AN ABSTRACT OF THE DISSERTATION OF

Yan Campbell for the degree of Doctor of Philosophy in Biochemistry &
Biophysics presented on May 23, 2013

Title: The Effect of Dietary Compounds on Human Cathelicidin Antimicrobial
Peptide Gene Expression Mediated Through Farnesoid X Receptor and Its
Potential Role in Gastrointestinal Health.

Abstract approved:

_____

Adrian F. Gombart

The human cathelicidin antimicrobial peptide (CAMP) is a broad spectrum
microbicidal agent and modulator of both the innate and adaptive immune
system. It is induced by 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) through
activation of the vitamin D receptor (VDR) and primary bile salts through
activation of the xenobiotic nuclear receptor farnesoid X receptor (FXR). Both
receptors are expressed by enterohepatic and gastrointestinal (GI) tissues and
play important roles in GI immunity and homeostasis. It has been
demonstrated by us and others that plant polyphenol xanthohumol (XN) acts
as an FXR ligand, but its regulation of CAMP gene expression has not been

determined. We hypothesize that plant polyphenols obtained in the diet act as ligands for FXR and regulate expression of the CAMP gene in the GI tract thereby promoting gastrointestinal health through improved barrier defense against infection and inflammation. In this study, we demonstrate that XN induces BSEP (bile acid export pump) and human CAMP promoter activity via FXR. This activation appears to require some combination of the vitamin D response element (VDRE) site in the CAMP promoter and a potential FXR response element (FXRE) located in the third exon of the gene. In addition, XN and its metabolite 8-prenylnaringenin (8-PN) induced the mRNA expression of several FXR target genes including: BSEP, SHP (small heterodimer partner), IBABP (ileal bile acid binding protein), CAMP and FXR in biliary carcinoma cell lines. Combinations of $1,25(OH)_2D_3$ and either XN or 8-PN cooperatively induced endogenous CAMP gene expression in cells. We conclude that the plant polyphenol XN and its metabolite 8-PN act as FXR ligands and regulate expression of the human CAMP gene alone or in combination with $1,25(OH)_2D_3$.

A second distinct project used immunohistochesmitry (IHC), Western blot and quantitative real-time polymerase chain reaction (qRT-PCR) to characterize a transgenic mouse that carries the human CAMP gene. In the transgenic mice fed a normal diet, the human CAMP gene is expressed in immune and epithelial barrier cells and treatment of tissues with $1,25(OH)_2D_3$, including those from the GI tract, induced expression of the human, but not the mouse gene. This study advances our understanding of human CAMP gene

regulation by FXR and also helps establish the mouse as a reliable model

system that can be used in future studies to determine the importance of

CAMP gene expression in human health.

The Effect of Dietary Compounds on Human Cathelicidin Antimicrobial Peptide

Gene Expression Mediated Through Farnesoid X Receptor and Its Potential

Role in Gastrointestinal Health

by

Yan Campbell

A THESIS

Submitted to

Oregon State University

In partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Presented May 23, 2013

Commencement June 2014

Doctor of Philosophy dissertation of <u>Yan Campbell</u> presented on <u>May 23, 2013.</u>

APPROVED:

_____

Major Professor, representing Biochemistry and Biophysics

_____

Chair of the Department of Biochemistry and Biophysics

_____

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes the release of my dissertation to any reader upon request.

_____

Yan Campbell, Author

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Adrian F. Gombart, for his mentorship over the past several years. Your enthusiasm in scientific research and patience in guiding students is deserving of great respect. I thank you for giving me the chance to work in your lab and your positive support during my time in your lab.

I would like to thank all my committee members for all your great advice, especially Dr. Andy Karplus and Dr. Kathy Magnusson. I thank Dr. Andy Karplus for your patience in answering my numerous ridiculous questions during your biophysics course and your constant support over the past several years, especially the effort you put into revising my thesis. I would like to thank Dr. Kathy Magnusson for letting me rotate in your lab and helping me analyze my immunohistochemical staining data.

I thank all the members in the Gombart lab for your family-like friendship and support both in and out of the lab. I thank my mom and dad for their support over the years, especially for their help in baby-sitting my newborn son and preparing delicious home-made food for the whole family. I also thank my dear husband Adam Campbell for your love and support.

Lastly but most importantly, I thank my son baby Alexander D. Campbell. You are the best piece of data I have ever generated. Thank you for keeping me awake at night during the last seven months, so I could spend more time working on my research and thesis. No words can express how grateful I am to have you here. I love you, son.

CONTRIBUTION OF AUTHORS

Adrian F. Gombart designed research, analyzed data, contributed reagents and edited the thesis; Yan Campbell performed and designed research, data analysis, and writing.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

Dedicated to

My son, Alexander.

The Effect of Dietary Compounds on Human Cathelicidin Antimicrobial Peptide Gene Expression Mediated Through Farnesoid X Receptor and Its Potential Role in Gastrointestinal Health

Chapter 1

Thesis Overview

Introduction

Vitamin D deficiency is a common problem worldwide. The importance of sufficient Vitamin D levels has increased with our understanding of its role in multiple sclerosis, rheumatoid arthritis, diabetes, cardiovascular disease and microbial infections (1). There are two forms of vitamin D that humans can utilize: vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) (2). Vitamin D_2 is synthesized primarily in fungi by a UVB mediated reaction that cleaves the B ring of ergosterol to generate pre-vitamin D_2 that undergoes a subsequent thermal isomerization to vitamin D_2 (1). Mushrooms that are exposed to the sunlight or UVB light contain high levels of vitamin D_2 (3, 4). Both vitamin D_2 and D_3 are hydroxylated by the cytochrome P450 enzyme CYP27A1 in the liver to become 25-hydroxyvitamin D which circulates in the blood and is used as a marker for determining serum vitamin D levels. The inactive 25-hydroxyvitamin D (25(OH)D) is hydroxylated at the C1 position by mitochondrial 1 α-hydroxylase enzyme (CYP27B1) becoming the active 1,25-dihydroxyvitamin D (1,25$(OH)_2$ D) via both renal and extra-renal pathways (5, 6) .

One main mechanism by which vitamin D is thought to protect against microbial infections is via the promotion of the expression of human cathelicidin antimicrobial peptide (CAMP) gene.

CAMP is a broad spectrum antimicrobial agent and modulator of both the innate and the adaptive immune system (1). It is a single copy gene in humans and located on chromosome 3 (3p21.3) (7). Within the cathelicidin gene promoter is an Alu-short interspersed element (SINE), primate-specific retro-transposable element, and the gene itself has four exons and three introns (Figure 1.1) (8). The vitamin D response element (VDRE) is located in the Alu-SINE and therefore, the regulation of CAMP gene expression by vitamin D is found only in humans and non-human primates (9). The 18 kDa pro-protein product of this gene is called hCAP (human cationic antimicrobial protein) 18 (10). The bactericidal peptide is derived by extracellular proteolysis of the variable C-terminal end from hCAP18 (11). It is released from the conserved N-terminal "cathelin" domain by elastase (12), stratum corneum tryptic enzyme (SCTE) (13) or proteinase 3 (14). In humans, the mature peptide, called LL-37, is 37 amino acids long of which the first two are leucine residues (Figure 1.1) (15). Throughout this thesis, cathelicidin or CAMP refers to the gene or mRNA, hCAP18 refers to the unprocessed 18 kDa pro-protein and LL-37 refers to the mature peptide.

LL-37 is a positively charged molecule with many arginine and lysine amino acid residues and forms an α-helical structure in solutions that mimic human plasma (16, 17). The LL37 peptide kills bacteria (1, 18, 19) and binds to and neutralizes LPS (20). This prevents LPS activation of TLR-4 (21) and subsequent NF-κB signaling and inflammatory cytokine release from host cells

(22), thereby protecting animals from LPS-induced sepsis (23, 24). LL-37 also acts as a chemoattractant for eosinophils and neutrophils via formyl-peptide receptors, suggesting it may play an important role in asthma and chronic obstructive pulmonary disease (COPD) (25).

The cathelicidin gene is expressed in squamous epithelia of the respiratory and digestive tract, including the mouth, tongue and intestines. In the immune system it is expressed in myeloid and lymphoid cells of the bone marrow and spleen (1). LL-37 and hCAP18 are secreted into sweat, saliva, wound fluid, breast milk and seminal plasma (26-29) and is critical for barrier defense against infection. Mice and humans lacking CAMP gene expression are more susceptible to bacterial infections (1, 18, 19). In humans, neutrophils produce and store hCAP18 in secondary (specific) granules. During infection, hCAP18 is released and subsequently cleaved by proteinase 3 to produce the active LL-37 (14). LL-37 is not detected in the plasma or saliva from patients with morbus Kostmann disease (a severe congenital neutropenia) and these patients suffer from severe periodontal disease (30).

In the following sections of this chapter, I will provide a brief summary of the focus of each of the remaining thesis chapters.

Overview of Chapter 2: Regulation of antimicrobial peptide gene expression by nutrients and byproducts of microbial metabolism

Antimicrobial peptides (AMPs) play multiple important roles in the human immune response (1). Cathelicidins, defensins and other AMPs are an evolutionarily conserved component of the innate immune system indicating they play a very important role in combating infection (31). AMPs have two or more positively charged residues such as lysine, arginine or histidine making them cationic and able to bind to the negatively charged membranes of both gram-negative and gram-positive bacteria, mycobacteria, fungi, enveloped viruses and cancer cells (16 2006, 32, 33). Binding of AMPs to pathogens initiates the host's innate immune response and eventual kills the pathogen by disruption of cell membranes. Two major classes of AMPs are cathelicidin (19, 34) and defensins (35). The focus of this review is the human cathelicidin, and the α- and β-defensins. In addition to their well-characterized antimicrobial activities, AMPs modulate the responses of both innate and adaptive immune cells by acting as chemokines themselves or inducing the production of other chemokines and cytokines (1)**.** Vitamin D, other nutritional compounds and microbial byproducts regulate the expression of AMPs (8, 36), suggesting that diet and/or consumption of nutritional supplements may be used to improve immune function. In Chapter 2, we review the regulation of the cathelicidin antimicrobial peptide gene and the defensin gene family by various nutritional

compounds and/or microbial byproducts and discuss their potential importance to human health.

Overview of Chapter 3: Regulation of the human cathelicidin antimicrobial peptide gene by the xenobiotic receptor FXR and its dietary ligands

Because of cathelicidin's importance to immune defense, there is much interest in understanding the transcriptional regulation of its expression. Armed with this knowledge, it may be possible to regulate endogenous CAMP expression to boost immune function. Regulation of human CAMP gene expression by vitamin D is via vitamin D receptor (VDR), a member of the nuclear receptor super family. The heterodimer formed by VDR and retinoic X receptor (RXR) binds to the VDRE in the human CAMP promoter and induces its expression in the presence of the active ligand $1,25(OH)_2D$ (8). Many other nuclear receptors, including the FXR, form heterodimers with RXR (Table 1.1). FXR was named for its weak activation by supra-physiological concentrations of farnesol, an intermediate in the mevalonate biosynthetic pathway (37). FXR plays important roles in various physiological and pathological processes, such as bile acid homeostasis (38, 39), lipid, carbohydrate and energy metabolism (40-44) and cancer (45). The FXR protein is approximately 69 kDa and consists of an N-terminal DNA-binding domain (DBD) linked to a 20 kDa ligand binding domain (LBD) (46, 47). FXR expression is highest in the liver

and intestines (48). FXR-deficient mice have increased levels of bacteria in the ileum, a compromised epithelial barrier (49) and are more susceptible to gastrointestinal injury (50), furthermore, aged FXR-/- mice develop spontaneous hepatocarcinogenesis (51). In humans, FXR expression is down-regulated in patients with hepatocellular carcinoma (HCC) (51). Taken together, these findings indicate that FXR is critical in maintaining liver and gastrointestinal health.

FXR is activated by its natural ligand, the primary bile acid chenodeoxycholic acid (CDCA), and induces expression of the human CAMP gene (52). The VDRE in the Alu-SINE of the human CAMP gene has been suggested to mediate FXR induction of the gene (52), however, the data presented is not compelling. FXR was shown to bind to a VDRE consensus sequence and activate a VDRE-consensus-sequence-driven promoter in a human biliary carcinoma cell line (52). However, in these studies, the natural VDRE sequence located in the human CAMP promoter was not tested and a requirement for the VDRE in the CAMP promoter was not demonstrated. In Chapter 3, studies we reported were designed to elucidate the mechanism involved in the induction of the human CAMP gene by dietary compounds that are ligands of FXR.

Numerous plant polyphenols are FXR ligands including xanthohumol (XN) (53). XN is a dietary prenylated chalconoid from hops (*Humulus lupulus L.*). It

inhibits the production of pro-inflammatory cytokines, nitric oxide (54) and IL-1β (55), IL-12 and reduces the severity of oxazolone induced chronic allergic contact dermatitis (56). 8-prenylnaringenin (8-PN), a metabolite of XN, is a phytoestrogen (57). 8-PN is generated either by the cytochrome P450 enzyme Cyp1A2 which catalyzes the O-demethylation of XN isomer isoxanthohumol (IX) in the liver (58) or by the metabolism of intestinal microbiota in the human gut (59). Deuterium-hydrogen exchange studies showed that XN, IX and 8-PN are all capable of interacting with the ligand binding domain of FXR suggesting they may also be natural ligands in vivo (Yang, et al., manuscript in preparation). The results reported in Chapter 3 show that XN is an FXR ligand and provide the first evidence that XN and its metabolite 8-PN regulate CAMP gene expression.

Overview of Chapter 4: Characterization of human CAMP gene transgenic mice

As noted earlier, the regulation of the CAMP gene by vitamin D is unique to humans and non-human primates (9). The determination of how vitamin D-mediated regulation of human CAMP gene expression participates in host defense would be facilitated by an appropriate and powerful animal model. For this purpose, therefore, Dr. Gombart and collaborators generated a strain of transgenic mice by inserting a 6.2 kb genomic fragment containing the human

CAMP gene into the mouse genome (unpublished data). Two founder lines were generated and we describe the initial characterization of expression of human CAMP in this "humanized" mouse in Chapter 4.

Overview of Chapter 5: General conclusions and outlook

In the Chapter 5, I will summarize the study and address the significance of this thesis in detail. In general, the research presented in this thesis is significant because it demonstrates that FXR is a bona-fide regulator of the CAMP gene and provides a mechanistic explanation of how dietary compounds regulate the CAMP gene and perhaps modulate barrier defenses against microbes. The characterization of transgenic mice shows this "humanized" transgenic mouse model is successful and will allow future studies to demonstrate that dietary compounds can modulate the expression of the CAMP gene via FXR *in vivo* leading to a deeper understanding of how dietary compounds and factors produced by gut microbes may modulate antimicrobial defenses in the gut lumen. Characterization of additional pathways that regulate the CAMP gene will provide the foundation for developing novel therapeutic treatments for gastrointestinal diseases.

Figure 1.1
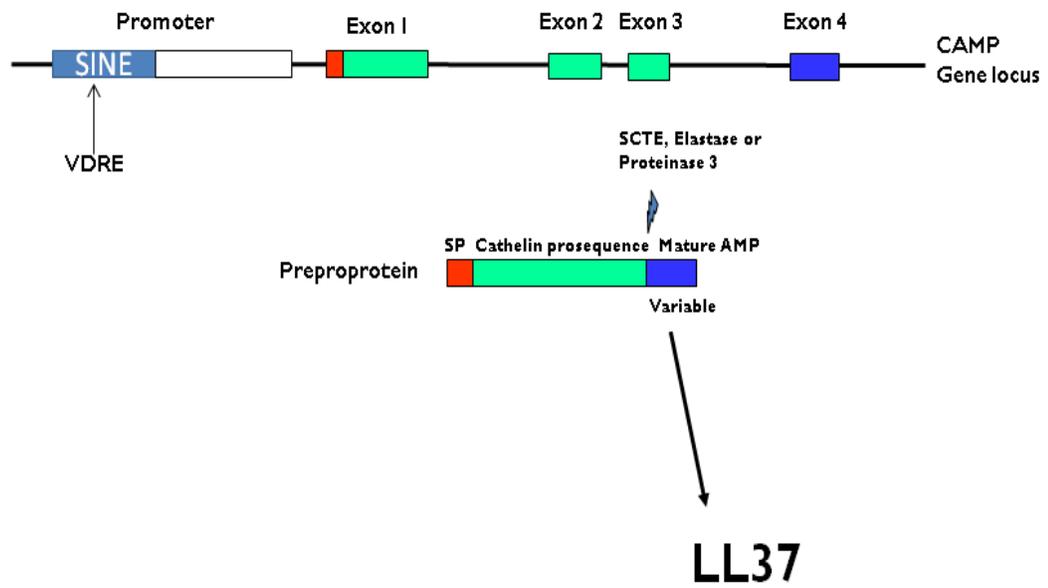
Figure 1.1. Structure of human cathelicidin gene and the processing of hCAP18. The promoter of the human cathelicidin gene contains an Alu-SINE and the gene has four exons and three introns. The propeptide is activated by cleavage at the C-terminal by proteinase 3, elastase or SCTE to generate the mature, active peptide-LL-37.

Table 1.1. Nuclear receptors that heterodimerize with RXR and their primary ligands.

Nuclear Receptor	Primary Ligand
T3R	Thyroidhormones
RAR	All-trans RA
VDR	$1,25(OH)_2D$
FXR	Bile Acids
PPARα	Fatty Acids
PPARγ	15d-PGJ2
EcR	Ecdysone
CAR	Androstane
LXR	Oxysterol
PXR/SXR	Xenobiotics

Regulation of antimicrobial peptide gene expression by nutrients and byproducts of microbial metabolism

Chapter 2

Yan Campbell, Mary L. Fantacone and Adrian F. Gombart

Abstract

Background

Antimicrobial peptides (AMPs) are synthesized and secreted by immune and epithelial cells that are constantly exposed to environmental microbes. AMPs are essential for barrier defense and deficiencies lead to increased susceptibility to infection. In addition to their ability to disrupt the integrity of bacterial, viral and fungal membranes, AMPs bind lipopolysaccharides, act as chemoattractants for immune cells and bind to cellular receptors and modulate the expression of cytokines and chemokines. These additional biological activities may explain the role of AMPs in inflammatory diseases and cancer. Modulating the endogenous expression of AMPs offers potential therapeutic treatments for infection and disease.

Methods

The present review examines published data from both *in vitro* and *in vivo* studies reporting effects of nutrients and byproducts of microbial metabolism on the expression of antimicrobial peptide genes in order to highlight an emerging appreciation for the role of dietary compounds in modulating the innate immune response.

Results

Vitamins A and D, dietary histone deacetylases and byproducts of intestinal microbial metabolism (butyrate and secondary bile acids) have been found to regulate the expression of AMPs in humans. Vitamin D deficiency correlates with increased susceptibility to infection and supplementation studies indicate an improvement in defense against infection. Animal and human clinical studies with butyrate indicate that increasing expression of AMPs in the colon protects against infection.

Conclusion

These findings suggest that diet and/or consumption of nutritional supplements may be used to improve and/or modulate immune function. In addition, byproducts of gut microbe metabolism could be important for communicating with intestinal epithelial and immune cells, thus affecting the expression of AMPs. This interaction may help establish a mucosal barrier to prevent invasion of the intestinal epithelium by either mutualistic or pathogenic microorganisms.

Introduction

Cathelicidins, defensins and other antimicrobial peptides (AMPs) are an evolutionarily conserved component of the innate immune system and play an

important role in combating infection (18, 35, 60, 61). AMPs have two or more positively charged residues in acidic environments and can bind to the negatively charged membranes of both gram (-) and gram (+) bacteria, mycobacteria, fungi and enveloped viruses which in turn kills the pathogens by disrupting their cell membranes (32, 62) .

The human cathelicidin antimicrobial peptide (CAMP) gene encodes a preproprotein with an ~30 amino acid long N-terminal signal sequence, a 94 amino acid cathelin domain and a 37 amino acid C-terminal cationic AMP domain (LL-37) (62). The CAMP gene is primarily expressed in myeloid bone marrow cells and the pro-protein, hCAP18, is packaged in neutrophil specific granules (63). CAMP is also expressed by epithelial cells in tissues exposed to environmental microbes and the protein is secreted in semen, saliva and sweat providing barrier protection to mammals (27-29). Mice and humans lacking CAMP are susceptible to bacterial infections in numerous tissues (1, 18, 19). High circulating levels of hCAP18 are found in human plasma (64). The hCAP18/LL37 peptide binds to and neutralizes LPS (20), thus preventing its interaction with the LPS-binding protein and subsequent activation of TLR-4 (21), NF-κB signaling and cytokine release from host cells (22). LL-37 protects against LPS-induced sepsis in animals (23, 24) and kidney dialysis patients with low serum levels of hCAP18 are twice as likely to die from infectious disease or sepsis than patients with higher levels (65). Therefore,

circulating hCAP18 or LL-37 may protect against both bacterial infection and sepsis.

Like cathelicidin, defensins are expressed by immune cells and epithelial cells of tissues that are exposed to the environment (35). Defensins have six highly conserved cysteines that form disulfide bonds (61). Human defensins are classified into two families: α- and β-defensins (66). All defensins are expressed as a biologically inactive preproproteins and activated by cleavage of a prosequence (66). To date, six α-defensins have been identified with HNP-1-4 packaged in human neutrophil primary granules and HD-5 and -6 expressed by Paneth cells in the small intestine (35); HD-5 is also expressed by epithelial cells of the female genitourinary tract (67). The α-defensins have antimicrobial activity against a range of bacteria, viruses and fungi (66).

β-defensins are the most widely distributed of the antimicrobial peptides. They are typically 38-42 amino acids long (68) and expressed in monocytes, macrophages and dendritic cells (DCs) (69) as well as epithelial cells in the respiratory and urogenital tracts, skin and tonsil (66, 70). Human β-defensin 1 (HBD-1) is constitutively expressed in many tissues while HBD-2, -3 and -4 expression is induced by inflammatory stimuli, such as bacterial infection, LPS, IL-1β, TNF- α or phorbol-myristate-acetate (PMA) (35, 61, 66, 68, 71). β-defensins have shown activity against both gram (-) and gram (+) bacteria, fungi, viruses and parasites (72-74).

In this chapter, we review the regulation of the CAMP gene and the defensin gene family by various nutritional compounds and/or microbial byproducts of metabolism and discuss the potential importance of this regulation for human innate immune function.

Nutritional regulation of AMP expression

Vitamin D

CAMP: There are two forms of vitamin D that humans can utilize: vitamin D_2 (ergocalciferol) from fungi and vitamin D_3 (cholecalciferol) from animal sources and synthesized in the skin (2). Expression of the human CAMP gene is induced by $1,25(OH)_2 D_3$ and its analogs in various cell lines and primary cells (75-77). Vitamin D induction of CAMP is solely a human and non-human primate phenomenon (8, 9) due to a vitamin D response element (VDRE) located within a primate-specific retro-transposable element (Alu-SINE) found in the upstream promoter region of the CAMP gene (9).

In subsequent studies, it was demonstrated *in vitro* that toll like receptor (TLR) activation induced CAMP expression via a vitamin D-dependent pathway in macrophages (78). In the proposed model, sufficient levels of circulating $25(OH)D_3$, the precursor to $1,25(OH)_2D_3$, are required for induction of CAMP gene expression and production of adequate LL-37 peptide levels to

effectively combat infection (Fig. 1A) (78). Cathelicidin expression is also increased in keratinocytes via a similar pathway after skin wounding (79).

Interestingly, the vitamin D-mediated induction of CAMP potentially boosts antimicrobial activity against pathogens through direct killing by LL-37 and enhancing phagosome maturation (80). In THP-1 and human primary monocytes, $1,25(OH)_2D_3$ triggers the formation of autophagosomes and autophagolysosomes via a hCAP18/LL-37-mediated pathway (80).

Defensins: The HBD-2 gene (DEFB4) was induced by $1,25(OH)_2D_3$ through a VDRE in the promoter, but the induction was not as robust as that observed for the CAMP gene (75). Also, the induction of the HBD-2 gene in macrophages requires TLR activation and the convergence of the IL-1β and vitamin D pathways (Figure 2.1B) (81). *In vitro* studies demonstrated that activation of intracellular pattern recognition receptor nucleotide-binding oligomerization domain protein 2 (NOD2) by its ligand muramyl dipeptide (MDP), a lysosomal breakdown product of peptidoglycan from both gram-negative and gram-positive bacteria, induced the expression of the HBD-2 gene (82). More recently, $1,25(OH)_2D_3$ was shown to strongly induce the expression of NOD2/CARD15/IBD1 in primary human monocytic and epithelial cells (83). In the absence of $1,25(OH)_2D_3$, the activation of NOD2 by MDP activates NF-κB and there is a modest induction of the HBD-2 gene; however, pre-treatment with $1,25(OH)_2D_3$ followed by MDP leads to a robust,

synergistic induction of the HBD-2 gene (Fig. 1B) (83). Activation of the

vitamin D pathway alone is not sufficient to induce robust expression of HBD-2

and additional signaling pathways are required (81, 83). Treatment of normal

human keratinocytes with $1,25(OH)_2D_3$ increased HBD-3 mRNA levels in a

dose-dependent manner (84). Interestingly, treatment of lesional psoriatic

plaques with the vitamin D analog calcipotriol increased IκB-α protein levels

inhibiting the NF-κB signaling pathway and blocked IL-17A-induced expression

of HBD-2 in the plaques (85). Down-regulation of HBD-3 was also observed in

the lesional psoriatic plaques after treatment with calcipotriol (85).

Primary and secondary bile acids

Bile acids play an important role in digestion and absorption of dietary fat and

nutrients by the digestive system. They bind to and activate many nuclear

receptors and thus modulate metabolism. In humans, the two major primary

bile acids are cholic acid (CA) and chenodeoxycholic acid (CDCA) (86).

CDCA binds to farnesoid X receptor (FXR) (87) and up-regulates cathelicidin

expression (Figure 2.1C) (52). This regulation may contribute to sterility of the

bile duct.

Lithocholic acid (LCA), a toxic secondary bile acid, is a byproduct of CDCA

metabolism by bacteria in the colon. It is a low affinity ligand for FXR,

pregnane X receptor (PXR) as well as the VDR (88-90). LCA binds to the

VDR and significantly increases both human CAMP transcript and protein levels in human primary keratinocytes *(NHEK)* in a time-and dose-dependent manner (91). In the human colonic epithelial cell line HT-29, LCA and butyrate act synergistically to induce human CAMP gene expression (92). The LCA derivatives LCA acetate and LCA propionate induced expression of human CAMP more efficiently than LCA itself and were less toxic in tissue culture cells and mice, suggesting they are more potent VDR agonists and might be safer potential therapeutic agents than LCA (93). Unlike LCA, the secondary bile acid ursodeoxycholic acid (UDCA) is not a ligand for the VDR, but rather increases the levels of VDR in the nucleus that, in turn, increases CAMP expression (Figure 2.1C) (52).

Butyrate

CAMP: Butyrate is a short chain fatty acid produced in the colon by fermentation of dietary fiber by anaerobic bacteria. It is found in many foods, such as butter and cheeses and is a histone deacetylase inhibitor (HDACi) (94). *Shigella* infections down-regulate LL37 and β-defensin-1 levels in the colon of adult patients (95) and rabbit CAP18 levels in colon surface epithelium are reduced during *Shigella* infection; however CAP18 can be restored after oral administration of sodium butyrate (96).

Because of its unpleasant smell, butyrate is rarely used in clinical trials and instead 4-phenylbutyrate (PBA), an odorless and palatable derivative of butyrate is used clinically. In several human cell lines PBA up-regulates CAMP gene expression more potently than butyrate (97). It was recently shown that PBA also counteracts the down-regulation of cathelicidin in both the colon and lung by *Shigella* in rabbits (98). Furthermore, butyrate and its derivatives up-regulate transcription of cathelicidin in human colon epithelial cells (99) and synergistically induces human CAMP mRNA levels with $1,25(OH)_2D_3$ in lung epithelial and myeloid cells (77, 97). A chemical analogue of PBA, α-methylhydrocinnamate (ST7), can also dramatically up-regulate human CAMP mRNA transcription (97). These studies support a potential role for sodium butyrate and its derivatives in the treatment of human infections.

Defensins: There are few publications reporting the regulation of defensins by butyrate; however, it has been shown to induce HBD-2 mRNA expression in colonocytes (100) and pretreatment of gingival epithelial cells with sodium butyrate significantly induced HBD-2 expression in response to bacterial challenge (101).

Sulforaphane

Defensins: Sulforaphane (SFN) is a dietary HDACi found in cruciferous vegetables (e.g. broccoli and broccoli sprouts) that reactivates epigenetically-

silenced genes (102). Recently, it was demonstrated that SFN induces HBD-2 mRNA and protein expression in the Caco-2 human colon cancer cell line in a time- and dose-dependent manner (100). Inhibition of VDR by an antagonist significantly blocked the SFN-induced HBD-2 mRNA expression in these cells, and SFN treatment increased the expression of VDR in both Caco-2 and HT-29 cell lines, indicating that induction of HBD-2 by SFN was mediated by the vitamin D pathway (100). Whether these observations were due to epigenetic changes was not examined. The MAPK/ERK and NF-κB signaling pathways were also involved (100). It has not been determined if cathelicidin expression is regulated by SFN.

Retinoic acid

Retinoic acid (RA) is a metabolite of vitamin A which is important in several aspects of immune function (103). The retinoic acid receptor (RAR) forms a heterodimer with the retinoid X receptor (RXR) and interacts with specific retinoic acid response elements (RAREs) in the promoters of target genes (104). All-trans RA was shown to induce both porcine cathelicidin, PR-39 expression (105) and hCAP18 promoter activity (106). In contrast, induction of HBD-2, -3 and -4 by Ca^{2+}, TNF-α, IL-1β, INF-γ, PMA or *P. aeruginosa* in human keratinocytes is inhibited by RA (107). The promoter regions of all the inducible human β-defensin genes have several possible AP-1 binding sites

(107) and RA may suppress expression of these genes by antagonizing AP-1 (c-Jun/c-Fos)-mediated gene expression pathways (108). Therefore, RA may impair the innate immune response in human skin, thus increasing susceptibility to infections during RA therapeutic treatment (107). On the other hand, α-defensin HNP-1 was induced by both all-trans RA and 9-*cis*-RA in a dose-dependent manner, suggesting that retinoic acid may be important for myeloid cell expression of HNP-1 (109).

Human Health

Increasing endogenous cathelicidin and defensin expression may be particularly useful in the treatment of infections. Clinical studies have shown that asthma patients have reduced hCAP18 levels (110). High levels of circulating hCAP18 in hemodialysis patients at the beginning of their treatment was indicative of a significant decrease in 1-year mortality and there was a modest correlation with $1,25(OH)_2D_3$ levels, but not with $25(OH)D$ levels (65). In sepsis patients, lower $25(OH)D$, vitamin D binding protein (DBP) and cathelicidin levels were associated with severe illness and a positive correlation between $25(OH)D$ and cathelicidin levels was seen in all subjects (111). Additional studies are needed to substantiate these latest findings and determine if supplementation of vitamin D-deficient individuals with vitamin D or therapy with active analogs of $1,25(OH)_2D_3$ would boost plasma levels of cathelicidin and thus increase protection against infection and sepsis.

Supplementation may increase cathelicidin and defensin expression in tissues and immune cells, thus enhancing barrier function. Atopic dermatitis patients suffer from frequent skin infections; therefore, induction of CAMP expression in the skin may increase protection from infection (112). Patients supplemented with 4,000 IU/d of oral vitamin D for 21 days showed increased cathelicidin expression in skin lesions and a mild increase in unaffected skin, but a decrease in skin infection was not determined (112). *Ex vivo* infection of urinary bladder biopsies from post-menopausal women after vitamin D supplementation resulted in an increased induction of the CAMP gene and protein expression when compared to biopsies taken prior to supplementation (113). The studies to date would argue that it is important for individuals to have sufficient serum levels of 25(OH)D to allow for the production of adequate levels of cathelicidin during infection.

Epidemiological studies link vitamin D deficiency and increased rates of respiratory infections and there is interest in using vitamin D supplementation to reduce influenza infections and treat tuberculosis (114). In a small randomized trial of school age children, the vitamin D-supplemented group showed a nearly two-fold reduction in influenza A rates than the placebo group (115). In another study with participants from different racial groups it was shown that the maintenance of serum 25-hydroxyvitamin D levels at 38 ng/ml or higher was correlated with a two-fold reduction in the incidence of acute viral respiratory tract infections (116). In a double-blind randomized control

trial of tuberculosis patients starting treatment, vitamin D supplementation did not significantly affect the time that patient sputum cultures converted from positive to negative for *M. tuberculosis* growth in the study population as a whole; however, a significantly shortened time of conversion was observed in a sub-group of participants with the tt genotype of the TaqI vitamin D receptor polymorphism (117). The latter study demonstrates the importance that genetic differences among individuals may play in the outcomes of trials involving supplementation.

In contrast to the positive findings above, a number of negative studies with vitamin D have been reported. A randomized controlled trial of vitamin D3 supplementation showed that there was no obvious difference in the incidence and duration of severity of upper respiratory tract infections (URIs) between vitamin D (2000 IU/day) and placebo groups although 25(OH)D level increased significantly after 12 weeks in the vitamin D group compared to the placebo group (118). Another study found that there was no difference in serum 25(OH)D levels between groups of patients aged 1-25 months admitted to hospital with uncomplicated acute lower respiratory tract infection (ALRI) and healthy, similarly aged patients without a history of hospitalization for ALRI. This study suggested that vitamin D status was not a risk factor in hospitalization for ALRI (119). In a randomized, double-blind, placebo-controlled trial in TB clinics, the intervention and placebo groups were given 100,000 IU of cholecalciferol or vegetable oil at inclusion and again at five and

eight months after the start of treatment, but no significant difference was observed between the groups on mortality in patients with TB (120).

Very recently, a randomized, double-blind, placebo controlled clinical trial to determine the efficacy of sodium butyrate as an adjunct therapy with antibiotics in the treatment of shigellosis in patients was performed. Reduced rectal luminal content of inflammatory cells and pro-inflammatory cytokines, increased expression of LL-37 in the rectal epithelia and improved rectal histopathology as compared to the placebo group was evident (121). Nevertheless, efficacy of sodium butyrate treatment in clinical recovery was not observed as in studies with rabbits (96, 121). Important differences between the animal and human studies included oral delivery in rabbits versus delivery by enema in the humans and the lack of antibiotics in rabbits (121). It remains to be determined if oral delivery of butyrate compounds would be more efficacious as the treatment would not be eliminated as quickly as it is with an enema due to repeated bouts of diarrhea (121).

Conclusions

Accumulating evidence demonstrates that nutrients and microbial byproducts derived from the metabolism of dietary factors may play a critical role in modulating the innate immune response via regulation of AMP gene expression. The byproducts of gut microbe metabolism are potentially

important for increasing AMP expression by epithelial cells of the luminal lining of the digestive tract, thus establishing a mucosal barrier preventing contact of microbes and pathogens with the intestinal epithelium (1, 92). Increasing the consumption of dietary fiber, thus increasing short chain fatty acid production, and/or food containing dietary HDAC inhibitors could increase the expression of AMPs in the digestive tract. This in turn would increase barrier protection in the gut lumen and reduce the rate or severity of intestinal infections. This is nicely demonstrated in the studies using a rabbit model of shigellosis (96, 98) and has shown some promise in a human trial (121).

The potential for VDR and FXR to bind to the VDRE in the human CAMP gene offers an abundance of new compounds that can be synthesized or obtained from the diet to produce potent ligands for these steroid hormone receptors and used to enhance AMP expression. For example, the therapeutic use of active vitamin D is hampered by the toxic side-effects of hypercalcemia (1) and although synthetic analogs reduce these side-effects they still exist. On the other hand, analogs of LCA have been shown to lack this side-effect, but activate VDR-target genes like CAMP (93).

The VDR may act as a receptor for additional nutritional ligands including curcumin and polyunsaturated fats including α-linolenic acid, docosahexaenoic acid, eicosapentaenoic acid and arachidonic acid (122); however, we recently demonstrated that in tissue culture only curcumin modestly induced CAMP gene expression through a yet uncharacterized VDR-independent mechanism

(Guo, 2012). Identification of additional nutritional compounds and their synthetic analogs would provide further options for increasing AMP gene expression for therapeutic uses.

AMPs are critical to the barrier defense provided by the innate immune system and deficits in AMP production can increase susceptibility to infections. As discussed, numerous nutritional compounds and microbial byproducts from metabolism of dietary factors have been shown to regulate the expression of AMPs. The possibility of improving the innate immune response or boosting barrier defenses toward infection by increasing the consumption of food items rich in these nutrients is an exciting prospect, but well-designed clinical and animal model studies need to be performed to demonstrate that compounds obtained from the diet can improve immune function by increasing AMP levels.

Figure 2.1
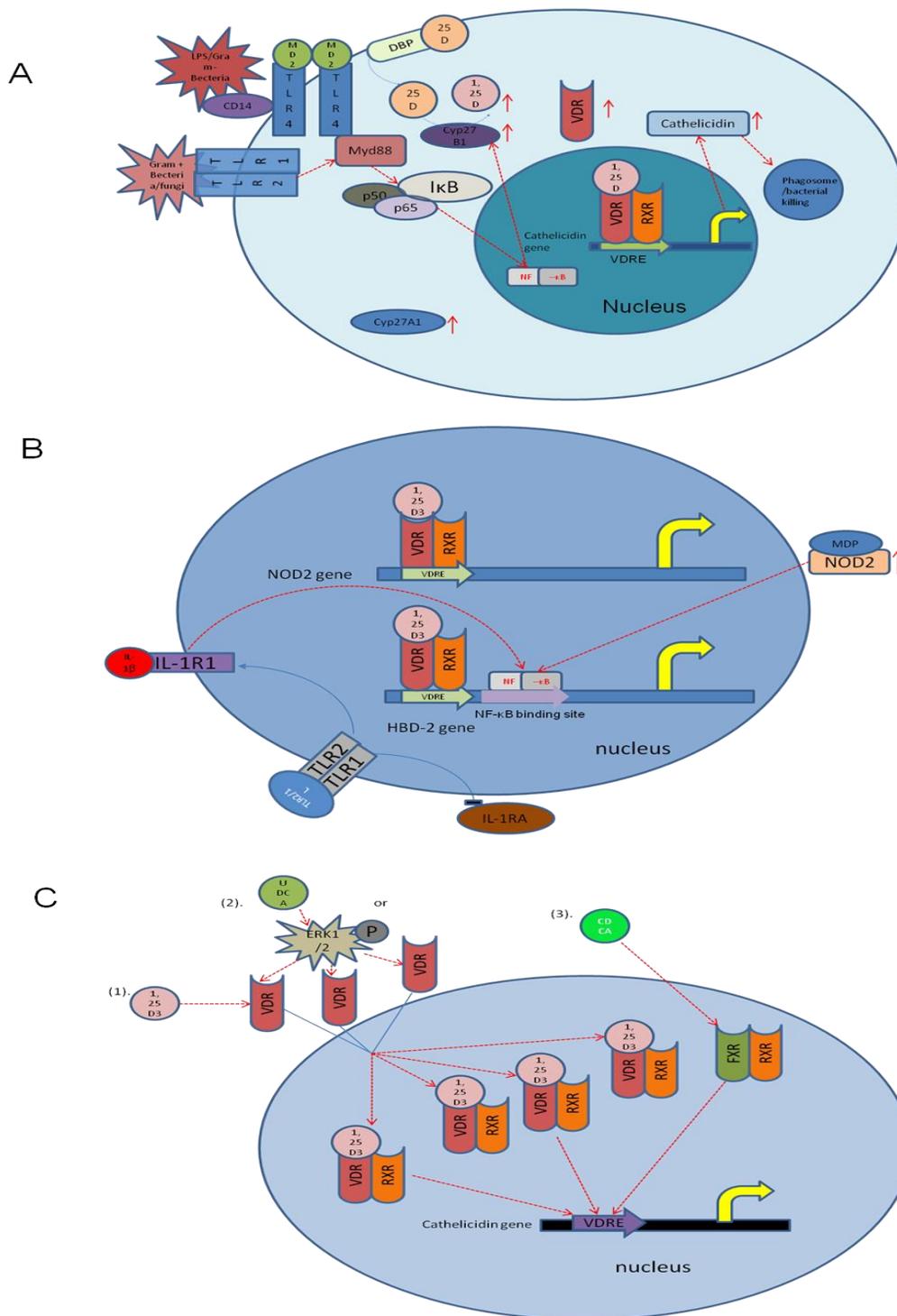
Figure 2.1. Regulation of AMP gene expression through the VDR or FXR pathways. (A) Vitamin D-pathway dependent TLR activation of CAMP gene expression. TLR-signaling activates NF-κB binding and induces VDR and CYP27B1 expression, the enzyme that catalyzes the conversion of 25(OH)D to 1,25(OH)$_2$D. The expression of the vitamin D receptor (VDR) is increased. In the presence of locally high levels of 1,25(OH)$_2$D, ligand-bound VDR:RXR heterodimers translocate into the nucleus and bind to the VDRE in the promoter of the human CAMP gene inducing its expression. (B) Vitamin D-mediated regulation of the human DEFB4 gene. (1) Direct induction: TLR stimulation activates the vitamin D-pathway as described in Figure 2.1A. Also it up-regulates expression of IL-1β and IL-1R1 and down-regulates expression of IL-1R antagonist (IL-1RA). IL-1R1 activates the NF-κB transcription factor which binds to the promoter proximal NF-κB binding site in the DEFB4 gene and induces HBD-2 expression together with the VDR:RXR heterodimer that binds to the VDRE in the promoter of DEFB4 gene. Indirect induction: In the presence of 1,25(OH)$_2$D$_3$ the VDR:RXR heterodimer that binds the VDRE in the NOD2 gene promoter and induces expression of the NOD2 protein. Activation of NOD2 by its agonist muramyl dipeptide stimulates the NF-κB transcription factor that binds to the promoter proximal NF-κB binding site in the DEFB4 gene to induce its expression. (C) Regulation of human CAMP gene expression by vitamin D and bile salts in biliary cells. 1) 1,25(OH)$_2$D$_3$ activates the VDR:RXR heterodimer that then binds to the VDRE in the CAMP gene promoter; 2) UDCA activates the ERK1/2 signaling pathway that, in turn, induces VDR protein expression and induction of CAMP gene expression in the presence of 1,25(OH)$_2$D$_3$ and 3) CDCA binds to the FXR:RXR heterodimer that binds to the VDRE in the CAMP gene promoter.

Authors' Contributions

YC performed the initial literature search, wrote the preliminary document, assisted with and completed the final version. MLF modified the preliminary document. AFG directed and augmented the literature search as well as modified and revised the preliminary document.

Regulation of human cathelicidin antimicrobial peptide gene by the xenobiotic receptor FXR and its dietary ligands

Chapter 3

Abstract

The human <u>c</u>athelicidin <u>anti</u>microbial <u>p</u>eptide (CAMP) is a broad spectrum microbicidal agent and modulator of both the innate and adaptive immune system. It is induced by 1,25-dihydroxyvitamin D (1,25(OH)$_2$D$_3$) through activation of the <u>vitamin D</u> receptor (VDR) and primary bile salts through activation of the xenobiotic nuclear receptor <u>f</u>arnesoid <u>X</u> receptor (FXR). Both receptors are expressed by enterohepatic and gastrointestinal (GI) tissues and play important roles in GI immunity and homeostasis. The plant polyphenol xanthohumol (XN) may act as an FXR ligand; therefore, we hypothesized that XN would regulate FXR target gene expression including the human CAMP gene. XN increased BSEP (bile salt export pump) promoter-driven luciferase activity in transfected HEK293 cells and required FXR and the FXR-binding site. Also, XN induced the mRNA expression of FXR target genes BSEP, SHP (small heterodimer partner), IBABP (ileul bile acid binding protein) and FXR in biliary carcinoma cell lines. Furthermore, XN required FXR to activate a CAMP promoter-luciferase reporter construct and it induced endogenous CAMP gene expression in biliary carcinoma cells. Additionally, the XN metabolite 8-prenylnaringenin (8-PN) induced endogenous expression of CAMP, SHP and FXR. Combinations of 1,25(OH)$_2$D$_3$ and either XN or 8-PN synergistically induced endogenous CAMP gene expression in cells. We conclude that the plant polyphenol XN and its metabolite 8-PN act as FXR ligands and regulate expression of the human CAMP gene alone or in combination with

$1,25(OH)_2D_3$. Future studies will elucidate the importance of this regulatory mechanism in contributing to GI tract barrier defense and health.

Introduction

The human cathelicidin antimicrobial peptide (CAMP) is a potent microbicidal agent that is active against a wide range of pathogens and plays an important role in both innate and adaptive immunity (1). The human CAMP gene encodes an 18 kDa preproprotein (hCAP18) comprised of an N-terminal signal peptide, a 94-amino acid-long cathelin domain and a 37 amino acid long AMP domain (LL-37) at its C-terminus that is activated upon cleavage from the cathelin domain by proteases (62). The CAMP gene is mainly expressed in myeloid bone marrow cells and the protein gets packaged into neutrophil-specific granules (63). Also, the hCAP18 protein is expressed by epithelial cells and organs that are exposed to environmental microbes, such as the respiratory, digestive and reproductive tracts. It is secreted into semen, saliva and sweat (27-29).

Two nuclear receptor family members, the vitamin D receptor (VDR) and the farnesoid X receptor (FXR) induce expression of the human CAMP gene (8, 52, 109). 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) and its synthetic analogs act as high affinity ligands, while the secondary bile acid lithocholic acid and it analogs act as low affinity ligands for the VDR to induce CAMP gene

expression (8, 93). Primary bile acid-chenodeoxycholic acid (CDCA), the natural ligand for FXR (123), induced CAMP gene expression in human biliary carcinoma cells (52). Although the VDR binding site (VDRE) was implicated, experimental evidence demonstrating it functioned as the binding site for FXR-mediated activation of the gene was not provided.

In addition to bile acids, several plant polyphenols have been identified as FXR ligands (53, 124-128). Xanthohumol (XN) is a dietary prenylated chalconoid isolated from hops (*Humulus lupulus*) and was suggested to be an FXR ligand based on its ability to activate a bile salt export pump (BSEP)-firefly luciferase reporter construct and its ability to affect lipid and glucose metabolism *in vivo* (53). A requirement for FXR or the FXR-binding site in the BSEP promoter was not demonstrated. Therefore, it remains to be determined if XN actually mediates its effects through the FXR.

Numerous biological activities are attributed to XN, including effects on the immune system. It inhibits the production of pro-inflammatory cytokines, nitric oxide, IL-1β and hepatic inflammation and fibrosis (54, 55, 129-131). XN treatment also inhibits IL-12 production and reduces the degree of oxazolone induced chronic allergic contact dermatitis (56). These findings suggest a potential use for XN in preventing or treating inflammatory diseases of the gastrointestinal tract. The goal of this research was to (1) identify the molecular mechanism by which XN mediates its effects on gene expression by

demonstrating that it is a bona-fide ligand for FXR, (2) demonstrate that it activates binding of FXR to the FXRE in the BSEP promoter and induces other endogenous FXR target genes in cell culture, and (3) demonstrate that the human CAMP gene is an FXR target gene that is induced by XN. Using a combination of promoter-luciferase constructs and induction of endogenous target gene expression, we provide strong evidence that XN and its metabolite 8-prenylnaringenin (8-PN) are bona fide agonists for FXR. Furthermore, both compounds cooperatively induce CAMP gene expression with $1,25(OH)_2D_3$ suggesting a synergy between the two compounds. Our findings demonstrate that XN and 8-PN act on FXR as selective bile acid modulators that are capable of regulating an important antimicrobial barrier defense molecule.

Materials and Methods

Reagents

GW4064 and Z-guggulsterone were purchased from Tocris Bioscience (Bristol, UK). CDCA was purchased from Sigma-Aldrich (St. Louis, MO). XN was isolated from female inflorescences of hops (*H. lupulus*) (132). 8-PN was synthesized by prenylation of naringenin (133). The BSEP promoter-reporter construct p-1445/Luc (wild type BSEP-Luc), p-1445/Luc (mutant BSEP-Luc) with either a wild type CMX-FXR or empty expression vector for human FXR was kindly provided by Dr. M. Ananthanarayanan (Yale University School of

Medicine, New Haven, CT)(134). To generate the human CAMP-luciferase reporter construct, the firefly luciferase gene was amplified using primers that incorporated SanD1 restriction enzyme sites on each end of the resulting PCR product. The cloned luciferase insert was sequenced to verify its integrity. A 6.2 kb EcoRI-KpnI genomic fragment encompassing the human CAMP gene was isolated from a lambda FIX II phage library and subcloned into pGEM-3Z. The luciferase insert was ligated into a unique SanD1 site in exon 2 of the human CAMP gene to generate an in-frame fusion of hCAP18 with luciferase (CAMP6.2-Luc, Figure 3.1). Serial deletions from the 5'-end (Figure 3.1) were generated using an Erase-a-Base system as instructed by the manufacturer (Promega Corporation, Madison, WI USA). The VDRE and FXRE site directed mutations (Figure 3.1) were generated using a QuikChange II system as directed by the manufacturer (Agilent Technologies, Santa Clara, CA USA).

Antibodies

The three antibodies used were purchased: mouse monoclonal anti-FXR (D-3; cat. # sc-25309; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.); mouse monoclonal anti-GAPDH (cat.# 10R-G 109a, Fitzgerald Industries International, Acton, MA 01720 USA); goat anti-mouse HRP conjugated (cat.# 1858413; Thermo Scientific Inc. Rockford, IL USA 61101); rabbit polyclonal anti-hCAP18 (3.6mg/ml, a gift from Dr. N. Borregaard, University of Copenhagen, Denmark).

Cell Culture

The human biliary carcinoma cell lines Mz-ChA-1, KMBC and HuCCT-1 were generously provided by Dr. G. J. Gores (Mayo Clinic, Rochester, MN). HEK293 cells were kindly provided by Dr. B. Frei (Oregon State University, Corvallis, OR). Cells were treated with compounds as described in the figure legends. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Mediatech Inc. Manassas, VA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% Pen/Strep (Life Technologies Corporation, Carlsbad, CA). Cell cultures were incubated at 37°C in a humidified 5% CO_2 incubator.

Western Blot

Protein lysates were prepared using RIPA buffer (50mM Tris-HCl PH8.0, 1% NP-40, 0.5% DOC, 150mM NaCl, 0.1% SDS) from HEK293 cells transfected with either the CMV-FXR or empty expression vector. Protein concentrations were determined using a modified Bradford reagent (Bio-Rad Life Science, Hercules, CA). Proteins were electrophoresed through a 10% SDS-polyacrylamide gel. For each sample, 100 μg of protein was loaded per lane. After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was then blocked in 5% non-fat dry milk for 30 min and incubated overnight at 4^oC with primary mouse anti-FXR

antibody (diluted 1:400) in PBS+Tween-20. GAPDH was used as a loading

control, visualized with a mouse anti-GAPDH antibody (diluted 1:10,000) in

PBS+Tween-20 containing 5% non-fat dry milk. The goat anti-mouse HRP

secondary antibody was diluted 1:10,000 in PBS-Tween 20 with 5% non-fat

milk and the blot was incubated at room temperature for 2h. Signal was

detected using Thermo SuperSignal West Femto Chemiluminescent Substrate

(Thermo Fisher Scientific Inc., Rockford, IL USA).

HEK293 cell transfection and luciferase reporter assay

Initially, the induction of the wild type CAMP6.2-Luc reporter construct by XN

was not observed following standard transfection conditions with Dulbecco's

modified Eagle's medium (DMEM; Mediatech Inc. Manassas, VA)

supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and

1% Pen/Strep (Life Technologies Corporation, Carlsbad, CA). As this may

have been due to potential FXR ligands present in the serum, transfection

conditions were optimized in serum-free medium to eliminate any possible

FXR ligands that could increase the baseline luciferase activity of the wild type

hCAMP6.2-luc reporter construct. Different reduced or serum-free conditions

were tested and the final optimized conditions involved seeding HEK293 cells

in 12-well plates at a density of 6 x 10^5 cells/well in Opti-MEM I reduced serum

media (Life Technologies Corporation, Grand Island, NY 14072, USA) and

culturing overnight before transfection. Cells were transfected using

Lipofectamine 2000 transfection reagent according to the manufacturer's

instructions (Life Technologies Corporation, Grand Island, NY 14072, USA).

Briefly, transfection mixtures for each well contained 2 μl Lipofectamine 2000,

0.59 μg of p-1445/Luc (wild type), p-1445/Luc (mutant) reporter constructs or

wild type CAMP6.2-Luc (WT), mutant VDRE-CAMP6.2-Luc (VDREmt), mutant

FXRE-CAMP6.2-Luc (FXREmt), p-1057△CAMP6.2-Luc (2-136), p-

151△CAMP6.2-Luc (2-139), 0.3 μg of CMX-FXR or empty expression vector,

0.1 μg phTKRenilla and 0.01 μg EGFP to monitor transfection efficiency. Cells

were cultured in transfection media at $37^{o}C$ for 18 h. The transfection medium

was replaced with fresh Opti-MEM I medium and 24 h post-transfection cells

were treated with compounds as described in the figure legends and

incubated at $37^{o}C$ for an additional 18 h. Cells were harvested in passive lysis

buffer and dual-luciferase assays were performed as instructed by the

manufacturer (Promega Corporation, Fitchburg, WI 53711, USA).

RNA isolation and quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the cultured cells using TRIZOL Reagent

according to the manufacturer's instructions (Life Technologies Corporation,

Grand Island, NY 14072, USA). cDNAs were synthesized using iScript

Reverse Transcription Supermix (Bio-Rad Life Science, Hercules, CA 94547,

USA). qRT- PCR reactions were performed using Ssofast Evagreen Supermix or Ssofast Probe Supermix as instructed by the manufacturer (Bio-Rad Life Science, Hercules, CA 94547, USA). Sequences for primers and probes for the genes analyzed by qRT-PCR are shown in Table 1.

Data Analysis

All samples for luciferase assays were measured in duplicate or triplicate. All cDNA samples were measured in triplicate. The results were represented as the mean value +/- the standard deviation (S.D.). Statistically significant differences were determined using a Dunnett's ANOVA (Statgraphics Centurion software; Statpoint Technologies, Inc., Warrenton, Virginia).

Results

XN induces FXR target gene BSEP promoter activity

To determine if XN functions as an FXR ligand in cell culture, we transfected HEK293 cells, which do not express endogenous FXR (Figure 3.2 A), with a BSEP luciferase-reporter construct containing either a wild type or non-functional mutant FXRE together with a wild type FXR or empty expression vector (Figure 3.2B). The transfected cells were treated with known natural FXR ligands CDCA and guggelsterone, and synthetic ligand GW4064, as

positive controls (135). The wild type FXRE-containing BSEP promoter construct was not activated by the various ligands in the absence of transfected FXR (Figure 3.2 B, wild type BSEP-Luc). Expression of FXR significantly increased BSEP promoter activity in the absence of ligand, suggesting that the overexpressed FXR undergoes some binding to the FXRE even in the absence of an added exogenous ligand. Nevertheless, the addition of ligand significantly increased BSEP promoter activity above this baseline level, about two-fold for CDCA and guggelsterone and about six-fold with GW4064 compared with untreated cells (Figure 3.2 B, wild type BSEP-Luc+FXR). XN induced BSEP promoter activity to a similar level as observed for CDCA and guggelsterone in the presence of FXR, but not in its absence (Figure 3.2 B, wild type BSEP-Luc). Mutation of the FXRE abrogated BSEP promoter activity both in the presence or absence of FXR upon treatment with the different ligands and XN, although expression of FXR slightly increased mutant BSEP promoter activity in a ligand independent manner (Figure 3.2 B, mutant BSEP-Luc and mutant BSEP-Luc+FXR), but this slight induction was not statistically significant when compared to the transfections lacking FXR (Figure 3.2 B, mutant BSEP-Luc-FXR; $p > 0.05$). Taken together, these data demonstrate that in the presence of FXR and a functional FXRE, XN activates the BSEP promoter to levels comparable with known ligands. These data are consistent with XN functioning as a ligand for FXR.

XN induces endogenous expression of FXR target genes in biliary carcinoma cell lines.

To further demonstrate that XN acts as a ligand for FXR, we determined the expression of four known FXR target genes SHP, BSEP, IBABP and FXR in the biliary carcinoma cell lines Mz-ChA-1, KMBC and HuCCT-1 after treatment with either vehicle, CDCA, or XN (136-138). Expression of each mRNA was determined by qRT-PCR.

Statistically significant induction of the FXR, BSEP, SHP, and IBABP mRNAs by XN was observed in most cell lines (Figure 3.3 A-C) with the exceptions being BSEP and IBABP in KMBC cells (Figure 3.3 B). Somewhat similar results were observed for CDCA (Figure 3.3 A-C), but there were many more exceptions. These were BSEP in Mz-ChA-1 cells (Figure 3.3 A), IBABP in KMBC cells (Figure 3.3 B) and FXR, BSEP and SHP in HuCCT-1 cells (Figure 3.3 C). Interestingly, these results show that XN consistantly induced expression of the target genes to similar or even higher levels than CDCA. These data further demonstrate that XN is capable of inducing FXR-target gene expression as well as currently known agonists indicating that it functions as a strong ligand for FXR.

XN and its metabolite 8-PN induce human CAMP gene mRNA level

expression

The human CAMP gene was shown to be induced by CDCA through FXR

(52). To determine if XN treatment could also induce expression of the human

CAMP gene, we treated three biliary cell lines: Mz-ChA-1, HuCCT-1 and

KMCH-1 with 10 µM XN. The qRT-PCR results showed that XN significantly

induced expression of CAMP more effectively than CDCA in all the cell lines

tested (Figure 3.4).

Human intestinal microbiota metabolize XN into 8-PN, a potent phytoestrogen

(59) and is known to activate both estrogen receptor α and β (139, 140). 8-PN

also can interact with the ligand binding domain of FXR (Yang, et al.,

manuscript in preparation). Because the expression of VDR is induced by 17β-

estradiol (E2) in colon and breast cancer cells (141), we hypothesized that 8-

PN may induce human CAMP expression either through increased VDR

expression via the activation of estrogen receptor or through FXR activation or

both. To test this hypothesis, we treated Mz-ChA-1 cells with 100 µM 8-PN

alone or in combination with either 1 nM or 10 nM $1,25(OH)_2D_3$, respectively.

The qRT-PCR results showed that 8-PN weakly induces human CAMP

expression alone (4-fold), but not quite reaching statistical significance in this

experiment. However, it shows a cooperative enhanced response when

combined with either 1 nM (18-fold) or 10nM $1,25(OH)_2D_3$ (40 fold) (Figure 3.5 A).

In separate studies, neither 8-PN nor estradiol induced VDR expression in these cells (data not shown) suggesting that the cooperative induction was not due to signaling through the estrogen receptor. Furthermore, estradiol treatment did not induce CAMP either when added alone or in combination with $1,25(OH)_2D_3$ (Figure 3.6).

Similar to 8-PN, XN alone was a weak inducer of CAMP expression, but showed strong cooperativity in CAMP induction when added with $1,25(OH)_2D_3$ (Figure 3.5 A). In further studies, both XN, 8-PN similarly induced expression of the FXR target genes FXR and SHP, but in these cases no cooperative induction with $1,25(OH)_2D_3$ was observed (Figure 3.5 B and C). Based on these findings, we conclude that rather than mediating its effects through estrogen receptor, 8-PN acts very much like XN, as an FXR agonist.

XN induction of human CAMP driven luciferase activity requires FXR

To determine if XN induced human CAMP gene expression through FXR, we co-transfected HEK293 cells with the wild type CAMP6.2-Luc (Figure 3.1) construct with or without an FXR expression vector. Without FXR, XN treatment failed to induce luciferase activity; however, co-transfection with FXR and treatment with XN at either 1 µM or 10 µM concentrations significantly induced luciferase activity (Figure 3.7 A). It was further tested if

guggulsterone, a potential antagonist of FXR (125) could block the activation of the wild type 6.2CAMP-Luc construct by XN. Incubating transfected HEK293 cells with 10 µM guggulsterone indeed abrogated induction of CAMP promoter-driven luciferase activity by 10 µM XN, further supporting the hypothesis that XN induces human CAMP expression through FXR (Figure 3.7 B).

XN-mediated induction of human CAMP expression may require both the VDRE in the Alu-SINE and an FXRE in the third exon

The VDRE localized in the Alu-SINE was implicated in the induction of the human CAMP gene by FXR, but definitive experimental data were not provided (52). We have identified two other possible FXR binding sites including one in the 5'-promoter region and an IR1-type site in exon 3 of the human CAMP gene (Figure 3.1). To determine if these sites were involved in mediating CAMP gene expression through FXR, we generated site-directed mutations in the CAMP6.2-Luc construct (mutant VDRE-CAMP6.2-Luc and mutant FXRE-CAMP6.2-Luc) and two 5'-serial deletions p-1057ΔCAMP6.2-Luc (2-136), p-151ΔCAMP6.2-Luc (2-139) (Figure 3.1). In HEK293 cells, the wild type CAMP6.2-Luc was activated about 5-fold by XN and each of the mutant constructs were activated about 2.5-fold by XN (Figure 3.8). These results showed that although XN can still significantly up-regulate luciferase activity of the mutant and deletion constructs, the level of induction is

significantly lower than that is seen with the wild type CAMP6.2-Luc construct (Figure 3.8). This suggests that FXR mediated induction of the CAMP gene involves both the VDRE in the Alu-SINE and the IR1-type FXRE identified in the third exon of the CAMP gene. Further experiments with constructs combining the VDRE and the FXRE mutations need to be performed to determine if both sites are required for full activation of the CAMP promoter.

Discussion

FXR is expressed mainly in the liver and intestines where it functions to maintain the homeostasis of bile salt, fat and glucose (40, 49, 142, 143). FXR is also involved in liver (144) and intestinal (145) immunity, metabolism (40-44), cell apoptosis (146), proliferation (147) and differentiation (148). Fxr-/- mice display a proinflammatory and profibrotic phenotype in the colon under naive conditions (145) with enhanced expression of inflammatory genes and proteins of the extracellular matrix in the colon (145). Also, macrophages from fxr-/- mice express more IL-1β, IFN-γ, and TNF-α compared with macrophages from wild-type mice either under basal conditions or in response to LPS stimulation (145). Fxr-/- mice are highly susceptibility to drug induced colon inflammation (145), hepatitis (144) and gastric injury (50) and lack the protection provided by FXR ligands (50, 144, 145). FXR induces the expression of several genes related to gut mucosal defense in a mouse

model, therefore, it plays an important role in preventing bacterial overgrowth and promoting gut health (49).

Natural dietary products have been used in treating various human diseases throughout history. Our study aimed to gain insight into the binding of plant-derived compounds XN and its metabolite 8-PN to FXR and activation of downstream target genes. Studies using hydrogen/deuterium exchange mass spectrometry showed that XN and 8-PN interact with the FXR-ligand binding domain (Yang et al., manuscript in preparation) and one previous publication suggested that XN is an FXR ligand because it induced BSEP luciferase reporter activity (53); however, the study didn't prove that induction of BSEP expression required FXR or its binding to the FXRE in the promoter. Our data showed that XN only induced BSEP luciferase activity with FXR and a functional FXRE. In addition, we showed that XN induced expression of endogenous FXR target genes FXR, SHP, IBABP and BSEP in biliary epithelial carcinoma cell lines. In fact, XN induced several endogenous FXR target genes even more effectively than the natural ligand CDCA. Taken together, these results conclusively demonstrated that XN is a bona-fide ligand for FXR.

The primary bile salt CDCA induces expression of the human CAMP gene via FXR (52). Consistent with these findings, XN also induced endogenous CAMP gene expression in biliary carcinoma cell lines. In the original study, CDCA

was shown to increase the binding of FXR to a VDRE consensus sequence and to activate a VDRE-consensus-sequence-driven promoter in the human biliary carcinoma cell line Mz-ChA-1 (52); however, the actual VDRE sequence located in the human CAMP promoter was not tested and a requirement for the VDRE in the CAMP promoter was not demonstrated. Using a CAMP6.2-Luc reporter construct, we demonstrated that induction by XN is FXR-dependent and it could be blocked by FXR antagonist guggulsterone. Furthermore, mutation or deletion of the VDRE in the Alu-SINE in the CAMP promoter significantly reduced activation of the promoter by XN and FXR, but didn't completely abrogate it. Similar levels of induction were observed when the potential FXRE site in exon 3 was mutated or deleted. This observation indicated that XN induction of human CAMP expression requires both the VDRE in the Alu-SINE and FXRE in exon3. Future work will focus on determining if both sites are required for induction of the CAMP gene by FXR using a double binding site mutant luciferase construct and ChIP-sequencing experiments.

Human intestinal microbiota metabolize XN into 8-PN a potent phytoestrogen (59). Although the concentration of 8-PN in beer is considered too low to affect human health, its precursor isoxanthohumol (IX) is the prevailing prenylflavonoid in beer and is about 10–30 times more abundant than 8-PN (133). Therefore, metabolism of IX into 8-PN in the human intestine could increase intestinal 8-PN concentrations around 10-fold (59), which means the

uptake of active doses after a small amount of beer consumption could fall within the range of biological activities (149). 8-PN has been shown to interact with the FXR ligand binding domain by hydrogen/deuterium exchange mass spectrometry (Yang, et al., manuscript in preparation). Like XN, it strongly induced CAMP, FXR and SHP expression suggesting that it acts as a bona-fide ligand for FXR. Interestingly, cooperative induction of CAMP gene expression was observed when either XN or 8-PN were combined with $1,25(OH)_2D_3$. This was not observed for the FXR or SHP genes which are not known targets of the VDR. One possible explanation for the cooperative induction is that the activation of ER by 8-PN might increase VDR expression through an ERK1/2 signaling pathway (141). Our data does not support this mechanism as 8-PN did not induce VDR expression. Our results are consistent with the binding of both the VDR and FXR to the CAMP promoter to increase transcription.

The mouse and human cathelicidins are expressed primarily at the surface and upper crypts in differentiated cells of the normal colon and ileum (distal small intestine) with little expression observed within the deeper crypts and some expression observed in the proximal small intestine and duodenal Brunner's glands (150-152). Human cathelicidin is induced by short chain fatty acids (SCFA) like butyrate, isobutyrate and proprionate (152). Consistent with its expression in the colon, mice deficient in CAMP suffer from greater colonization of the colon surface, epithelial cell damage and systemic

dissemination of infection when challenged with *Citrobacter rodentium* (153). In contrast, treatment of rabbits with oral butyrate increased CAMP in the surface epithelium of the colon and reduced clinical illness, inflammation and bacterial load in the stool during *Shigella* infection (96). In humans, butyrate treatment via enema induced CAMP expression in rectal epithelial and improved rectal histopathology (121). In experimental colitis induced by dextran sulfate sodium (DSS), intrarectal administration of murine cathelicidin ameliorated the severity of disease (154). Furthermore, cathelicidin expression in the colon was reduced in TLR9-deficient mice with DSS-induced colitis and CAMP-deficient mice developed a more severe form of colitis (155). Our data suggests that the consumption of diets rich in plant polyphenols could result in high local concentrations of FXR ligands that would increase expression of CAMP by epithelial barrier cells in the GI tract. As tea catechins, grape seed procyanidin extract and soy-derived phytosterols have also been identified as FXR ligands (126-128), it will be interesting to determine if these compounds similarly regulate CAMP gene expression.

Table 3.1. Oligonucleotides used for qRT-PCR

Gene	Primers	Detection
BSEP	F: 5'- GGGCCATTGTACGAGATCCTAA-3' R: 5'-TGCACCGTCTTTTCACTTTCTG-3'	EvaGreen
SHP	F: 5'-GGTGCAGTGGCTTCAATGC-3' R: 5'-GGTTGAAGAGGATGGTCCCTTT-3'	EvaGreen
IBABP	F: 5'-TCAGAGATCGTGGGTGACAA-3' R: 5'-TCACGCGCTCATAGGTCA-3'	EvaGreen
FXR	F: 5'-CTACCAGGATTTCAGACTTTGGAC-3' R: 5-'GAACATAGCTTCAACCGCAGAC-3'	EvaGreen
β-actin	F: 5'-GTACATGGCTGGGGTGTT-3' R: 5'-TTCTACAATGAGCTGCGTGT-3'	5'-FAM-AGGTCTCAAACATGA TCTGGGTCATCTT[BHQ]-3'

Figure 3.1
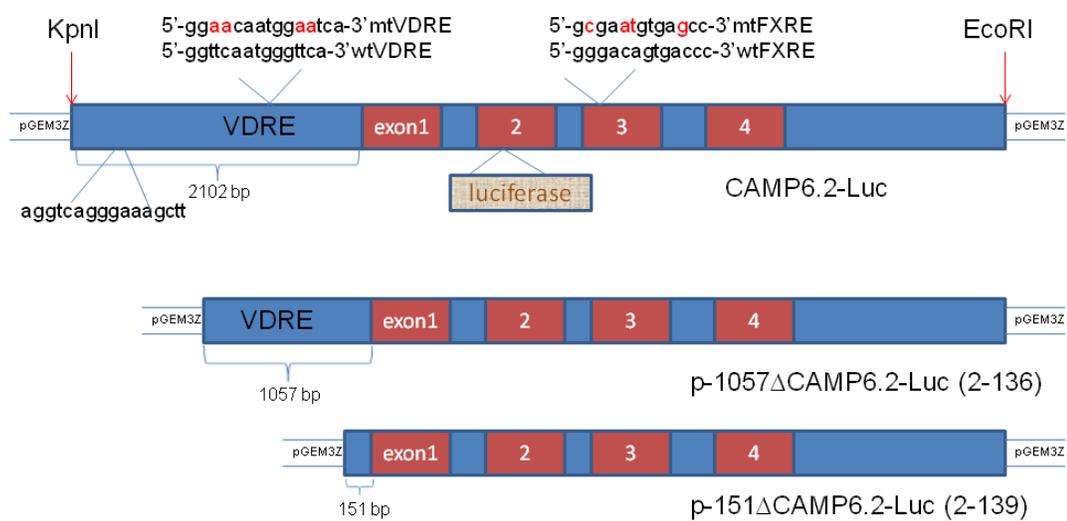
Figure 3.1 Structure of hCAMP6.2 luciferase reporter construct and its various deletion/mutation constructs. Luciferase gene was inserted into the 2nd exon of the human CAMP gene by in-frame insertion. Both WT and mutant VDRE/FXRE were shown. The deletion constructs p-1057∆CAMP6.2-Luc (2-136) and p-151∆CAMP6.2-Luc (2-139) has 1057 bp and 151bp of the promoter left respectively.

Figure3.2

A
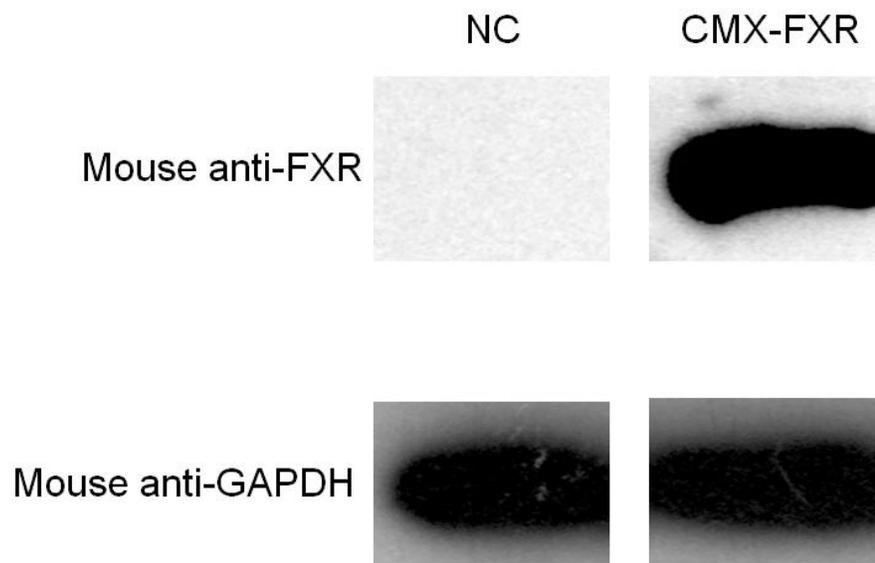
Figure 3.2 Part B
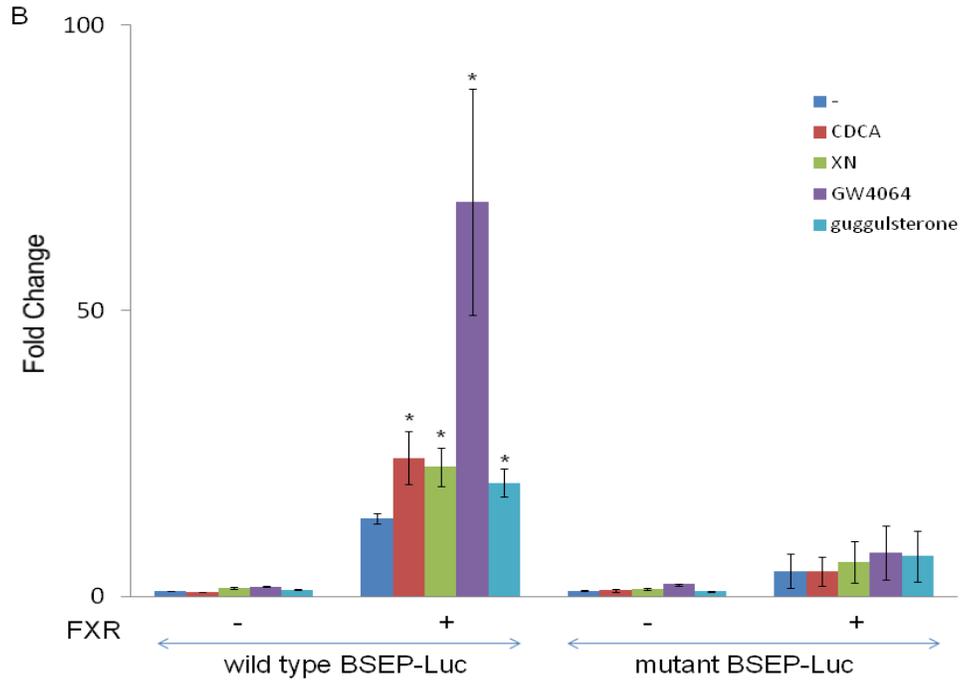
Figure 3.2. XN induces FXR target gene BSEP luciferase activity in HEK293 cells. A) HEK293 cells do not expression endougenous FXR (NC). FXR expression was observed after the transfection of CMX-FXR into HEK293 cells. Primary antibodies used were: mouse anti-FXR (1:400), mouse anti-GAPDH (1:10000). Exposure time: 25min for FXR, 10 seconds for GAPDH. B) Transfected HEK293 cells were incubated at $37^{o}C$ overnight with 100% EtOH, 10μM CDCA, 1μM XN, 1μM GW4064, 10μM guggulsterone respectively. The data shown represents the means of two sets of independent duplicated experiments with duplicated readings. Statistical significance was determined by ANOVA. * $P<0.05$ for all the treatment compared to the control group.

Figure 3.3
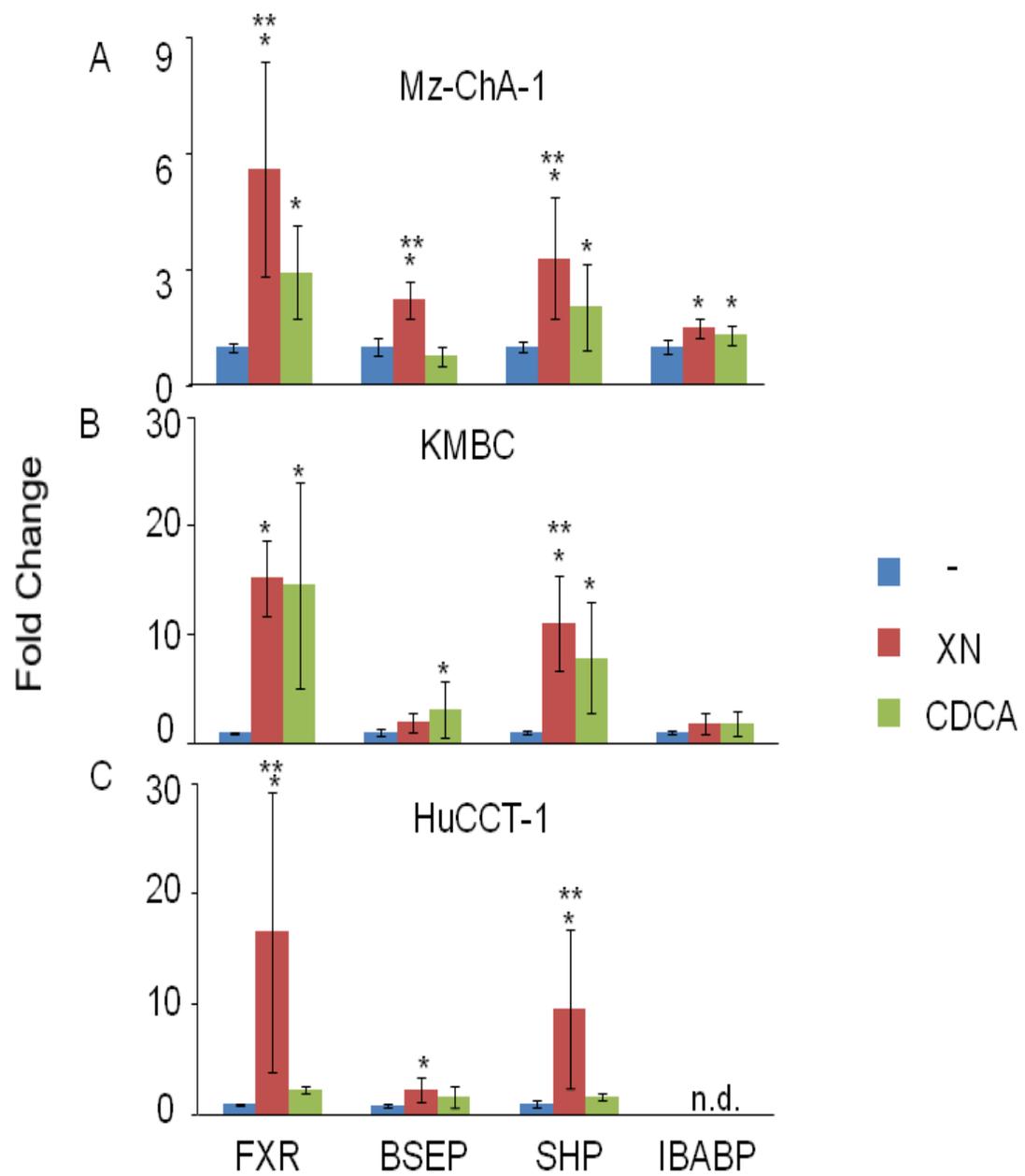
Figure 3.3. Induction of FXR target genes by XN and CDCA in cells. The biliary carcinoma cell lines Mz-ChA-1 (A), KMBC (B) and HuCCT-1 (C) were treated with either vehicle (100% EtOH), 10 µM XN or 100 µM CDCA for 18 hours. Expression of the FXR, BSEP, SHP and IBABP genes was determined by qRT-PCR. (A) Mz-ChA-1: Data represent means from five individual experiments for the FXR, BSEP and SHP genes and three individual experiments for the IBABP gene. B) KMBC: Data represent means from five individual experiments for SHP, four individual experiments for FXR and three individual experiments for both BSEP and IBABP. C) HuCCT-1: Data represent means from two individual experiments for FXR, BSEP and SHP. Data are normalized to the expression of beta-actin and shown in fold-change as compared to the control. Statistical significance was determined by ANOVA. *$P<0.05$ for XN or CDCA treated vs the control, ** $P<0.05$ for XN treated vs CDCA treated.

Figure 3.4
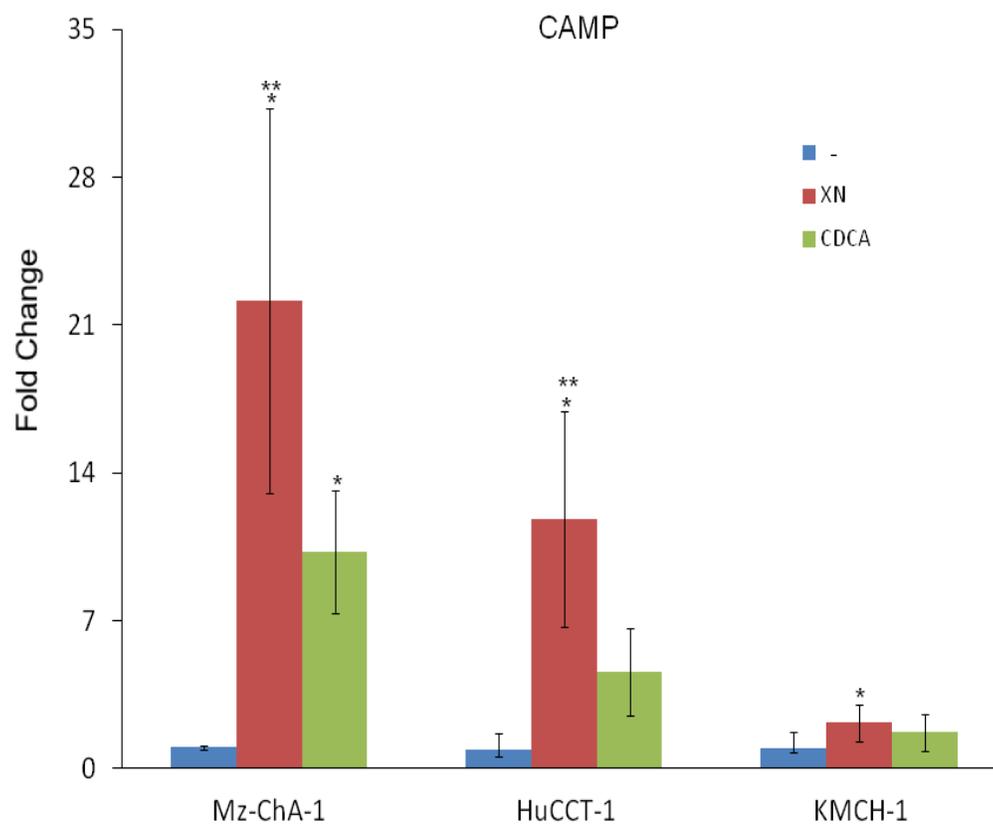
Figure 3.4. Human CAMP mRNA level expression in biliary epithelial cell lines. Biliary epithelial cell line Mz-ChA-1, HuCCT-1 and KMCH-1 cells were incubated with 10μM XN or 100μM CDCA for 18 hours. qRT-PCR for human CAMP gene expression was performed. The data was normalized to the expression of beta-actin. Data represent means from 5 individual experiments with triplicate readings for Mz-ChA-1 cells; 2 individual experiments with triplicate readings for HuCCT-1 cells and KMCH-1 cells. Statistical significance was determined by ANOVA. * $P<0.05$ for XN or CDCA treated vs control by ANOVA. **$P<0.05$ for XN treated vs CDCA treated.

Figure 3.5
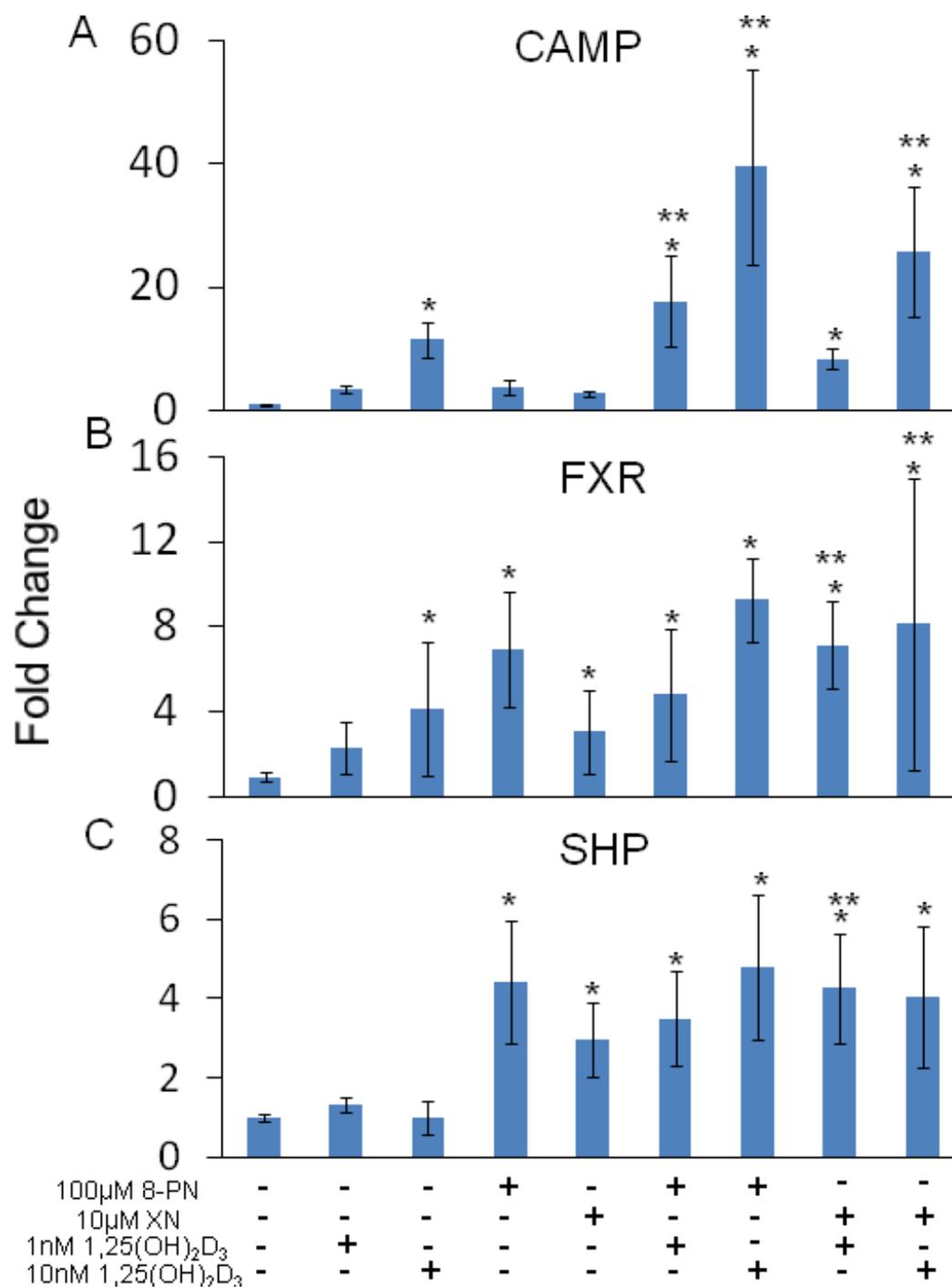
Figure 3.5. Effect of 8-PN, XN on inducing human CAMP mRNA expression with 1,25(OH)$_2$D$_3$ in the Mz-ChA-1 cell line. (A) 8-PN, XN induce human CAMP expression alone and cooperatively induce CAMP expression when combined with either 1nM or 10nM 1,25(OH)$_2$D$_3$. Mz-ChA-1 cells were incubated with 100 μM 8-PN, 10 μM XN with or without 1 nm or 10 nM 1,25(OH)$_2$D$_3$. The data was normalized to the expression of beta-actin and shown represents means of three sets of independent experiments with triplicate readings. Statistical significance was determined by ANOVA. * $P<0.05$ for all the treatment compared to the no treatment control group. ** $P<0.05$ for the combination group compared to the single treated group.

Figure 3.6

Figure: Bar chart titled CAMP showing Fold Change on the y-axis (0 to 20) for six treatment conditions.

10nM estradiol	-	-	-	+	+	+
1nM 1,25(OH)$_2$D$_3$	-	+	-	-	+	-
10nM 1,25(OH)$_2$D$_3$	-	-	+	-	-	+
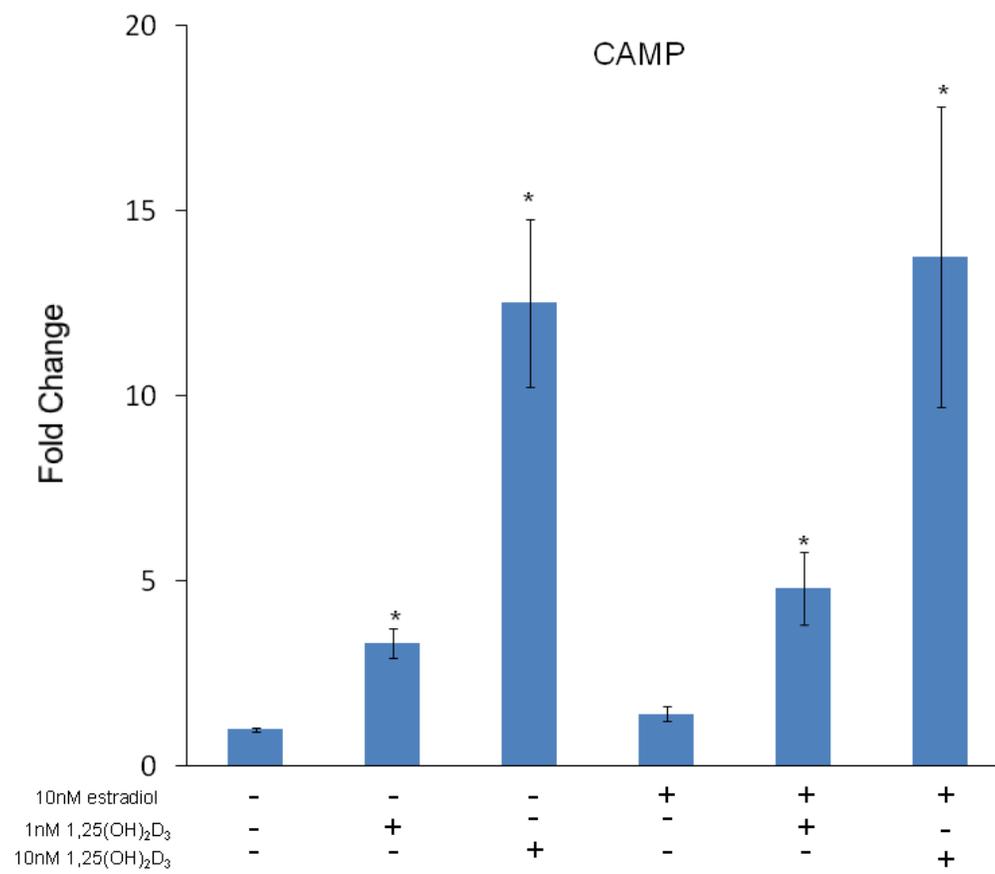
Figure 3.6. Effect of estradiol on inducing human CAMP mRNA expression with 1,25(OH)$_2$D$_3$ in the Mz-ChA-1 cell line. Mz-ChA-1 cells were incubated with 10 nM estradiol, 1nm or 10nM 1,25(OH)$_2$D$_3$ or the combination of 10nM estradiol with either 1nm or 10nM 1,25(OH)$_2$D$_3$. The data was normalized to the expression of beta-actin and shown represents means of two sets of independent experiments with triplicate readings. Statistical significance was determined by ANOVA. *P<0.05 for all the treated groups compared to the no treatment control group.

Figure 3.7
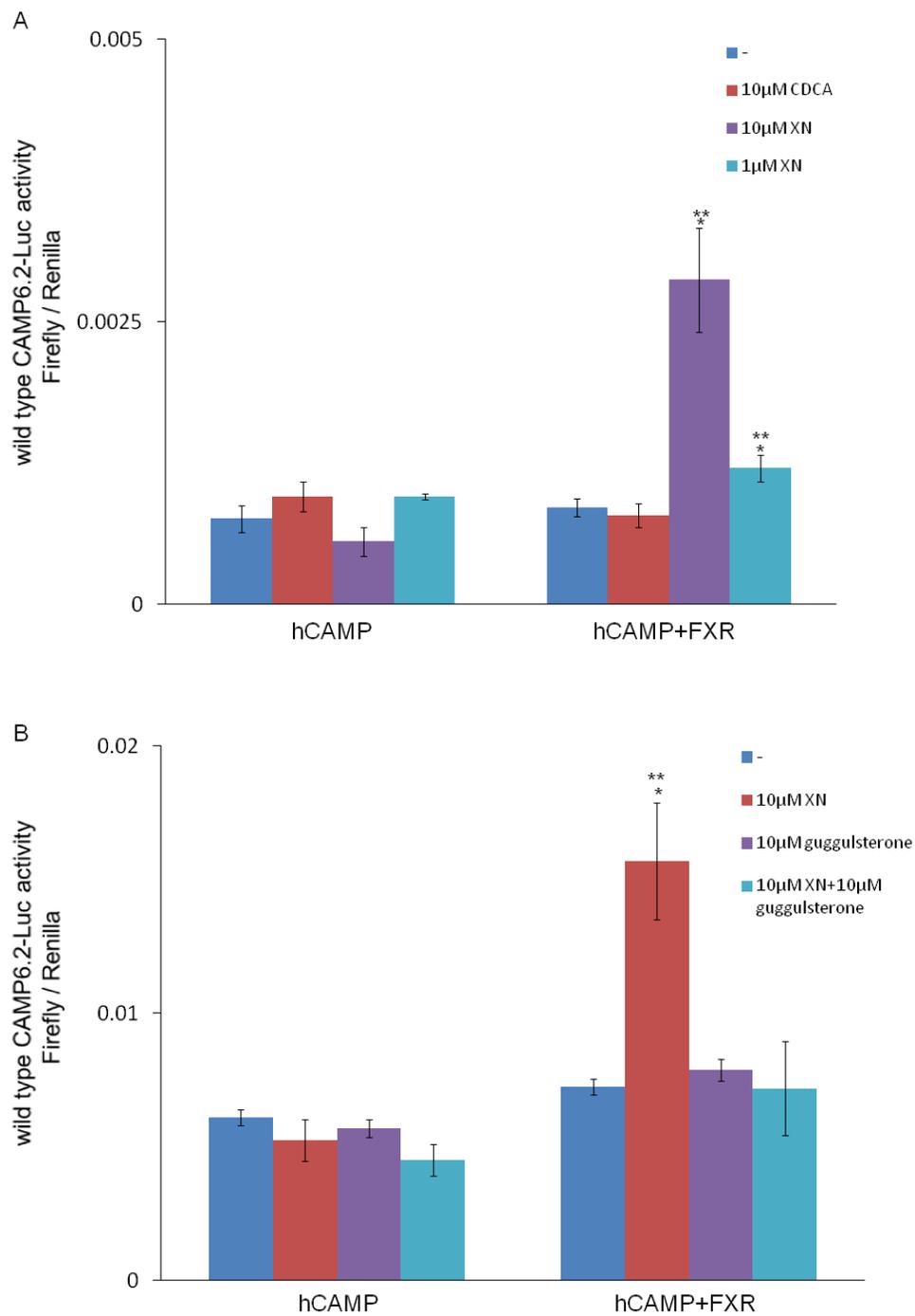
Figure 3.7. XN induces human CAMP promoter-luciferase activity through FXR in HEK293 cells. A) Transfected HEK293 cells were incubated at 37°C overnight with 100% EtOH, 10 μM CDCA, 10 μM XN or 1 μM XN, respectively. B) Transfected HEK293 cells were incubated at 37°C overnight with 100% EtOH, 10 μM XN, 10 μM guggulsterone or a combination of 10μM guggulsterone with 10μM XN, respectively. The data shown represents means of two independent transfections measured in duplicate. Statistical significance was determined by ANOVA. * $P<0.05$ for all the treatment compared to the control group.

Figure 3.8
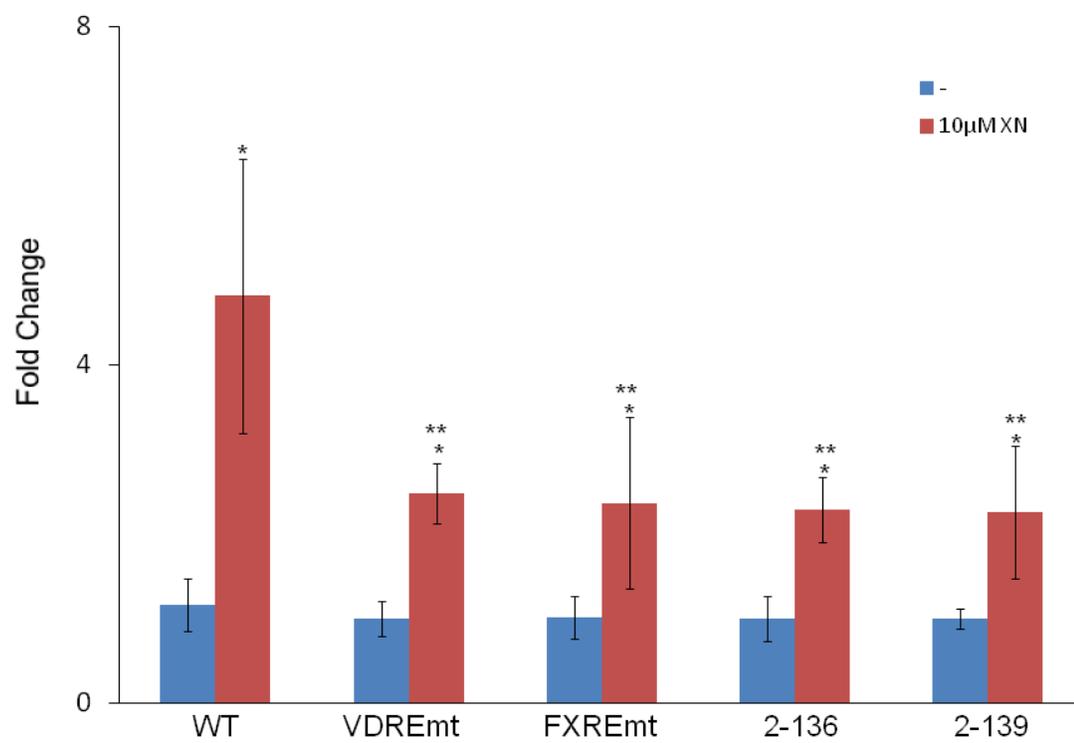
Figure 3.8. XN-mediated induction of human CAMP promoter-luciferase activity may require both VDRE and FXRE. HEK293 cells were co-transfected with an FXR expression vector and WT or various 5'-deletions or site directed mutants of the human CAMP luciferase reporter constructs. Transfected HEK293 cells were incubated at 37°C overnight with 100% EtOH or 10μM XN, respectively. The data shown represents means of four transfections measured in triplicate. Statistical significance was determined by ANOVA. * *P<0.05* for comparing each XN treated group to each untreated group. ** *P<0.05* for comparing XN treated group of VDREmt, FXREmt, 2-136, 2-139 compared to XN treated wild type CAMP6.2-Luc.

Characterization of human cathelicidin antimicrobial peptide gene expression in transgenic mice

Chapter 4

Introduction

The active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) and its analogs induce human cathelicidin antimicrobial peptide (CAMP) gene expression in numerous cells types through a vitamin D response element (VDRE) that is located in an Alu-short interspersed element (SINE) in the promoter region. Because the Alu-SINE is primate specific, it does not exist in the promoter of cathelicidin genes from other mammals, such as mouse, rat or dog (8). Only the human and non-human primate genes are regulated by vitamin D (1, 8).

Antimicrobial peptides (AMPs) play important roles in both innate and adaptive immunity; however, direct administration of AMPs is limited in clinical application due to the side effects of administering large doses of peptides, failure of delivery to the expected sites, or low physiological concentrations. Regulating endogenous human CAMP gene expression in a cell-and tissue-specific manner may be more effective, but requires comprehensive knowledge about how its expression is controlled in various cell types and tissues under different conditions. To accomplish this and determine how human CAMP participates in host defense at both the molecular and cellular level and ultimately design *in vitro* and *in vivo* experimental models to study human diseases, we generated a strain of transgenic mice by inserting a 6.2Kb genomic fragment containing the human CAMP gene (Figure 4.1) into

the mouse genome. Two founder lines were obtained and are referred to as Tg6 and Tg16. In this chapter, we present data from our characterization in the transgenic mice of human CAMP gene expression at the mRNA and protein levels.

Materials and Methods

Animals

All the mice used in this research were bred and maintained in Macrolon cages in temperature (23±1 ^{o}C), humidity (50–60%), and lighting (6:00 am to 6:00 pm) controlled rooms under specific pathogen free conditions. The hybrid transgenic mice were backcrossed onto the C57Bl6/J parental strain. Backcrossing means to breed a hybrid animal with an animal that is genetically similar to the parent and then breed the resulting offspring with the parental strain again. The repeated backcrossing of each generation will create an animal that genetically identical to the parent (in this case C57Bl6/J). The mice used in this study are referred to by lineage number (i.e. Tg6 or Tg16). The number of times a mouse was backcrossed onto the C57Bl6J strain is indicated after the lineage number (e.g. Tg16-9X for nine backcrosses). The eventual goal was to reach nine backcrosses that would produce mice that were pure for the C57Bl6/J strain. Some studies were

performed using mice that were backcrossed fewer than nine times, but in all cases litter mate animals were used as controls.

Vitamin D deficiency

Six month-old Tg6-5X mice were divided into two groups, one group (n=3) was fed a control diet with 2,200 IU vitamin D per kilogram (kg) and the other group (n=5) was fed a vitamin D-deficient diet for 60 days. Mice were euthanized and organs were collected at the end of the period. All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the OSU IACUC.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Fresh tissues were collected from both wild type (WT) and transgenic (TG) mice and placed in RNALater (Life Technologies Corporation) solution overnight to fix the tissue and preserve the RNA integrity. For *ex vivo* experiments, fresh tissues were incubated in Dulbecco's modified Eagle's medium (DMEM; Mediatech Inc. Manassas, VA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% Pen/Strep (Life Technologies Corporation, Carlsbad, CA) with 100 nM $1,25(OH)_2D_3$ at 37°C in a humidified 5% CO_2 incubator for 18-24 h. Total RNA was isolated from each tissue using TRIZOL Reagent (Life Technologies) by homogenization using nuclease free 1.6 mm stainless steel beads in a Bullet Blender (Next Advance,

Inc. Averill Park, NY 12018, USA). Samples were processed at power setting

8 until completely homogenized. qRT-PCR was performed as described

previously (36).

Antibodies

The primary rabbit anti-hCAP18 antibody (3.6 mg/ml was a gift from Dr. N.

Borregaard (University of Copenhagen, Denmark). The rabbit IgG used was

from the VECTASTAIN ABC KIT (Vector Labrotaries, Inc., Burlingame, CA

USA). The secondary goat anti-rabbit antibody-HRP Conjugate was

purchased (Cat#170-6515, Bio-Rad Hercules, CA 94547).

Immunohistochemistry (IHC)

Mouse tissue samples were collected from both wild type and transgenic mice

and fixed in 4% paraformaldehyde (PFA) solution for 40 minutes. The fixed

tissues were washed with phosphate buffered saline (PBS) twice followed by

incubation in PBS at 4^oC for 30 min. Tissues were then incubated in fresh PBS

at 4^oC overnight. PBS was removed and replaced with 25% sucrose in PBS

solution and tissues were incubated at 4^oC overnight. Tissues were placed in

fresh 25% sucrose in PBS the next day. Tissues were embedded in a

cryomold with clear frozen section compound (VWR, Radnor, PA, USA). The

tissue blocks were cut into 7 µm thick sections. Immunohistochemistry was

performed using a VECTASTAIN ABC KIT according to the manufacturer's

instructions (Vector Laboratories, Inc., Burlingame, CA USA). The primary

antibodies, rabbit anti-hCAP18 or rabbit IgG, were diluted 1:4000 before use. The secondary antibody (goat anti-rabbit-HRP) was diluted 1:10,000 before use. Bone was decalcified using 6% trichloroacetic acid (TCA) as described previously (156).

Western Blot

Mouse bone marrow, spleen, lung, duodenum, ileum, colon, tongue, salivary gland, gingival, skin, testes and kidney were collected from wild type, knock out, and transgenic mice. Protein was isolated from these tissues using RIPA buffer (50mM Tris-HCl PH8.0, 1% NP-40, 0.5% DOC, 150mM NaCl, 0.1% SDS) and protein concentrations were determined using a modified Bradford reagent (Bio-Rad Life Science, Hercules, CA). Protein electrophoresis was done using a 16% SDS-polyacrylamide gel. For each sample, 50 µg of protein was loaded per lane except the gingival tissue for which 40 µg was loaded due to the lower yield of total protein. After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was then blocked in 5% non-fat dry milk for 30 min and incubated overnight at 4°C with primary antibody (rabbit anti-hCAP18; 3.6 mg/ml) in PBS-Tween 20 containing 2.5% non-fat dry milk. A goat anti-rabbit secondary antibody was diluted 1:5000 in PBS-Tween 20 and the blot incubated 1.5 h at room temperature. Signal was detected by Thermo SuperSignal West Femto

Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL USA).

Results

Overview- The transgenic mice have been observed up to the age of 9-12 months, and have overall health and behavior patterns that are similar to those of wild type mice. We expected that the human CAMP transgene would be expressed in cells of the immune system and epithelial cells of the respiratory, digestive and urinary tracts and the reproductive system. Also, we predicted that the human CAMP transgene would be up-regulated by vitamin D in various organs of the transgenic mice. To determine expression of the human CAMP transgene in these mice, we used qRT-PCR to quantify mRNA levels and Western blot analysis and immunohistohemistical staining to identify the protein.

The human CAMP gene is expressed in tissues from transgenic mice

Quantitative PCR analysis of the genomic DNA revealed that the transgene in both lines was present at approximately 4-6 copies per haploid genome (Fantacone and Gombart, unpublished data). Human CAMP mRNA expression was detected by qRT-PCR in the tongue, salivary gland, lung, spleen, small intestine, colon, liver, kidney and brain of the transgenic, but not the wild type mice (Figure 4.2). It was especially high in the tongue, bone marrow, salivary gland, lung, spleen and intestines, all tissues that are

involved in barrier defense. Interestingly, the human CAMP transgene was expressed at higher levels in these tissues than was the mouse CAMP gene (also known as CRAMP). The exception was bone marrow where expression of mouse CRAMP gene and the human CAMP transgene were similar (Gombart and Saito, unpublished data). We do not have an explanation for this finding, but it may be the result of putting a human gene into a mouse background.

hCAP18 proprotein is expressed in tissues from the transgenic mice

To detect expression of hCAP18, the proprotein encoded by the human CAMP gene, in the different organs of transgenic mice, immunohistochemical (IHC) staining and Western blot analyses were performed. IHC staining detected hCAP18 expression in the tissues of the immune system (bone marrow and spleen), respiratory tract (trachea, bronchiole and lung), digestive tract (gingiva, tongue, salivary gland, small intestines and colon), endocrine system (kidney) and central nervous system (brain) and the skin (Figure 4.3). Details of the cells stain in each tissue are described in the figure legend.

Western blot analysis confirmed IHC results with hCAP18 expression detected in bone marrow, spleen, salivary gland, lung, intestines, kidney, skin, tongue, gingival tissues, testis (male) (Figure 4.4) and ovary (female) (data not shown). A 14 kDa band that was detected in the salivary gland (female, data

not shown) and skin (Figure 4.4) may occur due to the processing of hCAP18 to release LL37 plus a ~14 kDa N-terminal fragment.

An unexpected result was that the GAPDH protein chosen as a loading control was not detected in bone marrow. To rule out the possibility of loading mistake, we incubated the blot with antibody against the mouse CRAMP protein and it was detected at similar levels in both wild type and transgenic mice but not the knockout mouse, providing positive evidence that equal amounts of protein were loaded in the wild type and transgenic lanes. The reason for not detecting GAPDH in the bone marrow is unclear, but its expression has been noted to varies greatly across different tissues, and it may be too low for detection in the bone marrow (157). We will repeat the Western blot analysis using an antibody against a different reference protein with more similar expression levels across tissues. Taken together, the above results indicate that the human CAMP gene is expressed in the expected organs and tissues of the transgenic mice.

The human CAMP transgene is regulated by vitamin D

To determine if the human CAMP transgene is regulated by vitamin D, we performed both *ex vivo* and *in vivo* experiments. For the *ex vivo* experiment, various mouse tissues from transgenic mice were treated for 18-24 hours with 100 nM 1,25(OH)$_2$D$_3$. RNA was isolated and gene expression was determined by qRT-PCR. CAMP gene expression was significantly increased in the lung

and spleen by 1,25(OH)$_2$D$_3$ (Figure 4.5). In this experiment, a statistically significant increase was not observed in the salivary glands or kidney, but we have observed induction on other occasions. This may be due to variability in the diffusion of 1,25(OH)$_2$D$_3$ into the tissues during the *ex vivo* culture. It will be important to repeat these experiments three or more times with each tissue to more accurately determine the levels of CAMP gene induction.

To determine if vitamin D deficiency had an impact on expression of the human CAMP gene *in vivo*, we maintained transgenic mice on either control or vitamin D-deficient diets for eight weeks. The mice on the deficient diet had an average serum 25(OH)D level that was about 7% of those on the control diet (2.0 $\pm$1.5 ng/ml [n=9] vs 28.3 $\pm$10.6 ng/ml [n=6]) indicating that they were vitamin D-deficient. Tissues were collected from three mice on control diet and 5 mice on vitamin D deficient diet. RNA was isolated from the tissues and expression levels of CAMP mRNA were determined by qRT-PCR. Vitamin D deficiency dramatically reduced human CAMP gene expression in salivary glands of both male and female mice (Figure 4.6). In three other tissues tested (spleen, lung, colon), no statistically significant difference in expression was noted (data not shown). This result is very intriguing and will need to be verified with additional deficiency studies.

Discussion

Only the cathelicidin gene of humans and non-human primates is regulated by vitamin D; therefore, a major problem in studying the regulation of human CAMP gene expression by vitamin D and its role in treating various human diseases is the lack of an animal model. To overcome this hurdle, we generated a novel transgenic mouse with the human CAMP gene inserted into its genome. This unique mouse model will allow us to elucidate the importance of the vitamin D-cathelicidin pathway in the innate immune response against infections, in inflammation, wound healing and dermatological conditions, and in cancer. Also, the mouse strain could be used to identify additional transcription factors that regulate human CAMP expression by crossing it with transcription factor knockout mice.

In the transgenic mouse, the human CAMP gene was expressed at both mRNA and protein levels in the respiratory, digestive and reproductive tracts as well as immune system which are tissues that are most frequently in contact with environmental microbes or invading pathogens. In addition, the transgene was induced by vitamin D in some tissues both *ex vivo* and *in vivo*, and in vitamin D-deficient mice we observed significantly reduced CAMP expression in the salivary glands, but not other organs.

Although not a part of the studies presented in this thesis, we have shown that the transgenic mice have increased resistance to *Salmonella* infection as

compared to both wild type and knockout mice (Lowry and Gombart, unpublished observations). In future studies, we will use this model to define the role of vitamin D status on the induction of human CAMP gene expression during infection. The observed lack of expression in the salivary gland in vitamin D-deficient mice is interesting as patients with hypophosphatemic (vitamin D-resistant) rickets (VDR mutations) have increased rates of dental abscesses and caries (158, 159). In light of our data, it would be interesting to test the levels of hCAP18/LL-37 in the saliva of such patients, as low levels may predispose these patients to infection and cavity formation.

The mouse model will allow *in vivo* studies to determine the effect of the dietary compounds XN and 8-PN identified in Chapter 2 on human CAMP expression. *In vivo* administration of these compounds to transgenic mice followed by measuring human CAMP expression in different organs will provide insight into the potential benefit of these dietary constituents. Induction of human CAMP through VDR, FXR or other nuclear receptors by these compounds may help improve resistance to gastrointestinal infections or reduce the development of colon cancer induced by chemical carcinogens. FXR deficiency in mice leads to increased cell proliferation, inflammation and tumorigenesis in the intestine (160). This suggests that FXR ligands may be used to treat or reduce the rate of gastrointestinal cancers. Furthermore, LL-37 activates a GPCR-p53-Bax/Bak/Bcl-2 signaling pathway that triggers AIF/EndoG-mediated apoptosis in colon cancer cells (161) and is

hypothesized to reduce rates of colon cancer in humans where reduced expression of LL-37 was observed in tumors versus surrounding normal tissue (161). Mice lacking the CAMP gene are more susceptible to developing colon cancer (161). We hypothesize that FXR-mediated induction of LL-37 expression may reduce the development of colon cancer by promoting apoptosis of transformed cells. A recent study showed that imatinib, also known as Gleevec (Novartis Corporation, East Hanover, NJ), which is used to treat chronic myeloid leukemia, is an FXR modulator (162). It would be interesting to use our animal model to study induction of CAMP with this drug as well as natural FXR modulators and their potential to treat or prevent the development of colon cancer or increase resistance to gastrointestinal infection.

In conclusion, the human cathelicidin gene is successfully expressed in many organs and tissues in the human CAMP transgenic mice. This unique murine model may lead to new ways for promoting human health by providing a powerful tool for studying the regulation of human CAMP gene expression by many dietary compounds and the importance of such induced CAMP expression in the prevention of inflammatory diseases, autoimmune diseases or cancer.

Figure 4.1
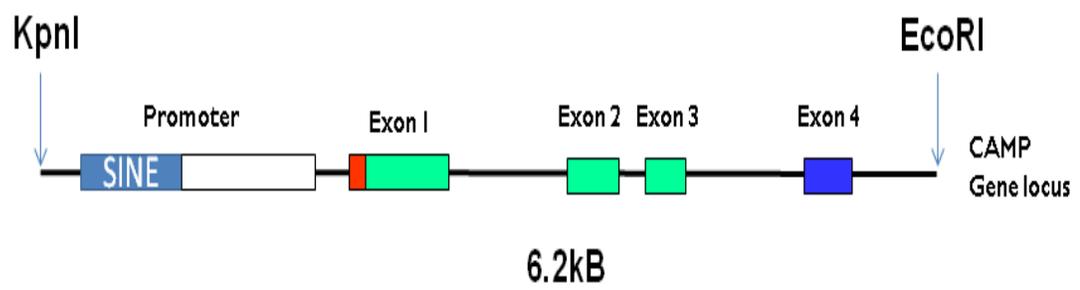
Figure 4.1. The structure of the 6.2 kB human CAMP gene construct which was inserted into the mouse genome.

Figure 4.2
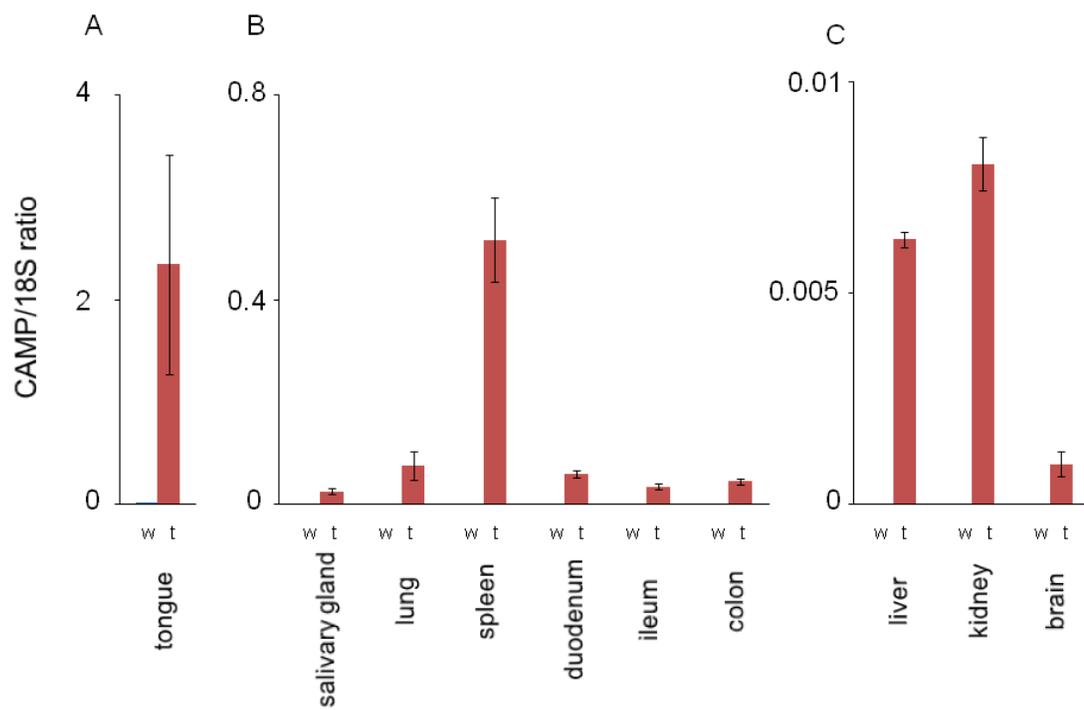
Figure 4.2. Human CAMP gene mRNA expression levels in select wild type and transgenic mouse organs. Total RNA was extracted from both wild type (w) and transgenic (t) mice and analyzed by qRT-PCR. The human CAMP gene was expressed in all the organs tested from transgenic mice as shown. No human CAMP was detected in wild type mice. The expression levels are reported as the ratio of the number of CAMP mRNA transcripts compared with the number of 18S rRNA transcripts (i.e. copies of CAMP/copies of 18S).

Figure 4.3

	WT	TG		WT	TG

tongue

gingiva

salivary gland

skin

duodenum

trachea

jejunum

bronchiole

colon

lung

kidney

bone marrow

brain

spleen
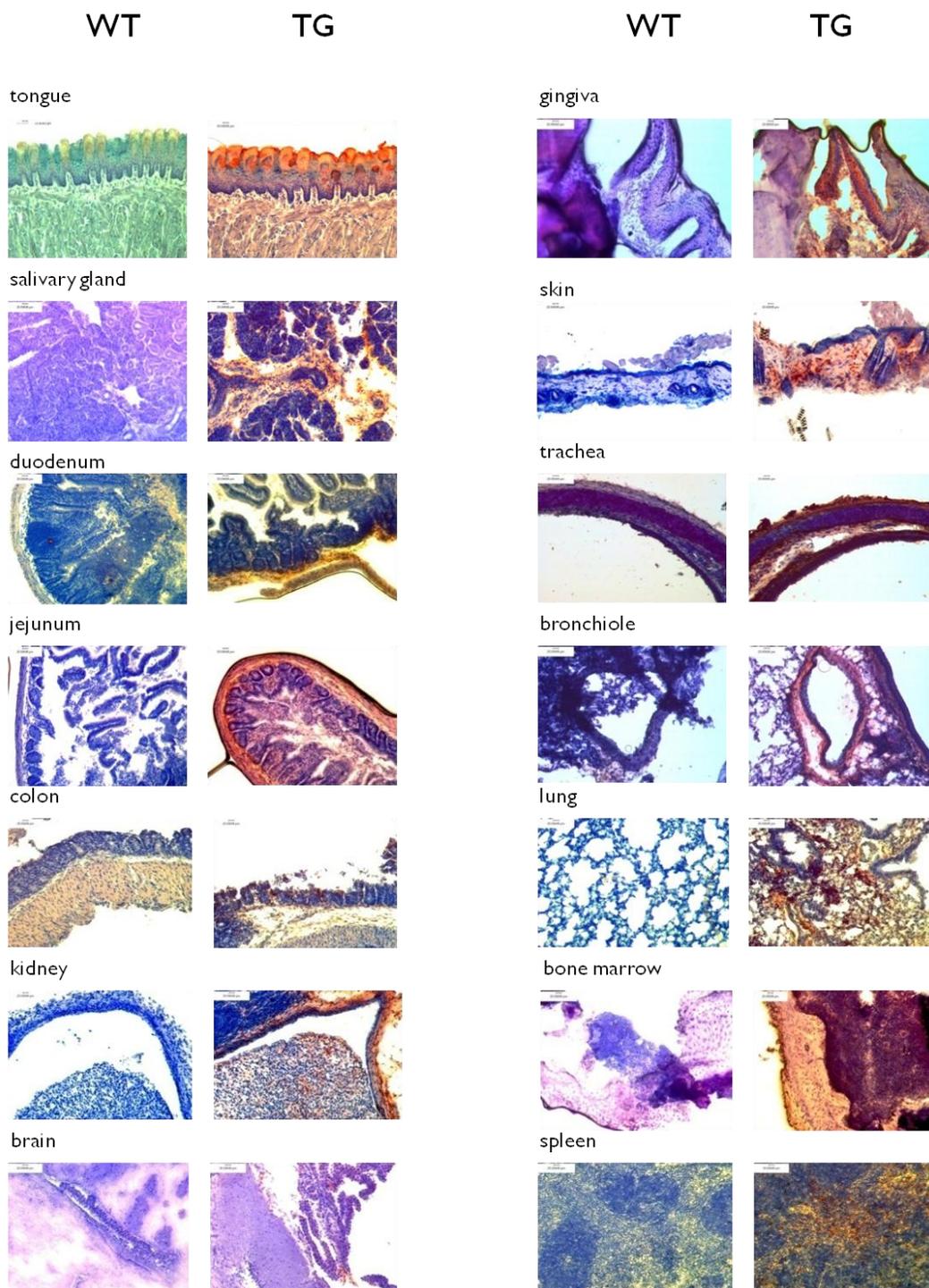
Figure 4.3. IHC detection of hCAP18 expression in various tissues of the transgenic mice. Novared staining (red color) was used in this experiment except for the trachea which used 3,3'-Diaminobenzidine (DAB; brown color). The expression of the hCAP18 protein was not detected in any tissues from the wild type (WT) mice. The expression of hCAP18 was detected in transgenic (TG) mice in all tissues tested: tongue (keratin and epithelial layer); salivary gland (connective tissue); duodenum, jejunum and colon (connective tissue and smooth muscle layer); kidney (connective tissue of both the renal medulla and renal pelvis); brain (choroid plexus); gingiva (epithelia and connective tissue surrounding the tooth); skin (connective tissue of dermis); trachea (deep into the cartilage); bronchiole (connective tissue); lung (epithelia to air duct); bone (bone matrix and bone marrow); spleen (red pulp).

Figure 4.4
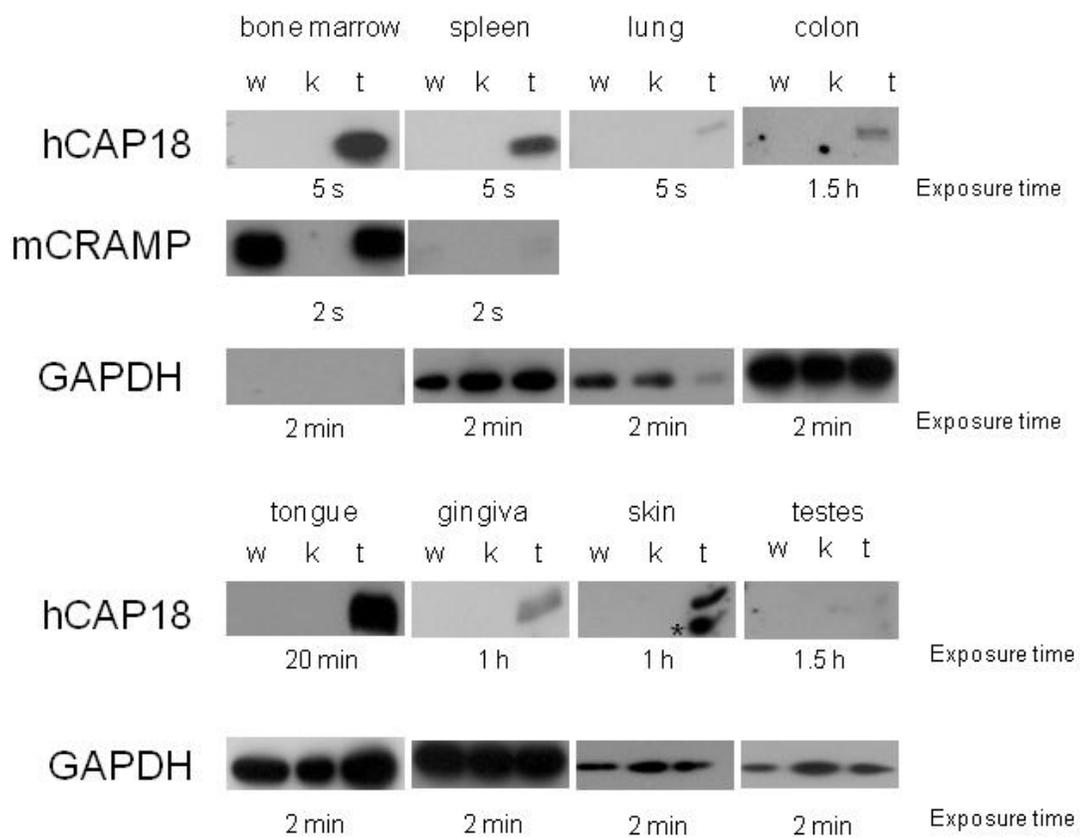
Figure 4.4. Western blot detecting hCAP18 expression in various organs of transgenic mice. Results are shown for wild type (w), knockout (k) and transgenic (t) mice. Expression of the hCAP18 protein was detected in tissues from the immune system (spleen and bone marrow), digestive tract (colon, tongue and gingiva), respiratory tract (lung), endocrine system (kidney and testis) and skin. GAPDH was used as a control for protein loading. The mouse protein (mCRAMP) was detected in the bone marrow and spleen as a control. The film exposure times are listed below the respective panels. The double band observed in the skin may indicate that hCAP18 is processed to release the peptide LL-37 and result in the detection of the 14 kDa N-terminal domain (*).

Figure 4.5
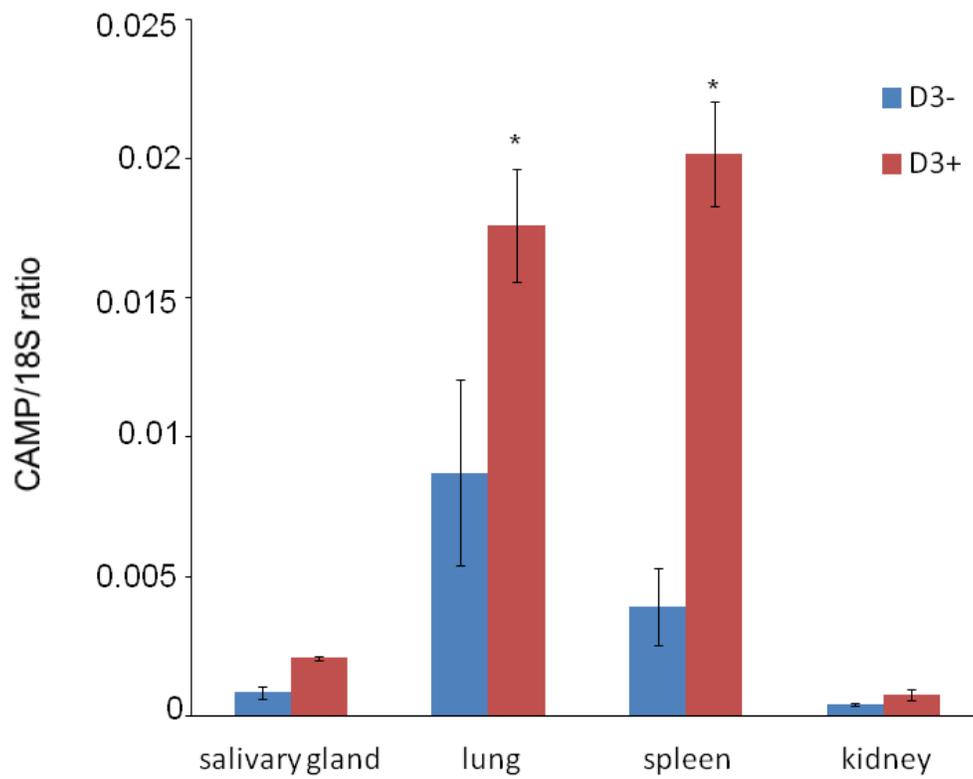
Figure 4.5. 1,25(OH)$_2$D$_3$ induces CAMP transgene expression *ex vivo*.
Tissues were isolated from transgenic mice, incubated with 100% EtOH
(vehicle, (D3-) or 100 nM 1,25(OH)$_2$D$_3$ (D3+). Human CAMP transgene
expression was measured by qRT-PCR. A copy number standard was used to
determine the number of CAMP and 18S rRNA transcripts per sample and the
ratio is reported (i.e. copies of CAMP/copies of 18S). Statistical significance
was determined by ANOVA. * $P<0.05$ for 1,25(OH)$_2$D$_3$ treated vs control in
each tissue.

Figure 4.6
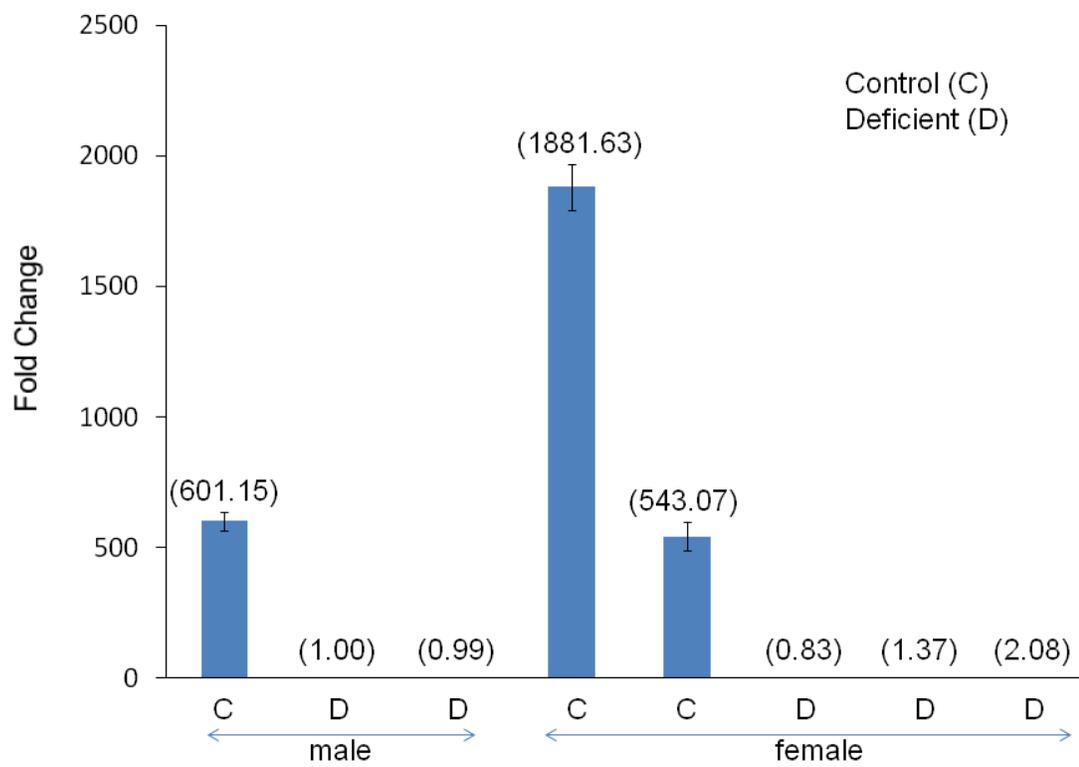
Figure 4.6. Vitamin D deficiency decreases the expression of the human CAMP gene in the salivary glands of transgenic mice. Salivary glands from transgenic mice which were fed control (C) and vitamin D-deficient (D) diet for eight weeks were collected, RNA was isolated and human CAMP expression measured by qRT-PCR. Within each tissue sample, CAMP levels were normalized to 18S rRNA levels, and the differences between samples are expressed as fold-change compared with the CAMP level from the first male on the deficient diet (i.e. the level in that male is set as one fold).

General conclusions and outlook

Chapter 5

Purpose of this study

The active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$), binds to and activates the vitamin D receptor (VDR), leading to its translocation from the cytosol into the nucleus where it forms a heterodimer with RXR and binds to the vitamin D recognition element (VDRE) in the promoter of the human CAMP gene to induce transcription (8).

Like the VDR, the farnesoid X receptor (FXR) is a member of the nuclear hormone receptor super family (37) and acts as a physiological bile acid receptor to regulate bile acid homeostasis (87, 163). It was shown to induce human CAMP expression in the presence of its ligand chenodeoxycholic acid (CDCA) (52); however, the exact mechanism of induction was not characterized fully. The vitamin D receptor binding site in the Alu-SINE of the CAMP gene was implicated as the binding site for FXR, but was not determined experimentally (52).

A number of potential non-bile acid ligands for FXR include plant polyphenols. Xanthohumol (XN) from hops (*Humulus lupulus L.*) was reported to be an FXR ligand (53). This was based on activation of a BSEP promoter reporter-luciferase gene by XN in HEP-G2 liver cells. It was not demonstrated that this activation required FXR or the FXR binding site in the BSEP promoter (53). Nevertheless, the potential for XN to act as an FXR ligand, led us to hypothesize that a dietary component could induce CAMP gene expression

through activation of FXR binding to the VDRE in the Alu-SINE. To test our hypothesis, we needed to 1) demonstrate that the VDRE was required for induction of the CAMP gene in the presence of bile acid ligands of FXR and 2) demonstrate that XN was a bona fide ligand of FXR. Accomplishment of these aims would show that plant derived plant polyphenols are capable of regulating human CAMP gene expression and provide an important biological understanding of the interactions between our diet and gut immunity. This knowledge would establish a foundation for developing natural therapies for protecting against gastrointestinal diseases.

As previously described in this thesis, a VDRE is located in an Alu-SINE in the promoter region of CAMP gene. Because the Alu-SINE is primate specific it does not exist in the promoter of cathelicidin genes from other mammals (8), therefore, only the human and non-human primate genes are regulated by vitamin D (1, 8). To perform *in vivo* studies addressing the biological importance of vitamin D or other dietary compounds like XN or 8-PN on human CAMP gene expression, we needed to generate and characterize a transgenic mouse model expressing the human CAMP gene.

Conclusions from this study

To summarize this thesis project, human cathelicidin gene expression can be regulated by various dietary compounds through activation of nuclear receptors and other transcription factors as described in Chapter 2. The work presented in Chapter 3, provides strong evidence that XN and its metabolite 8-PN act as ligands for FXR and induce several known target genes. In addition, we demonstrated that both compounds induce CAMP gene expression through FXR. This induction by FXR appears to involve both the vitamin D response element (VDRE) located in the Alu-SINE and a farnesoid X response element (FXRE) located in the beginning of the third exon of the human CAMP gene. The requirement for both elements remains to be determined by testing a VDRE/FXRE double mutation in the CAMP-promoter luciferase reporter construct and performing chromatin immunoprecipitation (ChIP) to identify the exact FXR binding sequence(s) in the human CAMP gene. Interestingly, both XN and 8-PN cooperatively induced CAMP gene expression when combined with $1,25(OH)_2D_3$, suggesting there might be a synergy between them. We predict that this cooperative induction is due to both active VDR:RXR and FXR:RXR complexes binding to their respective sites in the CAMP promoter and working together to induce CAMP gene expression (Figure 5.1).

From our work presented in Chapter 4, characterizing the human CAMP transgenic mouse model, we determined that the human CAMP transgene is

expressed strongly at both the mRNA and protein levels in the immune system, respiratory tract, digestive tract, reproductive system and central nervous system as expected. This unique transgenic mouse model will provide a powerful tool to study the *in vivo* importance of vitamin D-mediated regulation of the CAMP gene in the immune response to infection. Also, the regulation of CAMP by dietary compounds like XN can be studied *in vivo.* By oral administration or injection of dietary compounds into the transgenic mice, we can study the bioavailability of these compounds as well as their effects on human CAMP gene expression in the various organs under different physiological concentrations. This mouse model can also be used in gastrointestinal infection or carcinogenesis models to investigate the roles that dietary compounds play in preventing or treating these diseases.

Significance of this study

The work in this thesis project influences the immediate field of vitamin D, xanthohumol and immunity, but also provides insights into the fields of diet and optimal health, gastrointestinal microbiota, innate and adaptive immunity, prevention and treatment of infectious disease, cancer or autoimmune diseases, and even epidemiology. We will discuss the significance of this study for some of these fields.

Dietary compounds and byproducts of microbial metabolism. Our findings emphasize the potential importance of dietary components in modulating barrier defense in the gastrointestinal tract. In addition, byproducts from the metabolism by dietary components of gut microbiota could be important for communicating with intestinal epithelial and immune cells, thus affecting the expression of AMPs. For example, butyrate is a short chain fatty acid produced in the colon by fermentation of dietary fiber by anaerobic bacteria and is also a compound directly present in many foods, such as butter and cheeses (94). Oral administration of sodium butyrate restored the expression of rabbit CAP18 in colon epithelium after *Shigella* infection (96). Also, lithocholic acid (LCA), a bacterial metabolite produced from primary bile acids, induces CYP3A, a cytochrome P450 enzyme that detoxifies LCA in the liver and intestine and induces CAMP gene expression (88).

Finally, we showed in Chapter 3, that 8-PN, a microbial metabolite of xanthohumol, induces CAMP gene expression. This communication between host cells and the gut microbiota is likely very important in establishing a mucosal barrier preventing contact of microbes and pathogens with the intestinal epithelium (1, 92). In addition, accumulating evidence suggests a strong interaction between functional food components and the balance and/or composition of gut microbiota (164, 165).

Interestingly, probiotics which are nonpathogenic microorganisms that can restore balance to the gut microflora and benefit the host by promoting the recovery of damaged gut mucosa and prevent microbial translocation of the barrier (166, 167). Treating with *Lactobacillus plantarum* increased the expression of VDR and cathelicidin genes in cultured intestinal epithelial cells (168). These findings suggest that beneficial bacteria may also be important for up-regulation of the cathelicidin gene. Our transgenic mouse model provides an ideal tool to test this hypothesis *in vivo*.

Gut microbiota and homeostasis. The human gut harbors about 10^{14} bacteria consisting of 500-1000 different species (169). This microbiota plays important roles in many aspects of human health, including metabolism, chronic intestinal inflammation, obesity and immunity (170-173). The gut microbiota is a double-edged sword. On one hand it protects the host from colonization and/or invasion by pathogens and on the other, in the absence of host structural and immune barrier integrity, these beneficial intestinal bacteria have the potential to invade the host and cause various diseases [1]. Maintaining gut homeostasis depends on an intact epithelial barrier defense which can be affected by diet, gut microbiota and the host immune system [1].

Antimicrobial peptides (AMPs) directly kill bacteria. The two dominant bacterial phyla in the intestinal tract of mice and humans are the gram positive *Firmicutes* and the gram negative *Bacteriodetes* which together comprise up

to 80% of the total bacteria in the gut (174). Lack of Paneth cell defensins (deficiency) or expression of a human-specific Paneth cell defensin (α-defensin 5, *DEFA5*) (surplus) in mice caused significant defensin-dependent changes in the composition of the microbiota, but not in total numbers of bacteria in the small intestine (174). However, the impact of human AMPs on the gut microbiota is still largely unknown. Human CAMP is expressed in the epithelia of the digestive tract which is exposed to environmental microbes including the gut microbiota (1). Mice and humans lacking of CAMP expression are more susceptible to bacterial infections in various organs, including colon infection (18, 30, 153, 175-182). However, little is known about how CAMP expression will affect the composition of the gut microbiota. Our unique humanized transgenic mouse model provides a powerful tool to determine if vitamin D or other dietary compounds such as XN or 8-PN affect the composition of the gut microbiota through the regulation of cathelicidin expression.

Potential FXR ligands and target genes. As a nuclear receptor, activation of FXR by its ligands regulates the expression of many target genes, including BSEP, SHP, IBABP, human CAMP and FXR itself. Our data imply that the consumption of diets rich in plant polyphenols could result in high local concentrations of FXR ligands that would increase expression of CAMP by epithelial barrier cells in the GI tract. Many dietary compounds such as tea

catechins, grape seed procyanidin extract and soy-derived phytosterols have also been identified as FXR ligands (126-128). Ponasterone A and 20-hydroxyecdysone are both insect molting hormones also produced by plants (*Macrophyll Podocarpus* and *Cyanotis Vaga* respectively) to disrupt the development and reproduction of insects (183, 184). Since both are ligands for ecdysone receptor (EcR) which is an insect analogue to FXR, it is also possible that they may induce human CAMP gene expression through FXR and ultimately improve barrier function. This hypothesis may explain the role of these plants in traditional Chinese medicine for treating lung infections. The studies presented in this thesis are the first to investigate the role that these dietary compounds play in regulating human CAMP gene expression and our findings support further investigation of other plant polyphenols as FXR ligands to determine if they regulate CAMP gene expression like XN or 8-PN.

FXR is a selective bile acid sensor meaning that its ligands may act in a gene selective manner in vivo. For example, a ligand may act as an agonist (activator) for one gene, an antagonist (inhibitor) on a second gene and neutral (no effect) on a third gene. In our study, XN induced the CAMP gene to a higher level than CDCA in the Mz-ChA-1 and HuCCT-1 cell lines. Guggulsterone, an FXR antagonist, doesn't induce CAMP expression, but blocked the induction of human CAMP expression by XN. On the other hand, it induced BSEP gene expression in HEK293 cells. Because of these complex

regulatory patterns among genes and cell types, determining how these FXR

ligands regulate the expression of FXR target genes based on the

transcriptional and translational context will ultimately provide more knowledge

that may be useful in reducing or treating gastrointestinal infections and

cancer.

Gastrointestinal health and colon cancer. Cathelicidin is synthesized and

stored in the epithelial layer of the gastrointestinal tract and skin. In addition to

its antimicrobial activities, cathelicidin has anti-inflammatory and anti-cancer

activities in the GI tract. Colon cancer is one of the most common GI tract

cancers and is diagnosed in more than 130,000 people each year in the U.S.

alone. Therefore, research that aims to investigate the molecular pathogenesis

and possible treatments is necessary. Although genetics is one of the risk

factors contributing to colon cancer, greater than 75-95% of colon cancer

occurs in people with little or no genetic risk (185, 186). Other major risk

factors for colon cancer include: aging, gender, high intake of fat, alcohol, red

meat, obesity, smoking and a lack of physical exercise (185, 186). CAMP

mRNA expression was not detected by qRT-PCR in freshly isolated cells from

colon cancer tissue (187). In addition, colon cancer, hepatocellular and gastric

cancer cell lines express very low levels of CAMP (187). The lack of CAMP

expression may contribute to the development of these cancers, because LL-

37 activates a GPCR-p53-Bax/Bak/Bcl-2 signaling pathway that triggers

AIF/EndoG-mediated apoptosis in colon cancer cells (188) which is hypothesized to reduce rates of colon cancer in humans. Reduced expression of LL-37 was observed in tumors versus surrounding normal tissue and mice lacking the CAMP gene were more susceptible to developing colon cancer (188). A smaller synthetic peptide FK-17 (derived from amino acid residues 17-32 of LL-37) showed anti-cancer activity by inducing caspases-independent apoptosis and autophagy through the common p53-Bcl-2/Bax cascade in colon cancer cells (189). Taken together these observations and the findings from our own study suggest that dietary regulation of CAMP could be important in reducing the incidence of gastrointestinal cancers.

Our findings provide strong evidence that XN and its metabolite 8-PN are bona fide agonists for FXR and induce human CAMP gene expression. Both inhibit the growth of colon cancer cell lines by reducing proliferation and inducing apoptosis (190-192). It remains to be determined if CAMP induction contributes to the increased apoptosis. Although the XN and 8-PN concentrations that we used in our *in vitro* experiments were high (100 μM), this high concentration can be achieved in epithelial cells of the GI tract from diet or dietary supplements. In addition, although the concentration of 8-PN in beer is considered too low to affect human health, its precursor isoxanthohumol is the prevailing prenylflavonoid in beer and is about 10–30 times more abundant than 8-PN, which means the uptake of active doses after

a small amount of beer consumption could fall within the range of biological

activities (133). Vitamin D is important in reducing rates of colon cancer (193).

In light of the cooperative induction of CAMP by vitamin D and XN or 8-PN

observed Chapter 3, it is tempting to speculate that the combination of these

nuclear receptor ligands could be important in prevention of colon cancer.

Future studies will focus on determining if induction of CAMP by these

compounds prevents cancer cell growth.

In summary, in this thesis we identified XN and 8-PN as novel FXR ligands

that regulate the expression of FXR target genes including human CAMP and

genes involved in maintaining bile acid homeostasis. The latter reduce the

generation of toxic secondary bile acids that are involved in causing

gastrointestinal cancer, and the induction of CAMP gene expression may

induce the apoptosis of colon cancer cells that result from such insults. Thus,

the combination may work to reduce rates of colon cancer. In addition,

ingesting foods rich in plant polyphenols that act as ligands for FXR may be

critical for increasing the expression of CAMP that, in turn, may impact the

composition of the gut microbiota. Furthermore, even though dietary

compounds from daily food consumption do not achieve physiological

concentrations that are high enough to have any biological activity alone, we

showed that XN and 8-PN both can cooperatively induce CAMP gene

expression with $1,25(OH)_2D_3$. This suggests that two or more dietary

compounds may work together to induce gene expression even at low physiological concentrations. The consumption of a diet rich in these compounds and maintaining adequate vitamin D levels may reduce the rate and severity of intestinal infections, reduce the risk of cancer development, and maintain a healthy gut microbiota. In the long run, this will improve immune system function and gastrointestinal tract health (Figure 5.1).

Figure 5.1
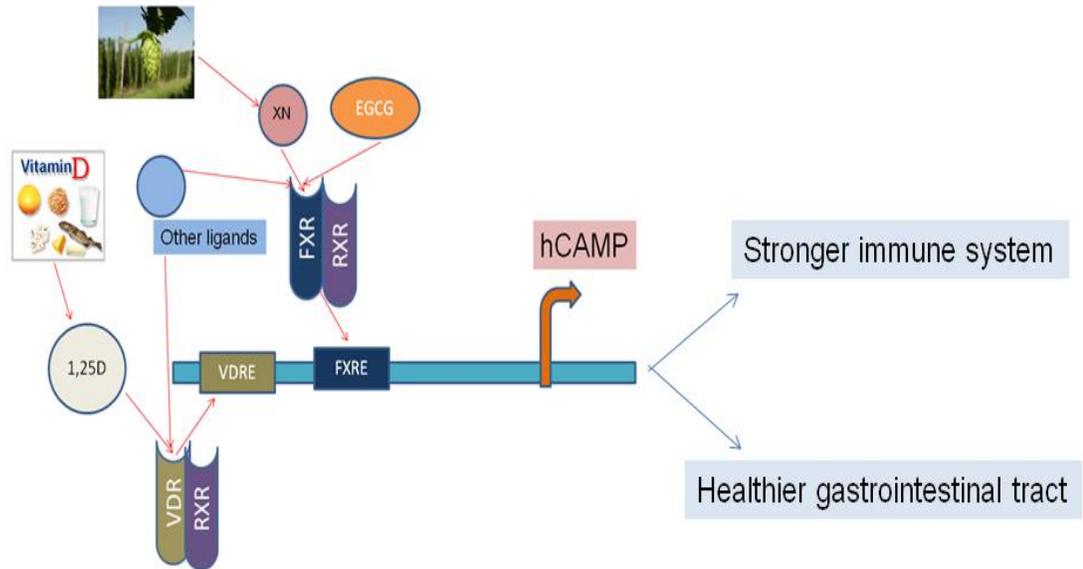
Figure 5.1. Summary of the regulation of human CAMP gene expression by various dietary compounds and potential role in the gastrointestinal tract immunity and health. 1,25(OH)$_2$D$_3$ binds to the VDR which heterodimerizes with RXR and interact with the VDRE located in the Alu-SINE sequence in the promoter region of human CAMP gene and induces CAMP expression. Other dietary compounds, such as XN from beer hops and its metabolite 8-PN activate FXR which binds to a yet unidentified FXRE in the CAMP gene promoter and induces CAMP gene expression. Induction of human CAMP transgene expression in mice may the enhance barrier function immune system and increase protection in the GI tract against bacterial infections or enhance GI tract health.

Bibliography

1.   Gombart AF. The vitamin D-antimicrobial peptide pathway and its role in protection against infection. Future Microbiol. 2009;4(9):1151-65. PMCID: 2821804.

2.   Bikle D. Nonclassic actions of vitamin D. J Clin Endocrinol Metab. 2009;94(1):26-34. PMCID: 2630868.

3.   Koyyalamudi SR, Jeong SC, Song CH, Cho KY, Pang G. Vitamin D2 formation and bioavailability from Agaricus bisporus button mushrooms treated with ultraviolet irradiation. J Agric Food Chem. 2009;57(8):3351-5.

4.   Lee GS, Byun HS, Yoon KH, Lee JS, Choi KC, Jeung EB. Dietary calcium and vitamin D2 supplementation with enhanced Lentinula edodes improves osteoporosis-like symptoms and induces duodenal and renal active calcium transport gene expression in mice. Eur J Nutr. 2009;48(2):75-83.

5.   Jones G, Strugnell SA, DeLuca HF. Current understanding of the molecular actions of vitamin D. Physiol Rev. 1998;78(4):1193-231.

6.   Prosser DE, Jones G. Enzymes involved in the activation and inactivation of vitamin D. Trends Biochem Sci. 2004;29(12):664-73.

7.   Larrick JW, Lee J, Ma S, Li X, Francke U, Wright SC, et al. Structural, functional analysis and localization of the human CAP18 gene. FEBS Lett. 1996;398(1):74-80.

8.   Gombart AF, Borregaard N, Koeffler HP. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. FASEB J. 2005;19(9):1067-77.

9.   Gombart A, Saito T, Koeffler HP. Exapation of an ancient Alu short interspersed element provides a highly conserved vitamin D-mediated innate immune response in humans and primates. BMC Genomics. 2009;10(1):321.

10.   Tjabringa GS, Rabe KF, Hiemstra PS. The human cathelicidin LL-37: a multifunctional peptide involved in infection and inflammation in the lung. Pulm Pharmacol Ther. 2005;18(5):321-7.

11.   De Smet K, Contreras R. Human antimicrobial peptides: defensins, cathelicidins and histatins. Biotechnol Lett. 2005;27(18):1337-47.

12.   Jacobsen F, Mittler D, Hirsch T, Gerhards A, Lehnhardt M, Voss B, et al. Transient cutaneous adenoviral gene therapy with human host defense peptide hCAP-18/LL-37 is effective for the treatment of burn wound infections. Gene Ther. 2005;12(20):1494-502.

13.   Yamasaki K, Schauber J, Coda A, Lin H, Dorschner RA, Schechter NM, et al. Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2006;20(12):2068-80.

14.   Sorensen OE, Follin P, Johnsen AH, Calafat J, Tjabringa GS, Hiemstra PS, et al. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. Blood. 2001;97(12):3951-9.

15.   Gudmundsson GH, Agerberth B, Odeberg J, Bergman T, Olsson B, Salcedo R. The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes. European journal of biochemistry / FEBS. 1996;238(2):325-32.

16.   Durr UH, Sudheendra US, Ramamoorthy A. LL-37, the only human member of the cathelicidin family of antimicrobial peptides. Biochimica et biophysica acta. 2006;1758(9):1408-25.

17.   Johansson J, Gudmundsson GH, Rottenberg ME, Berndt KD, Agerberth B. Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. The Journal of biological chemistry. 1998;273(6):3718-24.

18.   Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, et al. Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature. 2001;414(6862):454-7.

19.   Agerberth B, Gudmundsson GH. Host antimicrobial defence peptides in human disease. Curr Top Microbiol Immunol. 2006;306:67-90.

20.   Larrick JW, Hirata M, Balint RF, Lee J, Zhong J, Wright SC. Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. Infect Immun. 1995;63(4):1291-7. PMCID: 173149.

21.   Nagaoka I, Hirota S, Niyonsaba F, Hirata M, Adachi Y, Tamura H, et al. Cathelicidin family of antibacterial peptides CAP18 and CAP11 inhibit the expression of TNF-alpha by blocking the binding of LPS to CD14(+) cells. J Immunol. 2001;167(6):3329-38.

22.   Mookherjee N, Brown KL, Bowdish DM, Doria S, Falsafi R, Hokamp K, et al. Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. J Immunol. 2006;176(4):2455-64.

23.   Kirikae T, Hirata M, Yamasu H, Kirikae F, Tamura H, Kayama F, et al. Protective effects of a human 18-kilodalton cationic antimicrobial protein (CAP18)-derived peptide against murine endotoxemia. Infect Immun. 1998;66(5):1861-8. PMCID: 108136.

24.   Scott MG, Davidson DJ, Gold MR, Bowdish D, Hancock RE. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. J Immunol. 2002;169(7):3883-91.

25.   Tjabringa GS, Ninaber DK, Drijfhout JW, Rabe KF, Hiemstra PS. Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. Int Arch Allergy Immunol. 2006;140(2):103-12.

26.   Murakami M, Dorschner RA, Stern LJ, Lin KH, Gallo RL. Expression and secretion of cathelicidin antimicrobial peptides in murine mammary glands and human milk. Pediatric research. 2005;57(1):10-5.

27.   Andersson E, Sorensen OE, Frohm B, Borregaard N, Egesten A, Malm J. Isolation of human cationic antimicrobial protein-18 from seminal plasma and its association with prostasomes. Hum Reprod. 2002;17(10):2529-34.

28.   Murakami M, Ohtake T, Dorschner RA, Gallo RL. Cathelicidin antimicrobial peptides are expressed in salivary glands and saliva. J Dent Res. 2002;81(12):845-50.

29.   Murakami M, Ohtake T, Dorschner RA, Schittek B, Garbe C, Gallo RL. Cathelicidin anti-microbial peptide expression in sweat, an innate defense system for the skin. J Invest Dermatol. 2002;119(5):1090-5.

30.   Putsep K, Carlsson G, Boman HG, Andersson M. Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. Lancet. 2002;360(9340):1144-9.

31.   Pasupuleti M, Schmidtchen A, Malmsten M. Antimicrobial peptides: key components of the innate immune system. Crit Rev Biotechnol. 2012;32(2):143-71.

32.   Papagianni M. Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. Biotechnol Adv. 2003;21(6):465-99.

33.   Sitaram N, Nagaraj R. Host-defense antimicrobial peptides: importance of structure for activity. Curr Pharm Des. 2002;8(9):727-42.

34.   Lai Y, Gallo RL. AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. Trends Immunol. 2009;30(3):131-41. PMCID: 2765035.

35.   Ganz T. Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol. 2003;3(9):710-20.

36.   Guo C, Rosoha E, Lowry MB, Borregaard N, Gombart AF. Curcumin induces human cathelicidin antimicrobial peptide gene expression through a vitamin D receptor-independent pathway. J Nutr Biochem. 2012.

37.   Forman BM, Goode E, Chen J, Oro AE, Bradley DJ, Perlmann T, et al. Identification of a nuclear receptor that is activated by farnesol metabolites. Cell. 1995;81(5):687-93.

38.   Guo GL, Lambert G, Negishi M, Ward JM, Brewer HB, Jr., Kliewer SA, et al. Complementary roles of farnesoid X receptor, pregnane X receptor, and constitutive androstane receptor in protection against bile acid toxicity. J Biol Chem. 2003;278(46):45062-71.

39.   Gadaleta RM, van Erpecum KJ, Oldenburg B, Willemsen EC, Renooij W, Murzilli S, et al. Farnesoid X receptor activation inhibits inflammation and preserves the intestinal barrier in inflammatory bowel disease. Gut. 2011;60(4):463-72.

40.   Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. Cell. 2000;102(6):731-44.

41.   Sirvent A, Verhoeven AJ, Jansen H, Kosykh V, Darteil RJ, Hum DW, et al. Farnesoid X receptor represses hepatic lipase gene expression. J Lipid Res. 2004;45(11):2110-5.

42.   Yamagata K, Yoshimochi K, Daitoku H, Hirota K, Fukamizu A. Bile acid represses the peroxisome proliferator-activated receptor-gamma coactivator-1 promoter activity in a small heterodimer partner-dependent manner. Int J Mol Med. 2007;19(5):751-6.

43.   Savkur RS, Thomas JS, Bramlett KS, Gao Y, Michael LF, Burris TP. Ligand-dependent coactivation of the human bile acid receptor FXR by the peroxisome proliferator-activated receptor gamma coactivator-1alpha. J Pharmacol Exp Ther. 2005;312(1):170-8.

44.   Stayrook KR, Bramlett KS, Savkur RS, Ficorilli J, Cook T, Christe ME, et al. Regulation of carbohydrate metabolism by the farnesoid X receptor. Endocrinology. 2005;146(3):984-91.

45.   Wang YD, Yang F, Chen WD, Huang X, Lai L, Forman BM, et al. Farnesoid X receptor protects liver cells from apoptosis induced by serum deprivation in vitro and fasting in vivo. Mol Endocrinol. 2008;22(7):1622-32.

46.   Evans RM. The steroid and thyroid hormone receptor superfamily. Science. 1988;240(4854):889-95.

47.   Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, et al. The nuclear receptor superfamily: the second decade. Cell. 1995;83(6):835-9.

48.   Thomas AM, Hart SN, Kong B, Fang J, Zhong XB, Guo GL. Genome-wide tissue-specific farnesoid X receptor binding in mouse liver and intestine. Hepatology. 2010;51(4):1410-9.

49.   Inagaki T, Moschetta A, Lee YK, Peng L, Zhao G, Downes M, et al. Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor. Proc Natl Acad Sci U S A. 2006;103(10):3920-5. PMCID: 1450165.

50.   Fiorucci S, Mencarelli A, Cipriani S, Renga B, Palladino G, Santucci L, et al. Activation of the Bile Acid Sensor FXR Protects Against Gastrointestinal Injury Caused By ASA And NSAIDs in Mice. Br J Pharmacol. 2011.

51.   Liu N, Meng Z, Lou G, Zhou W, Wang X, Zhang Y, et al. Hepatocarcinogenesis in FXR-/- mice mimics human HCC progression that operates through HNF1alpha regulation of FXR expression. Molecular endocrinology. 2012;26(5):775-85. PMCID: 3355555.

52.   D'Aldebert E, Biyeyeme Bi Mve MJ, Mergey M, Wendum D, Firrincieli D, Coilly A, et al. Bile salts control the antimicrobial peptide cathelicidin through nuclear receptors in the human biliary epithelium. Gastroenterology. 2009;136(4):1435-43.

53.   Nozawa H. Xanthohumol, the chalcone from beer hops (Humulus lupulus L.), is the ligand for farnesoid X receptor and ameliorates lipid and glucose metabolism in KK-A(y) mice. Biochem Biophys Res Commun. 2005;336(3):754-61.

54.   Zhao F, Nozawa H, Daikonnya A, Kondo K, Kitanaka S. Inhibitors of nitric oxide production from hops (Humulus lupulus L.). Biol Pharm Bull. 2003;26(1):61-5.

55.   Cho YC, Kim HJ, Kim YJ, Lee KY, Choi HJ, Lee IS, et al. Differential anti-inflammatory pathway by xanthohumol in IFN-gamma and LPS-activated macrophages. Int Immunopharmacol. 2008;8(4):567-73.

56.   Cho YC, You SK, Kim HJ, Cho CW, Lee IS, Kang BY. Xanthohumol inhibits IL-12 production and reduces chronic allergic contact dermatitis. Int Immunopharmacol. 2010;10(5):556-61.

57.   Milligan SR, Kalita JC, Heyerick A, Rong H, De Cooman L, De Keukeleire D. Identification of a potent phytoestrogen in hops (Humulus lupulus L.) and beer. J Clin Endocrinol Metab. 1999;84(6):2249-52.

58.   Guo J, Nikolic D, Chadwick LR, Pauli GF, van Breemen RB. Identification of human hepatic cytochrome P450 enzymes involved in the metabolism of 8-prenylnaringenin and isoxanthohumol from hops (Humulus lupulus L.). Drug metabolism and disposition: the biological fate of chemicals. 2006;34(7):1152-9.

59.   Possemiers S, Bolca S, Grootaert C, Heyerick A, Decroos K, Dhooge W, et al. The prenylflavonoid isoxanthohumol from hops (Humulus lupulus L.) is activated into the potent phytoestrogen 8-prenylnaringenin in vitro and in the human intestine. J Nutr. 2006;136(7):1862-7.

60.   Zanetti M. The role of cathelicidins in the innate host defenses of mammals. Curr Issues Mol Biol. 2005;7(2):179-96.

61.   Ganz T, Lehrer RI. Defensins. Pharmacol Ther. 1995;66(2):191-205.

62.   Zanetti M, Gennaro R, Romeo D. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. FEBS Lett. 1995;374(1):1-5.

63.   Cowland JB, Johnsen AH, Borregaard N. hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules. FEBS Lett. 1995;368(1):173-6.

64.   Sorensen O, Cowland JB, Askaa J, Borregaard N. An ELISA for hCAP-18, the cathelicidin present in human neutrophils and plasma. J Immunol Methods. 1997;206(1-2):53-9.

65.   Gombart AF, Bhan I, Borregaard N, Tamez H, Camargo CA, Jr., Koeffler HP, et al. Low plasma level of cathelicidin antimicrobial peptide (hCAP18) predicts increased infectious disease mortality in patients undergoing hemodialysis. Clin Infect Dis. 2009;48(4):418-24.

66.   Diamond G, Bevins CL. beta-Defensins: endogenous antibiotics of the innate host defense response. Clin Immunol Immunopathol. 1998;88(3):221-5.

67.   Quayle AJ, Porter EM, Nussbaum AA, Wang YM, Brabec C, Yip KP, et al. Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. Am J Pathol. 1998;152(5):1247-58. PMCID: 1858596.

68.   Yang D, Biragyn A, Kwak LW, Oppenheim JJ. Mammalian defensins in immunity: more than just microbicidal. Trends Immunol. 2002;23(6):291-6.

69.   Duits LA, Ravensbergen B, Rademaker M, Hiemstra PS, Nibbering PH. Expression of beta-defensin 1 and 2 mRNA by human monocytes, macrophages and dendritic cells. Immunology. 2002;106(4):517-25. PMCID: 1782759.

70.   Harder J, Bartels J, Christophers E, Schroder JM. Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. J Biol Chem. 2001;276(8):5707-13.

71.   Chen H, Xu Z, Peng L, Fang X, Yin X, Xu N, et al. Recent advances in the research and development of human defensins. Peptides. 2006;27(4):931-40.

72.   Joly S, Maze C, McCray PB, Jr., Guthmiller JM. Human beta-defensins 2 and 3 demonstrate strain-selective activity against oral microorganisms. J Clin Microbiol. 2004;42(3):1024-9. PMCID: 356847.

73.   Zaalouk TK, Bajaj-Elliott M, George JT, McDonald V. Differential regulation of beta-defensin gene expression during Cryptosporidium parvum infection. Infect Immun. 2004;72(5):2772-9. PMCID: 387892.

74.   Underwood MA, Bevins CL. Defensin-barbed innate immunity: clinical associations in the pediatric population. Pediatrics. 2010;125(6):1237-47.

75.   Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, et al. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. J Immunol. 2004;173(5):2909-12.

76.   Weber G, Heilborn JD, Chamorro Jimenez CI, Hammarsjo A, Torma H, Stahle M. Vitamin D induces the antimicrobial protein hCAP18 in human skin. J Invest Dermatol. 2005;124(5):1080-2.

77.   Gombart AF, O'Kelly J, Saito T, Koeffler HP. Regulation of the CAMP gene by 1,25(OH)2D3 in various tissues. J Steroid Biochem Mol Biol. 2007;103(3-5):552-7.

78.   Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science. 2006;311(5768):1770-3.

79.   Schauber J, Dorschner RA, Coda AB, Buchau AS, Liu PT, Kiken D, et al. Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism. J Clin Invest. 2007;117(3):803-11. PMCID: 1784003.

80.   Yuk JM, Shin DM, Lee HM, Yang CS, Jin HS, Kim KK, et al. Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin. Cell Host Microbe. 2009;6(3):231-43.

81.   Liu PT, Schenk M, Walker VP, Dempsey PW, Kanchanapoomi M, Wheelwright M, et al. Convergence of IL-1beta and VDR activation pathways in human TLR2/1-induced antimicrobial responses. PLoS One. 2009;4(6):e5810. PMCID: 2686169.

82.   Voss E, Wehkamp J, Wehkamp K, Stange EF, Schroder JM, Harder J. NOD2/CARD15 mediates induction of the antimicrobial peptide human beta-defensin-2. J Biol Chem. 2006;281(4):2005-11.

83.   Wang TT, Dabbas B, Laperriere D, Bitton AJ, Soualhine H, Tavera-Mendoza LE, et al. Direct and indirect induction by 1,25-dihydroxyvitamin D3 of the NOD2/CARD15-defensin beta2 innate immune pathway defective in Crohn disease. J Biol Chem. 2010;285(4):2227-31. PMCID: 2807280.

84.   Dai X, Sayama K, Tohyama M, Shirakata Y, Hanakawa Y, Tokumaru S, et al. PPARgamma mediates innate immunity by regulating the 1alpha,25-dihydroxyvitamin D3 induced hBD-3 and cathelicidin in human keratinocytes. J Dermatol Sci. 2010;60(3):179-86.

85.   Peric M, Koglin S, Dombrowski Y, Gross K, Bradac E, Buchau A, et al. Vitamin D analogs differentially control antimicrobial peptide/"alarmin"expression in psoriasis. PLoS One. 2009;4(7):e6340. PMCID: 2709447.

86.   Chiang JY. Bile acids: regulation of synthesis. J Lipid Res. 2009;50(10):1955-66. PMCID: 2739756.

87.   Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, et al. Identification of a nuclear receptor for bile acids. Science. 1999;284(5418):1362-5.

88.   Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, et al. Vitamin D receptor as an intestinal bile acid sensor. Science. 2002;296(5571):1313-6.

89.   Prawitt J, Caron S, Staels B. Bile acid metabolism and the pathogenesis of type 2 diabetes. Curr Diab Rep. 2011;11(3):160-6.

90.   Reschly EJ, Krasowski MD. Evolution and function of the NR1I nuclear hormone receptor subfamily (VDR, PXR, and CAR) with respect to metabolism of xenobiotics and endogenous compounds. Curr Drug Metab. 2006;7(4):349-65. PMCID: 2231810.

91.   Peric M, Koglin S, Dombrowski Y, Gross K, Bradac E, Ruzicka T, et al. VDR and MEK-ERK dependent induction of the antimicrobial peptide cathelicidin in keratinocytes by lithocholic acid. Mol Immunol. 2009;46(16):3183-7.

92.   Termen S, Tollin M, Rodriguez E, Sveinsdottir SH, Johannesson B, Cederlund A, et al. PU.1 and bacterial metabolites regulate the human gene CAMP encoding antimicrobial peptide LL-37 in colon epithelial cells. Mol Immunol. 2008;45(15):3947-55.

93. Ishizawa M, Matsunawa M, Adachi R, Uno S, Ikeda K, Masuno H, et al. Lithocholic acid derivatives act as selective vitamin D receptor modulators without inducing hypercalcemia. J Lipid Res. 2008;49(4):763-72.

94. Karagiannis TC, El-Osta A. The paradox of histone deacetylase inhibitor-mediated modulation of cellular responses to radiation. Cell Cycle. 2006;5(3):288-95.

95. Islam D, Bandholtz L, Nilsson J, Wigzell H, Christensson B, Agerberth B, et al. Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. Nat Med. 2001;7(2):180-5.

96. Raqib R, Sarker P, Bergman P, Ara G, Lindh M, Sack DA, et al. Improved outcome in shigellosis associated with butyrate induction of an endogenous peptide antibiotic. Proc Natl Acad Sci U S A. 2006;103(24):9178-83. PMCID: 1482586.

97. Steinmann J, Halldorsson S, Agerberth B, Gudmundsson GH. Phenylbutyrate induces antimicrobial peptide expression. Antimicrob Agents Chemother. 2009;53(12):5127-33. PMCID: 2786349.

98. Sarker P, Ahmed S, Tiash S, Rekha RS, Stromberg R, Andersson J, et al. Phenylbutyrate counteracts Shigella mediated downregulation of cathelicidin in rabbit lung and intestinal epithelia: a potential therapeutic strategy. PLoS One. 2011;6(6):e20637. PMCID: 3108617.

99. Schauber J, Dorschner RA, Yamasaki K, Brouha B, Gallo RL. Control of the innate epithelial antimicrobial response is cell-type specific and dependent on relevant microenvironmental stimuli. Immunology. 2006;118(4):509-19. PMCID: 1782325.

100. Schwab M, Reynders V, Loitsch S, Steinhilber D, Schroder O, Stein J. The dietary histone deacetylase inhibitor sulforaphane induces human beta-defensin-2 in intestinal epithelial cells. Immunology. 2008;125(2):241-51. PMCID: 2561129.

101. Yin L, Chung WO. Epigenetic regulation of human beta-defensin 2 and CC chemokine ligand 20 expression in gingival epithelial cells in response to oral bacteria. Mucosal Immunol. 2011;4(4):409-19. PMCID: 3118861.

102. Nian H, Delage B, Ho E, Dashwood RH. Modulation of histone deacetylase activity by dietary isothiocyanates and allyl sulfides: studies with sulforaphane and garlic organosulfur compounds. Environ Mol Mutagen. 2009;50(3):213-21. PMCID: 2701665.

103. Hall JA, Grainger JR, Spencer SP, Belkaid Y. The role of retinoic acid in tolerance and immunity. Immunity. 2011;35(1):13-22.

104. Kamashev D, Vitoux D, De The H. PML-RARA-RXR oligomers mediate retinoid and rexinoid/cAMP cross-talk in acute promyelocytic leukemia cell differentiation. J Exp Med. 2004;199(8):1163-74. PMCID: 2211888.

105. Wu H, Zhang G, Minton JE, Ross CR, Blecha F. Regulation of cathelicidin gene expression: induction by lipopolysaccharide, interleukin-6,

retinoic acid, and Salmonella enterica serovar typhimurium infection. Infect Immun. 2000;68(10):5552-8. PMCID: 101505.

106.   Elloumi HZ, Holland SM. Complex regulation of human cathelicidin gene expression: novel splice variants and 5'UTR negative regulatory element. Mol Immunol. 2008;45(1):204-17. PMCID: 2121615.

107.   Harder J, Meyer-Hoffert U, Wehkamp K, Schwichtenberg L, Schroder JM. Differential gene induction of human beta-defensins (hBD-1, -2, -3, and -4) in keratinocytes is inhibited by retinoic acid. J Invest Dermatol. 2004;123(3):522-9.

108.   Schule R, Rangarajan P, Yang N, Kliewer S, Ransone LJ, Bolado J, et al. Retinoic acid is a negative regulator of AP-1-responsive genes. Proc Natl Acad Sci U S A. 1991;88(14):6092-6. PMCID: 52028.

109.   Wang N, Su Q, Boeckh-Herwig S, Yaneva M, Tempst P. Delayed-late activation of a myeloid defensin minimal promoter by retinoids and inflammatory mediators. Leuk Res. 2004;28(8):879-89.

110.   Xiao W, Hsu YP, Ishizaka A, Kirikae T, Moss RB. Sputum cathelicidin, urokinase plasminogen activation system components, and cytokines discriminate cystic fibrosis, COPD, and asthma inflammation. Chest. 2005;128(4):2316-26.

111.   Jeng L, Yamshchikov AV, Judd SE, Blumberg HM, Martin GS, Ziegler TR, et al. Alterations in vitamin D status and anti-microbial peptide levels in patients in the intensive care unit with sepsis. J Transl Med. 2009;7:28. PMCID: 2684740.

112.   Hata TR, Kotol P, Jackson M, Nguyen M, Paik A, Udall D, et al. Administration of oral vitamin D induces cathelicidin production in atopic individuals. J Allergy Clin Immunol. 2008;122(4):829-31.

113.   Hertting O, Holm A, Luthje P, Brauner H, Dyrdak R, Jonasson AF, et al. Vitamin D induction of the human antimicrobial Peptide cathelicidin in the urinary bladder. PLoS One. 2010;5(12):e15580. PMCID: 3001888.

114.   Ginde AA, Mansbach JM, Camargo CA, Jr. Vitamin D, respiratory infections, and asthma. Curr Allergy Asthma Rep. 2009;9(1):81-7.

115.   Urashima M, Segawa T, Okazaki M, Kurihara M, Wada Y, Ida H. Randomized trial of vitamin D supplementation to prevent seasonal influenza A in schoolchildren. Am J Clin Nutr. 2010;91(5):1255-60.

116.   Sabetta JR, DePetrillo P, Cipriani RJ, Smardin J, Burns LA, Landry ML. Serum 25-hydroxyvitamin d and the incidence of acute viral respiratory tract infections in healthy adults. PLoS One. 2010;5(6):e11088. PMCID: 2885414.

117.   Martineau AR, Timms PM, Bothamley GH, Hanifa Y, Islam K, Claxton AP, et al. High-dose vitamin D(3) during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial. Lancet. 2011;377(9761):242-50.

118.   Li-Ng M, Aloia JF, Pollack S, Cunha BA, Mikhail M, Yeh J, et al. A randomized controlled trial of vitamin D3 supplementation for the prevention of

symptomatic upper respiratory tract infections. Epidemiology and Infection. 2009;137(10):1396-404.

119. Roth DE, Jones AB, Prosser C, Robinson JL, Vohra S. Vitamin D status is not associated with the risk of hospitalization for acute bronchiolitis in early childhood. European Journal of Clinical Nutrition. 2009;63(2):297-9.

120. Wejse C, Gomes VF, Rabna P, Gustafson P, Aaby P, Lisse IM, et al. Vitamin D as supplementary treatment for tuberculosis: a double-blind, randomized, placebo-controlled trial. Am J Respir Crit Care Med. 2009;179(9):843-50.

121. Raqib R, Sarker P, Mily A, Alam NH, Arifuzzaman AS, Rekha RS, et al. Efficacy of sodium butyrate adjunct therapy in shigellosis: a randomized, double-blind, placebocontrolled clinical trial. BMC Infect Dis. 2012;12(1):111.

122. Jurutka PW, Bartik L, Whitfield GK, Mathern DR, Barthel TK, Gurevich M, et al. Vitamin D receptor: key roles in bone mineral pathophysiology, molecular mechanism of action, and novel nutritional ligands. J Bone Miner Res. 2007;22 Suppl 2:V2-10.

123. Wang H, Chen J, Hollister K, Sowers LC, Forman BM. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. Mol Cell. 1999;3(5):543-53.

124. Urizar NL, Liverman AB, Dodds DT, Silva FV, Ordentlich P, Yan Y, et al. A natural product that lowers cholesterol as an antagonist ligand for FXR. Science. 2002;296(5573):1703-6.

125. Cui J, Huang L, Zhao A, Lew JL, Yu J, Sahoo S, et al. Guggulsterone is a farnesoid X receptor antagonist in coactivator association assays but acts to enhance transcription of bile salt export pump. J Biol Chem. 2003;278(12):10214-20.

126. Carter BA, Taylor OA, Prendergast DR, Zimmerman TL, Von Furstenberg R, Moore DD, et al. Stigmasterol, a soy lipid-derived phytosterol, is an antagonist of the bile acid nuclear receptor FXR. Pediatr Res. 2007;62(3):301-6.

127. Del Bas JM, Ricketts ML, Vaque M, Sala E, Quesada H, Ardevol A, et al. Dietary procyanidins enhance transcriptional activity of bile acid-activated FXR in vitro and reduce triglyceridemia in vivo in a FXR-dependent manner. Mol Nutr Food Res. 2009;53(7):805-14.

128. Li G, Lin W, Araya JJ, Chen T, Timmermann BN, Guo GL. A tea catechin, epigallocatechin-3-gallate, is a unique modulator of the farnesoid X receptor. Toxicol Appl Pharmacol. 2012;258(2):268-74. PMCID: 3259191.

129. Gao X, Deeb D, Liu Y, Gautam S, Dulchavsky SA, Gautam SC. Immunomodulatory activity of xanthohumol: inhibition of T cell proliferation, cell-mediated cytotoxicity and Th1 cytokine production through suppression of NF-kappaB. Immunopharmacol Immunotoxicol. 2009;31(3):477-84. PMCID: 2759314.

130.   Lee IS, Lim J, Gal J, Kang JC, Kim HJ, Kang BY, et al. Anti-inflammatory activity of xanthohumol involves heme oxygenase-1 induction via NRF2-ARE signaling in microglial BV2 cells. Neurochem Int. 2011;58(2):153-60.

131.   Dorn C, Massinger S, Wuzik A, Heilmann J, Hellerbrand C. Xanthohumol suppresses inflammatory response to warm ischemia-reperfusion induced liver injury. Exp Mol Pathol. 2013;94(1):10-6.

132.   Stevens JF, Page JE. Xanthohumol and related prenylflavonoids from hops and beer: to your good health! Phytochemistry. 2004;65(10):1317-30.

133.   Stevens JF, Taylor AW, Deinzer ML. Quantitative analysis of xanthohumol and related prenylflavonoids in hops and beer by liquid chromatography-tandem mass spectrometry. J Chromatogr A. 1999;832(1-2):97-107.

134.   Ananthanarayanan M, Balasubramanian N, Makishima M, Mangelsdorf DJ, Suchy FJ. Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. J Biol Chem. 2001;276(31):28857-65.

135.   Yang D, Yang J, Shi D, Xiao D, Chen YT, Black C, et al. Hypolipidemic agent Z-guggulsterone: metabolism interplays with induction of carboxylesterase and bile salt export pump. J Lipid Res. 2012;53(3):529-39. PMCID: 3276476.

136.   Chen F, Ananthanarayanan M, Emre S, Neimark E, Bull LN, Knisely AS, et al. Progressive familial intrahepatic cholestasis, type 1, is associated with decreased farnesoid X receptor activity. Gastroenterology. 2004;126(3):756-64.

137.   Lew JL, Zhao A, Yu J, Huang L, De Pedro N, Pelaez F, et al. The farnesoid X receptor controls gene expression in a ligand- and promoter-selective fashion. J Biol Chem. 2004;279(10):8856-61.

138.   Xu G, Pan LX, Li H, Forman BM, Erickson SK, Shefer S, et al. Regulation of the farnesoid X receptor (FXR) by bile acid flux in rabbits. J Biol Chem. 2002;277(52):50491-6.

139.   Milligan SR, Kalita JC, Pocock V, Van De Kauter V, Stevens JF, Deinzer ML, et al. The endocrine activities of 8-prenylnaringenin and related hop (Humulus lupulus L.) flavonoids. J Clin Endocrinol Metab. 2000;85(12):4912-5.

140.   Choi Y, van Breemen RB. Development of a screening assay for ligands to the estrogen receptor based on magnetic microparticles and LC-MS. Comb Chem High Throughput Screen. 2008;11(1):1-6. PMCID: 2736128.

141.   Gilad LA, Bresler T, Gnainsky J, Smirnoff P, Schwartz B. Regulation of vitamin D receptor expression via estrogen-induced activation of the ERK 1/2 signaling pathway in colon and breast cancer cells. J Endocrinol. 2005;185(3):577-92.

142.   Inagaki T, Choi M, Moschetta A, Peng L, Cummins CL, McDonald JG, et al. Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. Cell Metab. 2005;2(4):217-25.

143.   Hollman DA, Milona A, van Erpecum KJ, van Mil SW. Anti-inflammatory and metabolic actions of FXR: insights into molecular mechanisms. Biochim Biophys Acta. 2012;1821(11):1443-52.

144.   Mencarelli A, Renga B, Migliorati M, Cipriani S, Distrutti E, Santucci L, et al. The bile acid sensor farnesoid X receptor is a modulator of liver immunity in a rodent model of acute hepatitis. J Immunol. 2009;183(10):6657-66.

145.   Vavassori P, Mencarelli A, Renga B, Distrutti E, Fiorucci S. The bile acid receptor FXR is a modulator of intestinal innate immunity. J Immunol. 2009;183(10):6251-61.

146.   Catalano S, Malivindi R, Giordano C, Gu G, Panza S, Bonofiglio D, et al. Farnesoid X receptor, through the binding with steroidogenic factor 1-responsive element, inhibits aromatase expression in tumor Leydig cells. J Biol Chem. 2010;285(8):5581-93. PMCID: 2820785.

147.   Huang W, Ma K, Zhang J, Qatanani M, Cuvillier J, Liu J, et al. Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration. Science. 2006;312(5771):233-6.

148.   Abdelkarim M, Caron S, Duhem C, Prawitt J, Dumont J, Lucas A, et al. The farnesoid X receptor regulates adipocyte differentiation and function by promoting peroxisome proliferator-activated receptor-gamma and interfering with the Wnt/beta-catenin pathways. J Biol Chem. 2010;285(47):36759-67. PMCID: 2978604.

149.   Prestwood KM, Kenny AM, Kleppinger A, Kulldorff M. Ultralow-dose micronized 17beta-estradiol and bone density and bone metabolism in older women: a randomized controlled trial. JAMA. 2003;290(8):1042-8.

150.   Gallo RL, Kim KJ, Bernfield M, Kozak CA, Zanetti M, Merluzzi L, et al. Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse. J Biol Chem. 1997;272(20):13088-93.

151.   Hase K, Eckmann L, Leopard JD, Varki N, Kagnoff MF. Cell differentiation is a key determinant of cathelicidin LL-37/human cationic antimicrobial protein 18 expression by human colon epithelium. Infect Immun. 2002;70(2):953-63. PMCID: 127717.

152.   Schauber J, Svanholm C, Termen S, Iffland K, Menzel T, Scheppach W, et al. Expression of the cathelicidin LL-37 is modulated by short chain fatty acids in colonocytes: relevance of signalling pathways. Gut. 2003;52(5):735-41. PMCID: 1773650.

153.   Iimura M, Gallo RL, Hase K, Miyamoto Y, Eckmann L, Kagnoff MF. Cathelicidin mediates innate intestinal defense against colonization with epithelial adherent bacterial pathogens. J Immunol. 2005;174(8):4901-7.

154.   Gnacadja G, Shoshitaishvili A, Gresser MJ, Varnum B, Balaban D, Durst M, et al. Monotonicity of interleukin-1 receptor-ligand binding with

respect to antagonist in the presence of decoy receptor. J Theor Biol. 2007;244(3):478-88.

155.   Koon HW, Shih DQ, Chen J, Bakirtzi K, Hing TC, Law I, et al. Cathelicidin signaling via the Toll-like receptor protects against colitis in mice. Gastroenterology. 2011;141(5):1852-63 e1-3. PMCID: 3199285.

156.   Begum F, Zhu W, Namaka MP, Frost EE. A novel decalcification method for adult rodent bone for histological analysis of peripheral-central nervous system connections. J Neurosci Methods. 2010;187(1):59-66.

157.   Nygard AB, Jorgensen CB, Cirera S, Fredholm M. Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. BMC Mol Biol. 2007;8:67. PMCID: 2000887.

158.   Douyere D, Joseph C, Gaucher C, Chaussain C, Courson F. Familial hypophosphatemic vitamin D-resistant rickets--prevention of spontaneous dental abscesses on primary teeth: a case report. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2009;107(4):525-30.

159.   Rabbani A, Rahmani P, Ziaee V, Ghodoosi S. Dental problems in hypophosphatemic rickets, a cross sectional study. Iran J Pediatr. 2012;22(4):531-4. PMCID: 3533156.

160.   Maran RR, Thomas A, Roth M, Sheng Z, Esterly N, Pinson D, et al. Farnesoid X receptor deficiency in mice leads to increased intestinal epithelial cell proliferation and tumor development. J Pharmacol Exp Ther. 2009;328(2):469-77. PMCID: 2682273.

161.   Nielsen N, Wetterslev J, al-Subaie N, Andersson B, Bro-Jeppesen J, Bishop G, et al. Target temperature management after out-of-hospital cardiac arrest-a randomized, parallel-group, assessor-blinded clinical trial-rationale and design. American Heart Journal. 2012;163(4):541-8.

162.   Steri R, Achenbach J, Steinhilber D, Schubert-Zsilavecz M, Proschak E. Investigation of imatinib and other approved drugs as starting points for antidiabetic drug discovery with FXR modulating activity. Biochem Pharmacol. 2012;83(12):1674-81.

163.   Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, et al. Bile acids: natural ligands for an orphan nuclear receptor. Science. 1999;284(5418):1365-8.

164.   Laparra JM, Sanz Y. Interactions of gut microbiota with functional food components and nutraceuticals. Pharmacol Res. 2010;61(3):219-25.

165.   Sanz Y, Santacruz A, Gauffin P. Gut microbiota in obesity and metabolic disorders. Proc Nutr Soc. 2010;69(3):434-41.

166.   Isolauri E, Rautava S, Kalliomaki M, Kirjavainen P, Salminen S. Role of probiotics in food hypersensitivity. Curr Opin Allergy Clin Immunol. 2002;2(3):263-71.

167.   Cong Y, Konrad A, Iqbal N, Elson CO. Probiotics and immune regulation of inflammatory bowel diseases. Curr Drug Targets Inflamm Allergy. 2003;2(2):145-54.

168.   Yoon SS, Sun J. Probiotics, nuclear receptor signaling, and anti-inflammatory pathways. Gastroenterol Res Pract. 2011;2011:971938. PMCID: 3144714.

169.   Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. Science. 2006;312(5778):1355-9. PMCID: 3027896.

170.   DuPont AW, DuPont HL. The intestinal microbiota and chronic disorders of the gut. Nat Rev Gastroenterol Hepatol. 2011;8(9):523-31.

171.   Sartor RB. Microbial influences in inflammatory bowel diseases. Gastroenterology. 2008;134(2):577-94.

172.   Tehrani AB, Nezami BG, Gewirtz A, Srinivasan S. Obesity and its associated disease: a role for microbiota? Neurogastroenterol Motil. 2012;24(4):305-11. PMCID: 3303978.

173.   Tilg H, Kaser A. Gut microbiome, obesity, and metabolic dysfunction. J Clin Invest. 2011;121(6):2126-32. PMCID: 3104783.

174.   Salzman NH, Hung K, Haribhai D, Chu H, Karlsson-Sjoberg J, Amir E, et al. Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol. 2010;11(1):76-83. PMCID: 2795796.

175.   Huang LC, Reins RY, Gallo RL, McDermott AM. Cathelicidin-deficient (Cnlp -/-) mice show increased susceptibility to Pseudomonas aeruginosa keratitis. Invest Ophthalmol Vis Sci. 2007;48(10):4498-508.

176.   Chromek M, Slamova Z, Bergman P, Kovacs L, Podracka L, Ehren I, et al. The antimicrobial peptide cathelicidin protects the urinary tract against invasive bacterial infection. Nat Med. 2006;12(6):636-41.

177.   Yu FS, Cornicelli MD, Kovach MA, Newstead MW, Zeng X, Kumar A, et al. Flagellin stimulates protective lung mucosal immunity: role of cathelicidin-related antimicrobial peptide. J Immunol. 2010;185(2):1142-9. PMCID: 3038689.

178.   Ong PY, Ohtake T, Brandt C, Strickland I, Boguniewicz M, Ganz T, et al. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. N Engl J Med. 2002;347(15):1151-60.

179.   Yang YH, Zheng GG, Li G, Zhang B, Song YH, Wu KF. Expression of LL-37/hCAP-18 gene in human leukemia cells. Leuk Res. 2003;27(10):947-50.

180.   An LL, Ma XT, Yang YH, Lin YM, Song YH, Wu KF. Marked reduction of LL-37/hCAP-18, an antimicrobial peptide, in patients with acute myeloid leukemia. Int J Hematol. 2005;81(1):45-7.

181.   Steinstraesser L, Oezdogan Y, Wang SC, Steinau HU. Host defense peptides in burns. Burns. 2004;30(7):619-27.

182.   Kovach MA, Ballinger MN, Newstead MW, Zeng X, Bhan U, Yu FS, et al. Cathelicidin-Related Antimicrobial Peptide Is Required for Effective Lung Mucosal Immunity in Gram-Negative Bacterial Pneumonia. J Immunol. 2012.

183.   Hikino H, Kohama T, Takemoto T. Biosynthesis of ponasterone A, an insect-moulting substance from Podocarpus macrophyllus. Chem Pharm Bull (Tokyo). 1969;17(2):415.

184.   Santos AC, Chua MT, Eufemio N, Abela C. Isolation of commisterone, a new phytoecdysone from Cyanotis vaga. Experientia. 1970;26(10):1053-4.

185.   Watson AJ, Collins PD. Colon cancer: a civilization disorder. Dig Dis. 2011;29(2):222-8.

186.   Cunningham D, Atkin W, Lenz HJ, Lynch HT, Minsky B, Nordlinger B, et al. Colorectal cancer. Lancet. 2010;375(9719):1030-47.

187.   Schauber J, Iffland K, Frisch S, Kudlich T, Schmausser B, Eck M, et al. Histone-deacetylase inhibitors induce the cathelicidin LL-37 in gastrointestinal cells. Mol Immunol. 2004;41(9):847-54.

188.   Ren SX, Cheng AS, To KF, Tong JH, Li MS, Shen J, et al. Host immune defense peptide LL-37 activates caspase-independent apoptosis and suppresses colon cancer. Cancer research. 2012;72(24):6512-23.

189.   Ren SX, Shen J, Cheng AS, Lu L, Chan RL, Li ZJ, et al. FK-16 Derived from the Anticancer Peptide LL-37 Induces Caspase-Independent Apoptosis and Autophagic Cell Death in Colon Cancer Cells. PLoS One. 2013;8(5):e63641. PMCID: 3659029.

190.   Miranda CL, Stevens JF, Helmrich A, Henderson MC, Rodriguez RJ, Yang YH, et al. Antiproliferative and cytotoxic effects of prenylated flavonoids from hops (Humulus lupulus) in human cancer cell lines. Food Chem Toxicol. 1999;37(4):271-85.

191.   Li H, Chen F, Shang Q, Pan L, Shneider BL, Chiang JY, et al. FXR-activating ligands inhibit rabbit ASBT expression via FXR-SHP-FTF cascade. Am J Physiol Gastrointest Liver Physiol. 2005;288(1):G60-6.

192.   Allsopp P, Possemiers S, Campbell D, Gill C, Rowland I. A comparison of the anticancer properties of isoxanthohumol and 8-prenylnaringenin using in vitro models of colon cancer. Biofactors. 2013.

193.   Evans SR, Shchepotin EI, Young H, Rochon J, Uskokovic M, Shchepotin IB. 1,25-dihydroxyvitamin D3 synthetic analogs inhibit spontaneous metastases in a 1,2-dimethylhydrazine-induced colon carcinogenesis model. Int J Oncol. 2000;16(6):1249-54.