The role of vitamin D and vitamin D analogues in gene regulation and potentiation of immune response to *Mycobacterium tuberculosis.*

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.List of Abbreviations

Abbreviation	Expression	Abbreviation	Expression
1,25D	1,25-dihydroxyvitamin D3	GAPDH	glyceraldehyde-3-phosphate dehydrogenase
25D	25-hydroxyvitamin D3	HAT	histone acetyltransferase
Alu	transposable element	hCAP-18	hCAP-18/LL-37 proprotein
AMP	amtimicrobial peptide	HDAC	histone deacetylase
BUT	butryate	HDI	histone decacetylase inhibitor
Ca2+	calcium	H37Ra	attenuated Mycobaterium tuberculosis strain
CAMP	cathelicidin antimicrobial peptide	H37Rv	virulent Mycobaterium tuberculosis strain
CF	cystic fibrosis	IBS	irritable bowl syndrome
ChIP	chromatin immuno-precipitation	IL-1β	interleukin-1 beta
CO2	carbon dioxide	iNOS	nitric oxide synthase
CYP24	24-hydroxylase	L. saha	1,25D analogue, Long saha
CYP27A1	vitamin D 25-hydroxylase	LL-37	human cathelicidin antimicrobial peptide
CYP27B1	1α-hydroxylase	M. smeg, M. smegmatis	Mycobacterium semgmatis
DEFB2 (DEFB4, BDEF2)	defensin beta-2	Mtb, M. tuberculosis	Mycobacterium tuberculosis
DR3	direct repeat with 3 bp spacing	NHBE	normal human bronchial epithelial cells
DNA	deoxyribonucleic acid	NO	nitric oxide
E. Coli	Escherichia coli	NOD2	nucleotide-binding oligomerization domain containing 2
ER6/8	everted repeat with 6/8 bp spacing	P. aeruginosa	Pseudomonas aeruginosa
FP	flourescence polarization	pDCs	plasmatoid dendritic cells
FXR	farnesoid X receptor	PMA	phorbol-12-myristale-13-ace

Abbreviation	Expression	Abbreviation	Expression
PO4	phosphate	TB	tuberculosis
qPCR	quantitative real time polymerase chain reaction	TLR	Toll-like receptor
RNA	ribonucleic acid	UV	ultraviolet
RT-PCR	reverse transcriptase polymerase chain reaction	VDR	vitamin D receptor
RXR	retinoid X receptor	VDRE	vitamin D response element
SCFA	short-chain fatty acid	WHO	World Health Organization
SINE	short interspersed element		

Abstract

1,25-dihydroxyvitamin D3 (1,25D) is the hormonally active form of vitamin D, a key factor in calcium (Ca2+) homeostasis. 1,25D has also been shown to mediate a number of other physiological mechanisms. Here we pay special attention to the immunoregulatory properties of 1,25D, and its role in stimulating immune responses against pathogenic infection. The innate branch of the immune system is responsible for the rapid, non-specific host response against pathogenic infection. 1,25D induces the expression of antimicrobial peptides, which serve as the vanguards of innate immune response as predicted *in vitro* by human monocytic (THP-1), as well as intestinal epithelial cell lines (SW480, HT-29). Furthermore, antimicrobial peptides regulate a number of immune responses such as wound healing and cytokine induction. Butyrate is a histone deacetlyase inhibitor (HDI), and a natural constituent of the gut, which is known to possess significant gene regulatory properties, including the induction of antimicrobial peptide expression. The purpose of this study is to investigate how therapy, combining 1,25D with the underlying gene regulation activity of butyrate, may function in stimulating immune responses against infection, particularly with regard to *Mycobacteria tuberculosis*. Given the potential that either butyrate or 1,25D alone promotes the production of antimicrobial peptides, this combination therapy shows promise in developing a novel approach to treat infection by enhancing antimicrobial gene expression. By modifying the side chain of 1,25D to possess HDI-like properties, it may be possible to develop bifunctional vitamin

D analogues with enhanced therapeutic value to be used against mycobacterial infection.

Résumé

1,25-dihydroxyvitamin D3 (1,25D) est la forme active de l'hormone vitamine D, un facteur clé dans l'homéostasie du calcium (Ca2 +). 1,25D est aussi un facteur clé dans plusieurs autres mécanismes physiologiques. Dans cette étude, nous sommes particulièrement intéressés par les propriétés immuno-régulatrices de 1,25D et son rôle dans la stimulation de réactions immunitaires contre les infections pathogènes. La branche innée du système immunitaire produit la réaction immédiate non-spécifique de l'hôte contre l'infection pathogène. 1,25D provoque l'expression de peptides antimicrobiens qui constituent l'avant-garde de la réaction immunitaire innée dans les cellules monocytes humaines (THP-1), ainsi que les cellules épithéliales intestinales (SW480, HT-29). En outre, les peptides antimicrobiens régissent un nombre important de réactions immunitaires telles que la cicatrisation de plaies et la production de cytokines. Butyrate est un inhibiteur d'histone déacetylase (IDH) et une substance naturelle de l'intestin, connu pour posséder d'importantes propriétés régulatrices de gènes y compris l'expression de peptides antimicrobiens. Le but de cette étude est d'examiner comment un traitement combinant 1,25D avec l'activité régulatrice génétique sous-jacente de butyrate peut agir comme stimulant de réactions immunitaires contre des infections, en particulier en ce qui concerne la bactérie *Mycobacterium tuberculosis.* Étant donné le potentiel individuel que le butyrate ou la 1,25D possède à favoriser la production de peptides antimicrobiens, cette conjugaison de thérapies pourrait aider au développement d'une nouvelle approche pour traiter les infections en renforçant l'expression des gènes

antimicrobiens. En modifiant la chaîne latérale de 1,25D de sorte qu'elle possède des propriétés similaires à l'IDH, il serait possible de développer des analogues bi-fonctionnels de la vitamine D ayant une valeur thérapeutique améliorée conte l'infection mycobactérienne.

Literature Review

1. Vitamin D

Vitamin D may be somewhat of a misnomer, in that it is not a true vitamin by classical definition [1]. A vitamin is an essential substance required for proper physiological function, but is not produced in sufficient quantity by the body [2]. More accurately, vitamin D refers collectively to a group of fat-soluble prohormones that undergo a series of hydroxylation reactions, converting them into hormonally active forms. It is the hormonally active forms of vitamin D that serve as regulators of gene expression.

The past 30 years research has elucidated substantial evidence revealing how vitamin D regulates gene expression. Vitamin D has long been known for its role in calcium homeostasis and bone maintenance, but more recent research has established vitamin D's involvement in a broad-spectrum of physiological systems such as nervous system function, cell growth and differentiation, and the potentiation of immune system responses. Much of the research today has refined the focus on vitamin D and its role as a potent modulator of gene expression.

2. Early history of nutritional vitamin D therapy

Nutritional vitamin D therapy can be traced back to the early pharmacology of cod-liver oil use. Cod-liver oil was first described as a medicinal agent for the treatment of chronic rheumatism in 1789 [3]. Throughout the next century, medical literature documented its effectiveness for treating a number of prevalent conditions such as gout and scrofula, a form of tuberculosis

which infects the lymph nodes [3]. Beginning in the 1820's, studies showed that administering doses of cod-liver oil to afflicted children could cure rickets [3], a nutritional disease characterized by a lack of vitamin D or calcium leading to bone softening and deformity. However, it would not be until several decades later that the active compound in cod-liver oil could be identified as vitamin D. By 1849, the list of conditions treatable with cod-liver oil would grow to include tuberculosis infection (TB) [4, 5].

Initially, vitamin D was thought solely to be a nutritional component of the diet. However, several independent observations culminating with research published by the 1930's revealed links between sunlight, cutaneous vitamin D synthesis, and the induction of immune function. This is highlighted by such findings as; (I) a relationship between the prevalence of rickets and availability of sunlight (II) treatment of rickets with UV light therapy (III) treatment of tuberculosis by UV light, and (IV) feeding ultraviolet irradiated skin to rats provided the same protective effects against rickets as does a diet supplemented with cod-liver oil [1, 3-6]. These studies supported the significant health benefits given by vitamin D treatment, and helped to establish an association between vitamin D deficiency and the prevalence of certain diseases.

3. Vitamin D deficiency

Today vitamin D deficiency is regarded as a widespread, and completely preventable, health concern. Although there is no strict definition, vitamin D deficiency is characterized by low circulating vitamin D metabolite levels, caused

by seasonal variations in sunlight and inadequate dietary consumption. In more temperate climates, this phenomenon is commonly referred to as vitamin D winter. For example, one survey found widespread vitamin D deficiency among female populations across Northern Europe [7]. Another intriguing study found that a significant portion of African-American women living in the United States are seriously vitamin D deficient [8].

While correlations between vitamin D deficiency and disease go back for over a century, more recent studies have established a direct connection between the two conditions. Since the association between tuberculosis and vitamin D deficiency was described nearly 20 years ago [9], numerous epidemiological studies have also linked vitamin D deficiency to increased rates of cancer, multiple sclerosis, type I diabetes, Crohn's disease, and infection [10-12].

4. Vitamin D signaling and mechanism of action

Hormonally active vitamin D is generated primarily by the photochemical action of UV light (wavelengths between 270–300 nm) in skin (Fig. 1), and is obtained from limited dietary sources such as fish oils and fortified dairy products. It is derived from one of two precursor forms known as vitamin D_2 (ergocalciferol), or vitamin D_3 (cholecalciferol) [13]. Vitamin D undergoes a series of tightly regulated hydroxylation and dehydroxylation reactions, required for both biological activation and deactivation of the molecule. First, 25-hydroxylation is catalyzed by the enzymes CYP2R1 or CYP27A1, producing 25-hydroxyvitamin D (25D), the major circulating vitamin D metabolite [14-18].

This occurs primarily in the liver, but may also happen in the skin. Then, 1α–hydroxylation of 25D is catalyzed by the enzyme CYP27B1, producing hormonally active vitamin D, 1,25-dihydroxyvitamin D (1,25D) [14, 17, 18]. 1α–hydroxylation sites are regulated in a tissue-specific manner in the kidney, peripheral tissues, and several cell types of the immune system. For example, renal 1α–hydroxylation is regulated by calcium homeostatic inputs such as parathyroid hormone (PTH). Regulating of extra-renal CYP27B1 activity is independent of calcium homeostasis, and includes a number of biomolecular triggers such as innate immune inputs for macrophages.

Hormonal 1,25D serves as the molecular ligand of the vitamin D receptor (VDR). Production of 1,25D triggers expression of CYP24, the enzyme that catabolyzes 25D or 1,25D via a 24-hydroxylation reaction to generate 24,25-dihydroxyvitamin D or 1,24,25-trihydroxyvitamin D, in a physiological negative feedback loop. Metabolites of 24-hydroxylation undergo further catabolism to calcitroic acid, which is then excreted in urine [14, 17, 18].

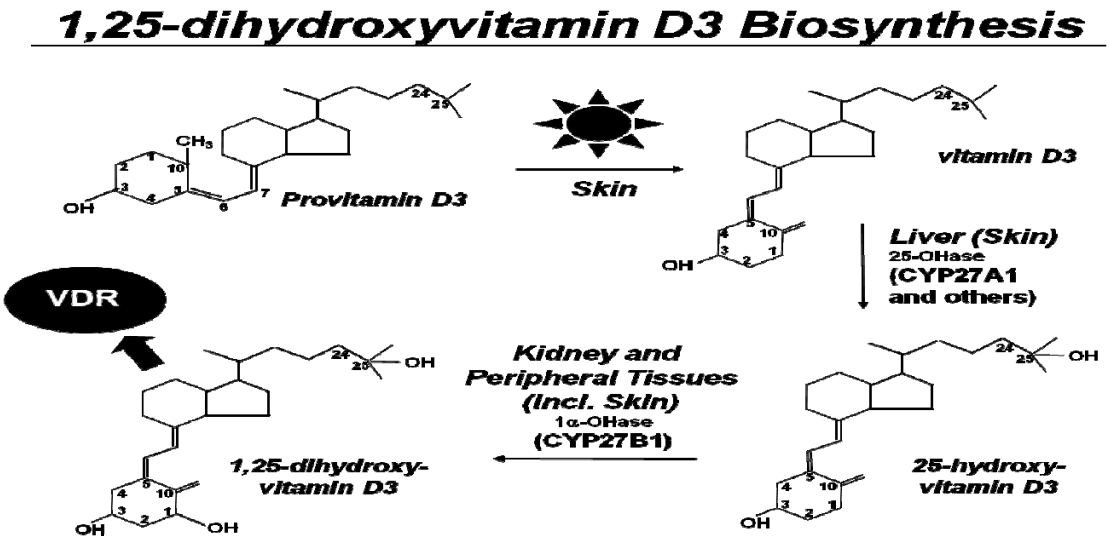


Fig. 1: Biosynthesis of 1,25-dihydroxyvitamin D3.

Molecular regulation of gene expression occurs when 1,25D binds to the VDR. The VDR is a nuclear receptor and transcription factor that is part of a larger group of ligand-regulated transription factors, which include receptors for hormonal vitamin A and steroid hormones. Nuclear receptors are composed of highly conserved DNA and ligand binding domains [14, 19, 20]. Upon ligand binding, the VDR undergoes a conformational change triggering dimerization with related nuclear retinoid X receptor (RXR). The VDR/RXR heterodimer complex is essential for high affinity binding with vitamin D response elements (VDREs), specific DNA sequences located in the regulatory regions of VDR target genes. VDREs are dimers of hexameric nucleotide motifs composed of direct (DR) or everted (ER) repeats of PuG(G/T)TCA motifs, characterized by their spacing of either 3 (DR3), 6 (ER6), or 8 (ER8) nucleotides [6, 21]. Binding specificity is conferred by the arrangment of two cognate DNA sequence motifs that are required for heterodimeric VDR/RXR receptor binding. The VDR/RXR complex binds to DR3 elements with the DNA binding domains in a heel-to-toe orientation (Fig. 2) [15, 19, 20], whereas inverse palindromic (everted) motifs are bound in a "toes out" arrangement (Fig. 2) [6].

After DNA binding the heterodimer nuclear receptor complex recruits a series of co-regulatory proteins, which regulate the effects of transcription on adjacent target genes. Co-regulators are a group of proteins that interact directly with both the nuclear receptor complex and the basal transcription machinery, regulating the processes of histone modulation, chromatin remodeling, and transcriptional initiation [22-24]. Notably, co-regulators may function as either

co-activators, co-repressors, or some combination of both. Current molecular evidence suggest that the DNA-bound RXR/VDR complex can modulate transcription at distances up to 75kb from the start site of the intended target gene [25].

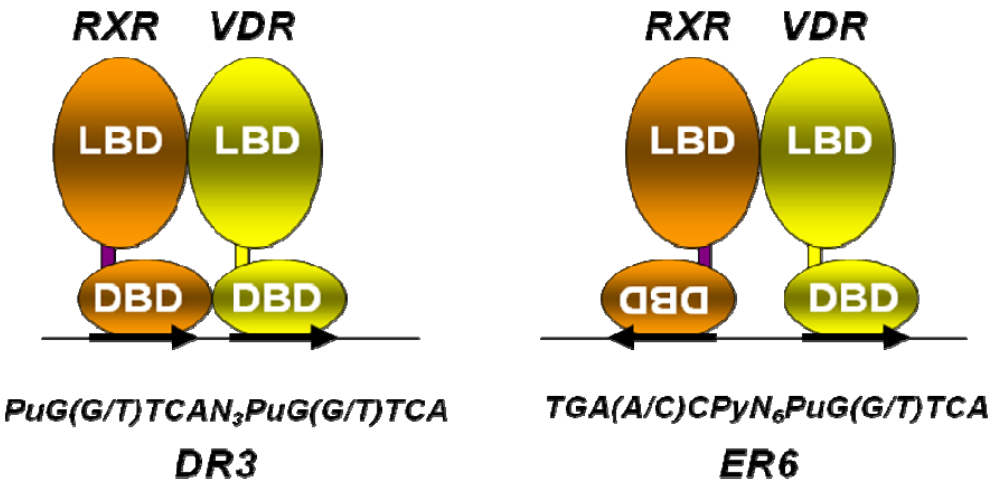


Fig. 2: VDR-RXR dimeric binding to the VDRE of the type DR3 (left) or ER6 (right) [6].

5. Classical properties of vitamin D

Traditionally, vitamin D is well known for it role in maintaining blood calcium (Ca2+) and phosphate (PO4) levels underlying bone metabolism. The regulation of bone metabolism is highly complex and requires several hormonal inputs, including parathyroid hormone (PTH), calcitonin, and vitamin D. Although each hormonal contribution is significant to overall bone health and maintenance—and in turn, each worthy of their own chapter—vitamin D remains the preeminent subject of focus.

Vitamin D regulates bone metabolism by stimulating calcium reabsorption in both the intestines and kidneys. After 1-α hydroxylase conversion of 25D, 1,25D enters the circulating bloodstream and travels to VDR receptors highly expressed within renal and intestinal epithelial cells. Dimerization of the VDR with the related RXR, allows high-affinity binding with VDREs of genes encoding for TRPV6 and TRPV5, and subsequently increases their expression. TRPV5 and TRPV6 represent a pair of homologous epithelial Ca2+ channels that allow for the active reabsorption of calcium into the blood stream [26, 27]. Notably, both TRPV channels have multiple VDREs in their gene promoters, and are highly selective for Ca2+ ions as compared to other members of the TRP family. This selectivity for the calcium ion underlies their mechanistic activity in active Ca2+ reabsorption [28, 29].

In order to enter the blood stream, Ca2+ needs to travel against its concentration gradient. Current theory proposes that active Ca2+ transport is a highly complex process requiring the extensive cooperation of cellular machinery. Beginning in the lumen, a transmembrane electrochemical gradient drives the influx of Ca2+ ions through TRPV6 channels along the apical membrane of enterocytes and into the cell. While calcium is an important signaling molecule in normal metabolism, an excess of intracellular Ca2+ leads to cell-signaling interference and alterations in metabolic function. [30]. 1,25D directly regulates Ca2+ transport by modulating the expression of TRPV6, PMCA, and calbindin-D9k—a calcium-binding protein with two functional Ca2+ binding sites [31-34]. PMCA is a Ca2+ ATPase pump believed to be activated by its calmodulin-

binding domain, upon binding with calbindin-D9k. Activation of PMCA allows for the facilitation of Ca2+ across the basolateral membrane of enterocytes, and delivery into the circulating blood supply. Furthermore, enterocytes express an auxiliary sodium (Na+)/Ca2+ exchanger (NCX) that aids basolateral transport of Ca2+ into the bloodstream, which is independent of VDR regulation [26, 32, 33, 35]. Current opinion also suggests that circulating calbindin proteins may directly secret Ca2+ into the bloodstream [26-29, 32, 35, 36].

Similarly, vitamin D regulates Ca2+ reabsorption in the distal kidney tubule [26]. Although the names of the particular proteins involved have changed, mechanisms between intestinal and renal Ca2+ reabsorption share the same basic underlying physiology. Within kidney epithelial cells, 1,25D induces TRPV5, PMCA1b (a Ca2+ ATPase pump), and calbindin-D28k (another Ca2+ binding protein) expression, driving calcium reabsorption from the lumen back into the circulating blood supply. Furthermore, the kidney expresses its own version of a VDR-independent sodium (Na+)/Ca2+ exchanger (NCX1), which aids basolateral Ca2+ transport back in the bloodstream (Fig. 3) [26, 31, 33, 37].

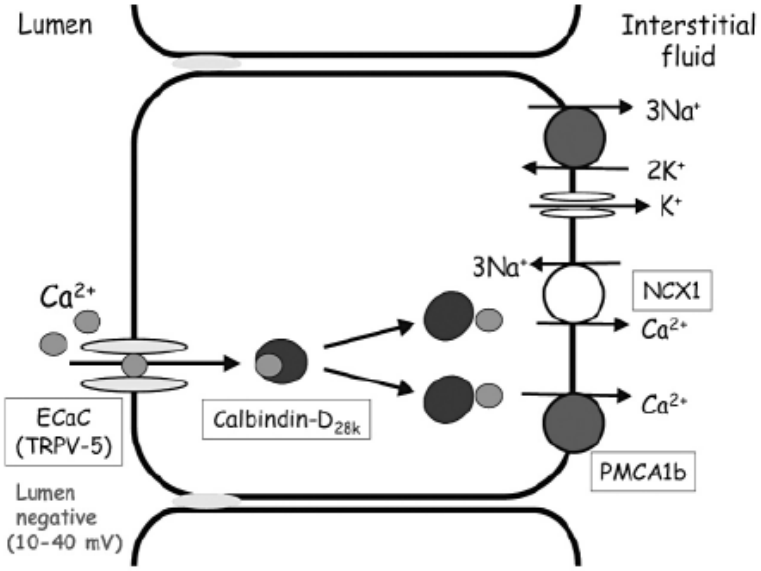


Fig. 3: Diagram representing Ca2+ reabsorption in the distal tubule of the kidney [37].

6. Tuberculosis

Vitamin D deficiency has been linked to immune susceptibility and increased rates of pathogenic infection, including tuberculosis [12]. Tuberculosis (TB) is an infectious disease caused by exposure to members of the mycobacteria genus of bacteria. TB infection occurs by inhalation of microbial-contaminated airborne droplets, and is easily transferable between hosts. World Health Organization (WHO) statistics for the year 2007 estimate that there were approximately 9.3 million new cases of TB, 14 million chronically active cases, and roughly 1.4 million TB related deaths [38]. TB infection has become so widespread today, it is estimated that roughly one third of the world's population has been infected with the tubercle bacillus, with the vast majority of TB incidences occurring in Asia and Sub-Saharan Africa (Fig. 4) [38, 39].

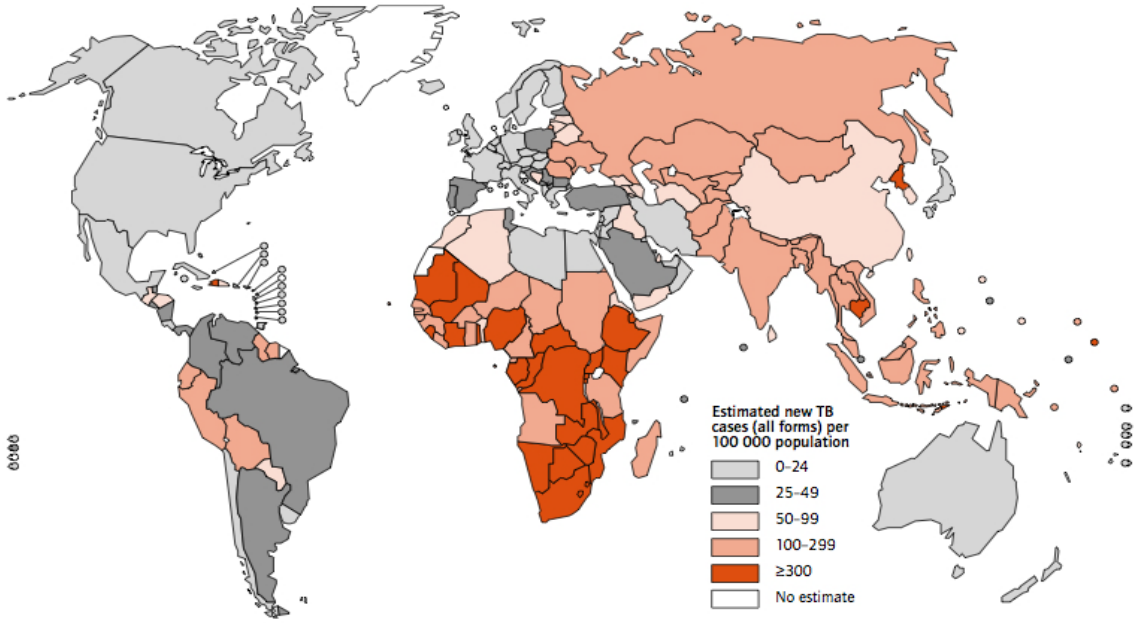


Fig. 4: 2007 estimates for rates of TB infection, by country [38].

Mycobacteria are a genus of pathogens that cause a number of serious host infections such as tuberculosis and leprosy. Although generally classified as Gram-positive due to their lack of an outer cell-membrane, mycobacteria technically represent an independent group of microbes due to the unique lipid content of their cell wall that prevents crystal violet staining [40]. This cell wall contains high concentrations of mycolic acid, a lipid unique to mycobacteria. The relative thickness of the mycobacterial cell wall lends to the hardy nature of these pathogens, rendering them resistant to the penicillin group of antibiotics that degrade cross-linking components of the bacterial cell wall. Furthermore, mycobacteria have been shown to withstand extended exposure to acids, alkalis, detergents, and oxidative bursts [40]. TB infection is primarily caused by exposure to *Mycobacterium tuberculosis* (Mtb). Other mycobacteria that can cause TB infection include *M. bovis, M. africanum,* and *M. canetti.*

Since doctor Robert Koch first described *Mycobacterium tuberculosis* in 1882, the biology of TB, as well as the pathogenesis of infection, have been studied in great detail. Mtb infects host organisms through the respiratory pathway. After inhalation, the pathogen settles in the lungs where it is taken up by alveolar macrophages, dendritic cells, and monocytes from the bloodstream [41]. Upon phagocytosis, Mtb has evolved several inherent mechanisms allowing the pathogen to remain undetected and undigested in host immune cells. Specifically, Mtb interferes with cell signaling underlying phagosomal maturation and vacuolar fusion with intracellular lysosomal compartments [41]. By arresting these normal host-pathogen recognition and response pathways, Mtb creates a hospitable intracellular environment for microbial proliferation within infected hosts.

Currently, Mtb is primarily studied utilizing two strains cultured for laboratory use. H37Rv is the original virulent strain from which H37Ra (attenuated strain) was derived in 1935; however, the basis of virulence attenuation is not yet completely understood [42]. In 1998 the genome of the H37Rv strain was published [43], yielding a powerful new tool in better understanding the biology of Mtb. Subsequently, genetic research has shown how Mtb induces host-immune response by activating toll-like receptors (TLRs) [44, 45] and NOD2 [46, 47] in humans. Toll-like receptors and NOD2 are molecular pattern recognition receptors that recognize motifs specific to bacterial cells wall components and stimulate immune response.

7. Vitamin D and the connection with TB

Historically, sunlight and TB treatment have a long association. In fact, Hippocrates, "the Father of Medicine," recommended that those suffering from tuberculosis be sent to the hills of Ancient Greece where treatment consisted of plenty of rest, fresh air, and sunshine [48]. Much more recently, in 1903, Niels Finsen won the Nobel Prize in medicine for his work describing how UV light can cure disease, such as cutaneous TB [49]. During the early part of the 20th century, common therapy for various tuberculosis infection consisted of extended stays in sanatoriums regimented with ample sunbathing, known as heliotherapy [50]. Soon thereafter, scientific exploration began investigating whether tropical sunlight could be more effective in treating TB than light from northern climates [51]. However, understanding the antibacterial actions of sunlight, and its intricate association with vitamin D, would remain unknown for a better part of the 20th century.

The link between vitamin D induced antimicrobial activity and TB viability would remain elusive until the 1980s. Beginning in 1975, several *in vitro* studies documented the immunoregulatory role of 1,25D in promoting the maturation of human immune cells [52, 53]. By utilizing this knowledge, Rook [54] and Crowle [55] did pioneering work establishing the antimycobacterial role of 1,25D in treating TB infection. Notably, these studies showed how treatment with 1,25D promoted the rapid protection against, and the reduced viability of, Mtb in infected macrophages. Furthermore, this protection was seen in as little as 72 hours of treatment. These two studies demonstrated the first direct association

between vitamin D and TB viability, and advanced a novel approach for treating tuberculosis infection utilizing 1,25D therapy.

8. Vitamin D as a regulator of antimicrobial gene expression

Today, modern genomic techniques have shifted the focus of vitamin D into the fields of cell physiology and molecular biology. The recently recognized benefits of vitamin D have their roots in how 1,25D modulates gene expression. To obtain a better understanding of the underlying mechanisms of vitamin D action, a genomic approach is best suited for examining the VDR, and its cognate VDREs. As mentioned above, the VDR is a direct regulator of gene transcription with a well-defined binding site [6]. Therefore, initial identification of 1,25D target genes began by utilizing a combination of microarrays and genomic wide screens for VDREs, yielding significant results [56-59]. Of particular interest, these screens identified consensus DR3 VDREs were located in the promoter region of two genes encoding the antimicrobial peptides (AMPs) defensin β2 (*DEFB2, HBD2*), and cathelicidin antimicrobial peptide (*CAMP*) [60]. Chromatin immunoprecipitation assays (ChIP) further confirmed 1,25D promotion of the *CAMP* and *DEFB2* genes as site specific, and being directly regulated by the VDR binding to consensus VDREs. However, 1,25D induction of *DEFB2* was initially noted to be significantly more modest than that of *CAMP* [60]. Several additional studies have confirmed 1,25D induction of AMPs in a number of human immune cells and tissues [61, 62]. These findings are highly significant

because AMPs govern the basis of pathogen eradication in innate immune response.

9. Innate immunity

There are two main branches of the immune system: innate and adaptive immunity. Innate immune responses are constitutively present and comprise the front line of defense to combat pathogenic infection in a non-specific manner. However, unlike adaptive immunity, innate responses are immediate and do not confer long lasting protection for the host. Innate immunity is found across a wide variety of plant and animal species. In humans, research has shown that regulation of both innate and adaptive immune response pathways are highly sensitive to circulating levels of vitamin D [11, 12]; however, the innate immune system remains the preeminent subject of focus. Furthermore, this body of work will more specifically focus on the promotion of antimicrobial peptides in innate immune response pathways *in vitro*.

10. Antimicrobial peptides

Antimicrobial peptides are a group of small proteins with intrinsic antibiotic properties that are synthesized and secreted in cells and tissues exposed to environmental pathogens. Much of the effectiveness of antimicrobial peptides stems from their ability to eradicate a broad-spectrum of pathogens including bacteria, fungi, and viruses without triggering antibiotic resistance [63-65]. Additionally, being a natural constituent of the host innate immune system,

antimicrobial peptides have been discovered to hold additional immunoregulatory properties, lending to the term: immune effector molecules. These regulatory properties include the alteration of gene expression, cytokine induction, and the modulation of dendritic cell responses [63-66]. As pathogens continue evolving into increasingly antibiotic resistant strains, antimicrobial peptides hold promise as a potent method in the control of microbial infection.

Cathelicidins are a family of antimicrobial polypeptides characterized by a highly conserved N-terminal cathelin region, with a variable C-terminal antimicrobial domain. The cathelin domain shares in similar sequence homology and function with the cystatin family of cysteine proteinase inhibitors [67]. Cathelicidins serve as a critical component of innate immune response providing rapid defense against infection. While genes encoding for cathelicidins are widely conserved amongst vertebrates [67-69], the human gene remains the subject of focus.

In humans, a single gene encodes the cathelicidin precursor protein hCAP-18/LL-37 (hCAP18), widely expressed throughout cells of the immune system. Proteolytic cleavage of the proprotein releases LL-37, more commonly known as human cathelicidin antimicrobial peptide (*CAMP*; Fig 3). LL-37 is a linear polypeptide with a net positive charge due to an excess of basic amino acids [70]. The cationic nature of cathelicidin provides the basis of its antimicrobial activity. Bacterial cell membranes contain high lipid compositions with a negatively charged surface, thereby creating a selective preference for the positively charged AMP. Upon binding to bacterial cell surfaces, cathelicidins fold into amphipathic

α-helical structures, leading to insertion of the protein and interference with cell membrane integrity. Increasingly, *in vivo* studies have documented a more complete role for cathelicidin beyond the direct eradication of microbes. Cathelicidins have been shown to mediate a number of immune system responses, including chemotaxis, alteration of transcription, directing inflammatory response, and promotion of wound healing [67-70].

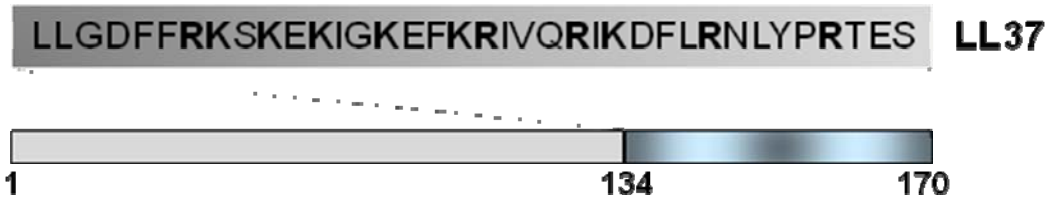


Fig. 5: A schematic representation of the hCAP18/LL-37 precursor protein and LL-37 cleavage product is shown. Basic amino acids in the LL-37 peptide are noted in bold.

Defensins represent another family of 1,25D-induced AMPs, which contribute to innate immune defense. Similar to cathelicidins, defensins are small polypeptides with cationic properties that are integral to their antimicrobial activity. Found in both vertebrates and invertebrates, defensins are also well recognized for their dual capacity to both eradicate microbes, and modulate immune function [71, 72]. Structurally, defensins are characterized by a cysteine-rich backbone forming β-pleated sheets stabilized by disulphide bonds [71]. Classified by their orientation of disulfide bonds to cysteine residues, two subfamilies of defensins exist in humans: α-defensins commonly produced by

neutrophils and Paneth cells, and β-defensins, which are secreted by epithelial tissues [72].

1,25D induction of *DEFB2* has recently been linked with cytokine production during immune response. As mentioned above, promotion of *DEFB2* expression by 1,25D was diminished when compared to the *CAMP* gene, particularly as seen in the human SCC25 cell line [60]. However, initial research showed that fold induction of *DEFB2* could be elevated with the addition of Interleukin-1 beta (IL-1β) to the incubation media. IL-1β is a cytokine important to the process of inflammatory response, and is produced by immune cells. In increasing AMP expression, the combined activity of 1,25D and IL-1β suggested some crosstalk with other underlying signaling pathways during innate immune response. Expanding upon these results, Liu *et al* [62] demonstrated the synergistic activity of IL-1β with 1,25D in the potentiation of antimicrobial response, as induced by TLR signaling. This convergence of VDR and IL-1β signaling into a single pathway denotes a novel mechanism for intracellular pathogen control, as mediated through elevated levels of defensin beta 4 (DEFB4, formerly HBD2) expression.

11. 1,25D regulation of CAMP is human and primate specific

The induction of antimicrobial gene expression by 1,25D does not appear to be conserved among mammals. Work by Gombart *et al* [61] found that 1,25D is a direct inducer of antimicrobial gene expression in humans. However, the VDREs for the *CAMP* and *DEFB2* genes are not conserved in mice. Indeed, a

comparison of several mammalian genomes shows the mechanism in 1,25D induction of *CAMP* is conserved specifically in humans and non-human primates [61, 73]. Lack of conservation of the *CAMP* promoter can be attributed to location of the VDRE within a short interspersed element (SINE) element of the Alu subfamily that is primate and human specific, suggesting lineage from a common progenitor element during evolution [73]. This specific conservation of the Alu repeat containing the *CAMP* VDRE can be traced to an insertion event dating back between 55-60 million years. Additionally, the insertion event leading to Alu-mediated divergence originated prior to the primate lineage leading to humans, apes, and old and new world monkeys.

Vitamin D regulation of the *CAMP* gene is believed to hold a selective advantage in delineated species. Evolutionary conservation for the mechanism of VDR regulated *CAMP* expression holds functional significance in innate immune response. This significance is highlighted by a number of findings: (I) The VDR is found in most immune cells, including T-lymphocytes, neutrophils, dendritic cells, and macrophages [74-78], (II) Studies linking VDR induction of AMP production with toll-like receptor signaling in innate immune responses [44, 79-83], and (III) VDR signaling is important to both the process of pathogen recognition, and antimicrobial response during tissue injury [84].

As mentioned above, toll-like receptors are a family of cell-surface pattern recognition receptors that recognize molecular motifs specific to pathogens and stimulate immune system responses. TLRs have long been studied for their roles in microbial infection, and have recently been linked to VDR induction of innate

immune response. By 2006, Liu *et al* [44] established that TLR2 activation of human or murine macrophages directly stimulates antimicrobial activity against TB infection. However, in mice and humans the mechanisms underlying antimicrobial activity remains highly distinct between the two species. In mice, antimicrobial activity is dependant upon the induction of nitric oxide synthase (iNOS), and the production of nitric oxide (NO) in infected tissues [44, 80]. Work also showed that iNOS inhibitors were able to block this induction of antimicrobial activity. Furthermore, Liu *et al* [44] also demonstrated that NO induced antimicrobial activity is mediated through TLR signaling in mice. However, this study demonstrated that human macrophages do not depend upon the production of NO for their antimicrobial activity.

Subsequent research has since confirmed the induction of AMPs as the basis for TLR-induced antimicrobial activity in human macrophages and linked VDR signaling to AMP production [80]. Utilizing microarray studies, Liu *et al* [44, 80] found that TLR-stimulation significantly up-regulates the VDR as well as CYP27B1 expression in human monocytes. These findings are significant because they show that macrophages react to pathogens by acquiring the capacity to convert circulating 25D into hormonal 1,25D. The functional significance of this discovery was demonstrated by observation of a direct reduction in *Mycobacterium tuberculosis (Mtb)* viability in infected monocytes, as mediated by VDR induction of AMPs and other downstream VDR targets. Notably, there also appears to be a direct correlation between serum 25D levels and TLR activation of VDR dependant antimicrobial response. Owing to a darker

pigmentation and the subsequent reduction in cutaneous vitamin D synthesis, African Americans are known to possess reduced serum 25D concentrations compared to Caucasians [81, 82]. This is significant because studies with monocytes containing low serum levels of 25D resulted in a pronounced abatement of TLR activated antimicrobial response, particularly with regard to *CAMP* expression. Furthermore, this study [80] found that normal physiological function could be restored through proper supplementation of 25D, demonstrating the dependence of innate immune response to circulating 25D levels.

Complimenting these results, Schauber *et al* [84] confirmed the link between 1,25D and TLR signaling in human keratinocytes. However, unlike monocytes, keratinocytes maintain a unique amplification loop whereby 1,25D induces TLR2 expression, which in turn leads to elevated 1,25D levels in response to tissue injury (Fig. 6).

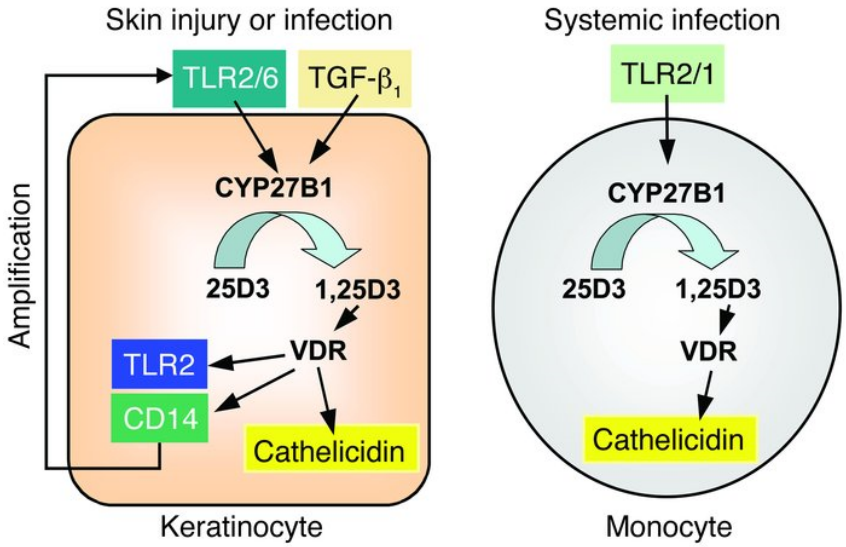


Fig. 6: Model of 1,25D regulated immune function in human keratinocytes and monocytes [84].

To summarize, the above findings are meaningful for a number of reasons. First, they clearly establish the role of vitamin D signaling in innate immune response. Secondly, they emphasize the importance of maintaining vitamin D sufficiency for optimal immune function, and suggest why ethnicities with darker skin tones may be more predisposed to certain conditions. Finally, they demonstrate the importance of CYP27B1 function in vitamin D immunoregulation.

12. Broad implications for 1,25D regulation of CAMP expression

Moving beyond *in vitro* studies, vitamin D has a well-established role in a number of physiological applications. Expression of the VDR is widespread among proliferating cell types, and 1,25D signaling has been shown to play a regulatory role in a wide array of pathophysiological functions.

The skin occupies a key position in vitamin D physiology. Epidermal keratinocytes express the hydroxylase enzymes CYP27A1 and CYP27B1, allowing for the localized synthesis of 1,25D. Additionally, keratinocytes express the VDR, making them responsive to vitamin D inputs. Owing to their dual nature as both the site of 1,25D synthesis and response, epidermal keratinocytes demonstrate a more complete role for vitamin D signaling in innate immunity and wound healing. Under conditions of tissue damage, keratinocytes undergo a number of 1,25D-mediated events, including changes in cell proliferation, differentiation, cytokine production, and gene expression [85-88]. Cell signaling

events underlying wound healing have also been shown to lead to a notable induction of CYP27B1, and subsequently increase *CAMP* expression levels. Moreover, *CAMP* induces keratinocyte migration vital to re-epithelialization of the wound by transactivating epidermal growth factor receptors (EGFRs) responsible for cellular architecture [86, 87].

1,25D induction of dermal cathelicidin expression is not always beneficial, as elevated *CAMP* expression has been implicated in several known inflammatory skin conditions. For example, rosacea is a condition characterized by the abnormal processing of hCAP-18 peptides leading to an abundance of altered or truncated cathelicidin peptide expression in keratinocytes [89]. These truncated cathelicidin peptides contribute to chronic erythema, or redness of the skin in afflicted patients. Furthermore, UV light aggravates rosacea, most likely in response to elevated *CAMP* expression as induced by cutaneous 1,25D synthesis.

Psoriasis serves as another example of how altered *CAMP* expression can have a negative physiological impact. Cathelicidin expression is normally limited to keratinocytes exposed to injury, stimulating immune responses and promoting wound healing [86, 87]. In cases of psoriasis this tightly regulated process has been altered, leading to an overexpression of the peptide LL-37, and subsequently triggering immune responses by activating plasmatoid dendritic cells (pDCs) [90]. Plasmatoid dendritic cells are specialized skin cells that sense for viral and bacterial DNA through TLR signaling. With particular cases of autoimmune disease, a breakdown of the sensory apparatus occurs, allowing for self-DNA to trigger an immune response. In psoriatic skin, Lande *et al* [90] showed that

CAMP is the mediating factor triggering pDC activation. Lending to the cationic, amphipathic nature of protein, LL-37 directly binds to DNA in pDCs, forming aggregated and condensed structures that are delivered to TLR-9 receptors, thereby activating immune response mechanisms, and leading to chronic skin inflammation.

VDR regulated signaling is not limited to dermal epithelial cell types. One recent study showed both the VDR and *CAMP* are expressed in hepatic biliary epithelial cells [91]. Under normal physiological conditions the biliary tract maintains a number of defense mechanisms, preserving a microbial-free environment. As demonstrated by this study, the antibacterial activity of the biliary tract is maintained by the signaling actions of bile salts through the VDR, and a related farnesoid X receptor (FXR). FXR is a member of the nuclear receptor family, activated by biliary salts. Remarkably, the VDR has also been established as a functional bile acid receptor [92]. Activation of either the FXR or VDR by bile acids has been found to mediate innate immune function through the induction of cathelicidin in both hepatic epithelial cells and normal human keratinocytes [91, 93]. Furthermore, the synergistic enhancement of *CAMP* expression is induced by a combination of vitamin D and bile salts, suggesting a novel therapeutic approach to treating inflammatory biliary disease [91].

Similarly, VDR regulation of innate immune response is essential for maintaining lung homeostasis. The epithelial linings of the lungs are constantly exposed to a variety of environmental pathogens; and induction of antimicrobial gene expression is vital in providing a front line of defense against inhaled

microbes. 1,25D has shown to strongly induce *CAMP* expression in Calu-3 cells, a human epithelial airway cell line [60]. Moreover, treatment with 1,25D yielded a significant reduction in the microbial activity of either *Escherichia coli (E. Coli)* or *Pseudomonas aeruginosa (P. aeruginosa)* in infected Calu-3 cells. *P. aeruginosa* is an opportunistic pathogen associated with the vast majority of respiratory infections in patients afflicted with cystic fibrosis (CF) [94]. Notably, patients with CF have also been observed to have low circulating serum 25D levels [95]. Expanding upon these results, Yim *et al* [96] documented the presence of the VDR, as well as a robust induction of cathelicidin by 1,25D, in normal human bronchial epithelial (NHBE) cells. Furthermore, this study demonstrated the direct contribution of cathelicidin on antimicrobial activity through preincubation of infected NHBE cells with an anti-LL-37 (*CAMP*) antibody.

Active vitamin D metabolism has been established in the endometrium and placenta during gestation. Beginning in the 1980s, studies conducted with rats connected vitamin D deficiency with significantly diminished rates of reproduction [97-99]. Recently, one intriguing study linked high levels of placental 1α–hydroxylase expression during early gestation, with the pleiotropic actions of vitamin D signaling beyond fetal musculoskeletal development [100]. Expanding upon these results, another study correlated vitamin D signaling with attenuated immune function during the early stages of pregnancy in maternal decidual tissue [101]. In upholding an immunosuppressive role, local synthesis of 1,25D was found to modulate maternal immune function, supporting implantation

at the fetal-maternal interface. Furthermore, Liu *et al* [102] documented the 1α–hydroxylase conversion of 25D to 1,25D, as well as robust cathelicidin induction, in placental human trophoblast cells. However, unlike human macrophages, *CAMP* induction in trophoblasts is independent of TLR-activation. Taken together, these results clearly demonstrate the broad spectrum of *in vivo* activity for vitamin D mediated signaling, moving beyond calcium homeostasis and bone metabolism.

13. HDAC inhibitors

In eukaryotic cells, DNA and complementary proteins form a highly organized complex required for its packaging within the confines of the nucleus, called chromatin. Within chromatin, nucleosomes represent the basic fundamental repeating unit. Nucleosomes are comprised of segments of progressively folding negatively charged DNA, coiled around small positively charged proteins, called histones. This high degree of organization within nucleosomes allows for greater compaction of linear DNA, and serves as a regulatory control during gene expression.

During transcription histone proteins are the targets of many post-translation events, including ubiquitination, phosphorylation, and acetylation. Acetylation at core N-terminal lysine residues neutralizes the positive charge of histones, thereby reducing their DNA affinity. This loosens the tightly wound chromatin structure, allowing the transcriptional apparatus and RNA polymerase easier access to DNA, which promotes gene expression [103]. Remarkably,

histone acetylation has also been linked to the transcriptional processes of DNA repair and recombination [104, 105].

Histone acetylation is regulated by two fundamental groups of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). As their name suggests, HATs are enzymes that acetylate N-terminal lysine residues on histone proteins, leading to transcriptional activation. Conversely, HDACs are a group of enzymes that remove terminal acetyl groups, lending to transcriptional repression.

The inhibition of HDACs favors increased HAT activity, facilitating the process of histone hyperacetylation. HDAC inhibitors (HDIs) are compounds that trigger histone hyperacetylation in order to loosen chromatin structure and modulate gene expression. However, increased histone acetylation and alterations to chromatin structure are not always associated with elevated transcription levels. Research has shown that HDIs may either inhibit or induce gene expression, with studies suggesting that HDI activity repressing nearly as many genes as may be induced [106, 107].

Within the HDAC family, enzymatic activity owes to conservation of a catalytic domain containing a zinc ion within the active site [108]. HDIs function by binding the zinc ion and preventing access to the active site, thereby inhibiting the enzyme. The molecule sodium butyrate (butyrate) is an ester of butyric acid (Fig. 7), and one of the first compounds discovered to possess HDI activity [109]. Since the initial discovery, butyrate has been extensively investigated for its role in maintaining colonic homeostasis and regulating gene expression. Butyrate is a

short chain fatty acid (SCFA) that is the naturally occurring end product of intestinal microbial fermentation of dietary fiber. Within the gut, butyrate is found in millimolar concentrations [110]. In millimolar concentrations, butyrate has been shown to exert potent effects on colonic function such as decreasing oxidative stress, inhibition of inflammation, and reinforcing constitutive defense mechanisms, including the induction of antimicrobial peptides [110, 111]. As a dynamic modulator of gene expression, butyrate provides a novel approach for developing therapeutics utilizing HDAC inhibitory activity.

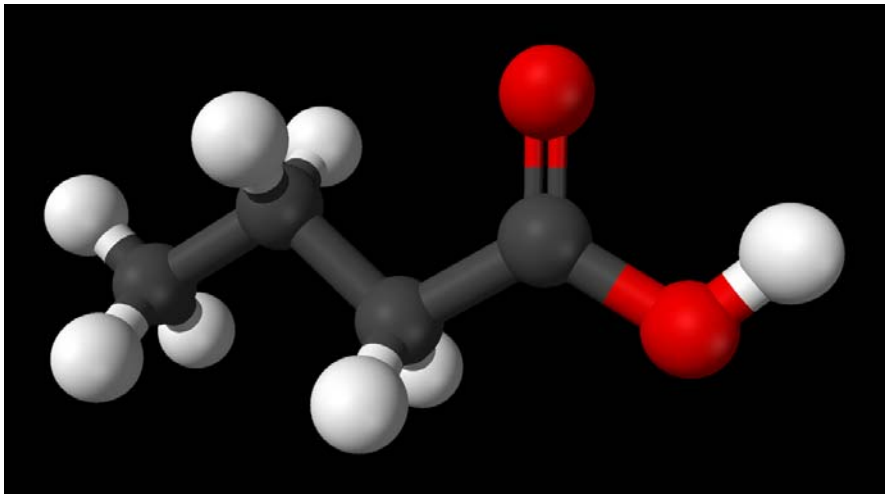


Fig. 7: Ball-and-stick model of butyric acid. Black: carbon, white: hydrogen, red: oxygen.

A number of HDIs have been developed and are currently used in various treatment protocols, most notably for psychiatric conditions and epilepsy. Studies have also shown promise for HDI use in cancer therapy [112], and the treatment of neurodegenerative disorders such as Alzheimer's and Huntington's diseases

[113]. One intriguing study that came out of our laboratory showed that a nuclear receptor agonist could be synthesized to possess HDI activity [114]. By fusing together the 1,25D backbone with a side chain possessing functional HDAC properties (Fig. 8), the idea was to combine butyrate-like HDI activity with VDR induced gene enhancement within the same molecule. These molecules serve as functional 1,25D analogues.

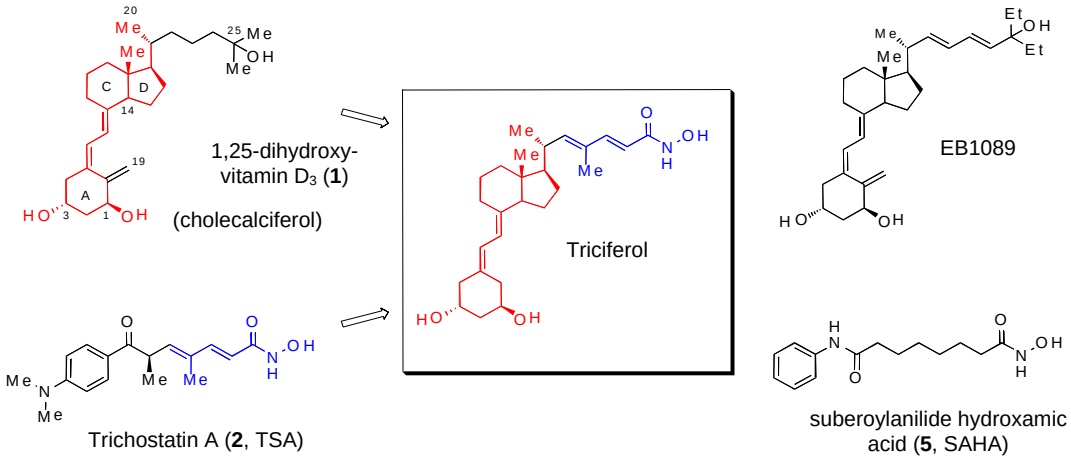


Fig. 8: Incorporation of histone deacetlyase inhibition into the molecular structure of 1,25D [114].

14. Therapeutic role of vitamin D analogues

Vitamin D analogues have shown considerable therapeutic promise across a number of applications. The potential of these analogues stems from their ability to mediate VDR signaling without inducing the calcemic effects associated with elevated hormonal 1,25D. Cancer serves as a prime example for selectively targeting vitamin D regulation for gene therapy. The anti-tumor activities of 1,25D analogs have long been established across a range of cancers [115].

Although mechanisms underlying 1,25D signaling in cancer therapy are highly complex, the anti-cancer properties of vitamin D are generally attributed to arresting cell growth and controlling proliferation. Indeed, a vast array of *in vivo* studies have linked treatment with vitamin D analogs to decreased tumor activity in colon [116], breast [117], prostate [118], and pancreatic cancers [119] while minimizing known calcemic effects. Moreover, vitamin D analogues have shown considerable promise prolonging the survival of mice inoculated with leukemia, without altering calcium serum levels [120].

The immunoregulatory properties of 1,25D also serve as a platform for vitamin D analogue development. Amongst autoimmune conditions, psoriasis is one of the few disorders currently treated by vitamin D therapy. Calcipotriol, a vitamin D analogue approved for treatment of psoriasis in both the United States and Europe, has demonstrated its effectiveness by stimulating terminal differentiation in keratinocytes [115]. Indeed, a number of vitamin D analogues have been approved for treatment of psoriasis, without significant calciotropic effects. Additionally, clinical studies have shown progress in analog development for several autoimmune conditions such as type I diabetes and arthritis [121, 122].

Taken collectively, the above findings strongly support vitamin D therapy for a number of applications. However, these studies only represent a small sample within the growing field of vitamin D related research. As evidence accumulates, increasing numbers of immune disorders serve as candidates for analogue treatment, and warrant further investigation. Indeed, alterations to VDR mediated signaling appear to play a profound role in the pathophysiology of

irritable bowl syndrome (IBS), Crohn's disease, and skin cancer [12, 115]. Unlocking the enormous potential of vitamin D analogues shows considerable promise for novel therapeutic development while minimizing the associated side effects.

Project Proposal

1,25D and related analogues govern a wide-spectrum of gene expression activity. In particular, studies have established that 1,25D is a potent inducer of antimicrobial peptide expression and innate immunity [60, 61, 102]. The induction of these peptides serves as a front line of defense in host-pathogen recognition and response pathways against *Mycobacterium tuberculosis* [44, 80]. Compounds that are HDAC inhibitors have also been shown to regulate a wide variety of gene expression activity, including the stimulation of immune responses [103-107]. Recent studies have shown that esters of the HDI compound butyric acid (butyrate, phenylbutyrate) directly induce antimicrobial gene expression in epithelial derived cell lines [111, 123]. Based upon these observations, this project investigated the *in vitro* effects of combining 1,25D and butyrate-like HDI activity could have on promoting antimicrobial gene expression levels, in addition to stimulating immune responses as measured through microbial viability in macrophages (THP-1) infected with Mtb.

Vitamin D analogue development has been driven by the idea that compounds can be developed to promote 1,25D receptor agonism while minimizing calciotropic side effects. Given that certain HDIs can modulate gene transcription and directly induce antimicrobial gene expression, by combining the activity of HDIs with VDR induction of gene expression, it is possible to develop series of 1,25D analogues that possess novel therapeutic value to treat pathogenic infection. These compounds were developed with cooperation from Dr. Jim Gleason (Department of Chemistry, McGill University), and were designed to

incorporate an HDI component to replace the 1,25D side chain (Fig 8; see above). Thus, this project assessed the value of developing functional vitamin D analogues to compare with 1,25D, and the combined treatments of butyrate and 1,25D.

Materials and Methods

Cell culture

All cell lines were cultured under recommended conditions. THP-1, SCC25, SW480, and HT-29 were obtained from (American Type Culture Collection, Manassas, VA) and human adult and neonatal primary keratinocytes from (BioWhittaker, Walkersville, MD). Cells were cultured in 100mm petri dishes (SCC25, HT-29) or 75cm^2 tissue culture flasks (THP-1, SW480, and primary keratinocytes) with recommended media (ATCC, Manassas, VA; Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 100U/mLpenicillin and 100 mg/mL streptomycin (Wisent, St-Bruno, QC). Cells were cultured at 37C, 100% humidity, and 5% CO2 for all cell lines except the SW480 cell line, where no CO2 was added. For cell treatments, cells were split, 24h later medium was changed to 10% charcoal-stripped FBS medium, and 24h after that medium was changed to stripped media supplemented with 100nM-1uM 1,25D (Sigma-Aldrich, Oakville, ON), or compound. For macrophage differentiation, THP-1 monocytes cells were cultured in the presence of 100 nM PMA (Sigma-Aldrich, Oakville, ON) for 24 hours.

RT-PCR analysis

PCR primers were designed using Primer-BLAST software at http://www.ncbi.nlm.nih.gov/tools/primer-blast/ and are listed below. After treatment, cells were washed with PBS, homogenized with 1ml TRIzol Reagent (Invitrogen, Carlsbad, CA), kept at room temperature for 2-5min, collected and

kept at -80 for at least 1h. Solution was thawed, and 0.2ml chloroform was added. After vigorous mixing and storing for 10min, mixtures were centrifuged and the upper layer was transferred to a new tube. 0.5ml isopropanol was added and the new solution was mixed. After centrifuging at 4^{o}C, the supernatant was discarded and 1ml of 75% ethanol was added. After centrifuging and discarding the supernatant, the pellet was air-dried for 5-10min, and then dissolved in ddH_2O. 1-3μg of RNA was used for reverse transcriptase reactions (total volume: 20μl). 80μl ddH_2O was added to the RT-product, and 1.5μl of that was used as template for the PCR reaction. Reverse transcriptase (Super Script II) was purchased from Invitrogen (Carlsbad, CA). DNA polymerase and dNTP's were ordered from Fermentas (Glen Burnie, MD).

qPCR analysis

RNA was isolated using Trizol Reagent (Invitrogen), and cDNA was synthesized using the iSCRIPT cDNA Synthesis Kit (BioRad, Hercules, CA). The primer sequences for GAPDH, NOD2, HBD2 and CAMP were designed by Primer-BLAST as previously described.

Mycobacteria growth and maintenance

M. tuberculosis (attenuated strain H37Ra) and *M. smegmatis* were grown in suspension containing Middlebrook 7H9 broth (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 0.05% Tween-80 (Sigma, St. Louis, MO, USA) and 10% Middlebrook ADC enrichment (Becton Dickinson). Aliquots

from logarithmically growing cultures were frozen in 7H9 culture containing 20% glycerol.

Infection and treatment of macrophages for CFU

THP-1 macrophages we maintained and differentiated as previously described. Macrophages were infected for 4 hours with suspensions of *M. tuberculosis* and *M. Smegmatis* at a multiplicity of infection of (MOI) 5:1. Since infected macrophages were adherent to the plastic 6-well plates, extracellular bacteria were removed by vigorous washing with RPMI for 3 cycles. After washing, THP-1 cells were incubated in RPMI media supplemented by 10% charcoal-stripped FBS medium for 24 hours (*M. smegmatis*) or 72 hours (*M. tuberculosis*). Triplicate sample were maintained, and between 100nM-1uM 1,25D, or compound, was added to the incubation media for every 24 hours of treatment.

Quantification of mycobacterial growth

After treatment period, all supernatant was collected from individual wells and spun down at 5000 rpm for 10 min. To measure survival of viable bacilli the infected cells were lysed with 1mL filtered, sterile ddH_2O with 1% Triton X-100 (Roche, Indianapolis, IN) to release intracellular bacteria. Lysates of infected cells were centrifuged down at 5000 rpm for 10 min and resuspended in 1mL Middlebrook 7H9 broth with reciprocal pellets collected from the supernatant. After resuspension, individual samples were vigorously shaken and further diluted

in (1/10) serial dilutions of filtered, sterile 1% PBS solution. Between 50-100uL of individual serial dilutions were enumerated for viable colony forming units (CFU) on Middlebrook 7H10 plates supplemented with 10% Middlebrook OADC enrichment (Becton Dickinson) and BD Bactec Panta/F antibiotics (BD Biosciences, Sparks, Maryland) after 4 days for *M. smegmatis*, or 21 days for *M. tuberculosis*.

PCR primers

Gene	5' Primer Sequence	3' Primer Sequence
CAMP	GGGTCACTGTCCCCATACAC	TCGGATGCTAACCTCTACCG
CYP24	TATTTGCGGACAATCCAACA	GGCAACAGTTCTGGGTGAAT
DEFB2 (HBD2)	AGGGCAAAGACTGGATGAACA	GGTGTTTTTGGTGGTATAGGCG
GAPDH	GATCTCGCTCCTGGAAGATG	GAGTCAACGGATTTGCTCGT
NOD2	CCTGTTCAGAGAAGCCCTTG	AACCTTTGATGGCTTTGACG

Results

Results presented below indicate that treatments combining 1,25D with butyrate-like HDI activity promote the robust induction of antimicrobial gene expression in a number of human epithelial and immune derived cell lines. Furthermore, analogues of 1,25D can successfully be synthesized that bind to the VDR, and actively stimulate innate immune response pathways. These analogues have been shown to induce antimicrobial peptide expression, and promote a measurable reduction in pathogen viability in macrophages exposed to mycobacteria and treated with our compounds. The effects of these analogues were tested and compared to an untreated control: vehicle (DMSO), 1,25D, butyrate, and a combination of 1,25D and butyrate in the human derived cell lines THP-1, HT-29, SW480, SCC-25, as well as normal human keratinocytes.

Originally, over 40 vitamin D analogues were developed in collaboration with Dr. Jim Gleason's laboratory, McGill University. Design of these analogues incorporated the backbone of 1,25D, combined with side chain modifications possessing HDAC inhibitory qualities, within the same molecule. These compounds were labeled with two sets of nomenclature for identification purposes, with each compound receiving both a numeric and alphabetic identifier (see results below).

Initially, roughly a dozen compounds were developed for testing. This first group of compounds was assessed by fluorescence polarization (FP) assay in our laboratory for their affinity for the VDR (Fig. 9; work by Basel Dabbas and Russell Spingarn). The FP assay effectively measures the concentration at which

one-half of maximal VDR sites were saturated (EC50), with compounds measuring lower concentration values showing a greater affinity for the VDR. As per the results, relative affinity of these compounds for the VDR ranged from near equivalence with 1,25D (Compound 371; P) to a compound X (compound 243), which required roughly 40 times the concentration of 1,25D to saturate half of the available VDR sites.

Expanding upon our binding affinity results, this initial group, along with newly developed compounds, was tested for their ability to induce 1,25D-like VDR agonistic activity. As mentioned in the literature review, CYP24 is the enzyme responsible for 1,25D catabolism. Since CYP24 is induced by the 1,25D substrate, compounds that up-regulate *CYP24* expression also possess intrinsic 1,25D-mediated gene expression activity. Induction of *CYP24* expression was tested by reverse transcriptase-PCR (RT-PCR) analysis after treating the individual cell lines with a range of [100nM-1uM] 1,25D, butyrate @ [1mM], a combination of both (1,25D + butyrate), or compound for a 24-hour period. All assays were replicated in triplicate. Results of our *CYP24* assays showed a number of compounds were able to induce 1,25D-like VDR activity to varying degrees in SCC25 (Fig. 10), normal human keratinocytes (Fig. 11), SW480 (Fig. 12), and THP-1 (Figs. 13 & 14) cells. In consideration that RT-PCR is a semi-quantitative analysis, visualization of band intensity provided a relative comparison of gene induction levels. As expected, universal up-regulation of *CYP24* was found in all cell lines among the 1,25D and (1,25D + butyrate) treated

samples. Additionally, a number our compounds triggered the robust induction of *CYP24*.

Similarly, the gene for the intracellular pattern recognition receptor NOD2 was also examined. NOD2 is a downstream target in 1,25D-regulation of gene expression [11], and as previously mentioned, serves as a critical component of host-pathogen recognition and response pathways. Regulation of *NOD2* was found to be highly variable between samples among the cell lines tested (Figs. 11-14). No clear pattern of up-regulation was noted correlating *NOD2* with either *CYP24* or AMP (*CAMP, DEFB2*) promotion, nor was any real consistency in *NOD2* expression levels noted between our treatments from the various cell lines tested. However, one consistent observation through RT-PCR analysis found that *NOD2* gene expression was induced by nearly all treatment samples to a greater extent than the untreated control. These results are seen most prominently in assays conducted with THP-1 macrophages (Figs. 13 & 14).

Induction of antimicrobial peptide expression for the *CAMP* and *DEFB2* genes was also assessed by RT-PCR analysis. Similar to the promotion of *CYP24*, AMP gene expression is dependent upon several factors, including VDR activation of innate immune pathways. Accordingly, up-regulation of either *CAMP* or *DEFB2* expression by 1,25D was seen to somewhat mirror the results from our *CYP24* analysis in SCC25 (Fig. 10), normal human keratinocytes (Fig. 11), SW480 (Fig. 12), and THP-1 (Figs. 13 & 14) cells. Notably, only a small fraction of the compounds tested even modestly enhanced AMP peptide expression in keratinocytes (Fig. 11) or SW480 (Fig. 12) intestinal epithelial cells.

A more prominent induction of the *CAMP* gene was seen when testing THP-1 cells (Figs. 13 & 14), and the SCC25 head and neck carcinoma cell line (Fig. 10), with a very limited selection of our analogues eliciting a robust response. These analogues were effectively known by their alphabetic nomenclature as compounds Q, R, M, and P (Fig. 23).

Further assessment of these four compounds began with a simple assay comparing gene expression levels to relative control samples for a 24-hour period. Semi-quantitative RT-PCR analysis showed that all four compounds, when treated at equivalent concentrations of 1,25D, induced *CAMP* expression in THP-1 cells to similar levels as seen from the control sample (Fig. 15). Quantitative PCR (qPCR) was then utilized to ascertain the variable degree of differences in up-regulation of the *CAMP* gene from these samples (Fig. 16). All four compounds significantly up-regulated the expression of *CAMP*, with 1,25D and vehicle (DMSO) serving as baseline controls. Treatment from one sample, compound P (371), showed a slightly favorable enhancement of *CAMP* expression compared to 1,25D (Fig. 16).

Based upon these promising results, compounds Q, R, M, and P were selected to test for their capacity to suppress mycobacterial replication in infected macrophages. Bacterial viability was measured utilizing colony-forming unit (CFU) assays in THP-1 cells. Initial antimicrobial assays were tested employing *Mycobacteria smegmatis*, a relatively non-pathogenic, fast growing strain of mycobacteria, as the agent of infection. Results from the initial assays showed no significant reduction in mycobacterial viability in THP-1 macrophages treated

with either [100nM] of compound or control (Fig. 17). A ten-fold increase in our treatment concentrations [1uM] was then employed, with 43% (compound P) to 60% (compound R) reductions in mycobacterial viability seen when compared to appropriate controls (Fig. 18). Remarkably, treating THP-1 macrophages with 1,25D saw no reduction in *M. smegmatis* viability when compared to the untreated control—a result that would require further troubleshooting for clarification.

Performing CFU assays with *Mycobacteria tuberculosis* (H37Ra) at [1uM] treatment gave rise to some significant reductions in microbial viability. CFU assays were carried out by infecting differentiated THP-1 cells with Mtb at ratio of 5:1, then following with treatment for a 72 hour period. The initial assay showed roughly a 50% reduction in Mtb viability in 1,25D treated samples, as compared to our negative control: vehicle [1uM] DMSO (Fig. 19). Statistical evaluation of this reduction in pathogen viability was found to be significant when comparing double the standard deviation values between the two samples. Notably, this 50% reduction was also comparable with results seen from samples treated with compounds Q, R, or P. However, two samples treated compound R became contaminated; leaving a single sample for evaluation, leading to lack of data for statistical significance as a measure of standard deviation.

The most significant results in the first assay were seen with treatment from compound M. Results with compound M showed a 75% reduction in Mtb viability as compared to the untreated control. This 75% reduction in microbial viability was statistically significant when compared to our 1,25D sample, as a

measurement of standard deviation. More remarkably, however, individual colony sizes of Mtb harvested after treatment with compound M were noticeably smaller than those from our controls or other treatment samples (Fig. 20).

Two additional CFU assays were carried out utilizing Mtb strain H37Ra infecting THP-1 cells at a ratio of 5:1. However, the overall reduction in microbial viability was limited when comparing results from the initial assay (Figs. 19, 21, & 24). Furthermore, results from the second assay did not reveal any reduction in mycobacterial viability in samples treated with 1,25D (Fig. 21), leading to a lack of statistical analysis. This result stands out as an anomaly in comparison to the other CFU assays conducted with strain H37Ra.

Based on results seen from the initial H37Ra assay, compound U was substituted for compound Q for the second CFU assay (Fig. 21). Selection of compound U for the second assay was based on a comparison of the similar molecular make-up in the side-chains structures of compounds M and U. As illustrated in (Fig. 23), these two compounds share a number features, with the side chain of compound U extended to include two additional carbon molecules. Remarkably, both compounds M and U exhibited a similar reduction in bacterial colony sizes, as seen in samples treated with M from the H37Ra assays preformed (Figs. 20, 22).

To further measure the efficacy of compound M, a sample was aliquoted and donated to Rob Kozak in the laboratory of Dr. Marcel Behr (Department of Medicine, McGill University). Mycobacteria tuberculosis strain H37Rv was then utilized as a pathogen to infect THP-1 cells (5:1) in triplicate samples with an

untreated control, 1,25D, and compound M treatments at [1uM]. This experiment was performed in the level 3 containment facility in the Behr laboratory, McGill University. From our initial experiment (Fig 25), results showed a modest reduction in mycobacteria viability in both treatment samples, with compound M showing slightly more favorable results than 1,25D. A comparison of our samples show approximately a 40% reduction in the samples treated with 1,25D, compared to nearly a 50% reduction treated with compound M. However, in contrary to experiments carried out with H37Ra, no noticeable differences in colony sizes were reported between all three-treatments from the H37Rv infected samples.

CFU assays are a useful tool to comparably measure reductions in microbial viability of infected THP-1 cells treated with compound. However, a new assay would be required as a control in order to measure any direct antimicrobial effects these compounds may have on mycobacteria growing in culture before THP-1 cell infection. For these assays, *Mycobacterium tuberculosis* (H37Ra) was cultured in isolated samples treated with [1uM] compound directly into the culture media 24 hours prior to THP-1 infection. CFU assays were then carried out in a manner similar to those mentioned above, but treatment was withheld from the incubation media after bacterial uptake by THP-1 cells. Results from these assays indicate that the compounds have little or no direct effect on mycobacterial growth or replication in culture, but rather they stimulate immune responses in THP-1 cells infected with Mtb (Figs. 26 & 27).

Additionally, these experiments did not show any noticeable differences in colony sizes between any of the treatments.

Figures

Fluorescence polarization (FP) assay
VDR–ligand interactions
1,25D and 11 vitamin D analogues
*Work by Basal Dabbas and Russell Spingarn (J. White lab, McGill University)

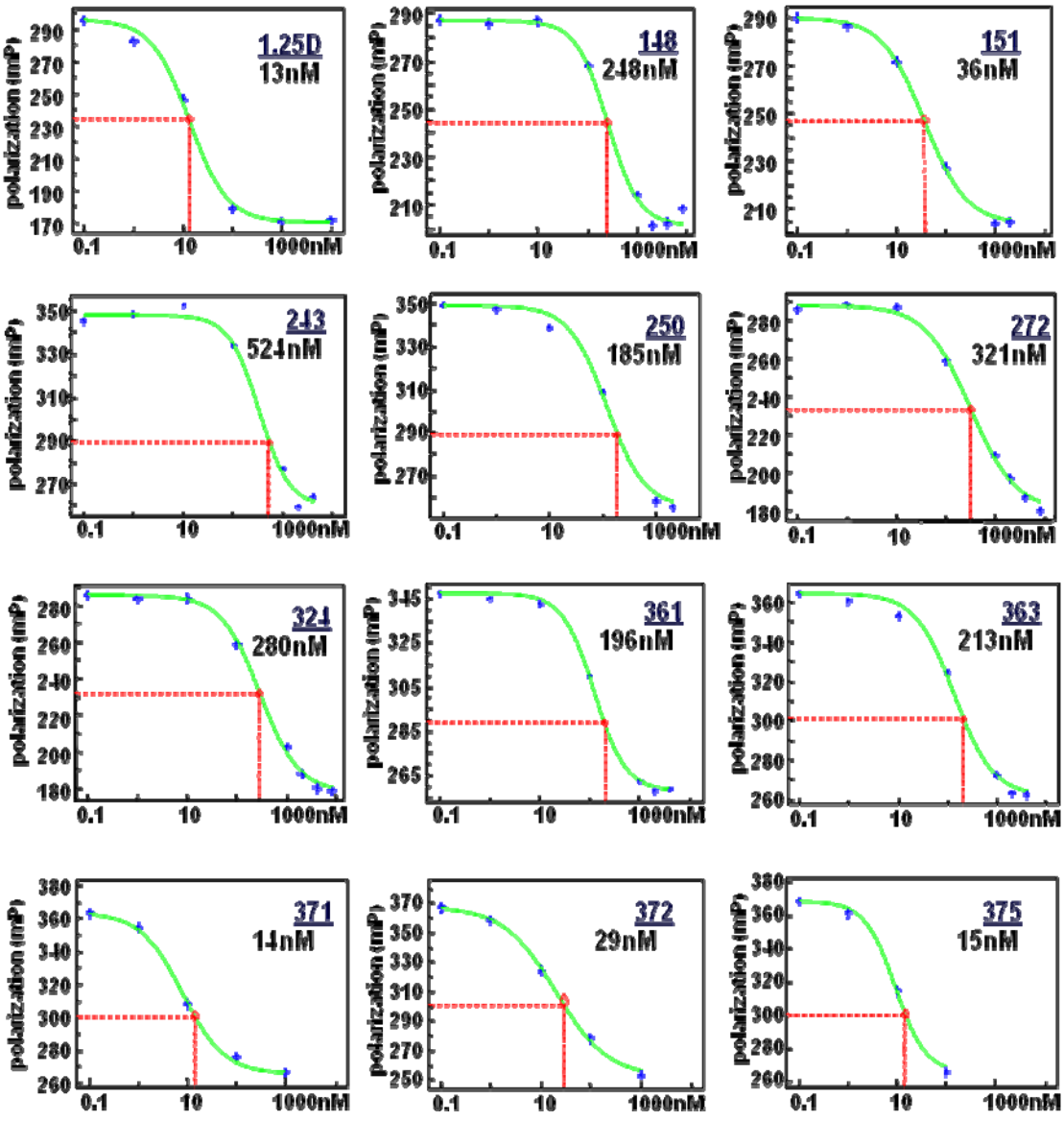


*Fig. 9: Receptor-ligand binding FP assay of 1,25D and the eleven initial 1,25D analogues developed. Key: Compound nomenclature **(blue)** Concentration at ½ maximal receptor site saturation **(EC50; black).***

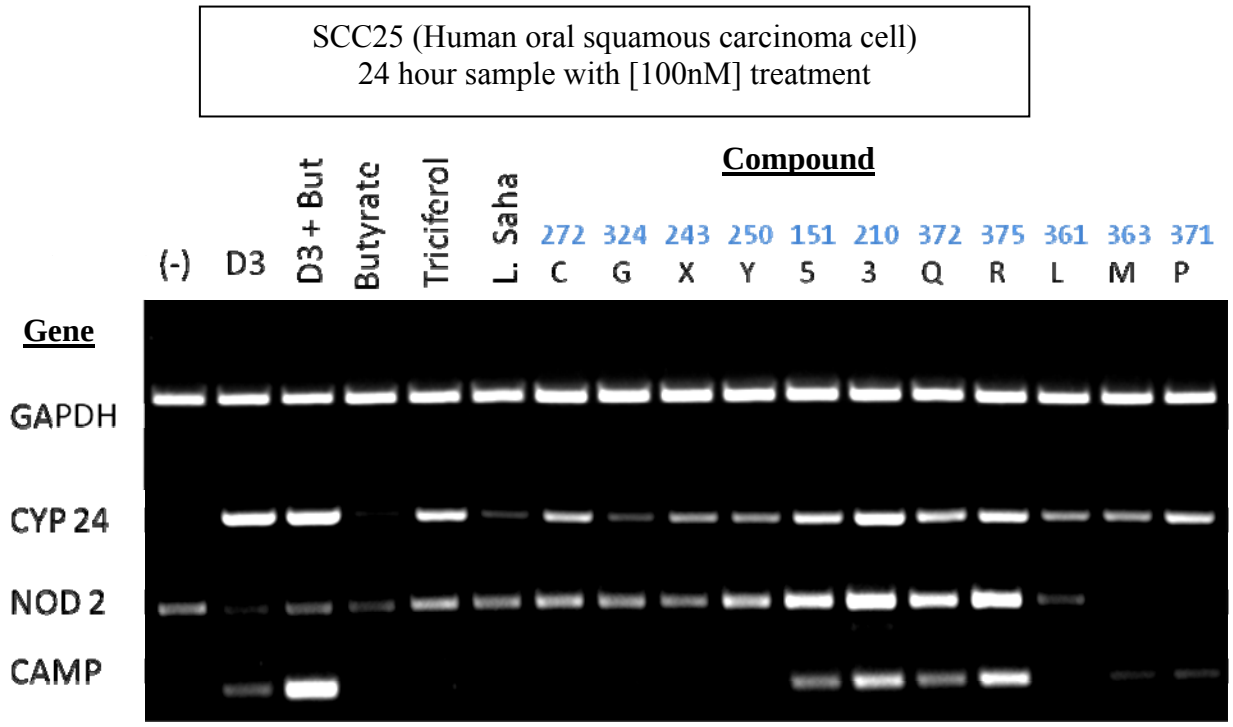


Fig. 10: RT-PCR analysis of SCC25 cells with a single dose [100nM]treatment for 24 hours. Samples are listed from left to right based upon their respective individual (except for the combined D3+Butyrate) treatment type. ***Black = alphabetic nomenclature.*** ***Blue = numeric nomenclature.***

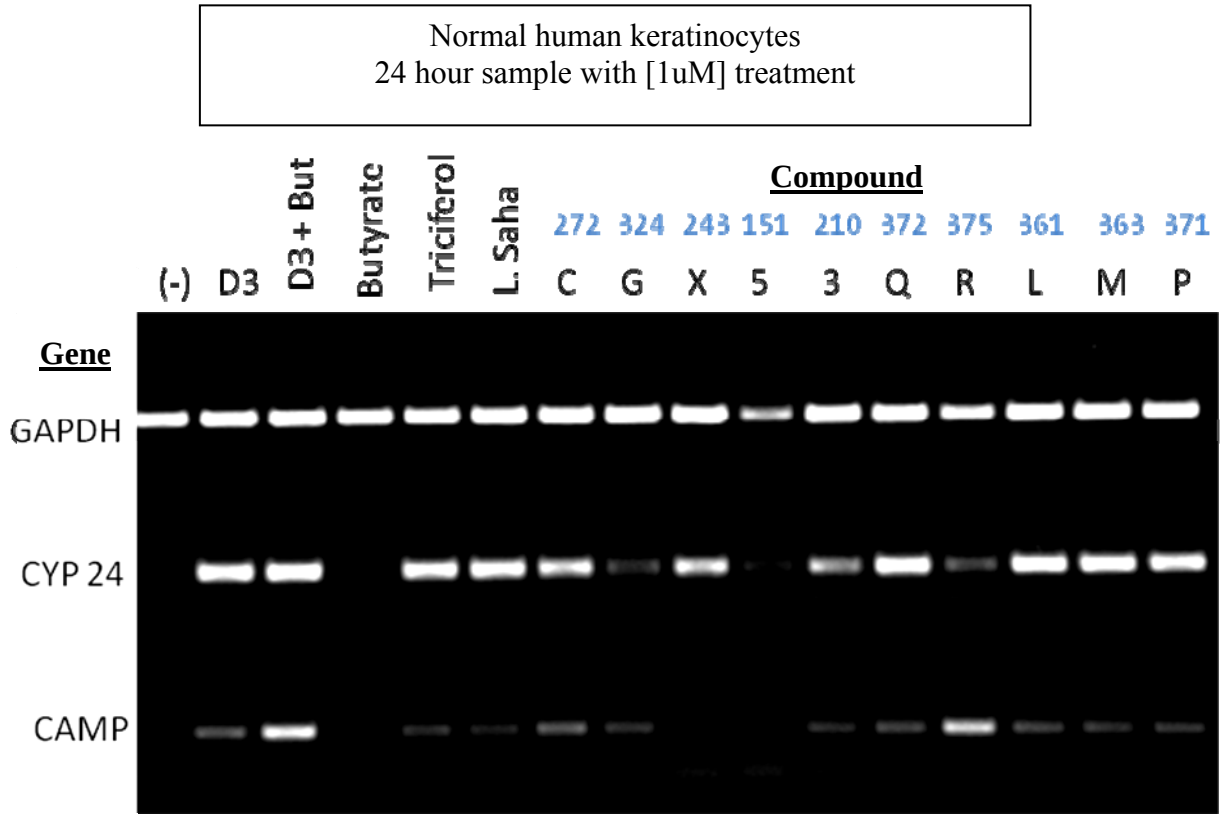


Fig. 11: RT-PCR analysis of Normal Human Keratinocytes with a single dose [1uM] treatment for 24 hours.

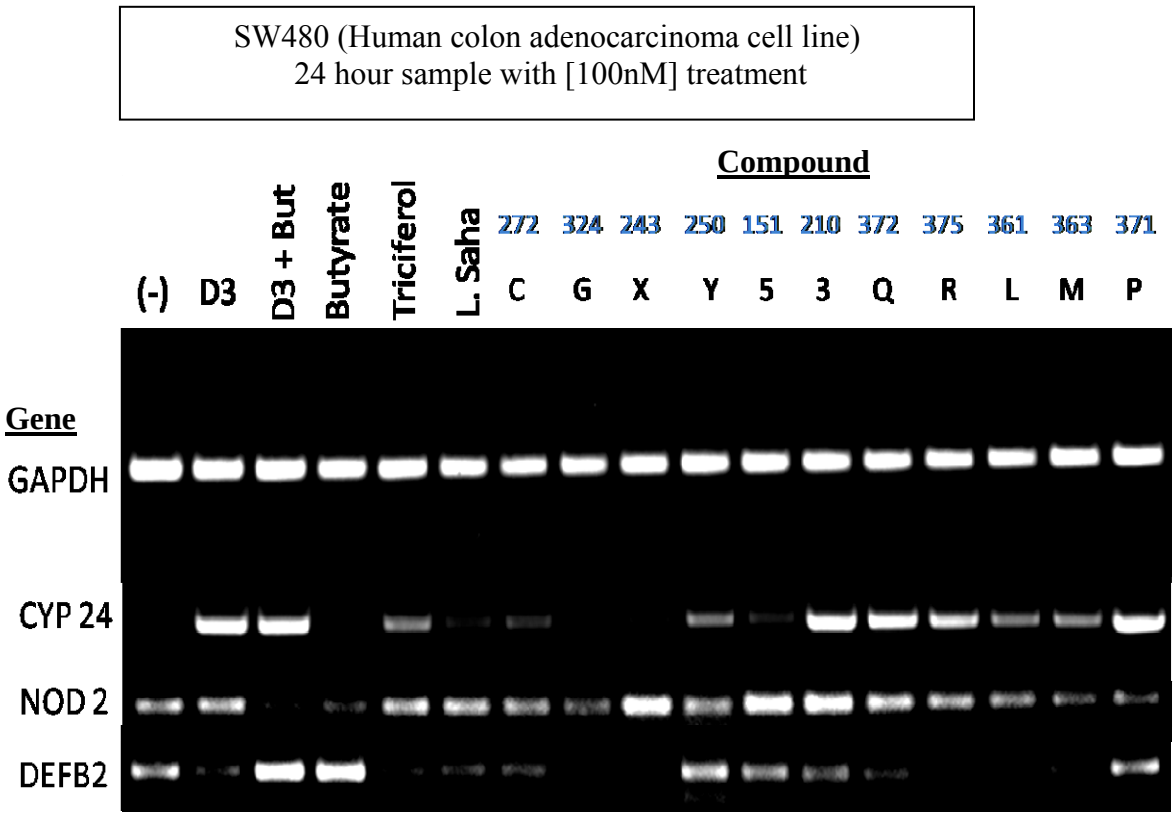


Fig. 12: RT-PCR analysis of SW480 cells with a single dose [1uM] treatment for 24 hours.

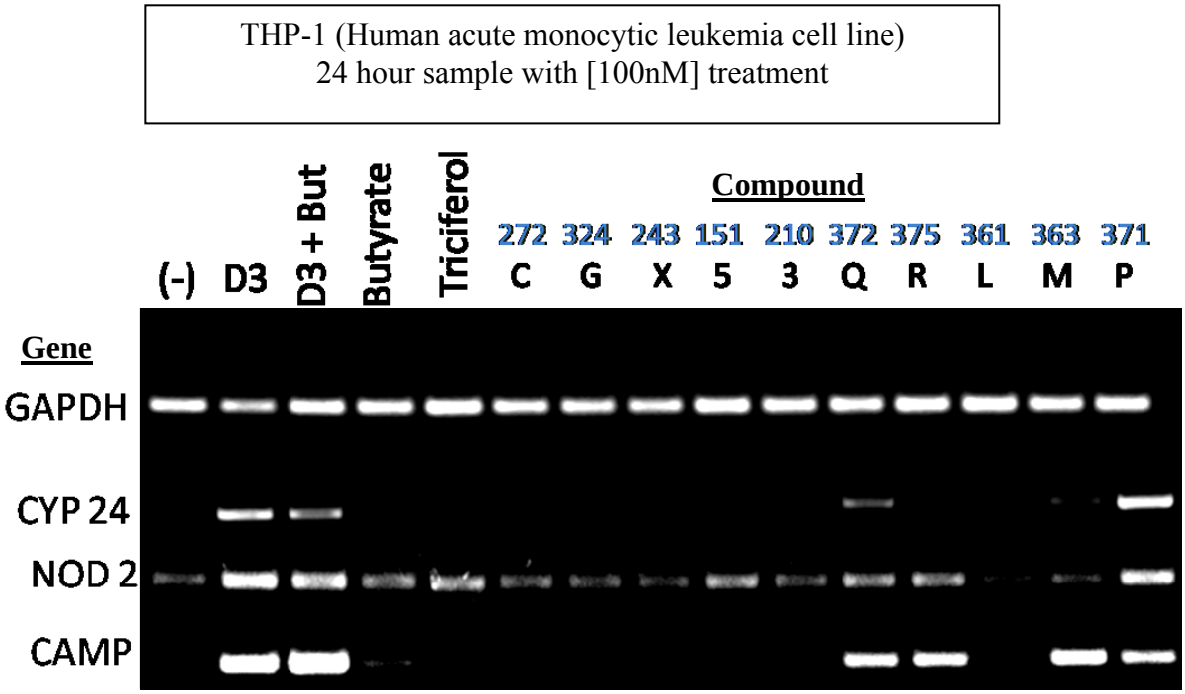


Fig. 13: RT-PCR analysis of THP-1 cells with a single dose [100nM] treatment for 24 hours.

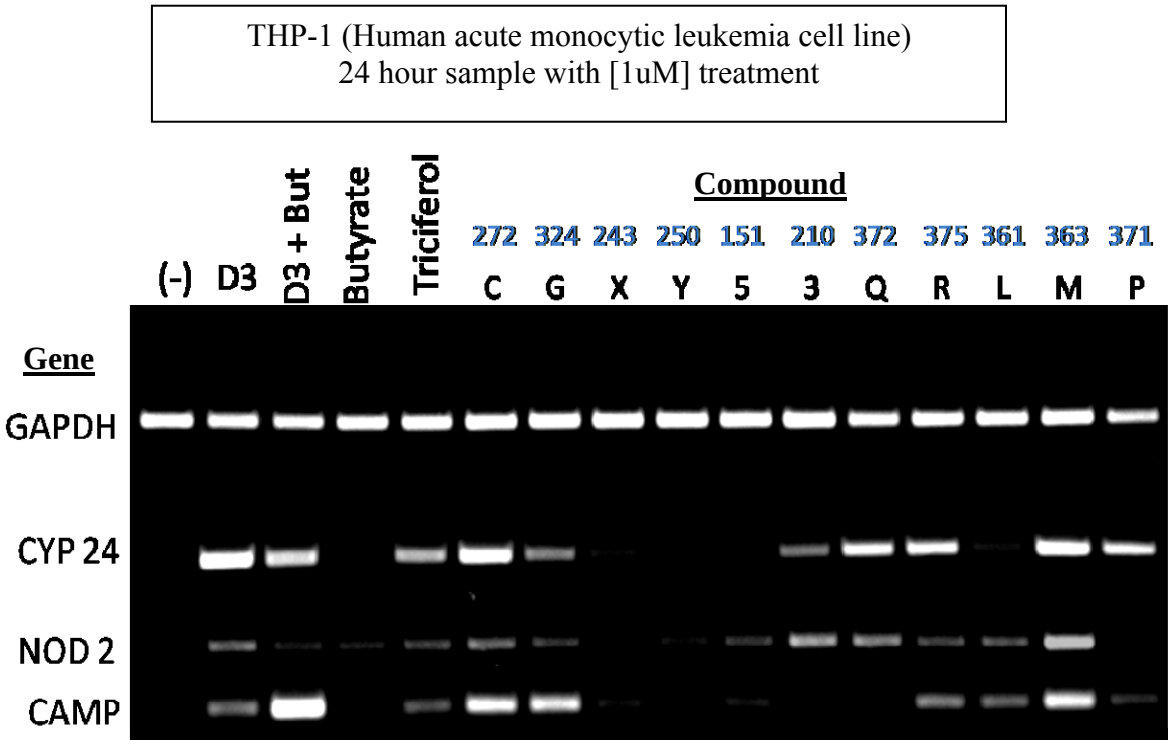


Fig. 14: RT-PCR analysis of THP-1 cells with a single dose [1uM] treatment for 24 hours.

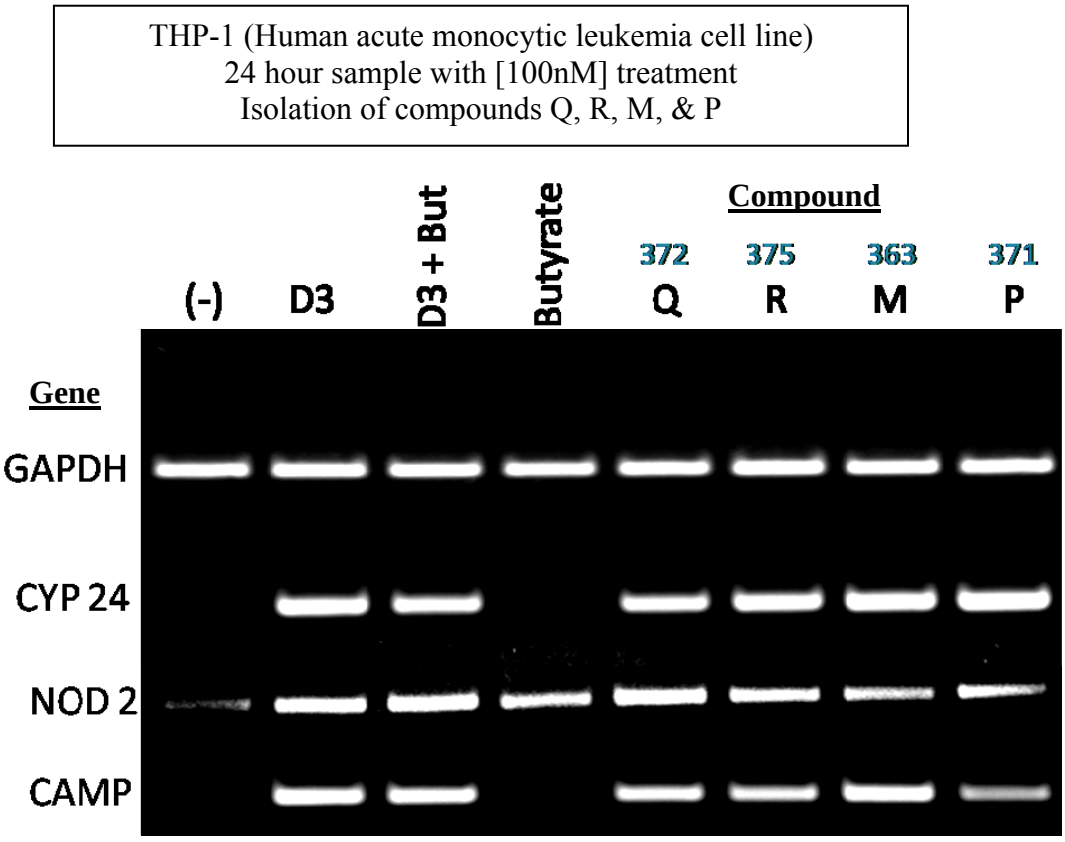


Fig. 15: RT-PCR analysis of THP-1 cells with a single dose [100nM] treatment (compounds Q, R, M, or P) for 24 hours.

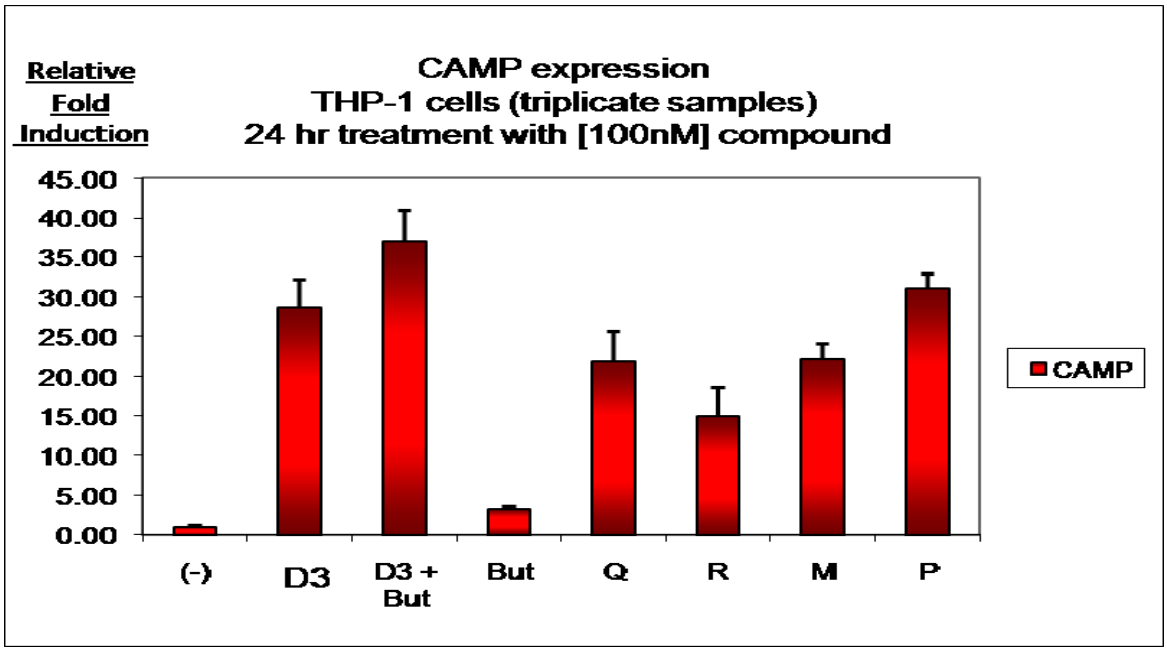


Fig. 16: qPCR analysis of THP-1 cells with a single dose [100nM] treatment (compounds Q, R, M, or P) for 24 hours. **(n=1)**

THP-1 (Human acute monocytic leukemia cell line)
CFU (10^4) assay – *Mycobacteria smegmatis* infection
24 hour sample with [100nM] treatment with compounds Q, R, M, or P

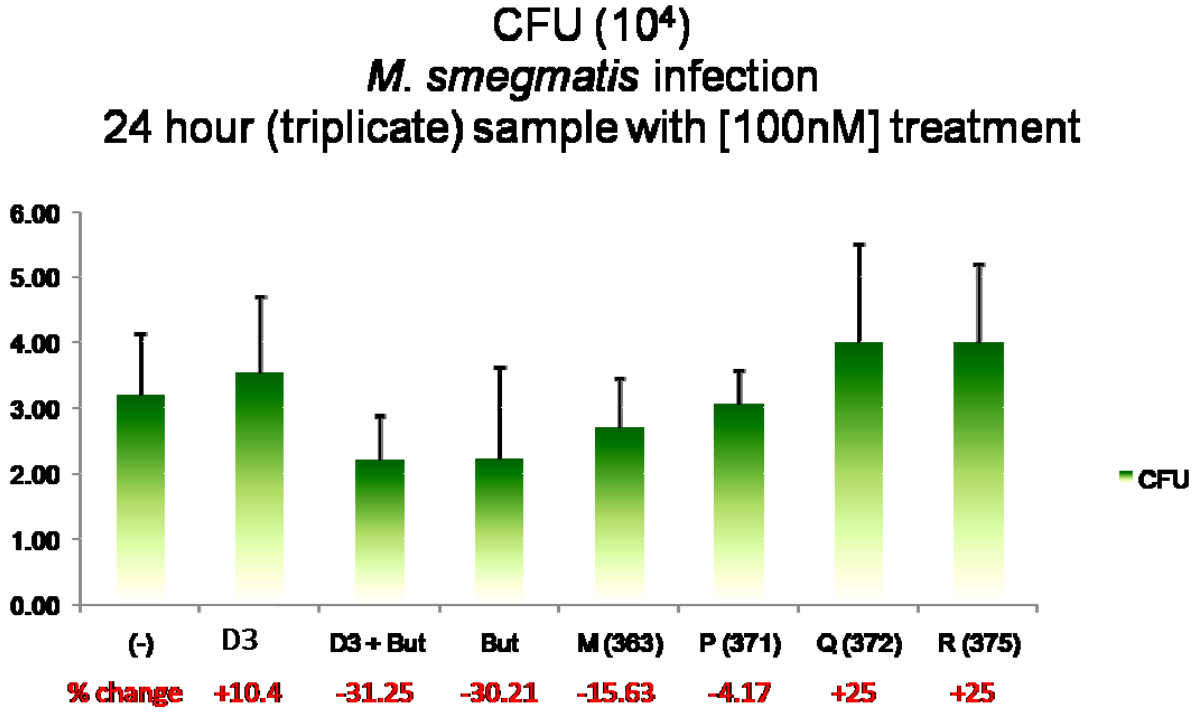


Fig. 17: CFU assay with M. Smegmatis. 24 hour [100nM] single treatment with compounds Q, R, M, or P.

THP-1 (Human acute monocytic leukemia cell line)
CFU (10^4) assay – *Mycobacteria smegmatis* infection
24 hour sample with [1uM] treatment with compounds Q, R, M, or P

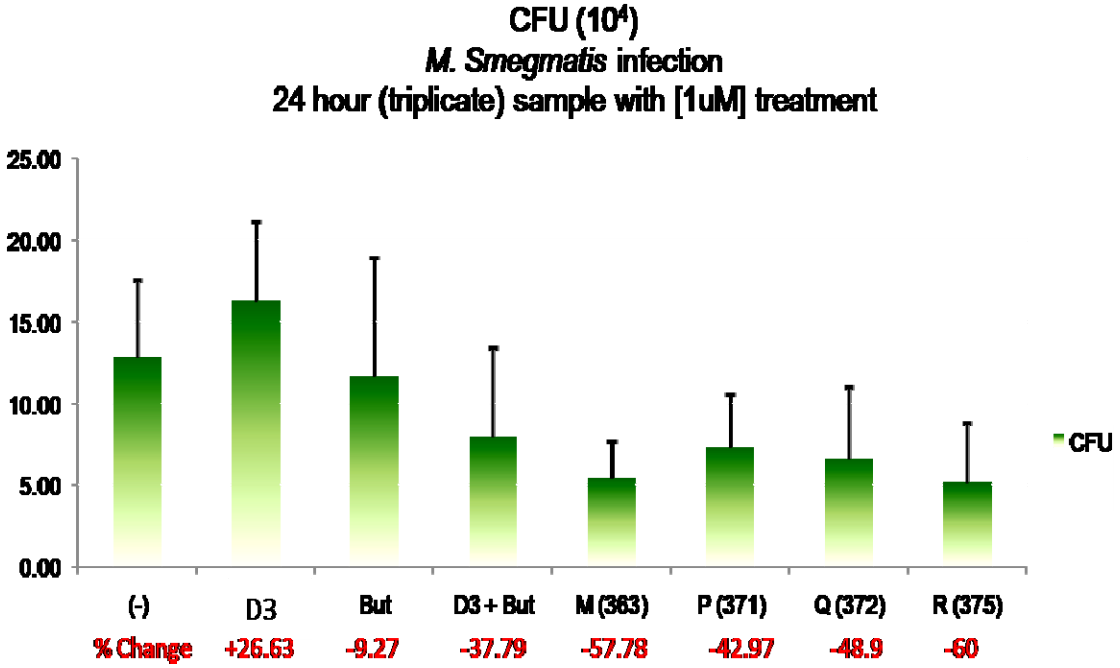


Fig. 18: CFU assay with M. Smegmatis. 24 hour [1uM] single treatment with compounds Q, R, M, or P.

THP-1 (Human acute monocytic leukemia cell line)
CFU (10^4) assay – *Mycobacteria tuberculosis* (H37Ra) infection #1
72 hour sample with [1uM] treatment with compounds Q, R, M, or P

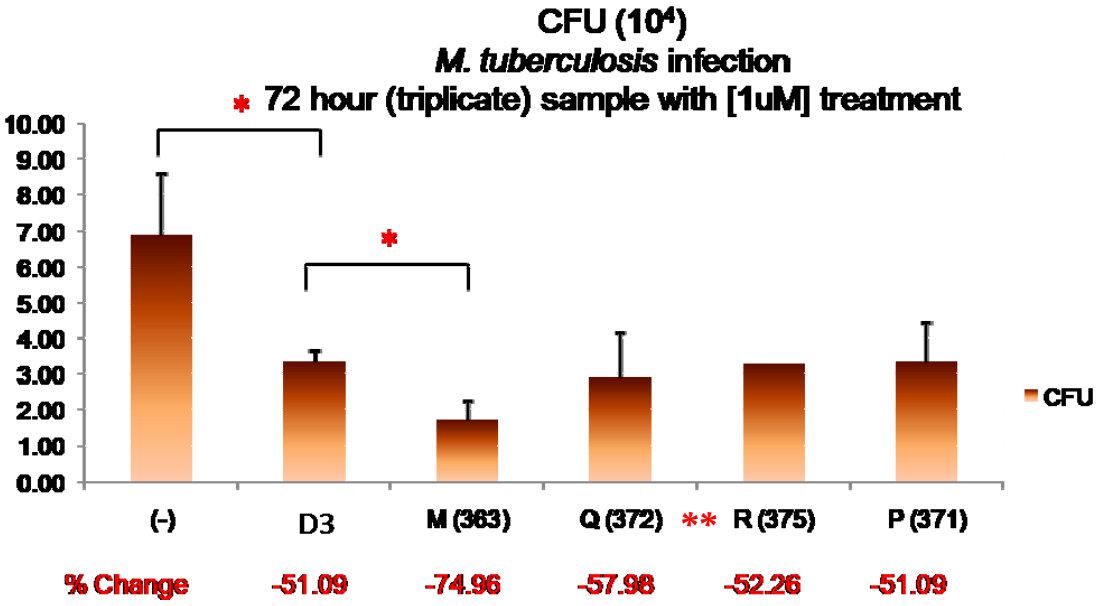


*Fig. 19: CFU assay with M. tuberculosis. 72 hour [1uM] single treatment with compounds Q, R, M, or P. *Statistically significant reduction between samples as a measurement of standard deviation. **Lack of error bars for compound R due to contamination of multiple samples.*

THP-1 (Human acute monocytic leukemia cell line)
CFU assay – *Mycobacteria tuberculosis* (H37Ra) infection #1
Agar plates of samples treated with [1uM] of 1,25D or compound M

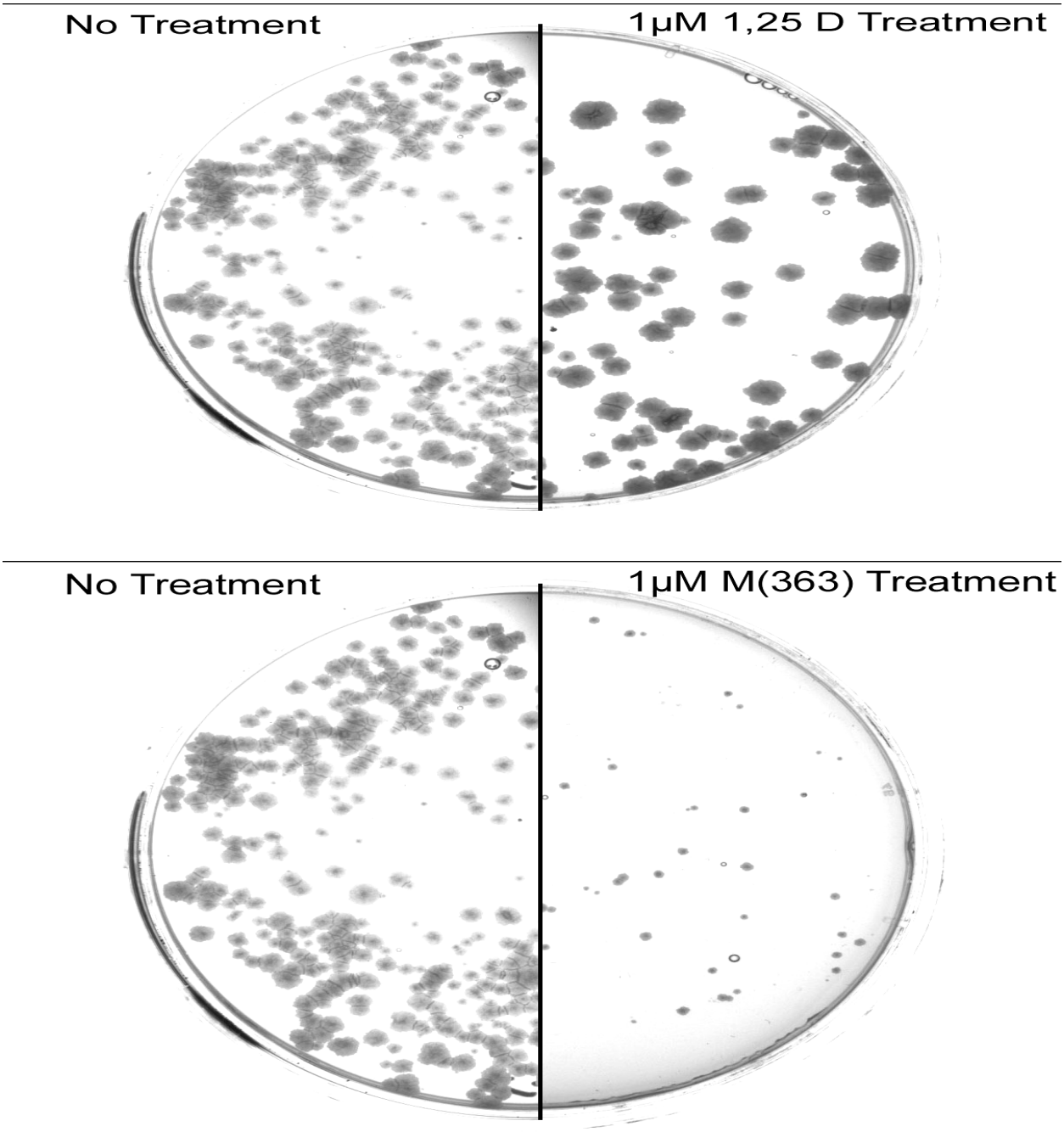


Fig. 20: Photos of M. tuberculosis H37Ra colonies grown on agar plates at 21 days after harvesting bacteria.

THP-1 (Human acute monocytic leukemia cell line)
CFU (10^4) assay – *Mycobacteria tuberculosis* (H37Ra) infection #2
72 hour sample with [1uM] treatment with compounds M, P, R, or U.

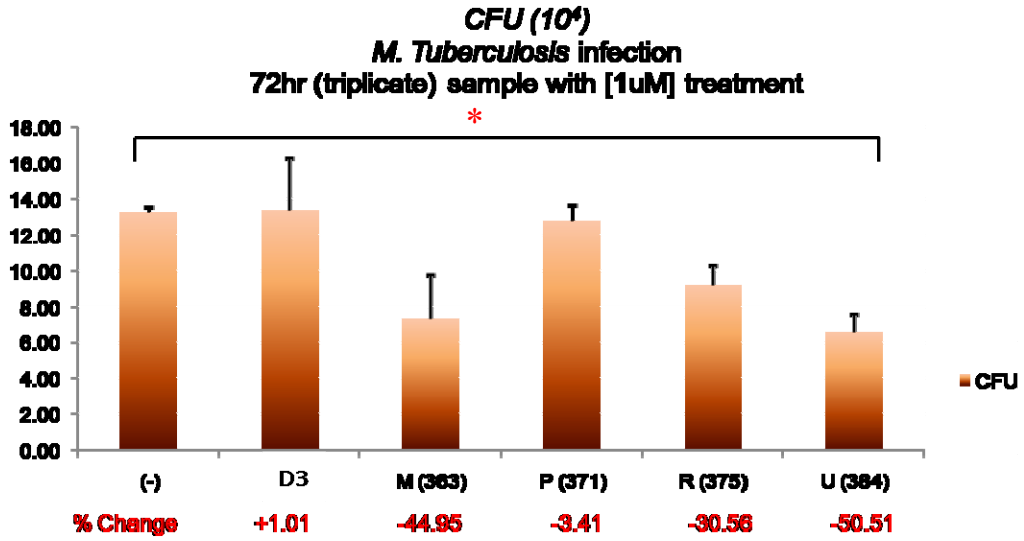


*Fig. 21: CFU assay with M. tuberculosis. 72 hour [1uM] single treatment with compounds M, P, R, or U. *Statistically significant reduction as a measurement of standard deviation.*

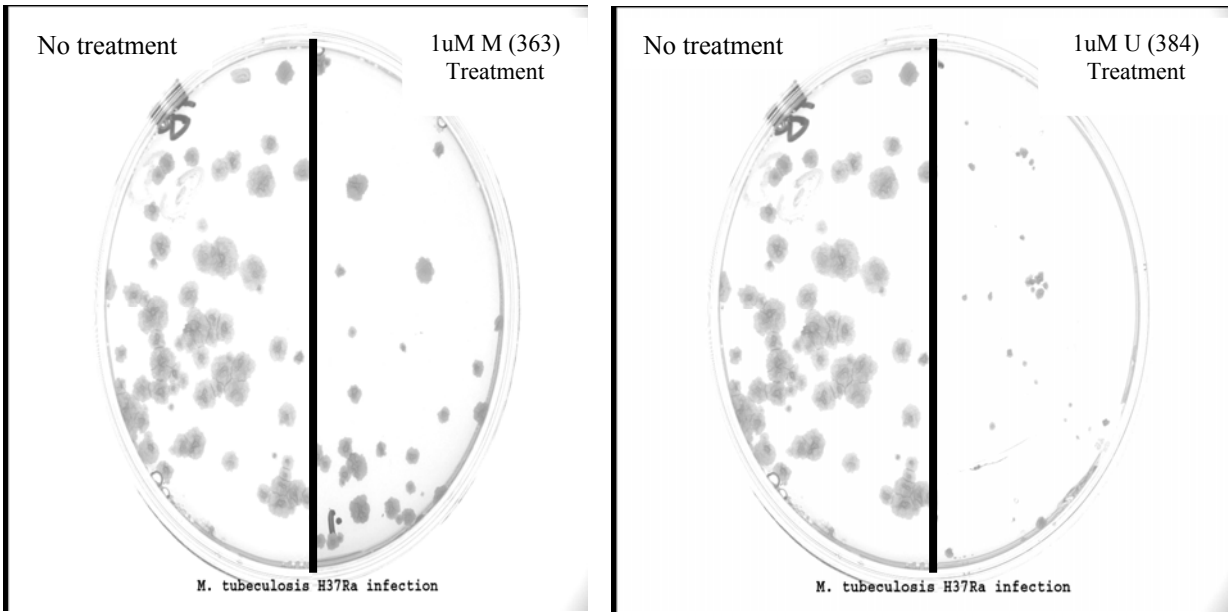
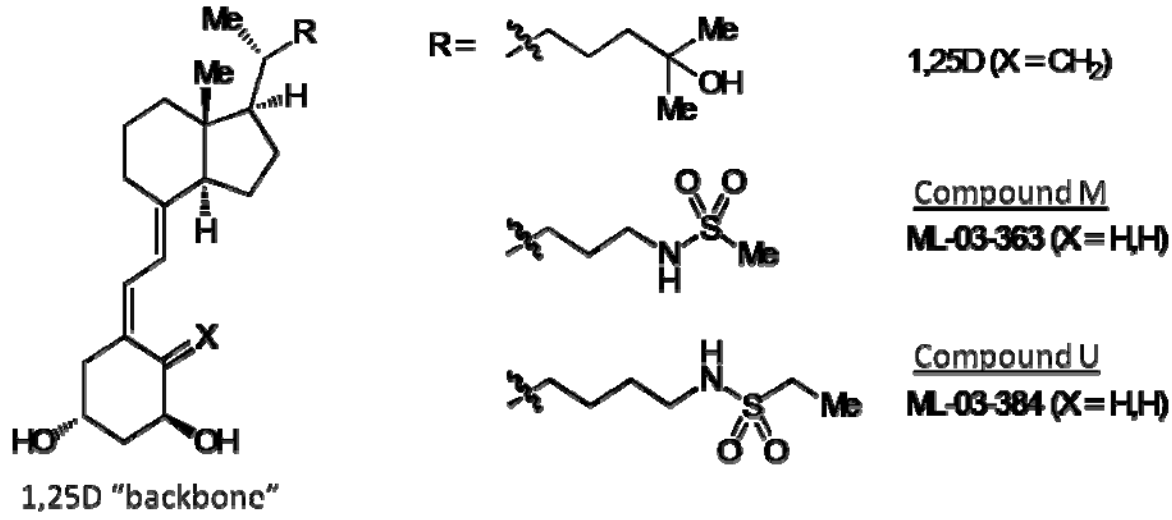


Fig. 22: Photos of M. tuberculosis H37Ra colonies grown on agar plates at 21 days after harvesting bacteria.

Molecular composition of select 1,25D analogues
Top: Side chain comparisons of compounds M & U
Bottom: Structures of compounds M, P, Q, & R



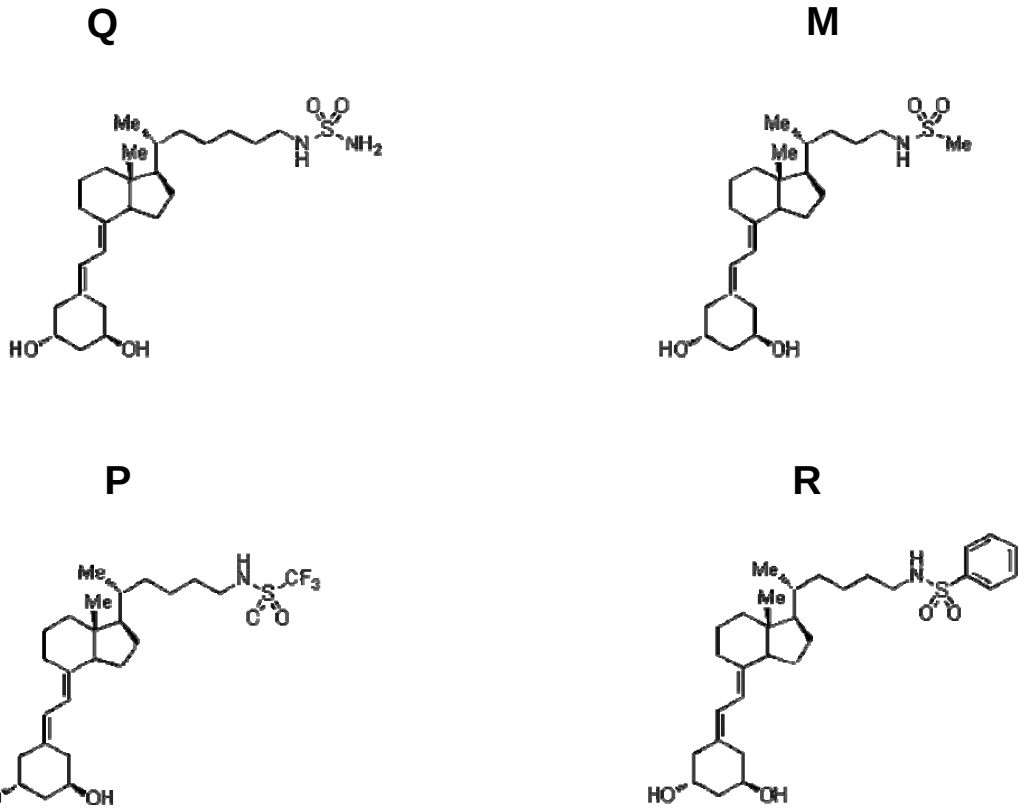


Fig. 23: (Top) Molecular comparison of compound M and compound U. (Bottom) Molecular structure of compounds Q, R, M, & P.

THP-1 (Human acute monocytic leukemia cell line)
CFU (10^4) assay – *Mycobacteria tuberculosis* (H37Ra) infection #3
72 hour sample with [1uM] treatment with compound M, or (D3 + butyrate)

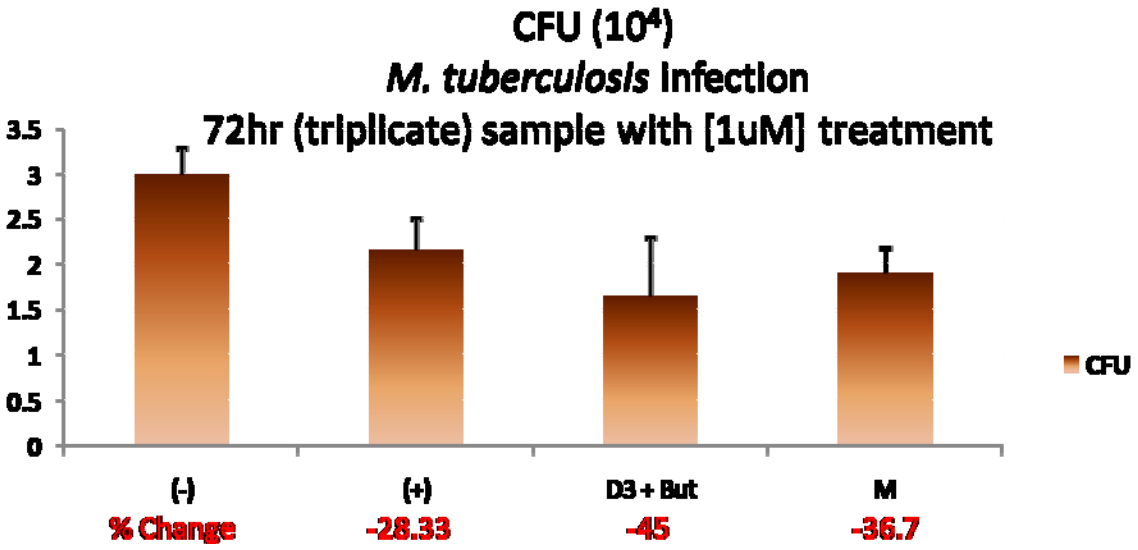


Fig. 24: CFU assay with M. tuberculosis. 72 hour [1uM] single-dose treatment with compound M or combined (D3 + butyrate).

THP-1 (Human acute monocytic leukemia cell line)
CFU (10^4) assay – *Mycobacteria tuberculosis* (H37Rv) infection
48 hour sample with [1uM] treatment with 1,25D or compound M
*Work thanks to Rob Kozak (M. Behr lab, McGill University)

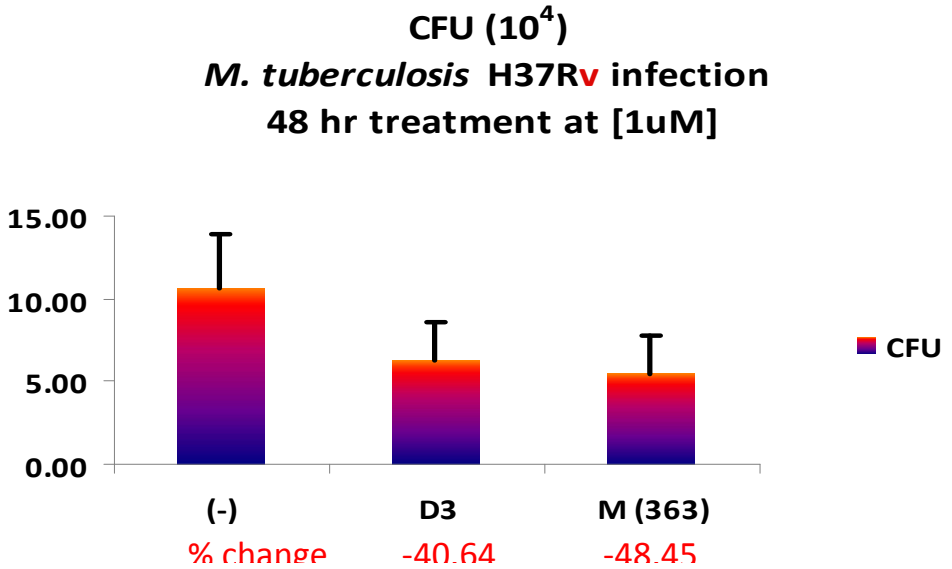


Fig. 25: CFU assay with M. tuberculosis strain H37Rv. 48 hour [1uM] single treatment with 1,25D or compound M.

THP-1 (Human acute monocytic leukemia cell line)
CFU (10^4) assay – *Mycobacteria tuberculosis* (H37Ra) infections #1 & 2
72 hour sample with 24 hour [1uM] pretreatment only

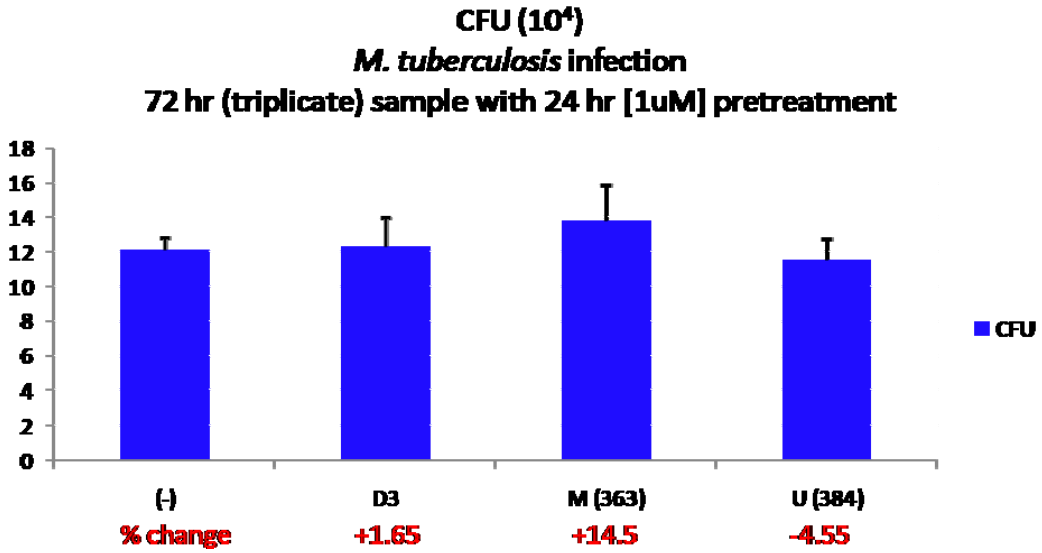


Fig. 26: Pretreatment and CFU assay #1 with M. tuberculosis strain H37Ra. Single 24 hour [1uM] pretreatment with 1,25D, compound M, or compound U.

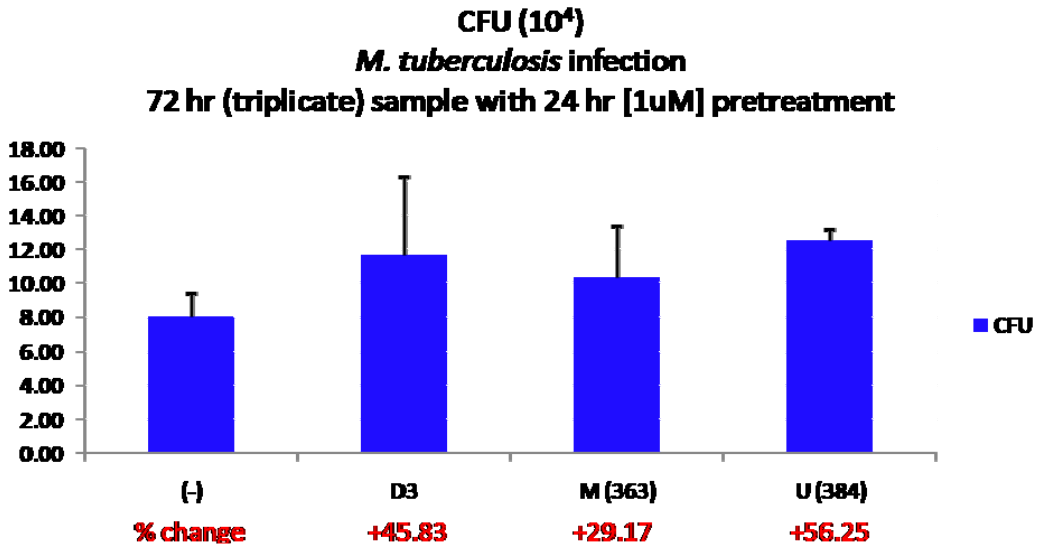


Fig. 27: Pretreatment and CFU assay #2 with M. tuberculosis strain H37Ra. Single 24 hour [1uM] pretreatment with 1,25D, compound M, or compound U.

Discussion

Analogues of 1,25D that stimulate innate immune responses *in vitro* can be successfully synthesized. These analogues possess various novel therapeutic properties, including affinity for the VDR, and vitamin D-like gene-regulation activity. The variability of these compounds to effectively stimulate specific VDR-regulated, innate immune mechanisms are attributed to the side-chain modifications intrinsic to each analogue. All compounds share a similar homologous molecular backbone, but each analogue contains a unique side-chain containing an HDI component.

Based upon our EC50 results from the fluorescence polarization assay, the initial group of developed analogues was tested at two predetermined concentrations for their ability to induce VDR-like gene expression activity. Our prior knowledge of physiological relevant 1,25D levels led to the selection of [100nM] and [1uM] of compound as the standard measurements for treatment. Notably, [1uM] of 1,25D is in excess of normal physiological serum concentrations—[75-80nM] is considered sufficient for circulating vitamin D levels [12]—however, it was selected as a standard measurement because we estimated effective concentrations of these analogues could be elevated when compared to 1,25D, with minimal associated calcemic side effects.

The selection of SCC25 cells for testing was due to our previous work with this cell line [60] and their sensitivity to treatment with 1,25D. Treatment at [1uM] showed a near universal induction of *CYP24* gene expression with the first

group of compounds tested (results not shown). By lowering the effective concentration of treatment to [100nM], differences between key VDR-regulated genes, including *CYP24, NOD2, and CAMP* became more visually apparent (Fig. 10). A closer examination of *CAMP* expression showed that combining 1,25D with [1mM] butyrate remained the only treatment sample that noted any significant promotion of antimicrobial peptide expression. Some of our analogues moderately induced *CAMP* in SCC25 cells, with compound R showing the most promise from all the analogues tested in this cell line.

Similarly, prior experience by our laboratory has also shown that normal human keratinocytes are highly sensitive to treatment to 1,25D [11]. In our assays conducted with keratinocytes, robust induction of *CYP24* was seen in nearly all the samples treated with [100nM] of compound or 1,25D (results not shown). However, little or no promotion of AMP expression was seen during the course of treatment. Analogue and control concentrations were then elevated to [1uM], with samples treated with (1,25D + butyrate) or compound R (Fig. 11) showing only modest promotion of the *CAMP* gene. Notably, assays conducted at [1uM] with compound R witnessed a morphological change, and lead to greater percentage of cell death, during three consecutive experiments. Reasons for any underlying responses in samples treated with compound R remain unclear, as the promotion of *CAMP* has been shown to suppress apoptosis in keratinocytes [124]. Furthermore, compound R did not induce any notable side effects in other cell lines tested, thus these results remain puzzling and require further investigation.

Observing only a modest promotion of antimicrobial peptide expression in SCC25 and normal human keratinocytes by our analogues, the decision was made to begin testing in various intestinal epithelial cell lines known for their sensitivity to 1,25D treatment. This lead to our selection of intestinal-derived cell lines HT-29 (results not shown) and SW480 cells for testing. Results from these cell lines showed little or no up-regulation in *DEFB2* expression, even when treated with high concentrations [1uM] of compound. The only samples that showed any significant promotion in *DEFB2* expression were treated with [mM] butyrate (Fig. 12). These results were somewhat expected, as they were consistent with work seen by Schauber *et al* [111].

After achieving fairly limited success at promoting AMP expression in epithelium-derived cell lines when treated with our analogues, testing began in the human monocytic leukemia cell line THP-1. THP-1 cells are a derivative of normal human monocytes, and are highly sensitive to treatment with 1,25D [60]. Additionally, exposure to phorbol-12-myristate-13-acetate (PMA) activates THP-1 monocytic differentiation into a macrophage-like lineage, prepping the cells for infection with *Mycobacterium tuberculosis* and providing an *in vitro* model for (intra)cellular responses.

In our testing with THP-1 cells, nearly half of the developed 1,25D analogues were eliminated from further analysis due to any lack of VDR binding activity, even at concentrations of [1uM] or greater. Of those compounds deemed efficacious at [1uM], testing at [100nM] continued to narrow the field down to four that appeared comparable to 1,25D in their ability to induce *CAMP*

expression (Fig. 15). qPCR analysis (n=1) confirmed these results, as well as illustrated expression levels of *CAMP* induced by these compounds relative to appropriate controls (Fig. 16). These four compounds were effectively known by their alphabetic nomenclature: Q, R, M, and P.

Induction of *CAMP* is considered the basis for TLR-induced antimicrobial activity in human macrophages [80]. Taking this into consideration, a set of CFU antimicrobial assays were utilized to test the effectiveness of these compounds at promoting VDR-stimulated immune responses. CFU assays have long been a standard to measure viable bacterial cell numbers/counts [54, 55]. The basic design of a CFU assays begins by first infecting macrophages with mycobacteria, and then treating with compound, it enables our assessment of THP-1 macrophages for clearance of intracellular microbes through stimulation of immune response.

Initial CFU testing began by exposing THP-1 cells to *Mycobacterium smegmatis*. Unlike other gram-negative bacteria such as *E. coli*, mycobacteria have notoriously slow replication times, which are measured in hours or even days, and not minutes. *M. smegmatis* was selected for these initial CFU assays because they are generally considered non-pathogenic, and possess relatively quick replication times of 4-6 hours per cycle. It should be mention that *M. smegmatis* is not the ideal pathogen for data collection in THP-1 infections due to their relatively infirm nature. However, working with this 'fast' growing mycobacterium was necessary to create and refine existing protocols [125] for infecting and treating THP-1 macrophages in a timely fashion. Turnaround time

for CFU antibacterial assays conducted utilizing *M. smegmatis* was approximately 1 week.

While our gene expression data showed that compounds Q, R, M, and P were effective at inducing AMP expression in THP-1 cells, treatment with [100nM] yielded no significant reduction in mycobacteria viability during the initial assays. The decision was then made to increase treatment ten-fold to [1uM] in order to try and stimulate a more robust response. At [1uM], compounds M and R both exhibited roughly 60% reductions in viable *M. smegmatis* cell counts when compared to the untreated sample (Fig. 18). These results indicated some promise from our newly developed analogues; however, they could not be considered statistically significant due to the lack of viable positive control during the course of our experiments.

M. smegmatis is generally not considered a robust microorganism, and treatment with 1,25D from any of our CFU initial assays failed to achieve the expected reduction in microbial viability or clearance through stimulation innate immune pathways in THP-1 cells. These results remained puzzling for some time, but troubleshooting would later reveal that at least one aliquot of 1,25D obtained from the manufacturer was found to be inferior in quality. Notably, other members of our laboratory team found drastic differences in 1,25D quality obtained from different manufacturers, but these results would not be confirmed until several round of CFU assays had already been conducted. After conferring with other members of our team, measures were taken to ensure the consistency and quality of 1,25D to be used with all future experiments.

After establishing protocol guidelines through our *M. smegmatis* experiments, CFU assays were then conducted utilizing *Mycobacterium tuberculosis* strain H37Ra. Notably, *M. tuberculosis* possesses a slower replication time of approximately twenty hours. Hence, these assays, conducted from start to finish, take roughly one month to complete.

The initial CFU assay conducted with *M. tuberculosis* strain H37Ra showed some remarkable and promising results. Results measured the average colony numbers taken from triplicate samples, and showed that treatment with select analogues were at least as effective as 1,25D in stimulating immune response(s) to Mtb infection. Moreover, all three samples treated with compound M stood prominent from others not only because of a significantly greater reduction in colony counts, but the bacteria colony sizes grown on agar plates were noticeably smaller when compared to control samples (Figs 19, 20). These results were intriguing in that compound M may possibly simulate alternative immune response pathways other than the promotion of AMP expression.

After obtaining results from the initial experiment with H37Ra, a closer examination of the side-chain structure of compound M was assessed to better understand this increase seen in antibacterial activity. Compound M contains a sulfonamide group within its side-chain configuration (Fig. 23). Sulfonamides are found in a class of antibiotics with broad-spectrum antibacterial activity. Moreover, sulfonamide antibiotics are generally considered bacteriostatic, and not bactericidal through their mechanism of action [126]. Hence, this raised the possibility that the sulfonamide group of compound M may have antimicrobial

activity, leading to the temporary arrest of H37Ra, and the smaller colonies that were visualized. However, a closer examination of the literature revealed that *M. tuberculosis* is resistant to treatment with sulfonamide antibiotics [126]. Additionally, when consulting with the Dr. Jim Gleason (the chemist and designer of these analogues), the molecular composition contained in side-chain of compound M lacks the substitutions necessary for function of sulfonamide antibiotics.

With no clear understanding of why compound M proved to be efficacious at inducing immune responses in THP-1 macrophages, several additional experiments were designed. First, a molecular comparison of all analogues was carried out for any noticeable similarities in structure. Compound U was found to be akin to compound M in molecular structure (Fig. 23). Based on this information an additional CFU assay was performed, with compound U substituted for compound Q. However, results from this second CFU assay were not entirely conclusive (Fig. 21). Initial results showed a reduction in Mtb viability from macrophages stimulated with M (45%) or U (51%; and significant compared to the no treatment sample) but our positive control (1,25D) did not show any reduction in bacterial viability. These results were puzzling, and inconclusive with those seen from the first experiment. Additionally, the results were inconsistent with our prior knowledge of 1,25D-stimulated immune response to Mtb infection [80]. Further testing and RT-PCR analysis (results not shown) conducted in THP-1 cells revealed that the vitamin D treated sample was rendered ineffective due to the poor quality of 1,25D obtained for use during the course of

this experiment. This information would also help explain the lack of productive results seen in prior experiments.

Although CFU reduction and colony count numbers were inconclusive for the second assay due to a lack of positive control, a comparison of Mtb colonies harvested from THP-1 macrophages treated with compounds M or U shows a similar reduction individual colony sizes when compared to our control samples (Fig. 22). These results closely resembled those from the first experiment (Fig. 20), and suggested that THP-1 stimulation by either compound M or U activated unknown immune response pathways, potentially leaving the mycobacterium in an altered metabolic state upon initial harvesting. Moreover, similar findings were seen from the third CFU assay conducted with H37Ra (results not shown); but these results would require further analysis.

Taking into consideration that a number of intracellular pathways drive mycobacterial cell growth and replication, additional testing with compounds M and U are suggested. A logical first step for further analysis would begin by conducting similar CFU-driven experiments with multiple treatments of compound administered at 24hr time points. It is also worth taking a closer examination of gene expression levels of the mycobacteria after phagocytotic uptake by THP-1 cells, and 72 hours of treatment(s) with compound. However, bacterial gene expression analysis would require ramping up large-scale experiments to isolate adequate levels of microbial RNA. Furthermore, extensive analysis into the genome in M. tuberculosis, with a focus on known markers of cellular replication, would also be required.

In parallel with our second H37Ra CFU assay, aliquots of 1,25D and compound M were donated to Rob Kozak of Dr. Marcel Behr's laboratory for testing with *M. tuberculosis* strain H37Rv under biosafety level 3 precautions. Tuberculosis strains H37Ra and H37Rv are highly genetically equivalent. However, minor genetic differences between these two strains of the pathogen has lead to increased resistance of the virulent strain to anti-tuberculosis chemotherapy, among many other adaptations [127]. Accordingly, strain H37Ra is considered both useful and fairly safe to work with when initially exploring anti-tuberculosis therapies, but strain H37Rv remains the standard for advancing research.

Testing with *M. tuberculosis* strain H37Rv showed some encouraging results (Fig. 26) from treatment with compound M. From the one experiment preformed, results show roughly a 40% reduction with 1,25D treatment, and roughly a 50% in pathogen viability with compound M treatment. These results were similar to those achieved with H37Ra experiments; however, they do not yield statistically significant values as a measure of standard deviation between any of the treatment samples. Nonetheless, this initial assay still shows some measure of promise, and is worthy of continued investigation.

After examining all previous results a final experiment was needed to clarify whether these analogues were stimulating THP-1 macrophage immune response pathways, or if the compounds had any direct effect to the mycobacteria themselves. For these experiments *M. tuberculosis* strain H37Ra was grown in culture media treated with [1uM] vehicle (DMSO), 1,25D, compound M, or

compound U. After 24 hours samples were assessed by optical density (OD) measurement for equivalent bacterial cell counts, and CFU assays were performed without any additional treatment added to the incubation media. Results from the pretreatment assays suggests that compounds have little or no effect directly on the mycobacterium (Figs 26, 27), and any reductions in mycobacteria viability are more likely due to the stimulation of immune responses in THP-1 macrophages. Additionally, colony sizes between all treatments were observed to be nearly equivalent from samples (results not shown), further lending credibility to the hypothesis that compounds M and U activate unknown/alternative immune response pathways in THP-1 cells. If metabolic pathways of the mycobacterium were affected directly by any intrinsic antibacterial effects these compounds possessed, this pretreatment assay could serve as one possible control to help eliminate such a hypothesis.

Concluding Remarks

Looking back on these results there are three major questions worth asking. (I) Can analogues of 1,25D be developed that stimulate immune responses, i.e. the induction of AMPs? (II) Do any of these analogues stimulate antimicrobial peptide expression to a greater extent than 1,25D? (III) Do any of these analogues show greater promise at promoting immune response(s) in THP-1 cells to eradicate intracellular *Mycobacterium tuberculosis* than 1,25D?

The answer to our first question was a resounding, yes. *In vitro* evidence in multiple cell lines shows that analogues of 1,25D can be developed to induce antimicrobial peptide expression. Since AMPs serve as vanguards of innate immune response, development of these compounds as an alternative or complimentary treatment to pathogenic infection is worthy of our investigation.

The answer to the second question remains as a possibility. As seen from some of our gene expression assays (Figs. 15, 16), induction of AMPs by these compounds was seen to be nearly equivalent, or greater than 1,25D, in THP-1 cells treated with similar concentrations of compound. These results show some promise; however, work needs to continue to develop compounds that induce AMP expression to a greater extent than 1,25D. Further refining our search for highly effective treatments to tuberculosis infection requires that samples using D3 + butyrate remains the threshold to which all compounds should be measured against. Moreover, by taking a closer examination of the compounds that have shown the most promise (M, U), while creating alternate molecular forms containing similarities in side chain structure, we can continue testing for 1,25D-

like induction of immune response in THP-1 cells. This process of compound design and development increases our odds of finding select analogues that safely and effective modulates immune response to infection.

The answer to the third question also remains as a possibility. While nothing completely conclusive may be drawn from these results, our research suggest a select few of these compounds (notably M and U) are certainly worthy of further investigation. Admittedly, many questions remain. For example, it remains unclear why there is a reduction in colony sizes seen in sample treated with M and U compared to vehicle or 1,25D. However, these results were seen in all the CFU experiments conducted with *M. tuberculosis* strain H37Ra. Furthermore, a greater reduction in the percentage of Mtb viability (when compared with 1,25D) was seen by these two compounds during several of our CFU assays. These results may possibly indicate something profound, and only further testing with continued refinement of these analogues can eliminate ambiguities, as well as help address some of the issues encountered during conduction of this work.

This body of research suggest that development of 1,25D analogues is a worthwhile pursuit, especially when taking into consideration that 1,25D analogues can be combined with existing treatments to create more effective therapies for treating infection, while limiting potential calcemic side effects associated with 1,25D.

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