Gene expression in mammalian innate immunity

4-PBA and ST7 reduce *CRAMP* gene expression in murine lung epithelial cells

Carolin Huehnken

Supervisor
Guðmundur Hrafn Guðmundsson

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UNIVERSITY OF ICELAND
FACULTY OF LIFE AND ENVIRONMENTAL SCIENCE
DEPARTMENT OF BIOLOGY

Yfirlýsing

Hér með lýsi ég því yfir að ritgerð þessi er samin af mér og að hún hefur hvorki að hluta né í heild verið lögð fram áður til hærri prófgráðu.

Carolin Huehnken

Reykjavík, January 16th 2009

Abstract

Antimicrobial peptides are an important component of the human and mammalian innate immunity. These peptides greatly contribute to the protective barrier against microbes found on all epithelial surfaces and therefore enable us and all other multicellular organisms to live harmoniously with those intruding microbes. Antimicrobial peptides consist of two families, the defensins and the cathelicidins. Only one cathelicidin has been identified in humans, CAMP/hCAP18/LL-37. *CAMP* gene expression has been shown to be induced by a variety of different agents, such as vitamin D_3, butyrate and phenylbutyrate. These findings are of great interest due to the increasing number of drug-resistant bacteria in recent years. There are hopes that in the near future induced *CAMP* expression, mediated by topically applied agents, would become a good alternative to common antimicrobials used today.

Here we investigate the effects of 4-PBA and ST7 treatment on the *CRAMP* expression in murine lung epithelial cells. We show that several heat shock proteins are involved in VDR mediated transcription. The treatment with 4-PBA and ST7 causes a reduction of the cellular CRAMP concentration because the expression levels of several VDR-associated heat shock proteins are affected as well.

Acknowledgements

I would like to thank Prof. Guðmundur Hrafn Guðmundsson for giving me the opportunity to work on this project and for his supervision. I would also like to thank Jónas Steinmann and especially Skarphéðinn Halldórsson for teaching me all the different techniques in the lab - without your help I probably would have been a little lost. Eggert Gunnarsson and Helga Erlendsdóttir for their help on the mouse expreiment.

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Abbreviations

AMP	Antimicrobial peptide
BCP	Bromochloropropane
CAMP	Cathelicidin antimicrobial peptide
cDNA	Complementary DNA
CF	Cystic Fibrosis
CRAMP	Cathelicidin-related antimicrobial peptide
DEPC	Diethylpyrocarbonate
dNTP	Deoxyribonucleotide
ER	Endoplasmic reticulum
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GRP78	Glucose regulated protein 78
Hop	HSP70/HSP90 organizing protein
HSC70	Heat shock cognate 70
HSP	Heat shock protein
HSP40	Heat shock protein-40
HSP70	Heat shock protein-70
HSP90	Heat hock protein-90
LCA	Lithocholic acid
LPS	Lipopolysaccharide
mRNA	Messenger RNA
NMRI	Naval Medical Research Institute
SCFA	Short chain fatty acid
SD	Standard deviation
ST7	α-methylhydocinnamate
TLR	Toll-like receptor
VDR	Vitamin D receptor
VDRE	Vitamin D response element
$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
$25(OH)D_3$	25-hydroxyvitamin D_3
4-PBA	Sodium 4-phenylbutyrate

Introduction

Our innate immune system is responsible for keeping us healthy by quickly repelling constant microbial assaults. It is a very important defense system against a broad spectrum of microbes, including Gram-positive and Gram-negative bacteria, fungi and certain viral pathogens [3,5,12,17]. The great diversity and diverse combination of antimicrobial peptides (AMPs) comprises the major component of the mammalian innate immunity. They are of great importance for mammals and all other multicellular organisms in order to live harmoniously with microbes [5,17].

The mammalian AMPs have two distinct roles. First, they function as signaling molecules, which link innate and adaptive immunity [4]. Second, they act as active antimicrobial effectors of innate immunity. These effectors are constitutively expressed by epithelial cells to protect the mucosal covered tissues and other tissue surfaces from invading microbes [3,6,11]. In case of inflammation and infection of those mucosal surfaces, AMP expression and secretion are known to be induced [10]. Certain mammalian leukocytes contain granules that can hold vast amounts of AMP precursors, which are proteolytically cleaved into active AMPs upon secretion to directly kill the invading microbes [3]. They are able to do this, not only by exploiting the distinct differences between microbial and eukaryotic membranes, but also by adopting an amphipathic conformation by arranging clusters of hydrophilic, hydrophobic and cationic amino acids spatially in discrete sectors of the molecule. The resulting α-helical or β-sheet conformation enables the AMPs to specifically

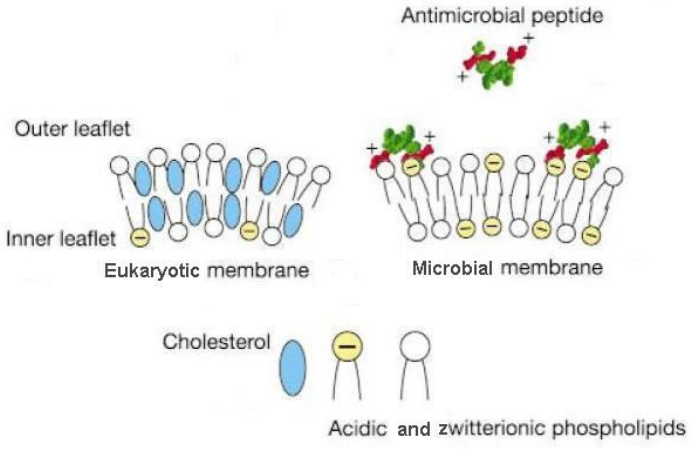
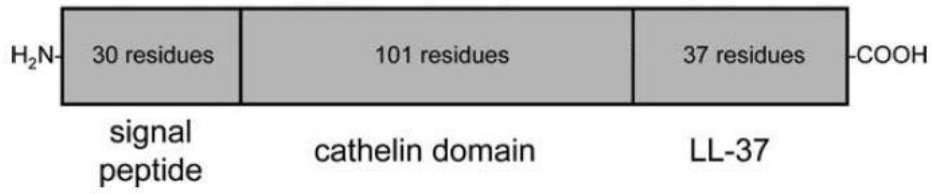


Figure 1: Composition and arrangement of eukaryotic cell membranes and bacterial membranes. Schematic representation of the interaction between AMPs and the negatively charged phospholipid headgroups. (Taken and modified from Zasloff, 2002)

target and distinguish between microbial membranes and the eukaryotic cell membranes, due to the fact that the outer leaflet of membrane bilayer is heavily populated by lipids with negatively charged phospholipid headgroups (Figure 1). These charged headgroups encourage strong electrostatic and hydrophobic interactions between themselves and the AMPs. However, these negatively charged and at the same time acidic phospholipids are not found on the outer leaflets of the eukaryotic cell membranes. They are in fact only found on the inner leaflet which faces the cytoplasm. The outermost leaflet of those membranes consists of lipids with no net charge and large amounts of cholesterol, which greatly reduces the activity of AMPs by stabilizing the membrane layer by reducing the lipid disorder [3,17]. The interactions between the intruding α-helical AMPs and the acidic phospholipids usually result in lipid replacements and therefore in the alteration of the membrane structure. These alterations include the depolarization and disturbance of the microbial membrane and intracellular functions, creation of physical holes that cause leakage of cellular content or activation of cell wall degrading hydrolases. In most cases these membrane alterations are followed by the physical disruption and the collapse of the microbial membrane [17].

There are two distinct classes of mammalian AMPs - the defensins and the cathelicidins [3,11]. The family of defensins comprises α-defensins and β-defensins [5]. Human α-defensins have been found in ganulocytes and in a variety of epithelia where as β-defensins have been only found in epithelial cells. Just like α-defensins cathelicidins have been found in granulocytes and in a variety of surface epithelia [2]. Cathelicidins are a family of precursor proteins that contain a highly conserved 100 residue N-terminal cathelin domain which is flanked by a 30 residue signal sequence on its N-terminus and by an antimicrobial domain on its C-terminus [3,11]. The signal peptide has the purpose of serving as target sequence and directing the tanslating ribosome to the surface of the endoplasmic reticulum (ER). This signal sequence is thought to be cleaved off once the ribosome has arrived on the ER. After this cleavage the translation of the cathelicidin AMP and the translocation into the ER continues. Post-translational processing takes place during which disulfide-bridges are formed to stabilize the proprotein. Via the Golgi apparatus the proprotein is then transported to its storage sites and/or secretion sites. The antimicrobial domain becomes active when it is cleaved from the highly conserved cathelin domain [3].

a)



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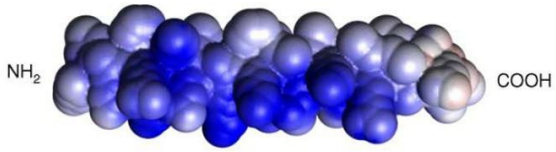

Figure 2: a) Schematic representation of the human cathelicidin hCAP18. b) Three-dimensional model of LL-37 in a hypothetical perfect α-helical conformation in physiological pH. (Taken and modified from Dürr et al., 2006)

Although there have been found several cathelicidins in other mammalian species, only one cathelicidin has been found in humans. CAMP is the only human cathelicidin-derived AMP and is also known as hCAP18/LL-37 [3,5,11]. CAMP maps to chromosome 3p21, with four exons and three introns [4]. CAMP gives rise to the LL-37 peptide, an amphipathic α-helical 37 residue AMP, when it is proteolytically cleaved from its cathelin propart (Figure 2) [3,10,11]. The primary structure of LL-37, the amino acid sequence LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES, reveals the highly charged properties of this amphipathic antimicrobial peptide [3]. The CAMP propeptides are mostly found to be stored in peroxidase-negative granules of neutrophils from which it can be quickly released at sites of infection. CAMP is synthesized and secreted by various squamous epithelia, including the tissues of the mouth, esophagus, lungs, intestine, cervix and the vagina. These tissues are constantly exposed to the environmental microbes. It has been also shown that CAMP is synthesized in certain glands which results in the secretion of the peptide in sweat, airways surface fluids, seminal plasma, and maternal milk [4,5,16]. Apart from its antimicrobial activity, LL-37 has been associated with anti-sepsis and the chemoattractation of neutrophils, monocytes and a few T cells in lung epithelia. The peptide is also thought to be involved in promoting angiogenesis, modulation of adaptive immunity, and epithelial wound repair and healing by promoting the LPS neutralization, activation of epithelial cells, and chemotactic activity towards neutrophils, monocytes, T cells and mast cells [3,5,10,15,16].

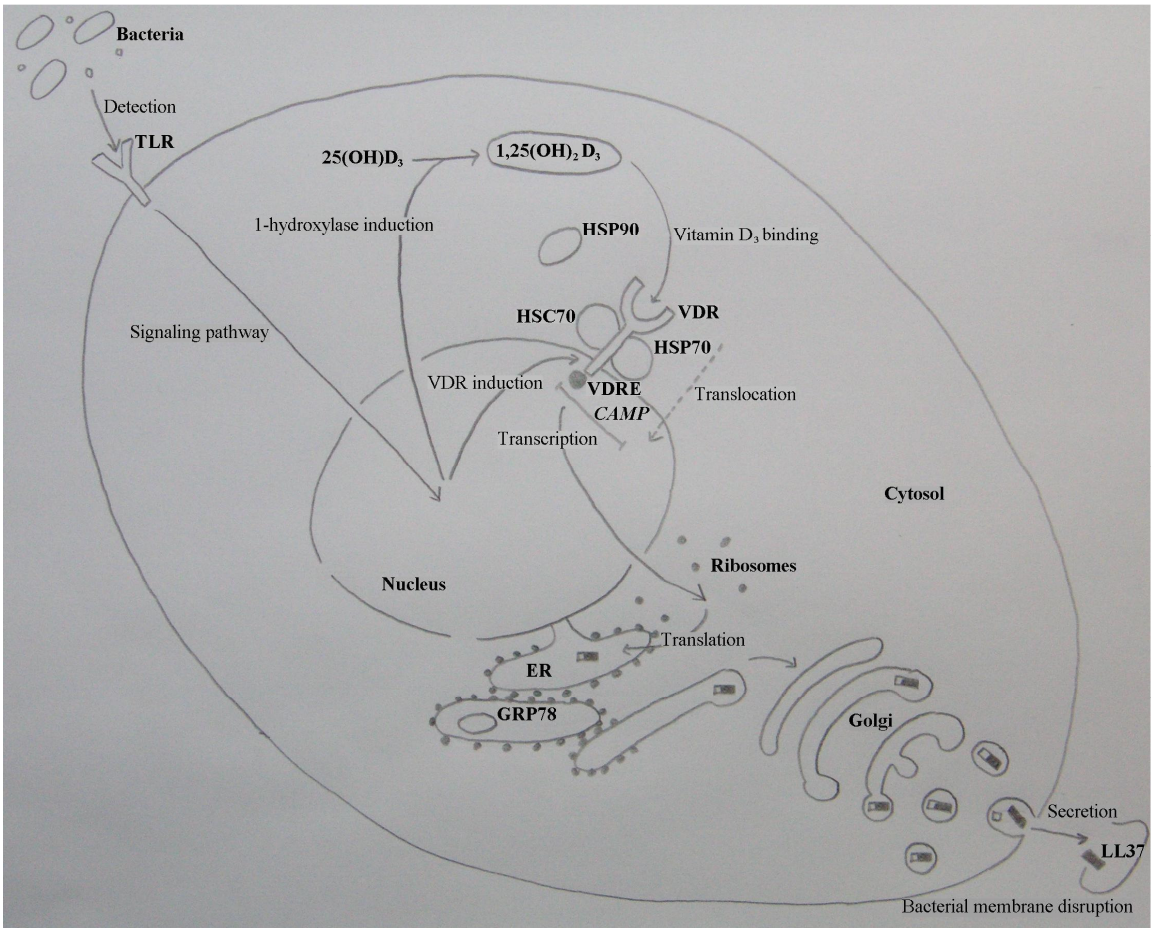

Figure 3: A possible *CAMP* expression up-regulation mechanism in a human cell. Bacterial products are recognized and detected by the Toll-like receptor (TLR), resulting in an induction of vitamin D_3 receptor (VDR) expression as well as 1-hydroxylase expression. The 1-hydroxylase enzyme catalyzes the inactive form of vitamin D_3 to the active form, which binds the VDR. The vitamin D_3-VDR complex translocates into the nucleus and activates the transcription of the *CAMP* gene via the in the promoter region located vitamin D_3 response element (VDRE). The *CAMP* mRNA is translated by free ribosomes in the cytosol. The target sequence is thought to be cleaved from the cathelin part of the peptide once it has directed the translating ribosome to the endoplasmic reticulum (ER). The growing polypetide chain translocates into the ER. After translation the peptide is transported via the Golgi apparatus to storage and/or secretion sites. Just before the secretion the LL-37 peptide is activated by PR3 serine protease-mediated cleavage from its cathelin propart. LL-37 targets bacterial membranes and displaces membrane lipids, which results in a collapse of the membrane and kills the bacteria.

The induction of LL-37 expression in epithelia by bacterial products is an indication that the peptide plays an important role in the innate immune system [3]. The human transcriptional mechanism which regulates *CAMP* gene expression in the tissues is yet not entirely known. But this regulation mechanism is thought to be actively regulated because the peptide expression has been shown to be tightly controlled during infection and inflammation [4]. *CAMP* expression is therefore regulated by extracellular signals and downstream transcription factors that are involved in the

activation of transcription of the *CAMP* gene [5]. It is known that the human innate immune system uses Toll-like receptors (TLRs), which are located on the extracellular cell membrane surface, to specifically recognize and bind microbial products, such as LPS, LCA, and SFCAs (Figure 3) [17]. The ligand-binding by the TLR stimulates the up-regulation and induction of the vitamin D_3 receptor (VDR) expression as well as the expression of vitamin-D-1-hydroxylase, which catalyzes the conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$, a steroid hormone and the active form of vitamin D_3 [8,14]. It has been shown that the *CAMP* gene expression is then activated by $1,25(OH)_2D_3$, which associates with and activates the VDR, a class II nuclear steroid hormone receptor acting as transcription factor by binding a specific ligand and interacting with regulatory sequences in the promoter region of the target genes. In addition, vitamin D_3 also regulates the intracellular VDR content [1,5,8]. Upon association this vitamin D_3-VDR complex translocates into the nucleus. Within the nucleus the VDR associates with the consensus vitamin D_3 response element (VDRE), which is situated in the promoter region of the *CAMP* gene and has been shown to be necessary for *CAMP* induction by vitamin D_3 [5,8,14]. The *CAMP* transcript is exported from the nucleus into the cytosol. There the transcript is translated by free ribosomes which are guided to the surface of the ER by the signal sequence. This signal sequence is thought to be cleaved from the cathelin part of the CAMP protein as soon as it arrives in the ER [2,3]. In keratinocytes, CAMP was found to localize in the Golgi apparatus which suggest a Golgi-mediated transport to the membrane. After stimulation, LL-37 is then activated by the PR3 protease-mediated cleavage of the pro-protein which liberates LL-37 (in neutrophils). The now active LL-37 is secreted into the extracellular environment, where it binds peripherally to the membranes of the microbes and effectively kills them by causing the collapse of the microbial membranes [4].

Prior activation, nuclear receptors are thought to exist as a multi-protein chaperone complex consisting of several heat shock proteins (HSPs) and other co-chaperones (see Figure 3). However, it is still not entirely known to what extent HSPs influence VDR function. In a bacterial model (*E. coli*) the VDR has been shown to bind HSP70, HSP40, and HSC70 and to be associated with HSP90, which seems to regulate the degree of responsiveness to the active form of vitamin D_3 and therefore influences the vitamin D_3-mediated transcriptional activity. Both HSP90 and HSC70 seem to be essential for correct VDR function. There are indications that HSP90 is

additionally involved in maintaining the receptor stability as well as being an important factor for VDR-VDRE binding. However, whether HSP90 also affects the VDR-ligand binding, the receptor translocation into the nucleus and the recruitment of transcription factors still remain to be determined. HSC70 seems to be necessary for the binding of HSP90 in a two-step assembly mechanism, which activates the receptor. The functional role of HSP70 in vitamin D_3 function still remains unclear [1,8].

SCFAs, like butyrate, are derived from the bacterial fermentation of undigested dietary fiber in the colon. Butyrate is an important energy source to the colon epithelial cells and that it is readily metabolized by the epithelial mucosa. It has been observed to increase the expression of the *CAMP* gene in colon epithelial cells [10,11]. Butyrate has been found to enhance the activity of the CAMP possibly by activating proteases that cleave the CAMP precursors to mature antimicrobial peptides. This is consistent with the observation, that CAMP in combination with butyrate is far more efficient in killing bacteria than CAMP alone [10]. Additionally, butyrate is known to affect the expression of cellular chaperones. In CF bronchial epithelial cells it has been shown to induce the expression of *HSP70*, *HSC70*, *Hop*, which has been found to be involved in the assembly of the HSP70/HSP90 heterocomplex as well as in the chaperone-mediated protein folding machinery, and *GRP78*, which is involved in the folding and assembly of proteins in the ER [13].

Unlike bacterial resistance to the conventional antibiotics, such as penicillin, a resistance to AMPs is highly unlikely due to the multiplicity of AMPs that are expressed by the cells [17]. They have therefore been proposed as useful therapeutics [16]. It is also unlikely because it would be a difficult and very costly solution for the microbes to develop resistance against AMPs [5,17]. However, there are a few AMP-resistant bacterial strains, such as *Morganella* and *Serratia*, which have been found to have evolved a reduced density of acidic phospholipids in the outer leaflet of their membranes, which greatly reduces peptide-lipid binding. Other resistant species, such as *Porphyromonas gingivalis*, destroy the peptides by secreting digestive proteases [17]. In this context, butyrate has been shown to significantly decrease the symptoms of *Shigella* infections, which themselves significantly down-regulate the expression of AMPs in the intestinal surface epithelium straight from the beginning of the course of infection. However, the mechanism by which *Shigella* down-regulates *CAMP* expression is not entirely known, but it is suspected that *Shigella*

does that by directly and immediately blocking some crucial protein in the signaling pathway for the *CAMP* expression like the down-regulation of innate immunity effectors. Thereby they can avoid the antimicrobial activities of this peptide and provide a niche for bacterial proliferation at the epithelial surface [6,10].
Because there are technical difficulties in the large-scale production of AMPs, this modulation by topically applied agents, like butyrate and vitamin D_3, is a big hope to help to strengthen the natural innate immune system against microbial infections when a treatment with traditional antibiotics fails [16].

The goal of this study was to investigate what effects a treatment with 4-PBA and ST7 might have on the antimicrobial peptide expression. We used murine lung epithelial cells that originated from mice treated with both substrates, respectively. The effects of the treatment were determined by measuring the cellular amount of CRAMP mRNA, the mouse analogue to the human LL-37. In addition, we also investigated the effects of 4-PBA and ST7 on the expression of heat shock proteins, because, as they are associated with the vitamin D_3 receptor, a reduction or an imbalance of the interacting factors could negatively affect the *CRAMP* expression.
Here we show that 4-PBA and ST7 do in fact affect the expression of *CRAMP*. After 4-PBA and ST7 treatment CRAMP was found to be reduced in both cases, respectively. Our results indicate that the HSPs might play a major role in the VDR-mediated *CRAMP* regulation and that they fragilely depend on being present in the right amounts in order to be able to function correctly. The treatment with 4-PBA and ST7 induced slight changes in the HSP expression, which seem to cause the HSPs to function incorrectly and therefore to affect the transcriptional activity of the VDR in a negative way.

Materials and Methods

Mouse experiment
15 female NMRI mice, 6-8 weeks old, were ordered from Taconic DK. The mice were looking viable and healthy, and had a weight between 26-30 g at arrival in Reykjavík, Iceland. They were divided into three groups of five, independently in

regard to their weight. To allow recognition, the mice were color-tagged according to their experimental groups (black = control (saline), blue = 4-PBA and red = ST7). Individual mice within each group were serially labeled (unlabeled – IIII) to allow further distinction. In addition, every mouse was given a number from 1 to 15 to simplify further data handling (1-5 = control (saline), 6-10 = 4-PBA and 11-15 = ST7). Sodium 4-phenylbutyrate (4-PBA) and α-methylhydrocinnamate (ST7) concentrations were based on pharmacokinetic studies by Pace *et.al*, 2002. The aim was to achieve plasma levels of approximately 4 mM of both the substrates, respectively. This concentration corresponds to the levels used in the *in vivo* experiments. To reach such levels, 500 mg of drug would have to be given per kg of mouse. Dilutions of each substrate were prepared by assuming a standard weight of 30 g per mouse. Therefore 15 mg of 4-PBA, ST7 and NaCl were dissolved in distilled water, respectively. To maintain physiological salt concentrations (9%), the final volume of both the substrate solutions was 600 µl and the concentration was 150 mM. The saline solution was used as control. All the mice were intraperitoneally injected with 0.6 ml of saline or one of the substrate solutions according to their experimental grouping. Injections were continuously given every six hours for the next 24 hours, resulting in a total of five doses per individual mouse. Other than the saline mice, the 4-PBA and ST7 mice were showing clear signs of sickness shortly after every injection, manifesting in spastic movements, cramps and reduced activity. These symptoms were observed to pass during the first two hours after injection and the 4-PBA mice as well as the ST7 mice were indistinguishable from the saline mice by the time of the next injection. Two hours after the final injection, the mice were killed by cervical dislocation. Lungs as well as other organs, such as liver, kidneys, spleen and heart were immediately removed. The organs were snap-frozen in n-hexan on dry-ice and placed in color-labeled cryo-tubes submerged in liquid nitrogen for transport. The organ tissues were then ground in liquid nitrogen in a mortar and stored in tubes at -80°C.

However, it should be noted here, the lung epithelia that was removed from mouse 12 was lost and nowhere to be found. The results of the ST7 group were obtained by analyzing the changes in expression in only four mice, whereas the results of the other two groups were obtained by analyzing the change in expression in five mice each.

RNA isolation from tissue

Total RNA was isolated from tissue by using the TRI Reagent® (Sigma-Aldrich). Lung tissue samples were homogenized in 1 ml TRI Reagent® solution. After incubation at room temperature for 5 min 100 µl BCP were added, mixed well and incubated for 10 min at room temperature. After centrifugation at 12.000 xg for 15 min at 4°C the aqueous transparent upper phase was transferred into a fresh tube. 500 µl isopropanol (100%) were added and mixed by vortexing briefly. The solution was incubated for additional 10 min at room temperature and then centrifuged at 12.000 xg for 8 min at 4°C. The solution was discarded without disturbing the pellet. To wash the RNA crystals, 1 ml ethanol (75%) was added. The solution was centrifuged at 7.500 xg for 5 min at 4°C. The ethanol was removed, the RNA pellet air dried and then dissolved in 50 µl of DEPC-treated water.

Total RNA measurement

The total RNA concentrations were measured by using the Quant-iT™ RiboGreen® RNA assay kit (Invitrogen). High range assays were performed. Each sample was measured in duplicates. 1x TE working solution was prepared by diluting the provided concentrated TE buffer 20-fold in nuclease-free water. Total RNA was diluted in 1x TE working solution to result in a 1/500 dilution. The RNA standard curve was prepared by diluting the 100 µg/µl RNA stock solution in 1x TE working solution resulting in a 2 µg/ml solution of RNA in TE which was further diluted into 1 µg/ml, 500 ng/ml, 100 ng/ml and finally 20 ng/ml. The Quant-iT™ RiboGreen® reagent solution was prepared by diluting the agent 200-fold in the 1x TE working solution. 100 µl of the diluted RNA sample were placed into each used well of the 96-well plate. 100 µl of the Quant-iT™ RiboGreen® reagent solution were added to the RNA samples in the wells, mixed and protected from light incubated, for approximately 5 min at room temperature. The plate was read by the Tecan at an excitation wavelength that was 480 nm and an emission wavelength at 520 nm.

cDNA synthesis

cDNA was synthesized by using the RevertAid™ first strand cDNA synthesis kit (Fermentas). On ice, 2 µg of total RNA were mixed with 1 µl random hexamer primer (0.2 µg/µl) and DEPC-treated water to add up to a total of 12 µl. The mixture was gently mixed, briefly spun down, incubated at 70°C for 5 min and then chilled

on ice. 4 µl 5x reaction buffer were added as well as 0.5 µl RiboLock™ Ribonuclease inhibitor (20 u/µl), 2 µl dNTP mix (10 mM), 1 µl DEPC-treated water and 0.5 µl RevertAid™ M-MuLV Reverse Transcriptase (200 u/µl). This mixture was gently mixed, briefly spun down and incubated at 25°C for 5 min, at 42°C for 60 min and finally at 70°C for 10 min.

Real-Time Quantitative PCR

The PerlPrimer v1.1.14 was used to find primers for the genes of interest. The primers were ordered from TAG Copenhagen A/S.

Gene		**Sequence of forward and reverse primer**
mGAPDH	fwd	5'-GAAGGGCTCATGACCACAGTC-3'
	rev	5'-CATCACGCCACAGCTTTCC-3'
mCRAMP	fwd	5'- GGTGGTGAAGCAGTGTATGGG-3'
	rev	5'- TGCACCAGGCTCGTTACAG-3'
mHSP70	fwd	5'-GAAGCACAAGAAGGACATCAG-3'
	rev	5'-GATGTGTAGAAGTCGATGCC-3'
mHSC70	fwd	5'-CTCTTTCCCTTGGTATTGAAACTG-3'
	rev	5'-CACCTTCATACACCTGAATGAG-3'
mHSP90	fwd	5'-TATCACTGGTGAGAGCAAAGAG-3'
	rev	5'-GTCAATTAGGCTCAGTCATATACAC-3'

The cDNA was analyzed with the 7500 Real-time PCR system (Applied Biosystems). The Power SYBR® Green PCR Master Mix (Applied Biostystems) was used to detect double-stranded DNA. A mixture was blended, containing 2 µl cDNA, 5 µl SYBR® Green Master Mix, 0.5 µl of both forward and reverse primer (1:1000) and 4 µl DEPC-treated water. This mixture was incubated at 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min, during which the amplification data was collected. Each sample was analyzed in duplicate. mGAPDH was used as endogenous control for relative quantitation. The results were analyzed by using the Comparative C_T Method ($\Delta\Delta C_T$) and are expressed as fold-induction relative to the control samples (saline).

Results

4-PBA and ST7 treatment affects CRAMP gene expression

Three groups of five mice where administered different solutions, by intraperitoneal injections, to investigate the effects of 4-PBA and ST7 on the *CRAMP* gene expression in murine lung epithelial cells. The treatment with 4-PBA as well as with ST7 had reducing effects on the expression of the *CRAMP* gene (Figure 4 and Table 1). The *CRAMP* expression was reduced by almost 60% in the 4-PBA-treated mice, while the ST7-treated mice were showing a reduction of expression by approximately 45%.

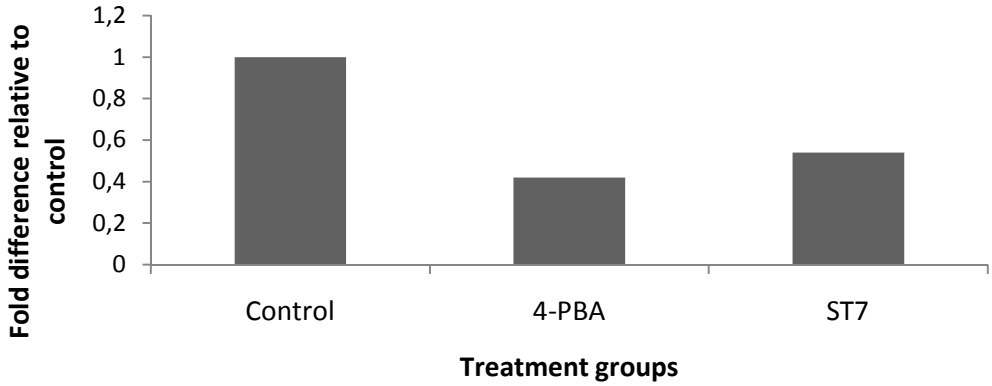

Figure 4: Fold change in expression of *CRAMP* in murine lung epithelial cells after treatment, determined by real-time PCR and the $\Delta\Delta C_T$ method. The treatment groups were treated with five doses of saline (Control), sodium 4-phenylbutyrate (4-PBA), and α-methylhydrocinnamate (ST7), respectively. Each dose had a volume of 600 µl and a concentration of 150 mM. The doses where administered over the course of 24 h.

We then decided then to investigate how 4-PBA and ST7 might affect the expression of the HSPs, since these proteins are thought to be associated closely to the VDR and are therefore thought to influence the function of this receptor. However, the treatment-induced changes in HSP expression were less distinct.

The expression of the *HSP90* gene was only slightly induced after treatment (Figure 5 and Table 2). Compared to the expression levels of the control group, there was only a 6% induction of *HSP90* in the 4-PBA group and a 16% induction in the ST7 group.

The expression of the *HSP70* gene was oppositely and a little more affected by the 4-PBA and ST7 treatment than the *HSP90* gene expression (Figure 6 and Table 3). 4-PBA treatment caused a slight reduction of *HSP70* expression. The results of the ST7

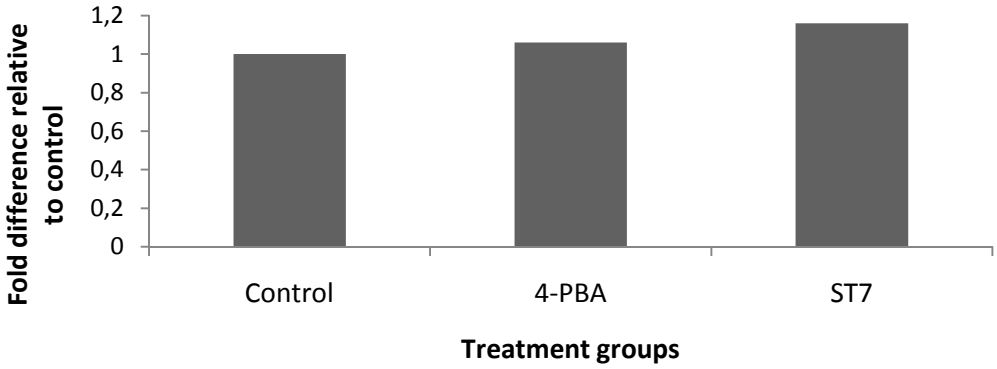

Figure 5: Fold change in expression of *HSP90* in murine lung epithelial cells after treatment, determined by real-time PCR and the $\Delta\Delta C_T$ method. The treatment groups were treated with five doses of saline (Control), sodium 4-phenylbutyrate (4-PBA), and α-methylhydrocinnamate (ST7), respectively. Each dose had a volume of 600 µl and a concentration of 150 mM. The doses where administered over the course of 24 h.

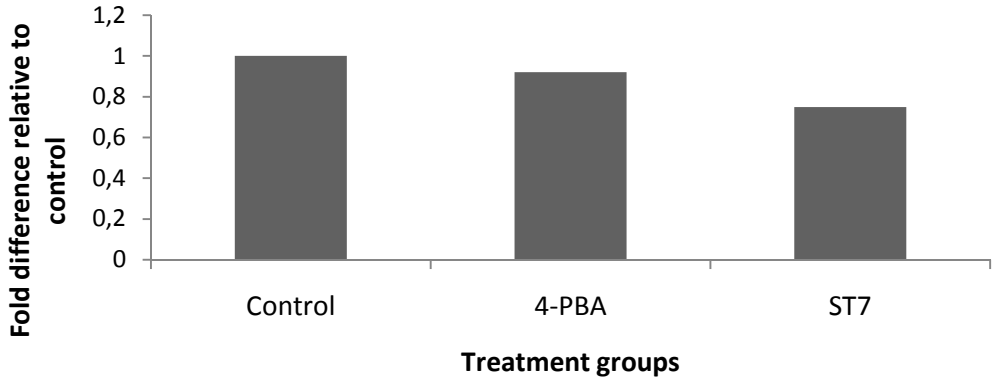

Figure 6: Fold change in expression of *HSP70* in murine lung epithelial cells after treatment, determined by real-time PCR and the $\Delta\Delta C_T$ method. The treatment groups were treated with five doses of saline (Control), sodium 4-phenylbutyrate (4-PBA), and α-methylhydrocinnamate (ST7), respectively. Each dose had a volume of 600 µl and a concentration of 150 mM. The doses where administered over the course of 24 h.

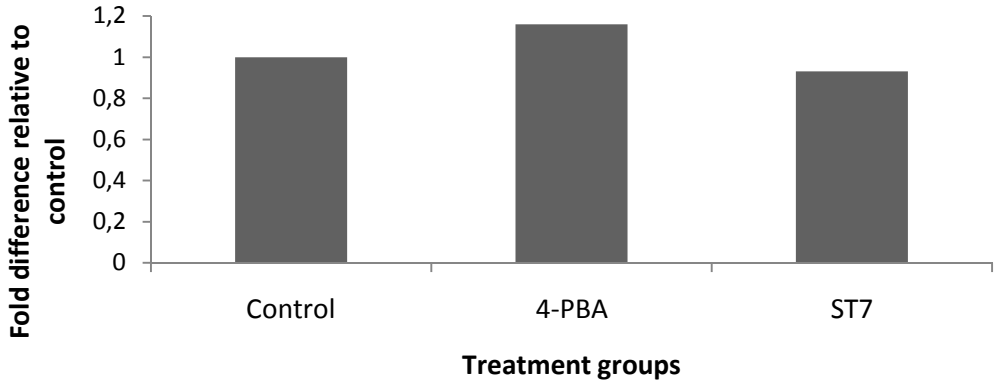

Figure 7: Fold change in expression of *HSC70* in murine lung epithelial cells after treatment, determined by real-time PCR and the $\Delta\Delta C_T$ method. The treatment groups were treated with five doses of saline (Control), sodium 4-phenylbutyrate (4-PBA), and α-methylhydrocinnamate (ST7), respectively. Each dose had a volume of 600 µl and a concentration of 150 mM. The doses where administered over the course of 24 h.

treatment showed a more significant reduction of expression by 25%.
The *HSC70* expression was affected in the way that there was an induction of *HSC70* gene expression by 16% after 4-PBA treatment and a reduction gene expression by approximately 10% after the treatment with ST7 (Figure 7 and Table 4).

Discussion

In an earlier study butyrate has been shown to greatly affect the expression of *CAMP*. It has been shown that butyrate was the best substrate to stimulate *CAMP* expression in the colon epithelial cells significantly [11,14].
Our results show that the treatments with 4-PBA as well as ST7 reduce the expression levels of the *CRAMP* gene compared to the *CRAMP* expression level in our control samples.
The mechanism that regulates the induction of cathelicidin expression by vitamin D_3 is still not entirely known. Earlier studies have shown that the VDRE in the cathelicidin promoter region is a necessary and important factor for the induction of expression of VDR target genes, such as the *CAMP* gene, when stimulated by vitamin D_3 [16]. But it seems that this mechanism is only conserved in primates because the VDRE is found to be absent in other mammals, including mice [5]. Consequently, *CRAMP* expression in murine lung epithelial cells was not found to be induced by vitamin D_3-mediated stimulation of the VDR. But the absence of the VDR-bound VDRE in the *CRAMP* promoter alone could not cause such highly reduced results.
It has been shown in earlier studies that HSPs in fact closely interact with the VDR. The expression of the HSPs has been shown to affect the transcriptional activity of the VDR. VDR has been demonstrated to interact with the HSP70 homolog, the Ssa1 protein in yeast cells [8]. Their findings show a reduction of the Ssa1 protein concentration resulting in the reduction of the intracellular amounts of the nuclear VDR. The reduction of the VDR of course results in the reduction of expression of VDR dependent genes. Both the treatment with 4-PBA and ST7 resulted in a reduction of *HSP70* expression which lets one conclude that there has been a reduction in VDR concentration as well. This of course would result in a lower

amount of the VDR dependent CRAMP, which could be an explanation for the highly reduced amount of CRAMP mRNA.

HSP70, HSP90 and HSC70 are required factors for VDR function. They can influence VDR stability and function. HSP90 has been also shown to be involved in the maintenance of ligand-binding conformation, translocation into the nucleus, interaction with DNA, receptor cycling at the promoter, and regulation of $1,25(OH)_2D_3$ responsiveness. It has been also suggested that HSP90 might be an important factor for VDR-VDRE binding. HSC70 is thought to bind HSP90 to the VDR [1]. It is therefore quite clear, that a reduction of HSP90 would most likely result in a reduced vitamin D_3-mediated transcriptional activity via the VDR. Our results show, that both the cellular concentrations of HSP90 and HSC70 have been induced as a result of 4-PBA treatment, which would result in the induction of *CRAMP* expression. The results of the ST7 treatment show an induction of HSP90 but a reduction of HSC70, which would most likely result in a reduction in *CRAMP* expression.

In summary, the reduction of HSP70 caused an overall reduction of VDRs. It therefore does not matter, that both HSP90 and HSC70 have been induced by 4-PBA treatment, because there is only a certain cellular amount of VDRs – so a reduction in *CRAMP* expression is very plausible. The ST7 treatment resulted in a less optimal concentration of both HSP90, which was induced, and HSC70, which has been reduced. Here is again only a limited concentration available, in this case the concentration of HSC70, which permits only a limited interaction between VDR and HSP90 and makes again a reduction of *CRAMP* expression very plausible.

In conclusion, the *CRAMP* expression in murine lung epithelial cells is considerably reduced and affected by 4-PBA as well as ST7 treatment. Not only is the transcriptionally important VDRE absent in murine cells but also the VDR stabilizing and transcriptionally influencing HSPs are clearly affected by the both treatments.

References

[1] **G. Angelo, S. Lamon-Fava, L.A. Sonna, M.L. Lindauer and R.J. Wood (2008).** Heat shock protein 90β: A novel mediator of vitamin D action. *Biochemical and Biophysical Research Communications*, 367: 578-583.

[2] **M.H. Braff, A.D. Nardo and R.L. Gallo (2005).** Keratinocytes store the antimicrobial peptide cathelicidin in lamellar bodies. *Journal of Investigative Dermatology*, 124: 394-400.

[3] **U.H.N. Dürr, U.S. Sudheendra and A. Ramamoorthy (2006).** LL-37, the only human member of the cathelicidin family of antimicrobial peptides. *Biochimica et Biophysica Acta* 1758: 1408-1425.

[4] **H.Z. Elloumi and S.M. Holland (2007).** Complex regulation of human cathelicidin gene expression: Novel splice variants and 5'UTR negative regulatory element. *Molecular Immunology*, 45: 204-217.

[5] **A.F. Gombart, N. Borregaard and H.P. Koeffler (2005).** Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D_3. *The FASEB Journal*, 19: 1067-1077.

[6] **D. Islam, L. Bandholtz, J. Nilsson, H. Wigzell, B. Christensson, B. Agerberth and G.H. Gudmundsson (2001).** Downregulation of bactericidal peptides in enteric infections: A novel immune escape mechanism with bacterial DNA as potential regulator. *Nature Medicine*, 7: 180-185.

[7] **P.T. Lui, S. Stenger, H. Li, L. Wenzel, B.H. Tan, S.R. Krutzik, M.T. Ochoa, J. Schauber, K. Wu, C. Meinken, D.L. Kamen, M. Wagner, R. Bals, A. Steinmeyer, U. Zügel, R.L. Gallo, D. Eisenberg, M. Hewison, B.W. Hollis, J.S. Adams, B.R. Bloom and R.L. Modlin (2006).** Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science*, 311: 1770-1773.

[8] **W. Lutz, K. Kohno and R. Kumar (2001).** The role of heat shock protein 70 in vitamin D receptor function. *Biochemical and Biophysical Research Communications*, 282: 1211-1219.

[9] **B.S. Pace, G.L. White, G.J. Dover, M.S. Boosalis, D.V. Faller and S.P. Perrine (2002).** Short-chain fatty acid derivatives induce fetal globin expression and erythropoiesis in vivo. *Blood*, 100: 4640-4648.

[10] **R. Raqib, P. Sarker, P. Bergman, G. Ara, M. Lindh, D.A. Sack, K.M.N. Islam, G.H. Gudmundsson, J. Andersson and B. Agerberth (2006).** Improved outcome in shigellosis associates with butyrate induction of an endogenous peptide antibiotic. *PNAS*, 103: 9178-9183.

[11] **J. Schauber, C. Svanhol, S. Termén, K. Iffland, T. Menzel, W. Scheppach, R. Melcher, B. Agerberth, H. Lührs and G.H. Gudmundsson, (2003).** Expression of the cathelicidin LL-37 is modulates by short chain fatty acids in colonocytes: relevance of signaling pathways. *Gut*, 52: 735-741.

[12] **J. Schauber, R.A. Dorschner, K. Yamasaki, B. Brouha and R.L. Gallo (2006).** Control of the innate epithelial antimicrobial response is cell-type specific and dependent on relevant microenvironmental stimuli. *Immunology*, 118: 509-519.

[13] **O.V. Singh, N. Vij, P.J. Mogayzel Jr., C. Jozwik, H.B. Pollard and P.L. Zeitlin (2006).** Pharmacoproteomics of 4-phenylbutyrate-treated IB3-1 cystic fibrosis bronchial epithelial cells. *Journal of Proteome Research*, 3: 562-571.

[14] **S. Termén, M. Tollin, E. Rodriguez, S. H. Sveinsdóttir, B. Jóhannesson, A. Cederlund, J. Sjövall, B. Agerberth and G.H. Gudmundsson (2008).** PU.1 and bacterial metabolites regulate the human gene CAMP encoding antimicrobial peptide LL-37 in colon epithelial cells. *Molecular Immunology*, 45: 3947-3955.

[15] **G.S. Tjabringa, D.K.Ninaber, J.W. Drijfhout, K.F. Rabe and P.S. Miemstra (2006).** Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. *International Archives of Allergy and Immunology*, 140: 103-112.

[16] **S. Yim, P. Dhawan, C. Ragunath, S. Christakos and G. Diamond (2007).** Induction of cathelicidin in normal and CF bronchial epithelial cells by 1,25-dihydroxyvitamin D_3. *Journal of Cystic Fibrosis*, 6: 403-510.

[17] **M. Zasloff (2002).** Antimicrobial peptides of microcellular organisms. *Nature*, 415: 389-394.

Appendix

Table 1: The fold change in *CRAMP* gene expression after treatment according to the treatment groups, calculated by the $\Delta\Delta C_T$ method. SD stands for Standard deviation. Mouse 12 sample N/A.

Sample	Treatment group	*CRAMP* Average C_T	*GAPDH* Average C_T	Δ C_T	$\Delta\Delta$ C_T	Fold difference in *CRAMP* relative to control
Mouse 1	Control	26,10±1,06	17,67±0,15	8,43±1,07	0,00±1,07	1,00 SD (0,48-2,10)
Mouse 2						
Mouse 3						
Mouse 4						
Mouse 5						
Mouse 6	4-PBA	28,30±1,56	18,66±1,41	9,68±2,10	1,25±2,10	0,42 SD (0,10-1,80)
Mouse 7						
Mouse 8						
Mouse 9						
Mouse 10						
Mouse 11	ST7	27,31±2,44	17,99±0,97	9,32±2,63	0,89±2,63	0,54 SD (0,09-3,34)
Mouse 13						
Mouse 14						
Mouse 15						

Table 2: The fold change in *HSP90* gene expression after treatment according to the treatment groups, calculated by the $\Delta\Delta C_T$ method. SD stands for Standard deviation. Mouse 12 sample N/A.

Sample	Treatment group	*HSP90* Average C_T	*GAPDH* Average C_T	Δ C_T	$\Delta\Delta$ C_T	Fold difference in *HSP90* relative to control
Mouse 1	Control	19,77±0.93	17,67±0,15	2,09±0,94	0,00±0,94	1,00 SD (0,52-1,92)
Mouse 2						
Mouse 3						
Mouse 4						
Mouse 5						
Mouse 6	4-PBA	20,63±1,56	18,66±1,41	2,00±2,10	-0,09±2,10	1,06 SD (0,25-4,56)
Mouse 7						
Mouse 8						
Mouse 9						
Mouse 10						
Mouse 11	ST7	19,87±1,14	17,99±0,97	1,88±1,50	-0,21±1,50	1,16 SD (0,41-3,27)
Mouse 13						
Mouse 14						
Mouse 15						

Table 3: The fold change in *HSP70* gene expression after treatment according to the treatment groups, calculated by the $\Delta\Delta C_T$ method. SD stands for Standard deviation. Mouse 12 sample N/A.

Sample	**Treatment group**	***HSP70* Average C_T**	***GAPDH* Average C_T**	**Δ C_T**	**$\Delta\Delta$ C_T**	**Fold difference in *HSP70* relative to control**
Mouse 1	Control	21,78±1,45	17,67±0,15	4,10±1,46	0,00±1,46	1,00 SD (0,36-2,75)
Mouse 2						
Mouse 3						
Mouse 4						
Mouse 5						
Mouse 6	4-PBA	22,85±1,88	18,63±1,41	4,22±2,35	0,12±2,35	0,92 SD (0,18-4,69)
Mouse 7						
Mouse 8						
Mouse 9						
Mouse 10						
Mouse 11	ST7	22,51±1,60	17,99±0,97	4,52±1,87	0,41±1,87	0,75 SD (0,21-2,74)
Mouse 13						
Mouse 14						
Mouse 15						

Table 4: The fold change in *HSC70* gene expression after treatment according to the treatment groups, calculated by the $\Delta\Delta C_T$ method. SD stands for Standard deviation. Mouse 12 sample N/A.

Sample	**Treatment group**	***HSC70* Average C_T**	***GAPDH* Average C_T**	**Δ C_T**	**$\Delta\Delta$ C_T**	**Fold difference in *HSC70* relative to control**
Mouse 1	Control	18,10±0,74	17,67±0,15	0,42±0,76	0,00±0,76	1,00
Mouse 2						
Mouse 3						
Mouse 4						
Mouse 5						SD (0,59-1,69)
Mouse 6	4-PBA	18,83±1,54	18,63±1,41	0,21±2,09	-0,26±2,09	1,16 SD (0,27-4,94)
Mouse 7						
Mouse 8						
Mouse 9						
Mouse 10						
Mouse 11	ST7	18,53±1,10	17,99±0,97	0,54±1,47	0,11±1,47	0,93 SD (0,33-2,56)
Mouse 13						
Mouse 14						
Mouse 15						