AN ABSTRACT OF THE THESIS OF

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Title: Antimicrobial Peptide Regulation by Small Molecules in Humans and other Primates

Abstract approved:

Adrian F. Gombart

Antimicrobial peptides (AMPs) play an important role in the innate immune system. Determining the pathways by which these proteins are regulated allows for modulation of their expression for better health. Two families of antimicrobial peptides have been studied in humans: cathelicidins and defensins. There is a single cathelicidin in humans called human cathelicidin antimicrobial peptide (CAMP). Defensins are divided into two families in humans, the alpha and beta defensins. In the beta defensin family, defensin beta 4 (DEFB4) is an inducible antimicrobial peptide. Both CAMP and DEFB4 play integral roles in maintaining barrier defenses and health.

The human cathelicidin antimicrobial peptide gene is regulated by a wide array of small molecules; however, there are still many untested small molecules. We proposed a high throughput screen to find additional compounds that regulate antimicrobial peptides. After screening nearly 5,500 small molecules in the NIH Clincal Compound Library and the ChemBridge DIVERSet libarary, two stilbenoids were found that regulate cathelicidin expression. When combined with 1,25 dihydroxy vitamin D3 both stilbenoids synergistically induced cathelicidin gene expression in U937 cells.

DEFB4 is an antimicrobial peptide induced by inflammatory responses and during infections. Several studies observed that DEFB4 is regulated by 1,25 dihydroxy vitamin D_3 either through a vitamin D response element (VDRE) in the promoter or by an indirect pathway that activates NF-kB. It is unclear if the vitamin D receptor directly regulates the DEFB4 gene by binding to its promoter. We hypothesized that if vitamin D induces DEFB4 by the VDR binding to the promoter, then the putative VDRE would be evolutionarily and functionally conserved in humans and primates. To test this hypothesis, we obtained the promoter sequences from 11 primates and investigated the conservation of the VDRE. The sequence was conserved in primates which suggest the VDRE sequence was selected for over 50-60 million years of evolution. This supports a role for the vitamin D pathway in the regulation of the DEFB4 gene, but functional assays have

failed to clearly demonstrate a response of the DEFB4 gene to 1,25 dihydroxy vitamin D in tissue culture systems. Additional experiments are required to elucidate the role of the vitamin D pathway in regulating the DEFB4 gene.

A thorough understanding of antimicrobial peptide gene expression will lay the foundation for therapeutic approaches to strengthen the innate immune system.

Antimicrobial Peptide Regulation by Small Molecules in Humans and other Primates

by
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APPROVED:

Major Professor, representing Biochemistry and Biophysics

Head of the Department of Biochemistry and Biophsyics

Dean of the Graduate School

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

Brian D Sinnott, Author

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I move forward cherishing my time with everyone.

CONTRIBUTION OF AUTHORS

TSTA – hCAMP – LUC was provided by Adrian Gombart. Malcolm Lowry prepared samples and ran the FACS to determine protein expression. Brenda Nui assisted in the screening of the NIH Clinical Compounds Library, ultimately screening the majority of those compounds. Jing Chen assisted in the cloning the DEFB4 promoter for the different primates. Elena Rosoha helped with Q-PCR.

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Chapter 1

Introduction

Antimicrobial proteins (AMPs) are nearly ubiquitous in all vertebrates and are so named because of their primary or secondary antimicrobial functions. Though not containing a strict consensus motif, a majority of these proteins have a well-conserved preproregion that regulates location and activation [1]. The mature proteins are usually approximately 12-58 amino acids. Antimicrobial peptides have a variety of secondary structures, but they consistently have patches of amphipathic hydrophobic and cationic regions. This design is targeted against the cellular membrane of pathogens and makes adaptive resistance to these proteins difficult. The exact mechanism by which different AMPs function is still unknown, but accumulating evidence supports either increasing the permeability of the membrane, permeating through the membrane to attack internal targets, or a combination of both. The broad range of protection and overall inability of microbes to develop resistance has drawn a great deal of attention to developing AMPs as therapeutic agents.

1.1 – The Vitamin D Receptor and the Vitamin D Response Element

The active form of vitamin D is $1,25(OH)_2\ D_3$. It is obtained by conversion of 25-hydroxyvitamin D_3 to $1,25(OH)_2\ D_3$ by 1-α-hydroxylase (Cyp27b1) [2]. $1,25(OH)_2\ D_3$ binds the Vitamin D receptor (VDR), a

transcription factor of the nuclear receptor superfamily. The VDR regulates genes by heterodimerizing with the retinoid X receptor to bind vitamin D response elements (VDREs) and recruiting transcription factors [3]. The putative VDRE consensus sequence consists of two direct repeats of RGKTCA (IUPAC code in Fig 14) separated by three base pairs [4]. We and others have shown that the VDR regulates the production of the human CAMP gene through a VDRE located in its promoter [5, 6].

1.2 – Cathelicidin

The family of antimicrobial peptides known as cathelicidins were originally found in their mature form in bovine neutrophils [7-9]. Soon afterwards, it was determined that these peptides came from a precursor that had a well conserved preproregion that had originally been discovered in pig leukocytes called the cathelin domain [8, 10-13]. Cathelicidins are found in most vertebrates and found as far back evolutionarily as hag fish. In humans there is only one cathelicidin called the human cathelicidin antimicrobial peptide (CAMP) [12, 14]. In humans, CAMP is primarily expressed in and secreted by cells important to innate immunity, such as epithelial cells, macrophages and neutrophils [15-25].

The CAMP gene encodes the 18-kDa human cathelicidin antimicrobial peptide proprotein, hCAP18. The proprotein is cleaved by proteases to release a 37 amino acid peptide called LL-37. This peptide has potent antibacterial activity and functions in wound healing, angiogenesis

and chemotaxis of immune cells [12]. The antimicrobial properties of LL-37 are effective against a range of different pathogens including gram-negative and positive bacteria, as well as some viruses and fungi [26-30]. These effects are seen even at very low concentrations (1-10 μM), but it is not very specific and has a narrow range of function, and so is toxic to mammalian cells at concentrations above 13-25 μM [31-34]. LL-37 is not only effective at killing bacteria, but also at inhibiting their virulent properties, such as biofilm formation in *Pseudomonas aeruginosa* and dampening inflammation from LPS [32, 35-39]. Neutrophils are also recruited by LL-37 [40].

1.3 – Defensin Beta 4

The defensin family is a large group of antimicrobial peptides that have been found in a wide variety of multicellular organisms [41]. These are classified into three different families based upon secondary structure: α, β, and θ defensins. Beta defensins are differentiated from alpha defensins by their disulfide bond arrangement, while theta defensins are cleaved and circularized. Alpha defensins are found in neutrophils in humans, but also expressed in a diverse set of locations in other mammals and theta defensins are expressed in rhesus macaques and baboons [42, 43]. Currently, humans are known to have seventeen different β defensins [44]. Defensin β4 (DEFB4) was first discovered in 1997 in the skin of a patient with lesional psoriasis [45]. Found in a variety of epithelial cells, it is also expressed in macrophages and monocytes [41, 46, 47]. DEFB4 is a 41

amino acid long cationic peptide that contains 6 cysteines that form disulfide bonds [45].

Proper DEFB4 transcriptional and translational regulation plays an important part in innate immunity. Though not constitutively expressed, it is quickly induced upon invasion by yeast, gram-negative and gram-positive bacteria and is highly effective at killing these pathogens [45]. There is also some evidence for increased expression by and protection against viruses [48]. Regulated by many warning signals, DEFB4 is known to be induced by inflammatory cytokines such as IL-1, TNF-α, IL-17, and IL-22 [45, 49-58]. These appear to work predominately through the pathways that activate NF-kB stimulation. Another molecule known to regulate innate immunity, $1,25(OH)_2$ D_3, functioning through the vitamin D Receptor (VDR), can regulate DEFB4 [5, 59]. There are numerous signals that control DEFB4, and improper regulation of DEFB4 has been linked with diseases, such as ulcerative colitis and Crohn's disease [60-64].

1.4 - Objective of our Study

Antimicrobial peptides are important in innate immunity, linking the innate and adaptive immune systems and are regulated by a number of small molecules. Cathelicidin is induced by $1,25(OH)_2$ vitamin D_3 in skin and monocytes [6, 65]. Butyrate is a short chain fatty acid that induces cathelicidin expression in the colon [66, 67]. Lithocholic acid, a secondary bile acid, also increases cathelicidin levels [68]. Some evidence supports

expression of DEFB4 by 1,25$(OH)_2$ D_3 in SCC25 cells [5]. Human beta defensin 3 message is induced by 1,25$(OH)_2$ vitamin D_3 in primary keratinocytes [69]. AMPs are effective at killing many microbes and there are few reports of pathogens developing resistance toward AMPs [41, 70, 71 2010]. Due to the growing number of drug-resistant microbes, boosting our own immune system's defenses to fight infections is promising. Based on the literature showing that several small molecules can modulate endogenous AMP expression, we hypothesize there are additional undiscovered compounds that will modulate AMP expression. The long term goal of our research is to discover small molecules that could safely and effectively strengthen the innate immune system.

Our research is focused on two different AMPs, human cathelicidin antimicrobial peptide (CAMP) and defensin beta 4 (DEFB4). Both have potent antimicrobial properties making them effective agents against a variety of pathogens. By understanding the regulatory mechanisms that modulate the expression of CAMP and DEF4, we hope to develop therapies that will improve human health.

Chapter 2

HTS for inducers of the CAMP gene

Brian Sinnott[1], Malcolm Lowry[2], Brenda Nui[3], and A. F. Gombart[4]

[1]Department of Biochemistry and Biophysics, [2]Department of Microbiology, Oregon State University, Corvallis, Oregon 97331, [3]Department of Biological Sciences, University of Southern California, Los Angeles, California 90089, and [4]Linus Pauling Institute, Department of Biochemisty & Biophysics, Oregon State University, Corvallis, Oregon 97331

2.1- Introduction

Antimicrobial proteins (AMPs) play an important role in innate immunity, the front line defense against infectious disease. Complications from infections, such as severe sepsis, affects approximately 750,000 patients each year [72]. The cost of sepsis is staggering in both life and resources; the prognosis for death of a patient with severe sepsis is about 28-50% and approximately $17 billion dollars is spent annually to fight sepsis worldwide [73]. Antibiotics are still the primary means of treating infections, but with increased resistance to antibiotics alternative treatments are needed. AMPs are a prospective candidate with their antimicrobial function and inherent resistance to bacterial adaption [74]. The cathelicidin antimicrobial peptide (CAMP) is already known to be regulated by a number of different small molecules such as lithocholic acid, butyrate, and $1,25(OH)_2 D_3$ [6, 68, 75-77]. Due to the number of compounds known to regulate CAMP, we predict that there are additional small molecules that may modulate CAMP expression. Identifying compounds that increase expression of human CAMP *in vivo* could be an effective method to boost the innate immune system.

There are a few approaches that are currently being pursued to increase CAMP concentrations, but we believe small compounds are the best option. Synthetic peptides are being produced for use, but these are expensive to prepare and injection of large doses of peptide may have

unwanted side effects [39, 74]. By utilizing endogenous CAMP regulation we believe small molecule induction of CAMP to be an inexpensive and possibly safer alternative. Even problems with drug toxicity due to high concentrations could be dealt with by synergy between two compounds, reducing the dose required to obtain similar activation of CAMP. Aside from the health benefits, this study's possible scientific contributions include the discovery of novel CAMP regulatory mechanisms.

To discover regulatory compounds we developed a high throughput screen (HTS) with the CAMP promoter linked to a luciferase reporter to test two small molecule libraries for activators of the promoter. In the screen, we discovered that both resveratrol and pterostilbene induced the expression of the hCAMP mRNA and protein. Also, when combined with low levels of $1,25(OH)_2D_3$, they synergistically induced expression of human CAMP.

2.2 - Materials and Methods

2.2.1 - Cell Culture

U937 cells were grown in RPMI 1640 supplemented with 10% FBS. HT-29 cells were cultured in DMEM supplemented with 10% FBS. All cells were cultured with antibiotics (100 units penicillin/streptomycin; Invitrogen, Carlsbad, CA). For QRT-PCR cells were treated for 16 hours with compounds. Resveratrol, tetraethylthiuram disulfide, calcipotriol, linezolid (Sigma-Aldrich Corporation, St. Louis, MO),

2-{[2-(1,3-benzothiazol-2-ylsulfonyl)ethyl]thio}-1,3-benzoxazole, 8-quinolinyl (2,4-dichlorophenoxy)acetate

N-(2-bromophenyl)-2-(1H-indol-3-yl)-2-oxoacetamide

2-[5-(5-bromo-2-chlorophenyl)-1,2,4-oxadiazol-3-yl]pyridine,

4-bromo-2-[2-(2-quinolinyl)vinyl]phenyl, acetate

2-(4-bromophenoxy)-N-(2,2,6,6-tetramethyl-4-piperidinyl)propanamide,

N-(4-methyl-2-pyridinyl)thieno[3,2-b][1]benzothiophene-2-carboxamide,

5-isopropyl-2-methoxy-N-(3-methylbutyl)benzenesulfonamide

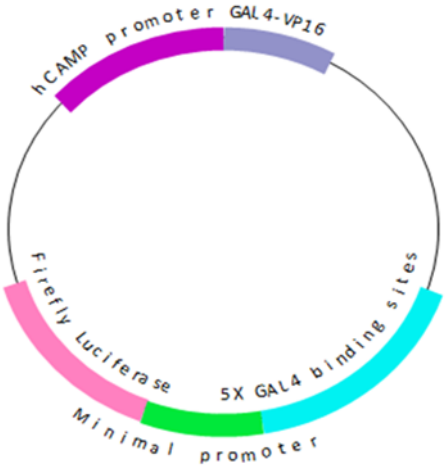
(Chembridge, San Diego, CA) were tested with or without 10^{-9} M 1,25$(OH)_2$ D_3 (Sigma).

2.2.2 - High Throughput Screen

In a Tip-100 5 x 10^7 U937 cells were transfected with 5 µg of the promoterless TSTA-vector or the TSTA-CAMP-Luc (Fig 1) using the Neon System (1400v, 30ms, 1pulse) as described by the manufacturer (Invitrogen) and incubated with FBS supplemented with 10% FBS and no antibiotics. At 8 hours post transfection the cells were seeded into 96 well plates w/ antibiotics and treated with control compounds (DMSO, 10^{-7}M 1,25$(OH)_2$ D_3 and EtOH) or test compounds from either the ChemBridge DIVERSet Library (ChemBridge) or the NIH Clinical Collection (NCC-003) (BioFocus DPI, Inc, Little Chesterford, Saffron Walden, CB10 1XL, United Kingdom) at a 1 x 10^{-5} M concentration. At 24 hours post-transfection, Dual-Glo Luciferase assays (Promega Corporation, Madison, WI) were

performed as instructed by the manufacturer with a SpectraMAXL luminometer (Molecular Devices, Sunnyvale, CA).

A



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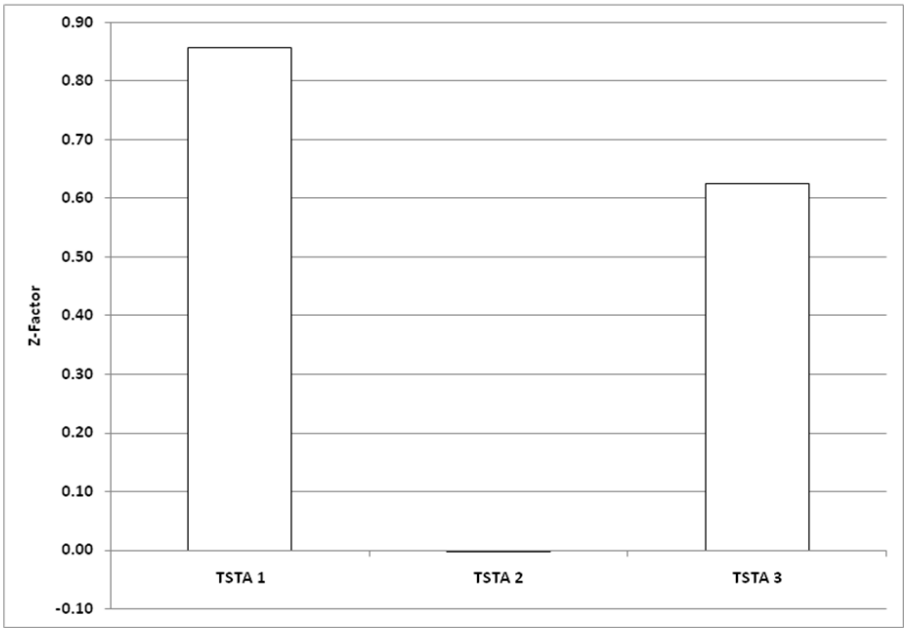

Fig 1: A) TSTA-CAMP-Luc plasmid map with an 800 bp promoter containing the VDRE 5' to a GAL4-VP16 gene. Induction of the hCAMP promoter results in GAL4-VP16 being expressed, which binds strongly to the 5X GAL4 binding site upstream of a firefly luciferase gene, amplifying the hCAMP promoter signal. B) Z-Factors for 3 different TSTA-CAMP-Luc constructs tested in triplicate. Z-factors above 0.80 are robust.

2.2.3 - RNA isolation and quantitative real-time PCR (QRT-PCR)

Total RNA from 2 x 10^6 U937 cells was prepared with Trizol as described by the manufacturer (Invitrogen). All cDNAs were synthesized from 2 µg of RNA using Superscript III reverse transcriptase as described by the manufacturer (Invitrogen). The cDNAs were analyzed by Q-PCR using Taqman probes specific for human CAMP (5'FAM-ACCCCAGGCCCACGATGGAT -BHQ-1-3'), Cyp42A1 (5'FAM-TGCGCATCTTCCATTTGGCG-BHQ-1-3') or 18S (5'FAM-AGCAGGCGCGCAAATTACCC -3' BHQ-1) at a final concentration of 300 nM per reaction. Primers against human CAMP (forward, 5'-GCTAACCTCTACCGCCTCCT -3' and reverse, 5'-GGTCACTGTCCCCATACACC -3'), Cyp24A1 (forward, 5'-GAACGTTGGCTTCAGGAGAA -3' and reverse, 5'-TATTTGCGGACAATCCAACA -3') or 18S (forward, 5'-AAACGGCTACCACATCCAAG -3' and reverse, 5'-CCTCCAATGGATCCTCGTTA -3') were used at 600 nM per reaction. PCR was performed using HotMaster™ Taq polymerase (5 Prime, Inc., Gaithersburg, MD) on a CFX96 Real Time System (Bio-Rad Laboratories, Hercules, CA). The protocol was 95°C, 1 min followed by 45 cycles of 95°C, 15 s and 60°C, 1 min. PCR was performed in triplicate for each sample and

fold change was calculated using ddCT values (treatment versus untreated) and normalized to 18S.

2.2.4 - Flow Cytometery

Treated cells U937 cells with 10^{-8} M $1,25(OH)_2$ D_3 or 10^{-5} M resveratrol for 48 hours. Cells were fixed, permeabilized/blocked, and stained for primary and secondary or secondary antibody alone. The primary antibody for hCAP-18 is a rabbit (a kind gift from N. Borregaard) and the secondary antibody is a Dylight 649 Fab' 2 donkey anti-rabbit (Jackson Immunoresearch, Pike West Grove, PA, USA).

2.3 - Results

2.3.1 - High Throughput Screen

Three different TSTA-CAMP-Luc constructs were screened as candidates for the HTS. The TSTA-CAMP-Luc reporters contained an 800bp region of the hCAMP promoter, including the VDRE (Fig 1). The Z-Factors were compared between the three different constructs (Fig 2). TSTA-CAMP-Luc #1 proved the best candidate and was selected for screening the compounds with a Z-factor above 0.86 and an above 4-fold change in mRNA levels in samples treated with 10^{-7} M $1,25(OH)_2$ D_3 compared to vehicle (Data not shown). A HTS assay that has a Z-factor above 0.8 will be sensitive enough to detect the difference between control and test compounds.

After screening approximately 5000 compounds from the ChemBridge DIVERSet Library and 480 from the NIH Clinical Collection, those that activated the promoter construct 2-fold or greater compared to DMSO treated samples, without significantly decreasing renilla luciferase activity, were tested again in triplicate. Any compound whose averaged values still met the previous criteria was screened for activation of the promoterless TSTA vector. The compounds from the NIH Clincal Collection were also tested in combination with 10^{-7} M $1,25(OH)_2$ D_3 to identify those compounds that could either suppress or cooperate with vitamin D in CAMP gene induction. Those compounds that increased CAMP promoter activity greater than 2-fold are listed in Table 1.

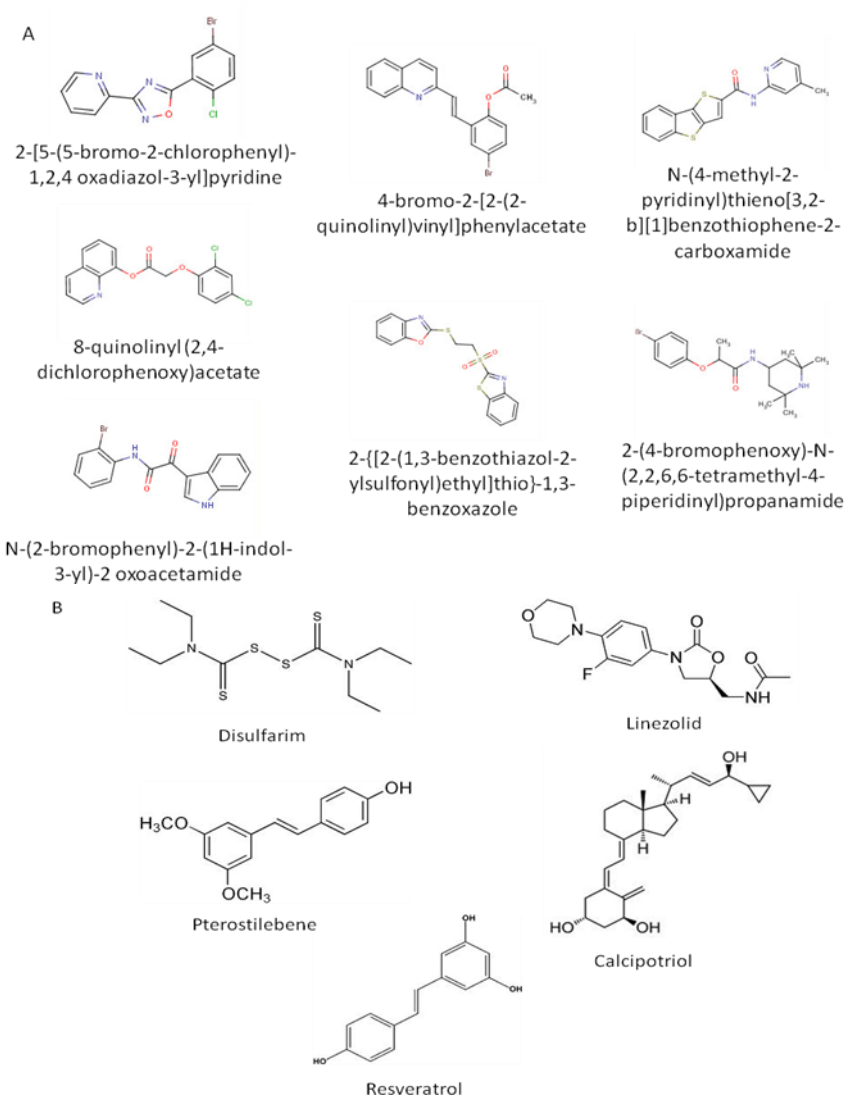

Fig 2: Names and structures of compounds that we tested for activation of the endogenous gene. A) Compounds purchased from the ChemBridge DIVERSet Library. B) Compounds purchased from the NIH Clinical Compound Library.

Compound	**Average Fold Change**
Cytarabine	4.00±0.25
Disulfarim	3.04±0.41
Calcipotriol	9.92±0.02
PTEROSTILBENE	3.24±0.03
LINEZOLID	7.45±0.82
Nitazoxanide	3.36±0.88
Resveratrol	2.88±0.15
2-{[2-(1,3-benzothiazol-2-ylsulfonyl)ethyl]thio}-1,3-benzoxazole	4.19±0.86
8-quinolinyl (2,4-dichlorophenoxy)acetate	2.85±0.36
N-(2-bromophenyl)-2-(1H-indol-3-yl)-2-oxoacetamide	2.53±0.60
2-[5-(5-bromo-2-chlorophenyl)-1,2,4-oxadiazol-3-yl]pyridine	3.05±0.33
4-bromo-2-[2-(2-quinolinyl)vinyl]phenyl acetate	2.92±0.15
2-(4-bromophenoxy)-N-(2,2,6,6-tetramethyl-4-piperidinyl)-propanamide	5.27±1.28
N-(4-methyl-2-pyridinyl)thieno[3,2-b][1]benzothiophene-2-carboxamide	2.36±0.09
5-isopropyl-2-methoxy-N-(3-methylbutyl)benzenesulfonamide	10.03±1.28

Table 1: Normalized RLU average fold change of transfected cells treated with compounds in the HTS that activated the CAMP promoter. Compounds were tested at 10^{-5}M in triplicate in U937 cells transfected with the TSTA-CAMP-Luc.

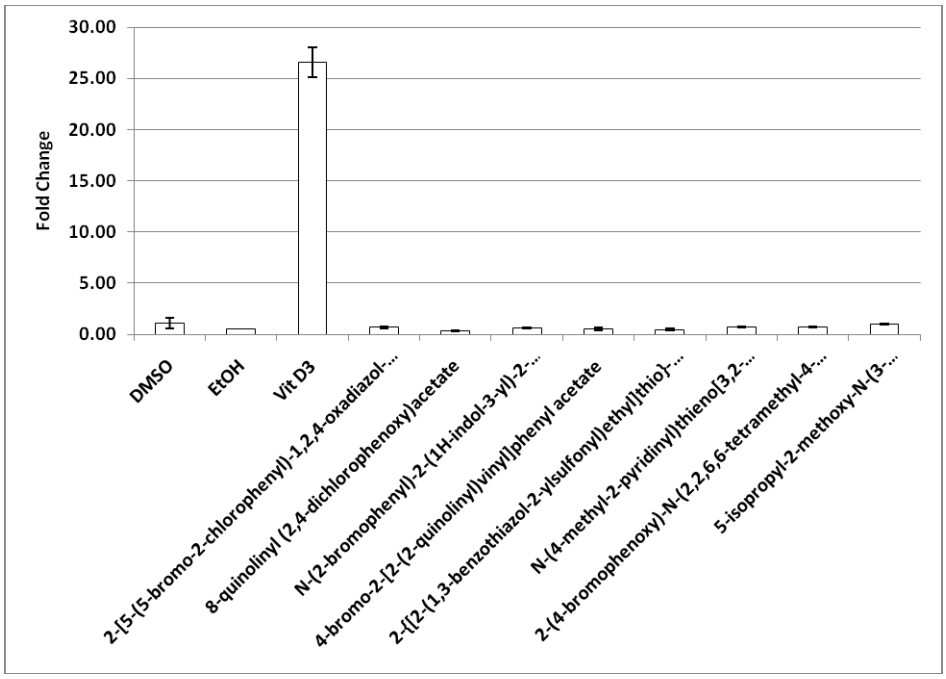

Fig 3: Q-PCR for CAMP with ChemBridge DIVERSet Library Compounds. U937 cells were treated with each compound at 10^{-5} M for 18 hours. DMSO and EtOH (vehicle for the compounds) were included as negative controls and $1,25(OH)_2 D_3$ as a positive control.

2.3.2 - QRT-PCR for HTS Compounds

A set of compounds from Table 1 were purchased for further testing, their names and structures are shown in Fig 2. U937 cells treated with the compounds from the DIVERSet did not activate the endogenous gene when analyzed by Q-PCR (Fig 3). Reseveratrol, calcipitriol (a $1,25(OH)_2 D_3$ analog), and disulfarim (Fig 4) from the NIH Clinical Collection were able to increase endogenous CAMP mRNA expression 4-fold or

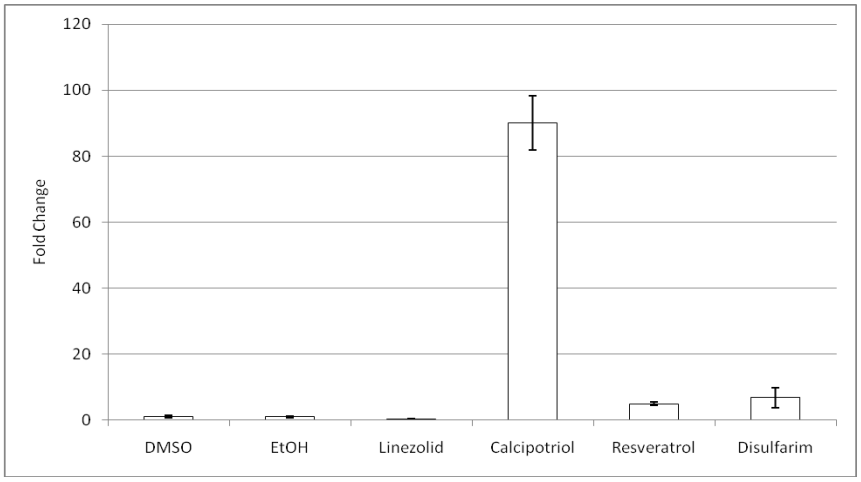

Fig 4: Q-PCR for CAMP with NIH Clinical Compound Library Compounds. U937 cells were treated with each compound at 10^{-5} M for 18 hours. DMSO and EtOH (vehicle for the compounds) were included as negative controls and 1,25 $(OH)_2$ D_3 as a positive control.

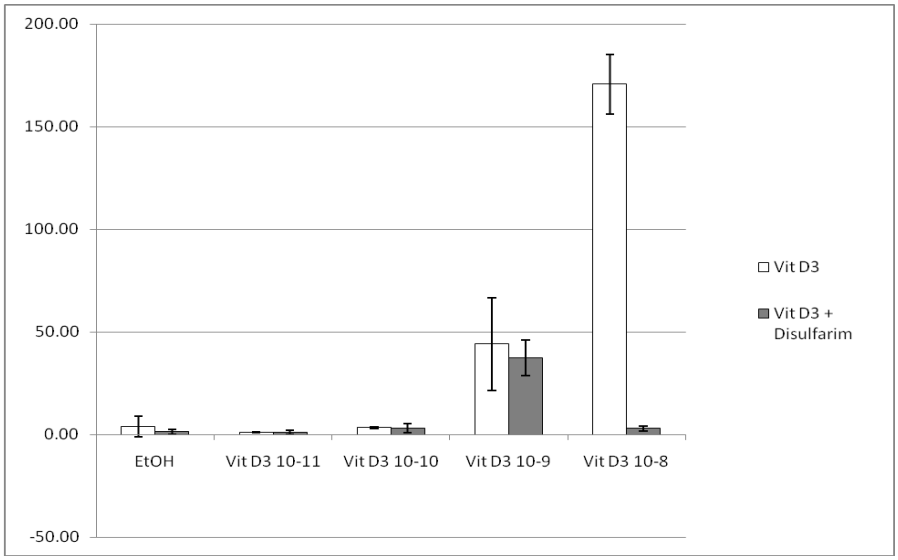

Fig 5: Q-PCR for CAMP with increasing doses of 1,25$(OH)_2$ D_3 with 10^{-5} M disulfarim in U937cells after 18 hours of treatment. Fold change normalized to EtOH.

greater as compared to samples treated with vehicle. Disulfarim did not consistently induce CAMP expression and had no synergistic effect on CAMP mRNA levels when combined with $1,25(OH)_2$ D_3 (Vit D3) (Fig 5). Resveratrol (Res) at concentrations of 10^{-5} M induced CAMP gene expression 4 to 5-fold over untreated cells (Fig 6). Also, when resveratrol was combined with $1,25(OH)_2$ D_3 it increased expression of CAMP 2 to 3-fold higher than samples incubated with $1,25(OH)_2$ D_3 alone (Fig 7).

To determine if resveratrol could induce the expression of other VDR target genes, we measured the levels of Cyp24A1 (24-hydroxylase) by QRT-PCR. Surprisingly, resveratrol alone did not induce Cyp24A1 even at 10^{-5} M concentrations (Fig. 8). This suggests that resveratrol specifically induces CAMP, but not other VDR target genes suggesting a mechanism independent of the VDR. Interestingly, Cyp24A1 expression was synergistically induced when 10^{-5} M resveratrol was combined with $1,25(OH)_2$ D_3 at 10^{-8} M, but not at 10^{-10} M and 10^{-9} M (Fig. 8). This was evidence that other transcriptional targets of the VDR were affected by resveratrol when combined with the vitamin D.

Having confirmed resveratrol induction of endogenous CAMP, the other stilbenoid that activated the CAMP promoter in the HTS, pterostilbene, was tested (Fig 9). CAMP mRNA levels in U937 were induced 5-fold by pterostilbene. Transcriptional activation by pterostilbene and $1,25(OH)_2$ D_3 together increased mRNA expression 205-fold, nearly 2-fold

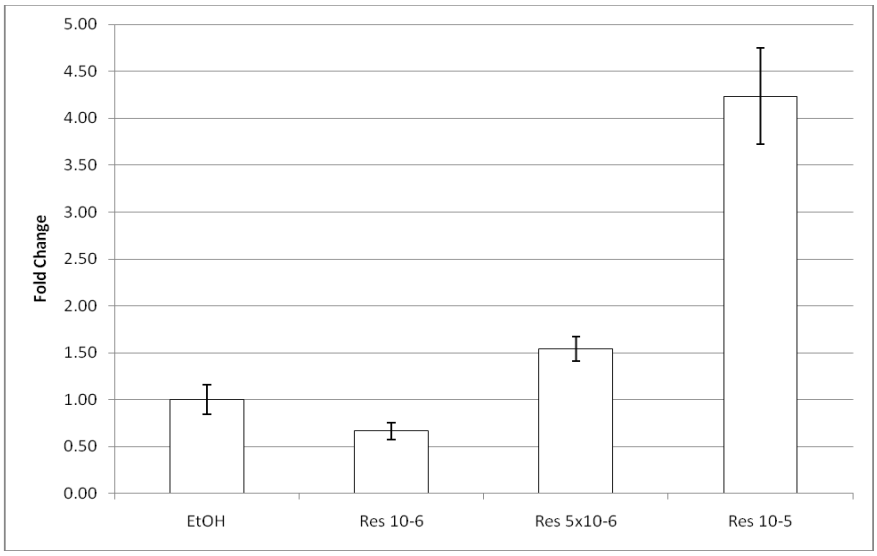

Fig 6: Q-PCR for CAMP with increasing concentrations of resveratrol in U937 cells after 18 hours of treatment

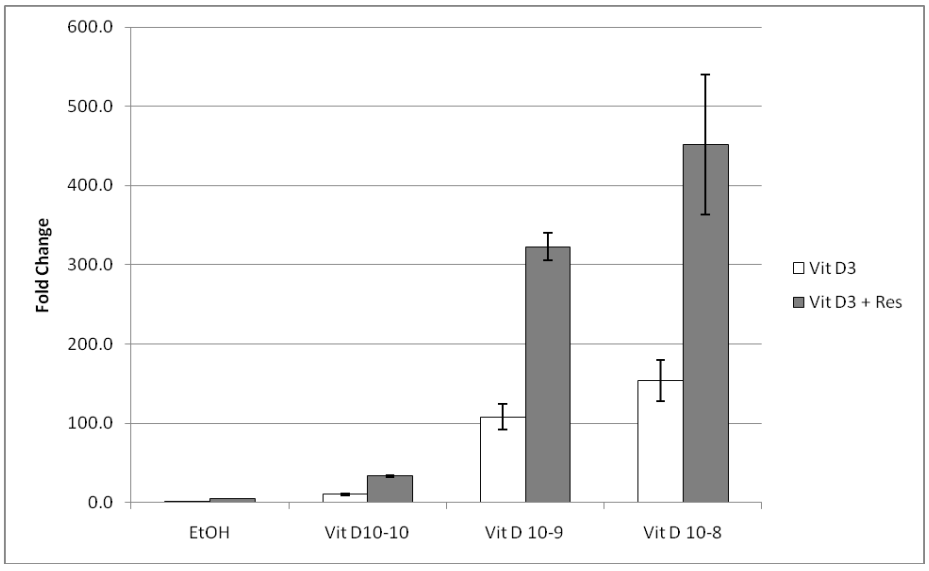

Fig 7: Q-PCR for CAMP with increasing doses of 1,25$(OH)_2$ D_3 with 10^{-5}M resveratrol in U937 cells after 18 hours of treatment.

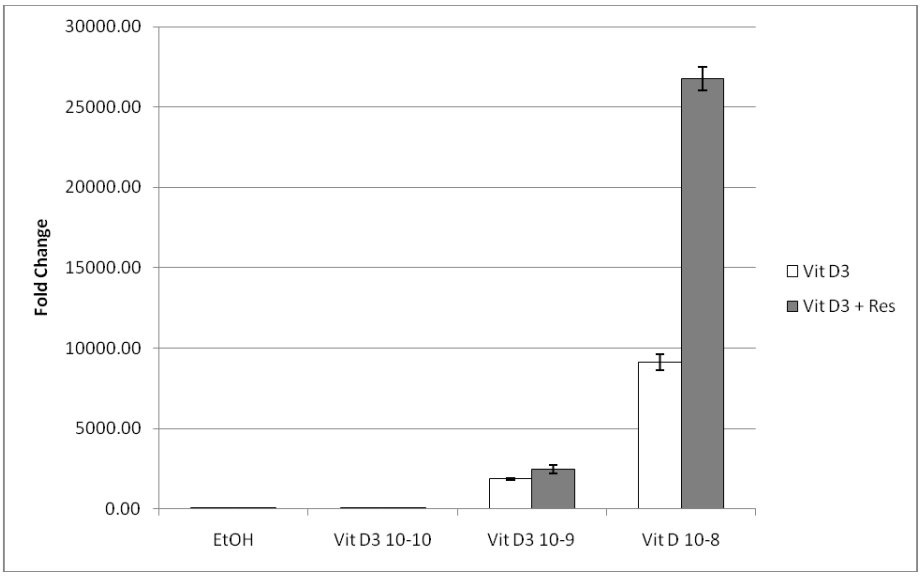

Fig 8: Q-PCR for Cyp24A1 with increasing doses of 1,25$(OH)_2$ D_3 with 10^{-5}M resveratrol in U937cells after 18 hours of treatment.

that of 1,25$(OH)_2$ D_3 alone. The induction of CAMP and synergy with 1,25$(OH)_2$ D_3 is similar to that of resveratrol.

2.3.3 - Protein Expression

To determine if resveratrol induced CAMP protein (hCAP18) levels, intracellular staining and FACS for hCAP18 was performed with cells treated with resveratrol for 24 h. A significant peak shift was observed in cells treated with resveratrol at 10^{-5} M (Fig 10). A shift was not observed at lower concentrations (data not shown). These results are consistent with the induction of CAMP mRNA.

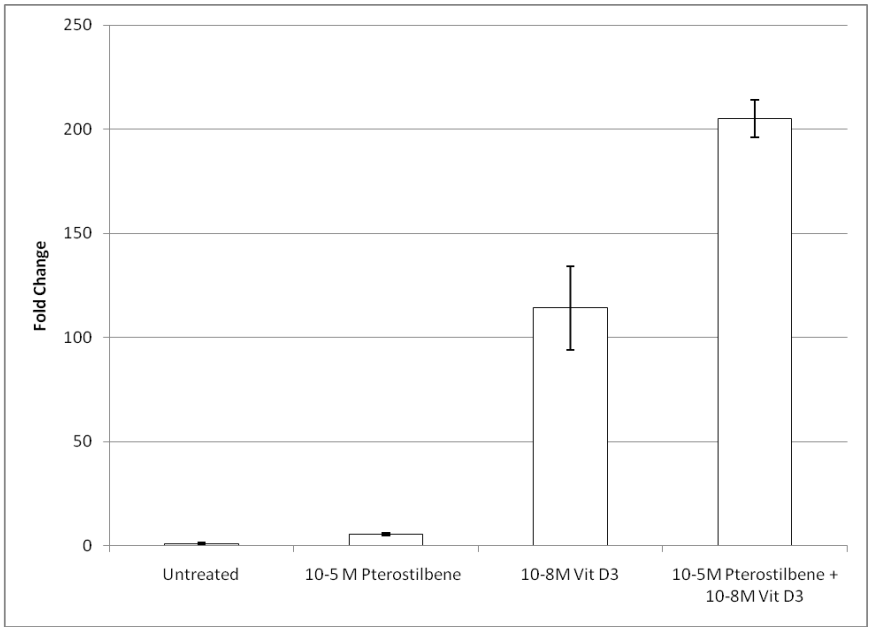

Fig 9: Q-PCR for CAMP with pterostilbene at 10^{-5} M and 1,25$(OH)_2$ D_3 in U937 cells after 18 hours of treatment.

2.4 - Discussion

Two activators of the endogenous CAMP gene were identified by the HTS and Q-PCR. The TSTA-CAMP-Luc was activated by approximately 125 compounds from the ChemBridge DIVER Set and 45 from the NIH Clinical Compounds library. After validating these compounds we reduced the total to 8 candidates from the DIVERSet and 6 from the NIH Clinical Library. The inability of compounds to activate the endogenous gene that activated the CAMP promoter in the HTS may be attributed to the 800bp promoter sequence taken out of the context of the endogenous gene. This sequence may lack important regulatory elements.

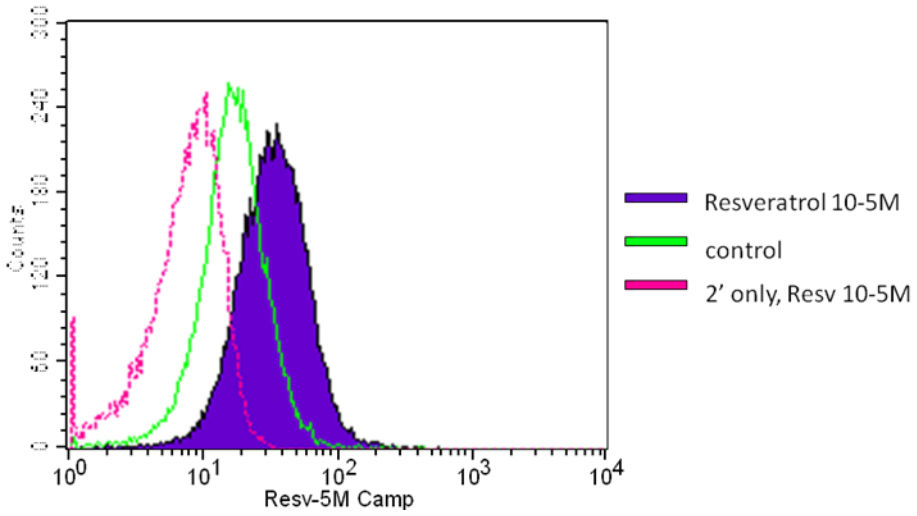

Fig 10: FACS for CAMP protein in U937 cells treated for 48 hours. Cells were treated with EtOH (control) or resveratrol. A group of resveratrol treated cells were also incubated with 2' antibody alone to obtain background fluorescence.

The biological importance for induction of hCAMP by resveratrol and pterostilbene is currently unknown. High concentrations of both compounds were required; those lower than 10^{-5} M did not induce CAMP mRNA or protein levels in U937 cells. Most individuals only maintain nanomolar concentrations of resveratrol in blood when taking 25-50 mg daily. Recently, it was demonstrated that orally administered pterostilbene showed greater bioavailability (80 % versus 20% for resveratrol) and total plasma levels of both the parent compound and metabolites than resveratrol [78]. Pterostilbene may be more biologically active *in vivo* than resveratrol.

Resveratrol, a polyphenolic phytoalexin and phytoestrogen of the stilbene class of compounds, is found at high concentrations in grape seeds. Resveratrol has a variety of confirmed and hypothesized functions through which CAMP induction may occur. Resveratrol is able to activate estrogen receptor α (ERα) in breast cancer cell lines [79]. It is known to indirectly regulate sirtuins, a class III histone deacetylase (HDAC), possibly by activating the NAD+/NAM recovery pathway that SIRT-1 requires for deacetylation [80, 81]. Of the sirtuins regulated by resveratrol, sirtuin 1 (SIRT1) regulates FOXO proteins and p53 [82, 83]. Interestingly, SIRT1 and the FOXO proteins are able to interact with the VDR [84]. Resveratrol causes ATF-6 processing, part of the unfolded protein response (UPR), which may induce CAMP [85].

Pterostibene is a stilbenoid, a dimethyl ether analog of resveratrol, which increased CAMP expression in the HTS, and subsequently endogenous gene expression. Pterostilbene is found in blueberries and other berries, as well as peanuts and grapes [86, 87]. Similar in structure, pterostilbene and resveratrol possibly function through a similar mechanism, but research on pterostilbene lags behind resveratrol. There is no evidence for pterostilbene activating sirtuins or NAD+/NAM. Pterostilbene has anti-inflammitory properties similar to resveratrol, such as NF-kB and COX-2 inhibition [88].

Resveratrol is known for its indirect activation of sirtuins 1, 2, and 7 [78, 89-95]. SIRT1 activation by high concentrations of NAD+ is known to deacetylate forkhead box O1(FOXO1), which leads to increased transcription of target genes by increasing recruitment of C/EBP α (Fig 11) [83, 96-98]. Analysis of the CAMP promoter revealed a C/EBP α binding site [99]. Transfection of C/EBP α increases cathelicidin promoter activity in U937 cells in reporter assays and C/EBPα and -ε regulate expression of the CAMP gene (Gombart et al., data not shown) [100]. One possible mechanism would be SIRT1 deactylation of Foxo1 leading to a recruitment

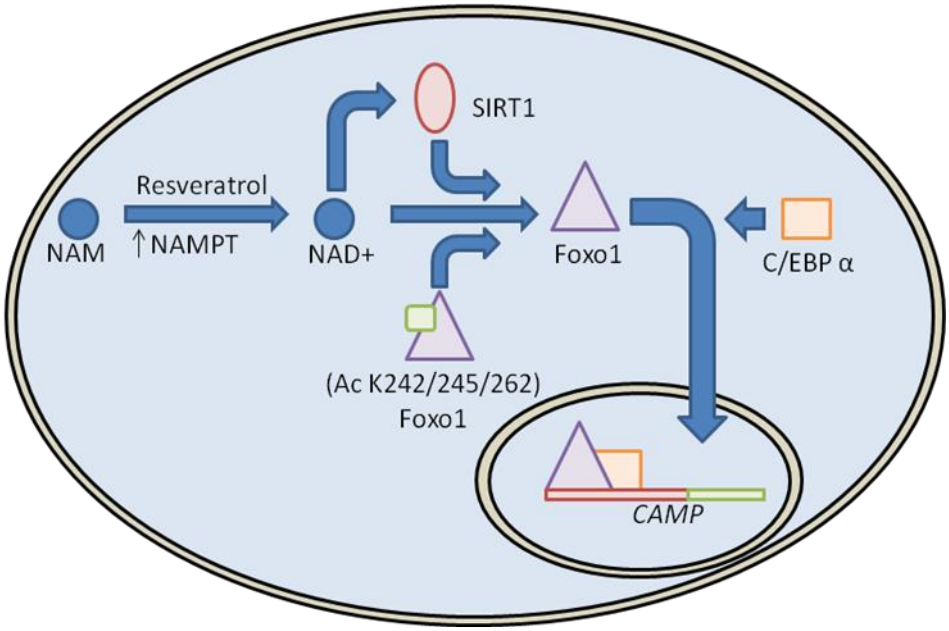

Fig 11: SIRT1 dependant induction of CAMP. Resveratrol enhances NAMPT activity, raising NAD+ concentration and increasing SIRT1 activity. FOXO1 then recruits C/EBP to the CAMP promoter and increases transcription.

of C/EBPα to the CAMP promoter. Further work, using short interfering RNA or sirtinol to abolish sirtuin dependant deacetylation, could help confirm our models [101].

Sirtuins are class III HDACs, and another model for CAMP induction is by histone deacetylation [102]. This silencing may decrease transcription of a repressor of CAMP expression. Contrary to this, HDAC inhibitors (HDACi) such as butyrate induce CAMP in colon cells and keratinocytes [67, 103]. Classical HDACi's such as butyrate and TSA do not

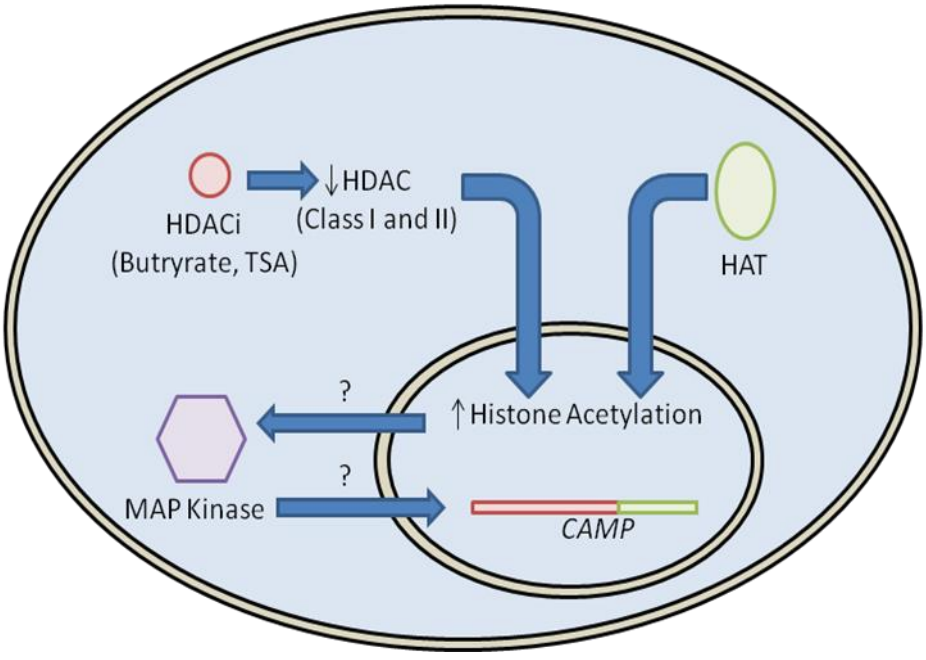

Fig 12: HDACi dependant induction of CAMP. HDACi's induce camp by decreasing HDAC activity, resulting in increased acetylation of histones. Then, through a MAP-Kinase dependant pathway, cathelicidin induction is increased.

inhibit class III HDACs [104]. These appear to function through a mitogen activated protein (MAP) kinase pathway. The HDACi or HDAC mediated induction of CAMP may be cell specific.

The estrogen receptor could also induce the CAMP gene. Resveratrol is a phytoestrogen that activates ERα [79, 105]. Evidence suggests that, though there are no putative estrogen receptor binding sites, the VDR gene has several SP1 sites upstream of exon 1c that estrogen and resveratrol regulate. Mutational analysis of the six SP1 sites found a specific SP1 site that when ablated, abolished the increased VDR promoter induction by resveratrol. Therefore, resveratrol and possibly pterostilbene may be increasing VDR expression, resulting in greater induction of genes containing VDREs. This explanation fits with the minor activation of CAMP by either stilbenoid alone, and the significantly larger synergistic activation with $1,25(OH)_2\ D_3$. Further experiments using tamoxifen, a competitive inhibitor of the estrogen receptor, can clarify this by negating stilbenoid induction of CAMP via ERs.

Future goals in our study are to determine the biological pathways these compounds utilize and develop a method to boost the innate immune system *in vivo* by elevating CAMP levels. The use of $1,25(OH)_2\ D_3$ along with these drugs could synergistically provide increased AMP production. Problems exist though, such as the poor absorption of resveratrol by the body and rapid glucuronation to its predominate form:

trans-resvratrol-3-*O*-glucuronide [106, 107]. Pterostilbene has a similar issue, though uptake and metabolism compared to resveratrol are much better [107]. The capacity of either stilbenoid to induce CAMP *in vivo* remains to be determined. Further research is still required to determine their mechanism of action and ability to boost the innate immune response. The development of more potent stilbenoid analogues combined with vitamin D analogs may provide therapeutically beneficial treatments for infections.

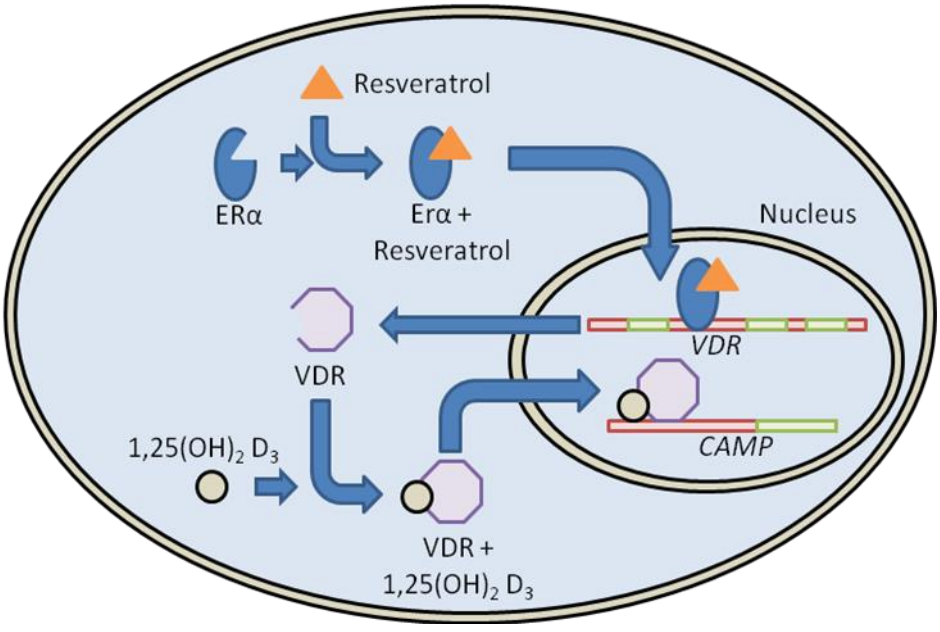

Fig 13: ER receptor mediated induction of CAMP. ERα is bound by resveratrol, which binds the VDR upstream of exon 1c. Extra VDR means more bond $1,25(OH)_2\ D_3$ and increased transcription of CAMP mRNA.

Chapter 3

Conservation of Vitamin D mediated induction of DEFB4 expression in humans and other primates

Brian Sinnott[1], Jing Chen[2], and A. F. Gombart[3]

[1]Department of Biochemistry and Biophysics, [2]Department of Pharmacy, Oregon State University, Corvallis, Oregon 97331, and [3]Linus Pauling Institute, Department of Biochemisty & Biophysics, Oregon State University, Corvallis, Oregon 97331

3.1 - Introduction

Defensin β 4 (DEFB4), an antimicrobial protein, plays an integral part in maintaining the innate immune system barriers against invading pathogens. Furthermore, abnormal levels of DEFB4 has have been linked with inflammatory bowel diseases (IBD) [60-64]. Understanding how DEFB4 is transcriptionally regulated could give greater insight into why these diseases occur and how the innate immune system functions. As such, researchers have discovered multiple inflammatory regulatory elements in the DEFB4 gene promoter [108, 109]. Along with these, a vitamin D response element (VDRE) upstream of DEFB4 was discovered. DEFB4 was induced by 1,25$(OH)_2$ D_3 through the Vitamin D Receptor (VDR), but the induction was not robust [5]. Currently, it is unclear if 1,25$(OH)_2$ D_3 induction of DEFB4 transcription by binding of the VDR to the VDRE in the DEFB4 promoter is important for DEFB4 transcriptional regulation *in vivo*. Elucidating the role that the vitamin D pathway plays in the regulation of DEFB4 expression will clarify the physiological importance of sufficient levels of vitamin D in the innate immune system's response against infection and disease.

In silico and experimental analysis of the DEFB4 promoter revealed a VDRE at approximately -1200 bp from the translational start site [5]. 1,25$(OH)_2$ D_3 treatment only modestly induced DEFB4 message and protein in epithelial and monocytic cells [5, 59, 110] . DEFB4 induction with

$1,25(OH)_2\ D_3$ and the VDR is not robust like for the CAMP gene (personal observation) [5]. The convergence of additional signaling pathways is required for efficient induction of the gene. In monocytes, activation of TLR2/1, expression of IL-1β and $1,25(OH)_2\ D_3$ treatment maximally induce DEFB4 expression [59]. Wang et al., demonstrated induction of NOD2 expression by $1,25(OH)_2\ D_3$, which synergistically acted with NOD2 ligands to up-regulate DEFB4 induction [110]. In both cases, activation of the NF-κB pathway was important. These results have raised questions about the importance of the vitamin D_3 pathway in the direct regulation of DEFB4.

NF-kB transcription factors regulate a variety of host inflammatory and apoptotic responses, and are inactivated by inhibitory I-kB [111]. Inflammatory signals, such as TLR activation from pathogenic invasion, phosphorylate I-kB proteins, which release NF-kB. Activated NF-kB has nuclear localization sequences which bring it into the nucleus where it can bind NF-kB sites in the genome. The DEFB4 promoter contains two proximal (NFkB1, 205 to -186; NFkB2, -596 to -572) and one distal (NFkB3, -2193 to -2182) NF-kB binding sites. Of the three sites the most proximal NF-kB binding sequence has been found to be critical in NF-kB regulation [108, 109, 112]. Mutations in the NF-kB1 binding site in reporter constructs abrogate luciferase activity.

The goal of this study was to elucidate the importance of the vitamin D pathway in regulating the expression of the DEFB4 gene. We

hypothesized that the VDRE in the DEFB4 promoter would be structurally and functionally conserved over the 50-60 million years of primate evolution if it was critical for regulation of the gene. Evolutionary conservation of this regulation in the primate lineages would provide strong evidence that the vitamin D-DEFB4 pathway evolved as a biologically important mechanism for regulating the innate immune response protecting human and non-human primates against infection.

To test our hypothesis, we amplified a ~1600 bp promoter region from the translational start codon in the DEFB4 gene of the human and 11 other primates. Analysis of both cloned sequences and those obtained from the database demonstrated a high degree of conservation of the VDRE in all primates. These findings demonstrate that the VDREs are evolutionarily conserved and suggest that the vitamin D pathway is required for the proper regulation of DEFB4 gene expression.

3.2 - Materials and Methods

3.2.1 - Genomic DNA samples, PCR amplification, sequencing and cloning

The human and primate genomic DNAs (gDNA) used for this study were described previously [6]. The human and primate DEFB4 promoter sequences were amplified using the following primers: forward, 5'-CTGACCCAGCCCTCTCTTT-3' (-1678 to -1659) and reverse 5'-GGCTGATGGCTGGGAGCTTC -3' (+17 to +36) (Fig 14). We amplified this region in humans, *Homo Sapiens (H. Sapiens)*, as well as ten different

primates, *Pan troglodytes (P. troglodytes), Pan paniscus (P. paniscus), Gorilla gorilla (G. gorilla), Pongo pygmaeus (P. pygmaeus), Macaca mulatta (M. mulatta), Macaca nemestrina (M. nemestrina), Cercopithecus aethiops (C. aethiops), Lagothrix lagotricha (L. lagotrihca), Ateles geoffroyi (A. geoffroyi) , Aotus trivirgatus (A. trivirgatus*). The primers were located outside the VDRE and after the translational start site in regions that showed the highest homology among human, chimpanzee, gorilla, and orangutan sequences in the database. The PCR conditions for amplification included 1X Failsafe buffer E (Epicentre Biotechnologies, Madison, WI), 300 nM forward and reverse primers, 300nM dNTPs, 300ng of gDNA, and Failsafe Taq polymerase. The PCR amplification conditions were 94°C for 2 min, followed by 25 cycles of 94°C for 20sec, 55°C for 20 seconds, and 65°C for 2 mins, followed by 10 minutes at 65°C. PCR products were isolated after electrophoresis through a 1% agarose gel and purified by spin column (Zymo Research, Orange, CA). The purified bands were cloned into

Fig 14: Illustration of amplified DEFB4 promoter region showing the VDRE, SP1, and NF-kB binding sites, along with location where the primers annealed.

the pGEM-T Easy vector (Promega Corporation, Madison, WI). Sequencing was performed by the CGRB at Oregon State University.

The consensus sequences used for comparison of transcription factor binding sites, except for the VDRE, were obtained from the JASPAR database (http://jaspar.genereg.net/). It is important to note that these represent *in vitro* binding for the transcription factors and may not function *in vivo*.

3.2.2 - Cell Culture

U937 cells were grown in RPMI 1640 supplemented with 10% FBS and antibiotics (100 units penicillin/streptomycin; Invitrogen, Carlsbad, CA). Cells were treated with 1,25$(OH)_2$ vitamn D_3 (10 nM), IL-1B (50ng/mL) for 16 hours and harvested.

3.2.3 - Reporter Assays, RNA isolation and QRT-PCR

U937 cells were transfected using the Neon system as described by the manufacturer (Invitrogen). In a Tip-100 $5x10^7$ U937 cells were co-transfected (1400v, 30ms, 1pulse) with 5 µg of the pGL4-Luciferase vector (Promega) with or without the DEFB4 promoter. At 24 hours post-transfection, cells were lysed and dual-luciferase assays were performed as instructed by the manufacturer (Promega Corporation) with a SpectraMAXL luminometer (Molecular Devices, Sunnyvale, CA).

For quantitative real-time PCR (QRT-PCR), total RNA was prepared with Trizol reagent (Invitrogen) and cDNAs were synthesized by reverse

transcription using Superscript III reverse transcriptase with 2 µg of RNA as described by the manufacturer (Invitrogen). The cDNAs were then analyzed by Q-PCR using a Taqman probe specific for DEFB4 (5'-d CAL Fluor Red 610-TCCTGATGCCTCTTCCAGGTGTTT-BHQ-1-3') or 18S (5'FAM-AGCAGGCGCGCAAATTACCC -3' BHQ-1) at a final concentration of 100 nM per reaction. Primers against DEFB4 (forward, 5'-GACTCAGCTCCTGGTGAAGC -3' and reverse, 5'-GAGACCACAGGTGCCAATTT -3') or 18S (forward, 5'-AAACGGCTACCACATCCAAG -3' and reverse, 5'-CCTCCAATGGATCCTCGTTA -3') were used at 600 nM per reaction. PCR was performed using HotMaster^{TM} Taq polymerase (5 Prime, Inc., Gaithersburg, MD) on a CFX96 Real Time System (Bio-Rad Laboratories, Hercules, CA). The protocol was 95°C, 1 min followed by 45 cycles of 95°C, 15 s and 60°C, 1 min. PCR was performed in triplicate for each sample and fold change was calculated using ddCT values (treatment versus untreated) and normalized to 18S.

3.3 - Results

3.3.1 - The VDRE is conserved in the promoter of DEFB4 in Primates

The alignment of the human and 10 other primates (Fig 15) shows evolutionary conservation of the VDRE with few nucleotide differences between primates. We used a consensus motif from ChIP-seq data of VDR binding sites in a human lymphoblastoid cell line [113]. We compared the

ChIP-Seq VDRE sequence to the VDRE in the DEFB4 promoters of different primates to determine the degree they matched. The human DEFB4 VDRE perfectly matched the consensus sequence generated from the ChIP-Seq VDRE. Of note, the DEFB4 gene was not bound by the VDR

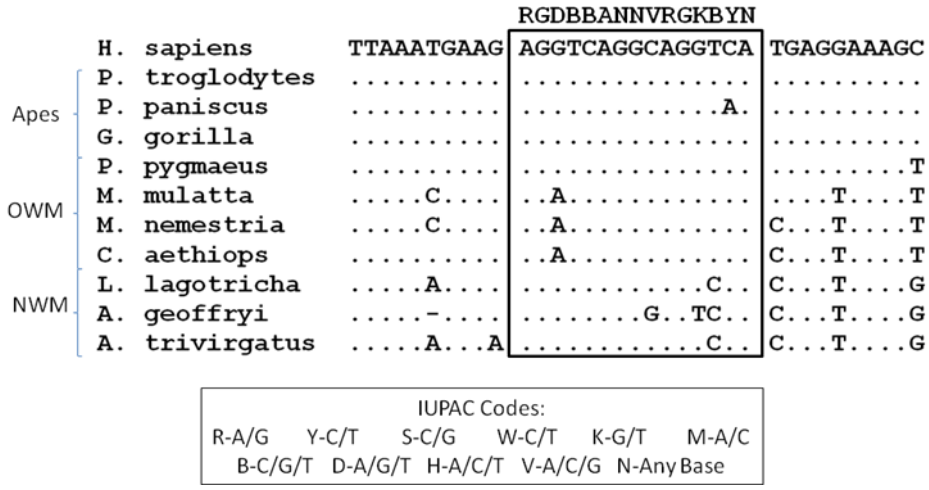

Fig 15: Alignment of DEFB4 promoter of the VDRE to the human sequence compared to 10 primates. With only one exception theses sequences fit the consensus sequence. P. paniscus is the only species with a base pair that differs from the consensus sequence in an essential region.

in the ChIP-seq experiments. The ChIP-seq data may not have identified all possible transcriptional target genes of the VDR because different sets of genes are regulated by the VDR in different cell types. As such, the DEFB4 gene may not be expressed in lymphoblastoid cells treated with vitamin D. The fit of the VDRE in the DEFB4 promoter to the consensus VDRE

sequence suggests that it would be a functional VDR transcription factor binding site.

There were few notable differences between the VDRE from the human and the different primates. The OWM's, excluding *P. pygmaeus*, possessed an A in place of a G at position 3 (Fig 12); this change is consistent with the consensus sequence and would not be expected to affect binding of the VDR. The NWM's have a change from a T to a C at position 13, which still fits the A/C/T predicted in the consensus sequence. In the apes, only *P. paniscus* differed from the human sequence with a C to an A change at position 14, which does not fit the consensus sequence. One base pair change in the NWMs differs from the ChIP-Seq consensus sequence of the VDRE in *A. geoffryi*, which has a C to a G at position 9. This is in the three base-pair spacer sequence and is unlikely to affect VDR binding. Another mutation in *A. geoffryi* from G to T at position 12 is in agreement with the consensus sequence. The sequence differences from the consensus sequence in the DEFB4 VDRE in humans and primates are minimal and would not be expected to impact functionality of the VDRE. The VDRE has been well conserved over 50-60million years of evolution. This implies that the transcription factor binding site is important for proper regulation of the DEFB4 gene. Further work testing the VDREs of these primates in tissue culture for conservation of vitamin D mediated induction is still required.

3.3.2 - Conservation of other Transcription Factor Binding Sites

DEFB4 is induced by NF-κB binding to the promoter. In the 1600 bp fragment that we cloned from the different species there are two NF-KB sites (Figure 16). This does not include a third NF-KB site upstream at ~2.2kb that does not affect NF-kB mediated function [112]. The proximal NFKB1 site is the best conserved of the three with minimal changes. All three NWMs have a change from a T to a G in position 7 that is in the spacer region. Two of the NWMs (*L. lagothrica* and *A. trivirgatus*) have a change from a T to a C in the second direct repeat at position 20; this is closer to the NF-KB consensus sequence. NFKB2 is not as well conserved as the NFKB1. Mutational analysis showed that NFKB2 is a weak inducer of DEFB4 whereas NFKB1 mutation abrogates promoter activity [109, 114]. The changes in the first direct repeat of NFkB2 are in the variable spacer region of the NF-KB consensus sequence at position 5 and 6. In *A. trivirgatus*, the second NF-KB binding site has a change from a G to a C at position 1 which does not match the consensus sequence. In *P. pygmaeus* the replacement of a T to a C matches the consensus sequence. Our data agrees with previous studies showing the importance of the first proximal NF-kB binding site for transcriptional regulation of DEFB4, and the reduced importance of the second NF-kB site in enhancing expression.

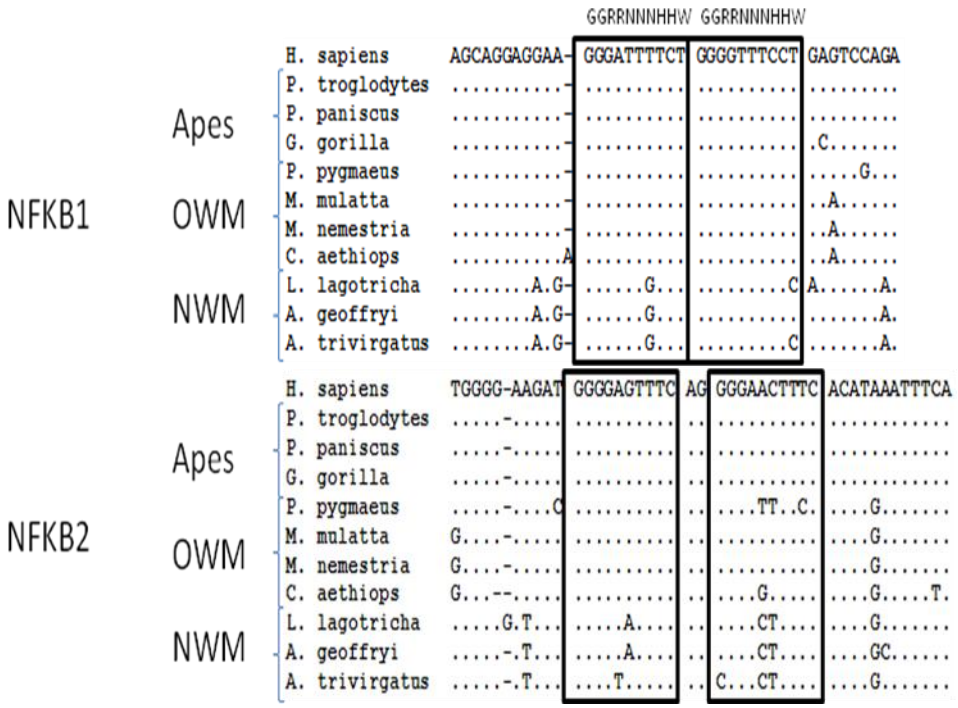

Fig 16: Alignment of DEFB4 promoter of the two NF-kB sites to the human sequence compared to 10 primates. The first NF-kB binding site (NFKB1) is an essential binding site for NF-kB mediated transcription and is well conserved. NFKB2 has limited effect on NF-kB mediated induction and is less conserved.

The most striking conservation is found at the AP1 binding site (Fig 17). AP1 is strongly linked with immunological function. The AP1 site is entirely conserved in all primates, without exception. Previous studies have concluded that mutations in the promoter of the AP1 site in a reporter construct reduced luciferase activity, though not nearly as potently as the removal of the first proximal NF-κB site [109, 114].

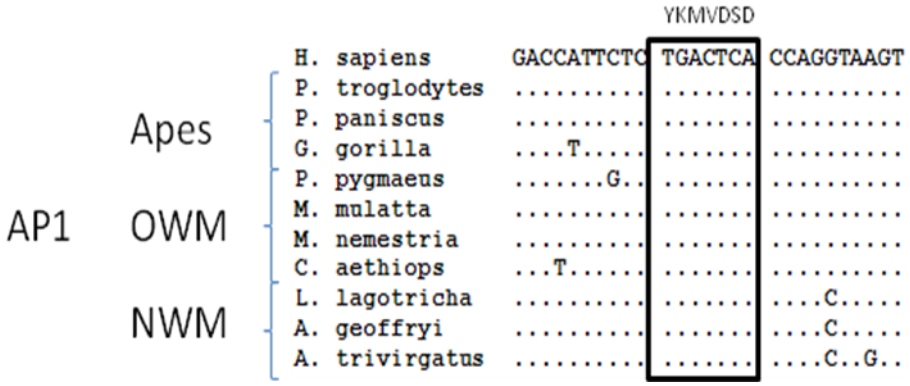

Fig 17: Alignment of DEFB4 promoter of the Ap1 site to the human sequence compared to 10 primates. There is no variation through-out the 50-60 million years of evolution.

3.4 - Discussion

DEFB4 regulation by 1,25$(OH)_2$ D_3 is still not fully understood. DEFB4 induction in macrophages required three different signals; IL-1B, 1,25$(OH)_2$ D_3, and a TLR2 ligand [59]. Others have shown that 1,25$(OH)_2$ D_3 alone increases expression of DEFB4 in reporter assays in COS cells [5]. Like-wise, experiments using 1,25$(OH)_2$ D_3 increased expression of NOD2 that was activated by internalized muramyl dipeptide, a NOD2 ligand, which, in turn, stimulated NF-kB and increased DEFB4 transcription. Although the importance of the VDRE in regulation of the DEFB4 gene is not clear, multiple mechanisms by which 1,25$(OH)_2$ D_3 indirectly induces DEFB4 transcription are known.

We determined conservation of the VDRE in the promoter sequence and it's retention in primates over 50-60 million years of primate evolution.

The VDRE in the human DEFB4 promoter matches the VDRE consensus sequence. Similarly in primates, there were few changes from the human sequence. These altered base pairs still correspond with the VDRE consensus sequence. This high level of sequence conservation supports our hypothesis that the VDRE is maintained throughout evolution because it plays an important role in DEFB4 expression.

The data presented suggests that the VDRE in the DEFB4 promoter plays an important role in regulating vitamin D mediated induction; however, our experimental results do not provide support for this hypothesis. When U937 and HT-29 cells were treated with $1,25(OH)_2\ D_3$, IFN-γ, FSL, IL-1β, or with combinations of the four, the expression of the DEFB4 gene was not induced (data not shown). This contrasts with prior studies that showed induction of DEFB4 with a combination of IL-1β, TLR2 ligand, and $1,25(OH)_2\ D_3$. It should be noted that in these prior studies primary macrophages incubated with autologous human serum were used and our work was done with leukemia cells lines with fetal bovine serum. The different cell types and media may explain the contrasting results. We are planning to transfect our constructs into primary macrophages and test activation of the CAMP promoter by $1,25(OH)_2\ D_3$ in combination with IL-1β and TLR2 ligand. This may provide a model for us to demonstrate a role for vitamin D in regulation of DEFB4 gene expression.

We showed that the VDRE in the DEFB4 promoter is evolutionarily conserved, but have not yet demonstrated binding of the VDR to this site and subsequent transcriptional regulation. Further refinement to our system is required before a conclusion is drawn. If possible, we would like to show the effects of $1,25(OH)_2\ D_3$ on the DEFB4 promoter by cloning the DEFB4 promoters from the human and primate genomic DNAs into firefly luciferase reporter vectors. Alternatively, using ChIP for VDR target genes in macrophage or epithelial cells treated with $1,25(OH)_2\ D_3$ would provide evidence for the VDR directly binding the VDRE in the DEFB4 promoter. Further experimentation is required to understand the exact role that the vitamin D pathway plays in regulating DEFB4 gene expression.

Chapter 4

Conclusion

These studies focused on transcriptional regulation of two separate important and frequently studied antimicrobial proteins, cathelicidin and DEFB4, and their regulation by small molecules.

In the U937 cell line we were able to induce CAMP expression by treatment with two stilbenoids found in a HTS. Both compounds increased CAMP mRNA levels at 10^{-5}M concentration and had synergistic mRNA expression with $1,25(OH)_2\ D_3$. Resveratrol by itself increased protein expression after 48 hours treatment. The mechanism of induction by these compounds may involve either estrogen receptor induction of VDR levels that in turn increases the response to vitamin D or indirect stimulation of sirtuin HDAC activity. Uncovering the mechanism by which these small molecules function will give greater insight into cathelicidin regulation.

This work also verified the VDRE in the DEFB4 promoter is conserved throughout primate evolution. Our *in vitro* experiments were not able to increase DEFB4 mRNA levels by vitamin D_3. We are working to develop a system in primary cells that might recapitulate the *in vivo* role of vitamin D in regulating DEFB4. Using this model we intend to show conservation of DEFB4 induction by $1,25(OH)_2\ D_3$ using a luciferase reporter containing the human or one of ten different primate DEFB4 promoters.

Antimicrobial peptides play an important role in the capacity of the innate immune system to combat infection and maintain health. Understanding the transcriptional regulation of AMP genes will provide the foundation necessary for developing therapeutic approaches that allow induction of endogenous genes to boost the innate immune response and possibly treat and/or prevent diseases. Our work has further increased the understanding of the transcriptional regulation of two different antimicrobial peptide genes.

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