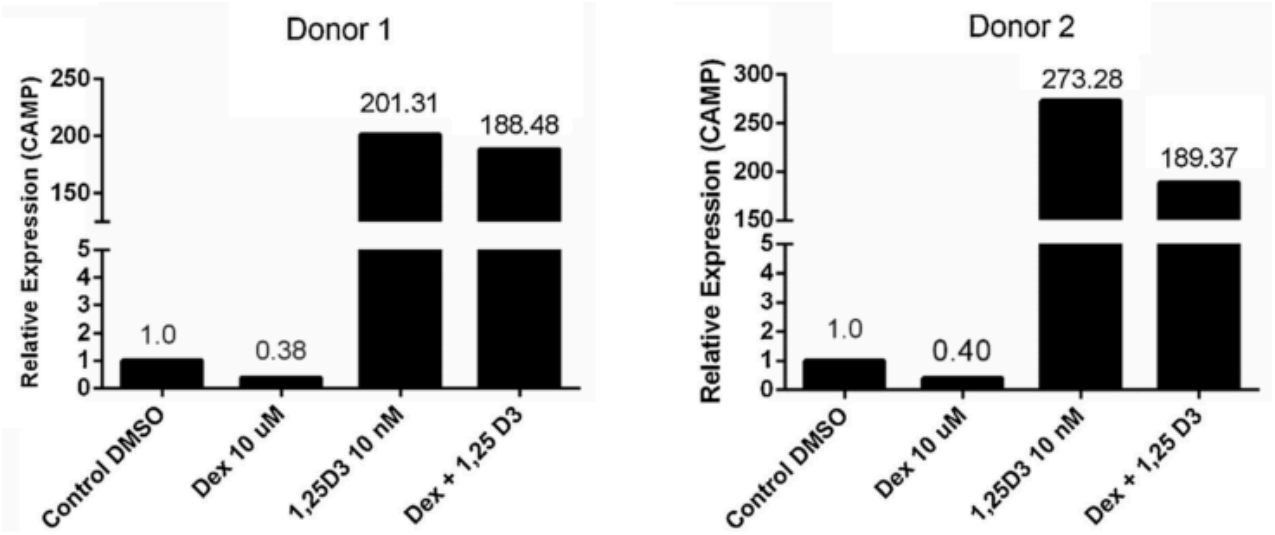

Figure 5 - Effects of Dex and Vitamin D on primary monocytes. Dexamethasone (Dex) seems to take down both basal and 1,25D3 induced *CAMP* expression in primary monocytes. The monocytes were isolated from buffy coats from two healthy donors.

4.2 Butyrate and PBA induction of *CAMP*

In the second part of my project I studied how butyrate induction of the *CAMP* gene can be linked to VDR. Earlier it was shown that PBA was working through VDR in its induction of innate immune response (Nikhil N Kulkarni et al., 2014). I sought to verify if this was also true for butyrate and I also wanted to shed some light on how these chemicals worked through VDR, if it was a direct ligand, simply HDAC inhibition allowing access through less tightly packed chromatin, if it was working through some phosphorylation or if there are some other factors in play.

4.2.1 Effects of inhibiting MAPKs on *CAMP* & *HIF1a* expression

The first experiment I did was to inhibit the MAP kinases: p38, JNK and ERK. It has been shown that butyrate mediated up-regulation of VDR works through p38 MAPK signaling pathway in Caco-2 cells (Daniel et al., 2004).

I used the bronchial epithelial cell line VA10 for this experiment. I treated the cells with inhibitors for p38, JNK and ERK individually and with inducers; including PBA, butyrate and vitamin D individually and in combinations of PBA and vitamin D and butyrate and vitamin D. I then used RT-qPCR to examine mRNA expression of *CAMP*.

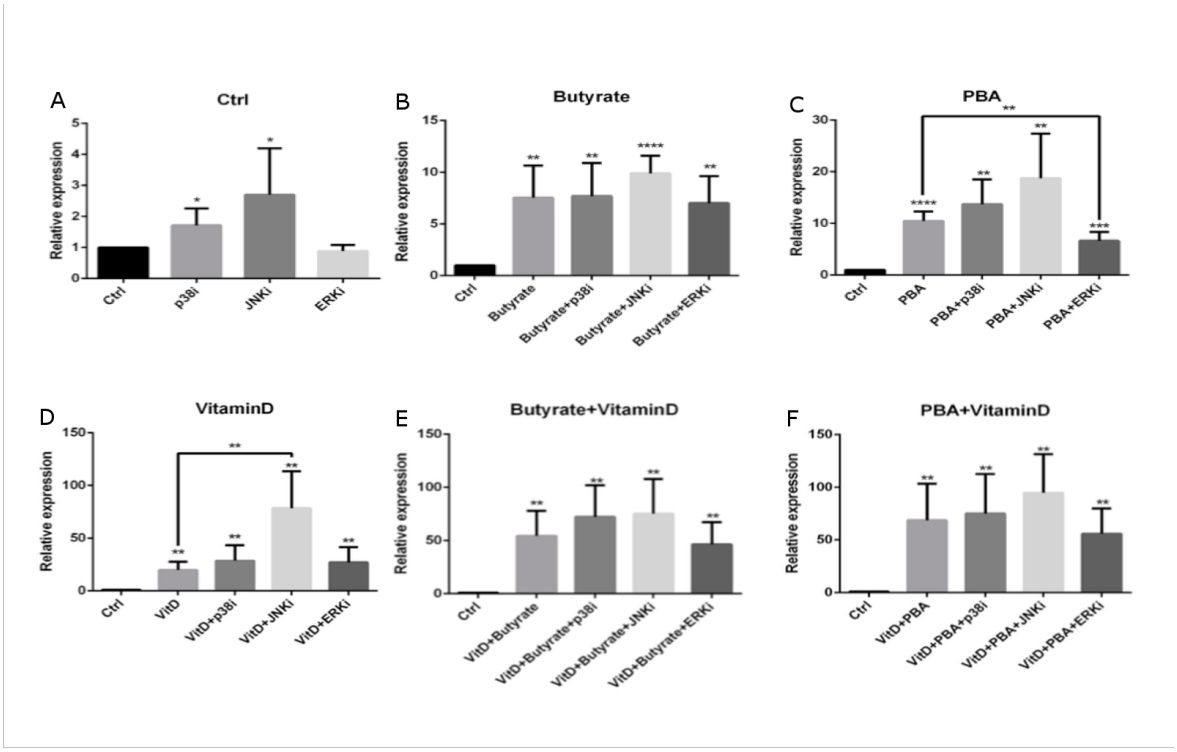

Figure 6 - MAPK inhibitors effect on *CAMP* induction in VA10 cells. (A) p38 and JNK inhibition affect basal expression of *CAMP* in a positive manner. (B) Butyrate induces *CAMP* expression and the MAP kinase inhibition has no significant effect on this induction, (C) PBA induced *CAMP* expression however is down-regulated with ERK inhibition. (D) Vitamin D (1,25D3) induced *CAMP* expression is synergistically up-regulated by JNK inhibition. (E&F) The MAP kinase inhibitors had no significant effect on *CAMP* expression induced by the combination of the SCFA and vitamin D (*=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001).

The results gotten from these analyses were as follows. Firstly, butyrate and PBA do not work in the exact same way; seeing that inhibition of ERK reduced the PBA induction of *CAMP* while it did not influence the butyrate-mediated response significantly. Secondly, JNK inhibits classic vitamin D induction of *CAMP*; for JNK inhibition significantly and synergistically increases 1,25D3 mediated *CAMP* induction. Thirdly p38 and JNK affect base expression of *CAMP*, both in a inhibiting fashion (Fig 6).

At this point in time we came upon interesting articles linking LL-37 and HIF-1α in *Candida albicans* infection prevention (Fan et al., 2015). For this reason we decided to look at *HIF1a* expression in the same settings as in Figure 6.

The effects on *HIF1a* expression were that JNK and ERK inhibition significantly induced *HIF1a* expression and butyrate induced it as well and significantly more in combination with p38 and ERK inhibition. PBA however did not induce *HIF1a* except when in combination with JNK inhibition. Vitamin D induced *HIF1a* individually as well as in combinations with the SCFAs (Fig 7). These results further support the difference in butyrate and PBA effects.

HIF1α role in *CAMP* regulation should be analyzed in more details in lung epithelia.

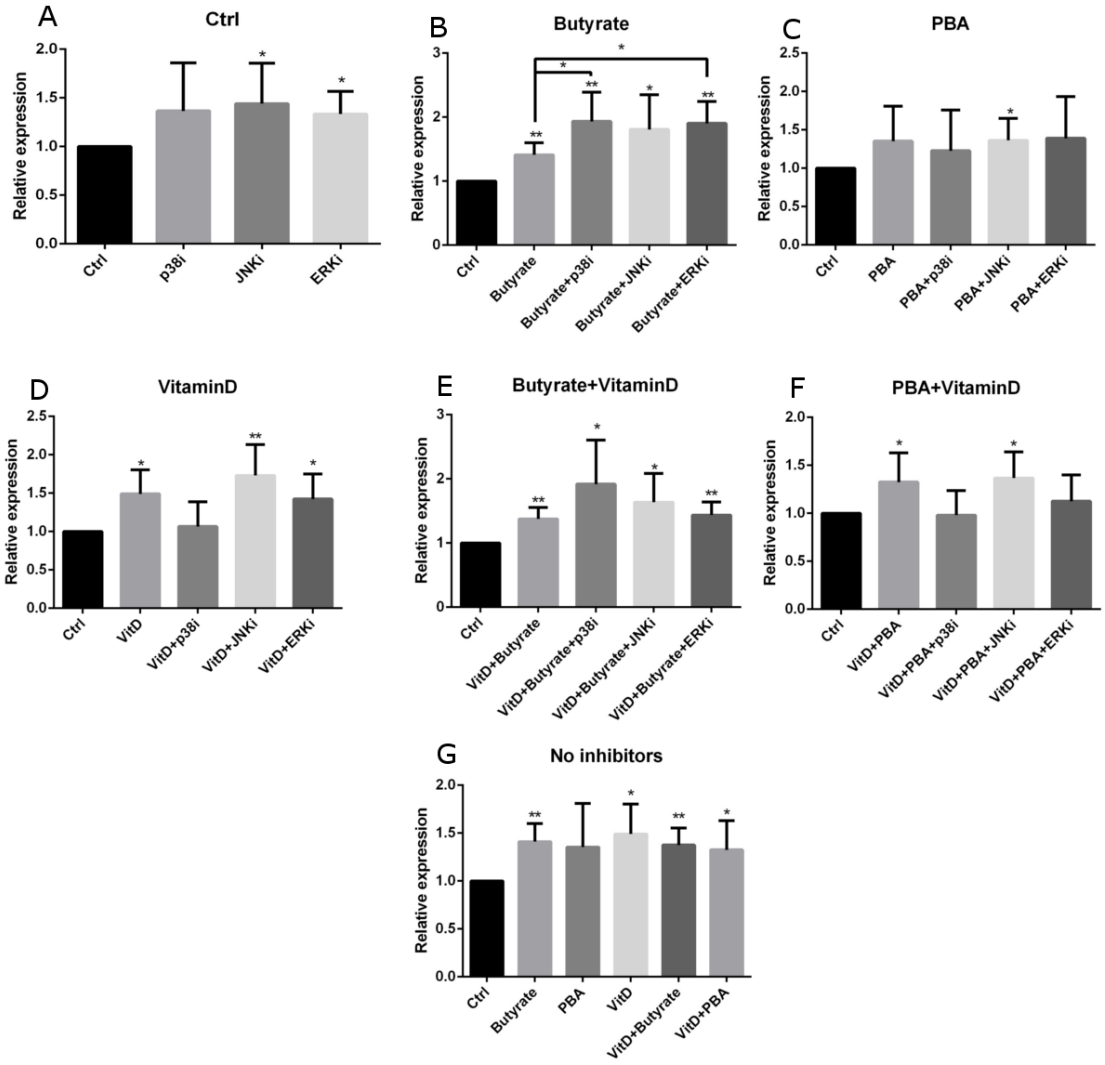

Figure 7 - MAPK inhibition affects *HIF1A* expression in VA10. (A) Inhibition of MAPKs JNK and ERK significantly increases base expression of *HIF1A*. (B) Butyrate up-regulates *HIF1A* expression and inhibition of p38 and ERK further increase the expression. (C) PBA does not affect *HIF1A* expression significantly but when combined with JNK inhibition a significant increase in *HIF1A* expression was measured. (D) 1,25D3 induced *HIF1A* expression and so did its combination with JNK and ERK inhibition but when combined with p38 inhibition there was not a significant effect. (E&F) The MAPK inhibition had no significant effect on *HIF1A* expression induced by vitamin D in combination with either of the SCFA in question. (G) *HIF1A* expression was significantly increased when the cells were introduced to butyrate, 1,25D3 and the combination of 1,25D3 and the SCFAs (butyrate and PBA). When induced with PBA alone there was not a significant effect however (*=P<0.05; **=P<0.01).

4.2.2 Attempted VDR knockout HT-29 cell line

Next experiments I did on an attempted CRISPR/Cas9 VDR knockout HT-29 cell line that we got from our collaborators in Karolinska Instituted in Stockholm. The cell line had been shown to have a reduced VDR function but the knockdown had not been confirmed with sequencing or protein analysis. We were unable to confirm the knockout but I did an experiment with the cells comparing them with untreated HT-29 cells. The results I got were clear, important activity in both butyrate and PBA mediated *CAMP* induction is missing (Fig 8). Both the vitamin D induction and its synergistic induction with butyrate and PBA are completely gone in the attempted knockout. I did a western blot to see if VDR was in fact knocked out and found that it was not. However there was a faint band just under the VDR band that showed only in the untouched cell line but not in the attempted knockout cell line (Fig 9).

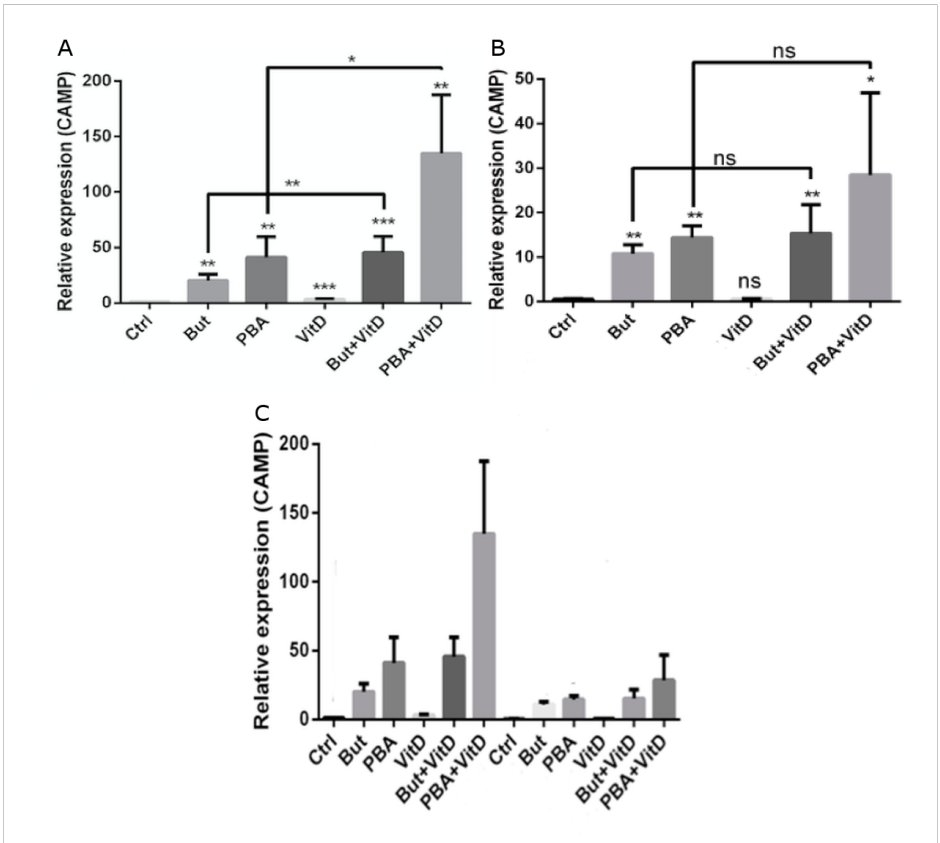

Figure 8 - *CAMP* expression in attempted dVDR HT-29 cells. (A) The induction on normal HT-29 cells. Butyrate and PBA induce *CAMP* expression as well as 1,25D3 in HT-29 although the 1,25D3 induction is a fraction of what is often seen in other cell types. 1,25D3 works in synergy with both Butyrate and PBA in *CAMP* induction. (B) The attempted knockout VDR HT-29 cells induced. Butyrate and PBA induce *CAMP* expression in the presumed ΔVDR HT-29 cells although the induction is significantly weaker than in the untouched cell line. All effect of 1,25D3 on *CAMP* expression is however gone, both individually and in combination with the SCFAs. (C) When compared on the same scale the effect is clear. HT-29 is on the left side and its ΔVDR counterparts on the right. All inductions are significantly lower in the ΔVDR compared to their WT HT-29 counterparts (*=P<0.05; **=P<0.01; ***=P<0.001).

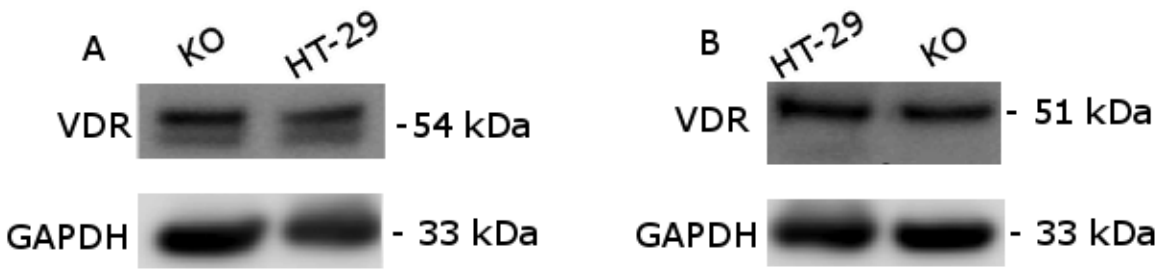

Figure 9 - Western blot analysis on the attempted VDR knockout HT-29 cell line. In (A) VDR expression was analyzed using a polyclonal rabbit antibody. There are clear bands visible at a little larger size than would be expected for VDR (48 kDa). There is no apparent difference between the attempted knockdown (KO) and the untouched cell line (HT-29). In (B) VDR expression was analyzed using a monoclonal mouse antibody. This analyzes showed equal expression in the knockout (KO) compared to the untouched cell line (HT-29). In both pictures a mouse monoclonal GAPDH antibody was used to control for equal loading of samples.

4.2.3 Docking simulations

To approach the mechanism of VDR's role in butyrate and PBA mediated *CAMP* we decided to look in to the possibility that butyrate and PBA are direct ligands to VDR. To look at this idea we collaborated with Aron T. Skaftason and Talentum (two separate sources) to do a docking simulation *in silico* with butyrate, PBA and vitamin D as possible ligands and VDR as the receptor. Aron also looked at binding with PPARγ as the receptor. The results of the simulations were that PBA and butyrate could both bind to VDR's binding site and PPARγ. Vitamin D was able to bind to all VDR complexes that were checked but not PPARγ (Table 4). The reason for including PPARγ was that it had been shown to bind butyrate earlier (Alex et al., 2013). The Talentum team assessed however that this binding was likely to weak to activate the receptor. Furthermore the synergy between vitamin D and the SCFAs cannot be explained by these results for the binding to the pocket was a competitive one. For these reasons we decided not to pursue the idea that butyrate and PBA were direct ligands to VDR but this could be tested further for example with radioactive label on butyrate and immune precipitation or luciferase based reporter assay.

Table 4 - Calculated affinity values in kcal/mol for ligands to different receptor complexes. In this table the strongest affinity values calculated for each binding are listed. As expected 1,25D3 has the strongest binding to all VDR complexes as it served as a positive control. All the ligands bind to the ligand-binding domain of VDR but 1,25D3 does not bind PPARγ.

Ligands	VDR-VDR	VDR-RXR	VDR-RXR-DNA	PPARγ
1,25D3	-13	-7,4	-6,5	No binding
Butyrate	-4,6	-4,6	-3,1	-3,5
PBA	-7,1	-6,8	-5	-5,9

4.2.4 CRISPR/Cas9 knockout of VDR in BCi cells

We sought to knock out VDR expression in BCi cells to have a stable model to analyze VDR effects in the cells. To do this we designed two different sets of gRNA pairs (listed in materials and methods). We managed to integrate one of these pairs on a plasmid (VDR-gRNA20) and managed to select out colonies of transfected cells on a plate using blasticydin selection. However we were not able to culture single cell clones from these colonies, all cells died after being separated.

4.2.5 shRNA knockdown of VDR

A lentiviral shRNA knockdown of VDR was performed on VA10 cells. The transfection was performed to develop a staple model of cells with reduced VDR activity to see the effects of inducing these cells compared with inducing regular VA10 cells and to analyze the role of VDR in differentiation of lung epithelia. First results show that the transfection was successful, showing reduced level of mRNA expression for *VDR* (Fig 10). This knockdown has to be further confirmed on protein level before experiments are initiated.

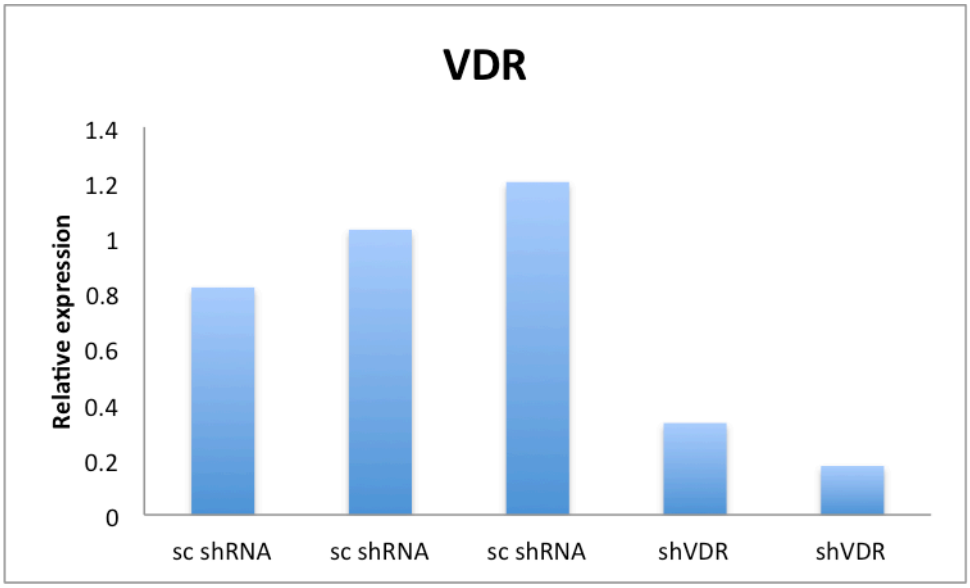

Figure 10 - qRT-PCR results showing mRNA expression of VDR. Three different transfected populations of scrambled shRNA control and two of the VDR shRNA both are VA10 cells. The VDR expression in the VDR knockdown cells seems to be 20-30% of the scrambled controls expression. The results are not proven to be significant as this is a single run.

5 Discussion

In the first part of my project we found that the glucocorticoid steroid dexamethasone (Dex) suppresses *CAMP* expression, both the basal expression and 1,25D3 induced expression in monocytes and bronchial epithelial cell line. However the vitamin D does counter the down-regulation as the expression of *CAMP* when the cells are treated with both Dex and vitamin D is higher or equal to the basal expression.

After the publishing of our article there was another article published that showed that Dex treatment induced *CAMP* expression in macrophages (Steiger et al., 2016). That is the same results as we got and we had a figure showing this in the supplementary of our article although when the macrophages were pretreated with Dex the vitamin D induction of *CAMP* was suppressed.

The RNA mimic poly I:C is a Toll-like receptor 3 ligand and is known to induce inflammatory response similar to viral infection (Marshall-Clarke et al., 2007). It was used to confirm the anti-inflammatory effect of Dex. Dex inhibited the induction of both *IP10* and *IL1B*. Interestingly vitamin D treatment enhanced poly I:C induced *IP10* induction and dexamethasone eliminated that enhancement.

These findings do suggest that vitamin D supplement aid should be considered a promising co-therapeutic option with GC treatment, countering the AMP suppression and strengthening innate immunity. This could be a good addition to the current treatment used for asthma and other inflammatory disorders.

In the second part of my project we have found that MAPKs (p38, JNK and ERK) inhibition affects basal expression of *CAMP* in the case of JNK and p38. There were also effects on induced expression in the cases of PBA induced expression and ERK inhibition and JNK inhibition in 1,25D3 induced expression (Fig 6). These effects taken together show that PBA and butyrate induction are not identical and this is further supported by the information gathered on *HIF1A* expression in the same settings as in figure 6 (Fig 7). There we saw an up-regulation of *HIF1A* expression with butyrate induction that was further enhanced by p38 and ERK inhibition. In contrast with PBA alone, PBA induced *HIF1A* expression in cells where JNK had been inhibited. Vitamin D induced *HIF1A* expression but the MAPK inhibition had no significant effects on that induction (Fig 7). These effects on *HIF1A* are interesting as HIF1 is a transcription factor so the effects could potentially have widespread effects within the cell and HIF1 induces *CAMP* for example. These results give a reason to look further into the connection between *HIF1A* and MAPK.

The attempted knockout VDR HT-29 cell line yielded clear results in showing that the altered cell line had impaired induction of *CAMP* when induced with butyrate, PBA and vitamin D (Fig 8). Similar effects had been showed before with PBA induction of *CAMP*

in VA10 bronchial epithelial cell line using siRNA knockdown of VDR (Nikhil N Kulkarni et al., 2014). These results show that VDR is the central transcription factor in butyrate and PBA mediated *CAMP* induction. However protein analysis on the attempted knockout showed that VDR was not successfully knocked out (Fig 9) the qPCR analysis did not show any difference in *VDR* transcription. This could mean that the VDR that is expressed has impaired function, possibly due too an indel mutation that did not take the gene out of frame for translation. The only way to be certain is to make cDNA and amplify the gene region and finally sequence the area of interest.

The collaboration with Aron T. Skaftason and Talentum gave interesting results. The computed docking simulation showed that both PBA and butyrate could bind to the ligand-binding site in VDR. The Talentum team suggested however that this binding was likely too weak to activate the receptor. It is however interesting that according to Aron's calculations the binding of butyrate was stronger to VDR than PPARγ and butyrate is a known PPARγ ligand (Alex et al., 2013). The synergy butyrate and PBA have with vitamin D cannot however be explained by these results as the binding to the pocket was a competitive one. There is a possibility that butyrate and PBA are direct ligands that activate VDR but to determine that further research has to take place. This could be done for example with a radioactive label on butyrate and PBA and immune precipitation analyzing if the radioactivity gets pulled down with VDR. Other way to test the binding would be to set up a luciferase based reporter assay specific for the ligand-binding pocket of VDR, similar to what was done to asses butyrate's binding to PPARγ (Alex et al., 2013).

The next step I would take in the project to approach the mechanism would be to use immune precipitation to see if butyrate and PBA induce dimerization of VDR and RXR. If this would be the case that would indicate direct binding to VDR, rather than just the unwinding of chromatin allowing access. It could either be with direct ligand activity that would not explain the synergy, but could non-the less still be a factor, or it could be through some different means like acetylation on VDR or other proteins. Recent reports have shown that acetylation is an important post transcriptional modification on PXR modifying its activity (Cui et al., 2015; Pasquel et al., 2015). PXR is closely related to VDR (Haussler et al., 2008) so it would surprise me if acetylation is not a factor in VDRs activity.

References

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