EXPRESSION AND MODULATION OF THE UGT1A FAMILY OF PHASE II METABOLISM GENES VIA LIGANDED VITAMIN D RECEPTOR

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Research conducted within

The School of Biomedical Science

Faculty of Life and Health Sciences

Ulster University

A Thesis submitted for the degree of

Doctor of Philosophy

September 2018

I confirm that the word count of this thesis is less than 100,000 words

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Acknowledgements

I would like to thank the following people:

Dr Paul Thompson, Dr Patricia Rodriguez and Dr Declan McKenna for all their time, support and patience throughout my PhD studies.

I would also like to acknowledge my Mum, Dad and Sister, of whom I am eternally grateful and would like to take this opportunity to thank; because without their consistent encourage and support I would never have seen the light at the end of the tunnel.

Abstract

Phase two metabolic genes are primary targets for a range of nuclear receptors within the body. These include but are not limited to PXR, FXR, and LXR all of which create a complex network of crosstalk pathways between members to establish and maintain efficient sensing, resulting ultimately in the elimination of potentially harmful endogenous/exogenous toxin. The Vitamin D Receptor (VDR) is a member of the nuclear receptor super family and is activated in a ligand dependent manner. VDRs neoclassical potential has been well established in transactivation roles within phase one and three metabolic gene families such as Cytochrome P450 members, and ATP-binding Cassette transporters, however very little is understood of VDRs prospective role within phase two metabolism.

Uridine 5'-diphospho-glucuronosyltransferase (UGT) are one of the major phase two metabolic gene families and are highly expressed within the liver, lower intestinal and kidney to name a few. The extra hepatic activity of UGT members' may now have an influence on the body's pharmacokinetic potential (Nakamura et al., 2008b). tract chief among which is *UGT1A1*. *UGT1A1* is involved in the glucuronidation of endogenous and exogenous compounds including steroids, chemotherapeutics such as Irinotecan used to treat colon cancer and small cell lung cancer, and bile acids, generating more hydrophilic metabolites. In many cancerous disease states including liver and biliary, *UGT1A* expression is down regulated leading to impaired metabolic functioning (Strassburg et al., 1997). This is also relevant in genetic diseases such as Gilbert's Syndrome and Crigler-Najjar Syndrome.

VDRs role in regulating UGTs was assessed through qRT-PCR analysis, exposing LS180 (colon), and HepG2 (liver) respectively to a range of VDR agonists, using

known regulators of UGT as positive controls. Further investigation was undertaken to identify the VDRE within the promoter of *UGT1A1* and assessing its functionality as a nuclear receptor motif through site directed mutagenesis. It was noted that the isolated VDRE also is the binding motif for PXR therefore the loss of this motif resulted in the loss of both VDR and PXR as a regulator of *UGT1A1*.

To conclude we have shown through luciferase activity that VDR has the potential to positively regulate *UGT1A1* to a comparable level of already known nuclear receptor regulator PXR. This could lead to the potential regulation of *UGT1A1* through diet supplementation of vitamin D using both natural and synthetic sources, in a range of inflammatory and genetic disease states allowing for a potential therapeutic to be established.

Abbreviations

ABC	ATP Binding Cassette
ATP	Adenosine Triphosphate
ATCC	American Tissue Culture Collection
bp	Base Pair
BSA	Bovine Serum Albumin
CA	Cholic Acid
CAR	Constitutive Androstane Receptor
CDCA	Chenodeoxycholic Acid
cDNA	Complementary Deoxyribonucleic Acid
CoA	Co-Activator
CtBP	C-terminal binding protein
DBD	DNA Binding Domain
DCA	Deoxycholic Acid
DMEM	Dulbecco's Modified Eagles Media
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
dNTP	2' deoxynucleoside 5'-triphosphate
DTT	Dithiothreitol

ECACC	European Collection of Cell Cultures
EDTA	Ethylenediamine Tetraacetic Acid
EGFR	Epithelial Growth Factor Receptor
ER	Estrogen Receptor
EtBr	Ethidium Bromide
FBS	Foetal Bovine Serum
FXR	Farnesoid X Receptor
g	Gram
GIT	Gastrointestinal Tract
GR	Glucocorticoid Receptor
HAT	Histone Acetyltransferase
HDAC	Histone Deacetyltransferase
HEPES	N-Cyclohexyl-2-aminoethanesulfonic acid
IFN	Interferon
IL	Interleukin
kb	Kilobase
LBD	Ligand Binding Domain
LCA	Lithocholic Acid
LXR	Liver X Receptor

MDR	Multi Drug Resistance
MEM	Modified Eagle's Media
mg	Milligram
min	Minute
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
MRP	Multi Drug Resistance Protein
NaCl	Sodium Chloride
nM	Nanomolar
NR	Nuclear Receptor
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
P-gp	P-glycoprotein
PPAR	Peroxisome Proliferator-Activated Receptor
PTH	Parathyroid Hormone
PXR	Pregnane X Receptor
RAR	Retinoid X Receptor

RNA	Ribonucleic Acid
rpm	Revolutions per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RXR	Retinoid X Receptor
SDS	Sodium Dodecyl Sulphate
SENP	Sentrin Specific Protease
SNP	Single Nucleotide Polymorphism
SULT	Sulfotransferase
TBS	Tris-Buffered Saline
TEMED	N,N,N',N'-Tetramethylethylenediamine
TR	Thyroid Receptor
UGT	Uridine 5'-diphospho-glucuronosyltransferase
US	United States
UVB	Ultraviolet Light B
V	Volts
VEGF	Vascular Endothelial Growth Factor
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element
µg	Microgram

μm	Micrometer
μM	Micromolar
μl	Microlitre
%	Percentage

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Chapter 1: General Introduction

1.1 Vitamin D

Vitamin D is a fat-soluble hormone that is synthesized through the skin when exposed to UVB radiation generated from sunlight exposure (Kulie et al., 2009). The pre hormone, once synthesized in the skin requires two metabolic transformations. The active hormone produced is $1,25(OH)_2D_3$, binds to the cognate Vitamin D Receptor (VDR) modulating the genetic expression of a number of biological networks. The established 'classical' role of vitamin D encompasses maintenance of calcium and phosphate homeostasis to ensure bone mineralization. In addition, vitamin D has been shown to play a fundamental roles in cellular differentiation, immune function, and apoptotic pathways (Aranow, 2011). Vitamin D deficiency has been well studied and documented and as such has been linked with a number of disease phenotypes with the most established in a clinical context being the risk of developing rickets in children or osteomalacia in adults (Sahay and Sahay, 2012). Furthermore vitamin D deficiency has been associated with an increased risk of developing certain cancers, auto immune diseases, type one and two diabetes, hypertension and heart disease (Ke et al., 2015).

1.2 Vitamin D biosynthesis, calcium/phosphate homeostasis

7-Dehydrocholesterol is a cholesterol precursor and undergoes an ordered sequence of metabolic alterations to form the active metabolite $1,25(OH)_2D_3$, also known as calcitriol (Wacker and Holick, 2013). It is the presence of this precursor in human skin that eventually enables the generation of $1,25(OH)_2D_3$ from exposure to ultraviolet in sunlight, initially generating an intermediary metabolite, cholecaliferol, before further down stream metabolic activities within the liver and kidney to generate the final and physiologically active form of vitamin D (see figure 1.1).

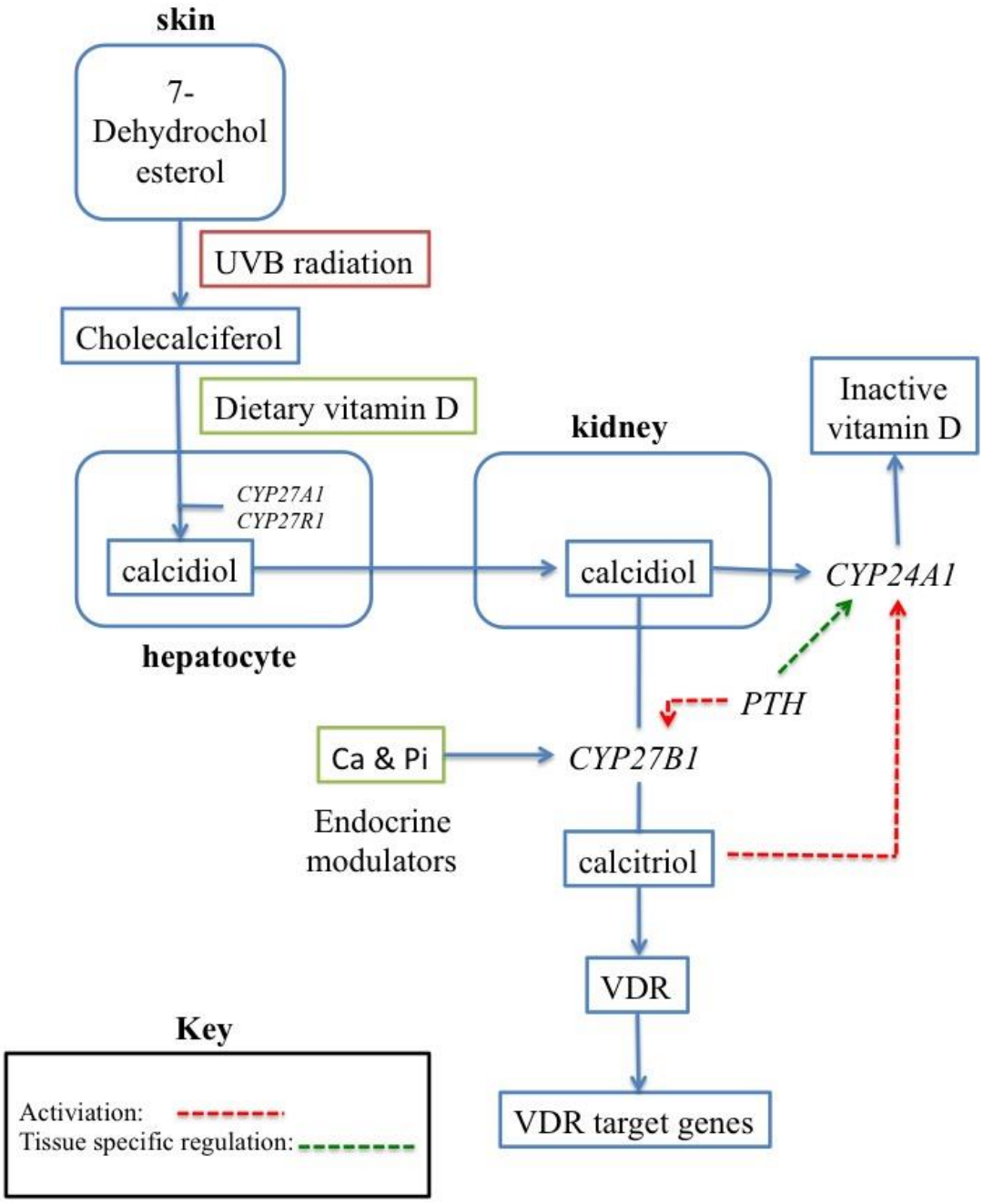


Figure 1.1: Overview detailing the biosynthesis of the active metabolite of vitamin D through UVB radiation and subsequent metabolic turn over.

Photochemical synthesis of the intermediary metabolite occurs within the cutaneous layers of the skin were 7-dehydrocholesterol is converted to cholecaliferol on exposure to UVB radiation. It is this metabolite that is found in the diet and can be used to fortify a range of foodstuffs ensuring adequate levels are present to reduce the risks of developing vitamin D deficiencies. Almost 100% of circulating vitamin D metabolites are bound to a vitamin D binding protein (DBP) which can act as a reservoir of vitamin D that under the right conditions can release ligand to stimulate VDR mediated biological responses. DBP works in this way to avoid unwanted toxicity of vitamin D accumulation through controlling its access to target tissues.

Once cholecaliferol has been formed it is hydroxylated in the liver at carbon 25 to form $25(OH)D_3$ or Calcidiol. This process of hydroxylation is carried out through the actions of the cytochrome P450 super family of enzymes that include *CYP27A1*. $25(OH)D_3$ is the major metabolite associated with studies of vitamin D status (Holick, 2003). The action of 1-α hydroxylase located within the kidney is primarily controlled through the endocrine actions of parathyroid hormone (PTH), calcium and phosphate levels, and fibroblast growth factor 23 (FGF23). Biologically active $1,25(OH)_2D_3$ generates a feedback loop inhibiting the production and secretion of PTH (Khundmiri et al., 2016). FGF23 on the other hand inhibits renal transformation of vitamin D through inhibition of the 1-α hydroxylase pathway while simultaneously increasing the expression and activity of 24-α hydroxylase resulting in the generation of $24,25(OH)_2D$, an inactive form of vitamin D.

The importance of vitamin D to bone health has been well studied since the 1930's with the maintenance of calcium and phosphate homeostasis to ensure adequate bone

matrix formation remaining the fundamental and classical function of vitamin D. The trans-cellular uptake of calcium is highly controlled through VDR medicated activity orchestrating the expression of calcium uptake genes such as members of the transient receptor potential cation channel sub family; *TRPV6* which highly expressed within the duodenum of the intestinal tract and enables the entry of calcium into enterocytes.

1.3 Vitamin D deficiency, defined

The definition of vitamin D deficiency is a topic of ongoing debate. The World Health Organisation (WHO) defines vitamin D insufficiency as serum levels lower than 20ng/ml. The National Institute for Health and Care Excellence (NICE) guidelines state that levels lower than 10ng/ml qualify as deficient.

The diagnosis of low vitamin D levels can be achieved through assessing the levels of circulating 25-hydroxyvitamin D within the blood. Although there are many differences of opinion of what constitutes vitamin D deficiency vs. insufficiency most authorities agree that levels lower than 20ng/ml (50nmol/L) is suboptimal for skeletal human health.

In 2016 the European Food Safety Authority (EFSA) derived dietary reference values for vitamin D. It is now considered that the serum 25(OH)D concentration, reflecting both the dietary and cutaneous derived sources to be a valid biomarkers of vitamin D status children and adults. It is therefore considered that a serum concentration of 50nmol/L is a suitable target through the acquisition of 15μg/day based on meta regression analysis studies.

1.4 Extra-renal production of $1,25D_3$

$1,25(OH)_2D_3$ can be synthesized outside those organs classically associated with its production and biotransformation. In these instances the active vitamin D metabolite is being synthesized in a PTH independent fashion and can only elicit an affect on the tissue that synthesized it. In other words the $1,25(OH)_2D_3$ synthesis outside of the kidneys does not circulate away from its site of production. PTH- independent extra-renal synthesis of $1,25(OH)_2D_3$ from 25(OH)D has been witnessed in activated macrophages in the lung and lymph nodes due to the expression of 1- α hydroxylase activity (Zhang et al., 2012). Extra renal production of $1,25(OH)_2D_3$ as in keratinocytes and macrophages is under different control, being stimulated primarily by cytokines such as tumour necrosis factor alpha (TNFa) and interferon gamma (IFNg) (Gil et al., 2018).

However the most well recognized extra renal expression of 1- α hydroxylase remains that of patients suffering from granulomatous, (inherited primary immune deficiency disease (PID)). Through the massive localization of immune cells, physiologically active $1,25(OH)_2D_3$ can spill into the peripheral circulation. Therefore it is this evidence bolstered with the 1- α hydroxylase expression in other extra renal sites that has prompted the idea that auto and paracrine synthesis of $1,25(OH)_2D_3$ can contribute to normal physiological functioning, with a key role appearing to be within innate and acquired immune responses (Hewison et al., 2007).

1.5 Pathophysiology of vitamin D deficiency

$1,25(OH)_2D_3$ opens calcium channels within the gut, stimulates the formation of calcium binding proteins in intestinal cells facilitating the uptake of calcium and phosphate from the gut. The action of bone mineralization is a passive process,

occurring once sufficient calcium and vitamin D is available. In a vitamin D deficient state, a less than optimal amount of vitamin D is available; as a result less calcium will be available for bone mineralization. This results in a cascade of endocrine reactions, ultimately leading to an increase in parathyroid hormone (PTH) production, stimulating the hydroxylation of 25(OH)D by the kidney in an effort to re establish adequate circulating levels of active vitamin D, however the increase in serum PTH results in increase bone turn over and eventual bone loss as a result due to the hypocalcaemic state in the body's attempts to re establish a normocalcemic environment. If chronic vitamin D deficiency occurs bone loss can be increased leading to the onset and development of related disease states with an increasing chance of bone fracture predominantly witnessed in the hip, vertebrae and forearms (Hill and Aspray, 2017) (figure 1.2).

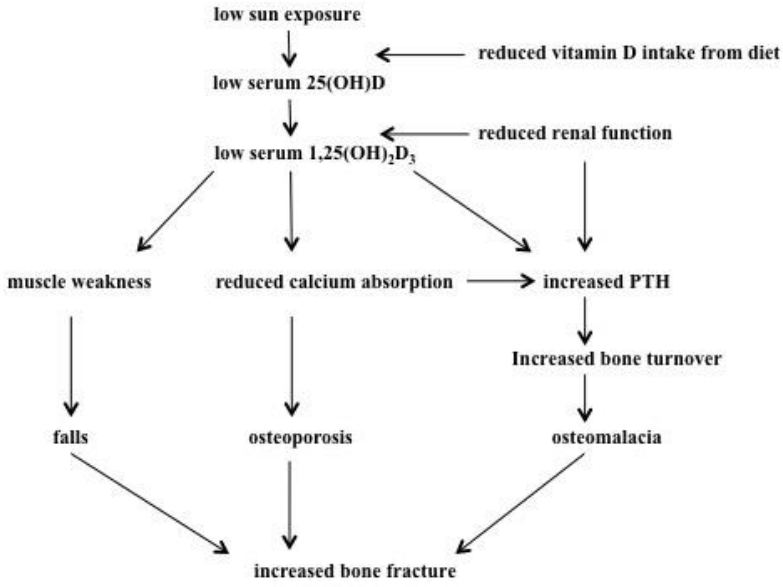


Figure 1.2: The pathophysiological pathways from vitamin D deficiency to osteoporosis, osteomalacia, resulting in falls and fractures, adapted from (Lips and van Schoor, 2011).

1.6 Vitamin D & rickets

Rickets is most commonly witnessed in children and like osteomalacia develops due to a chronic deficiency of active vitamin D in conjunction with hypocalcaemia or hypophosphatemia. Both rickets and osteomalacia share many similarities yet they have distinct differences; in rickets there is deficient mineralization at the growth plate whereas osteomalacia has a deficiency of bone mineralization within the matrix structure. The pathogenesis of rickets occurs within the growth plate of the bone, were there is a build up of osteoid, usually mineralized through calcium salts, however in osteomalacia the mineralization defect leads to a build up of osteoid below the growth plate resulting in bone weakness and eventual bowing in the major load bearing bones (Ozono, 2016). A number of population-based studies have been carried out to assess the prevalence of vitamin D insufficiency amongst children in Great Britain. A number of these studies have concluded that the diagnosis vitamin D deficiency has markedly increased over the last decade in the United Kingdom (Basatemur et al., 2017). The associated higher risk of Vitamin D insufficiency) VDI was witnessed in those children who exercised less outside, were overweight and watched more TV, this also highlighting potential modifiable risk factors that could be used to reduce the risk of developing complete vitamin D deficiency (Absoud et al., 2011).

1.7 Vitamin D, beyond bone health

Vitamin D has long been known and understood to be a requirement for overall health that is highly evidenced through many diseases phenotypes associated with a reduction in vitamin D status and activity. An appreciation for how vitamin D achieves its range of biological effects requires an understanding of the molecular mechanisms employed by VDR. (Christakos et al., 2013). Vitamin D has been the focus of a number of epidemiological studies that have made a direct correlation with a number of cancerous states including prostate and breast cancer (Moukayed and Grant, 2013). Those areas with increased natural sunlight exposure have a reduced incidence rate of prostate and breast cancers, due the overall increase in vitamin D production within these populations (Zhalehjoo et al., 2016). Furthermore VDR has been associated with other esoteric effects such as immune response, cellular differentiation (Eyles et al., 2005). Many tissues have been found to express VDR to varying degrees. It is this broad affinity of expression across many tissue types that have led much research into the additional roles VDR may play outside that of bone health and structure.

1.7.1 Immune response

Vitamin D is now regarded by many as an immune modulator due to its defined molecular effects within the innate and adaptive immune responses. A number of studies have highlighted how impaired vitamin D status has important effects on the immune response to infection and inflammation associated activities, linked with autoimmune disorders (Agmon-Levin et al., 2013).

VDR expression has been witnessed in many cells within the immune system including monocytes, dendritic cells and B/T-lymphocytes. The interaction between

vitamin D and immune cells indicates that these cells are no longer constrained to the effects mediated through the endocrine actions. A subclass of the T cell super family known as helper T cells, express VDR lending more credence to VDR's influence within the immune system (Cantorna et al., 2015). These cells are involved in antigen presentation as well as the production of many factors involved in overall immune response including interleukins two and five (Il-2, IL-5), and tumour necrosis factor (TNF) allowing for the potential influences that VDR could have spread across many pathways within immune regulation. Vitamin D has been observed to reduce the amount of interleukins produced by the helper T cells controlling cellular proliferation (Cantorna et al., 2015). Furthermore vitamin D has been seen to be involved in a number of autoimmune disorders, in such conditions as rheumatoid arthritis and inflammatory bowel disease. Those individuals who have reduced vitamin D status have significantly more aggressive phenotypes (Kempinska-Podhorodecka et al., 2017).

Macrophages, another key member of the immune response have been deemed an extra renal source of the enzymatic activity of 1-alpha-hydroxylase, giving these cells the ability to produce biologically active $1,25(OH)_2D_3$ acting upon T and/or B cells impacting immune regulation. For example studies of innate immunity have shown intracrine induction of antimicrobial events through vitamin D as a pivotal requirement for macrophages response to infection (Hewison, 2010). Renal enzyme activity is predominantly under the homeostatic control of PTH and $1,25(OH)_2D_3$ itself whereas immune cell production of the active metabolite seems to be beyond the control of PTH.

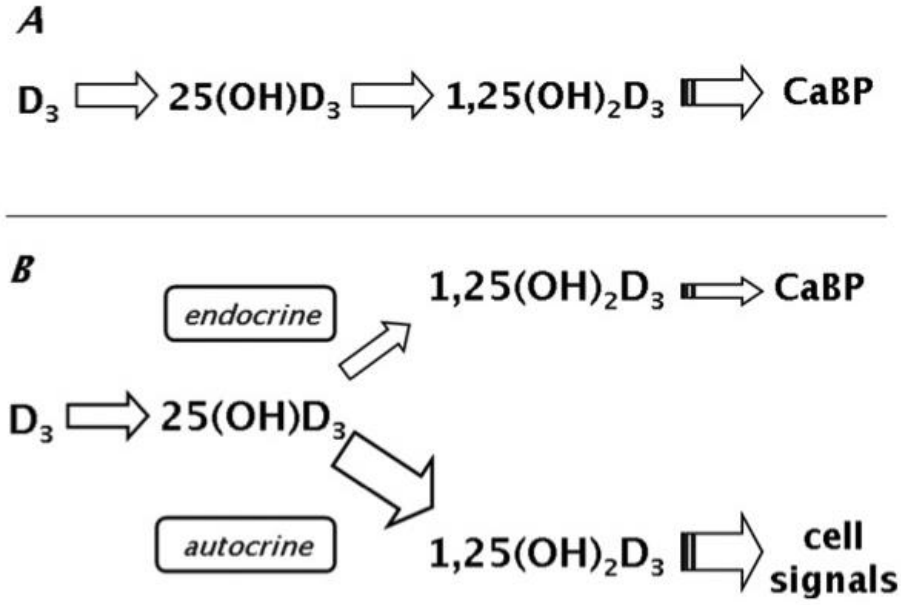


Figure 1.3: (A) Indicates the scheme by which vitamin D exerts its effect on the body BEFORE 1-alpha-hydroylase was recognized outside of the kidney. (B) Shows the incorporation of extra renal hydroxylation (Heaney, 2008).

It has become clear in recent years that many tissues involved in the autocrine systems have the ability to synthesis $1,25(OH)_2D_3$. This ability to synthesis $1,25(OH)_2D_3$ serves as a key link in signaling pathways that connects extracellular stimuli to downstream genomic responses. When target tissues and cells are exposed to signals requiring genomic action to produce a protein in response, the presence of intracellular vitamin D facilitates the efficient transcriptional responses. Without the presence of vitamin D the ability of many cells to respond to pathologic and physiologic signals is effected. An example of which is the ductal epithelium of breast tissue that requires vitamin D to respond to cyclic variations in estrogen and progesterone (Zinser et al., 2002). Further to this many of the epithelial structure in

the body use vitamin D to signal down stream transcriptional events that regulate cellular differentiation, proliferation and apoptosis (Heaney, 2008).

Vitamin D plays an essential function in the innate antimicrobial immune response leading to a cascade of downstream antimicrobial mechanisms to defend against invading pathogens. A major pathway in humans is the activation of Toll-like receptor (TLR) superfamily. TLR 1 and 2 binding leads to increased downstream binding of VDR mediated through vitamin D dependent induction of antimicrobial proteins. This culminates in giving $1,25(OH)_2D_3$ the ability to bind VDR forming the associated complex of $1,25D_3$:VDR: Retinoid X Receptor (from this point referred to as RXR) which is a heterodimeric partner of many nuclear receptor family members including VDR, allowing for the direct association and subsequent binding of DNA response elements within target genes such genes as cathelocidin and beta defensin 4 (Liu et al., 2006b), (Lykouras et al., 2016). Interleukins have also been shown to play important roles within innate antimicrobial response. Interleukins (IL) are secreted proteins known as cytokines that are signaling molecules produced by CD4 lymphocytes, macrophages, monocytes and endothelial cells. IL-14 and 15 are of particular interest as these cytokines are able to induce the expression of *CYP27B1* allowing the facilitated conversion of 25-hydroxy-vitamin D3 to its biologically active state leading to VDR activation of genes such as cathelicidin (Krutzik et al., 2008).

Cathelicidin is an antimicrobial peptide found within the lysosomes of macrophages and plays a critical role in the defense against bacterial infections. Cathelicidin peptides were originally discovered in neutrophils but have since been found to be expressed in a number of different cell types within the innate immune system (Liu et al., 2006a).

1.8 Vitamin D, a 21st century perspective

The capacity of vitamin D to impact upon biological processes that extend beyond bone health has motivated much research as to its molecular function and interactions with other signaling pathways. Vitamin D is known to effect the regulation of a number of signaling pathways including those involved in insulin production (Alvarez and Ashraf, 2010), tumour necrosis factor alpha (TNF-alpha) synthesis and cytokine expression (Yu-Ting et al., 2010). Furthermore having the ability to regulate processes within the cell cycle, apoptosis, actin remodeling and cell adhesion lends credence to the integral role vitamin D may play in overall genome stability (Berridge, 2015). It has been hypothesized that vitamin D has anti aging effects having the ability to influence the length of telomeres within cells and has been linked lower associated risks of developing a number of chronic disease states (Mazidi et al., 2017).

Supporting this are the functional interactions that have been defined between vitamin D, VDR and alpha Klotho hereafter referred to as Klotho and FGF23 as well as Nrf2 pathways.

Fibroblast growth factor 23 or FGF23 was initially characterized in 2000 and is a member of the fibroblast family of genes which are responsible for phosphate and vitamin D metabolism and is readily secreted by osteocytes in response to elevated levels of $1,25(OH)_2D_3$. It has been shown to have a direct influence on vitamin D levels within the body through feedback mechanisms controlling the regulation of renal 1-alpha-hydroxylase, ultimately effecting calcium absorption within the body (Christakos et al., 2015). FGF23 was originally defined as a phosphaturic bone-derived hormone, however recent advances have shed light on major functions of

FGF23 and Klotho signaling in renal tissue, heart, and bone. It is now apparent that FGF23 is understood to be a pleiotropic endocrine and auto-/paracrine factor not only involved in phosphate homeostasis, but also in calcium and sodium metabolism and homeostasis. Furthermore additional associations have been made with regards bone mineralization as well as in the development of cardiac hypertrophy.

Klotho is a transmembrane protein and discovered originally as an anti aging factor (Kuro-o et al., 1997), as mutations in the mouse counterpart lead to syndromes resembling 'aging'.

Klotho functions as a co receptor with FGF23 involved in phosphate homeostasis. Further pleiotropic functions have been attributed to Klotho including protection against oxidative stress, inhibition of apoptosis, and promotion of vascularisation and angiogenesis (Lim et al., 2015).

Age related phenotypes associated with Klotho deficient mice are characterised by age related phenotypes that include the onset of osteomalacia, and ectopic calcifications (Haussler et al., 2012). FGF23 has become in recent years identified as a novel phosphate regulator within the body. $1,25(OH)_2D_3$ has been shown to induce the release of FGF23 from oesteocytic cells as well as working in close harmony with klotho acting as a co-receptor, which remains a bona fide longevity factor primary expressed in the distal tubule of the renal structures.

The major activity of FGF23 are to repress the expression of CYP27B1 when in an hyperphosphataemic state, ultimately creating a negative feedback loop repressing vitamin D bioactivation in the kidney. This process is further heightened through the activation of CYP24A1 to further lower the levels of biologically active $1,25(OH)_2D_3$ through increased degradation. The relationship between vitamin D/FGF23 and

Klotho remains an area of intense research. Further speculation suggests that the relationship between the bone and kidney through the unique interactions may be crucial for *healthful* aging by reducing the risk of developing chronic disease states. Bone remains the major source of FGF23, and the kidney is the major site of control of both klotho and 1,25D, ultimately benefiting from mineral metabolism, but perhaps expanding into the immune system and vasculature (Haussler et al., 2016).

1.9 Nuclear receptors, a molecular overview

The nuclear hormone receptor super family include receptors associated with the following lipophilic molecules; steroid hormones, thyroid, retinoids, and vitamin D (Aranda and Pascual, 2001b). Nuclear receptor (NR) superfamily, many of which are ligand activated transcription factors that bind and respond to a wide variety of ligands. Furthermore there degrees of commonality across all members, through the presence of a number of key elements that include a central DNA binding domain (DBD) which allow the targeting of DNA response elements. Towards the C terminal of the nuclear receptor protein lies the ligand-binding domain (LBD), lending the capacity to associate with ligand and ensure the specificity and selectivity of physiological response.

The effect nuclear receptors exert on transcriptional activity is directly mediated through the recruitment of co regulators and transcriptional machinery to towards the start site of target genes. When nuclear receptors are not bound to activating ligands, most are associated with co repressors such as nuclear receptor co repressor 1 (N-

CoR-1) or silencing modifiers for retinoid and thyroid hormone receptors (SMRT) allowing further recruitment of deacetylases, therefore tightening chromatin structure resulting in transcriptional repression.

Upon ligand binding the nuclear receptor undergoes conformational changes shifting the receptor to a transcriptionally active state allowing association with co activators such as histone acetyltransferase allowing further recruitment of transcription factors to associated at bind at the transcriptional start site of the target gene (Rastinejad et al., 2013).

These protein complexes allow for the unraveling of the chromatin super structure enabling the recruitment of a number of transcription factors associated with the formation of the pre-initiation complex. This overall recruitment of co activators to the promoter of target genes causes chromatin unwinding and transcriptional activation to occur (Aranda and Pascual, 2001b)

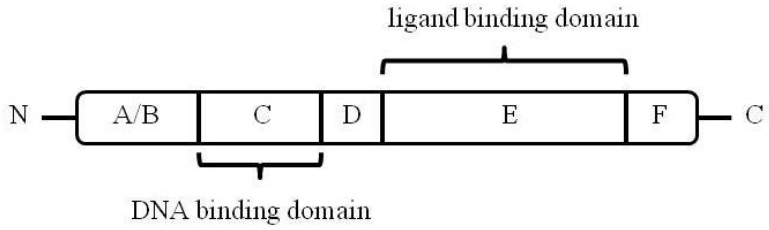


Figure 1.4: Schematic representation of a nuclear receptor. Most nuclear receptors are comprised of number functional domains. The conserved DNA-binding domain (DBD) is responsible for the recognition of specific DNA sequences. Whereas the E domain contains the ligand binding domain

1.10 Hormone response elements

Hormone response elements are sections of DNA that contain conformational signatures that are used to control chromatin binding and accessibility to DNA response elements within target genes (Umesono et al., 1991). The allosteric effects of DNA response elements on nuclear receptor function are essential for differential gene expression (Sever and Glass, 2013).

Dimeric response sites can be configured as palindromes either inverted (IPs) or direct (DRs). These are generated through a series of diverse genome wide repeats derived from transposable elements. A number of nuclear receptors including VDR recognize a direct repeat palindrome separated by three nucleotide base (DR3) this formation presents optimal binding for the VDR: RXR complex suggesting that the asymmetry of the conformation provides the most efficient interface for the DNA binding domain of nuclear receptor complex to interact with, making the DR3 response element the predominant and preferential form of binding the VDR: RXR heterodimer (Orlov et al., 2012).

1.10.1 Nuclear Hormone Receptors PXR & VDR

All nuclear receptors play a key role in maintaining adequate levels of expression of key regulatory genes concerned with such fundamental processes that include cellular proliferation, differentiation, metabolism and immune response (Sonoda et al., 2008). The NR family also encode for a number of metabolic sensors and regulators including the pregnane X receptor (PXR). Many nuclear receptors members control a number of central pathways having fundamental repercussions within a wide range of pathophysiology's including cancers and metabolic disease states. Nuclear hormone

Receptors have a high conserved ligand-binding domain with a great range of genes influenced through their activation. It is this trait that has led much research into defining specific ligands to up regulate a certain number of key genes without affecting the expression of others (Huang et al., 2010).

The nuclear receptor superfamily is further sub classified into four smaller families based on their DNA binding, ligand binding and dimerization properties.

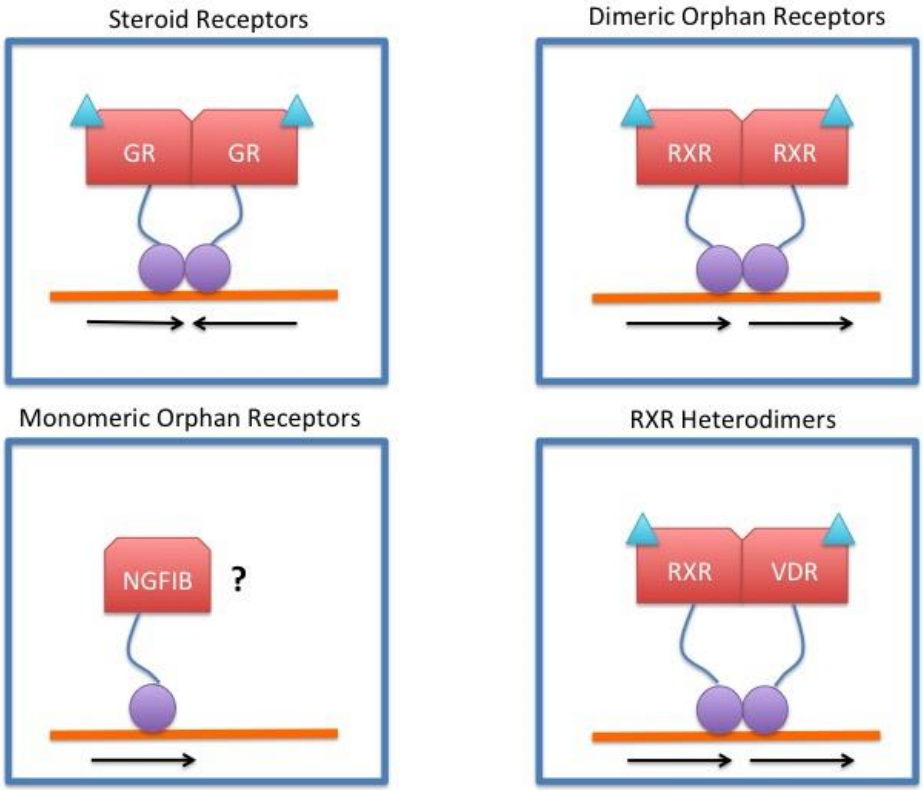


Figure 1.5: Summarizes the subdivisions of the nuclear receptor superfamily based on ligand binding and dimerization properties.

The sub class of interest within this thesis is that which includes the RXR-containing heterodimers. This sub family of nuclear receptor include such members as PXR, constitutive androstane receptor (CAR) and Farnesoid X receptor (FXR). The importance of this sub family of nuclear receptors is their importance in overall metabolism within the body. PXR is highly expressed within the liver and intestine and therefore plays an integral role in xenobiotic and endobiotic metabolism through the regulation of drug metabolizing enzymes and transporters.

PXR fulfills this role through the binding of DNA response elements as a heterodimer with the RXR leading the recruitment of a host of co-activators to the transcriptional start site of target genes. PXR has been deemed the master regulator of metabolism due to PXRs ability to be activated by a wide variety of agonists and indeed having a broad range of down stream implications as a result, ranging from drug metabolism, drug-drug interactions to the homeostasis of a number endobiotics such as glucose, steroids, lipids, bile acids, bilirubin and retinoic acid (Ihunnah et al., 2011).

Xenobiotic metabolism is the biochemical manipulation and further modification of substrates catalysed through enzymatic regulated activity; In essence it is the conversion of hydrophobic substrates to more hydrophilic metabolites allowing for the eventual excretion from the body (Guengerich, 2001).

The term “xenobiotic” comes from the Greek *xenos* for “stranger” and *biotic* meaning “living beings”. The phases of metabolism combines to form a complex network of inter relating metabolic pathways that modify the chemical structure of xeno and endobiotics, including drugs, steroids and mutagens (Watkins, 1990). These pathways come together in the core process of biotransformation and are present in all

groups of organisms within the phylogenetic tree of evolution (Caetano-Anollés et al., 2009).

The net result of biotransformation in the context of xenobiotic metabolism is the overall detoxification of potentially hazardous substrates. However it must be noted that a number of intermediate metabolites can be more hazardous than the parent substrate causing more harm as a result of metabolic transformation (Meyer, 1996). The fundamental processes that governs xenobiotic metabolism are of particular interest to researchers developing personalized medicine tactics, allowing for the achievement of individually tailored treatments (Jackson and Chester, 2015), (Chalmers et al., 2013).

Many of the substances hazardous to the body are highly lipophilic; this attribute allows for the diffusion across cellular membranes enabling the substrates to reach the effector target site (Xu et al., 2005). The process of metabolic clearance has evolved to a point in which it is sub divided into three distinct phases; all of which work in unison to facilitate the efficient modification and efflux of substrates from the body (Rushmore and Kong, 2002).

1.11 PXR in xenobiotic metabolism

The most well established function of PXR is to act as a xenosensor in the biological protection against exogenous chemicals. PXR is highly expressed in the liver, kidney and intestine with lower expression levels witnessed in other peripheral tissues including breast, brain, heart, bone marrow and monocytes (Zhang et al., 1999). It is

this broad spectrum of locals as sites of expression that allows PXR to be well suited to play its role through the induction of detoxifying enzymes and transporters.

1.11.1 Phase I

Efficient metabolism of exogenous and endogenous compounds is essential for normal physiological functioning and as such PXR has the ability to modulate these process through induction of phase I cytochrome P450 enzymes (CYPs). CYPs are a superfamily of haem dependent monooxygenases that catalyze the first steps in the detoxification of lipophilic compounds (Poulos, 2004). CYPs are highly expressed in the liver and intestine and use hydroxylation reactions to convert target compounds into more water-soluble metabolites that are more easily excreted from the body (Lehmann et al., 1998a).

Since PXR has the ability to control the transcription of a large number of CYPs including but not limited to *CYP3A4*, *CYP3A5*, it stands to reason that it must be able to be activated by a huge range of xenobiotic compounds. PXR has indeed been shown to be activated by a large range of pharmaceutical drugs as well as a wide range of environmental pollutants (Chang, 2009). It is interesting to note that PXR however does not have the capacity to regulate *CYP24A1* this is supported through the works Kenneth Thummel, (Zheng et al., 2012), furthermore the work contained within this thesis further corroborates the lack of regulatory ability PXR has over *CYP24A1* expression.

1.11.2 Regulation of Phase II enzymes

PXR has also been shown to regulate phase II metabolism-related genes including UDP-glucuronosyl transferase (UGT), sulfotransferase (SULT) and glutathione S-transferase (GST) enzymes. These phase II metabolic enzymes add on polar molecules xenobiotics/endobiotics producing water-soluble metabolites. A major role of PXR mediated phase II metabolic enzyme regulations is the metabolism and subsequent detoxification of bile acids, thyroxin, xenobiotics, oestrogens, and carcinogens (Xie et al., 2003).

1.11.3 PXR and drug transporters (Phase III)

Xenobiotic metabolism and disposition are also regulated by cellular uptake and efflux transporters controlling intestinal and hepatic absorption, as well as renal re-absorption, along with biliary and urinary excretion. These phase III transporters work in close concert with phase I and II systems creating an efficient and effective metabolic pathway. The ATP binding cassette family (ABC) are the major transporters regulating by PXR, and are expressed in hepatocytes, kidney, enterocytes and regulate the cellular movement of drugs in and out of cells. Examples of PXR targeted ABC transporters include multi drug resistance 1 or p-glycoprotein (MDR1/P-gp), and multi drug resistance proteins (MRP2, 4, 3 and 5) (Mills et al., 2004).

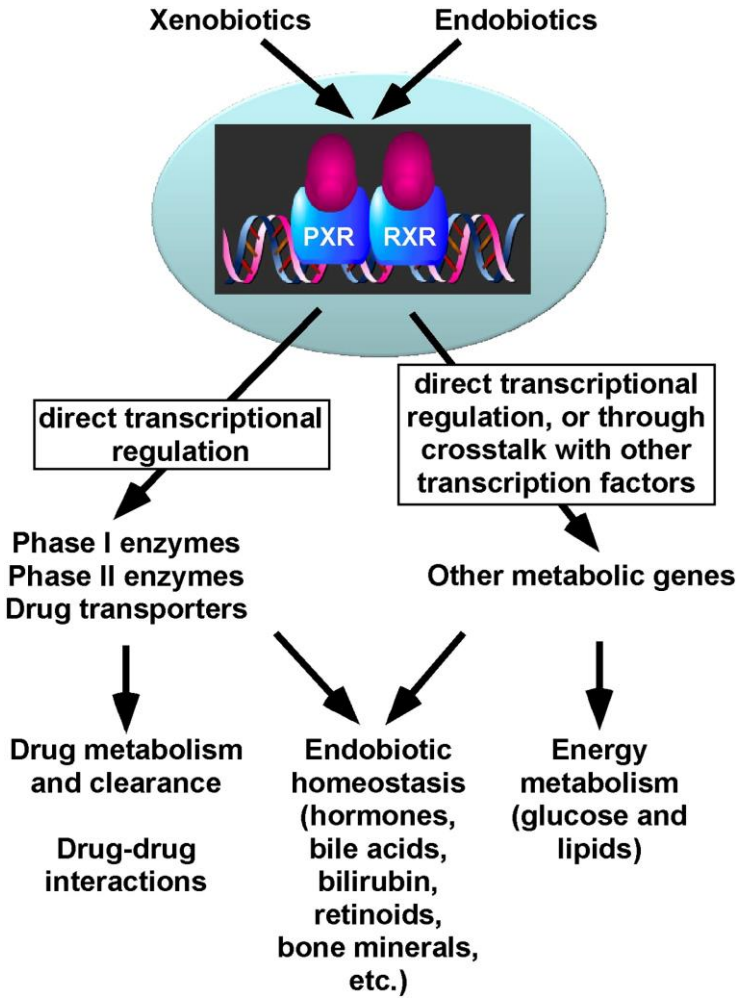


Figure 1.6: Summary of the transcriptional pathways associated with PXR activation sourced from (Ihunnah et al., 2011)

As detailed above PXR plays a fundamental role on the ability of the body to protect against xenobiotic and endobiotic assault. The vitamin D receptor is another member of the nuclear receptor sub family and as such shares approximately 95% homology with PXR. Due to PXRs ability to widely influence the expression of a range of metabolic and detoxification genes associated with all three phases of metabolism and due to the closeness its genetic composition between itself and its family member VDR possess, does VDR have the same capabilities as PXR to regulate metabolic gene expression across all three phases of metabolism?

The evolution and origins of the nuclear receptor subfamily that includes PXR and VDR is still under intense research and debate. However it does appear very probable that a single nuclear receptor gene duplicated early in vertebrate evolution. These two genes then diverged from each other to become VDR and PXR that we find. Further mutations and duplications have resulted in multiple PXR and VDR variants expressed among a number of different species.

From the phylogenic study of nuclear receptors it is well established that that nuclear receptors emerged long before the divergence of vertebrates and invertebrates. Since the advent of the human genome project and the genetic sequencing of the entire human genome 48 nuclear receptor genes have been identified (Zhang et al., 2004).

Phylogenetic tree of nuclear receptors resulted in the classification of human nuclear receptors into six evolutionary groups. The largest group contains the receptors VDR (NR1I1) and PXR (NR1I2) along with other VDR-like members (Evans and Mangelsdorf, 2014). A key similarity exists between each of the DNA binding sites among the members of the phylogenetic tree. There is an ancestral link between the evolution of PXR and VDR that remains of great interest. The close homology seen between these two nuclear receptors allows for the postulation of potential cross talk and direct interaction between nuclear receptor binding sites with the potential of DNA sites being shared between the receptors. This relationship has not been researched in great detail and remains a key and fundamental area of research investment today (Germain et al., 2006).

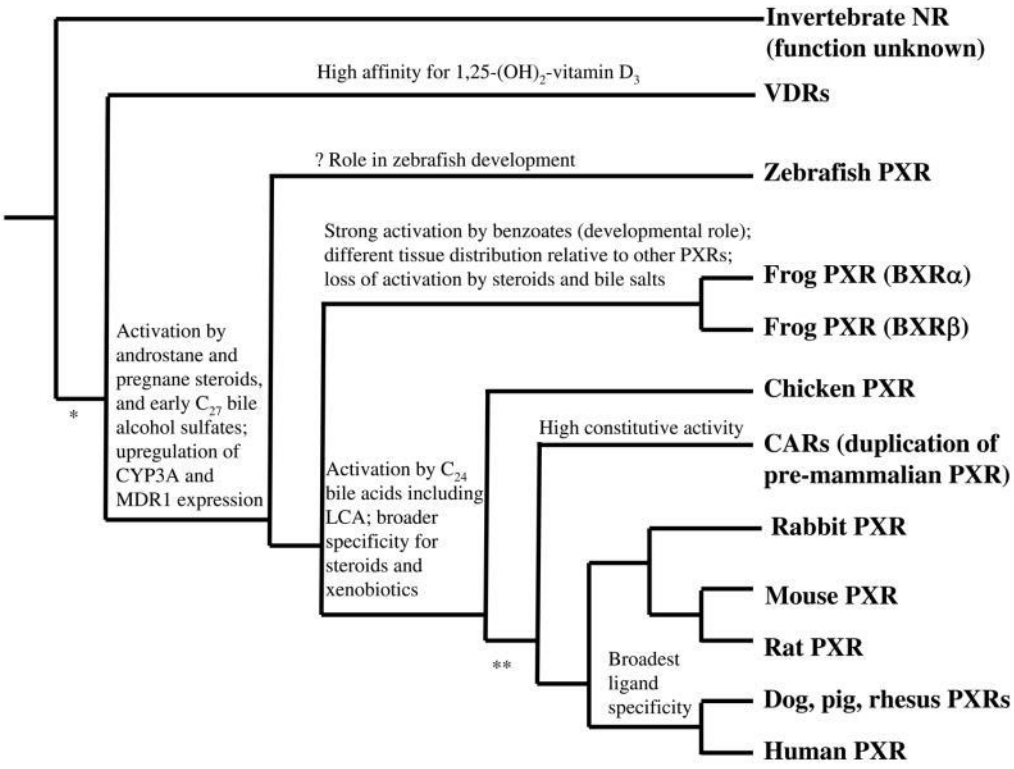


Figure 1.7: Phylogeny of PXR highlighting functional characteristics and close relationship between VDR and PXR sourced from (Iyer et al., 2006)

1.12 Gene targeting by VDR

As previously discussed the classical role of $1,25(OH)_2D_3$ and VDR is the regulation of calcium/phosphate homeostasis and bone mineralization.

Microarray analysis has indicated that between two and six hundred genes are targets of the vitamin D receptor and respond in a cell specific fashion. Through genome wide analysis more that 1600 Vitamin D response elements (from this point known as VDRE) have been identified (Hii and Ferrante, 2016).

VDR has been seen to regulate the expression of the major calcium channel gene within the intestine, *TRPV6*, as well as the sodium phosphate transport protein

SLC34A2. Further VDR targets include the up regulation of the fibroblast growth factor *FGF23,* discussed previously. VDR has also been seen to induce tumour necrosis factor *RANKL* leading to increased oesteoclast formation (Barthel et al., 2007).

The most responsive VDR-target gene identified to date remains *CYP24A1*, whose enzymatic gene product is key to the catabolism of $1,25(OH)_2D_3$. The activation of this gene generates a negative feedback loop controlling the levels of circulating vitamin D. VDR binds preferentially to a vitamin D response element consisting of two hexanucleotide (AGGTCA or similar) direct repeats, separated by a three nucleotide spacer; direct repeat 3 or DR3. Furthermore an everted repeat of the hexanucleotide motif with a six nucleotide spacer (everted repeat 6; ER6)is an additional configuration that allows VDR binding. A number of VDREs, including DR4, ER7, 8 and 9 motifs have been identified in cyclin c and insulin like growth factor binding protein. In addition gene involved in inflammatory pathways, and cell growth such as tumour necrosis factor alpha, interlukin 2 and c-Myc are negatively regulated by VDR activation. It is therefore imperative that our understanding of gene regulation of VDR is well understood and established as the potential therapeutic effects of VDR ligands in the treatment of cancer and autoimmune diseases are based on both positive and negative regulation of target genes (Saramäki et al., 2006), (Maruyama et al., 2006).

1.12.1 VDR and metabolic gene regulation

VDR also has an effect on other metabolic gene members of the Cytochrome p450 family. *CYP3A4* contains a DR3 motif as well as an everted ER6 proximal to the start site of the gene and has been shown to be greatly induced through the activity of VDR binding (Thompson et al., 2002) and has been considered a primary target of VDR activity. Due to the ability of VDR to impact on the transcriptional activity of *CYP3A4*, VDR may have an impact on the metabolism of prescription drugs as *CYP3A4* is involved in the metabolism of over 60% of drugs available today as will be discussed in later chapters. Furthermore VDR has the ability to act as an intestinal bile acid sensor, as secondary bile acids have shown affinity towards VDR binding (Chiang, 2009). The ability of VDR to bind secondary bile acids gives the potential for effective turn over/metabolism of potentially harmful substrates, expediting their removal from the lower intestinal tract.

Further investigation is required into the precise role VDR has over the transcriptional activity of metabolic gene activity, as this area has not yet been fully explored. VDR has been shown to affect phase one metabolic genes, as well as phase three in the form of members of the ATP binding cassette (ABC) superfamily, and multi drug resistance proteins all of which are involved in the expulsion of xenobiotics from cellular compartments including P-glycoprotein and MDR1 (Saeki et al., 2008). To this end very little research has been conducted in the area of phase two metabolism, and the potential role VDR may have at controlling gene activity.

Phased metabolism is regulated through the activity of nuclear receptors such as PXR and CAR. PXR and VDR share approximately 90% homology with each other. Furthermore PXR and CAR are already established inducers of phase two metabolic gene activity; however nothing is documented on the ability of VDR to regulate UGT gene expression. The major family of genes within phase two metabolism is Uridine 5'-diphospho-glucuronosyltransferase (UGT) and it is this family of genes that will be the focus of this thesis.

1.13 Phase II metabolism and UGTs

Phase II metabolism is characterized through conjugation reactions, with the use of glucuronic acid or sulphate groups that drastically increase the molecular weight and therefore the water solubility of the metabolized substrate. This conjugation system aids in eventual removal of metabolites from the body (Jancova et al., 2010).

Cytochrome P450 (phase one) and Uridine 5'-diphospho-glucuronosyltransferase (UGT, phase two) work in conjunction with each other to form easily excretable glucuronides metabolites, through conjugation with glucuronic acid (Guillemette, 2003). The original isolated glucuronides can be traced back to rural India in the acquisition of the Indian yellow dye attributed to Schmidt in 1855. The dye is a glycoside and is a conjugate of the aglycone euxanthone with glucuronic acid generating the more water-soluble chromophore. As with the majority of phase one proteins; phase two enzyme production and activity was discovered to be highly localized in the liver by Lipschitz and Bueding et al**.**

UGTs where later localized to the microsomal fraction of cell lysates, and it was then hypothesized that UGT proteins resided within the endoplasmic reticulum (Ikushiro et al., 1997), a finding later confirmed by Shepherd et al 1989. It was during this time of discovery that the roles of UGTs within the body where being discovered. Glucuronide formation through UGT activity was driven towards a number of endogenous hormones, steroids and pharmaceutical drugs including paracetamol, morphine, and phenobarbitone (Fisher et al., 2001), (Crettol et al., 2010), (De Gregori et al., 2012).

The most important discovery of UGTs was the identification that these proteins where the sole enzyme involved in the detoxification of bilirubin in 1956 (Sticova and Jirsa, 2013). Bilirubin is a breakdown product of haemoglobin, which, when unbound can cause unconjugated hyperbilirubinaemia, and if left untreated can lead to neuronal toxicity and eventual death (Tukey and Strassburg, 2000), (Fujiwara et al., 2010).

During the early years of UGT discovery it was postulated that there was only one UGT enzyme that had a promiscuous activation domain, which was responsible for the wide range of conjugation events within the body (Strassburg et al., 1997b). However this was contradicted by other researchers who hypothesized the heterogeneity of the UGT proteins was due to the presence and activity of many transferase enzymes not just a single enzyme; therefore each transferase had varying degrees of binding affinity depending on the substrate being conjugated. This line of reasoning was supported by the works of Kasper and Gorski et al who developed a range of affinity chromatography techniques allowing for the purification of UGT proteins. This in addition to the work of (Tukey and Strassburg)allowed for the confirmation that UGT proteins are integrated into the endoplasmic reticulum membrane. With this increase in understanding and a clear method developed to

purify homogenous and active UGT proteins, resulted in the isolation of substrate specific UGTs for a range of steroids found within the body including oestrogens, testosterones (Guillemette et al., 2004). This research culminated in the proposal that there is a heterogeneous population of UGTs.

With this proposal in mind a number of mouse and rat UGT isoforms were identified providing clear evidence for the heterogeneity of UGT family of genes. In 1991 the first series of UGT nomenclature was published. Many of the UGT isoforms share >80% homology however a clear presence of two distinct UGT families exists: *UGT1* and *UGT2*.

The *UGT1A* locus is located on chromosome 2 and comprises thirteen distinct first exons followed by four common exons as depicted in figure 1.8.

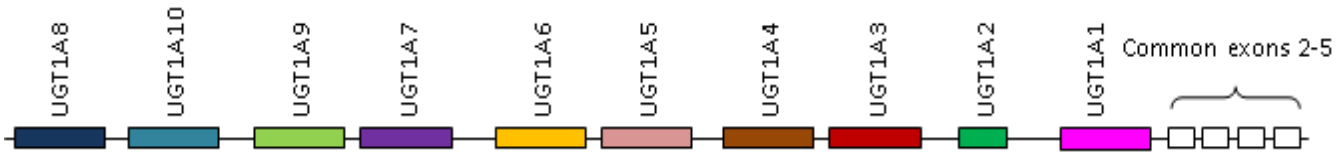


Figure 1.8: The *UGT1A* gene spans 220 kilo bases located within chromosome 2q37 and contains 13 cassette exons. There are a total of 9 functional exons each with unique regulatory promoter regions enabling independent RNA transcription, and 4 pseudogenes (p). The process by which each unique primary transcript is generated is referred to as exon sharing. Adapted from (Tukey and Strassburg, 2000).

UGT1A expression is a highly controlled, and expressed in a tissue specific manner with the majority of expression present in the liver however other tissues of note include the colon, kidney, intestine and brain (Zhang et al., 2007). The liver has long been designated the major organ associated with metabolism and this is further supported by the abundant expression of genes across all three phases of metabolism present throughout hepatic tissue including *CYP3A4* and *CYP3A5* (Kuehl et al., 2001). The liver expresses many members of the *UGT1A* family including A1, A3, A4, A6, A9. The latter four members are expressed in a solely extra hepatic manner (Strassburg et al., 1997). It is now understood that the *UGT1A* family of genes undergo epigenetic regulation through methylation events aiding in tissue specific expression (Choudhuri et al., 2010). This holds true for *UGT1A* expression in the kidney. The *UGT1A1* gene is hyper methylated, silencing the expression of the gene within the tissue (Oda et al., 2013). In contrast the colon has the most abundant isoform expression second only to the liver, expressing all the *UGT1A* isoforms except *UGT1A7* which is localized to the upper intestinal tract. Further *UGT1A* specificity is highlighted in the brain, and is thought to be involved in the metabolism of certain neuronal hormones such as serotonin as well as xenobiotic substrates including morphine (Ohno et al., 2008), (Armstrong and Cozza).

UGT1A1 is the sole metabolizer of bilirubin within the body and as such is associated with a number of genetic disease phenotypes, a number of which if left untreated can be fatal (Kamisako et al., 2000). Evidence also supports the increased risk factors *UGT1A1* polymorphisms may have on the development of coronary heart disease and diabetes (Lingenhel et al., 2008).

1.14 UGTs and bilirubin

Bilirubin is the breakdown product of aged erythrocytes and therefore a result of haem metabolism. Bilirubin is generated through the activity of biliverdin reductase on biliverdin lending bilirubin to have anti oxidative properties within a biological system (Sticova and Jirsa, 2013).

As with most endogenous substrates bilirubin is metabolized within the liver and as such is known as conjugated or direct bilirubin depending on the measurement being assessed (Shiomi et al., 1999). The process of conjugation with glucuronic acid makes the resulting metabolite more hydrophilic in nature lending itself to increased water solubility. The conjugated metabolite then moves into the small intestine were bile acid reabsorption occurs via the enterohepatic circulation (Lester et al., 1961), direct bilirubin however passes through into the colon, and is not reabsorbed.

1.15 UGTs in disease

The *UGT1A* family of enzymes play important roles in metabolism and as such a disruption in these enzymes results in disease states of varying degrees of complexity.

Interruption of *UGT1A1* can result in high levels of circulating bilirubin known and hyperbilirubinaemia. The most common genetic variation in *UGT1A1* is an elongated TATA sequence within the proximal promoter manifesting phenotypically as Gilbert's Syndrome (Burchell and Hume, 1999).

1.15.1 Gilbert's Syndrome (*UGT1A1*28*)

Gilbert's Syndrome is a hereditary and chronic presence of elevated unconjugated bilirubin levels as a result of impaired hepatic bilirubin clearance that can lead to jaundice under certain stresses including infection and fasting, but otherwise the condition is usually asymptomatic with normal liver functionality. The disease was first documented in 1901 but the main characteristics of the syndrome where not described until 1920 by a German physician Meulengracht coining the term *icterus interminus juvenilis*. With this mutation present the activity of the *UGT1A1* enzyme is reduced to 30%, resulting in a 70% overall reduction in the livers ability to conjugate bilirubin (Radu and Atsmon, 2001).

1.15.2 Crigler-Najjar Syndrome

Crigler-Najjar Syndrome is a rare autosomal recessive disorder and like Gilberts Syndrome has a detrimental inability to metabolize bilirubin. It is subdivided into two categories, with each subclass having distinct yet similar features. Type I and II Crigler-Najjars sufferers have very distinct phenotypes. Type I sufferers exhibit intense jaundice and is seen soon after birth remaining thereafter (Jansen, 1999), (Nair et al., 2012). A complete lack of *UGT1A1* expression also accompanies the disease with a percentage of sufferers having a mutation within the common exons (2 to 5) leading to an inability to metabolize phenobarbital as well as a range of other drugs and xenobiotics (Trotman et al., 1983). Children who are diagnosed with this genetic disease are at risk of developing bilirubin encephalopathy accompanied with many other complications including deafness, ocular palsy and lethargy. The risk of neurological defects can be very serious and has been documented to affect the basal ganglia and hippocampus (Gourley, 1997).

Type II, results in a milder manifestation of hyperbilirubinaemia that does respond to phenobarbital treatment a known prototypical ligand dependent activator of *UGT1A1*.

1.16 Xenobiotic Regulation of UGTs

UGTs are highly tissue specific in nature as indicated through varying degrees of expression seen throughout the human body; to this end adding an additional tier of complexity is the ability of xenobiotics to regulate UGT activity through the binding and subsequent activation of a large number of allelic polymorphisms found within UGTs, allowing for the determination of individuals glucuronidation profiles (Kim et al., 2014).

A number of early studies focused on localized transcription factors within the liver as the prime source of UGT activity. Liver rich transcription factors such as the hepatocyte nuclear factors or (HNFs), has been shown to be an essential requirement of a number of UGTs found within the liver such as *UGT1A3* and *1A4* (Gardner-Stephen and Mackenzie, 2007). Conserved response elements have been mapped highlighting palindromic motifs within a number of key *UGT1A* family members. UGT dependence on HNF as pre requisite for activation in a number of circumstances indicates the transcription factors play a pivotal role in the cross regulation of expression, further modifying an individual's expression profile of drug metabolizing enzymes.

1.17 Conclusion & aims

UGT1A1 has been shown to play a key role in hepatic metabolism through its ability to metabolism bilirubin as well as other xenobiotic substrates including chemotherapeutics and painkillers. However the extra hepatic role of *UGT1A* remains unclear and the potential for its expression to be modulated through the activities of the nuclear hormone receptor VDR to regulate its expression has not been documented before.

Therefore, the aims of this thesis can be categorized as follows:

- Identify which members of the *UGT1A* family are directly regulated by VDR.
- Establish the effectiveness of VDR activation through comparison to known and established prototypical inducers.
- Delineate the mechanistic action of VDR targeted *UGT1A* members, through analysis of novel vitamin D response elements within the promoter/enhancer module regions.
- Assess further the potential implications VDR regulation could have on detoxification gene activity.

Chapter 2: Expression Analysis of Detoxification Genes

In Extra Hepatic & Hepatic Cell Line Models.

2.1 Introduction

The liver has long been established as the major detoxification organ associated with toxin, steroid, and xenobiotic transformation (Almazroo et al., 2017). However more evidence brought to light the important role that intestinal detoxification plays within the bodies overall ability to metabolize potential harmful substrates (Li and Apte, 2015). Metabolism beyond hepatic tissue remains a relatively new area of study, however much research is now geared towards the potential protective roles intestine; small, large and colonic sources could play in genome wide protection against carcinogenic and environmental assault. To this end, a deeper understanding of metabolic control is required to clearly understand hepatic and extra hepatic roles. Nuclear receptors are long been known to facilitate the homeostatic regulatory balance within the liver, and indeed their role far surpasses that of a hepatic nature. Vitamin D and by extension the Vitamin D Receptor (VDR), is known to play classical roles in calcium and phosphate ion homeostasis ultimately effecting bone mineralization, strength and formation (Veldurthy et al., 2016). This functional role that vitamin D plays within the skeletal system was once thought to be its only major role. This line of thought was entirely accepted until VDR was cloned from a primitive fish lacking the ability to mineralize tissue (Doherty et al., 2015). This in itself led to speculation over the potential role VDR has beyond that of bone mineralization. Our understanding of VDR is ever increasing; its role within the overall detoxification goes beyond that of the liver and expands into all three phases of metabolism. Many target genes have been identified over the past decade involved in detoxification events. VDRs ability to regulate detoxification events outside the primary hepatic environment is supported through the observation that VDR can be activated and elicit a transcriptional response whilst bound to secondary bile acids

such as lithocholic acid and its derivatives supports the role the VDR may play in detoxification within biliary (major site of bile acid transport), intestinal and colonic tissue environments (Ogura et al., 2009), (Han et al., 2010) .

2.2 Hepatic metabolism

The historical source and key organ associated with environmental, and pharmaceutical detoxification remains the liver. Within hepatic tissues resides a network of phased metabolic systems working in concert to achieve efficient removal of potentially harmful substrates from the body. It is only through these three metabolic systems working together, can toxins be metabolized in a fashion that limits the damage potential of the metabolizing substrate (Strnad et al., 2017), (Xu et al., 2005).

All three phases of metabolism are controlled through the actions of nuclear receptors that are superfamily of metabolic and endocrine sensors with the ability to activate and repress transcriptional responsiveness of target genes depending on the hepatic environment at the time.

2.3 Nuclear receptor control of hepatic & enterohepatic metabolism

Nuclear receptors are primarily ligand activated transcription factors involved in a wide range of processes within the cell. In the hepatic and enterohepatic circulation, bile acids and derivatives of such, act as agonists for nuclear receptors eliciting transcriptional responses from target genes involved in the elimination and further metabolism of such substrates as primary bile acids (BA) and cholesterols. LXR and FXR play key roles in maintaining bile acid levels within the body and it is through

BA binding of nuclear receptors like FXR that lead to an up regulation of CYP450 members in an attempt to increase the metabolic turnover of primary BA accumulation. FXR remains the key metabolic sensor associated with BA metabolism within the liver and intestine (Gonzalez et al., 2016). All of the class I nuclear receptors perform their actions with the heterodimeric partner RXR alpha, this indeed holds true for additional members including PXR and VDR. Due to the close homology shared among nuclear receptor members, secondary bile acids have been seen to elicit VDR responsiveness, suggesting a potential role in maintaining a homeostatic balance within the enterohepatic circulation and other tissues outside the liver as VDR has limited expression in hepatocytes (Zollner et al., 2006).

This master control of many metabolic related genes gives PXR unprecedented control over hepatic and extra hepatic metabolism. This is evidenced through the ability of PXR to control bile acid metabolism. When LCA binds to PXR it results in the increase in expression of *CYP3A* members allowing for hydroxylation events to take place, facilitating the conversion of the metabolic substrate to be more hydrophilic in nature, allowing for the re entry into the circulation as a secondary bile acid or primes the metabolite for further transformation for rapid clearance from the body through the urine of faeces. This up regulation of *CYP3A* activity is thought to be an adaptive, protective response to potentially harmful levels of bile acid accumulation. PXR, CAR, and FXR act concordantly to mediate the exposure of liver to bile acid assault. PXR, CAR and FXR have been seen to induce the expression of *UGT1A* members within the liver as a result if BA binding facilitating the addition of a glucuronile group to the substrate. The ability of VDR in this instance remains extra hepatic in nature, as VDR is not highly expressed within the liver, however VDR has been seen to control detoxification genes outside the liver

including genes involved in phase I (CYP450) and III (drug efflux proteins; *ABCB1*), (Tachibana et al., 2009), (Chae et al., 2016).

Therefore if VDR has the ability to regulate metabolic genes within phase one and three pathways, to what extend does VDRs control play in phase two systems?

2.4 Extra hepatic detoxification & VDR expression

VDR is not highly expressed within core hepatocytes that make up greater than 90% of the livers mass however stellate, biliary epithelial cells, kupffer cells and sinusoidal endothelial cells all express higher levels of the vitamin D receptor. The extra hepatic expression and metabolism remains an area of key interest to those investigating the role of VDR in overall metabolism and detoxification. Tissue specific expression of VDR within cells outside of the liver suggest that the liver as a whole *could* be responsive to vitamin D. This hypothesis is supported through hepatic stellate cells expressing high levels of VDR furthermore stellate cell have been recently shown to repress TGF-Beta activation (Ding et al., 2013). VDR expression has been seen to be expressed throughout the body and have implication on disease pathophysiology including Crohn's, IBD and diabetes (Cantorna, 2006). Intestinal and colonic expression has been of great interest in recent years, and impairment of VDR expression within these tissues has been linked with disease progression including Crohn's disease (White, 2016) , colorectal cancers (Thompson et al., 2002c), (Meeker et al., 2016) and inflammatory bowel disease (Froicu et al., 2003), (Kong et al., 2008). Indeed, mouse model studies have shown that VDR-*null* mice can be rescued from severe intestinal colitis and death with the introduction of hVDR transgene intestinal epithelial cells (Liu et al.). The epithelial expression of VDR has been shown to

inhibit inflammatory pathways such as NF-kB, with an overall reduction in pro apoptotic pathways. With the ability of VDR going beyond what was once thought to be it's only role within the body, that of bone mineralization through calcium and phosphate homeostasis, allows further questions to be sought. The nuance of VDRs ability to effect disease progression is that reduced circulating vitamin D has an impact on disease states (Aggarwal and Kallay, 2016). Vitamin D deficiency has been diagnosed in many patients who suffer from inflammatory conditions including irritable bowel disease (IBD) and Crohn's Disease (CD) (Sadeghian et al., 2016). VDR expression has been associated with an increase risk of developing neurodegenerative disorders such as Alzheimer's Disease (AD) and therefore vitamin D deficiency and by extension VDR expression and activity has been linked with disease development and progression.

2.5 *UGT1A* hepatic expression

UDP-glucuronosyltransferases (UGT) are phase two metabolism enzymes, and act as homeostatic regulators aiding in biotransformation of potentially harmful substrates. These enzymes achieve this through conjugation reaction, fusing glucuronic acid to the metabolizing substrate ultimately transforming it into a more hydrophilic metabolite. This family of enzymes play major roles in overall detoxification through their role in bilirubin metabolism (haem break down product), as well as a wide range of other steroids, hormones, and xenobiotics. As a result a number of nuclear receptors including PXR, FXR, and CAR have DNA response elements within a wide number of the *UGT1A* gene family members proximal and distal promoters, and it is the presence of all these response elements that lend the *UGT1A* family of enzymes to

be highly inducible in hepatic models such as the HepG2 and HuH7 cell lines (Nakamura et al., 2008a) and (Fasinu et al., 2012). *UGT1A1* has been highly researched due to its major and sole role in bilirubin metabolism, a number of KO mouse models have been generated allowing for further investigative analysis of the role *UGT1A1* plays in the overall detoxification pathways within a biological system. Polymorphisms within the *UGT1A1* genes have also been linked with disease states including Gilberts Syndrome (*UGT1A1*28*), phenotypically manifesting as hyperbilirubinaemia, due to impairment in the catalytic activity of the *UGT1A1* gene. This also has further implications towards the ability of *UGT1A1* to act upon other exogenous and endogenous substrates including Irinotecan and morphine.

2.6 *UGT1A1* intestinal expression

The extra hepatic expression of *UGT1A1* remains an area of keen interest for many researchers. It has been seen; mouse models with a K.O. of hepatic *UGT1A1* expression exhibit a compensatory strategy with regards intestinal *UGT1A1* expression. Furthermore a selective K.O. of intestinal expression results in the cessation of life within the animal model paradigm (Chen et al., 2013). This further indicates the potential importance intestinal expression of *UGT1A1* could have in base line survival and genome wide protection against external and internal assault. Cancer of the gastrointestinal tract shows a marked reduction on metabolically active genes including members of the *UGT1A* family (Vogel et al., 2002), (Drozdzik and Oswald, 2016). Efficacy of Irinotecan metabolism is also affected with impairment of *UGT1A1* intestinal activity. A reduction in intestinal expression has also been noted to lead to the development of hyperbilirubinaemia and a condition known as bilirubin

induced neurologic dysfunction (BIND). BIND is characterized as a toxic build up bilirubin in the brain most commonly seen in neonates ultimately leading to neurotoxicity if left untreated.

In this chapter the focus will be to evaluate if the vitamin D receptor can influence the transcriptional expression levels in; an enteric cell model (LS180) and hepatic cell model (HepG2) following exposure to a number of VDR agonists. In addition to this we want to further delineate if activation via VDR ligands are as potent inducers as previously identified agonist of *UGT1A* activity, as mediated through PXR activation. This will be achieved through a number of PCR based approaches allowing for both a qualitative and quantitative view to be established and assessed. In addition to this vector transfection studies will be employed to delineate further which specific members of the *UGT1A* family are regulated through VDR activity, using known and established nuclear receptors as a comparison in both hepatic and extra hepatic environments.

Chapter 2: Materials and Methods

2.2.1 Cell Culture

All cell lines used within this research where obtained from the American Type Culture Collection (ATCC), (Rockville, MD, USA), unless otherwise stated.

2.2.2 LS180 cell line model & maintenance

The LS180 cell line was kindly gifted to the Thompson lab by Professor J Wesley Pike (Madison, Wisconsin). These cells were maintained in Minimum Essential Media (MEM), 10% foetal bovine serum, 1% sodium pyruvate, 1% non-essential amino acids, and 1% penicillin/streptomycin (Gibco, Paisley, UK). The LS180 cell line model is a well-established and highly used epithelial colonic adenocarcinoma model and represents an excellent cell line for exploring underlying transcriptional mechanisms as the mRNA output from these cells are abundant and highly reproducible.

2.2.3 LS180 passaging

The cells were washed with PBS and incubated with Minimum Essential Media containing 1M EDTA (10ml media: 50µl EDTA). The cells were then replaced back into 37°C, 5% CO_2 and 95% air, with humidified atmosphere and allowed to incubate for between 3 and 5 minutes, no longer as EDTA can become toxic if left on cells for an extended period of time. To ensure complete removal of the cells from the flask a cell scraper was used to mechanically remove adherent cells.

2.2.4 HepG2 cell line model & maintenance

The HepG2 cell line has been used within the research to represent a liver cell model. The cell line is derived from a hepatocellular carcinoma and is again epithelial in nature. The cells were maintained in Dulbecco's Modified Eagles Media (DMEM), high glucose (4.5g), supplemented with 15% foetal bovine serum, and 1% penicillin/streptomycin (Gibco, Paisley, UK).

All medias and supplements detailed above were sourced from GIBCO (Invitrogen, Paisley, UK), unless otherwise stated.

2.2.5 HepG2 cell passaging using trypsin dissociation

Complete growth media was removed and the cell monolayer was washed with 1x Phosphate Buffered Saline (PBS) (Oxid, Hampshire, UK), to remove any residual growth media from the flask. 5ml of 1 x Trypsin was added to the flask and allowed to incubate for 3-5 minutes at 37°C, 5% CO_2 and 95% air. With gentle tapping of the flask the adherent cells were dislodged forming a cell suspension. Double the amount of complete growth media was added to the Trypsin and cell suspension, this inactivates the Trypsin. The cell suspension was then centrifuged at 12000rpm for 5 minutes to create a cell pellet followed by careful removal of the supernatant. The resulting pellet was resuspended in complete growth media to an appropriate dilution of cells to ensure optimal continuation of growth.

2.2.6 Cell counting

To ensure cell viability and cell seeding density throughout all experimental procedures a haemocytometer was used to ensure accurate cell counts.

Cells where collected as outlined above and spun at 12000rpm using a bench top centrifuge at room temperature. The supernatant was carefully removed and the cell pellet resuspended in between 5-10ml of complete growth media (depending on the size of the cell pellet). An aliquot (12µl) of the resulting cell suspension was taken and mixed 1:1 with Trypan Blue stain (Sigma-Aldrich, Dorset, UK). The resulting stained suspension was then pipetted up and place at the edge of a cover slipped haemocytometer, and allowed to flow via capillary action across the counting chambers. Viable cells do not take up the Trypan blue stain therefore allowing for cell viability to be determined counting the unstained cells in the outer 4 squares and the middle square, viewed under inverted phase contrast light microscopy (40x magnification).

The same process was conducted using the 2^{nd} counting chamber on the haemocytometer giving a total of 10 squares counted allow for an average to be calculated. The total number of cells in the suspension from which the aliquot was taken was determined using the following equation: cells/ml = (n) x 2 x 10^4, where

n = average total number of cells counted in 10 squares, 2 = the dilution factor with Trypan blue stain and 10^4 is the multiplication factor to obtain the number of cells in 1 ml of suspension.

2.2.7 Freezing and thawing cell stocks

Frozen stocks of all cell lines were prepared as per the ATCC guidelines using either 95% FBS: 5% Dimethyl Sulfoxide (DMSO) or 95% complete media: 5% DMSO. A 175cm^3 confluent flask of cells was pelleted and a cell count performed allowing for the freezing of 4x10^6 cells/ml in freeze down solution and placed in -80°C. For long-term storage and to ensure no loss in cell viability a number of cryovials were subsequently stored in liquid nitrogen.

Cell thawing was achieved through rapid defrosting using a water bath at 37°C. Upon complete defrost of the cell stock the cells were transferred to a universal and 5ml of complete growth media was added drop wise to the cells and freeze down mixture. The suspension was then spun at 12000rpm for 5 minutes. The supernatant was discarded and the pellet washed in 1x PBS before being resuspended in 1ml of complete growth media and transferred to a large 175cm^3 flask. The flask was then incubated overnight at 37°C, 5% CO_2 and 95% air, and humidified atmosphere. The following day non-adherent, dead cells were washed away using 1x PBS and fresh complete growth media replaced.

Ligands/Solvents used in Gene Expression Studies

Ligand/Solvent	Final Working Conc.	Solvent	Source
Ethanol	<0.095%	-	Rathburn, Scotland
DMSO	<0.099%	-	Sigma
Methanol	<0.099%	-	Sigma
1,25-$(OH)_2D_3$	10nM	Ethanol	Enzo Scientific
EB1089	10nM, 1nM	Ethanol	Enzo Scientific
Bexarotene	1μM	Ethanol	
Rifampicin	20μM	Methanol	Sigma
GW474064A	1μM	DMSO	Glaxosmithkline
T0901317	1μM	DMSO	Sigma Aldrich

The above table details the ligands and their respective solvents used within the gene expression studies. Each of the ligand treatments are controlled against their respective vehicle only counterpart (itemized above) from which to extrapolate fold induction of gene expression achieved through exposure to ligand over levels observed with vehicle alone.

2.2.8 RNA extraction & Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

All gene expression studies were conducted using 6 well plates across all cell types, this allowed for adequate isolation of RNA from the cell models used. When 70% confluency was reached cells were washed in 37°C PBS, collected either via trypsin disassociation or mechanical removal of cells via a cell scraper, counted and seeded and 2-2.5x10^5 cell/well, depending on cell model used. Cells were allowed to adhere for 24 hours before treatment. Cells were treated for 24 hours at which point total RNA extraction was performed using the Qiagen RNeasy Plus Kit per manufacturers protocol (Qiagen, Sussex, UK). The procedure involved collection of the cells using trypsin or cell scraper (cell line dependent), and re suspending the cell pellets in 350µl of RLT buffer containing 6µl – β-mercaptoethanol. The resulting homogenate was then passed through a Neolus® 20G needle (Terumo UK, Surrey, UK), 5 times, before being expressed into a gDNA eliminator column. The resulting flow through underwent a series of ethanol precipitation and wash steps with the aid of RNA spin column technology, the total RNA was eluted into 50µl of RNase-free water. The Total RNA concentration was then determined using the NanoDrop® ND-1000, and stored at -80°C.

cDNA synthesis was performed using SuperScript™ II (Invitrogen, Paisley, UK). Between 1-2µg of RNA was used depending on the RNA concentration and quality. The RNA was reverse transcribed using random $OligoDT_{12-18}$ primers (Invitrogen, Paisley, UK) using manufacturers protocol. Total RNA, $OligoDT_{12-18}$ and dNTPs to a final volume of 12µl, were heated to 65°C for 5 minutes. 7.5µl of a master mix made up of first strand reaction buffer, 0.1M DTT, and PCR water was added to each

sample respectively, before being heated for 2 minutes at 42°C. Finally, 0.5µl of SuperScript II Reverse Transcriptase was added to each sample and placed back on the on the heat block for one cycle of 50 minutes at 42°C, and 15 minutes at 72°C (inactivation), before holding the samples at 4°C degrees. All the reverse transcription reactions were carried out in 0.2ml PCR tubes on a multi block Techne-5000. The cDNA was subsequently diluted to 10ng/µl using PCR grade water and stored at -20°C.

2.2.9 End Point Polymerase Chain Reaction (End Point-PCR)

The end point PCR reactions were achieved using the GoTaq® Flexi DNA Polymerase technology, (Promega, Madison, USA). Each reaction mix contains 1 x Green Flexi Buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.2µM of sense and antisense primers respectively. To this 15.9µl of PCR grade water was added, along with 0.5µl of GoTaq® DNA Polymerase creating a final volume of 25µl. The resulting PCR mixture was spun briefly to ensure all the reagents were at the bottom of the PCR tube.

Hypoxanthine-guanine phosphoribosyltransferase (HPRT) is used within these experiments as a housekeeping gene control as this gene is not effected by any of the ligands being used within the study.

End Point PCR Primers

Primer	Sense (5' to 3')	Antisense (5' to 3')
HPRT	GACCAGTCAACAGGGGACAT	AGCTTGCGACCTTGACCAT
UGT1A1	GTTGATCCCAGTGGAATGGCAG	AAGTGGGAACAGCCAGACAA
UGT1A3	AGGTGTCAGTGGTGGATATTCT	ACAGGACTGTCTGAGGGATTTTG
UGT1A4	CTGGCTACACTCAAGGGTT	TGGCAAATGTAGGACAGGGC
UGT1A5	GCTGGGTTCACACTCAATCGT	CAAAGGTAGGACAGGGCCAG
UGT1A6	TCAGGGGTTTTCCGTGTCC	GGCTTCAAATTCCTGAGACAAGT
UGT1A7	GTTTTTGCCGATGCTCGCTG	GGCAAAATATTCCCCTGGCG
UGT1A8	GCCCCATTCCCCTATGTGTTTC	ATGAATCCCGGTCCAGATCC
UGT1A9	TCCAACACCTGTTACGGAG	CATCGGGTGACCAAGCAGAT
UGT1A10	CAATGGAAAGCACAGGCACAA	CATAGGAAAGAGGAGCAGGGCA

Thermocycler conditions

All end point PCR reactions were as follows:

Initial Denaturation	2 minutes, 94°C
Denature	30 seconds, 94°C
Annealing	30 seconds, 55°C
Extension	1.15 minutes, 72°C
Final Extension	10 minutes, 72°C
Hold	10°C

The cycle number applied to achieve amplification for the listed primer sets were: *UGT1A10*, 40 cycles, *HPRT*, *VDR*, *PXR* and *UGT1A4*, *UGT1A5*, *UGT1A7*, was achieved at 35 cycles, *UGT1A6*, and *UGT1A8* at 30 cycles *UGT1A1*, 27 cycles.

The PCR products were separate on 1.5% agarose using electrophoresis, 1 x Tris Acetate (TAE) buffer, containing 10mg/ml ethidium bromide (Sigma, Dorset, UK). The agarose was dissolved in TAE buffer and poured into a casting cassette and allowed to solidify at room temperature. Once the gel was set the comb in removed and 1 x TAE is poured into the electrophoresis chamber until the gel is just submerged in buffer. 8µl of the appropriate ladder (100bp TrackIt DNA ladder), (Invitrogen, Paisley, UK) was loaded, followed by 12µl of each respective sample (half the reaction mix). The gels ran at 100 volts for 20-30 minutes depending on the migration speed before visualization under UV light exposure using a Kodak UV trans-illuminator.

2.2.10 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Quantitative Real Time PCR was used to establish endogenous messenger RNA levels of expression of target genes of interest. Therefore 2.5µl of cDNA (10ng/µl) was subject to a PCR reaction using Roche gene specific hydrolysis probes using the Roche Light Cycler® 480 analyzer. The reaction mix is detailed below.

	Volume (µl)
- Light Cycler Probes Master Mix	5
- Probe	0.5
- PCR grade H_2O (with kit)	2
- cDNA template (10ng/µl)	2.5 µl

Each reaction as described above underwent amplification on the Roche Light Cycler® 480. The thermocycle began with pre incubation at 95°C for 10 minutes followed by amplification for 50 cycles at 95°C for 10 seconds, 60°C for 30 seconds, 72°C for 1 second followed by a final cooling step at 40°C for 30 seconds. Once the PCR run was complete the raw Cp values underwent relative quantification using the built in analysis tools provided by Roche for data analysis, employed also was the Delta/Delta Ct methodology. This method of analysis was devised by Kenneth Livak and Thomas Schmittgen in 2001 and has been used and cited over 61,000 times. In brief the cycle threshold or Ct is the cycle number when the florescence generated by the PCR reaction is distinguishable from background interference or noise. The Delta

Ct is the difference in Ct values for the target gene of interest and the housekeeping gene (HPRT). This rationale normalized the gene of interest to a gene that is not affected by the experimental set up and procedure allowing for fold change to be calculated as a result.

RealTime ready Probe used in qRT-PCR analysis

Hydrolysis Probe	Cat RealTime Ready ID	Hydrolysis Probe	Cat RealTime Ready ID
HPRT	102079	*UGT1A7*	145839
CYP3A4	135760	*UGT1A8*	145843
CYP3A5	112269	*UGT1A10*	145837
CYP24A1	114955		
TRPV6	110452		
UGT1A1	138404		
UGT1A3	140686		
UGT1A4	140612		
UGT1A5	145870		

2.2.11 Transfection Methods

Calcium Phosphate Transfection

Within this project the calcium phosphate protocol was used for transient transfection of cells. This protocol employs 2M Calcium Chloride ($CaCl_2$) and 2 x HBS (50mM HEPES, 280mM NaCl, 1.5mM Na_2HPO_4), that can be prepared before hand sterile filtered through a 0.22μm filter and stored at -20°C.

Day One: HepG2 were collected from a confluent flask (~70-75%), and seeded into the appropriate plate size depending on the experiment being performed. The cells were then incubated overnight to allow the cells to adhere and establish within the plate.

Day Two: On the day of the transfection, 2M $CaCl_2$ and 2 x HBS was removed from storage and allowed to completely thaw and come up to room temperature before use. The cells being transfected were washed with warm 1 x PBS before fresh media was applied.

The transfection mixes containing DNA/$CaCl_2$/H_2O was then added in a 1:1 ratio to 2 x HBS, and allowed to precipitate at room temperature for 30 minutes before being applied to the cells in a drop-wise manner. The plates were then incubated between 16-18 hours, any longer than this and cell death can occur.

2.2.12 Nuclear Receptor vectors used within gene analysis studies

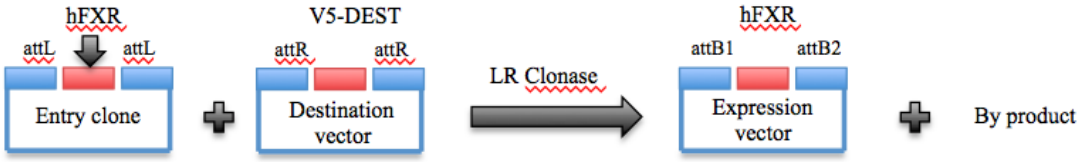
The HepG2 cell line model was transfected with expression vectors encoding human VDR, PXR, FXR and RXR alpha respectively. The VDR, FXR and RXR constructs were created using the commercially available gateway compatible pcDNA/V5-DEST vector backbone. The pcDNA vectors are designed for high level, constitutive expression in a range of mammalian cell lines. The pcDNA/V5 DEST vector contains Cytomegalovirus (CMV) immediate early promoter, which allows for efficient high level expression of recombinant protein; the presence of the c-terminal V5 epitope allowing for the detection of recombinant fusion proteins by anti V5 antibodies and the ampicillin resistance gene (Beta-lactmase), allows for the selection of transformants in E-coli.

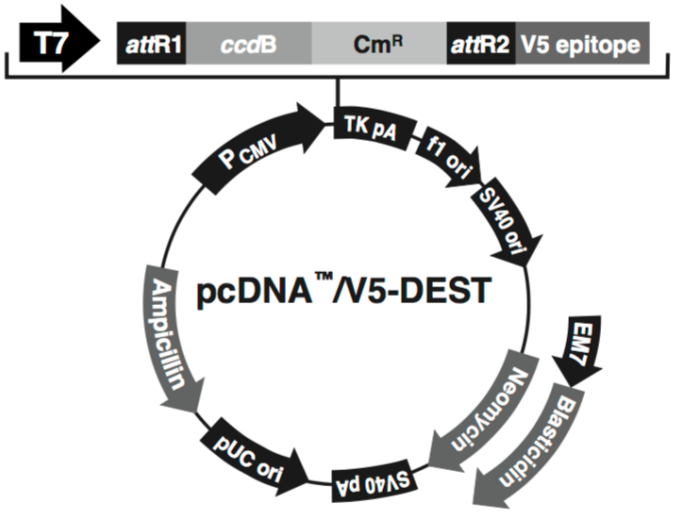
V5-hVDR and V5-hLXR had already been generated previously however in brief, from pSG5-hVDR that was also gifted by Professor Haussler the hVDR insert was amplified using PCR and cloned into the pDONER201 vector by *BP clonase*. This entry clone (pDONER201-hVDR) was then employed in down stream gateway reactions using the vector pcDNA3.2 V5-DEST vector facilitated via an *LR clonase* reaction, resulting in the generation of the V5-hVDR construct. A pDONER entry clone already existed for hFXR and therefore only underwent *the LR clonase* reaction to generate the complete V5-hFXR construct.

2.2.13 Gateway cloning technologies

A number of the constructs already existed within the laboratory (V5-hVDR,

V5-hLXR), however the generation of the V5-hFXR was solely generated within this project. The schematic below highlights the steps involved in the generation of a gateway compatible V5 based construct.





Vector map of the V5 DEST backbone used within this study

2.2.14 LR cloning reaction

The LR reaction was carried out as described below.

	Volume (µl)
- Entry clone (50ng)	1
- Destination vector (75ng)	1
- Gateway LR clonease II enzyme mix	1
- T.E. buffer pH 8.0	2
- **Final Volume**	**5**

The above reaction mix was then allowed to incubate at room temperature for 2 hours, after which 1µl of protease K was added to cease the clonase reaction (2µg/ µl, provided with the clonase kit) and incubated at 37°C for 10 minutes. The resulting expression vector was then transformed into library efficiency (LE) DH5α cells, detailed below), and colonies selected from Luria-Bertani (LB) agar plates (50µg/ml ampicillin), and grown in LB broth overnight. The plasmids subsequently underwent miniprep extraction using the Invitrogen Pure-Link™ Quick plasmid miniprep kit (Invitrogen, Cat: K2100-10). To ensure the entry clone was successful in ligating with the destination vector colony and sequencing PCR was performed.

Chapter 2: Results

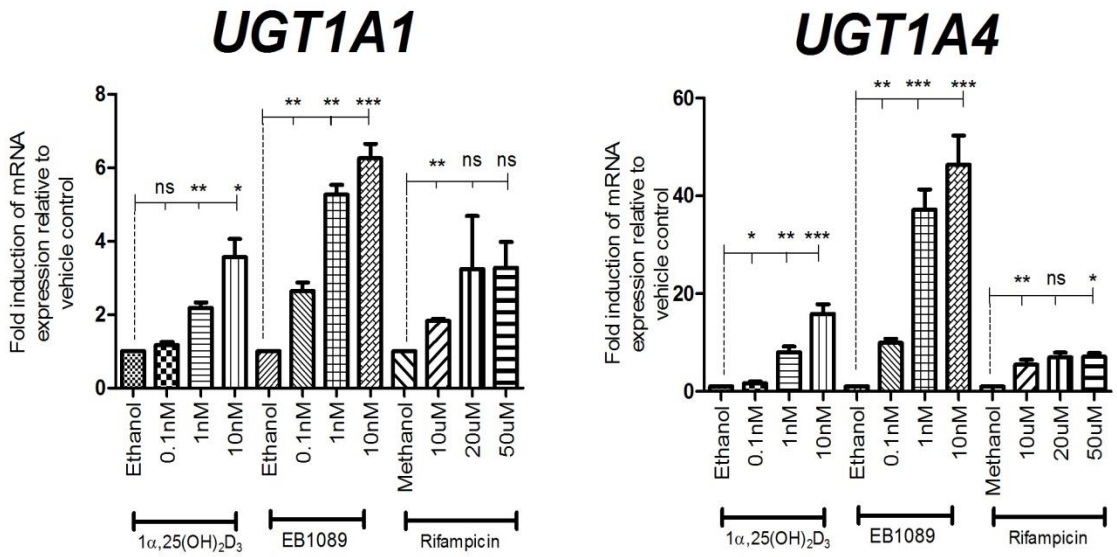


Figure 2.1 Dose dependent expression of *UGT1A* genes in response to VDR and PXR ligands: LS180 cells were treated for 24hrs with vehicle control or indicated concentration of test ligand. Extracted RNA was reverse transcribed subjected to qRT-PCR analysis as per manufacturer's protocol. Data are represented as means (± SEM) of three independent experiments, $p<0.05$ *, < 0.01 **, and <0.001 ***.

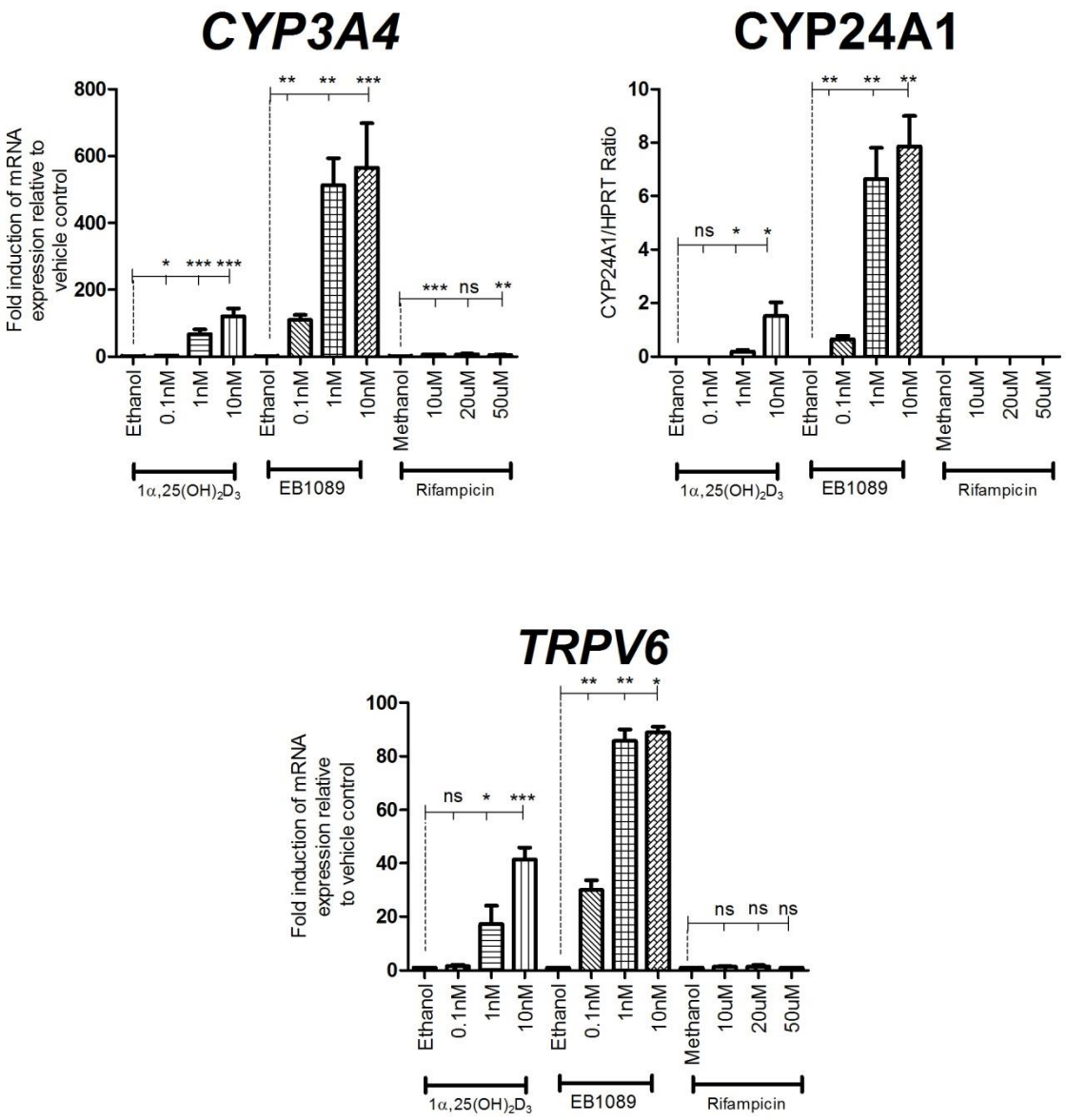


Figure 2.2 Established VDR target genes exhibit dose dependent responsiveness: LS180 cells were treated for 24hrs with vehicle control or indicated concentration of test ligand. Extracted RNA was reverse transcribed subjected to qRT-PCR analysis as per manufacturer's protocol. Data are represented as means (± SEM) of three independent experiments, $p<0.05$ *, < 0.01 **, and <0.001 ***.

2.3.1 *UGT1A* and known VDR target genes exhibit dose dependent responses

qRT-PCR analysis was carried out to monitor transcriptional response of *UGT1A* family members within LS180 cells following exposure to varying concentrations of ligands for VDR and PXR. Rifampicin was used as a PXR activator as this represents a known and established inducer of *UGT1A* activity (Mackenzie et al., 2003) allowing direct comparisons to those effects achieved through VDR.

Figure 2.1 depicts the dose dependent nature *UGT1A1* and *UGT1A4* exhibit when exposed to natural and synthetic VDR ligands. $1,25(OH)_2D_3$ at 1 and 10nM concentrations show a statistically significant induction of *UGT1A1* and *UGT1A4*. EB1089 or Seocalcitol is a synthetic analogue of $1,25(OH)_2D_3$ and does not have as strong an influence on calcium metabolism. Within this experimental set up EB1089 at a 10nM concentration is showing over 6-fold induction of the endogenous *UGT1A1* gene in LS180 cells with a three star significance vs. no statistical significance when compared to the PXR agonist Rifampicin at concentrations within the micro molar ranges.

EB1089 has been utilized as an anti cancer agent as it has been seen to inhibit growth, induce differentiation and induce apoptosis of cancer cell line models *in vitro* (Evans et al., 2002), furthermore it has been used in the clinic trials as a treatment against hepatocellular cancer, witnessing complete remission of the cancer (Hansen et al., 2000).

EB1089 induces target mRNA activity in LS180s giving an average induction of > 6 fold (*UGT1A1*) and >50 fold (*UGT1A4*) respectively. This trend of heightened induction through the actions of EB1089 is very promising as it highlights the potential therapeutic application of EB1089 upon the regulation of key *UGT1A* members, as the same ability has been witnessed in a number if *CYP450* members, (*CYP3A4* and *CYP3A5*).

In addition to this the comparison between PXR and VDR with the understanding that PXR is a known prototypical inducer o*f UGTA* activity shows a limited ability to induce mRNA production even at a micro molar level. This is supported with the observations that statistically significant responses were seen at a nano molar level when LS180 cells were exposed to VDR agonists both natural and synthetic.

Figure 2.2 depicts known VDR targets, including phase one genes (*CYP3A4, CYP24A1*) and calcium transporters (*TRPV6*). As with the *UGT1A* members there is a definite dose dependent response seen amongst these genes. *CYP3A4* has a highly inducible response when exposed to EB1089 (~500 fold), as does *TRPV6* (~100 fold) and *CYP24A* (~9 fold). Due to the high degree of variability and massive fold inductions witnessed upon *CYP24A1*, the above graph is plotted as a ratio compared to the housekeeping gene HPRT.

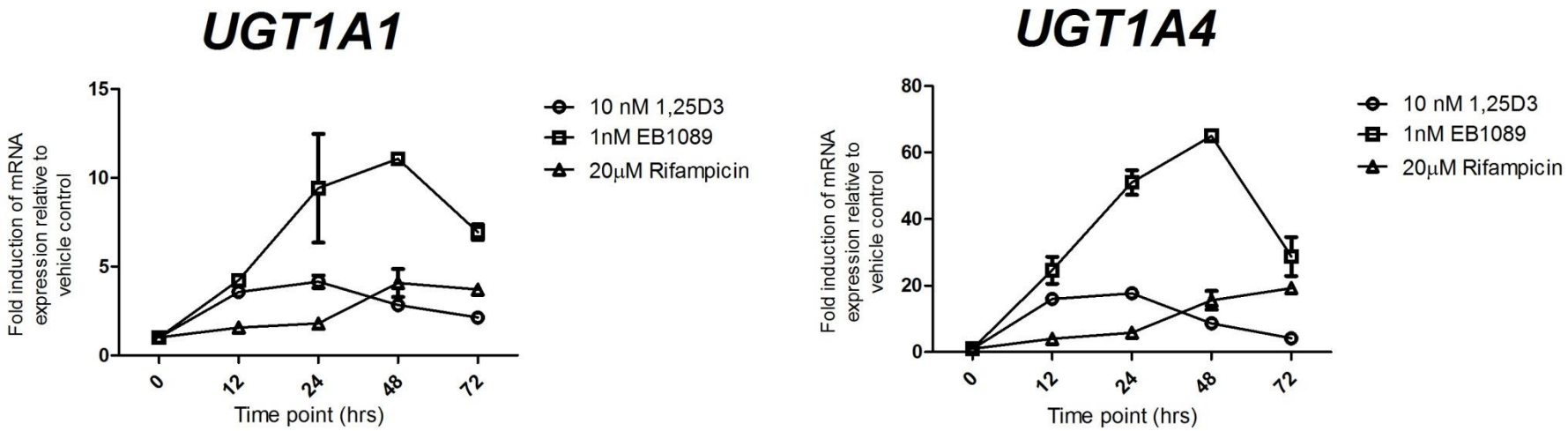


Figure 2.3 *UGT1A* members time course response within a 72hr time frame: LS180 cells were treated with indicated concentration of test ligand before RNA extraction was carried out at each time point as detailed above. Extracted RNA was reverse transcribed subjected to qRT-PCR analysis as per manufacturer's protocol. Data are represented as means (± SEM) of three independent experiments.

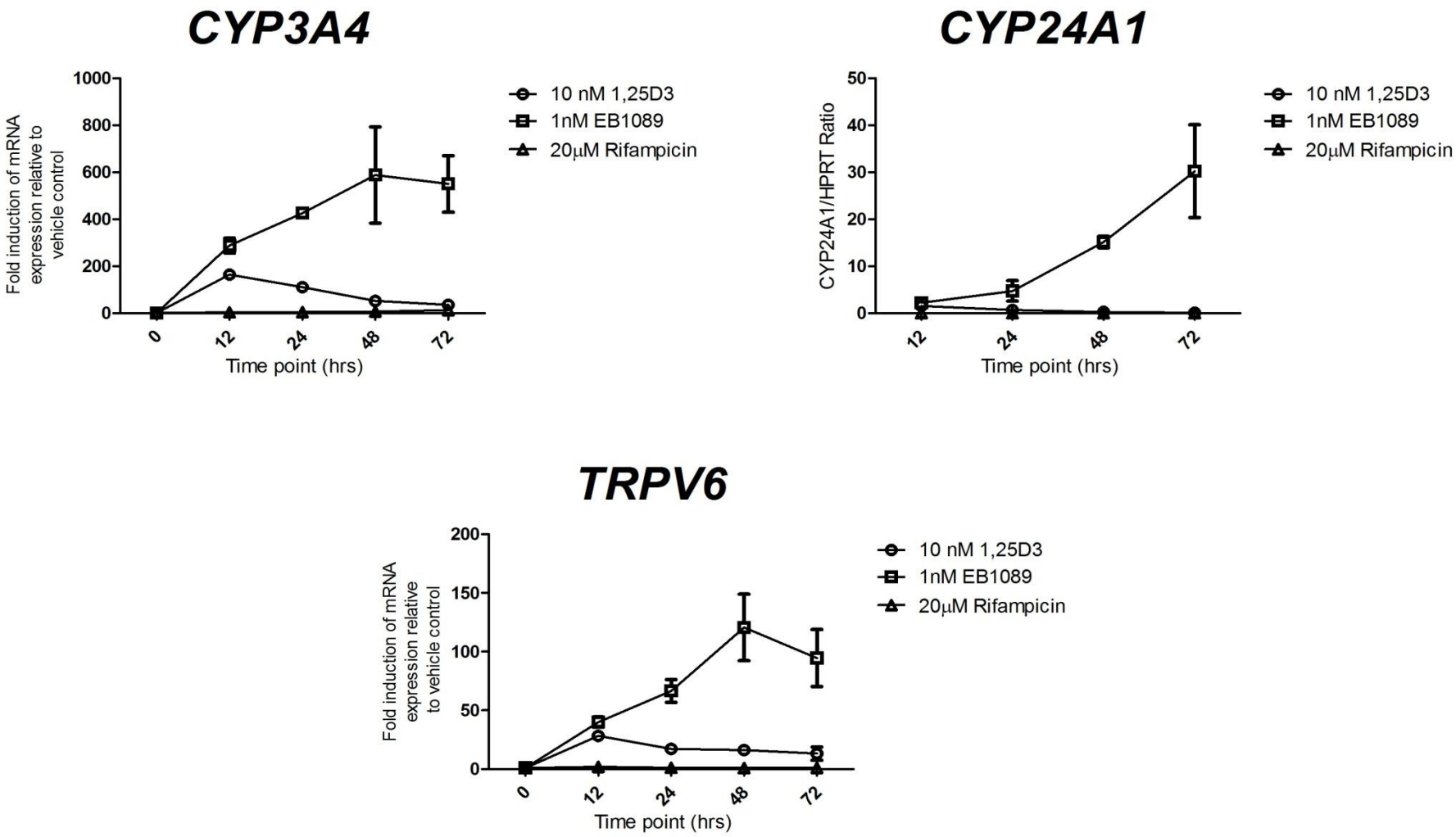


Figure 2.4 Established VDR target genes; time course response within a 72hr time frame: LS180 cells were treated with indicated concentration of test ligand before RNA extraction was carried out at each time point as detailed above. Extracted RNA was reverse transcribed subjected to qRT-PCR analysis as per manufacturer's protocol. Data are represented as means (± SEM) of three independent experiments.

2.3.2 Time course analysis in LS180 model

From the dose response study, appropriate concentrations of $1,25(OH)_2D_3$, EB1089 and Rifampicin were chosen for time course analysis to be conducted. 10nM of $1,25(OH)_2D_3$ was used as this concentration had previously been established with the laboratory and as is shown in the dose response gives clear statistical significance and reproducibility at this concentration. Using 1nM EB1089 again shows statistically significant induction vs. vehicle control. I also wanted to highlight even further the stark contrast in concentration of ligands to produce a significant transcriptional response, when PXR agonist Rifampicin is using concentrations in the micro molar ranges for less transcriptional responsiveness.

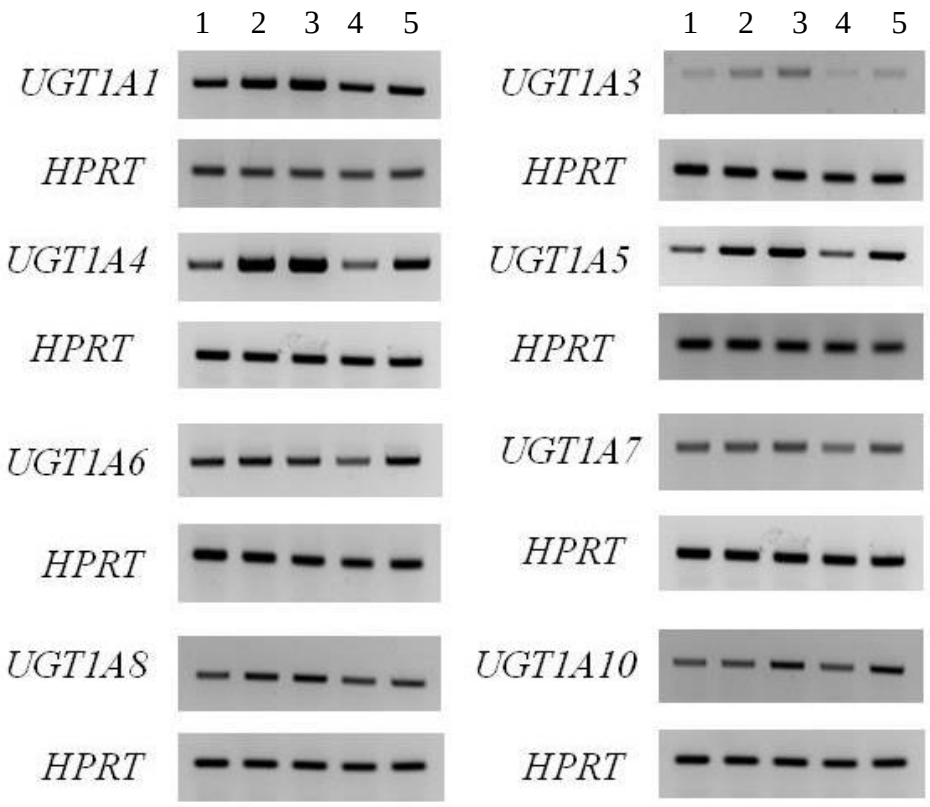
The LS180 cell line model was used again to establish more fully the effects these agonists have on mRNA expression over a time period extending up to 72 hours. Both *UGT1A1* and *UGT1A4* exhibit very similar trends in time course analysis. 1nM of EB1089 showing the most robust and prolonged transactivation of said targets, inducing mRNA levels of *UGT1A1* to > 10 fold and *UGT1A4* to > 60 fold at 48 hours after which a sharp decrease in activation is seen at 72 hours. $1,25(OH)_2D_3$ shows its peak being reached after 24 hours with a steady decline in activation thereafter. The decline post 24 hours could be attributed to the rate at which the LS180 cells metabolize the natural agonist. Rifampicin indicated a more progressive response reaching a peak at 48 hours where is levels off thereafter. It is interesting to note that when the comparison between VDR and PXR activation is made, the PXR agonist rifampicin achieves similar levels of activation however the time required to reach this point is longer than $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ achieves its peak transcriptional response at 12 hours with Rifampicin achieving its peak transcriptional expression at 24 hours.

EB1089 shows again a super induction of *CYP3A4*, *CYP24A1* and *TRPV6*, however *CYP24A1* having such a potent ability to be induced through VDR activity does not show a decrease in responsiveness within the 72 hour window of the experimental procedure.

CYP3A4 and *TRPV6* share very similar trends when compared together. *CYP3A4* when exposed to both $1,25(OH)_2D_3$ and EB1089 show a sharp increase in mRNA expression at 12 hours. At this point $1,25(OH)_2D_3$ activity declines, most likely due to metabolic turnover of available ligand whereas EB1089s effectiveness is witnessed climbing at a steady rate to 48 hours reaching an mRNA level of expression of approx. 600 fold. The same trend is seen in *TRPV6* with a fold induction of approx. 130 being witnessed at 48 hours when exposed to EB1089.

CYP24A1 is one of the key metabolic genes involved in the turnover of physiologically active vitamin D. Within the body a negative feedback loop is set up i.e. increase serum $1,25(OH)_2D_3$ results in a massive induction of *CYP24A1*. The *CYP24A1* graph is viewed as a ratio to HPRT due to the extreme variation in expression potential witnessed when ligand is introduced.

The induction seen within *UGT1A1* and *A4* show less activation potential when compared to *CYP3A4*, *CYP3A5* and *TRPV6*. However the ability of VDR agonists to induce transcriptional responses across all genes analysed remains the greatest when compared to PXR mediated activation. EB1089 shows potential promise as a therapeutic inducer of metabolic gene activity, however more research is required to establish the mechanism of action witnessed through EB1089's ability to regulate and induce target gene expression.



ID	Treatment
1	Ethanol
2	10nM 1,25D3
3	1nM EB1089
4	Methanol
5	20µM Rifampicin

Figure 2.5 Bench Top PCR Analysis of *UGT1A* Family Members: Colon derived LS180 cells were treated with ligands for 24 hrs, the RNA extracted and PCR conducted using Go-Taq® Flexi DNA Polymerase. Three independent experiments were performed and pooled for each treatment group. HPRT has been used and the housekeeping gene with solvent controls for each treatment identified as Ethanol (**ID1**) and Methanol (**ID4**).

2.3.3 Bench Top PCR Analysis of *UGT1A* Family Members

To assess *UGT1A* responsiveness, end point PCR was performed to allow a preliminary assessment of the potential action VDR agonists may have on endogenous levels of *UGT1A* expression in comparison to an established prototypical inducer PXR. Figure 2.5 highlights the potential of VDR ligands to manipulate the expression of the *UGT1A* gene family in LS180 cells.

UGT1A1, A3, A4, A5 and A10 were shown to be regulated by VDR agonists all of which play varying but important roles within metabolism. *UGT1A1* as previously described is directly linked with a number of metabolic disorders, due to mutations being present in the *UGT1A1* gene and or promoter. All of the remaining members having varying roles in the detoxification of many lipophilic substrates including steroids, hormones and xenobiotics, within a wide variety of tissues throughout the body.

There is a consistent pattern witnessed when exposed to VDR agonists. EB1089 shows the most transcriptional responsiveness as is most evident in *UGT1A1*, *UGT1A3* and *UGT1A4*.

It is interesting to note that the ability of VDR to regulate *UGT1A* members rivals that of PXR agonist Rifampicin. All the *UGT1A* family members showed some degree of activation when exposed to VDR agonists. Furthermore all the *UGT1A* members studied above showed transcriptional induction when exposed to the PXR agonist Rifampicin.

The above figure highlights the comparison between VDR and PXR, in their respective abilities to induce a transcriptional response. PXR ligands are used currently in the clinic to induce expression of *UGT1A1* an example of which is Irinotecan used in the treatment of colorectal cancer (Zhuo et al., 2014); therefore PXR remains a good benchmark from which to compare transcriptional activity responsiveness.

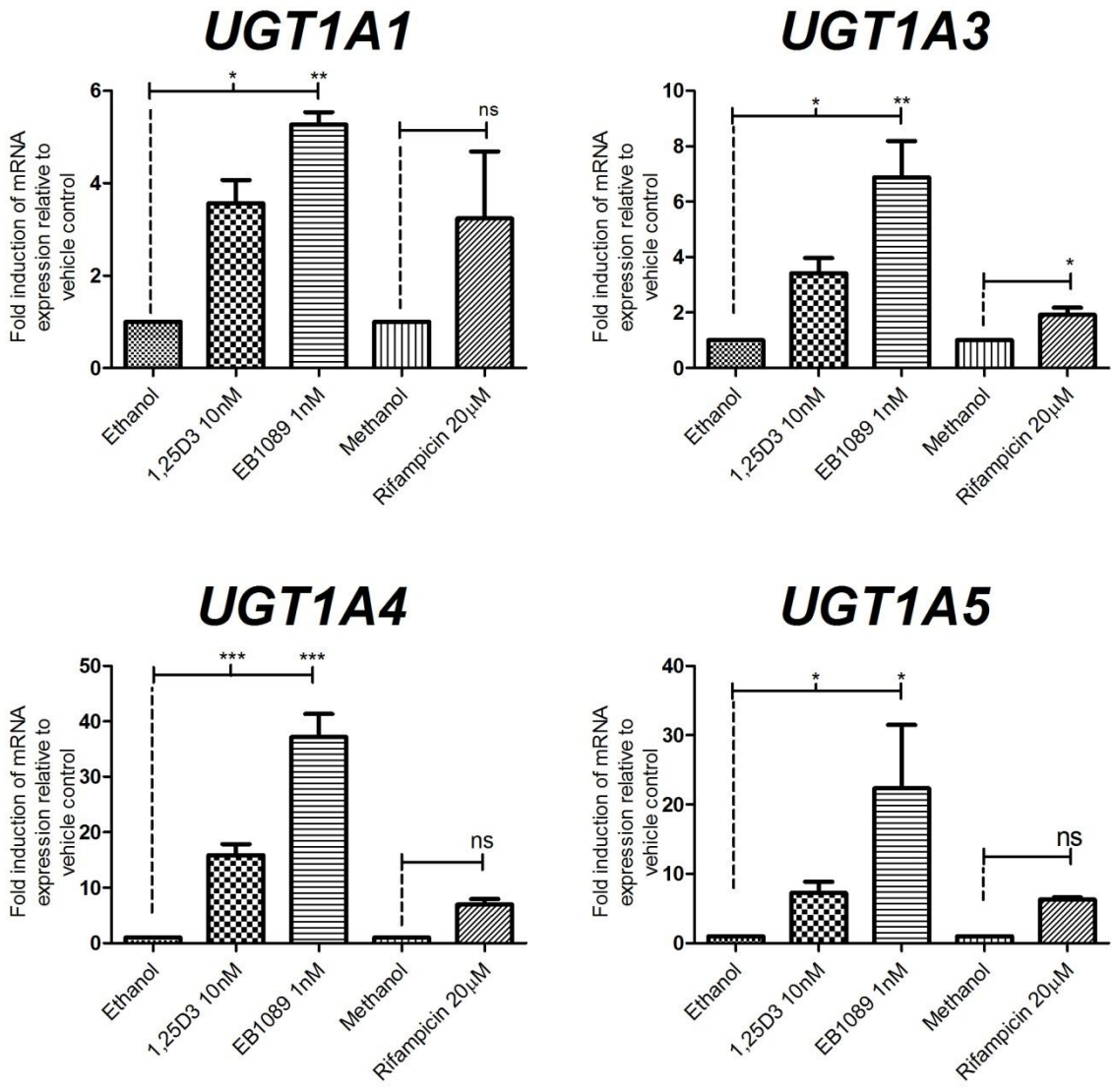


Figure 2.6 qRT-PCR Analysis of *UGT1A* Family Members: *UGT1A* expression profile elicited by VDR and PXR ligands. LS180 cells were treated for 24hrs with vehicle control or indicated concentration of test ligand. Extracted RNA was reverse transcribed subjected to qRT-PCR analysis as per manufacturer's protocol. Data are represented as means (± SEM) of three independent experiments, $p<0.05$ *, < 0.01 **, and <0.001 ***.

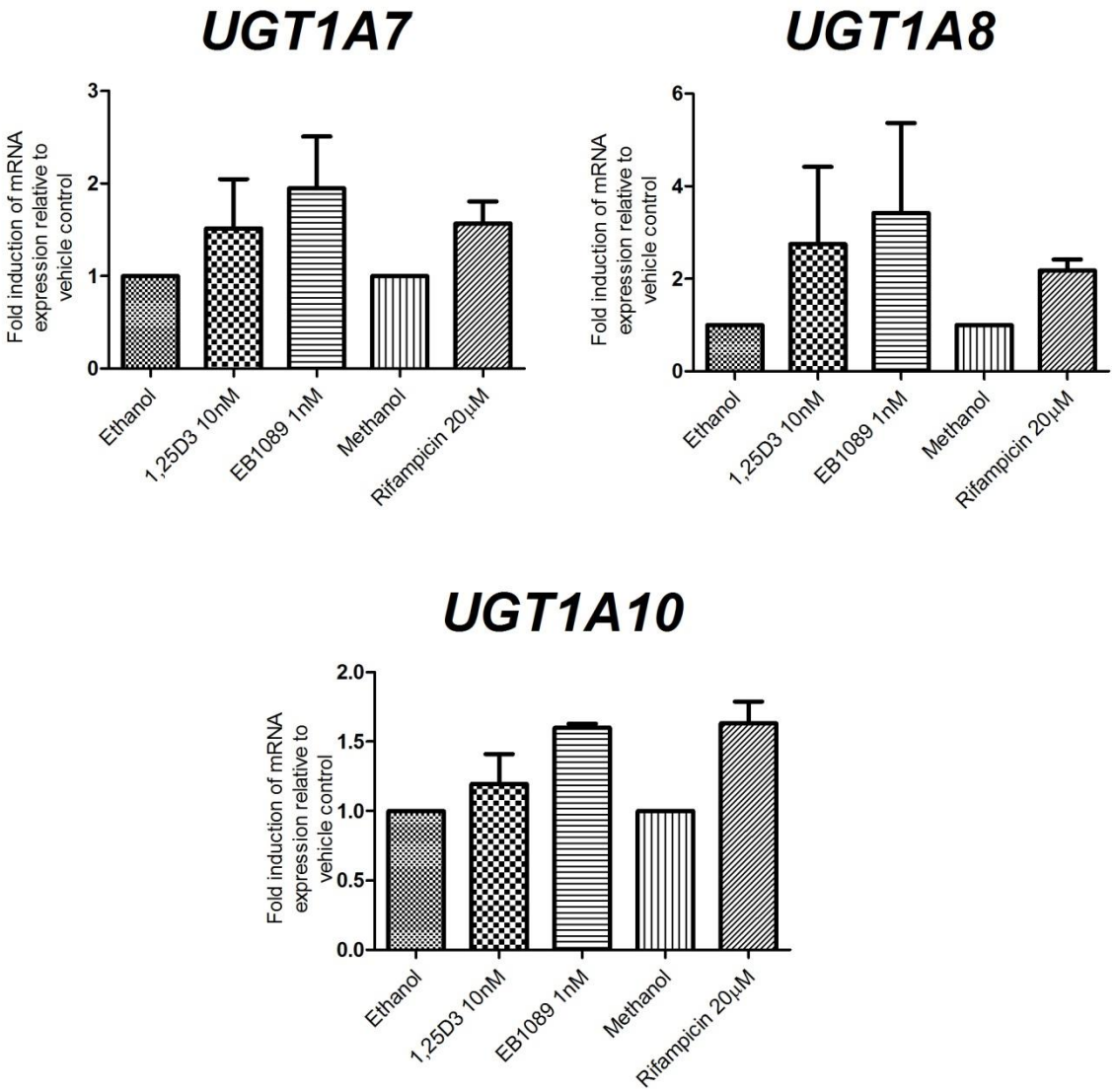


Figure 2.7 qRT-PCR Analysis of *UGT1A* Family Members continued: *UGT1A* expression profile elicited by VDR and PXR ligands. LS180 cells were treated for 24hrs with vehicle control or indicated concentration of test ligand. Extracted RNA was reverse transcribed subjected to qRT-PCR analysis as per manufacturer's protocol. Data are represented as means (± SEM) of three independent experiments. The UGT members detailed above showed no statistical significance.

2.3.4 Quantitative analysis of *UGT1A* members in the LS180 cell line model

To detail further the ability of VDR to regulate *UGT1A* members, qRT-PCR analysis was performed on key members of the *UGT1A* family. *UGT1A1, UGT1A3* and *UGT1A4* were chosen to continue on as subjects in subsequent experiments. These genes were chosen due to their clinical significance as well as their induction profiles witnessed through the dose response, time course and bench top PCR studies. In addition to this *UGT1A3* and *UGT1A4* have been implicated in an auto regulatory capacity within the vitamin D metabolic pathway (Wang et al., 2014b).

Transcriptional induction remains true when exposed to 1nM EB1089. This synthetic agonist has shown super inductive capabilities in a number of CYP450 members including *CYP3A4* and *CYP24A1* (Doherty et al., 2014a). A statistically significant induction does occur especially among *UGT1A1, A3, A4* and *A5* when exposed to both $1,25(OH)_2D_3$ and EB1089, with fold inductions of *UGT1A1* being seen at 4 and 5.5 fold respectively. *UGT1A3* and 4 show profound mRNA induction with *UGT1A3* showing at 8-fold change from control when exposed to E1089. *UGT1A4* indicates also a 40-fold change. The ability of VDR agonists to induce *UGT1A3* and *UGT1A4* lends support to their role in vitamin D metabolism and clearance. It does stand to reason that VDRE agonists would have the ability to induce expression of genes involved in agonist clearance to maintain homeostatic feedback mechanisms.

Furthermore although similar trends were seen in other *UGT1A* members (*1A7, A8, A10*), there was not any statistical significance to report.

UGT1A1, *A3* and *A4* show a greater induction through the actions of endogenous VDR vs. that of PXR, indicating the prospect that VDR induction could be more beneficial than PXR agonists in eliciting a transcriptional response in colon.

Due to the lack of statistical responsiveness seen *UGT1A5*, *7*, 8 and 10 they were excluded from further analysis, allowing for the focus to shift to *UGT1A1*, *A3* and *A4*.

qRT-PCR analysis in non transfected HepG2

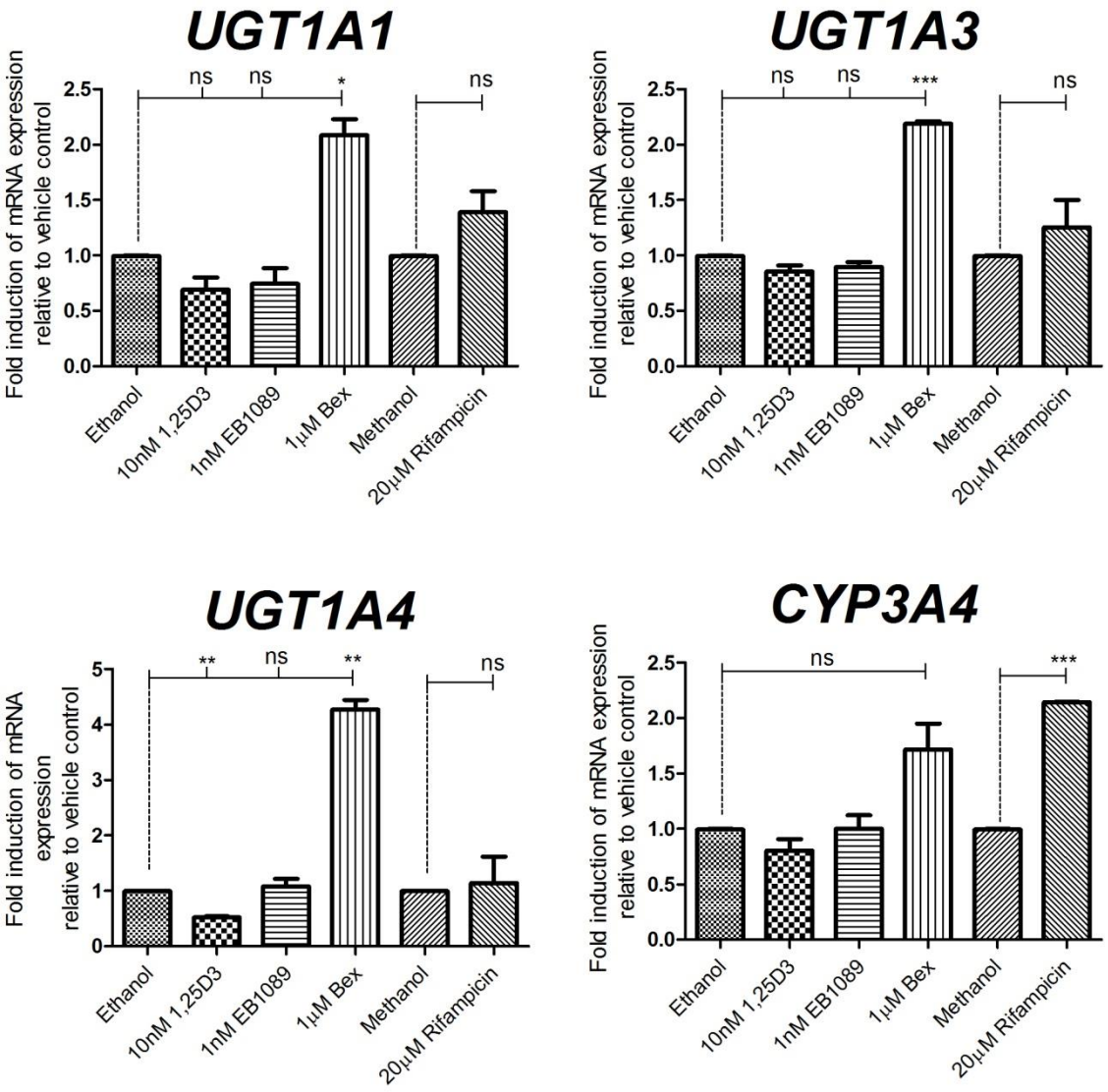


Figure 2.8 qRT-PCR Analysis of *UGT1A* Family Members vs. *CYP3A4* in non transfected HepG2: *UGT1A* expression profile elicited by VDR, RXR & PXR ligands. HepG2 cells were treated for 24hrs with vehicle control or indicated concentration of test ligand. Extracted RNA was reverse transcribed subjected to qRT-PCR analysis as per manufacturer's protocol. Data are represented as means (± SEM) of three independent experiments, $p<0.05$ *, < 0.01 **, and <0.001 ***.

qRT-PCR analysis in transfected HepG2

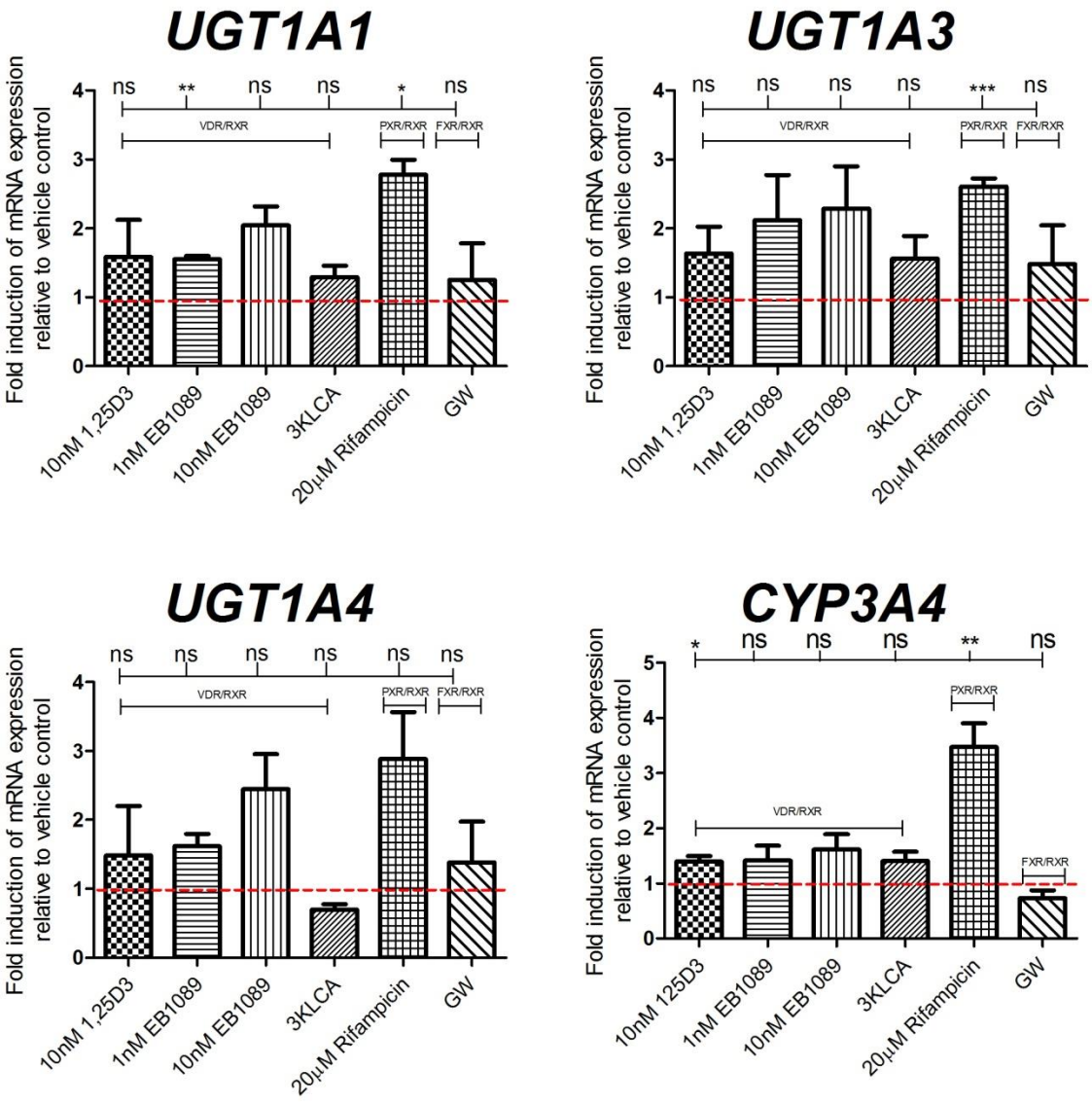


Figure 2.9 qRT-PCR Analysis of *UGT1A* Family Members vs. *CYP3A4* in nuclear receptor transfected HepG2: *UGT1A* expression profile elicited by VDR, FXR & PXR ligands. Each well was transfected using calcium phosphate methodology, with 2μg of V5-VDR, V5-FXR, or psg5-PXR respectively after which the cells were treated for 24hrs with vehicle control or indicated concentration of test ligand. Extracted RNA was reverse transcribed subjected to qRT-PCR analysis as per manufacturer's protocol. Data are represented as means (± SEM) of three independent experiments, $p<0.05$ *, < 0.01 **, and <0.001 ***.

2.3.5 Comparison of VDR and other related nuclear receptors to regulate *UGT1A* gene expression in a hepatic cell model

The HepG2 cell line model was employed as a representative, hepatic cell line model allowing for the comparison between LS180 (colon), and HepG2 (liver) to be carried out. The liver remains the primary site for biological detoxification within the body and as such boasts the widest range of metabolic genes. HepG2 cells do not express high levels of VDR; therefore the ability of VDR to regulate potential targets within the liver is limited. This is consistent with literature in that VDR has limited expression in hepatic tissue. Conversely PXR and a number other nuclear receptors are highly expressed throughout the liver (Lehmann et al., 1998b) and as such remain key regulators within the hepatic environment. HepG2 cells showed very little statistical induction of *UGT1A* members through invoking activation of endogenous VDR. Furthermore the ability of Rifampicin to elicit a response through endogenous PXR was also lacking. PXR however remains expressed at appropriate levels as induction was witnessed by the known PXR inducible gene *CYP3A4* giving an mRNA fold induction of 2 fold (figure 2.9).

The inclusion of bexarotene was considered, as RXR alpha (RXRα) is the heterodimeric partner that both VDR and PXR share to elicit their respective target responses. It is interesting to see that bexarotene as an RXR alpha inducer has the ability to also induce *UGT1A* members, perhaps warranting further investigation, however this is beyond the scope of this thesis.

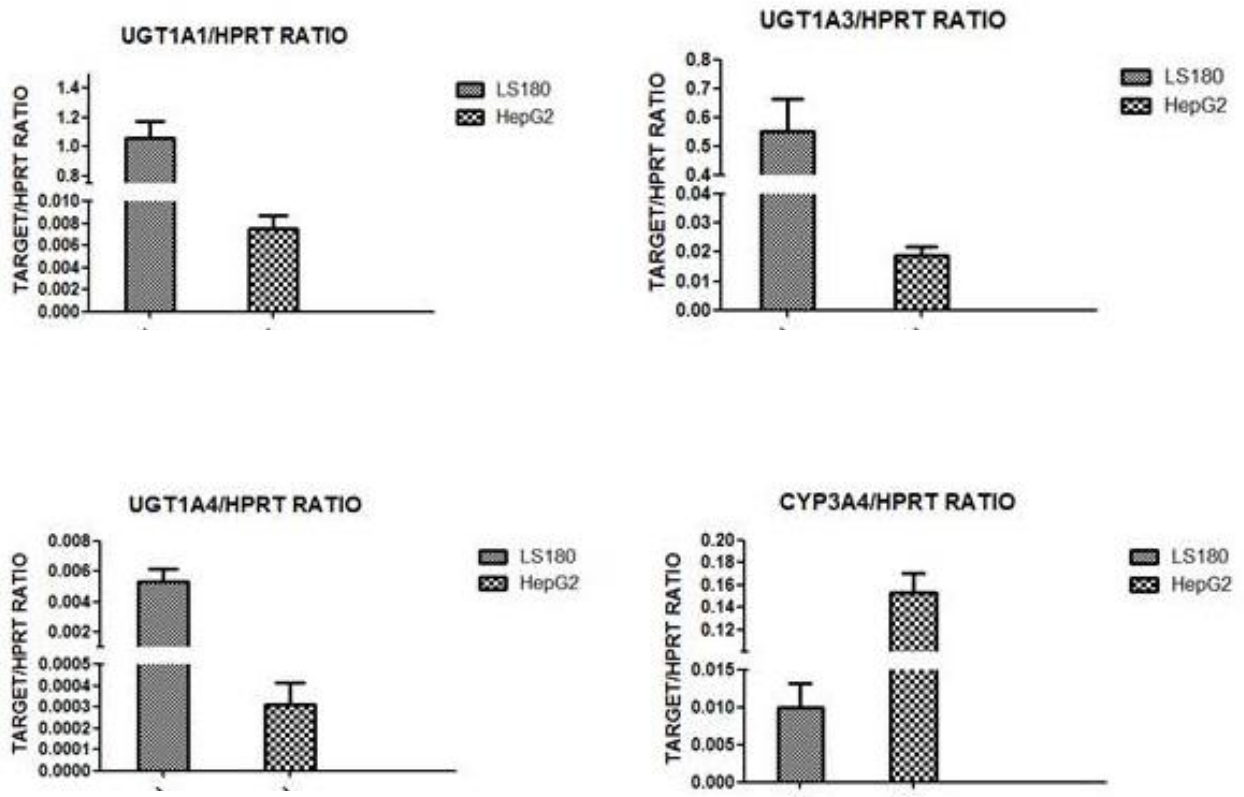


Figure 2.10 Absolute basal qRT-PCR Analysis of key *UGT1A* members vs. *CYP3A4*: The RNA was extracted from colon derived LS180 and hepatic HepG2 and RT-PCR conducted using SSRT (Invitrogen). qRT-PCR analysis was performed on the LC480 Light Cycler. Plotted is the relative target to housekeeper ratios across three independent experiments run in duplicate.

2.3.6 Basal qualitative analysis of *UGT1A* members in comparison to known VDR target gene CYP3A4 in the HepG2 and LS180 cell line models

Figure 2.10 highlights the relative basal expression levels of each target gene (*UGT1A1, UGT1A3, UGT1A4,* and *CYP3A4*) in the absences of activating ligand. This gives a sense of the expression levels relative to each of the cell lines being explored.

It is evident that *UGT1A* members are expressed more highly in the enteric cell line model vs. the hepatic model. This adds additional credence to the rationale that extra hepatic expression of *UGT1A* members, controlled through VDR mediation could potentially play a vital role in overall detoxification and genome wide protection in additional tissues outside that of the liver.

The reverse is seen in *CYP3A4* basal expression, showing an increase basal expression in liver cells over the colonic model; however the ability of VDR to regulate *CYP3A4* activity outside the liver has been documented through natural and synthetic vitamin D ligands (Doherty et al., 2014b) .

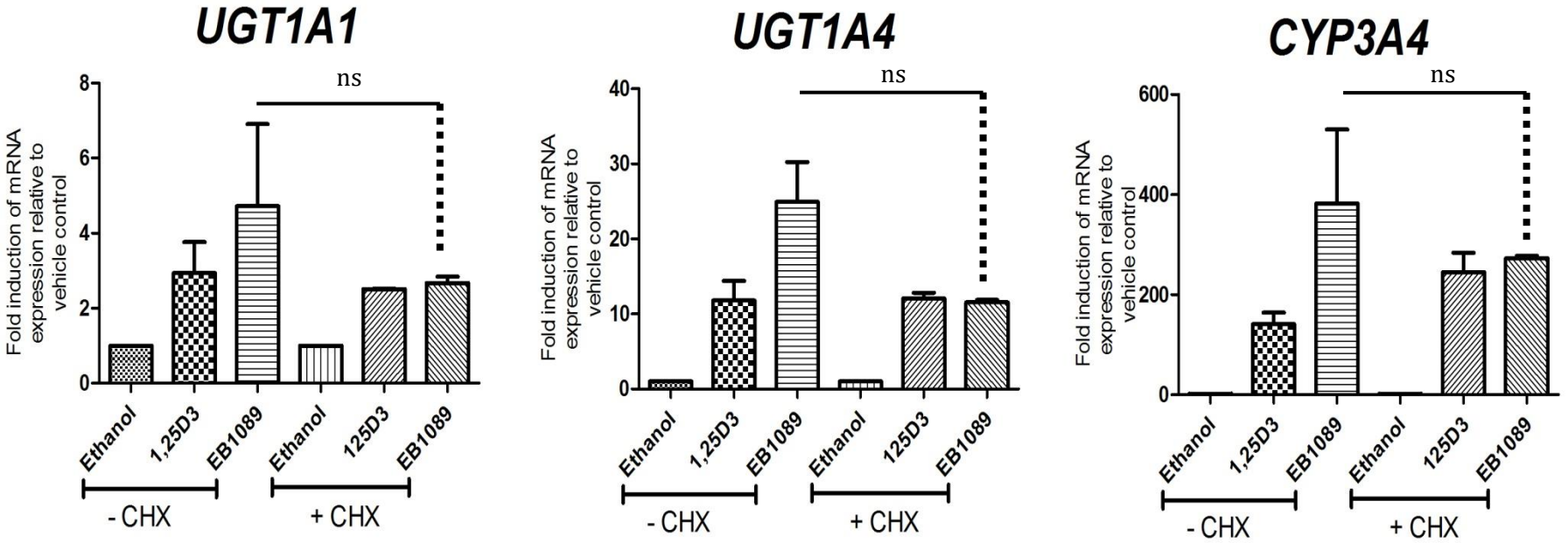


Figure 2.11 *UGT1A1* is a direct Vitamin D/Vitamin D Receptor target: LS180 cells were treated with VDR ligands 1,25D3 (10nM) and EB1089 (1nM) with (+) and minus (-) cyclohexamide (CHX). The extracted RNA was reverse transcribed and subjected to qRT-PCR analysis as per manufacturer's protocol. Data are represented as means (± SEM) of two independent experiments with each data point run in triplicate.

2.3.7 *UGT1A1* is a direct Vitamin D/Vitamin D Receptor target

Cyclohexamide is a protein synthesis inhibitor by interfering with the translocation steps in protein synthesis blocking the elongation process in translation.

Cyclohexamide was employed within this study to assess if VDR can regulate *UGT1A* members as a result of a direct transcriptional event and not through any secondary mode of regulation. LS180 cells were treated with the concentration indicated in figure 2.11 with the addition of cyclohexamide or solvent control.

There was no statistical significance noted with the addition of cyclohexamide. EB1089 did not loss its ability to induce expression of *UGT1A1*, *A4* or *CYP3A4,* giving mRNA level inductions comparable to the time course analysis at 24hrs, however it did seem to inhibit the previously witnessed 'super induction' of target genes. Perhaps there is a tertiary interaction between ligand affinity being interrupted with the introduction of cyclohexamide that reduced the efficacy of EB1089? This is an area that has potential for further investigation but is beyond the scope of this thesis.

What can be concluded from the cyclohexamide study is that no tertiary protein is generated that could subsequently act on the promoter regions of the VDR targets eliciting a response. It is through direct VDR activity and not a secondary protein generation that *UGT1A1*, *UGT1A4* and *CYP3A4* respond to VDR agonists.

Chapter 2: Discussion

2.4 Discussion

Many studies have shown the role vitamin D and the vitamin D receptor have in targeting a number of metabolic genes within a range of disease states including prostate (Williamson et al., 2013), breast (Fuady et al., 2016) and colon cancers (Audet-Delage et al., 2017). The finding that VDR can induce *UGT1A* expression in the LS180 cell line model further highlights the potential importance vitamin D and by extension, the vitamin D receptor in overall detoxification activity outside that of the hepatic environment.

This chapter explores the expression of *UGT1A* gene family members in one colon cancer cell line as well as a liver cell line model (HepG2) allowing comparisons to be drawn. Defining further the potential role vitamin D and by extension the vitamin D receptor could have in overall regulation of these phase two metabolic genes.

Microarray data has shown that VDR has the ability to induce phase two metabolic gene and stress response activity within the intestine as a result of the actions of $1,25(OH)_2D_3$ (Kutuzova and DeLuca, 2007), however this was the first study to explore VDR mediated activity of *UGT1A* genes, comparing the ability of VDR against other known and established inducers such as PXR. The microarray analysis carried out by (Kutuzova and DeLuca, 2007) witnessed a 1.7 fold increase in expression levels of *UGT1A* members after 6 hours of exposure to agonist.

It is also of great interest that the synthetic VDR ligand, EB1089, shows an ability to induce further *UGT1A1, UGT1A3,* and *UGT1A4* to a greater extent than natural VDR agonist, $1,25(OH)_2D_3$ and Rifampicin, a PXR agonist. EB1089 has been utilized within clinic trails as an anti cancer treatment (Hansen et al., 2000). Furthermore EB1089 has been shown to be used as an anti proliferative agent in some colon cancers (Akhter et al., 1997). EB1089 as a synthetic analogue of natural $1,25(OH)_2D_3$ has all the properties of its natural counterpart

without the hypercalcaemia influences witnessed when increased natural $1,25(OH)_2D_3$ is administered within the clinic. Within the Thompson laboratory we have witnessed an increased transcriptional response of other VDR target genes including *CYP3A4* and *TRPV6* when exposed to EB1089. Witnessing a similar trend within the *UGT1A* family indicates that VDR could have the potential to regulate expression and activity.

Furthermore the ability of VDR to induce a transcriptional response requires ligand concentrations at a nano molar (nM) level compared to a micro molar (µM) level when PXR agonist is used this could be caused by the confirmation of the nuclear receptor protein, requiring a higher concentration of ligand before the binding affinity threshold between ligand and receptor is reached due to the conformation of the ligand binding pockets (Mizwicki et al., 2004). This in itself shows the greater affinity *UGT1A* members have towards VDR mediated activation over PXR stimulation.

VDR agonists have the ability to elicit transcriptional responses at a nano molar level; this suggests that VDR activation of metabolic gene activity is a more deeply regulated process, maintaining a basal expression providing a homeostatic balance potentially generated basal genome protection through continual transcriptional signaling elicited through circulating VDR agonists at nano molar concentrations.

All the *UGT1A* members explored within the study exhibited some degree of response to VDR mediated activation with *UGT1A1, A3, A4* and *A5* exhibiting the greatest responses.

The ability of VDR to regulate *UGT1A3* and *UGT1A4* is of interest as glucuronidation of 1,25D3 has been witnessed through the activity of both these *UGT1A* gene members (Wang et al., 2014c). Furthermore the movement and transportation of vitamin D based glucuronide conjugates may contribute to the intestinal VDR responsive target genes including *CYP24A1* and *TRPV6*. Thus the delivery of glucuronide conjugates through the biliary tract into the

proximal intestine has been shown to serve as a possible endocrine/paracrine signaling loop of VDR dependent gene expression and activity (Wang et al., 2014a).

The real time analysis was carried out in LS180 and HepG2 cell lines, allowing for a direct comparison to be made between the ability of VDR to elicit a transcriptional response. HepG2 cells contain low endogenous levels of VDR and perhaps the concentration of ligand used in these experiments was not sufficient to elicit any statistically significant responses (Han and Chiang, 2009). This allows us to examine the impact of exogenous VDR upon the expression of these genes in a cell line derived in a tissue of metabolic functioning.

This in itself gives additional credence to the possible extra hepatic role VDR has in regulating *UGT1A* members, indicating that there may be tissue specific regulation of this gene family, however further investigation would be required to ascertain completely the tissue specific nature of the *UGT1A* gene family.

Furthermore to ascertain that adequate levels of VDR and PXR were transfected, a western blot approach could have been used.

Non-transfected HepG2 even when exposed to the PXR agonist Rifampicin, showed no statistically significant induction of *UGT1A* members. This was compared to *CYP3A4* highlighting the PXR response is present, indicating adequate levels of PXR being present to elicit certain target gene responses. Attributing to this could be the basal expression levels of *CYP3A4* vs. *UGT1A* members in the HepG2 cell line model. *CYP3A4* exhibits a higher basal expression over *UGT1A* members allowing for a greater activation potential to be achieved when exposed to agonist.

The lack of *UGT1A* activation could be due to the fact that HepG2 are a transformed cell line and perhaps primary hepatocytes would contain additional transcription factors that could enable a PXR mediated response of these phase two genes.

Additionally when the comparison of basal levels of metabolic gene expression was ascertained between LS180 and HepG2 there was a distinctly lower level of expression in HepG2 as is seen in figure 2.10. Basally, there is more expression of *UGT1A* members in colon vs. liver, conversely the expression of the phase one gene *CYP3A4* was higher in HepG2 over LS180; the real time data supports this observation. This finding highlighting basal expression between the LS180 and HepG2 cell line models, cements further, the potential extra hepatic role *UGT1A* members could play in overall body detoxification. Intestinal expression of key metabolic genes involved in detoxification as well as bile acid sensors have the ability to be regulated through the actions of VDR. VDR has already been shown to be highly expressed within the intestine and as such has the potential regulate *UGT1A* genes within the intestinal environment. Altered intestinal UGT expression resulting in increased glucuronidation activity has been shown to exacerbate bile acid injury within the colon (Zhou et al., 2014), indicating that nuclear receptors ability to regulate metabolic gene activity play vital roles in bile acid homeostasis and pathological development of colitis as well as other inflammatory based diseases of the intestine and colon, including IBD and Crohn's Disease.

Through the dose response and time course studies, we were able to observe the effect increasing concentration of both natural and synthetic ligands had on *UGT1A1* and *UGT1A4* responsiveness in comparison to known VDR target genes. Both *UGT1A1* and *1A4* exhibit dose dependent responses, producing similar trend profiles. *UGT1A4* shows a greater induction over *UGT1A1* eliciting approx. 20 fold induction when exposed to natural vitamin

D and 40-45-fold increase when challenged with EB1089. Both family members showed stronger responses to VDR activation over PXR mediated activation

In conclusion these results highlight the ability of VDR to elicit a transcriptional response to the same degree, if not more potently that known and established prototypical inducers such as PXR at a ligand concentration far lower than that of PXR agonists (nM vs. µM). Furthermore the lack of VDR expression in the liver indicates a more extra hepatic role vs. primary hepatic functionality. This is further supported through the increased basal expression of *UGT1A* members in the extra hepatic cell line LS180, used throughout this research.

Chapter 3: Identification and validation of a Vitamin D Response Element (VDRE) within the *UGT1A1* distal enhancer module.

3.1 Introduction

Nuclear receptors encompass a huge family of proteins that act as transcription factors binding lipophilic ligands ultimately mediating a transcriptional response of target genes, both activation and repression, through the recognition of specific stretches known as DNA response elements located within the promoter regions of gene. These response elements are recognized through the actions of the DNA binding domain of the nuclear receptor.

Nuclear receptors

Nuclear receptors belong to a super family of proteins and as such share closely related structures in their physical make up. All nuclear receptors contain a ligand binding domain, and a DNA binding domain. The DNA binding domain is comprised of a 66- residue conserved core and C terminal extension (Moras and Gronemeyer, 1998). Furthermore the DNA binding domain contains eight highly conserved zinc fingers, the function of these zinc residues are to maintain the integrity of the binding domain allowing for the structure to hold fast. DNA binding is achieved through the tetrahedral co-ordination zinc with four cysteine residues in two distinct extensions that form two structural "zinc fingers". Zinc fingers are common among gene regulatory proteins and the specificity of hormone response elements is achieved through the more conserved hydrophilic first zinc finger (C1), whereas the 2nd zinc finger is involved in dimerization and stabilizing the DNA binding through interactions with the DNA phosphate backbone. The removal of the zinc molecules from the DNA binding domain leads to the complete unfolding of the DNA binding domain, resulting in an inability to bind DNA response elements.

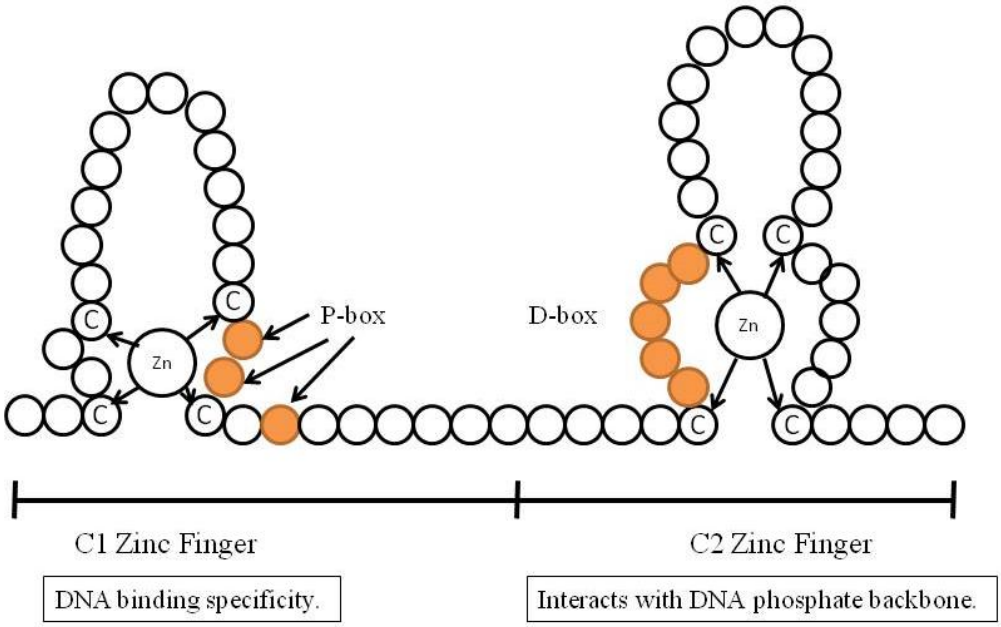


Illustration 3.1: Schematic showing zinc finger protein characteristics within the DBD structure of steroid hormone nuclear receptor family members.

The C1 zinc finger interacts directly with five base pairs of DNA determining the DNA sequence to be recognized by particular steroid nuclear receptors. The three orange amino acids highlighted above indicate the P-box region within the C1 motif. The shaded amino acids on the right indicate the D-box, involved in dimerization and contacts with the DNA phosphate backbone.

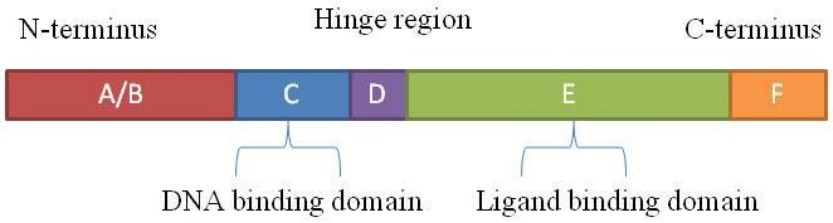


Illustration 3.2: 1 dimensional representation of nuclear receptor structure.

The promoter: DNA response elements

The transcription of DNA to messenger RNA is one of the most important processes controlled by steroid nuclear receptors. All genes within the body share a common basic structure and design. Structural regions include stretches of DNA that encode for specific amino acids, and a regulatory region that interacts with various proteins that control the overall rate and transcription. Within these regulatory regions several key elements must be present before transcriptional activation can occur. These elements are known as cis-acting elements, and are located near the 5' end of the gene, and consist of four main groups; 1) promoters, 2) hormone response enhancers 3) silencers and 4) hormone independent enhancers.

The promoter of a gene is located closest to the transcriptional start site. DNA that nuclear receptors recognize and bind to are known as DNA response elements (REs), and can be found both preceding and after the gene the element has a transcriptional effect on. The recognition and binding of response elements can be achieved through homo and heterodimeric interactions. There are a number of nuclear receptors that act as monomers, principally nuclear receptor subfamily 2 (Khorasanizadeh and Rastinejad, 2001).

Depending on which DNA binding mofit is targeted during nuclear receptor activation, will determine if the target gene is trans activated or repressed. The RAR/RXR heterodimer can elicit both outcomes (Aranda and Pascual, 2001a). Binding of a direct repeat separated with one nucleotide results in repression of transcription whereas the binding to a direct repeat separated by five nucleotides results in activation of the target gene.

There are a range of motifs that have extremely potent effects as a result of nuclear receptor binding. Direct repeats and everted repeats both remain highly effective at eliciting transcriptional responses upon NR binding (Aranda and Pascual, 2001a).

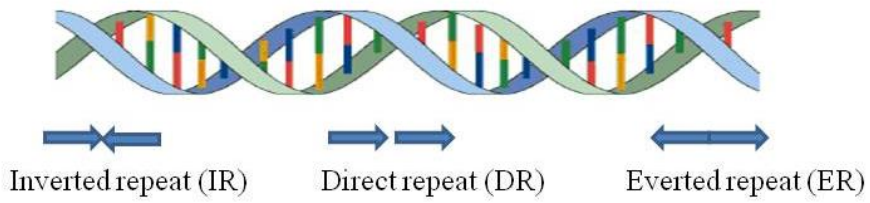


Illustration 3.3: Hormone response elements found within human DNA promoter regions.

Vitamin D response elements

Many of the genes that respond to the actions of the Vitamin D Receptor (VDR) contain within their promoter region direct repeat response elements separated by three nucleotide spacers (DR3) or everted repeats separated by six spacer nucleotides (ER6). VDR forms a heterodimeric complex with RXR alpha and forms said complex in a 5'-RXR/VDR-3' polarity where RXR occupies the upstream half site and VDR occupies the down remaining down stream half site (Schräder et al., 1995). VDR has been seen to act as a homodimer however little effect is seen and is therefore thought not to play a major role in transcriptional control (Cheskis and Freedman, 1994).

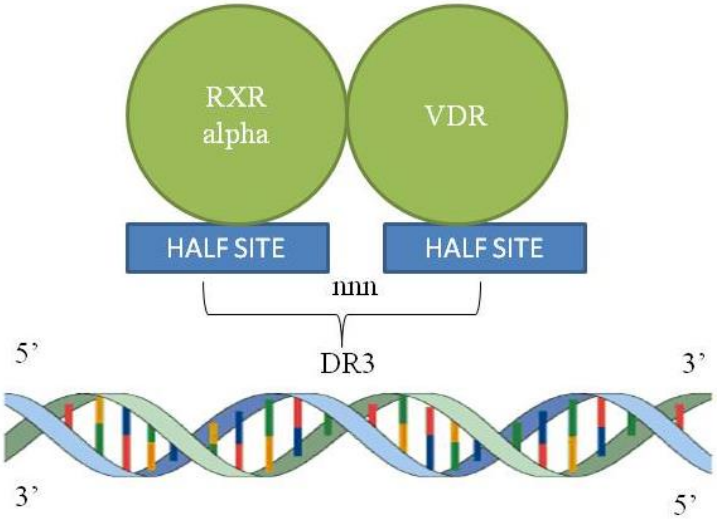


Illustration 3.4: Schematic highlighting the DR3 configuration within potential VDR targeted genes.

The homology shared between the nuclear receptors indicates toward the potential for crosstalk between each of the receptors members. As such the pregnane X receptor (PXR) has the ability to bind to a number of response elements such as the DR3 and ER6 motifs, highlighting potential for competitive binding to occur between the two receptors. This homology between PXR and VDR highlights the intriguing potential for VDR to regulate detoxification genes as PXR has been established as the master regulator of many metabolic genes both in a hepatic and extra hepatic capacity.

Chapter Rationale

Within this chapter VDR ligands were shown to have the ability to induce the phase two metabolic gene *UGT1A1*. To further characterize the induction profiles; comparison between known and established inducers of the *UGT1A1* gene promoter were used to ascertain a base line from which to compare the ability of VDR to induce *UGT1A1* at a transcriptional level. This was achieved through bioinformatic analysis ascertain the presence and precise location of any classical vitamin D response elements (VDRE) within the promoter enhancer module of the *UGT1A1* gene. Two promoter constructs were kindly gifted to our lab by Professor Masahiko Negishi (North Carolina, USA). One construct containing a 2 kilobase fragment of the *UGT1A1* promoter beginning from the start site of the promoter to include an enhancer region previously described by (Tukey et al., 2001). This enhancer module contains various response elements for a number of nuclear receptors previously reported. The second promoter vector contained only the 290bp enhancer module as described above. Both these constructs were created using the pGL3 basic backbone, commercially available through Promega. The human embryonic kidney cell or HEK-293 cell line and HepG2, previously described, was used throughout these experimental procedures. HEK-293 model has been well established and characterized. It is an easily transfectable cell line model and can be used as a 'blank' host for transfection/luciferase studies. Luciferase reporter assays were performed initially comparing both constructs to determine which vector responded better or potential VDRE motifs. This would allow us to focus primarily on a single vector construct moving forward. Luciferase activity was then coupled with mutagenesis reactions to assess identified motifs for their specificity and requirement for intact sequences for mediating a response to VDR ligand and role on the overall activation potential of the *UGT1A1* gene.

Chapter 3: Materials and Methods

3.2.1 Cell Culture

HEK-293 cells were maintained in Minimum Essential Media (MEM), 10% foetal bovine serum and 1% penicillin/streptomycin (Gibco, Paisley, UK) and kept at 37°C, 5% CO_2 and 95% air. Trypsin disassociation was employed as previously described in materials and methods, chapter 2.

3.2.2 Vectors used in study

Both the pGL3-UGT1A1-U2K, and pGL3-UGT1A1-290 were kindly provided by Professor Masahiko Negishi, Research Triangle Park, North Carolina.

The various nuclear receptors such as VDR-V5, FXR-V5, LXR-V5, PXR-psg5, RXR-psg5 were all generated in house using the V5 or psg5 backbone allowing for the V5 epitope to be identified later on using western blot technologies. The process of generating these constructs is detailed in section 2.16 and 2.17.

3.2.3 Luciferase activity assay in HEK-293 to measure VDR, FXR, PXR, LXR (V5)-RXR (psg5) mediated transcriptional activity using Calcium Phosphate transfection

24 well plate set up

Day One: $1x10^5$ cells were seeded in each well of a 24 well plate, 500µl final volume.

Day Two: Cells were transfected with 350ng, of reporter vectors, *UGT1A1*-290bp, or 500ng *UGT1A1*-U2K respectively, 100ng of V5-VDR, V5-FXR, V5-LXR, and 100ng psg5-RXR were also added; assay dependent. The total amount of DNA was kept constant using empty pgl3-basic vector (up to 1µg).

Day Three: Cells were washed with 1 x PBS and treated with the appropriate ligand and complete media mix for 24 hours.

Day Four: The cells were lysed in 150µl of 1 x reporter lysis buffer and placed in

a -20°C freezer to undergo a freeze thaw cycle, and subsequently placed on a moving platform for 45 minutes to ensure complete cell lysis. 20µl of the cell lysate was then loaded into an opaque 96 well plate. To each well 50µl of luciferase substrate (LARII), (Promega, Madison, USA) was added and luminescence was recorded using the FLUOstar Omega micro plate reader. The raw luciferase numbers were then normalised to total protein concentration as described previously.

3.2.4 Calcium Phosphate Transfection

Within this set of experiments the calcium phosphate methodology was used as the system had been previously optimized within the laboratory. This protocol employs 2M Calcium Chloride ($CaCl_2$) and 2 x HBS (50mM HEPES, 280mM NaCl, 1.5mM Na_2HPO_4), that can be prepared before hand sterile filtered through a 0.22µm filter and stored at -20°C.

Day One: HepG2 or HEK-293 were collected from a confluent flask (~70-75%), and seeded into the appropriate plate size depending on the experiment being performed. The cells were then incubated overnight to allow the cells to adhere and establish within the plate.

Day Two: On the day of the transfection, 2M $CaCl_2$ and 2 x HBS was removed from storage and allowed to completely thaw and come up to room temperature before use. The cells being transfected were washed with warm 1 x PBS before fresh media was applied.

The transfection mixes containing DNA/$CaCl_2$/H_2O was then added in a 1:1 ratio to 2 x HBS, and allowed to precipitate at room temperature for 30 minutes before being applied to

the cells in a drop-wise manner. The plates were then incubated between 16-18 hours, any longer than this and cell death can occur.

3.2.5 Site-directed mutagenesis reactions

Mutant primer design

All mutant primers were designed using the online Agilent Quick Change Mutant Primer design software. The online system uses built in parameters to generate optimum mutant primers for the input sequence provided. The mutagenesis PCR was used to create two base pair changes within a postulated VDRE located in the enhancer module of the *UGT1A1* gene promoter vector. The base change from Wild Type to mutant is detailed further in the results section of this chapter.

Mutant primers used within mutagenesis reactions

Primer Name	5' to 3' Sequence
VDRE Mutant	caaagaacattctaacggttcataaagtatattaggtgtaatgaggatgtgttatc gataacacatcctcatttacacctaatatactttatgaaccgttagaatgttctttg

Site-directed mutagenesis reaction

Two point mutations were introduced using the Agilent Quick Change site-directed mutagenesis kit (Agilent Technologies, Texas, Cat: 200519-5). The recipe below was used for the PCR amplification using reagents in the kit provided.

	Volume (µl)
- 10 x Reaction buffer	5
- Forward Primer (125ng)	x
- Reverse Primer (125ng)	x
- Template (50ng/µl)	1
- dNTP mix	1
- PCR grade H_2O	up to 50µl
- *Pfu* Turbo enzyme	1

After all the reagents were added to the PCR tube the reaction mix was briefly spun before being subject to PCR amplification under the following conditions.

Mutagenesis Thermocycler conditions

Initial Denaturation		30 seconds, 95°C
Denature	***12 cycles***	30 seconds, 95°C
Annealing		1 minute, 55°C
Extension		5 minutes, 68°C
Final Extension		***Disabled***
Hold		4°C

The extension time is determined by the length of the template being mutated i.e. 1 minute for every 1 kilobase of template. Upon completion of the amplification process 1µl of *Dpn I* restriction enzyme was added and incubated at 37°C for 1 hour. Once the digestion was complete, standard transformation protocol was followed to transform 1µl of the PCR mix into library competent DH5α cells using S.O.C. broth as recovery media. Minipreps were then made and confirmation of the mutant carried out via restriction digest and DNA sequencing as detailed above. Upon confirmation of mutated plasmid the miniprep was re-transformed using SE-DH5α cells and grown up to be subject to maxipreparation.

3.2.6 Whole Cell Protein Extraction

Transfected cells had media removed and washed in 37°C, 1 x PBS. 1ml of trypsin was then added to the plates and allowed to incubate for 5 minutes. 2mls of complete media was then added to neutralize the trypsin. The plates were washed to ensure complete removal of all cells into suspension, before being collected and spun at 2000 rpm in a universal. The resulting pellet was then washed in ice-cold 1 x PBS, before being spun at 2000 rpm reforming the cell pellet. The supernatant was then removed and the cell pellet resuspended in ~100-200µl of RIPA buffer (25mM Tris-HCl; pH 7.8, 150mM NaCl, 1mM EDTA; pH 8.0, 0.5% Igepal CA-630, 0.5% Sodium Deoxycholate, 0.1% SDS, 1mM DTT, 10% glycerol, and protease inhibitor cocktail), (Sigma), depending on the size of the cell pellet being resuspended. The lysis mixture was then maintained on ice for 30 minutes, with agitation every 10 minutes to ensure complete lysis of the cells. The lysis mixture was then spun at 12,000 rpm for 10 minutes at 4°C. The resulting supernatant was collected and stored at -80°C for future use.

3.2.7 Bio-Rad DC protein assay

The total protein yield from whole cell lysate extractions and reporter lysis buffer extractions were quantified using the Bio-Rad DC protein assay (Cat: 500-0116). Protein standard bovine serum albumin (BSA) was requisitioned from Sigma at a stock concentration of 2mg/ml and underwent a 2 fold serial dilution using 1 x PBS when the assay was being performed. The protein assay was performed in a tissue culture grade 96-welled plate, and standards were generated to a final volume of 10µl.

Whole cell lysate: These samples were thawed on ice and diluted 2µl of lysate in 8µl of 1 x PBS (1:5 dilution factor), creating a final volume of 10µl.

Reporter buffer lysis: A 10µl aliquot of lysate was loaded directly into a 96-welled plate.

To the 10µl standards and samples 25µl of A' was added. A': prepared by adding 20µl of reagent S (Bio-Rad Cat: 500-0115), and 1ml of reagent A (Bio-Rad Cat: 500-0113). This was then immediately followed by the addition of 200µl of reagent B to each well (Bio-Rad Cat: 500-0114). The plate was then allowed to incubate at room temperature for 15 minutes and the absorbance read at a wavelength 750nm using FLUOstar Omega microplate reader. All protein concentrations are calculated against the standards using the formula protein conc. = (absorbance-intercept)/slope.

3.2.8 Western blot analysis

Over expression of V5-based constructs were detected by western blot, using home made gradient gels (4-10%), and the Bio-Rad Trans-Blot semi dry transfer cell. 30-50µg of lysate was loaded along with LDS, reducing agent, and RIPA, as per laboratory protocol.

	Volume (µl)
- Cell lysate (30-50µg)	x
- NuPAGE LDS sample buffer (4x)	5
- NUPAGE Reducing Agent (10x)	2
- RIPA Buffer	made up to 20µl

Each sample along with any blanks were heated to 95°C for 5 minutes before being loaded into the wells of the gradient western gel. 5µl of Spectra™ Multicolour Broad Range Protein Ladder (Fermentas) was loaded into well number 1. The gel was run in SDS PAGE western blot running buffer (recipes highlighted below). The gel was run at 80 volts for 10 minutes to allow entry of the protein into the 4% stacking gel, before switching to 150 volts for 1 hour. After the electrophoresis had run, the gel was placed on the semi dry transfer cell to transfer the resolved proteins onto a nitrocellulose membrane (Amersham Biosciences). The transfer cell was run at 20 volts for 1 hour. To ensure complete transfer of proteins to the membrane was stained with Ponceau S solution (Sigma Cat: L7170). The stain was then removed by agitated washing in TBS-T (Tris Buffered Saline and Tween-20), this was followed by a 5% block using a milk powder solution (Marvel), made up using 1 x TBS-T, for 1 hour at room temperature. The blocked membrane was then washed in 1 x TBS-T for 10 minutes to remove any excess blocking solution before being placed in TBS-T containing primary antibody for overnight incubation and 4°C. The following day the membranes were washed

3 times for 10 minutes in 1 x TBS-T to remove any unbound primary antibody. Once the final wash was complete the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase in 1 x TBS-T for 1 hour at room temperature. This was then followed by another 3 washes in 1 x TBS-T each of 5 minutes. The membranes were then incubated for 5 minutes with Millipore Immobilon Western Chemiluminescent HRP substrate (Millipore; WBKLS0100) containing equal volumes of luminol solution and peroxide buffer; 500µl of each was sufficient. The luminescent signal was then detected using the Sygene G:BOX Chemi system.

Antibodies and their dilutions used in Western blot analysis

Primary Antibody		**Secondary Antibody**	
Mouse anti V5 (Invitrogen, Cat: 46-0705)	1:5000	Rabbit anti Mouse (Serotec, STAR13B)	1:3000
Rabbit anti HPRT (Serotec Cat: sc-20975)	1:1000	Goat anti Rabbit (Santa Cruz, sc-2004)	1:5000

Western blot reagents

SDS PAGE Western Running Buffer	**Amount**
Tris-Base	3g
Glycine	14.4g
SDS	1g
Make up to 1000ml using dH_2O and store at room temperature	
Semi Dry Transfer Buffer	
Tris-Base	6.9g
Glycine	28.8g
Methanol	400ml
Make up to 2000ml using dH_2O and store at room temperature	
10 x TBS	
NaCl	87.6g
Tris-Base	60.55g
Add 800ml of dH_2O and pH to 7.5 using conc. HCl, make up to 1000ml post pH.	
1 x TBS-T	
10 x TBS	200ml
dH_2O	1800ml
TWEEN-20	2ml

3.2.9 Mammalian-1-Hybrid/GAL4 based Transcription Assay

Principle

The mammalian-1-hybrid is an artificial system used to measure protein-DNA interactions. In this system the detection is based on the interaction of a single protein (known as the prey) with a bait DNA sequence position up stream of a reporter gene. The prey protein is fused to a transcriptional activation domain; therefore positive protein DNA interactions bring the fusion activation domain into close proximity with the promoter element resulting in activating down stream transcription of the report gene as illustrated below.

The mammalian-1-hybrid assay was conducted in HEK-293 cells and includes the ligand-binding domain of nuclear receptors (VDR, RXR, FXR) fused to the DNA binding domain (DBD) of the GAL4 construct. When the nuclear receptor binds the DBD, and LBD come into close proximity eliciting a transcriptional response. This response can be read through luciferase activity. The Thompson laboratory remains the only one to employ such technologies to determine ligand affinity for a given nuclear receptor.

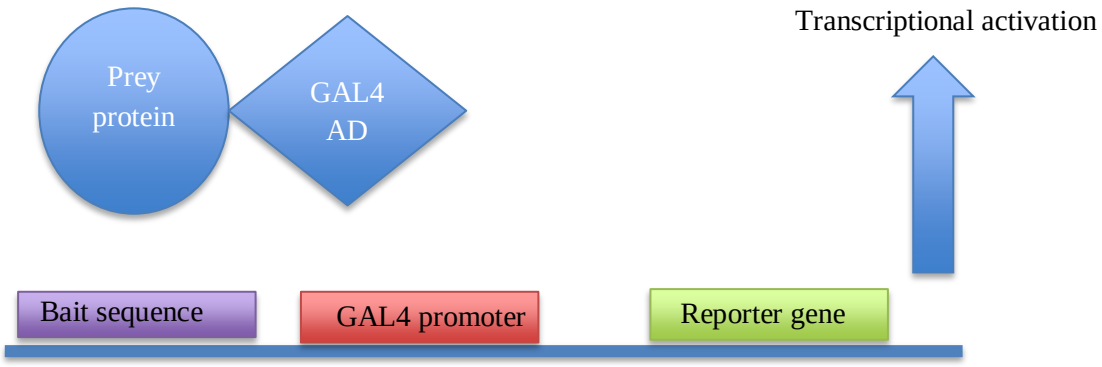
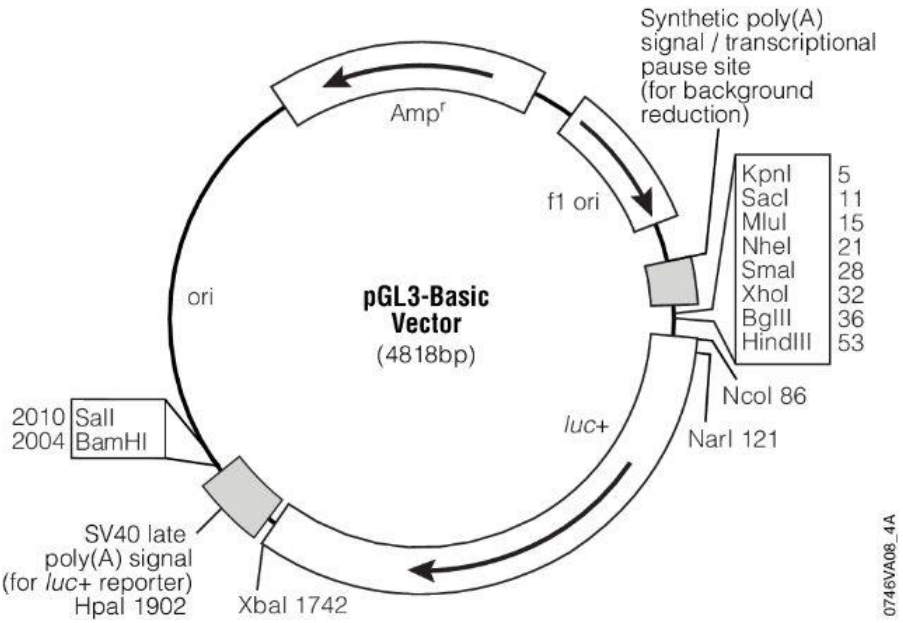


Illustration: Mammalian one Hybrid system principle

Chapter 3: Results

A.



B.

5′-caatacactagtaaaggtcactcaattccaaggggaaaatgattaaccaa

agaacattctaacggttcataaagggtattaggtgtaatgaggatgtgtt

atctcaccagaacaaacttctgagtttatataacctctagttacataacc

tgaaacccggacttggcacttggtaagcacgcaatgaacagtcatagtaa

gctggccaagggtagagttcagtttgaacaaagcaatttgagaacatcaa

aggaagtttggggaacagcaagggatccagaatggctaga-3′

Response Elements

LXRE VDRE/SXR/PXRE CARE AhRE FXRE HALF SITE

Figure 3.1 *UGT1A1*-290bp-pGL3 basic construct: (A) Schematic detailing the commercially available pGL3-basic vector (Promega). (B) Indicates the enhancer module within the multiple cloning site; present within this pGL3-basic construct.

3.3.1 *UGT1A1*-290bp-pGL3 basic construct

The *UGT1A1*-pGL3 basic constructs are artificially engineered vector constructs with endogenous promoters or sections of promoter regions ligated into the backbone of the pGL3basic vector that is commercially available from Promega. The two *UGT1A1* constructs used within this study contained 2kb (U2K) fragment while the other contained a 290bp enhancer module, (detailed in figure 3.1). Both the pGL3-*UGT1A1*-U2K, and pGL3-*UGT1A1*-290 were kindly provided by Professor Masahiko Negishi, Research Triangle Park, North Carolina. Figure 3.1 details within the 290bp stretch of promoter lies a number of nuclear receptor response elements. It was due to this presence that the 290bp fragment became the focus of this research study, as the U2K construct, although containing more promoter than the smaller construct showed negligible difference in expression profiles when exposed to agonist.

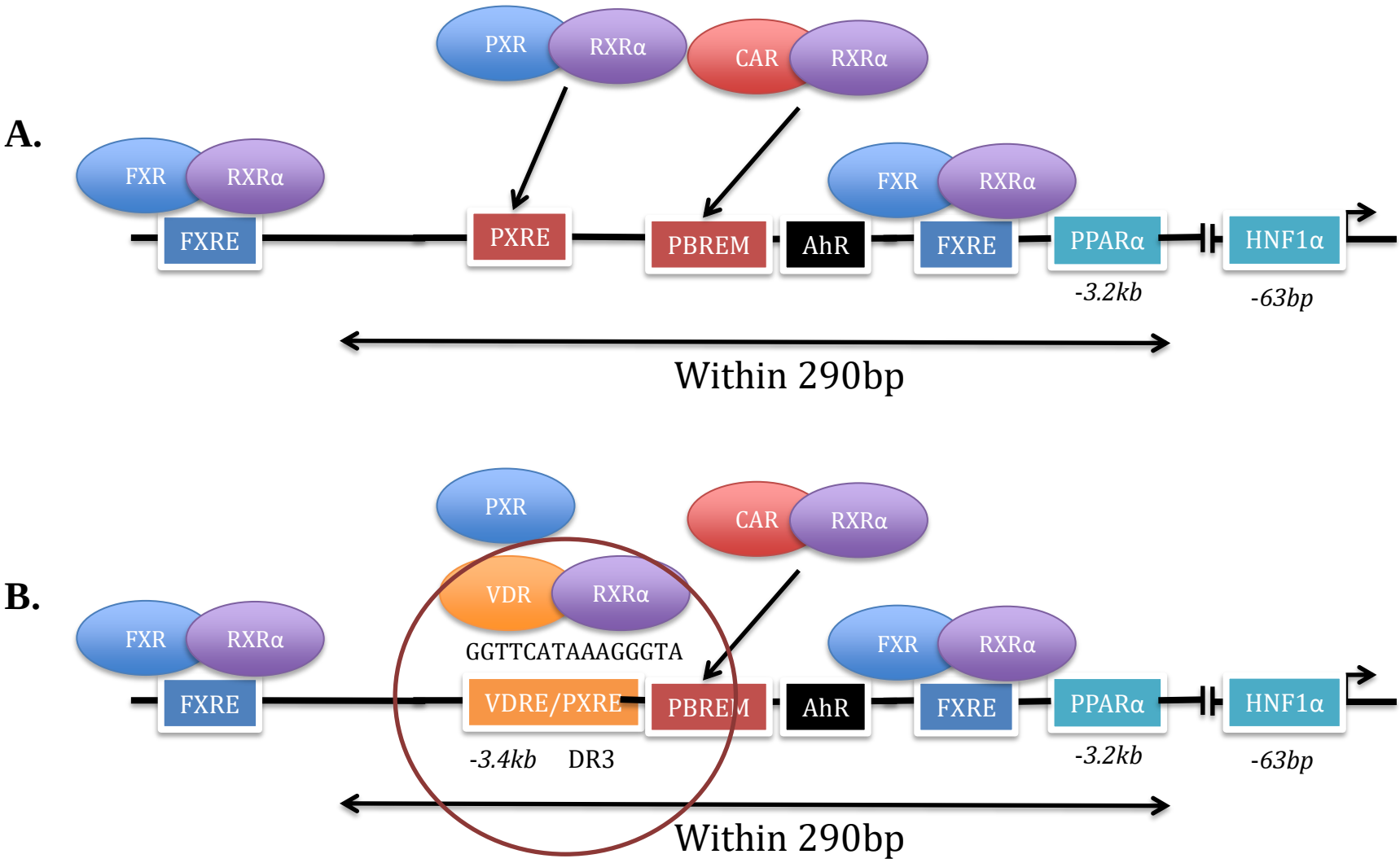


Figure 3.2 Schematic of the *UGT1A1* gene promoter: **(**A) highlights known and established inducers of the *UGT1A1* gene. (B) Indicated using the red circle shows the previously unknown VDRE element within the 290bp enhancer module, located distal to the start site.

3.3.2 Schematic of the *UGT1A1* gene promoter

Figure 3.2 details a schematic of the *UGT1A1* promoter beginning at the start site of the gene where there is an HNF1alpha binding site that is thought to be key requirement for the expression and activity of *UGT1A1* within the liver. The diagram further details the presence of CAR, PPAR, AhR and PXR binding sites within the 290bp module located 3.2kb upstream of the start. The elements highlighted in red indicate the previously unidentified VDRE. This being said Tukey, *et al* had previously explored the motif in question and identified it as an SXRE or PXRE. To date this motif has not been explored in its capacity to be a Vitamin D response element in addition to being a PXRE/SXRE.

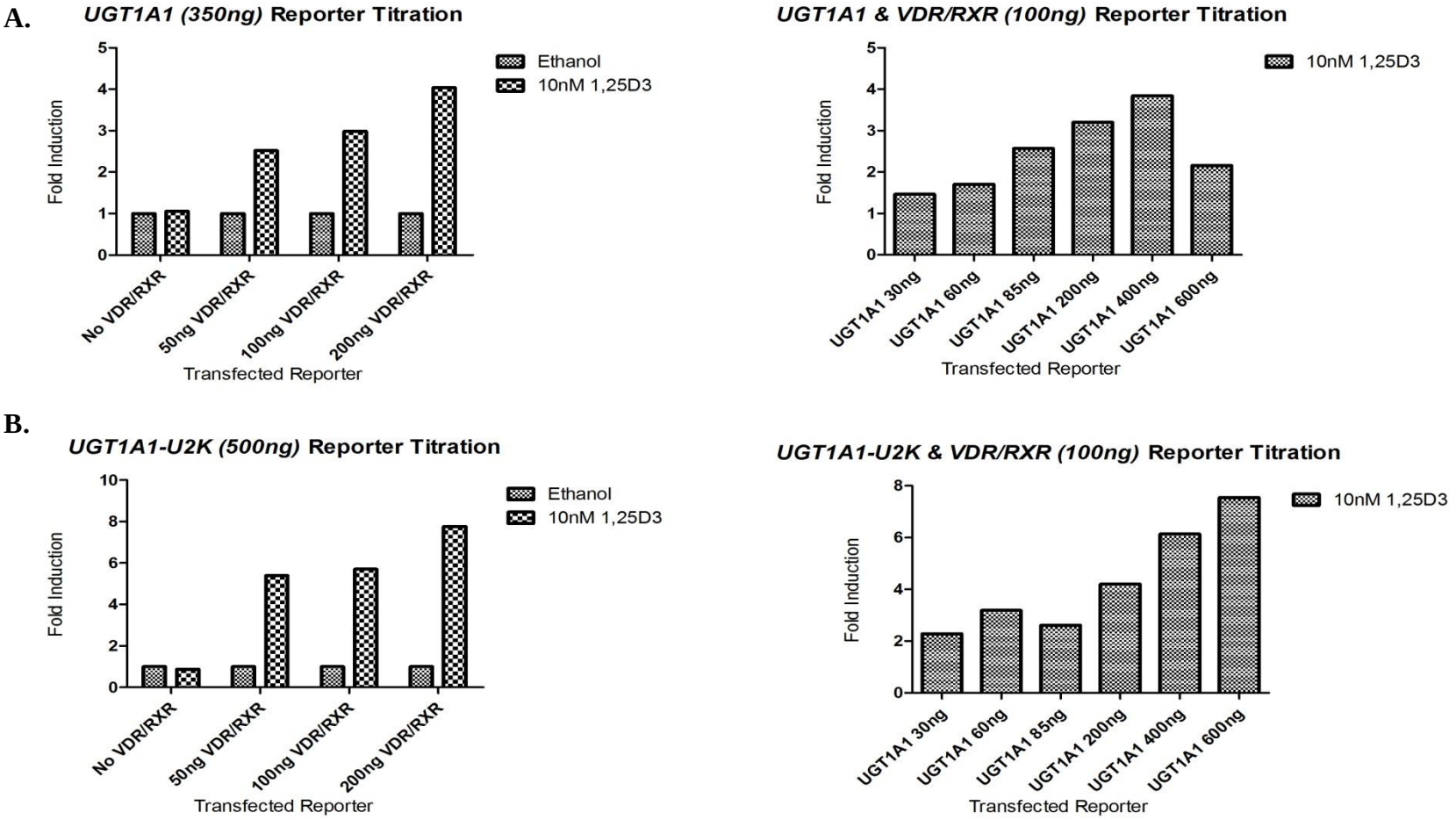
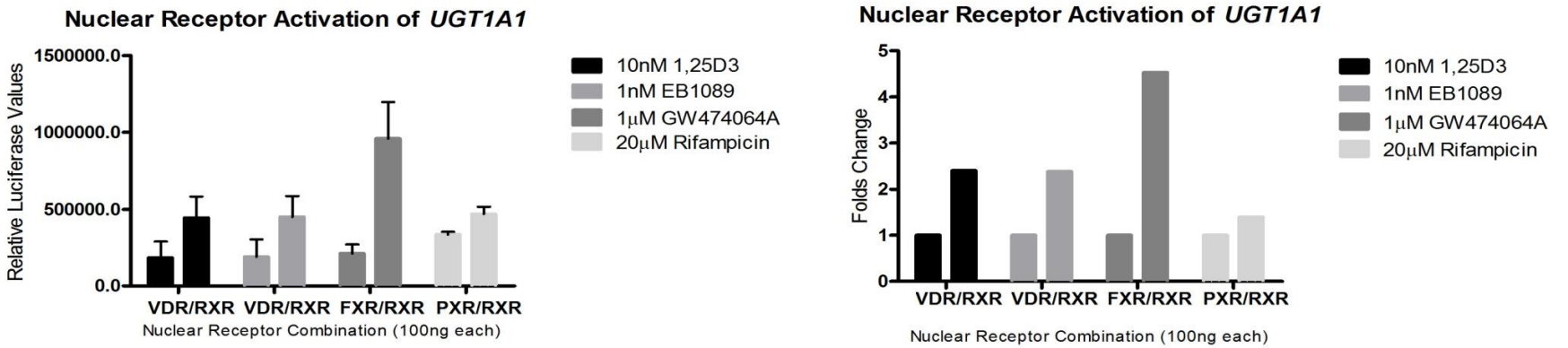


Figure 3.3 Preliminary luciferase report vector titrations: HEK-293 cells were transfected using calcium phosphate methodology with the above amounts of reporter vector (pGL3-basic) and nuclear receptor constructs (V5-VDR, psg5-RXR). The cells were then treated for 24hrs with 10nM 1,25$(OH)_2D_3$, after which a single glo luciferase assay was performed as per manufactures protocol, standardising to total protein concentration. The above preliminary experiments were ran as n=1 with each data point run in triplicate.

A.



B.

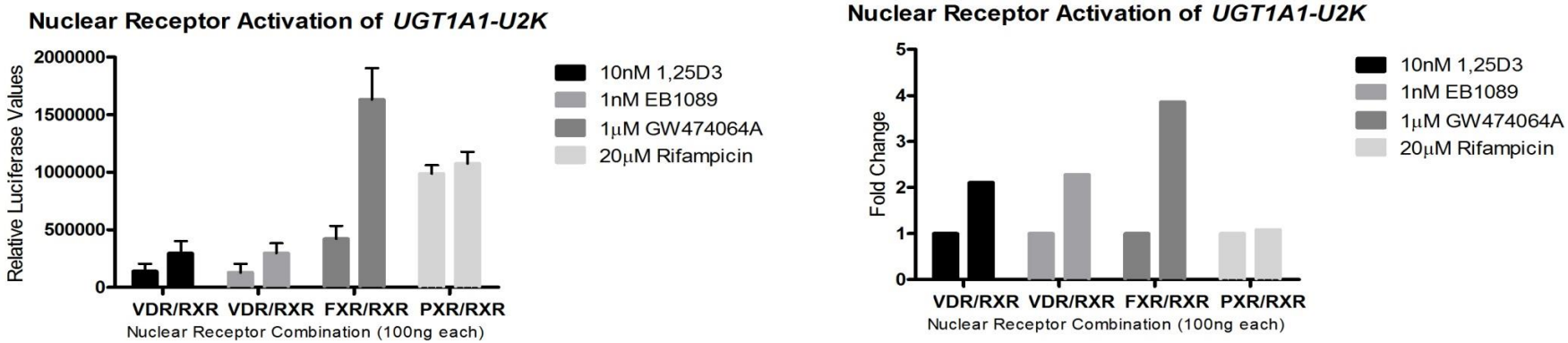


Figure 3.4 Preliminary luciferase activities, defining nuclear receptor effects on the *UGT1A1* promoter: HEK-293 cells were seeded at a density of $1x10^5$ cells/well and transfected using the calcium phosphate methodology with the indicated reporter (*UGT1A1*-290bp, 350ng, 2000bp, 500ng) and nuclear receptor (100ng) construct. The cells were treated for 24hrs with 10nM $1,25(OH)_2D_3$ 1nM EB1089, 1μM GW474064A & 20μM Rifampicin, after which a single glo luciferase assay was performed as per manufactures protocol, standardising to total protein concentration. The above preliminary experiments were ran as n=1 with each data point run in triplicate.

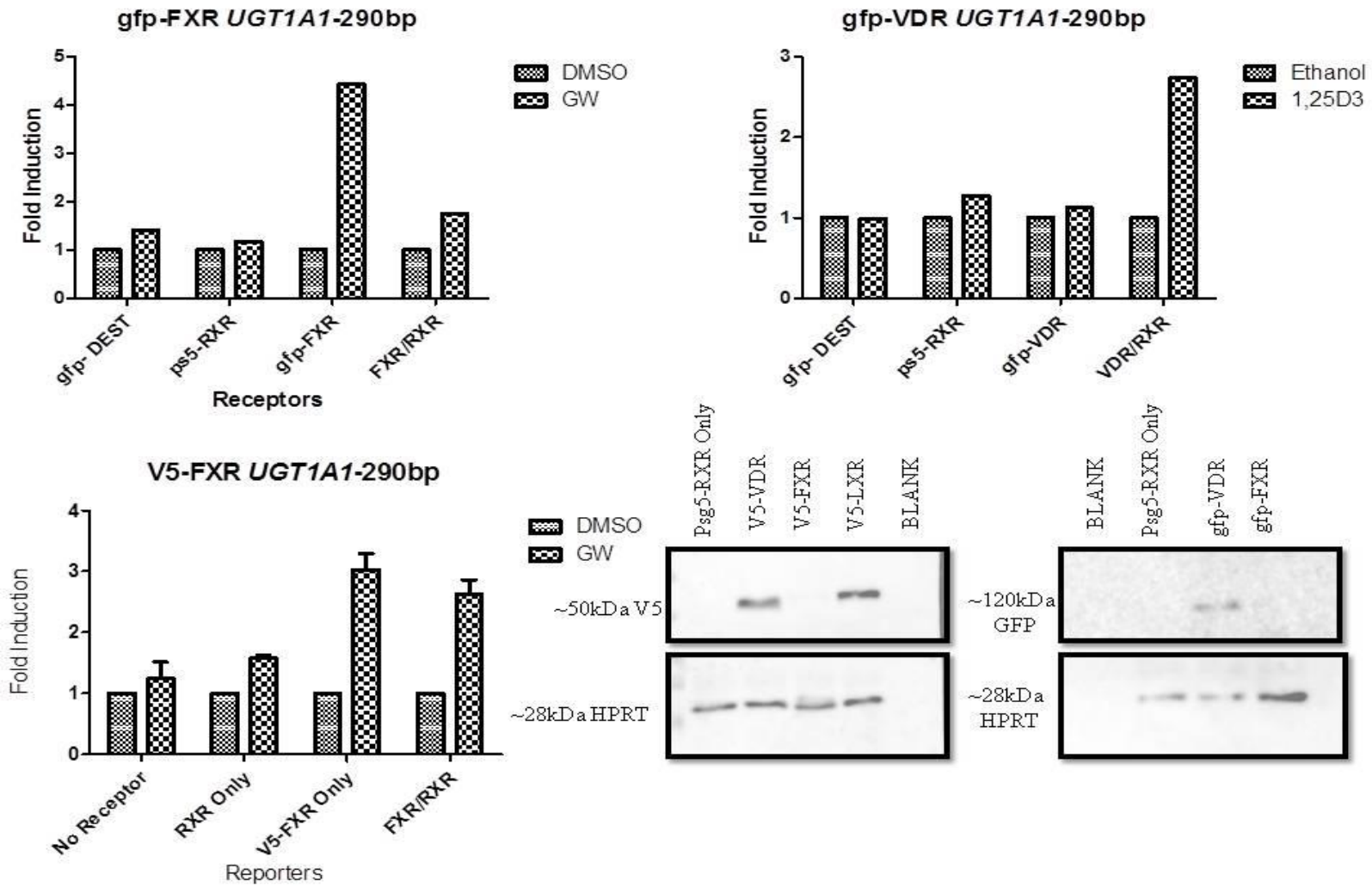


Figure 3.5 Preliminary luciferase, coupled with V5 and gfp western activity: HEK-293 cells were seeded at a density of $1x10^5$ cells/well and transfected using the calcium phosphate methodology each receiving 100ng of nuclear receptor vector as detailed above (gfp or V5-VDR, FXR, or psg5-RXR). The cells were treated for 24hrs with 10nM $1,25(OH)_2D_3$ or 1µM GW4704064A followed by a single glo luciferase assay being performed as per manufactures protocol, standardising to total protein concentration. Whole protein lysates were extracted from 100mm dishes seeded at $8x10^5$ and transfected with 1µg of each construct, with no treatment. The protein was quantified using Bio Rad technology and 50µg of lysate was loaded on a 4-12% polyacrylamide gel.

3.3.3 Preliminary luciferase studies

Preliminary luciferase studies were carried out to ascertain the appropriate amount of each of the reporter vectors and nuclear receptor vectors to be used in later experiments. As can be seen from figure 3.3 HEK-293 cells were used to establish vector concentrations required for optimal luciferase read outs. Section A of figure 3.3 represents a constant amount of the *UGT1A1*-290bp vector construct with varying amounts of nuclear receptor constructs VDR/RXR. The 2nd graph in section A details a constant amount of nuclear receptor construct (100ng of VDR and RXR respectively), with varying amounts of *UGT1A1*-290bp pGL3 basic construct. The 2nd graph shows a drop in luciferase activity when greater than 400ng of vector was added. This loss of luciferase activity could be down to a lack of transfection efficiency due the higher amount of vector being added to the cell models. Section B of figure 3.3 depicts the same format as previously described however it utilized the larger *UGT1A1*-U2K construct instead.

It was determined 350ng of the *UGT1A1*-290bp construct was adequate. 500ng of the U2K fragment was also deemed acceptable. Each of the preliminary studies was carried out using 10nM $1,25(OH)_2D_3$ as the agonist.

Figure 3.4 A utilizes the *UGT1A1*-290bp construct and is used to establish the activation profiles varying nuclear receptors have in the enhancer motif housed within the pGL3 basic construct. The first graph within 3.4 A; represents the relative luciferase values whereas the 2nd graph has been calculated to express the fold change, standardising the measurements to each of the respective vehicle controls. FXR was used within this experimental set, as a potential avenue for investigation through FXRs endogenous role in bile acid sensing and downstream metabolic regulator. These experiments where conducted as preliminary trials and as such only performed once with three technical repeats within each data set. As with

figure set 3.3B, 3.4B represents the same set up as described above only employing the larger *UGT1A1*-U2K vector construct. From these preliminary experiments, it was decided to focus soley on the 290bp fragment in later experiments, as the larger U2K fragments did not yield any greater luciferase activity when comparing fold change between both the *UGT1A1* constructs.

After witnessing the luciferase activity within figure 3.3 when the *UGT1A1* enhancer module was exposed to FXR agonist GW4704064A we decided to explore this slightly further resulting in a direct comparison between FXR and VDR activity. Originally the V5 constructs for both VDR and FXR were used however only VDR-V5 was detectable via western blot analysis as is shown in figure 3.5. Psg5-RXR was used as an internal control to ascertain anti-V5 specificity when probing the cellulose membrane. The V5 epitope may perhaps be hidden amongst the confirmation arrangement of the FXR protein restricting the ability of the V5 antibody to bind, making it undetectable via western blot. This resulted in the use of a gfp-based construct being attempted, utilizing gfp-VDR and gfp-FXR. Again FXR was undetectable. At a luciferase level, however the presence of FXR does elicit a response in activity on the *UGT1A1* promoter in comparison to no nuclear receptor being present.

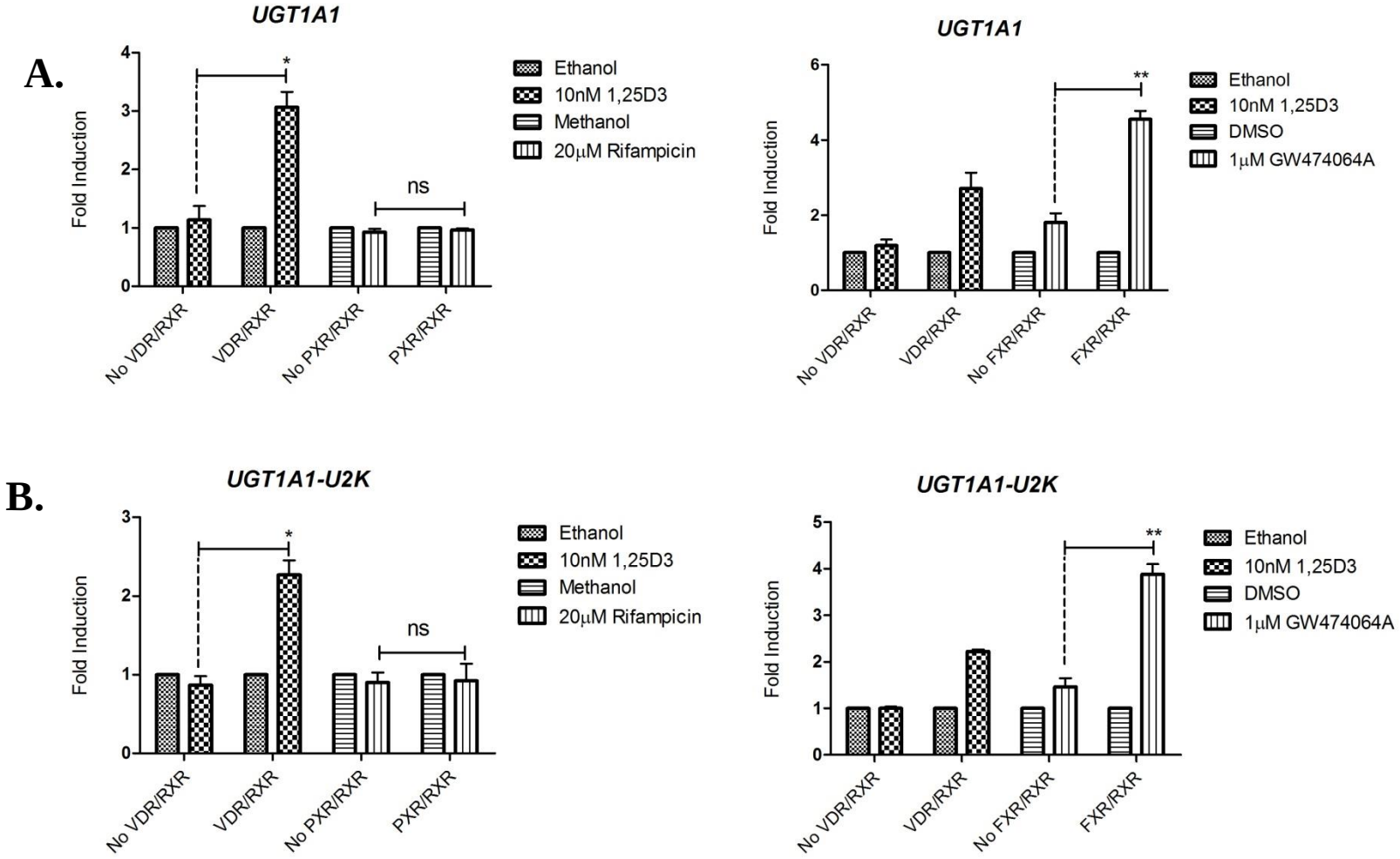


Figure 3.6 Comparative nuclear receptor activity of the *UGT1A1* (290bp (A) & 2000bp (U2K) (B)) promoter in HEK-293 cells: HEK-293 cells were seeded at a density of $1x10^5$ cells/well and transfected using the calcium phosphate methodology with the indicated reporter (*UGT1A1*-290bp, 350ng, 2000bp, 500ng) and either empty vector or 100ng of each nuclear receptor construct keeping DNA amounts constant. The cells were then treated for 24hrs with 10nM 1,25D3, 1μM GW4704064A or 20μM Rifampicin, after which a single glo luciferase assay was performed as per manufactures protocol, standardising to total protein concentration. Data are represented as means (± SEM) of two independent experiments, with each data point performed in triplicate (n=3) $p<0.05$ *, < 0.01 **, and <0.001 ***.

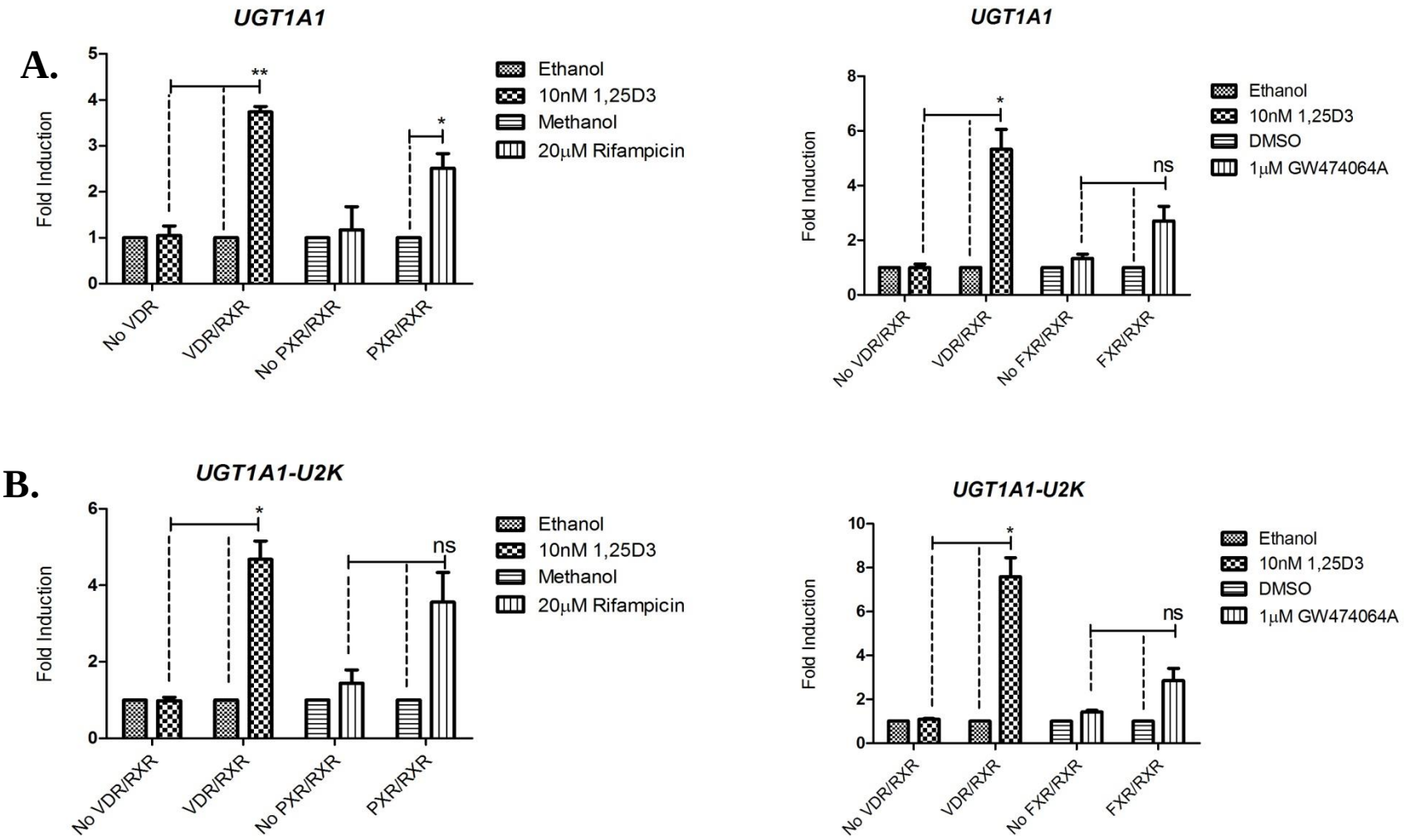


Figure 3.7 Comparative nuclear receptor activity of the *UGT1A1* (290bp (A) & 2000bp (U2K)(B)) promoter in HepG2 cells: HepG2 cells were seeded at a density of $1x10^5$ cells/well and transfected using the calcium phosphate methodology with the indicated reporter (*UGT1A1*-290bp, 350ng, 2000bp, 500ng) and either no nuclear receptor or 100ng of each construct. The cells were then treated for 24hrs with 10nM 1,25D3, 1μM GW4704064A or 20μM Rifampicin, after which a single glo luciferase assay was performed as per manufactures protocol, standardising to total protein concentration. Data are represented as means (± SEM) of two independent experiments, with each data point performed in triplicate (n=3) $p<0.05$ *, < 0.01 **, and <0.001 ***.

3.3.4 Comparative nuclear receptor activity of the *UGT1A1* (290bp & 2000bp (U2K)) promoter in HEK-293 vs. HepG2 cells

Figure 3.6 was carried out using the HEK-293 cell model transfecting each with a nuclear receptor construct comparing directly VDR activity against PXR and FXR. The upper panel of figure 3.6 utilizes the 290bp *UGT1A1* construct whereas the lower panel used the larger U2K fragment. Each well was then treated with their respective agonist as detailed in figure legend 3.6 for a period of 24 hours before luciferase activity was measured.

The comparison between VDR and PXR activation is of great interest as we hypothesized that VDR and PXR share the same binding motif, and that competitive binding may occur between the nuclear receptor constructs. With this in mind VDR seems to have preference over PXR, as is indicated through an almost 3.5 fold induction compared to a non-significant induction mediated through PXR.

FXR again shows a modest activation of the *UGT1A1* promoter and further investigation into its relationship amongst the other nuclear receptors should be pursued, however remains beyond the scope of this project.

The 2nd panel of graphs shows an identical set up as before only using the UGT1A1-U2K promoter vector. Very similar expression profiles are seen when the larger construct is used vs. the smaller 290bp construct.

Figure 3.7 uses the same experimental set up as described previous, however the cell model used is the HepG2 cell line. Within this data set the PXR response was seen when the PXR agonist Rifampicin was added. This is in direct contrast with the same experimental set up only using HEK-293. This in itself could indicate tissue specificity, perhaps eluding to the presence of addition transcription factors present in liver derived cells that may not be present in other cell models? Furthermore the VDR activation potential remains present in the HepG2 model even though PXR activation is also present. The FXR response is present, but muted, and deemed statistically insignificant.

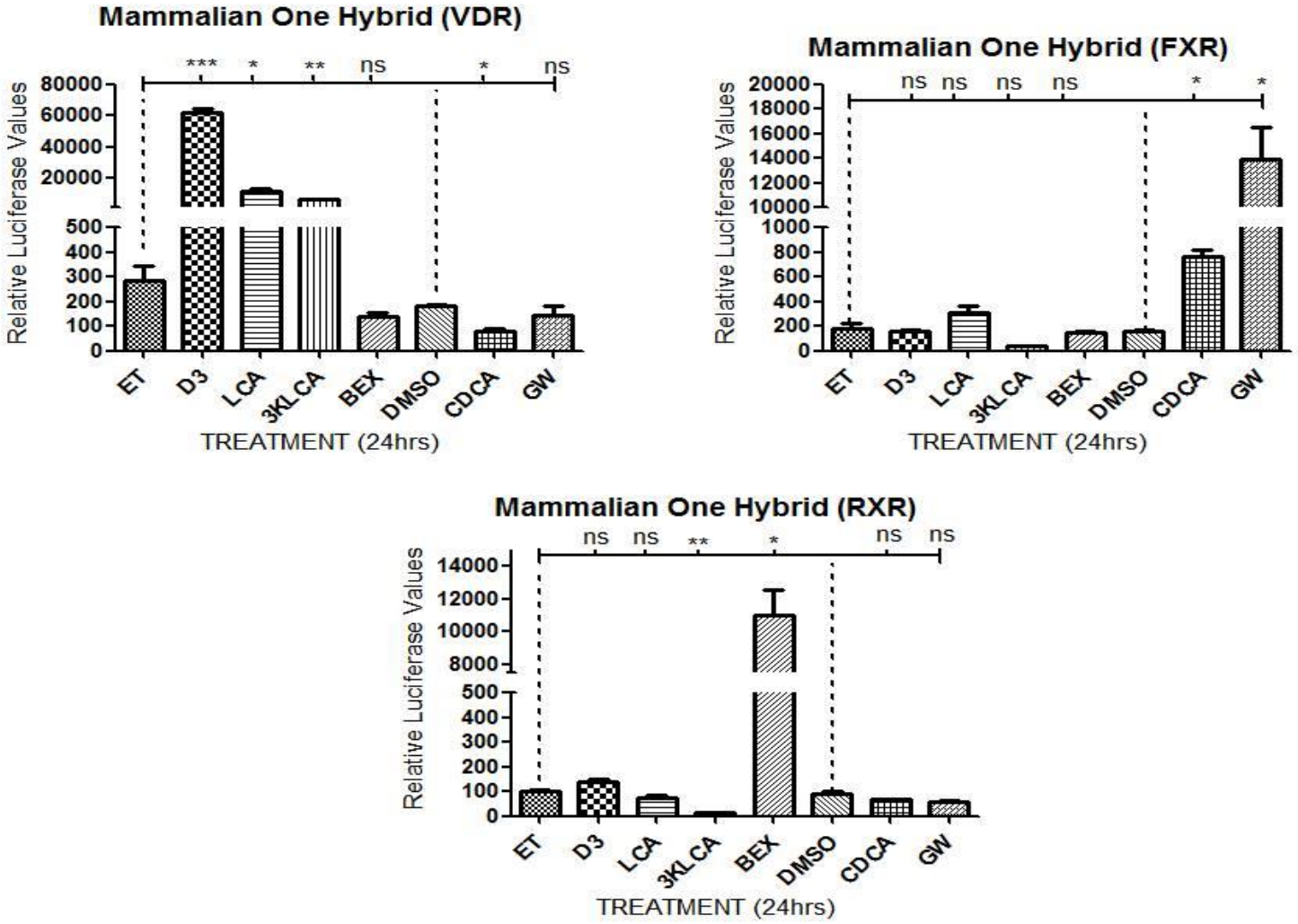


Figure 3.8 Mammalian One Hybrid, indicating ligand/receptor specificity in the HEK-293 cell line: HEK-293 cells were seeded @ $1x10^5$ cells/well and transfected with 640ng pFR-Luc, 30ng phRL-TK and 100ng of pCMV BD/NR_{LBD} (VDR, FXR or RXR). The cells were then treated with the above ligands for 24hrs before dual glo luciferase readings were taken. Data are represented as means (± SEM) of three independent experiments, with each data point performed in triplicate (n=3) $p<0.05$ *, < 0.01 **, and <0.001 ***.

3.3.5 Mammalian One Hybrid, indicating ligand/receptor specificity in the HEK-293 cell line

The Thompson laboratory remains the only one to utilize the mammalian one hybrid technology within the UK. This assay was designed to determine the affinity of different ligands towards nuclear receptor ligand binding domains (LBDs). The assay employs the GAL4 DNA binding domain fused to the nuclear receptor ligand-binding domain. Upon ligand binding/interaction the GAL4 DNA binding domain would bind to the GAL4 DNA response elements on the phFR-Luciferase promoter resulting in a transcriptional and therefore luciferase based response. It was used to ensure that all the ligands used within the study where indeed specific agonists to their respective nuclear receptors.

HEK-293 cells were transfected with vectors containing the nuclear receptor ligand binding domains for VDR, FXR and RXR. These were then treated with natural ($1,25(OH)_2D_3$), primary/secondary bile acids) and synthetic (Bexarotene and GW474064A) agonists.

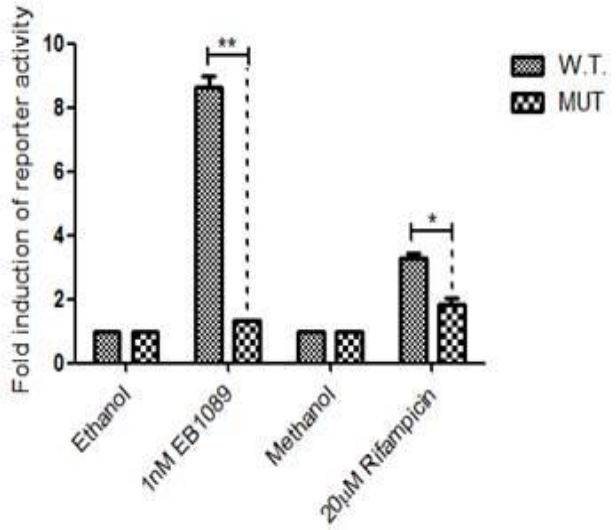
Figure 3.8 shows that secondary bile acids are agonists for both FXR and VDR, inducing both VDR and FXR activity in HEK-293 cells. Secondary bile acids however have an even more potent effect on VDR binding. Indicating perhaps the potential for VDR to be activated in a tissue specific manner as previous studies conducted within the laboratory detailed a lack of primary bile acids to induce VDR in Caco-2 cell model.

Wild Type

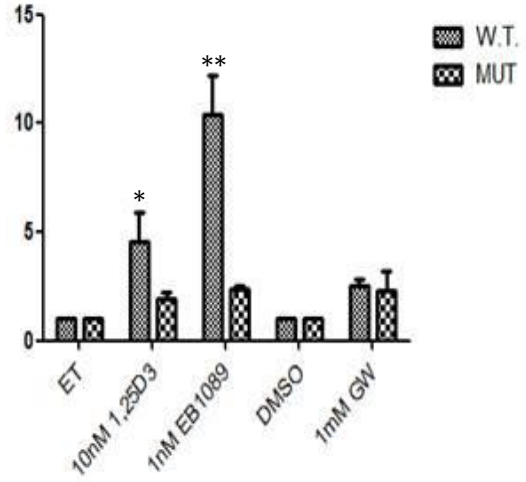
5’- ggttcataaagggta - 3’

Mutation

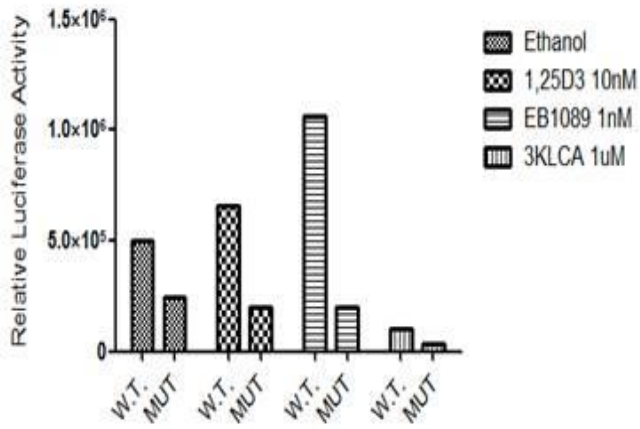
5’ - ggttcataaa**TA**gta - 3’



Mutant VDR vs. PXR



Mutant VDR vs. FXR



Wild type VDR vs. Mutant

Figure 3.9 *UGT1A1* 290bp Wild Type vs. VDRE mutant construct: HEK-293 cells were seeded @ $1x10^5$ cells/well and transfected with constructs harbouring intact or mutated versions of the putative VDRE sequence/pGL3-*UGT1A1*-290bp (350ng) and indicated receptor reporter vectors (100ng each) respectively. All transfected cells were then treated for 24 hours with VDR, PXR and FXR ligands, detailed above. Data are represented as means (± SEM) of two independent experiments, with each data point performed in triplicate (n=3) $p<0.05$ *, < 0.01 **, and <0.001 ***.

3.3.6 *UGT1A1* 290bp Wild Type vs. VDRE mutant construct

The mutant *UGT1A1* construct was created using the *UGT1A1*-290bp-pGL3 and specifically designed mutant primers created through the Agilent Technologies online primer design software. Figure 3.9 highlights the nucleotide change to create the mutant VDRE motif. The orientation of the mutation will was chosen as it will inhibit the binding of VDR to the VDRE motif as the heterodimer complex formed between VDR and RXR lies binds with the orientation as RXR:VDR (5' to 3').

As a result of the mutation VDR could no longer bind resulting in a loss of luciferase activity as is evidenced through figure 3.9. By using the synthetic analogue of vitamin D, EB1089, we can see the drastic loss in activity, in comparison the with Wild Type construct. Furthermore there is an additional loss of activity surrounding PXR binding adding additional credence to the hypothesis that the two nuclear receptors, VDR and PXR share the same binding motif within the enhancer module. The final graph details the wild type construct vs. the mutant construct against various VDR agonists, natural, synthetic and bile acid derived. The mutation is all instances results in a loss in luciferase activity.

Chapter 3: Discussion

3.4.1 Discussion

This chapter was used to identify, test and validate the putative VDRE located within the enhancer module of the *UGT1A1* promoter, to ascertain how ligands specific to VDR were inducing the gene at an mRNA level discussed in chapter three. Within this chapter luciferase based assays were optimized using two major cell in an attempt to compare the relative expressive abilities of the *UGT1A1* in two different tissue types. The VDRE being targeted within this chapter has the orientation known as a direct repeat or DR3 motif, which has previously been identified as a PXRE binding motif (Chen et al., 2012), however no one has identified the same binding site as a motif for the Vitamin D receptor. The homology shared between both PXR and VDR makes its very likely that they too would have the ability to competitively bind the same and similar DNA motifs with a given gene promoter (Laudet et al., 1992).

The luciferase technologies were used to assess how potent an induction $1,25(OH)_2D_3$, and EB1089, as VDR agonists, Rifampicin as a PXR agonist, and GW474064A as an FXR agonist were upon the expression of the *UGT1A1* promoter, while also allowing for a comparison to be made between agonists as PXR and FXR are already established prototypical inducers of the endogenous *UGT1A1* gene (Guo et al., 2003).

The luciferase technology used within this study has previously been established within the laboratory however the amount of promoter vector and nuclear receptor constructs were unknown at the time. Throughout this study the single glo luciferase assay was used, normalizing the absolute luciferase values to overall protein concentration using the Bio-Rad DC protein assay.

VDR has been shown to bind DR3 elements in many classically associated genes including but not limited to *CYP24A1*, *CYP3A4*, *CYP3A*, and *ABCB1* (Thompson et al., 2002a). Identifying the DR3 response element as a VDRE within these phase one genes further supports the hypothesis that the scope of VDR moves beyond that of bone remodeling and mineralization. It also raises the question does the ability of VDR to regulate metabolic gene transcription extend beyond of phase one and three?

Gene reporter testing

The HEK-293 cell is an epithelial derived human embryonic kidney cell that was transformed using adenovirus DNA (Graham et al., 1977). The kidney has long been known as a VDR target organ and HEK-293 have been well established with excellent transfection efficiency.

The HepG2 cell is an immortalised liver cell line and has been used within this study as a comparison for expression as the liver remains the major site of metabolism associated with overall detoxification. It is for these reasons that both these cell lines were used within this study.

The gene reporter titrations were carried out in HEK-293 cells. The cells were transiently transfected with the stated amounts and subsequently treated for 24 hours before the single glo luciferase assay was performed. The preliminary titrations to establish the amount of nuclear receptor to transfect was ascertained to be 100ng before an overall decrease as witnessed on luciferase activity. This could be due to an oversaturation of the promoter leading to a repressive effect on luciferase activity. The 2nd titration carried out was to establish the amount of promoter construct to transfect. 350ng (*UGT1A1*-290bp) and 500ng (*UGT1A*1-U2K) were deemed satisfactory before a loss of activity was witnessed. The subsequent loss of activity

may have been due to an increase in promoter amount depleted the transfected nuclear receptor resulting in loss of activation; therefore all the subsequent luciferase assays used the above established amounts of transfectable material.

The preliminary data also suggests that the presence of the nuclear reporter vectors is a pre requisite for transcriptional induction to occur, indicating the promoter to be nuclear receptor dependent in nature.

The *UGT1A1*-290bp construct transfected with VDR/RXR and exposed to 10nM of $1,25(OH)_2D_3$ resulted in a 3.5 fold induction, compared to a negligible induction via PXR agonists in the HEK-293 cell line. This result is interesting to note as it indicates a tissue specific response with regards the PXR related activation, even though both steroid nuclear receptors share approx. 90% homology with each other, PXR does not seem to boast any inductive capabilities of the *UGT1A1* promoter enhancer module outside the hepatic environment. This could be due to a lack of additional transcription factors such as HNF-1 alpha that is present in abundance within the liver, as it can be recruited to the promoter of a transcriptionally active gene as part of the pre initiation complex.

Mammalian one Hybrid

In conjunction with the luciferase assays we employed a mammalian one hybrid approach to assess the affinity each of the ligands have towards their respective receptors. Within the experimental design we exposed VDR, FXR and RXR to various ligands including primary and secondary bile acids. It is interesting to note that VDR was activated when exposed to $1,25(OH)_2D_3$, lithocholic acid (LCA), and 3-keto- lithocholic acid (3KLCA). Each of the receptor plates were exposed to various ligands for 24 hours before analysis. The VDR data is consistent with the

findings that 3KLCA and LCA show approx. the same affinity for VDR and therefore can produce a VDR mediated transcriptional response *outside* of the liver in a enterohepatic capacity, suggesting further an intestinal role in VDRs ability to regulate metabolic gene functionality (Makishima et al., 2002), (Jacobs et al., 2016). Both FXR and RXR reacted as expected, with FXR showing greatest affinity towards bile acid exposure, whereas RXR responded towards Bexarotene exposure. Bexarotene is an antineoplastic agent and is a third generation retinoid whose mechanism of action selectively activates the retinoid X receptor (RXR). By selectively activating RXR the drug is able to induce cell differentiation and apoptotic pathways preventing drug resistance. In addition to this the drug has anti angiogenic effects inhibiting cancer metastasis (Qu and Tang, 2010).

UGT1A1-290bp DR3 K.O

Once we has determined that VDR had the ability to induce *UGT1A1* expression through the activation of the DNA binding motif located within the enhancer module, we proceeded to conduct site directed mutagenesis on the DR3 motif. The 2-nucleotide base changes (figure 4.10) resulted in a complete inability of VDR to bind the DR3 response element negating its ability to induce transcriptional activity. It is interesting to note, that the PXR also lost its ability to bind the response element, indicating a shared site between both PXR and VDR. This indeed does stand to reason that two steroid nuclear receptors would in essence have dual compatibility with DNA binding sites. The loss of VDR activity within this region indicates that this is the only VDRE present within the enhancer module. It must however be noted that the assays performed within this study incorporated only a small segment of the

overall gene promoter, and may not include all the VDRE response elements that regulate *UGT1A1* transcriptional induction, however further mutagenesis needs to be performed within the 2K base pair vector as it is possible that potential VDRE elements could be present and missed otherwise. However in the context of the enhancer module the presence and confirmatory identification of the VDRE has never been observed before making this an interesting observation, opening up the possibility of further exploitation outside of the hepatic environment with the potential for therapeutic application.

EB1089

EB1089 also known as Seocalcitol has been shown through this project that it has the ability to induce VDR driven gene activity to a higher degree than $1,25(OH)_2D_3$. This has also been noted in other researchers within the group, but a deeper investigation into why EB1089 has this ability has yet to be explored. As EB1089 is an analogue of vitamin D it may not undergo the same metabolic degradation as the naturally occurring ligand, which is susceptible to metabolism through various phase one reactions chief among which remains *CYP24A1*. This then may result in EB1089 being able to elicit a response for a greater period of time resulting in a higher degree of promoter activation. Another concept for consideration is the binding affinity EB1089 may have on target gene promoters? Does EB1089 bind to response elements with greater affinity, at lower concentrations and still elicit highly responsive states of activation, witnessed at a transcriptional level? It would be of great interest to study further the effects EB1089 has on VDR target genes and delineate further its capabilities however this is beyond the scope of this project.

Chapter 4: Preliminary studies assessing the cross talk potential between nuclear receptor members, including inflammatory effects, and UGT glucuronidation activity.

4.1 Introduction

Nuclear receptors belong to a super family of proteins, and act as ligand activated transcription factors and are involved in a range of physiological processes. The nuclear receptors respond to ligands such as steroids, hormones and retinoids. This being said there is still a distinct group known as 'orphan' nuclear receptors that lack a defined endogenous ligand, therefore these receptors could in theory be regulated through other means such as post translation modification or SUMOlaytion (Chawla et al., 2001).

The homology shared among nuclear receptor members' stretches back through evolution. This homology between receptors has allowed the sharing of ligands that activate each receptors activity. This also leads to a potential cross talk between nuclear receptor members to ensure adequate and efficient physiological response i.e. metabolic turnover of harmful substrates.

Due to the presence of the enhancer module within the *UGT1A1* gene promoter, encompassing a number of nuclear receptors DNA binding motifs, we have hypothesized that a number of the receptors have an ability to interact and potentially affect the activation potential of *UGT1A1*.

Liver X Receptor (LXR)

LXR is a cholesterol and bile acid sensor and has been termed the master regulator of hepatic metabolism (Huang et al., 2010). Cholesterol is an essential part of all living beings, making up key elements within cellular membranes and is the precursor to all steroids and bile acids. Cholesterol levels are however tightly controlled; as excessive amounts in the body has been linked with the development of cardiovascular disease

and atherosclerosis. Transcriptional control of genes involved with cholesterol homeostasis is mediated in part by two nuclear receptors LXR and FXR. Both these receptors like VDR form heterodimers with RXR, and work concurrently in controlling many key lipid and bile acid regulated genes. LXR regulated genes are associated with the absorption, transportation, efflux and elimination of cholesterol, whereas FXR is bound by bile acids and acts as a bile acid sensor, controlling bile acid levels within the body. Bile acids are the major end products of cholesterol in the liver, with FXR playing a major role in absorption and enterohepatic circulation events of many bile acids and cholesterol metabolites.

Farenoid X Receptor (FXR)

FXR has emerged as a key modulator of a number of metabolic processes within the liver related to cholesterol and bile acid metabolism and balance. Much research has been carried out using synthetic FXR agonists to determine a selective subset of metabolic genes directly controlled through FXR activation. Such findings suggest an interrelationship and crosstalk between bile acid metabolism, cholesterol metabolism, insulin resistance and triglyceride metabolism (Goodwin et al., 2000). FXR having such a wide range of potential gene targets make it a possible target for therapeutic interventions, for a range of liver and bile acid disorders such as cholestasis or liver sclerosis.

Nuclear receptor crosstalk

Cross talk is defined as the interaction between one or more transcription factors resulting in a cooperative induction or inhibition of gene transcription. One of the major benefits of the crosstalk between transcription factors is that the gene specific responses are greatly increased in a cell type dependent manner. Nuclear receptors have a direct effect on target genes however they can also have the potential to interfere with many signaling pathways, including; NF-κβ or AP-1.

The cross talk between nuclear receptors such as LXR and FXR mean that cholesterol and bile acid levels within the body are tightly controlled and regulated. *UGT1A1* as a key metabolic gene involved in the metabolism of bilirubin, (break down product of haem), as well as other bile acids and exogenous substrates leads to the hypothesis of the potential cross talk between activating nuclear receptors, due to the close proximity each of the NR binding motifs have within the enhancer module.

VDR has been shown to activate the *UGT1A1* gene as effectively as other nuclear receptor activators and at far lower ligand concentrations. This leads on to question; do the nuclear receptor members that have been shown to induce *UGT1A1* work in concert and could they have the potential to generate a 'super state' of activation?

In a recent study PXR was shown to have crosstalk potential with constitutive androstane receptor also known as CAR. Evidence suggests that the cross talk is mediated through competition for common co activators or disruption of co activators. In this instance PXR-CAR interference has been reported and a number of CYP450 enzymes, phase II metabolic genes including *UGT1A1* and phase III transporters were shown to be down regulated after PXR activation (Pavek, 2016).

The presence of a number of nuclear receptor sites located within the 290bp range within the *UGT1A1* genes shows the presence of and FXRE binding site in close proximity to the established PXRE/VDRE (figure 3.2 p127) both up stream and down stream. With the FXRE sites being separate entities this suggests that there could be a synergistic or combinational effect witnessed when exposure to multiple agonists targeting *separate* nuclear receptor binding sites, which stands to reason throughout genetic evolution genes have developed a number of activation methods to maintain expression under a variety of stress condition. Previously in this thesis PXR and VDR have been shown to competitively bind a single nuclear receptor-binding site.

Therefore to what extent does the potential cross talk between nuclear receptors have on the activation of *UGT1A1*? Will LXR and FXR have a synergistic effect with VDR to elicit a *UGT1A1* transcriptional response? Finally does VDR activation of the *UGT1A1* gene have an effect on the rate of glucuronidation?

Within this chapter preliminary experiments were conducted to ascertain the potential impact VDR mediated activation may have on glucuronidation activity using the UGT Glo assay supplied by Promega.

In addition to this combinational studies using luciferase technology was used to investigate if the addition of multiple ligands for a number of nuclear receptor targets included VDR, FXR and LXR agonists) have a cumulative effect on *UGT1A1* promoter activity.

Finally real time analysis was carried out to assess the effects inflammatory driven pathways have on VDR mediated target gene expression. TNF alpha was used as an activator of systemic inflammation within the acute phase response.

Chapter 4: Methods and Materials

4.2.1 UGT-Glo glucuronidation assay

The UGT Glo assay was conducted using the LS180, colon adenocarcinoma cell line model. The cells were seeded at $8x10^5$ cell/100mm dish, and allowed to incubate overnight at 37°C, 5% CO_2 and 95% air. The cells were washed with 1 x PBS and subsequently treated with vehicle control or VDR ligands at indicated concentrations. Cell pellet were harvested 24 hours later using ice cold PBS and subject to standard whole cell lysate preparation, before whole cell protein concentration was determined using BCA protein quantification.

Each reaction consisted of 50µg of protein lysate, UGT-Glo buffer, 50µM UGT multi enzyme substrate and water to a total of 30µl. To this either 10µl of PCR grade water (corresponding negative controls) or 16mM UDPGA (final conc. of 4mM) was added. The reactions were conducted in triplicate and incubated at 37°C for 90 minutes. Only those reactions with the addition of UDPGA are able to glucuronidate the multi enzyme substrate. The plate was then removed from the incubator and 40µl of Luciferin Detection Reagent with the addition of D-Cysteine was added to all the wells and allowed to incubate at room temperature for 20 minutes, this allowed for the stabilization of the luciferase signal before a luciferase reading was taken.

4.2.2 TNF alpha treatment of LS180 cells

TNF alpha sourced from Gibco was reconstituted at 100μg/ml in sterile PBS containing 0.1% bovine serum albumin and subsequently sterile filtered through a 0.2μm filter, and frozen at -20 degrees. LS180 cells were seeded into 6 well plates as previously described and allowed to adhere to the plates overnight, following standard protocols. Each ligand treatment containing TNF alpha received a final concentration of 10ng/ml of cytokine. To this appropriate ligands were added or vehicle control. The plates were then allowed to incubate for 24 hours before standard RNA extraction and qRT-PCR analysis was performed, described previous.

Chapter 4: Results

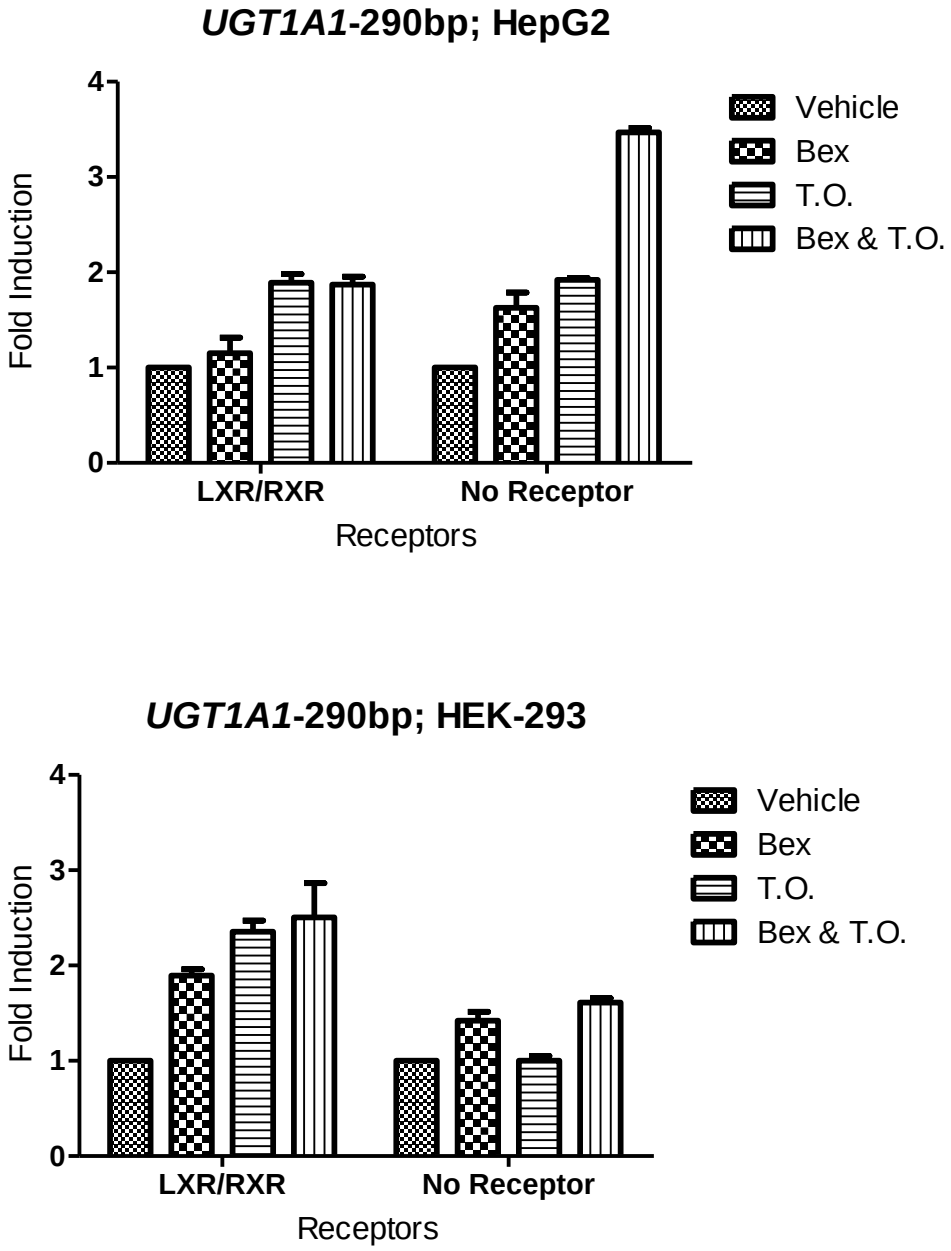
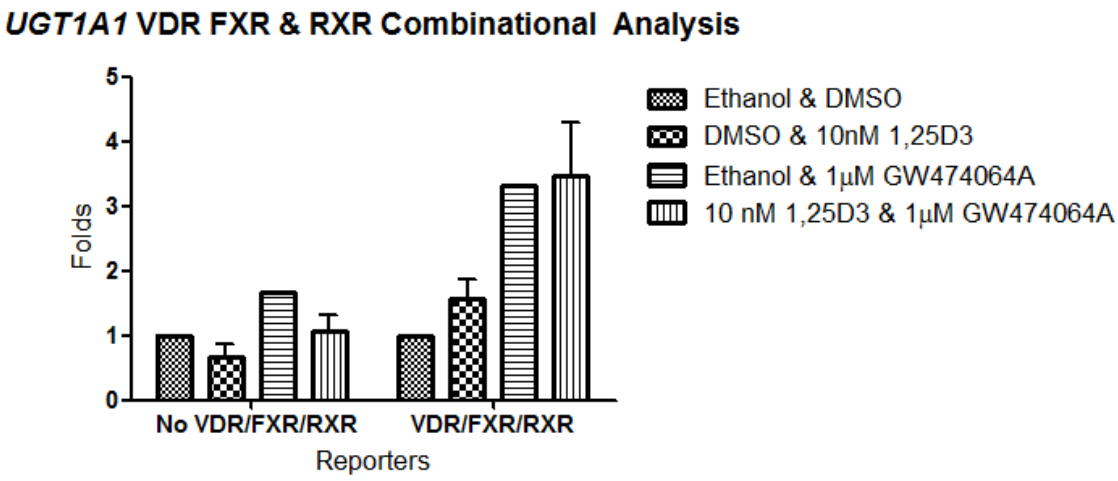


Figure 4.1: Preliminary luciferase experimentation: HepG2 & HEK-293 were transfected with pGL3-*290pb-UGT1A1* reporter vectors in combination with the indicated expression constructs for LXR/RXR empty vector combinations followed by treatment 24hrs with respective cognate receptor ligand or vehicle controls or combinations there of. Data are represented as means (± SEM) of two independent experiments, with each data point performed in triplicate (n=3).

A.



B.

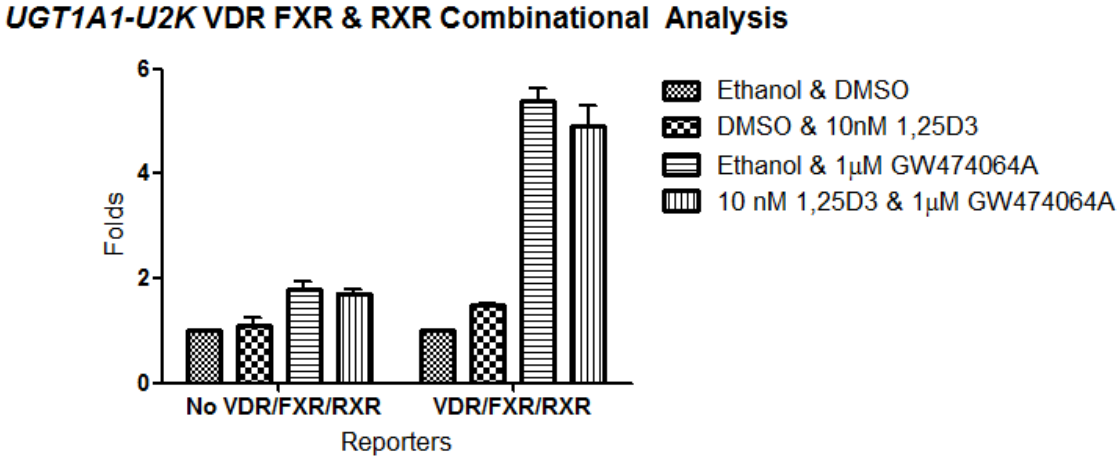


Figure 4.2: Preliminary luciferase experimentation: HEK-293 were transfected with pGL3-*290pb-UGT1A1* (A) or U2K (B) reporter vectors in combination with the indicated expression constructs for VDR/FXR/RXR or empty vector combinations followed by treatment 24hrs with respective cognate receptor ligand or vehicle controls or combinations there of. Data are represented as means (± SEM) of two independent experiments, with each data point performed in triplicate (n=3).

4.3.1 Combinational ligand analysis using *UGT1A1* promoter plasmid constructs

The preliminary luciferase studies were carried out to assess the potential cumulative nature of agonists on the activation and the *UGT1A1* promoter using Promega Single Glo system. Both HepG2 and HEK-293 cells were transfected with LXR/RXR plasmid constructs using calcium phosphate methodologies, followed by 24hr treatment with each respective ligand combination (LXR; T0901317 ((T.O), and RXR (Bex) agonists).

It has not been noted before the presence of an LXRE within the 290bp enhancer module of the *UGT1A1* gene. Figure 4.1 shows a definite response to LXR agonist indicating the presence of a potential full or partial LXRE previously undocumented.

As can be witnessed from figure 4.1 the presence of both the LXR and RXR plasmid constructs within the HepG2 have very little impact on the activity of the *UGT1A1* promoter. This could be due to an overall lack of additional promoter elements that may be required for LXR activation to occur effectively. It does seem to indicate from figure 4.1 when exogenous LXR RXR is absent the UGT1A-290bp vector produced a stronger luciferase signal in HepG2 cells. Perhaps there is sufficient endogenous LXR and RXR present within the cells to bind the promoter vectors and thus with the addition of exogenous constructs results in a squelching scenario rendering any activity mute? Indeed the reverse holds true in HEK-293. To achieve luciferase activity nuclear receptor vectors need to be introduced into the system. This being said there does not seem to be a cumulative effect amongst the treatments Bex and T.O. in the HEK-293 model. Perhaps again a liver derived model contains additional factors required for transcriptional responsiveness that are lacking in other extra hepatic models? As can be seen from the nuclear receptor illustration (figure

3.2 page 127) there is a HNF-1 binding site located approx. 63bp up stream from the start codon. Perhaps the presence of HNF is required for optimal transcriptional activation to occur in the hepatic environment?

In figure 4.2, HEK-293 cells were co transfected with RXR and FXR to cells containing both the full 2000bp and 290bp *UGT1A1* constructs there was very little difference between induction levels of the *UGT1A1* gene promoter constructs indicating the active regions for receptor binding must be within a common region shared between both constructs i.e. the 290bp enhancer region.

The combinational analysis between VDR and FXR agonists suggests no cumulative induction is present. Indeed in HEK-293 cells when exposed to the FXR agonist GW are shown to have a greater affinity for induction within this experimental system when compared to VDR agonistic activation.

Receptor	Ligand	*UGT1A* Target	Ref
PXR/RXR	**Rifampicin, Dexamethasone, Bile Acids**	**A1, A3, A4, A6**	**(Tukey, 2004)** **(Tukey, 2012)**
CAR/RXR	**Phenobarbital**	**A1, A6**	**(Sugatani, 2001)** **(Sngatani, 2008)**
FXR/RXR	**Bile Acids, (LCA, CDCA)**	**A1**	**(Barbier, 2006)**
PPAR/RXR	**Fibrates, Oxidised Fatty Acids**	**A1, A3, A9**	**(Barbier, 2003)** **(Tukey, 2006)** **(Morris, 2009)**
LXR/RXR	**oxysterols**	**A1 (possible), A3, A4**	**(Barbier, 2006)** **(Verreault,2006)**
RXR/RXR	**Bex**	**A1**	
ER/ER	**Estradiol**	**A3, A10**	**(Radominska, 2007)**
AhR/ARNT	**Aromatic hydrocarbons, Naphthoflavone**	**A1, A3, A4, A6**	**(Tukey, 2003)** **(Togawa, 2008)**
VDR/RXR	**1,25D3, EB1089**	**A1, A3, A4**	

Figure 4.3: Table summarizing members of the Nuclear Receptor superfamily with their associated ligands and target *UGT1A* members.

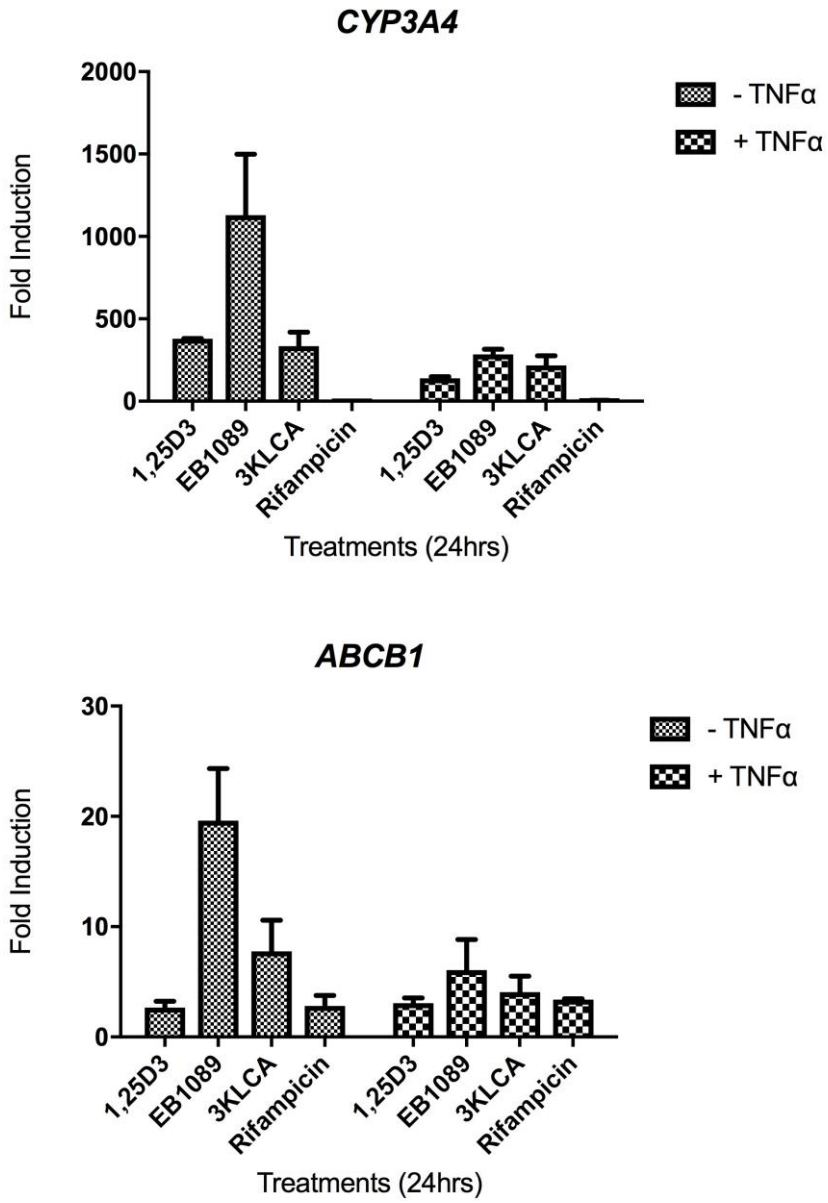


Figure 4.4 Established VDR target genes showing the effects TNF-alpha has on mRNA expression: LS180 cells were treated with previously established concentration of test ligand before RNA extraction was carried out plus/minus TNF-alpha treatment. Extracted RNA was reverse transcribed subjected to qRT-PCR analysis as per manufacturer's protocol. Data are represented as means (± SEM) of two independent experiments.

4.3.2 The effects cytokine TNF-alpha has on mRNA expression on established VDR targets in the LS180 cell line model.

TNF alpha is a cell signaling protein known as a cytokine, involved in systemic inflammation and is one of the cytokines involved in the acute phase response. A deregulation or dysfunction of TNF alpha has been associated with various diseases including IBD, cancerous states and Alzheimer's disease. TNF alpha has the ability to bind cell surface receptors that result in the downstream activation of the pro inflammatory pathway NF-Kapp-Beta or NF-kB is a protein complex that regulates the transcription of DNA. NF-kB plays a major role in regulating immune response and has been linked to cancer development and progression as well as inflammatory and autoimmune conditions. By utilizing a cytokine such as TNF alpha in this experimental set it has the potential to mimic, albeit at a basic level cells that have undergone a stress-inducing event, allowing analysis of gene activation profiles through qRT-PCR analysis. Figure 4.4 indicates that when the pro inflammatory cytokine TNF alpha is present it has a selective inhibitory effect on those genes regulated by the vitamin D receptor. PXR activation through Rifampicin in the case of *ABCB1* shows little change in the presence of TNF alpha, further hinting at the selective repression of VDR specific target genes. This inhibition is of great importance as it indicates that even acute responses that may potentially lead to the development of chronic inflammatory based conditions have a fundamental impact on the bodies ability to regulate key genes involved in a host of metabolic and homeostatic roles.

NK-kB transcription factor promoted immunity by controlling many genes involved in the inflammatory response. Cell surface binding of cytokines such as TNF alpha and pathogen associated molecular patters (PAMPs) stimulate a signaling cascade leading to the expression of target genes that regulate cellular proliferation and immune response. NF-kB was first characterized in cells present in the hematopoietic system however it has now come to light that NF-kB activation can occur through many cells types with significant importance in liver, adipose tissue and the central nervous system, all of which has links with the development of inflammatory associated metabolic diseases (Baker et al., 2011). However reduced VDR expression has been linked with the development of colonic inflammation as a result of systemic activation of NK-kB and disease progression. Therefore perhaps a prophylactic approach to maintain VDR levels may have the ability to reduce the activation of down stream inflammatory pathways thus inhibit the progression of inflammation driven pathology (Wu et al., 2010). Figure 4.3 summarizes the already established inducers of UGT1A target genes along with the nuclear receptor responsible for transcriptional activation. VDR has been added as it has been now shown through the work detailed in this thesis to induce a transcription of a number of *UGT1A* members using natural and synthetic analogues.

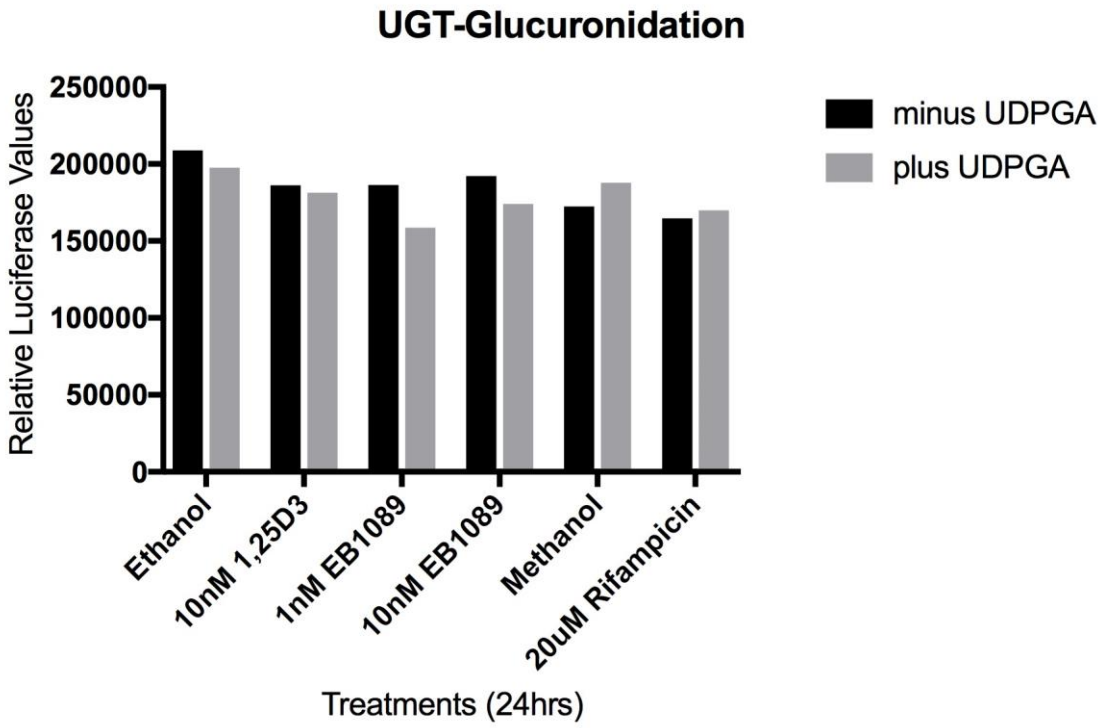


Figure 4.5: Preliminary UGT Glucuronidation Glo Assay: LS180 cells were seeded at $8x10^5$ cell/100mm dish, and allowed to incubate overnight at 37°C, 5% CO_2 and 95% air. The cells were washed with 1 x PBS and subsequently treated with vehicle control VDR or PXR ligands at indicated concentrations above. Only those reactions with the addition of UDPGA are able to glucuronidate the multi enzyme substrate. 40µl of Luciferin Detection Reagent with the addition of D-Cysteine was added to all the wells and allowed to incubate at room temperature for 20 minutes, before a luciferase reading was taken.

4.3.3 Preliminary UGT-Glo assay identifying if VDR agonists affect glucuronidation activity compared to established inducer PXR.

Being unsuccessful isolating detectable UGT protein in previous experimental set ups, the next logical step was to assess if VDR agonists increased the rate of glucuronidation activity using the UGT Glo system commercially available through Promega.

The assay allows for the analysis of the rate of glucuronidation activity under different stimulation events. However this study was only a preliminary analysis and very little can be gleamed from it, the assay suggests that when LS180 cells when exposed to VDR agonists such as $1,25(OH)_2D_3$ or EB1089 that there is a trend indicating an increase in glucuronidation activity.

The UGT-Glo Assay provides a luminescent method for measure UGT activity. The assay is designed to measure UGT activity from a number of sources including microsomes containing recombinantly expressed enzyme or microsomal preparations derived from mammalian tissue. The assay uses proluciferin substrates that are then modified by the UGT enzymes and then converted to luciferin derivatives using D-Cysteine during the detection steps. Without this conversion facilitated by the UGT enzyme activity the proluciferin substrate will not produce light when incubated with the luciferase chemical. It is of importance to note that the proluciferin substrate has specificities towards a number of *UGT1A* and *2B* members therefore would not be able to show the glucuronidation activity of one member alone. Light output is inversely proportional to UGT enzymatic activity. Unfortunately the assay development was in its infancy when performed. The assay is designed to be used with microsomal preparations containing active UGT enzyme sources commercially

or prepared from mammalian tissue sources. The assay performed within this experimental set up used whole cell lysates from LS180 cells, which I now feel the model used not produce adequate amounts of the active enzyme for analysis within the Glo system. Furthermore attempting to maintain stability of the enzyme activity during the protein extraction phase may have led to destruction of enzymatic activity that may have been present. The use of a gentler lysis method could have been considered in an attempt to maintain integrity.

There is a direct correlation between the amount of light emitted from the assay and the rate of glucuronidation activity. I.e. reduced light emitted equates to an increase in the rate of glucuronidation. An alternative approach would be to isolate glucuronidation metabolites and visualize using mass spectrometry.

However before any meaningful conclusions can be made, further work is required to ascertain the potential implications VDR agonists have on the rate of glucuronidation, this was however beyond the scope of this thesis. Furthermore this assay would allow the study and extrapolation of the varying mutant constructs generated within the study on the rate of glucuronidation activity. Sourcing microsomal preparation from mammalian tissue from murine sources could be the way to establish this assay within the laboratory and I feel is the next step in understanding the role VDR plays in UGT glucuronidation activity.

Chapter 4: Discussion

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4.4 Discussion

UDP-glucuronosyltransferases (UGTs) belong to the Phase II drug metabolizing enzyme super family. UGTs mediate the transfer of glucuronic acid, from UDP glucuronic acid to predominantly hydrophobic states, therefore increasing the rate of their detoxification and excretion from the body.

Deficiency in the expression and/or activity of UGTs has been shown to result in genetic and acquired diseases such as Crigler-Najjar syndrome and jaundice (inability to bread down bilirubin).

UGT genes show tissue-specific distribution patterns as indicated through real time analysis previously undertaken within the body of work. The underlying mechanisms for this tissue specificity are not fully understood. Emerging evidence further supported by this thesis have demonstrated that nuclear receptors (NR), such as pregnane X receptor (PXR), constitutive androstane receptor (CAR), Liver X Receptor (LXR), and Vitamin D Receptor (VDR) can regulate UGTs and this NR-mediated regulation may contribute to the tissue-specific expression pattern of UGTs (Yang et al., 2017).

In addition, UGTs have been shown to be subject to the regulation by aryl hydrocarbon receptor (AhR) and other tissue-specific transcription factors (Tukey, *et al*). Furthermore based on UGTs capacity to catalyse the glucuronidation of xeno and endobiotics UGTs play an important role in hormonal homeostasis, energy metabolism, bilirubin clearance, and xenobiotic detoxification (Strassburg et al., 2002). Therefore this chapter helps in elucidating UGT regulation by nuclear

receptors highlighting a broader significance in understanding UGT's functioning that may have further implications in a number physiological and pathophysiological states.

Inflammation is central for the progression of chronic liver disease as well as systemic inflammation and the promotion cancer progression and development (Park et al., 2014).

NRs can directly interact with classic pro-inflammatory transcription factors including nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1). In addition, NRs repress pro-inflammatory gene expression by interference with Toll-like receptor (TLR) signalling that regulates transcriptional pathways involved in the innate immune response. However, inflammatory processes can affect RNA expression and posttranslational modification of nuclear receptors and their target genes. Figure 4.4 highlight the impact systemic inflammatory pathway activation has on vitamin D receptor (VDR) dependant target genes is indicated.

LXRs inhibit genes involved in the innate immune response and simultaneously induce genes for lipid metabolism, therefore providing a bridge between lipid metabolism and systemic inflammation (Ito et al., 2015).

A number of studies has been performed in the field of atherosclerosis, focusing on macrophage function and LXRs ability to act as a cholesterol sensors allowing the increased expression of genes associated with reverse cholesterol transport, cholesterol conversion to bile acids, and intestinal cholesterol absorption (Arrese and

Karpen, 2010). LXRs exert anti-inflammatory effects by attenuating bacterial or LPS-induced expression of pro-inflammatory proteins through inhibition of NF-κB pathway activation. Recent studies suggest that LXR agonists also reduce inflammatory processes in chronic inflammatory liver diseases.

Reduced expression of VDR is associated with inflammatory rooted pathophysiology. Apart from its classical actions in the gut and bone, $1,25(OH)_2D_3$ possess potent anti-proliferative and immune-modulatory properties and is exerted through VDR. The presence of VDR in various tissues not including the gut and bone, along with their ability to regulate cellular differentiation, and anti-inflammatory action, has set the stage for therapeutic exploitation of VDR ligands for the treatment of a number of various inflammatory indications and cancers, and opens the potential prophylactic applications to ensure inflammatory based disorders are not able to manifest as readily.

Since the UGT family of enzymes is tightly regulated through the activity of the nuclear receptor superfamily, anything that may have a detrimental impact on NRs ability to regulate efficiently will have a direct impact on genes that rely on NR activation. Since UGTs have been shown to have a wide and varying impact throughout the body on many physiological process involved in xenobiotic metabolism the implication within disease development and progression is now widely accepted and supported therefore allowing the potential exploitation with regards UGTs regulation through NR activity may lend itself to maintaining basal genomic and homeostatic protection against pro inflammatory and external assault.

Chapter 5: General Discussion

5.1 General Discussion

Since the discovery of vitamin D as an essential component of healthy biological functioning, the physiological role of vitamin D goes far beyond that of its' classical roles in bone strength, mineralization, and calcium homeostasis (Bergwitz and Juppner, 2010). Within the last ten to fifteen years researchers understanding of vitamin D and its functionality within the body has evolved greatly to include roles within immune response, cellular differentiation, and extra hepatic metabolism (Aranow, 2011). A keen area of research at the moment remains the idea that vitamin D provides a baseline genome wide protection aiding in the overall protection of DNA from external assaults through the maintenance and baseline expression of certain key metabolic genes (Carlberg, 2014).

The hypothesis that VDR has the ability to regulate and maintain basal expression levels of key metabolic genes is bolstered through its evolutionary history. VDR is ubiquitously expressed throughout most human tissues and therefore are responsive to $1,25(OH)_2D_3$ (Wang et al., 2012).

VDR shares its main structural characteristics with other nuclear receptors i.e. a highly conserved DNA and ligand binding domain respectively (Mangelsdorf et al., 1995). The DNA binding domain of VDR along with its heterodimeric partner RXR (Sone et al., 1991) specifically contacts the hexameric consensus sequence within genomic DNA allowing for efficient binding to its target sites. The major binding motif, as determined by the steric constraints of the dimerizing DNA binding domains of both VDR and RXR is a direct repeat of two hexameric nuclear receptor (NR) binding motifs separated by three nucleotides (DR3) (Shaffer and Gewirth, 2004). Within the ligand binding domain of VDR there is a network of approximately forty

amino acids that form a ligand binding pocket, in which $1,25(OH)_2D_3$ and synthetic ligands bind with high affinity. Ligand binding results in a conformational change of altering drastically VDR's protein-protein interaction profile; changing from a repressor protein to an activator.

To further cement the hypothesis that VDR plays an integral role in overall genome protection, as well as basal activation of key regulatory genes, VDR has been shown to interact with the chromatin and histone remodeling proteins. Chromatin itself provides a repressive potential ultimately stopping transcription factors from interacting directly with DNA. This is contrasted with regions of the epigenome that show highly dynamic changes through the binding of VDR. These changes involve DNA methylation, posttranslational modifications, including acetylation and phosphorylation, allowing the activation of VDR target genes that are involved in key regulatory processes throughout the body.

A number of ChIP-chip analyses have been carried out through the works of Pike *et al*, and have identified a wide range of VDR specific target genes through ChIP analysis on enhancer and regulatory regions within mouse promoters including but not limited to; *Trpv6* (Meyer et al., 2006), *CYP24A1* (Meyer et al., 2010), and *Cbs*, (Kriebitzsch et al., 2011).

Vitamin D serum concentrations vary widely between one individual and the next. There are a number of factors that can affect this; 1) differences in diet 2) varying degrees of sunlight exposure, 3) age, and 4) genetic and epigenetic variations. As a result of chronically low vitamin D status, accelerated age related conditions could result including bone loss, and increased risk of falls and fractures.

This poses an important question, whether an insight into the transcriptome and genomic actions of $1,25(OH)_2D_3$ and by extension the vitamin D receptor can help in an individual's overall basal genome protection, and therefore an individual's responsiveness/need for vitamin D. A study by (Carlberg et al., 2013) was carried out using a cohort of 71 individuals who participated in a five-month vitamin D intervention study. They noted only a subset of individuals showed a significant correlation between genetic transcription events and serum $1,25(OH)_2D_3$ concentration, suggesting that at a molecular level not all the participants gained any benefit from the vitamin D supplementation. This could be due to a number of factors; 1) they had reached their optimal vitamin D status before the trial began or 2) they possess a polymorphism making them less responsive to vitamin D. Furthermore a negative correlation was noted between vitamin D status and the inflammatory marker interleukin 6 (IL-6), highlighting the potential role VDR may play in inflammatory response pathways.

Research into further understanding the role VDR plays within the body is still ongoing. Within this thesis we have identified that VDR has the ability to regulate the activity of members of the phase two metabolic gene family, *UGT1A*.

The *UGT1A* family members are involved in glucuronidation reactions i.e. the transfer of glucuronic acid to metabolic substrates making the substrate more hydrophobic in nature.

The *UGT1A* family of genes has been of great interest since the mid 1990's and have been identified as the most important phase II metabolic gene members. UGT's have been shown to be regulated through many nuclear receptors including but not limited to PXR, CAR and FXR (Bock and Köhle, 2004).

This thesis has identified that the vitamin D receptor also has the ability to regulate a number of *UGT1A* members, focusing mainly on *UGT1A1*, due to this genes involvement in a number of hereditary disease states involved in bilirubin metabolism as well as its role in extra hepatic tissues.

The major tissue associated with phased metabolism has always been the liver. Hepatic tissues express a plethora of metabolic genes across all three phases of metabolism. Liver tissue does express *UGT1A* family members however this thesis proposes that the extra hepatic expression of *UGT1A1* is as of equal importance if not more important in biotransformation and detoxification vs. hepatic expression. Tukey *et al* supports this observation. Through the development of K.O mouse models specifically knocking out the intestinal expression of *UGT1A1* resulted in death of the animal subject, comparatively a knockout of the hepatic expression resulted in the intestinal expression compensating for the loss hepatic expression (Fujiwara et al., 2012).

One of the major observations through this study is that VDR can directly bind and regulate the transcriptional activity of *UGT1A* members as it identified in chapter one. The ability of VDR to actively regulate the transcriptional activity of this family of genes has not been documented before. In addition to this VDR along with its heterodimeric partner RXR has been shown to directly bind DNA response elements and not require the use of tertiary protein interactions. This observation was achieved through our cyclohexamide studies.

This led us to question how effective is VDR compared to other nuclear hormone receptors in activating *UGT1A* members and *UGT1A1* specifically?

To this end a comparative analysis was carried out to assess the transcriptional potential of VDR against known prototypical inducers of *UGT1A1* activity. PXR and FXR have been well established and inducers of a number of phased two metabolic genes including *UGT1A1* (Kanno et al., 2016), (Fang et al., 2013).

Through the study and identification of the enhancer module within the *UGT1A1* promoter it has allowed for the precise location of the potential DR3 response element employed through VDR/RXR binding to be identified and assessed. Interestingly the DR3 motif has previously been identified by Tukey et al as a PXR binding site. Due to the homology shared between many of the steroid hormone receptors it stands to reason that a number of the receptors have the potential to share binding motifs. This evidently held true for this DR3 motif. When the ability of VDR was disrupted through site directed mutagenesis this not only knocked out VDR's ability to bind but PXR's ability also (Chen et al., 2012).

Furthermore, when considering the potency of the activation potential across the nuclear receptor activators it is interesting to note that the VDR based ligands, both natural and synthetic achieved transcriptional activation when exposing cell models to concentrations in the nano molar ranges compared to those in the micro molar ranges when we consider PXR and FXR agonists. That variance in itself could bode well for potential therapeutic application.

VDR also seems to achieve transcriptional responsiveness independent of other nuclear receptors. When combining VDR and FXR agonists there was no synergistic response, supporting that both receptors employ different DNA response elements within the enhancer module. Furthermore preliminary studies were conducted to assess if VDR activation of *UGT1A* members had an impact on the rate of

glucuronidation within a basic cell line model. To achieve this a luciferase based approach was employed, and the preliminary assessment looked promising as VDR agonists had an overall impact on glucuronidation activity, however further work is essential to assess completely the statistical significance as it was beyond the scope of this project.

It was also observed that VDR's ability to regulate *UGT1A* activity was far more effective in extra hepatic environments. HepG2 cells do not express adequate levels of VDR to achieve statistically significant transcriptional activity even when VDR was artificially transfected into the cells. (This could be due in part due to the transfection efficiency of the HepG2 cell line). Furthermore the transcriptional responses seen in the LS180 cell line model were far more transcriptionally responsive, and further supported by the overall basal expression of a number of *UGT1A* members compared across both HepG2 and LS180. Figure 5.1 details the proposed model of the potential role *UGT1A* members play in homeostatic genome protection. The increase in basal expression within the intestine supports our theory that *UGT1A1* could play a pivotal role in overall genome wide protection and detoxification within the body. Our data shows the presences of a never before identified VDRE site advancing the complexity and overall activation profile of the *UGT1A* gene family. VDR has now been shown to be expressed in varying tissues throughout the body, and the role of VDR beyond simply bone health has been an area of keen research for many years lends credence to the fact that genes controlled via liganded VDR have the potential to be exploited for areas beyond bone health and structure. The liver has long since been described as the primary detoxification organ within the body, however the intestinal tract, including the small intestine and colon are constantly under external stresses from a range of sources i.e. reactive oxygen

species, xenobiotics, primary/secondary bile acids and chemotherapeutics (Fang et al., 2013), and has been documented that it plays just as an important role in overall detoxification and the genetic profile witnessed in that gut are of pivotal importance to establish and maintain an active an functioning metabolism. It has also been documented that *UGT1A1* expression inhibits the downstream activation of the inflammatory pathway NF-kB, furthermore it has been witnessed that key metabolic genes in cancerous states are reduced including *UGT1A1* and *CYP3A4* (Ghosh et al., 2012). This opens up further potential therapeutic applications through the artificial raising of key metabolic gene expression levels in healthy tissue, increasing the overall rate of detoxification, enabling the more precise targeting and destruction of cancerous cells due to their reduced capacity to metabolize the chemotherapeutic.

UGT1A1 has been documented to also stabilize the expression of the 'gate keeper' of the genome, p53 (Shukla and Gupta, 2010), as well as activate downstream pathways including Nrf2 enabling the removal of reactive oxygen species (Wu et al., 2011).

Our understanding of how *UGT1A* genes are controlled and their role within the body both in a hepatic as well as extra hepatic is ever changing and requires further investigation, as there are many potentials for therapeutic manipulations that could be exploited in the years to come.

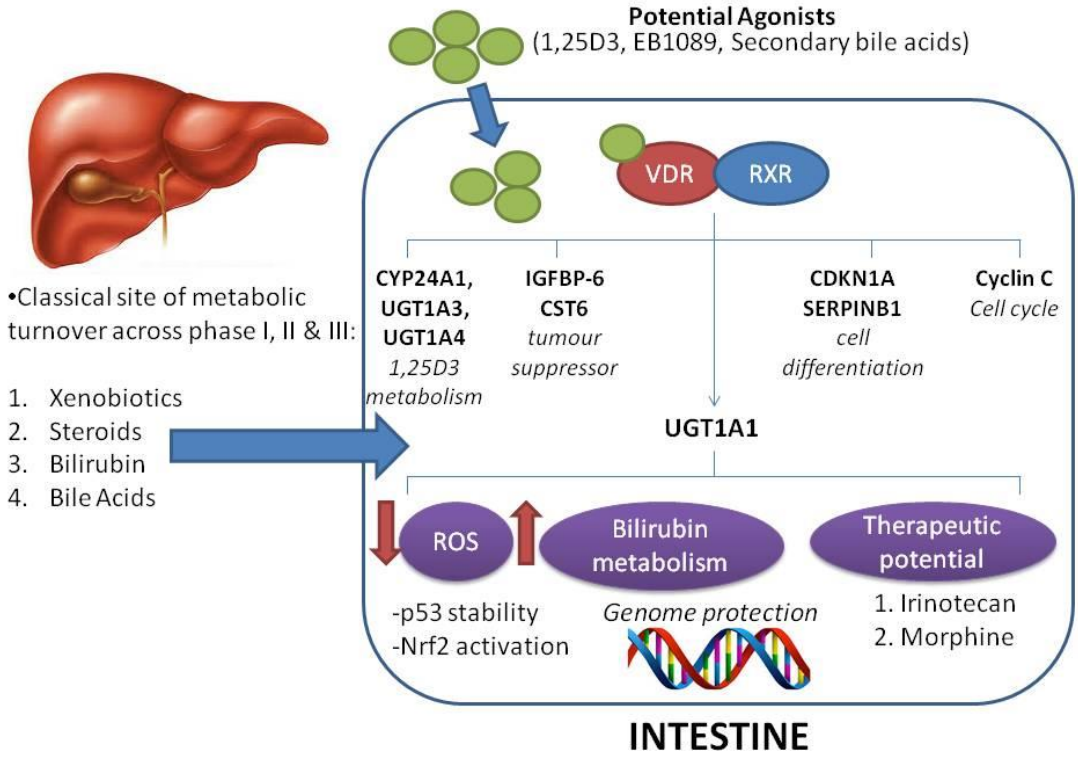

Figure 5.1: Proposed model of *UGT1A1's* potential ability to provide baseline protection against harmful xeno and endobiotic assault through direct regulation through the vitamin D receptor in healthy intestinal tissue, through the activation of downstream response pathways, with an additional prospective for therapeutic targeting to reduce non-specific cytotoxicity within healthy tissue.

Limitations

There were a number of limitations noted within this study. The major one I feel being a lack of translational responses through the use of western blot analysis. A number of attempts were made to isolate microsomal fractions for western blot analysis however this was trickier than first expected, and attempts at isolating this fraction failed. If isolated we would have been able to assess the *UGT1A* and *UGT1A1* at a protein level, allowing us to determine if VDR activation was producing an up regulation in protein production. The second limitation noted is the lack of glucuronidation activity assays. Having this within the study again would enable further deductions to be made regarding VDR's overall effectiveness to regulate *UGT1A* responsiveness to its agonists.

Future work

- Assess using ChIP analysis physical VDR interaction with DNA response elements.
- Further define and establish a fully function glucuronidation assay to assess the effectiveness of VDR agonists on the rate of glucuronidation in comparison the known agonists.
- Employ Mass spectrometry to isolate glucuronide metabolites to further ascertain the effectiveness of VDR activation of UGT activity.

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