

Non-Calcemic Actions of Vitamin D Receptor Ligands

Abbreviated Title: VDR

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Abbreviations: APC, Antigen presenting cell; CAT, Calcium transporter; CE, Cornified envelope; CIA, Collagen-induced arthritis; EAE, Experimental allergic encephalomyelitis; ECaC, Epithelial calcium channel; DBD, DNA binding domain; DR, Direct repeat; EGF, Epidermal growth factor; HAT, Histone acetyltransferase; IBD, Inflammatory bowel disease; IGFBP, Insulin-like growth factor binding protein; KS, Kaposi's sarcoma; LBD, Ligand binding domain; LXR, Liver X receptor; MBP, Myelin basic protein; MDS, Myelodysplastic syndromes; MMP, Matrix metalloproteinase; MOG, Myelin oligodendrocyte glycoprotein; MS, Multiple sclerosis; NGF, Nerve growth factor; NK, Natural killer; NOD, Nonobese diabetic; PBMC, Peripheral blood mononuclear cells; PEL, Primary effusion lymphoma; PLC, Phospholipase C; PPAR, Peroxisome proliferator activated receptor; PSA, Prostate specific antigen; RA, Rheumatoid arthritis; RANKL, Receptor activator of nuclear factor-kappa B ligand; RAR, Retinoic acid receptor; ROR, RAR-related orphan receptor; RXR, Retinoid X receptor; SCC, Squamous cell carcinoma; SLE, Systemic lupus erythematosus; SRC, Steroid receptor coactivator; TGase, Transglutaminase; Th, T helper; TR, Thyroid hormone receptor; TRIP, Thyroid receptor interacting protein; VDR, Vitamin D receptor; VDRE, Vitamin D response element.

Abstract

$1\alpha, 25$ -dihydroxyvitamin D₃ ($1,25$ -(OH)₂D₃), the active metabolite of vitamin D₃, is known for the maintenance of mineral homeostasis and normal skeletal architecture. However, apart from these traditional calcium-related actions, $1,25$ -(OH)₂D₃ and its synthetic analogs are being increasingly recognized for their potent anti-proliferative, prodifferentiative and immunomodulatory activities. These actions of $1,25$ -(OH)₂D₃ are mediated through vitamin D receptor (VDR), which belongs to the superfamily of steroid/thyroid hormone nuclear receptors. Physiological and pharmacological actions of $1,25$ -(OH)₂D₃ in various systems, along with the detection of VDR in target cells, have indicated potential therapeutic applications of VDR ligands in inflammation (rheumatoid arthritis, psoriatic arthritis), dermatological indications (psoriasis, actinic keratosis, seborrheic dermatitis, photoaging), osteoporosis (post-menopausal and steroid-induced osteoporosis), cancers (prostate, colon, breast, myelodysplasia, leukemia, head and neck squamous cell carcinoma and basal cell carcinoma), secondary hyperparathyroidism and autoimmune diseases (systemic lupus erythematosus, type I diabetes, multiple sclerosis and organ transplantation). As a result, VDR ligands have been developed for the treatment of psoriasis, osteoporosis and secondary hyperparathyroidism. Further, encouraging results have been obtained with VDR ligands in clinical trials of prostate cancer and hepatocellular carcinoma. This review deals with the molecular aspects of non-calcemic actions of vitamin D analogs that account for the efficacy of VDR ligands in the above mentioned indications.

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I. Introduction

The biological actions of the hormonally active form of vitamin D₃, 1,25-(OH)₂D₃ or calcitriol (Fig.1), and its synthetic analogs are mediated by the nuclear vitamin D receptor (VDR). VDR is a ligand dependent transcription factor belonging to the superfamily of steroid/thyroid hormone receptors (1), and has traditionally been associated with calcemic activities, namely, calcium and phosphorus homeostasis and maintenance of bone content. However, the observation that VDR is also present in cells other than those of the intestine, bone, kidney and parathyroid gland, led to the recognition of non-calcemic actions of VDR ligands. As a result, VDR is also known to be involved in cell proliferation, differentiation and immunomodulation. For example, activated T- and B-lymphocytes, rheumatoid arthritis synoviocytes and macrophages, Kaposi's sarcoma, prostate, breast and colon cancer cells, exhibit increased level of VDR protein when compared to their normal counterparts. This activation or disease-specific upregulation of VDR protein provides an opportunity to treat these conditions with VDR ligands. The expression of VDR in a variety of cell lines and primary cells, coupled with the increased evidence regarding the involvement of VDR in the processes of cell differentiation, inhibition of proliferation and immunoregulation, has prompted testing of the therapeutic effect of VDR ligands in several human diseases (Table 1) as well as in various animals models of diseases. These efforts have led to the development of VDR ligands for the treatment of psoriasis (2-4), secondary hyperparathyroidism (5) and osteoporosis (6, 7). In addition, VDR ligands have shown some efficacy in limited open clinical trials for prostate cancer, myelodysplasia (a precancerous state), psoriatic arthritis and rheumatoid arthritis. Examples of vitamin D analogs that have undergone clinical

trials with positive outcome are shown in Table 1. VDR ligands have also shown efficacy in the prevention and treatment of inflammatory and autoimmune diseases in various animal models. The goal of this article is to review the progress in the field of non-calcemic actions of vitamin D and its analogs with a particular emphasis on the current and potential therapeutic applications of VDR ligands. To limit the references to a reasonable number, many recent up-to-date reviews are included, sometimes in place of relevant articles. We apologize to our colleagues when a recent review article is mentioned instead of the multiple original references due to lack of space.

II. VDR and the regulation of gene expression

A. VDR and its functional unit

At the molecular level, 1,25-(OH)₂D₃ and its synthetic analogs modulate gene expression through a heterodimer between VDR and retinoid X receptor (RXR). RXR, a nuclear receptor for 9-cis retinoic acid, is an obligate partner of VDR in mediating 1,25-(OH)₂D₃ action (1, 8). In the absence of ligand and serum in cellular systems, most of the VDR is present in the cytoplasm (9). VDR ligand induces RXR-VDR heterodimerization and translocation of the complex into the nucleus (10, 11). The RXR-VDR heterodimer binds to vitamin D₃ response elements (VDREs) present in the promoter regions of responsive genes. Canonical VDREs are direct repeat (DR) of 5'-AGG/TTCA-3' motifs or a minor variation of this motif separated by three nucleotides and commonly referred to as DR-3 motifs (1, 8). However, non-canonical VDREs, such as an everted repeat of this hexanucleotide motif with a spacer of 6 base pairs (ER-6 motif) has been described in the promoter region of human Cyp3A4 gene (12). The VDR protein is modular in nature and like other nuclear receptors can be functionally divided

into three regions with well-characterized functions. The NH₂-terminal region contains a ligand independent transactivation function, AF-1. However, unlike other nuclear receptors, AF-1 is not well developed in VDR and whether the AF-1 region of the longer version of VDR plays a significant role in VDR-mediated transactivation, remains to be seen. The central region contains the DNA binding domain (DBD) consisting of two C2-C2 type zinc fingers, which target the receptor to VDREs. The C-terminal region of the receptor contains a multifunctional domain harboring the ligand binding domain (LBD), the RXR heterodimerization motif and a ligand dependent transactivation function, AF-2. A VDR ligand binds to the LBD of VDR and the ensuing conformational change results in the enhancement of VDR-RXR heterodimer formation (10). Unlike other nuclear receptors, there is only one isoform encoded by a single gene in humans and other organisms. However, 14 distinct transcripts of VDR have been reported that differ in their 5' termini and are produced as a result of alternative splicing and differential promoter usage. Most of the variant transcripts produce the same classical VDR protein of 427 amino acids (13).

B. Regulation of gene expression

VDR is a ligand-dependent transcription factor that can modulate the expression of vitamin D responsive genes by three different ways (Fig. 2). It can either positively regulate the expression of certain genes by binding to the VDREs present in their promoter regions (1, 8) or negatively regulate the expression of other genes by binding to negative VDREs (14, 15) or inhibit the expression of some genes by antagonizing the action of certain transcription factors, such as NF-AT and NF- κ B (16-18). Genes whose expression is induced by VDR ligands, and which are known to

contain a VDRE in their promoter are presented in Fig.2 along with their known function. These genes include osteocalcin, osteopontin, RANKL and carbonic anhydrase II, which are involved in extracellular bone matrix formation and bone remodeling (1, 19-24). Other genes, which contain a VDRE in their promoter region and show vitamin D-dependent upregulation in their expression, are the cell adhesion molecule $\beta 3$ integrin, tumor suppressor p21, calbindin-9k, 24-hydroxylase, human CYP3A4 and its rat and mouse CYP counterparts, involucrin, PLC $\gamma 1$ and IGFBP-3 (1, 12, 25-32). Genes that are downregulated in response to $1,25\text{-(OH)}_2\text{D}_3$ and its synthetic analogs are also listed in Fig.2. The known hyperproliferative and inflammatory functions of these gene products indicate that many of the therapeutic effects of $1,25\text{-(OH)}_2\text{D}_3$ and its analogs could result from their negative gene regulatory or transrepression activities. VDR ligands have been documented to inhibit the expression of cytokines, namely, IL-2 (T-cell lines and PBMCs) (33, 34), IL-12 (myelomonocytes) (35), TNF- α (PBMCs) (34), IFN- γ (PBMCs) (34) and GM-CSF (PBMCs) (36). Proliferation associated genes that are repressed by VDR ligands include, EGF-receptor (keratinocytes) (37), c-myc (keratinocytes) (37) and K16 (psoriatic plaques) (38). PTH (parathyroid cells) (39) and PTHrP (osteoblasts and keratinocytes) (40, 41) that are involved in mineral homeostasis are also downregulated by VDR ligands. Recently, rel B (NF- κ B component) showed vitamin D-dependent down-regulation in dendritic cells (14). Negative regulation of PTH (42), PTHrP (43) and rel B (14) gene expression appears to occur through a DNA motif, called negative VDRE (nVDRE). However, the mechanism of VDR-dependent inhibition of IL-2 and GM-CSF expression appears to be more complex than the involvement of positive or negative VDREs. In the case of these cytokines, VDR first competes with NF-AT1 for binding to

the composite NF-AT1-AP1 enhancer motif, and then it interacts with c-Jun. This apparent co-occupancy of the composite site by VDR-c-Jun leads to inhibition of activated IL-2 and GM-CSF expression (16, 17, 44). Both VDR monomers and VDR-RXR heterodimers are involved in inhibition of IL-2 and GM-CSF promoters.

C. VDR cofactors

Vitamin D-dependent transcription requires the binding of ligand occupied RXR-VDR heterodimers to VDREs present in the upstream regions of responsive genes. The ligand binding in general increases the affinity of VDR with various proteins called cofactors that act as a bridge between the RXR-VDR heterodimer and the basal Pol II transcription machinery. Using genetic and biochemical approaches, a number of cofactors that interact with VDR and other nuclear receptors, in a ligand dependent manner, have been identified. VDR-interacting cofactors are listed in Table 2. Cofactor proteins do not show any DNA binding activity but possess the capability to modulate gene expression in transfected systems.

Cofactors include two functionally distinct families of proteins, namely coactivators and corepressors. Coactivators mediate induction of transcription, whereas the reciprocal family of corepressors binds to the unliganded or antagonist occupied nuclear receptors and suppresses the expression of responsive genes. The identification of cofactors provided an increased understanding of the process of transcription, since these proteins provided either the chromatin modifying enzymatic activities or acted as a platform for the recruitment of histone destabilizing/stabilizing enzymatic activities or recruited basal transcription factors to the ligand responsive promoters. The SRC family of cofactors includes three members, namely SRC-1, SRC-2 and SRC-3 (Table 2). SRC

family members, along with CBP/p300 and pCAF, are histone acetyltransferases (HATs) that destabilize the nucleosomal core by catalyzing the acetylation of lysine residues present in the N-terminal tails of histones (45). TRIP1/Sug1 interacts with the VDR in a ligand-dependent manner and may act as a mediator for transcription or direct the receptor to ligand-dependent proteosomal degradation (46-48). Sug1 has also been found to have DNA helicase activity (49). Therefore, TRIP1/Sug1 may perform various roles by forming different complexes in a cell context-dependent manner (Table 2). SKIP/NcoA-62 synergized with SRC-1 and SRC-2 to induce RXR-VDR-mediated ligand-dependent transactivation. This synergy was explained by the observations that NcoA-62 formed a ternary complex with VDR and SRC-2, wherein NcoA-62 and SRC-2 interacted with VDR through helices H10 and H12, respectively (50, 51). The SRC family members, CBP/p300, NcoA-62 and TAFs, act as transcriptional coactivators and strongly potentiate ligand-dependent activation of transcription by VDR and other members of the nuclear receptor superfamily. VDR also directly interacts with certain components of the basal transcription machinery including TF-IIB, TF-IIA and TATA binding protein (TBP)-associated factors (TAFs), e.g., TAFII₁₃₅, TAFII₅₅ and TAFII₂₈ (52-54).

A complex of approximately 20 proteins called DRIP (VDR-interacting proteins)/SMCC/TRAP/ARC that interacts with VDR, other nuclear receptors and transcription factors, has been described (55). The DRIP complex was found to be sufficient for *in vitro* ligand-dependent transcription by the RXR-VDR heterodimer (55). The RXR-VDR recruits the complete DRIP complex by ligand-mediated interaction with DRIP 205, a component of the complex. The current working model for the vitamin D-mediated transcription is thought to require ligand-dependent targeted recruitment of

VDR-DRIP and VDR-SRC complexes to the VDRE in a sequential manner (56). The first step involves the recruitment of VDR-SRC or a VDR-HAT activity complex to a responsive promoter to facilitate the destabilization of the nucleosomal core. The unwound DNA then becomes a target for the VDR-DRIP complex, which contains mediator factors that are required for interaction with basal transcription factors and polymerase II. This two-step model is currently in vogue but may get more complex as additional VDR interacting proteins are discovered.

Three corepressors (Table 2) have been found to interact with VDR, and these are NcoR-1, NcoR-2 and Hairless (45). These corepressors recruit histone deacetylase activities, that deacetylate the lysine residues present in the N-terminally located histone tails, resulting in chromatin compaction and silencing of genes. Ligand binding induces a receptor conformation that creates a hydrophobic cleft, thus rendering the nuclear receptor receptive to interaction with coactivators through their NR boxes (LXXLL motifs). A related motif present in corepressors NcoR-1 and SMRT, called CoRNR box (I/LXXI/VI) was shown to be essential for the interaction of corepressors with the unliganded receptor (57, 58). Coactivators and corepressors provide “yin” and “yang” that is required for tighter control of transcription by physiological stimuli. The number of VDR interacting cofactors indicates that even combinatorial and parallel receptor-interacting complexes may exist. Understandably, with the discovery of a plethora of receptor interacting proteins, the transcription picture has become more complicated. But this scenario also provides an opportunity of increased understanding of tissue and gene-selective transcription by natural and synthetic ligands.

III. VDR crystal structure

Though structures for many nuclear receptors with or without ligands were available, obtaining a good model for 1,25-(OH)₂D₃ complexed VDR was challenging. The VDR LBD has an insertion domain from amino acid residues 165-215, which is poorly conserved between different species, with no obvious biological significance (59). Rochel et al., engineered a VDR LBD lacking this loop and characterized this mutant protein to prove that it was able to bind the natural ligand and was also capable of transactivation (60). This mutant VDR-LBD was crystallized to solve the structure of the ligand bound receptor. The crystal structure of the LBD of mutant VDR (60) resembles that of other nuclear receptors displaying three layers of 13 alpha helices and a short β -sheet of three strands (Fig. 3A and B). The nomenclature of helices is based on the description of the structure of RXR α LBD (61). Members of the nuclear receptor superfamily not only exhibit the same modular domain structure but also a moderately conserved LBD (60). The canonical folds of LBDs of all nuclear receptors consist of 10-13 α -helices, 2-5 β -sheets arranged in anti-parallel orientations and connecting loops of varying sizes (62). The relative position of N-terminal helix H1 is conserved among all nuclear receptors and it provides intramolecular contacts for the stabilization of the global structure of LBD. In VDR, helices H1 and H3 are connected by two short helices H2 and H3n, where H3n is predicted by secondary structure predictions in the place of a loop structure that is present in other nuclear receptors (60). The crystal structure of VDR LBD closely resembles that of RAR γ . However, a very conspicuous difference between VDR and RAR γ is that the loop connecting H1 and H3 (shown in green color for RAR γ in Fig. 4) wraps around the three-stranded short β -sheet in RAR γ (63), whereas it just passes by the one end of β -sheet in VDR (shown in pink color in Fig. 4), thus leaving the sheet exposed. Note that

the β -sheets of RAR γ are shown in blue and those of VDR are shown in red (Fig. 4). Concomitantly, the loop connecting the β -strand-2 (β 2) and β -strand-3 (β 3) in VDR depicted in red color is folded out of the 1,25-(OH) $_2$ D $_3$ -binding pocket, whereas the corresponding loop in RAR γ (depicted in blue color) is pointing towards the pocket, thus making the pocket smaller in RAR γ .

The structure of the liganded VDR LBD (60) gives an opportunity to understand possible interactions between the natural ligand and the receptor. The structure of receptor-ligand complex revealed that all the β -sheet residues contact the ligand. Trp-286 that is specific to VDR plays the crucial role of positioning the ligand. Trp-286 forms an intra-molecular hydrogen bond network with Ser-275, which in turn is hydrogen bonded to Met-272 (60). The ligand binding pocket is primarily composed of hydrophobic residues. The ligand curves around the helix H3 with its A ring interacting with the C-terminus of helix H5 and the 25-hydroxyl end close to helices H7 and H11 (Figs. 3 and 5). The 1-hydroxyl forms two hydrogen bonds with Ser-237 (H3) and Arg-274 (H5), while the 3-hydroxyl forms two hydrogen bonds with Ser-278 (H5) and Tyr-143 (Fig. 5) (60). A water channel is also observed with water molecules hydrogen bonded to Arg-274 leading to the solvent (Fig. 5). The conjugated diene connecting the A and C ring nicely fits against the hydrophobic planar Trp-286 stabilized by planar interactions. The α -face of the C ring contacts Trp-286, while the C18 methyl group points towards Val-234 that lies in helix H3 (Fig. 5) (60). The aliphatic chain adopts an extended conformation and is surrounded by hydrophobic residues. The 25-hydroxyl group is hydrogen bonded to His-305 (lies in the loop connecting H6 and H7) and His-397 (which lies in helix H11) (Fig. 5). It is also observed that the His-305 would be a

hydrogen bond acceptor while His-397 would be a donor, given the network of hydrogen bonds around this region (60).

The positioning of the helix H12 that is crucial for the coactivator binding and transactivation represents the agonist position in the published structure. This makes two direct Van der Waals contacts (Val-418 and Phe-422) with the methyl groups of the ligand, thus indicating the modulation of helix H12 conformation and positioning by the ligand (60). The position of helix H12 is also stabilized by a number of hydrophobic contacts and polar interactions (Val-234, Ile-268, His-397 and Tyr-401). The helix H12 residue Glu-420 that makes a salt bridge with Lys-264 of helix H4 has been implicated in ligand-dependent transactivation (64). Recently, computer docking of a VDR ligand specific for the non-genomic actions has resulted in the identification of an alternative ligand binding pocket (A-pocket) that partially overlaps the 1,25-(OH)₂D₃-binding pocket (G-pocket) identified in the VDR-LBD crystal structure (65). Although modeling studies showed this alternative ligand-binding pocket in the VDR, the crystal structure of estrogen receptor β (ER β) associated with 4-hydroxytamoxifen (4-OHT) revealed two molecules of 4-OHT bound to the protein (66). The ER β second ligand-binding pocket resembles the A-pocket of VDR and might be a unifying feature of the nuclear receptor superfamily. The elucidation of the crystal structure provides an opportunity for medicinal chemists to design and synthesize novel, non-secosteroidal, high affinity VDR ligands for the treatment of responsive indications.

IV. VDR knock-out animals

Gene knock out studies in mice and VDR mutations in humans have provided considerable insights into the physiological functions of vitamin D. Four groups have created VDR knockouts animals (67-71). VDR null mice were phenotypically normal at birth but developed hypocalcemia, hyperparathyroidism, rickets/osteomalacia and alopecia after weaning. Female null mutant mice also displayed uterine hypoplasia and impaired folliculogenesis. These mice died within 4-6 months of age. Similar symptoms occur in kindreds with VDR mutations in vitamin D-dependent rickets type II (VDDR II). Interestingly, feeding animals with a diet rich in calcium, phosphate and lactose normalized all the symptoms in null mice, but for hair abnormalities. These observations suggest that increased intestinal calcium absorption is critical for 1,25-(OH)₂D₃ action on bone and calcium homeostasis. The calcium homeostasis defect could be explained by the reduced expression of duodenal epithelial calcium channels CAT1 (ECaC2) and CAT2 (ECaC1) in VDR null mice (71).

Vitamin D deficient animals have also been shown to develop hypocalcemia, rickets and hyperparathyroidism, but unlike VDDR II patients, never developed alopecia. Thus, intact VDR is required for maintaining bone mineral homeostasis after birth as well as for normal hair development and hair follicle homeostasis. Hair-reconstitution studies in nude mice using VDR^{-/-} keratinocytes showed normal hair follicle morphogenesis but a defective response to anagen initiation phase of the hair cycle (72). These studies also indicated that the defect lies in keratinocytes. In accordance with this notion, targeted expression of human VDR transgene to keratinocytes of VDR null mice prevented alopecia (73). Therefore, hair loss

in VDR^{-/-} mice appears to be due to keratinocytes defective in epithelial-mesenchymal interactions that are required for normal hair cycling.

Similar hair cycle defect with total alopecia was also observed in inactivating mutation in hairless (hr) gene and in mice with temporally controlled RXR α mutations in the epidermis. Hairless gene product (Hr) acts as a corepressor of VDR, TR and ROR α (retinoic acid receptor related orphan receptor α), and associates with VDR *in vitro* and *in vivo* (74). It is tempting to hypothesize that VDR-Hr interaction may modulate hair cycling by controlling the expression of an inhibitor of hair cycle. Epidermis-specific temporal disruption of RXR α in mouse showed alopecia and skin abnormalities (75). Like VDR ablation, progressive alopecia in epidermal targeted RXR α ^{-/-} animals was attributed to defects in hair cycle. Since RXR is the heterodimer partner of other nuclear receptors (RAR, PPAR, LXR and TR) resident in skin, these results suggest that only RXR-VDR signaling pathway is involved in hair cycling. Further, the absence of keratinocyte differentiation abnormalities in the VDR null animals (76), but the presence of these defects in the temporally controlled RXR α mutation in mouse epidermis points towards the redundancy of RXR partner nuclear receptor functions in mouse skin. VDR knock out mice were also found to have impaired insulin secretory capacity (77).

VDR/RXR α compound null mutant mice have also been prepared and showed growth retardation, impaired bone formation, hypocalcemia and alopecia, features typical of VDR null animals (78). The growth plate development in compound knock out animals was more severely impaired in comparison to VDR null animals.

These findings indicate that both vitamin A and vitamin D signaling pathways are required for the normal development of growth plate chondrocytes.

V. Vitamin D action in autoimmune disease models

In addition to its central role in calcium and bone metabolism, 1,25-(OH)₂D₃ has potent immunomodulatory effects on many immune cell types including both innate and adaptive immune cells (79-81). Consistent with these effects, the VDR is widely expressed in most immune cell types such as antigen presentation cells (APCs: monocyte/macrophage, dendritic cells), NK cells (82, 83), T cells (84) and B cells (85). Furthermore, the intriguing effects of 1,25-(OH)₂D₃ have been demonstrated in several autoimmune disease models, namely, systemic lupus erythematosus (SLE) in *lpr/lpr* mice (86, 87), type I diabetes in nonobese diabetic (NOD) mice (88-92), collagen-induced arthritis (CIA) (93-95), experimental allergic encephalomyelitis (EAE) (96-101) and inflammatory bowel disease (102).

A. Multiple Sclerosis (MS)

MS is a chronic inflammatory autoimmune disease of the central nervous system (CNS) in which self-epitopes on myelinated nerve fibers are inappropriately recognized by adaptive immune cells of the host. The ensuing immune response recruits T cells and macrophages into the CNS, resulting in localized areas of inflammation and demyelination known as MS lesions. EAE is widely used as an animal model for human MS disease. Self-antigen reactive T helper I (Th1) cells have been demonstrated to play an essential role in both induction and effective phase of disease. Th1 cell differentiation is controlled by both antigen stimulation and cytokines, particularly IL-12 and IL-23, which subsequently induce synthesis of Th1 specific transcription factor, T-bet, and

drives Th0 cells towards Th1 differentiation (103). Therefore, controlling Th1 development by inhibiting IL-12 production will benefit the Th1-mediated disease, such as MS. VDR ligands have been shown to inhibit IL-12 p70 production in freshly isolated human monocyte or peripheral blood mononuclear cells that are primed with IFN- γ and stimulated with LPS in a dose-dependent manner (104). Furthermore, mitogen-induced differentiation of neonatal CD4⁺ T cells into Th1 cells (IFN- γ secreting T cells) *in vitro* is dramatically inhibited by 1,25-(OH)₂D₃ and its analogs. Interestingly, the inhibition of Th1 development seems to be specific, since Th2 (IL-4 secreting T cells) cell differentiation is largely unaffected by this treatment (104).

When mice were immunized with self-antigen peptide, myelin oligodendrocyte glycoprotein (MOG35-55), and treated with 1,25-(OH)₂D₃, disease induction is inhibited as evidenced by the reduction of inflammatory infiltration and reduced demyelination of brain and spinal cord, along with decreased antigen-induced T cell proliferation during an *in vitro* T cell recall response. More importantly, it also inhibited Th1 development (104), suggesting that inhibiting CD4 Th1 effector function is one of the mechanisms by which 1,25-(OH)₂D₃ inhibits EAE. In contrast to CD4⁺ T cells, the protection of EAE from 1,25-(OH)₂D₃ treatment does not involve CD8⁺ T cells (97), although CD8⁺ T cells have the highest levels of VDR and have been implicated as both suppressors and effectors of the inflammation associated with EAE. In addition to effects on T cells, 1,25-(OH)₂D₃ has also been shown to decrease macrophage accumulation in the CNS, which can contribute to its protective effect during EAE (99). When EAE was induced in B10.PL mice after immunization with myelin basic protein (MBP), administration of 1,25-(OH)₂D₃ not only prevented the induction of the disease,

but also ameliorated the disease when the treatment was administered at the appearance of the first disability symptoms. Interestingly, withdrawal of 1,25-(OH)₂D₃ resulted in the resumption of EAE. Deficiency of vitamin-D in the food led to the increased EAE susceptibility in mice, suggesting that daily vitamin D *in vivo* prevents the occurrence of the autoimmune disease (105). Furthermore, immunization of VDR-deficient mice led to EAE which was not suppressed by administration of 1,25-(OH)₂D₃, whereas EAE in wild type animals was completely blocked by this treatment, suggesting that the VDR is necessary and directly involved in 1,25-(OH)₂D₃-mediated suppression of EAE.

Although the mechanism of EAE inhibition by 1,25-(OH)₂D₃ is not completely understood, many studies have clearly implicated that a major effect of VDR ligands is on T cell functions, mainly through inhibiting Th1 cell development and function while enhancing Th2 cell development (106). Administration of 1,25-(OH)₂D₃ in mice increases IL-4 and TGF-β1 expression, which presumably play an important role in regulating T cell responses (100). Accordingly, VDR ligands were found to be less effective in reducing the progression of EAE in IL-4 null mice (102).

B. Rheumatoid Arthritis (RA)

RA, one of the most common chronic inflammatory diseases that affects about 1% of the population, is characterized by articular infiltration of neutrophils, macrophages, T and B cells, and dendritic cells, resulting in subsequent tissue damage (107). The collagen-induced arthritis (CIA) model is the most commonly used arthritis model for human RA. Immunization of mice with type II collagen induces arthritis, which could be prevented by dietary supplementation or oral administration of 1,25-(OH)₂D₃ in both mouse and rat (93-95). More interestingly, administration of 1,25-

(OH)₂D₃ or its analogs at the time of disease with early symptoms could prevent the progression to severe arthritis (92, 94). In addition, treated animals showed diminished serum levels of antibodies to rat collagen type II and reduced mitogen-induced proliferation of lymph node cells (94), suggesting that 1,25-(OH)₂D₃ suppresses RA by altering adaptive immunity.

Epidemiological studies have reported low serum levels of vitamin D, and its metabolites, in rheumatoid arthritis patients (108). In addition, an open-label clinical trial involving patients (Table 1) with psoriatic arthritis showed an improvement in disease symptoms following the oral administration of 1,25-(OH)₂D₃ (109). More significantly, in a 3 month open-label clinical trial in 19 RA patients being treated with standard DMARD therapy for acute RA (Table 1), high dose oral alfacalcidol (1 α hydroxyvitamin D₃; Fig. 1) therapy showed a positive effect on disease activity in 89% of the patients, and only 11% patient showed no improvement (110). This result provides strong evidence that VDR ligand can be used clinically for the treatment of RA.

1,25-(OH)₂D₃ has been detected in synovial fluid of arthritic joints and the expression of VDR has also been reported in rheumatoid synovial tissue and at the site of cartilage erosion. Matrix metalloproteinases (MMPs) play an important role in the chondrolytic process of rheumatoid lesion. Animal studies have shown that the production of some MMPs may be upregulated in rat chondrocytes by administration of 1,25-(OH)₂D₃ (111). Interestingly, treatment of rheumatoid synovial fibroblasts by 1,25-(OH)₂D₃ did not affect the expression of MMPs. However, when simultaneously stimulated with IL-1 β , the MMP production was reduced 50% compared with IL-1 β alone. Prostaglandins have a role in the immune system and inflammatory processes

associated with RA. Interestingly, IL-1 β -stimulated PGE2 synthesis is also completely inhibited by 1,25-(OH)₂D₃ treatment (112). Therefore, VDR ligand can suppress both the IL-1 β -stimulated production of MMP and PGE2 in rheumatoid synovial fibroblasts, suggesting that VDR-mediated biological processes are important in controlling RA. However, the molecular mechanism by which VDR ligands inhibit IL-1 β -stimulated MMP and PGE2 production is not known.

C. Inflammatory Bowel Diseases (IBDs)

IBDs (Ulcerative colitis and Crohn's disease) are immune-mediated pathologies with unknown etiology that target the gastrointestinal tract. T cells, particularly CD4⁺ Th1 cells, which preferentially produce inflammatory cytokines (IL-2, TNF- α , IFN- γ), have been shown to transfer Crohn's-like symptoms to naive mice, and the production of Th1 cytokines is associated with IBD in humans (113, 114). Interestingly, IL-10 deficient mice develop colitis within 5-8 weeks of life and one third of these mice die after the development of severe anemia and weight loss (115). In this model, lack of Vitamin D in the diet led IL-10 knockout mice to rapidly develop diarrhea, wasting disease, and mortality. In contrast, supplementation with 1,25-(OH)₂D₃ in diet significantly ameliorated the symptoms of IBD in IL-10 null mice. Similarly, treatment of animals with 1,25-(OH)₂D₃ blocked the progression and decreased the established IBD symptoms (115). 1,25-(OH)₂D₃ also inhibited the proliferation of rectal epithelial cells (116) and T cells (117) in active ulcerative colitis patients, suggesting the potential of VDR ligands in the treatment of IBDs.

D. Type I Diabetes

Type I diabetes is an autoimmune disease characterized by the immune-mediated destruction of insulin-producing β -cells of islets of Langerhans in the pancreas. Several cellular effector mechanisms leading to β -cells destruction have been identified, including $CD4^+$ and $CD8^+$ T cells and macrophages (118). The nonobese diabetic (NOD) mouse that spontaneously develops type I diabetes, is the most widely used animal model for type I diabetes (119). Based on the effect of VDR ligands on T cell and macrophage functions described earlier, it is reasonable to predict that type I diabetes would be modulated by VDR ligand. Epidemiological studies have shown an increase in the disease incidence when vitamin D deficiency was present in the first month of life in children. Moreover, in a recent study in NOD mice, vitamin D deficiency accelerated the onset of type I diabetes (120).

In fact, when $1,25-(OH)_2D_3$ was administrated at 3 weeks of age before the onset of insulinitis, it effectively prevented the progression of diabetes in NOD mice. However, treatment was ineffective if administrated at 8 weeks of age when insulinitis was well established (121). In addition to the natural ligand, a vitamin D analog, KH 1060 (Fig. 1), has also been shown to prevent the onset of type I diabetes in NOD mice (91). Vitamin D analogs were also effective in the treatment of ongoing type I diabetes in the adult NOD mice by effectively blocking the disease course (92), raising the possibility of treatment of type I diabetes with VDR ligands before β -cells are completely destroyed. Treatment of mice with a synthetic $1,25-(OH)_2D_3$ analog also inhibited IL-12 production and blocked the infiltration of Th1 cells into the pancreas. It should be noted that IL-12 is a direct target of $1,25-(OH)_2D_3$ (35). More interestingly, $CD4CD25^+$ Treg cells were also increased by this treatment in pancreatic lymph node (92). In fact, adaptive transfer of

pancreatic lymph node-derived Treg cells into NOD mice completely prevented spontaneous diabetes. Interestingly, Treg cells accumulated preferentially in the pancreatic lymph nodes and islets, but not other lymph nodes or the spleen (92). In addition, 1,25-(OH)₂D₃ could induce an autoantigen-specific T-helper Th1 to Th2 immune shift in NOD mice immunized with GAD65 (89), suggesting that VDR ligands might work on multiple level to modulate the immune system and prevent autoimmune diseases.

E. Systemic Lupus Erythematosus (SLE)

SLE is a systemic autoimmune disease. Patients with SLE produce autoantibodies to many tissue antigens including DNA, histones, red blood cells, platelets, and leukocytes, and as a result these individuals present with varying symptoms. The therapeutic potential of VDR ligands for the treatment of SLE was explored using an analog of 1,25-(OH)₂D₃, 22-oxa-1 α , 25-dihydroxyvitamin D₃ (Fig. 1). Symptoms of SLE were alleviated in MRL/lpr mice following treatment with 22-oxa-1 α , 25-dihydroxyvitamin D₃. In addition, at therapeutically effective doses of 22-oxa-1 α , 25-dihydroxyvitamin D₃, hypercalcemia was not observed in the autoimmune animals (122).

F. Transplant Rejection

Organ and tissue transplantation are increasingly being performed worldwide, and these procedures many times lead to long-term survival. The commonly used strategy for preventing transplantation rejection involves the use of immunosuppressive agents such as corticosteroids and cyclosporin. 1,25-(OH)₂D₃ exerts a variety of immunomodulatory effects such as inhibition of T Lymphocyte proliferation (123-125), down regulation of cytokines IL-2 and IFN- γ (124), and DCs maturation and

survival (126). Based upon these observations, 1,25-(OH)₂D₃ and its analogs have been tested either as single agent or in combination with other immunosuppressive agents such as cyclosporin in many experimental models, including heart (127, 128), liver (129), pancreatic islets (130, 131) and skin (132, 133). Interestingly, 1,25-(OH)₂D₃ treatment was shown to prolong allograft survival. For example, transplantation of rat islets under the kidney capsule in spontaneously diabetic NOD mice resulted in early graft failure in 4 out of 10 recipients (131). Single treatment of NOD mice with KH1060 (Fig. 1), a vitamin D₃ analog or cyclosporin did not result in statistically significant suppression of early graft failure. However, combination of both resulted in 100 % early graft success (131). Similar results were also reported in chronic allograft rejection, where adventitial inflammation and internal hyperplasia in rat aortic allograft was inhibited by treatment with the VDR ligand (134). In addition to its direct immunosuppressive function in transplantation, 1,25-(OH)₂D₃ and its analogs also demonstrated important impact on the prevention of bone loss and improvement on bone quality after organ transplantation (135-137). The mechanism of vitamin D₃ in preventing transplant osteoporosis is probably due to its effect on hyperparathyroidism caused by the usage of corticosteroids or cyclosporin. Moreover, the immunomodulatory properties of 1,25-(OH)₂D₃ may enable the reduction of cyclosporin and/or corticosteroid dosage, which could potentially contribute to an improvement in post transplantation-related bone loss.

In addition to its primary physiological role in regulating calcium homeostasis, vitamin D₃ has been demonstrated to have pleiotropic actions in immune system. 1,25-(OH)₂D₃ was found to inhibit antigen-induced T cell proliferation (138), and cytokine production (139). Antigen presenting cells (APC), in particular, dendritic cells (DCs) are

also the primary targets for the immunosuppressive activity of 1,25-(OH)₂D₃. They play a central role in regulating immune response to self and foreign antigens. During the normal immune response, T cell response and specificity is conferred through the clonal restricted T cell receptor, which recognizes MHC class I and class II molecule complexed with peptides. However, the potency of DC-mediated T cell activation also depends on their maturation status. Immature DCs express low levels of MHC class II costimulatory specific maturation markers, such as CD83, whereas mature DCs express high levels of these molecules in response to appropriate proinflammatory stimuli (140, 141). Immature DCs not only provide weak signal for activating T cells but also induce anergic and regulatory T cells (142). Mature DCs have considerably less antigen uptake, demonstrate increased antigen presentation and induction of costimulatory ligands on the surface. These cells also show enhanced production and secretion of immunomodulatory cytokines such as IL-12 (143). The maturation of DCs is regulated by many factors and cell-cell interactions. Among the factors that induce DC maturation are the components of pathological pattern recognition, which are recognized by cell surface Toll-like receptors and many cytokines including TNF- α . T cells can induce DC maturation through their intimate interactions by activating TNFR family members expressed on DC such as CD40. However, whether the preservation of DCs in an immature state results from the absence of maturational stimuli or is also actively maintained *in vivo* is unknown.

VDR is widely expressed in most cell types in the immune system such as APCs, monocyte/macrophage, NK cells and DCs (82, 83). 1,25-(OH)₂D₃ could affect DC function through differentiation, cytokine production, activation, maturation as well as

survival (126, 144). The most direct evidence of $1,25\text{-(OH)}_2\text{D}_3$ effect on DCs comes from VDR knockout mouse studies, where DCs from VDR deficient mice showed significantly higher level of maturation marker such as class II MHC, CD40, CD80 and CD86 on cell surface (144). Under differentiation conditions in the presence of GM-CSF and IL-4 from purified human CD14^+ monocytes, $1,25\text{-(OH)}_2\text{D}_3$ completely inhibited DC differentiation with low-level expression of IL-12 and maturation markers (144, 145). Consequently, $1,25\text{-(OH)}_2\text{D}_3$ -treated DCs lead to impaired alloreactive T cell activation *in vitro* and *in vivo* (144, 146). Furthermore, $1,25\text{-(OH)}_2\text{D}_3$ also affects maturing DCs, leading to decreased IL-12 secretion and enhanced IL-10 production, which subsequently affects T cell differentiation (126). Recent evidence also pointed out that $1,25\text{-(OH)}_2\text{D}_3$ not only exhibits immunosuppressive effects on T cells and APCs, but also leads to the induction of tolerogenic DCs, which subsequently enhance $\text{CD4}^+\text{CD25}^+$ regulatory T cells and protect the allograft rejection (147). In conclusion, all the preclinical studies suggest that $1,25\text{-(OH)}_2\text{D}_3$ and its analogs can be potentially used for the prevention of transplant rejection.

In summary, VDR ligands have pleiotropic effects on the immune system including both innate and adaptive immunity. In particular, their effect on dendritic cells is thought to induce tolerogenic dendritic cells resulting in T cell anergy. Their effect on T cells can lead to the formation of regulatory T cells as well as accelerate Th2 cell development. All of this evidence suggests that VDR ligands have great potential for the development of therapies for multiple human autoimmune diseases. The challenge for the future is to develop safer oral VDR ligands without the hypercalcemic side effect.

VI. Vitamin D action on keratinocytes and psoriasis

Psoriasis, a recurrent inflammatory skin disorder, affects approximately 2% of the population. In addition, 5-10% of the patients develop psoriatic arthritis, with inflammation and swelling in the hands, feet and large joints. Psoriasis is characterized by keratinocyte hyperproliferation, abnormal keratinocyte differentiation and immune-cell infiltration into the epidermis and dermis. The most common form of psoriasis is plaque psoriasis or psoriasis vulgaris. At the molecular level, psoriasis lesions show a prominent loss of loricrin and filaggrin in the suprabasal layers of the epidermis and abnormal overexpression of other differentiation markers such as involucrin, transglutaminase I (TGase I), psoriasin, migration inhibitory factor related protein-8 (MRP-8) and skin derived antileukoproteinase (SKALP). The expression of normal suprabasal keratins K1 and K10 is inhibited and replaced by the expression of the hyperproliferative keratins K6 and K16. Psoriatic epidermis also demonstrates an increased expression of IL-8 receptor, IL-6, IL-8, EGF-receptor (EGF-R), TGF α and amphiregulin. Lesions also show infiltration of IL-2, IFN- γ and TNF- α secreting CD8+ lymphocytes into epidermis and CD4+ lymphocytes into dermis, and these cytokines are thought to alter keratinocyte proliferation and differentiation.

The observations that keratinocytes and T-cells express VDR, and that 1,25-(OH) $_2$ D $_3$ is a potent stimulator of keratinocyte differentiation, provided a reasonable basis for the clinical use of VDR ligands for the treatment of psoriasis (82, 148). The first clinical evidence to support the use of vitamin D analogs was obtained fortuitously when a patient treated orally with 1 α -hydroxyvitamin D $_3$ (Fig. 1) for osteoporosis showed remarkable remission of psoriatic lesions (149). Subsequently, promising clinical results were obtained in studies using oral 1 α -hydroxyvitamin D $_3$, oral and topical 1,25-

(OH)₂D₃ (calcitriol) and topical 1,24-(OH)₂D₃ (tacalcitol; Fig. 1). In these clinical trials approximately 70-80% of the patients showed marked improvement and complete clearance of the target lesions was observed in 20-25 % of patients (150). A topical preparation of calcitriol/ (Silkis, 3 µg/g ointment) is being developed by Galderma Laboratories for the treatment of psoriasis. It has shown safety and efficacy at a calcitriol concentration of 3 µg/g ointment but resulted in increased risk of hypercalciuria at 15 µg/g concentration (151, 152). Therefore, it appears to show a therapeutic window of 5 between efficacy and side effect. Medicinal chemists have tried to develop 1,25-(OH)₂D₃ analogs with decreased hypercalcemia liability mostly by minor modifications of the secosteroidal backbone of vitamin D₃. Calcipotriol (Calcipotriene or Dovonex; Fig. 1), a synthetic 1,25-(OH)₂D₃ analog used topically for the treatment of psoriasis, was chemically engineered to be metabolized quickly in systemic circulation and as a result it is 100-200 times less calcemic than 1,25-(OH)₂D₃ (4). A comparison of hypercalcemic properties of calcitriol and calcipotriol is given in Table 3. A number of studies have confirmed the clinical efficacy of calcipotriol and significant improvement has been observed in approximately 70% of the patients after 6-8 weeks of topical therapy with twice daily application of the drug (4). The most common side effect of calcipotriol was cutaneous irritant reaction in approximately 20% of the patients (153). In comparative clinical trials, the efficacy of topical calcitriol was generally similar, and that of topical calcipotriol slightly better than potent topical steroids (154, 155). Steroids result in early onset of action in psoriasis but cannot be used for longer periods, because of their skin thinning (telangiectasia) side effect, which results because of the alteration in skin collagen metabolism (decrease in type I and type III collagen, TIMP 1 and TIMP 2

message levels) by this class of drugs (156). Therefore, a combination of topical calcitriol or calcipotriol with potent topical steroids has been tried in patients. These clinical studies indicated more rapid onset of action, increased efficacy and better tolerability of the combination regimens than the individual treatments (157, 158). As a result, a new formulation containing calcipotriol and betamethasone dipropionate (Daivobet) is under consideration by drug regulatory authorities for the treatment of mild-to-moderate plaque-type psoriasis (159).

The anti-psoriatic activity of VDR ligands could be attributed to their differentiation, anti-proliferative and immunomodulatory properties. VDR ligands exhibit multi-pronged effects in psoriatic lesions and affect the function of keratinocytes, T-cells and antigen presenting cells (APCs). VDR ligands promoted differentiation and inhibited the proliferation of keratinocytes (150, 160). Differentiation of keratinocytes results in the formation of cornified envelope (CE) that provides the barrier function of the skin. The expression of involucrin, a component of the CE, and TGase I, the enzyme that crosslinks the components of CE, was increased by 1,25-(OH)₂D₃ and other VDR ligands under certain conditions (161). Treatment of keratinocytes with the medium containing high calcium also stimulated keratinocyte differentiation by increasing the expression of involucrin and TGase I. 1,25-(OH)₂D₃ also promoted keratinocyte differentiation at least in part by increasing intracellular calcium by two separate mechanisms. 1,25-(OH)₂D₃ increased the expression of calcium receptor (CaR) and PLC- γ 1 in keratinocytes (162). 1,25-(OH)₂D₃ may also induce the expression of AP1 transcription factors by the induction of PLC- γ 1 (via second messenger IP3) and by inducing the expression of c-Fos (30) (Lu and Nagpal, unpublished observations). 1,25-(OH)₂D₃-mediated elevation of

AP1 activity may in turn induce the expression of keratin 1, involucrin, TGase I, loricrin and filaggrin, that are required for CE formation. Genes that are positively and negatively regulated by the treatment of keratinocytes by 1,25-(OH)₂D₃ are shown (Fig. 6). Expression of EGF-R, c-myc, and keratin 16 was downregulated in keratinocytes and/or psoriatic lesions following VDR ligand treatment (1).

VDR ligands decreased the expression/level of pro-inflammatory cytokines IL-2, IFN- γ , IL-6, IL-8, and GM-CSF (1, 33, 34, 36) in T cells, all of which play a role in cutaneous inflammation, and proliferation of T-lymphocytes and keratinocytes. Further, topical calcipotriol increased anti-inflammatory cytokine IL-10 and decreased IL-8 in psoriatic lesions (163), and 1,25-(OH)₂D₃ also increased the expression of IL-10 receptor in keratinocytes (164). As a matter of fact, oral less calcemic VDR ligands, which exhibit multi-pronged effect on all the major cell types involved in psoriasis, have the potential to replace more expensive biologic therapies (TNF- α antibodies, soluble TNF- α receptor, etc.), particularly in the face of recent observations that these therapies lose their effectiveness over time in a manner analogous to that of steroids.

APCs or dendritic cells also play an important role in psoriasis and autoimmune diseases since they are involved in autoantigen presentation. It appears that APCs are one of the major targets of 1,25-(OH)₂D₃-mediated immunosuppressive action and VDR ligands prevent the differentiation, maturation, activation and survival of dendritic cells leading to T-cell hyporesponsiveness (126). 1,25-(OH)₂D₃ also increased the expression of IL-10 and decreased the expression of IL-12, two major cytokines that are involved in Th1-Th2 balance (147). It is believed that the development of more

efficacious topical and oral VDR ligands, with improved side effect profiles, will further expand the treatment options for patients with psoriasis.

VII. Vitamin D action on prostate cancer cells

Prostate cancer is the second leading malignancy after skin cancer and it is also the second leading cause of cancer deaths among men in the United States (165). The discovery that the VDR is expressed in normal prostate, benign prostate hyperplasia (BPH), malignant prostate, and prostate cancer cell lines, led to the recognition that BPH and prostate cancer could be potential targets for VDR ligands (166-168). Epidemiological studies have indicated an inverse relationship between mortality rates due to prostate cancer and UV light exposure. UV light is required for the synthesis of vitamin D in skin (169). In fact, one of the major risk factors for developing prostate cancer was low serum level of 25-hydroxyvitamin D (170). Several *in vitro* studies have demonstrated that 1,25-(OH)₂D₃ and its synthetic analogs inhibited the proliferation of prostate cancer cell lines (171) and primary epithelial cells from normal prostate, BPH and prostate cancer (172, 173). VDR ligands also inhibited tumor cell growth and metastasis *in vivo* (174-177). VDR ligands demonstrate pleiotropic action on prostate cancer cells that include growth arrest at G0/G1 stage of the cell cycle, apoptosis, tumor cell differentiation and interaction with androgen signaling pathway. The growth inhibition of cancer cells by VDR ligands is achieved by reduction of cyclin-dependent kinase 2 (CDK2) activity as well as the induction of p21, p27, IGFBP-3 (insulin-like growth factor binding protein-3), IGFBP-5 and E-cadherin expression (Fig. 6) (173, 178-180). The 1,25-(OH)₂D₃-mediated induction of p21 causes a decrease in CDK2 activity that leads to decreased phosphorylation of retinoblastoma (Rb) protein and repression of

E2F transcriptional activity resulting in growth arrest at G1 stage of cell cycle. Induction of IGFBP-3 was found to be obligatory for 1,25-(OH)₂D₃-mediated growth inhibitory effect since IGFBP-3 antisense oligonucleotides or neutralizing antibodies abrogated the growth inhibition engendered by the VDR ligand in LNCaP cells. Further, 1,25-(OH)₂D₃-mediated induction of p21 expression in LNCaP cells was also blocked by IGFBP-3 neutralizing antibodies, thus demonstrating that at least in LNCaP cells, p21 does not appear to be a direct target of vitamin D action (181).

Recently, a cDNA microarray analysis was performed on LNCaP, normal prostate or prostatic adenocarcinoma of Gleason grade 3/3 treated with vehicle or 1,25-(OH)₂D₃ to identify VDR target genes and to elucidate the mechanism of action of 1,25-(OH)₂D₃ in prostate cancer cells (173). As expected, 24-hydroxylase was found to be maximally upregulated by 1,25-(OH)₂D₃ in both normal as well as primary cancer cells. However, in LNCaP cells, 24-hydroxylase expression was not induced by the VDR ligand. In LNCaP cells, the most vitamin D-responsive gene was found to be IGFBP-3, whereas primary cells did not show any upregulation of IGFBP-3 expression. In both normal and cancer primary cells, the expression of the dual specificity phosphatase 10 was maximally induced by 1,25-(OH)₂D₃. Dual specificity phosphatase 10 inactivates mitogen activated protein kinase (MAPK), indicating that inhibition of the growth promoting actions of MAPK may at least in part explain the growth inhibitory actions of 1,25-(OH)₂D₃ in primary prostate cancer cells (173). The anti-oxidant effects of 1,25-(OH)₂D₃ in prostatic primary cultures were highlighted by the induced expression of thioredoxin reductase 1 and superoxide dismutase 2 (Fig. 6). Metallothioneins, most of the intracellular protein thiols that are thought to be anti-apoptotic factors, show

differential 1,25-(OH)₂D₃-dependent regulation in normal and primary prostate cancer cells. They show VDR ligand-mediated upregulation in normal and down-regulation of expression in primary cancer cells, therefore indicating that 1,25-(OH)₂D₃ may induce anti-apoptotic proteins in normal and apoptotic pathways in cancerous cells (173).

1,25-(OH)₂D₃ also induced the expression of 24-hydroxylase that catalyzes the first step of 1,25-(OH)₂D₃ degradation pathway leading to calcitroic acid, which is excreted in bile. In prostate cancer cells, the extent of growth inhibition by 1,25-(OH)₂D₃ was found to be inversely proportional to 24-hydroxylase activity. DU145, a prostate cancer cell line that shows higher 1,25-(OH)₂D₃ induced expression of 24-hydroxylase, was less responsive to 1,25-(OH)₂D₃-mediated growth inhibition than LNCaP cells that showed very low basal and induced expression of 24-hydroxylase. Accordingly, liarozole, an inhibitor of P450 hydroxylases, elicited a significant 1,25-(OH)₂D₃-dependent growth inhibition in DU145 cells (182). Another cytochrome P450 hydroxylases inhibitor, ketoconazole, also augmented the growth inhibitory effects of 1,25-(OH)₂D₃ and its synthetic analog EB 1089 (Fig. 1) in primary human prostate cancer cells (183). Therefore, the use of 24-hydroxylase inhibitors may enhance the growth inhibitory activity of 1,25-(OH)₂D₃ and its synthetic analogs in prostate cancer. 1,25-(OH)₂D₃ has been shown to inhibit the proliferation of both androgen-dependent as well as androgen-independent prostate cancer cells. The growth inhibitory effects of 1,25-(OH)₂D₃ on LNCaP cells were androgen-dependent, since, casodex, an androgen receptor (AR) antagonist, blocked these effects. On the other hand, 1,25-(OH)₂D₃ also inhibited the growth of AR negative prostate cancer cells as well as cells derived from a patient with advanced androgen-independent prostate cancer (172, 184, 185).

Clinical trials with 1,25-(OH)₂D₃ in prostate cancer patients have highlighted the potential of VDR ligands either alone or in combination with other cytostatic agents. In a pilot study of 7 patients with recurrent prostate cancer, oral calcitriol (0.5-2.5 µg/day) treatment for 6-15 months resulted in a significant decrease in the rate of prostate specific antigen (PSA) rise during therapy (in comparison to PSA increase before therapy) in 6 out of 7 patients (Table 1). In the seventh patient, the decrease in PSA rise did not reach statistical significance. As expected, the use of maximally intended calcitriol therapy was limited by the development of dose-dependent hypercalciuria (186). Generally, cancers are treated by a therapeutic regimen involving a combination of drugs. Therefore, differentiating molecules like 1,25-(OH)₂D₃ may sensitize tumor cells to cytotoxic effects of other chemotherapeutic agents. This premise was tested in a number of *in vitro* and *in vivo* studies, where 1,25-(OH)₂D₃ enhanced the anti-tumor effects of other chemotherapeutic agents, including cisplatin, paclitaxel and adriamycin (187-189). These studies provided the basis for the use of combination therapy involving calcitriol and taxol in prostate cancer patients. In a clinical study, 37 patients with androgen-independent prostate cancer were treated with oral 1,25-(OH)₂D₃ (0.5 µg/kg) on day 1, followed by docetaxel (36 mg/m²) intravenously on day 2 and the regimen was repeated weekly for 6 consecutive weeks on an 8-week cycle (Table 1). Patients were maintained on a reduced calcium diet (400-500 mg calcium/day) and increased oral hydration. The primary end point was PSA response (50 % decrease in PSA) that was confirmed in a second evaluation after 4 weeks. Thirty of 37 patients achieved statistically significant PSA response and 22 patients showed >75 % reduction in PSA levels. This study demonstrated that the combination was better than the

docetaxel treatment alone when PSA response rate, number of patients with >75 % decrease in PSA, time to progression or overall survival was compared with contemporary phase II clinical trials involving the administration of docetaxel as a single agent in androgen-independent prostate cancer patients (190). It is clear from these and other studies that prostate cancer treatment could benefit from further development of non-calcemic vitamin D analogs.

BPH is the most common non-malignant tumor in the aging male, and its pathogenesis also involves the regulation of prostate cell growth by androgen-dependent and androgen-independent growth factors (191). This process involves both prostate stromal and epithelial cells, since the messages for growth factors IGF-1 and KGF (keratinocyte growth factor) were expressed in stromal cells, whereas their receptors were expressed in prostatic epithelial cells (192). VDR ligands inhibited the growth factor-stimulated proliferation of cells from human BPH (193, 194). Further, a synthetic less-calcemic vitamin D analog (BXL-628/Ro-26-9228/RS-980400, Fig. 1) inhibited the androgen-induced ventral prostate weight in a dose-dependent manner in both intact and castrated rats. The effect of the VDR ligand was compared to that of anti-androgen, finasteride (194). Hypercalcemic properties of BXL-628/Ro-26-6228 are compared with calcitriol and presented in Table 3. Therefore, VDR ligands have potential as a new first line therapy for the treatment of BPH.

VIII. Vitamin D action on breast cancer cells

Breast cancer strikes approximately 200,000 women in the US each year and nearly 40,000 succumb to the disease (195). Epidemiological studies have shown an inverse relationship between exposure to solar radiation and higher breast cancer

incidence and mortality (196, 197). Another plausible link between $1,25\text{-(OH)}_2\text{D}_3$ and breast cancer came to surface by the observation that chromosomal region 20q13.2 that contains 24-hydroxylase (CYP24) was amplified in breast cancer. Since 24-hydroxylase is involved in $1,25\text{-(OH)}_2\text{D}_3$ degradation, its amplification may lead to decreased serum $1,25\text{-(OH)}_2\text{D}_3$ levels, thus providing a microenvironment conducive for cell growth in the absence of vitamin D-mediated growth control (198). Further, serum $1,25\text{-(OH)}_2\text{D}_3$ levels were found to be reduced in advanced bone metastatic breast cancer patients than in early stage patients (199). The VDR is expressed in most breast cancer cell lines, carcinogen-induced rat mammary tumors, normal breast tissues as well as in primary breast cancer tumors. Further, increased RXR and VDR protein levels were found in breast cancer tissues than normal breast tissue (200, 201).

$1,25\text{-(OH)}_2\text{D}_3$ and its synthetic analogs have been shown to inhibit the proliferation of breast cancer cells *in vitro*, and tumor progression *in vivo* (1, 200). One of the most interesting aspects of the action of VDR ligands is their efficacy in both ER-positive (MCF-7, T-47D, ZR-75-1 and SKBR-3) and ER-negative (BT-20, MDA-MB-435, MDA-MB-231 and SUM-159PT) breast cancer cells (1, 150, 202). Although the exact mechanism underlying the growth inhibitory actions of vitamin D in breast cancer cells is not clear, the data support a multi-pronged effect involving growth arrest at G0/G1 stage, cell apoptosis, disruption of estrogen and other growth factor-mediated cell survival signals and angiogenesis. The G0/G1 cell cycle arrest effects of VDR ligands on breast cancer cells could be explained by their ability to induce the expression of cyclin dependent kinase inhibitors (CDKIs), $p21^{\text{CIP1/WAF1}}$ and $p27^{\text{KIP1}}$ in breast cancer and other epithelial cells (Fig. 6) (202-204). CDKIs inhibit the cell cycle progression by blocking

the activity of cyclin-cyclin dependent kinase (CDK) complexes, which are positive factors necessary for cell cycle progression. The identification of a VDRE in p21 promoter indicated it to be an early mediator of vitamin D-mediated cell cycle arrest (28). Vitamin D-dependent induction of p27 is not mediated via a VDRE but depends upon transcription factors Sp1 and NF-Y (205). In MCF-7 breast cancer cells, 1,25-(OH)₂D₃ also decreased the protein levels of CDK2, CDK4, cyclin D1 and cyclin A (Fig. 6) in a time dependent manner (203, 206). 1,25-(OH)₂D₃ prevented the activation of cyclin D1-CDK4 and also resulted in the loss of cyclin D3, which leads to repression of E2F transcription factors and decreased cyclin A expression (204).

The antiproliferative effects of vitamin D on breast cancer cells could also be mediated by the induction of transforming growth factor- β 1 (TGF- β 1) and suppression of proto-oncogene c-myc (207, 208). 1,25-(OH)₂D₃ inhibited c-myc by inducing the expression of HOXB4, that binds to a specific DNA sequence in the c-myc promoter and blocks its transcriptional elongation (209). The natural VDR ligand also blocked the mitogenic activity of insulin and IGF-1 on breast cancer cells most probably by inducing the expression of IGFBP-3 and IGFBP-5 (Fig. 6) (210, 211). The molecular events involving cell cycle proteins lead to vitamin D-dependent induction of apoptosis, bax redistribution, decreased bcl-2 levels, cytochrome c release, polyADP ribose polymerase (PARP) cleavage, external display of phosphatidylserine and DNA fragmentation. Apart from having direct growth inhibitory effects, VDR ligands also inhibit angiogenesis and decrease the metastatic/invasive potential of breast cancer cell *in vitro* and *in vivo* (212-214). Similar to the rationale described for prostate cancer, treatment of breast cancer could be expanded to include combination therapies of VDR

ligands with more common treatment regimes. As expected, 1,25-(OH)₂D₃ and its synthetic analogs augmented the cytotoxicity of doxorubicin, paclitaxel, adriamycin and irradiation in breast cancer cell cultures (188, 215-217). An *in vivo* xenograft study has also demonstrated the interaction between a VDR ligand and paclitaxel or cisplatin treatment. In this model, additive antiproliferative effects were observed following combination treatment with the vitamin D₃ analog CB 1093 (Fig. 1) with either paclitaxel or cisplatin (187). VDR ligands also have immense potential to act as chemopreventive agents since they blocked the progression of mammary carcinogenesis *in vivo* (218).

IX. Vitamin D action on colon cancer cells

Colon cancer, one of the most prevalent tumors in Western countries, is the second leading cause of cancer deaths in the USA. Epidemiological studies have suggested the involvement of vitamin D in the pathogenesis of colorectal tumors. An inverse association has been reported between calcium, vitamin D, milk intake, sunlight exposure, serum levels of 25-hydroxyvitamin D₃ and colon cancer incidence/mortality (219, 220). Increased VDR protein was found in colonic tumors than their normal counterparts (219). VDR^{+/-} heterozygote and VDR^{-/-} knock out animals showed increased PCNA activity, cyclin D1 expression and proliferation of cells in the colon descendens, thus indicating the importance of 1,25-(OH)₂D₃ in keeping a check on increased colonic proliferating activity (220). Apart from possessing VDR, colonic cells also possess the ability to synthesize 1,25-(OH)₂D₃ from its precursor 25-hydroxyvitamin D₃ by the action of 1 α -hydroxylase activity. All the above-mentioned observations indicate VDR to be a potentially very important therapeutic target for colorectal cancer prevention and treatment. 1,25-(OH)₂D₃ and its synthetic analogs inhibited the

proliferation of colon cancer cells by affecting multiple pathways involving G1 cell-cycle block, apoptosis and cell differentiation (221-223). 1,25-(OH)₂D₃ induced differentiation of colonic tumor cells and also potentiated butyrate/tributylin-induced differentiation of HT-29 colon cancer cells (224, 225). Interestingly, using a VDR antagonist, ZK 191732 (Fig. 1), it was discovered that butyrate-induced differentiation of Caco-2 cells was mediated by VDR (225).

In a study involving colon carcinoma cell lines (RG/C2, AA/C1, PC/JW, HT-29 and SW620), 1,25-(OH)₂D₃ and EB 1089 (Fig. 1) induced growth inhibition, differentiation marker (alkaline phosphatase) and apoptosis (226). Apoptosis occurred subsequent to differentiation and it was consistently associated with increased levels of proapoptotic protein, Bak in all the colon carcinoma cell lines tested. Decreased Bcl-2 was not observed in all the 5 cell lines and it was not associated with apoptosis. 1,25-(OH)₂D₃ and a synthetic analog, ZK 156718 (Fig. 1), also induced the expression of cyclin-dependent kinase inhibitors p21 and p27 (Fig. 6) in Caco-2 cells (227). In SW480 cells, 1,25-(OH)₂D₃-induced differentiation was accompanied with an increase in E-cadherin and other adhesion proteins (occluding, zonula occludens [ZO-1 and ZO-2] and vinculin) (Fig. 6). In these cells, 1,25-(OH)₂D₃ also promoted the translocation of β -catenin, plakoglobin and ZO-1 from the nucleus to the plasma membrane. Further, liganded VDR competed with T-cell transcription factor (TCF)-4 for interaction with β -catenin and as a result, 1,25-(OH)₂D₃ repressed β -catenin-TCF-4 transcriptional activity. VDR ligand also repressed the expression of β -catenin-TCF-4-responsive genes, c-myc, PPAR δ , Tcf-1 and CD44, and it induced the expression of ZO-1 (223). Therefore, inhibition of β -catenin-TCF-4 signaling may be one of the molecular pathways involved

in vitamin D₃ action in colon cancer cells. It was reported that the reduction of cyclin D1 levels was a key factor in the antiproliferative effects of two vitamin D₃ analogs on Caco-2 tumor cell growth (228). In a recent study involving the examination of 1,25-(OH)₂D₃-mediated genetic changes in SW480-ADH colon cancer cells using oligonucleotide microarrays, 1,25-(OH)₂D₃ up-regulated the expression of c-Jun, JunB, JunD, FREAC-1/Fox1, ZNF-44/Kox7, plectin, filamin, keratin 13, G₀S2 and putative tumor suppressors NES-1 (normal epithelial cell-specific 1 gene or kallikrein 10) and protease M (229) apart from the previously known regulated genes (Fig. 6). The regulation of these genes was confirmed by Northern or Western blotting or immunocytochemistry. Overall, the regulation of gene expression by 1,25-(OH)₂D₃ in colon cancer cells indicates the involvement of vitamin D in the processes of differentiation, growth inhibition, cell adhesion and apoptosis, resulting in the transformation of neoplastic to a normal epithelial phenotype.

Bettoun et al., identified a novel complex involving VDR-Ser/Thr protein phosphatase PP1c/PP2A and p70S6 kinase. In this complex, 1,25-(OH)₂D₃ induced the VDR-associated phosphatase activity. The modulation of PP1c/PP2A activity by VDR resulted in a ligand-dependent rapid and specific dephosphorylation and inactivation of their substrate p70S6 kinase. Since p70S6 kinase is essential for G1 to S phase transition, these results also provided one of the molecular pathways of 1,25-(OH)₂D₃-mediated G1 block in Caco-2 cells (221). In keeping with these molecular observations, VDR ligands not only reduced the proliferation of colon cancer cell *in vitro*, but they also inhibited the proliferation of normal and premalignant human rectal epitheliomas *in vitro*, and reduced tumorigenesis in xenograft, Apc^{min} mutant mouse and reduced chemically-induced

tumors *in vivo* (209, 226, 228, 230-234). Colonic mucosal hyperproliferation and aberrant crypt foci are considered as biomarkers of colon cancer *in vivo*. Azoxymethane, a chemical that induces colon carcinogenesis in rat, increased the proliferation of colon cells, and this was accompanied by an increase (5-fold) in cyclin D1 and a decrease (>50 %) in E-cadherin protein in aberrant crypt foci. A fluorinated synthetic vitamin D analog inhibited azoxymethane-induced crypt cell hyperproliferation, aberrant crypt foci development, and tumor burden and also normalized the levels of cyclin D1 and E-cadherin *in vivo* (234).

X. Vitamin D action on leukemic cells

Uncontrolled proliferation of hematopoietic cells, that are unable to further differentiate into mature cell types, results in leukemia. Maturation block is also a hallmark of myelodysplastic syndromes (MDS), a group of clonal hemopoietic stem cell disorders with abnormal cell differentiation. Therefore, agents that induce differentiation in the hemopoietic precursors hold considerable promise as therapeutic options for leukemias and MDS. Activated lymphocytes, monocytes, macrophages and myeloid cells express VDR, and 1,25-(OH)₂D₃ induced differentiation in murine and human myelomonocytic leukemia cells, and prolonged the survival of mice inoculated with leukemia cells (235, 236). VDR ligands also exhibited cooperative effects with retinoids, vitamin K2 and chemotherapeutic agents on differentiation of myeloid leukemic cells (237-239). Direct target genes of 1,25-(OH)₂D₃ action in U937 leukemic cells are CDK inhibitor p21, b-zip-HLH transcription factor Mad-1 and homeobox protein HOXA10. Overexpression of p21 and HOXA10 facilitated the differentiation of U937 cells to monocytes/macrophages (27, 28, 240). p21 is a direct transcriptional target of vitamin D

since it contains a functional VDRE in its promoter region (28). Major and sustained hematological responses have been observed in MDS patients treated with calcitriol and alfacalcidol (241-243). Although the response rate in these studies varied from 44-100% (with complete remission in only 6% of the patients), still the prevention of progression of MDS to overt leukemia transiently during drug administration is significant and augurs well for the development of more efficacious therapeutically active less-calcemic VDR ligands.

XI. Vitamin D action in squamous cell carcinoma

The presence of VDR in keratinocytes and the ability of 1,25-(OH)₂D₃ to induce keratinocyte differentiation and inhibit their proliferation, also indicated its potential usefulness in squamous cell carcinoma (SCC) of head and neck and aerodigestive tract. 1,25-(OH)₂D₃ inhibited the growth of SCC cells *in vitro* and *in vivo* (189, 244). However, unlike other tumor cells, 1,25-(OH)₂D₃ decreased the expression of p21 in a murine SCC model (189). Further, overexpression of RXR γ in human SCC cells and inhibition of p21 expression in keratinocytes was found to be necessary for differentiation (245, 246). VDR target genes have been identified by expression profiling in EB 1089 (Fig. 1) treated head and neck SCC25 cells. The expression profile provided the molecular basis for the antiproliferative, differentiative and genoprotective effects of the VDR in SCC. The expression of 24-hydroxylase, protease M, cystatin M, amphiregulin, stromelysin and collagenase I was found to induced and that of CRABP-II, N-cadherin and SCC antigen (SCCA) was inhibited by EB 1089 (Fig. 6) (247). A number of other genes involved in differentiation, cell growth inhibition and immunomodulation pathways were identified but their expression was not confirmed by other techniques.

XII. Vitamin D action in Kaposi's sarcoma

Kaposi's sarcoma (KS) is a highly vascular tumor that occurs predominantly in men with HIV infection. The herpesvirus associated with KS also causes B cell lymphoma and primary effusion lymphoma (PEL). KS cell lines, and primary KS and PEL tumor tissue showed high levels of expression of VDR mRNA and protein and their proliferation was inhibited by $1,25-(\text{OH})_2\text{D}_3$ *in vitro* and *in vivo* (248, 249). In an open clinical trial, topical treatment of KS lesions with calcipotriol showed anti-tumor activity in patients (249).

XIII. Vitamin D action in bone and osteoporosis

Osteoporosis is a common metabolic disease characterized by reduced bone mass and microarchitectural deterioration of bone tissue that results in increased bone fragility and the risk of developing fractures. Various conditions that may lead to osteoporosis are estrogen deficiency, androgen deficiency, glucocorticoid excess, immobilization, hyperthyroidism, hyperparathyroidism and space travel. Bone is one of the major target organs of vitamin D, and VDR ligands regulate both bone formation and resorption. Because of these actions, calcitriol and alfacalcidol (Fig. 1) have been used for the treatment of osteoporosis and osteomalacia, and several VDR ligands (ED-71, Ro-26-9228 and 2MD; Fig. 1) are under preclinical/clinical development for osteoporosis. VDR ligands may also exert their beneficial bone anabolic effects by enhancing intestinal calcium absorption and by inhibiting the synthesis of parathyroid hormone. However, the mechanism of bone anabolic effects of $1,25-(\text{OH})_2\text{D}_3$ is not clear. $1,25-(\text{OH})_2\text{D}_3$ is classically considered to be a stimulator of bone resorption because it induces osteoclastogenesis by enhancing the expression of the RANKL in bone marrow stromal

cells (250). Further, human and mouse RANKL gene promoters contains a functional VDRE that shows RXR-VDR heterodimer-mediated ligand-dependent activation in the context of a reporter construct (23, 24). However, *in vivo*, VDR ligands decrease bone resorption and increase bone formation in ovariectomized animals and osteoporotic women. Therefore, 1,25-(OH)₂D₃ may show bone resorption in normal state, and bone formation as well as anti-resorption activities in osteoporotic state. Similar paradox is also observed in skin, where VDR ligands inhibit keratinocyte proliferation in psoriatic skin but induce epidermal proliferation in normal skin (251). Therefore, it appears that the differential action of 1,25-(OH)₂D₃ in normal and disease state is part and parcel of vitamin D pharmacology.

VDR is expressed at high levels in primary osteoblasts and various osteoblast cell lines, and they may hold the key to explain the bone anabolic effects of 1,25-(OH)₂D₃ and its synthetic analogs (252). Osteoporosis involves the loss of both the organic and mineral contents of the bone. 1,25-(OH)₂D₃ increased the expression and/or protein levels of osteocalcin and osteopontin in osteoblasts, thus supporting its role in bone matrix formation (Fig. 2). A number of reports have shown prevention and decrease of vertebral fractures and an increase in total body and spine bone mineral density in osteoporotic women with 1,25-(OH)₂D₃ treatment (6, 253-255). In a three year study of 622 postmenopausal osteoporosis women (Table 1) who had at least one vertebral compression fractures, calcitriol (0.25 µg twice a day) was compared with treatment with supplemental elemental calcium (1g/day). A significant reduction in vertebral fractures was observed during the second (9.3 vs 25 fractures/100 patients) and the third (9.9 vs 31.5 fractures/100 patients) year of study in women taking calcitriol in comparison to

calcium supplementation (255). In another clinical study involving postmenopausal women (n=55), treatment with calcitriol (0.5 µg/day) for 4 years increased femoral neck bone mineral density (BMD) by 3 %, whereas it was decreased by 1.9 % in the control group (Table 1). Two years of treatment resulted in 57 % increase in intestinal strontium absorption (a measure of intestinal calcium absorption), 100 % increase in urinary calcium and a 32 % decrease in serum PTH levels. Of the 18 people treated with calcitriol, 2 developed hypercalciuria (6). However, the use of 1,25-(OH)₂D₃ as a treatment for osteoporosis is limited by its margin of safety which appears to be very narrow and there is a real risk of developing side effects (hypercalcemia and hypercalciuria) unless subjects are monitored closely, which even though is possible in a clinical setting, is impractical in a real world treatment scenario. These unwanted effects result from an increase in calcium absorption through the intestine leading to increased plasma and urine levels of calcium that can ultimately result in the mineralization of soft tissues and kidney stone formation.

Both prodrug and medicinal chemistry approaches have been explored in an effort to identify and synthesize less calcemic vitamin D₃ analogs suitable for the treatment of osteoporosis. The prodrug approach resulted in the identification of alfacalcidol (1α-hydroxyvitamin D₃), a precursor of 1,25-(OH)₂D₃, that gets enzymatically converted to the active hormone in liver by the action of 25-hydroxylase. As a result, its action on gut is somewhat reduced because enterocytes lack 25-hydroxylase and therefore, alfacalcidol does not induce intestinal calcium absorption in the first pass when it is absorbed from the intestine. It has been found to reduce the incidence of vertebral fractures and increase bone mass in several clinical trials (256).

Success in treating osteoporosis with alfacalcidol is believed to be the result of the ability to administer higher doses of this compound as compared to 1,25-(OH)₂D₃, prior to the detection of hypercalcemia (256, 257). It is currently an approved treatment for osteoporosis in Japan because Japanese diet contains less calcium and therefore its side effect is manageable in that population.

Several attempts have been made to synthesize analogs of 1,25-(OH)₂D₃ that exhibit a lower occurrence of hypercalcemia *in vivo*. ED-71, 1 α , 25-dihydroxy-2 β -(3-hydroxypropoxy) vitamin D₃ (Fig. 1), is one example of such a compound (258). In studies using normal, ovariectomized, and prednisolone-treated rats, ED-71 increased calcium absorption in the gut, decreased bone resorption and increased bone mineralization (259, 260). ED-71 has also been found to be as effective as PTH in ovariectomized rats. At a dose of 0.08 μ g/kg/day for 5 weeks, ED-71 decreased bone resorption and increased bone mass without inducing hypercalcemia. In phase I studies in healthy human male volunteers (n=40), ED-71 at oral doses of 0.1 to 1.0 μ g for 15 days, resulted in a dose-dependent increase in urinary calcium but none of the subjects showed sustained hypercalciuria above 400 mg/day or hypercalcemia over 10.4 mg/dl. Based upon these results, a phase II, open label clinical trial with ED-71 (0.25, 0.5, 0.75 and 1.0 μ g/day for 24 weeks) was started with 109 osteoporosis patients. There was a dose-dependent increase in lumbar spine and this effect was better than that obtained in historical studies with estrogen-treated patients. ED-71 was well tolerated in these patients, without causing any sustained hypercalcemia and a dose of 0.75 μ g/day was clinically effective (261).

Two secosteroidal vitamin D analogs, Ro-26-9228 and 2MD (Fig. 1) are under preclinical development for osteoporosis. Ro-26-9228, a VDR modulator, was less potent than 1,25-(OH)₂D₃ in inducing the expression of 24-hydroxylase, calbindin D-9k and plasma membrane calcium pump 1 (PMCA 1) in duodena of ovariectomized rats, whereas, it was as potent as 1,25-(OH)₂D₃ in enhancing the expression of osteocalcin, osteopontin, TGF β1 and TGF β2 in trabecular bone (262). Ro-26-9228 decreased bone resorption and increased the number of differentiated osteoblasts in ovariectomized rats and showed approximately 3-fold separation between bone protecting effect and hypercalciuria. Increase in bone mineral density was accompanied by a dose-dependent decrease in type I collagen degradation products (pyridinoline cross-links) in the urine. Tissue selective action of Ro-26-9228 was also observed *in vitro*, where Ro-26-9228 was less potent than 1,25-(OH)₂D₃ in inducing the expression of a VDRE containing reporter in intestinal Caco-2 cells, whereas they showed equipotent activities in osteoblastic MG-63 cells (262, 263). A synthetic analog of vitamin D, 2MD (2-methylene-19 nor-(20S)-1,25-(OH)₂D₃) has been described that was found to be a more potent stimulator of bone formation *in vitro* and *in vivo*. In a 7-day animal model fed low calcium diet, it was found to have approximately 30-fold difference between osteoblast-mediated calcium mobilization and intestinal calcium transport when compared to 1,25-(OH)₂D₃ (264). It showed increased bone mass at non-calcemic dose when ovariectomized rats were treated for 23-weeks. However, the extent of separation between bone efficacy and hypercalcemia is not clear since dose response curves were not shown. *In vitro*, 2MD was approximately 2-logs more active than 1,25-(OH)₂D₃ in stimulating the expression of Cyp24 (24-hydroxylase), osteopontin and RANKL and decreasing the expression of

osteoprotegrin (265). 2MD was also more potent than 1,25-(OH)₂D₃ in inducing the interaction of VDR with RXR and cofactors, SRC-1 and DRIP205.

However, there is still a safety issue with the current analogs because of their associated hypercalcemic activities. Optimal less calcemic vitamin D analogs have yet to be identified since most current 1,25-(OH)₂D₃ analogs have a small therapeutic window due to the development of hypercalcemia as a result of extended dosing.

XIV. Vitamin D action on blood pressure

Hypertension is a major contributor to morbidity and mortality associated with heart attack, stroke and end-stage renal disease. The renin-angiotensin system regulates blood pressure and salt/water homeostasis. Renin, a protease that is secreted by juxtaglomerular cells in nephrons, cleaves liver-derived angiotensinogen to angiotensin I. Angiotensin converting enzyme then cleaves angiotensin I to angiotensin II, which modulates mammalian blood pressure by stimulating the synthesis of aldosterone by adrenal zona glomerulosa. Aldosterone stimulates sodium absorption and potassium secretion in the distal nephrons, and this process results in the expansion of blood volume leading to hypertension. Epidemiological studies have shown an inverse correlation between UV light exposure or plasma 1,25-(OH)₂D₃ levels and blood pressure. Further, vitamin D supplementation and 1,25-(OH)₂D₃ treatment reduced blood pressure in hypertensive and hyperparathyroidism patients (218). VDR null mice also showed defects in the rennin-angiotensin system. Renin expression in the kidney and plasma angiotensin II production was increased in VDR knock-out mice than that of wild type littermates, leading to hypertension, cardiac hypertrophy and increased water intake (266). Treatment of VDR null animals with captopril, an angiotensin converting enzyme

inhibitor, led to a decrease in blood pressure, thus confirming that the hypertension in VDR null animals was due to rennin and angiotensin II elevation. Therefore, 1,25-(OH)₂D₃ appears to be a negative regulator of rennin-angiotensin system and may play a critical role in blood pressure homeostasis. Development of less calcemic VDR ligands could be useful therapeutically for the prevention and treatment of hypertension.

XV. Conclusions

In the past decade, numerous physiological, molecular, genetic, structural and biochemical studies performed by basic and applied researchers have enhanced our understanding of the vitamin D signaling pathway and paved the way for the therapeutic exploitation of the VDR biology by medicinal chemists and drug discovery scientists. These studies have not only provided the non-gut actions of vitamin D, but also exposed various target indications responsive to VDR ligands. As a result, the therapeutic efficacy of VDR ligands in osteoporosis, psoriasis and secondary hyperparathyroidism has been well established. Based upon a plethora of pre-clinical and clinical studies, arthritis, multiple sclerosis, type I diabetes, inflammatory bowel diseases, myelodysplasia, leukemia and cancers of prostate, colon, breast and skin have emerged as additional vitamin D-responsive indications. The potential beneficial effects of VDR ligands in these indications were further corroborated by epidemiological studies. In spite of this progress, still the major hurdle facing the translation of basic and applied research into therapeutic VDR ligands has been hypercalcemia/hypercalciuria. Although less-calcemic analogs have been synthesized, they have not yet provided the desired separation between the therapeutic and calcemic action and truly “non-calcemic” vitamin D analogs are still elusive. The calcemic liability of various vitamin D analogs is presented in Table 3.

Therefore, a structure activity relationship-based medicinal chemistry effort for the identification and development of truly less-calcemic ligands appears to be very important. Fortunately, increased understanding of the mechanism of vitamin D action has now provided the molecular and cellular tools necessary to identify tissue and gene-selective VDR modulators (VDRMs) for various disease indications. Further, combination of VDR ligands, especially new generation of VDRMs with other differentiation inducers, histone deacetylase inhibitors or chemotherapeutic regimens may offer additive or synergistic activities in target diseases.

Figure Legends

Fig. 1. 1,25-(OH)₂D₃ and its synthetic analogs. Structures, common names and chemical names of vitamin D analogs are presented in the figure.

Fig. 2. Regulation of gene expression by VDR ligands. A schematic representation of VDR-mediated regulation of gene expression is presented. All the genes with known VDREs are shown as positively (+) regulated genes. Genes that are negatively (-) regulated by vitamin D because of the presence of negative VDRE (nVDRE) in their promoter regions as well as genes that are negatively regulated by mechanisms involving transcription factor antagonism (anti-NF-AT and anti-NF-κB activities) are also shown. References are given in parenthesis.

Fig. 3. Topology of VDR LBD crystal structures (X-ray coordinates; Protein Data Bank ID code 1DB1). (A) Crystal structure of 1,25-(OH)₂D₃ bound to LBD of VDR is shown. The helices (H1-H12) are represented as cylinders (red color except helix-12 which is colored in pink), β sheets are represented as arrows in yellow and the ligand is shown in white. Positioning of the ligand in the ligand-binding cavity is clearly shown by making helices H3 and H2 translucent. (B) Ribbon diagram of the VDR LBD in stereo. The 1,25-(OH)₂D₃ is represented as ball and stick embedded in translucent surface in pink color. The helices are numbered as H1, H3, H5, H11 and H12. Few of the residues close to the ligand are displayed in stick model identified with one letter amino acid code and the residue number in blue color. The helix-7 (H7) is directly behind the ligand in this orientation and thus not visible. However, its location can be seen in figure 3A, which is represented as cylinders. These figures were produced using the published crystal structure coordinates (60).

Fig 4. Ribbon diagram of VDR and RAR γ LBDs. RAR γ (dark shaded) and VDR (light shaded) are optimally superimposed to show the important structural difference between them. The beta-sheet region (β 1, β 2 and β 3) for RAR γ is shown in blue and the corresponding region for VDR is shown in red. The loop connecting the helix-1 and helix-3 (which includes helix-2 in the case of VDR) is colored pink in VDR and the corresponding region is colored green for RAR γ . Total of 452 backbone atoms were used for superposition and the RMS deviation was 1.03Å°. This figure was produced using the coordinates from the published crystal structures of VDR (X-ray coordinates; Protein Data Bank ID code 1DB1) and RAR γ (X-ray coordinates; Protein Data Bank ID code 2LBD) (60, 63).

Fig. 5. Details of molecular interactions between 1,25-(OH) $_2$ D $_3$ and VDR ligand binding cavity. LIGPLOT (267) was used to detail the molecular interactions between 1,25-(OH) $_2$ D $_3$ and VDR ligand binding cavity. The interactions between amino acid residues lining the VDR ligand binding pocket and 1,25-(OH) $_2$ D $_3$ are schematically depicted herein. 1,25-(OH) $_2$ D $_3$ is shown in ball-and-stick in the middle. Residues that make potential hydrogen bonds to 1,25-(OH) $_2$ D $_3$ are shown in ball-and-stick. Ligand bonds are in violet and bonds in the protein are colored in reddish-yellow. Oxygen atoms except in water molecules are filled in red color, nitrogen atoms in blue and carbons in black. The water molecules (HOH) are shown in cyan. Red spikes denote the hydrophobic interactions between the ligand atoms and the protein residues. Hydrophobic contacts are discerned when the spikes from a ligand atom radiate out towards the protein residue (which is represented as arcs) and the spikes from that protein residue radiate out towards

the same ligand atom. In other words, the red spikes from a ligand atom and a protein residue facing each other, is indicative of the hydrophobic interaction between the two. Hydrogen bonds and hydrophobic contacts were calculated with HBPLUS (268).

Fig. 6. Vitamin D-regulated genes in epithelial cancer cells. Genes that are positively or negatively regulated by $1,25\text{-(OH)}_2\text{D}_3$ in prostate, colon, breast cancer cells and keratinocytes/squamous cell carcinoma are shown herein. The regulation of expression of these genes was confirmed by immunohistochemistry, Northern or Western blotting techniques.

Table 1. Vitamin D analogs in clinical trials with favorable outcome.

VDR Ligands	Indication	Outcome	Reference
Calcitriol	Osteoporosis	BMD increase (4 year trial)	(6)
Calcitriol	Osteoporosis	Reduced rate of vertebral fractures	(255)
EB 1089 (Seocalcitol)	HCC	2/33 patients complete response 12/33 stable disease	(269)
Calcitriol (oral)	Psoriasis	Improvement in 88 % of patients	(270)
Calcitriol (topical)	Psoriasis	Improvement in 79 % of patients	(155)
Calcipotriol	Psoriasis	Disease improvement	(153)
Tacalcitol	Psoriasis	Improvement in PASI score	(271)
Maxacalcitol (OCT)	Psoriasis	Improvement in PASI score	(272)
ED-71	Osteoporosis	BMD increase (phase II trial)	(261)
RS-980400	Osteoporosis	BMD increase (phase II trial)	(273)
Paricalcitol	2HPT	PTH suppression	(274)
OCT	2HPT	PTH suppression	(275)
Alfacalcidol	MDS	Leukemia-free survival	(241)
Alfacalcidol	Arthritis	Disease improvement	(110)
Calcitriol	PA	Disease improvement	(109)
Calcitriol	Prostate cancer	Decrease in PSA rise	(186)
Calcitriol	Prostate cancer	Survival and decrease in PSA rise	(190)
Calcipotriol	Kaposi's sarcoma	Decrease in tumor size	(249)

BMD: Bone mineral density; HCC: Hepatocellular carcinoma; 2HPT: Secondary hyperparathyroidism; MDS: Myelodysplastic syndromes; PA: Psoriatic arthritis; PASI: Psoriasis area and severity index; PSA: Prostate specific antigen; PTH, Parathyroid hormone

Table 2. VDR Interacting Co-factors

Cofactor	Function/Activity	Reference
Coactivators:		
SRC/p160 family	Chromatin modification	
SRC-1 (SRC-1/NCoA-1)		(276)
SRC-2 (GRIP1/TIF2/NCoA-2)		(277)
SRC-3 (pCIP/RAC3/ACTR/AIB1/TRAM-1/NcoA-3)		(278)
DRIP205/TRAP220/PBP/TRIP2	Mediator complex recruitment	(55)
TRIP1/SUG1	DNA Helicase	(49)
	Transcriptional mediator	(46)
	Proteasome-dependent proteolysis	(47)
CBP/p300	Chromatin modification	(279)
RIP 140	Not known	(280)
TIF1	Protein Kinase	(281)
NCoA-62/SKIP	Chromatin modification	(50)
TAF _{II} 135	Basal transcription factor	(52)
TAF _{II} 55	Basal transcription factor	(53)
TAF _{II} 28	Basal transcription factor	(54)
Corepressors:		
NcoR-1/RIP13	Chromatin remodeling	(282)
NcoR-2/SMRT/TRAC2	Chromatin remodeling	(282)
Hairless/TRIP8/KIAA1380	Chromatin remodeling	(74)

Table 3. Hypercalcemic activity of VDR ligands

Compound	Hypercalcemic dose	Model	Dosing period	Ref.
Calcitriol	15 µg/kg/day	BALB/c mice	3 days	(283)
Calcitriol	5 µg/kg/day	BALB/c mice	5 days	(283)
Calcitriol	0.2 µg/kg/day	OVX rats	3 weeks	(262)
ED-71	0.05 µg/kg twice a week	Rats	12 weeks	(259)
ED-71	0.2 µg/kg twice a week	OVX rats	3 months	(258)
Calcitriol	≥ 1333 pmol/kg/3X/week	OVX rats	23 weeks	(264)
2MD	18.7 pmol/kg/7X/week	OVX rats	23 weeks	(264)
Calcitriol	< 0.5 µg/kg/3X/week	Copenhagen rats	26 days	(175)
EB 1089	< 0.5 µg/kg/3X/week	Copenhagen rats	26 days	(175)
Ro-26-6228	> 14 µg/kg/day	OVX rats	3 weeks	(262)
Calcitriol	1 µg/kg/day	Rats	28 days (Topical)	(284)
Tacalcitol	1 µg/kg/day	Rats	28 days (Topical)	(284)
Calcipotriol	15 µg/kg/day	Rats	28 days (Topical)	(284)
BXL-628	300 µg/kg/day	Rats	2 weeks	(194)

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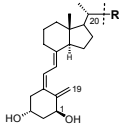
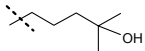
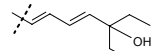
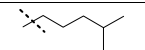
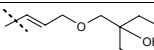
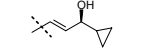
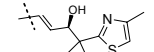
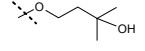
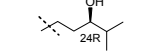
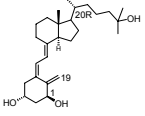
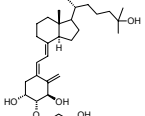
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Vitamin D Analogs			
			
Compound	R	Compound	R
1 α ,25-(OH) ₂ D ₃ (Calcitriol)		1 α ,25-(OH) ₂ -22,24-diene-24a,26a,27a-trihomo-D ₃ (EB 1089)	
1 α -(OH)D ₃ (Alfacalcidol)		1 α ,25-(OH) ₂ -22-ene-25-oxa-D ₃ (ZK 156718)	
1 α ,24-(OH) ₂ -24-cyclopropyl-D ₃ (Calcipotriol)		25-(4-methylthiazol-2-yl)-calcipotriol (ZK 191732)	
1 α ,25-(OH) ₂ -22-oxa-D ₃ (Maxacalcitol)		1 α ,24R-(OH) ₂ D ₃ (Tacalcitol)	
 1 α ,25-(OH) ₂ D ₃ (Calcitriol)		 ED-71 [1 α ,25-(OH) ₂ -2 β -(3-hydroxypropyl)D ₃]	

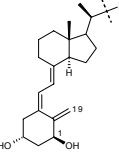
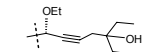
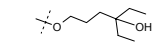
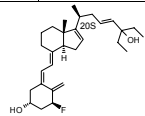
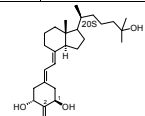
“20-Epi Vitamin D Analogs”			
			
Compound	R	Compound	R
20-epi-22-ethoxy-23-yne-24a,26a,27a-trihomo-1 α ,25-(OH) ₂ D ₃ (CB 1093)		20-epi-1 α ,25-(OH) ₂ D ₃ (KH 1060)	
 1 α -fluoro-25-(OH)-16,23E-diene-26,27-bishomo-20epi-cholecalciferol (Ro-26-6228, BXL-628, RS-980400)		 2-methylene-19-nor-(20S)-1 α ,25-(OH) ₂ D ₃ (2MD)	

Fig. 1

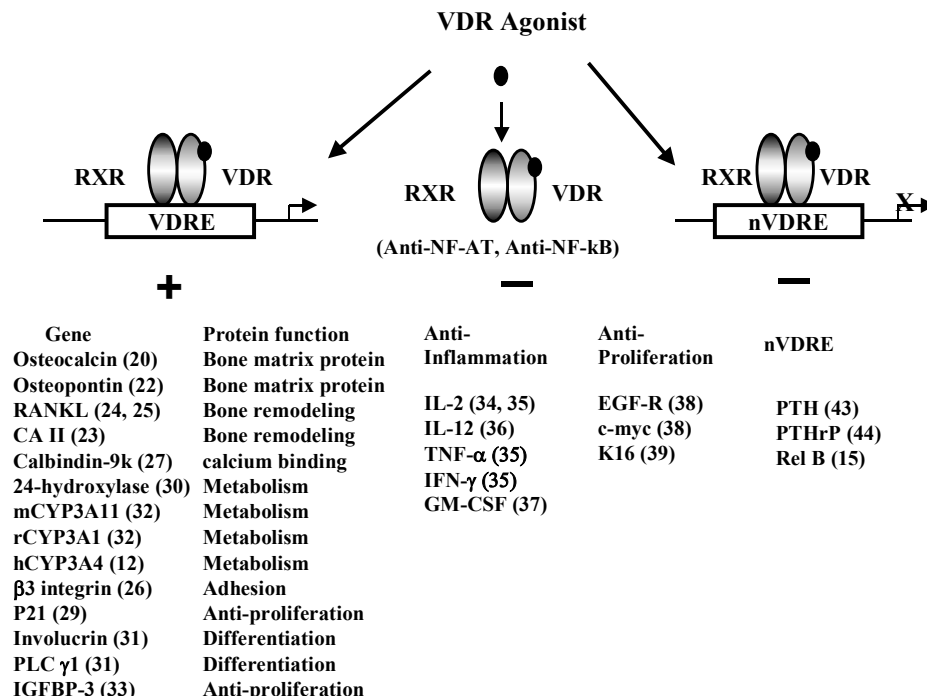
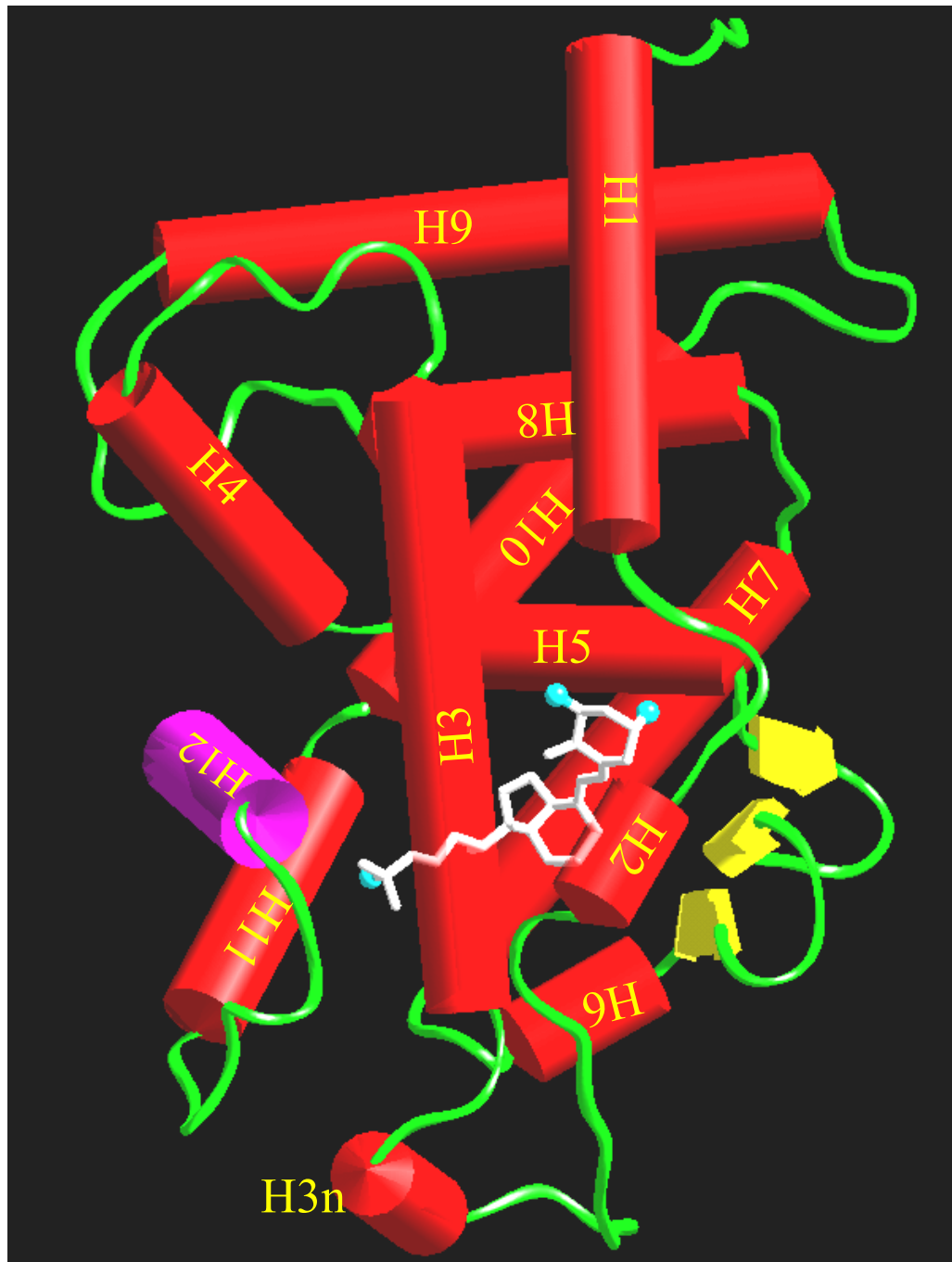


Fig. 2

Fig. 3A



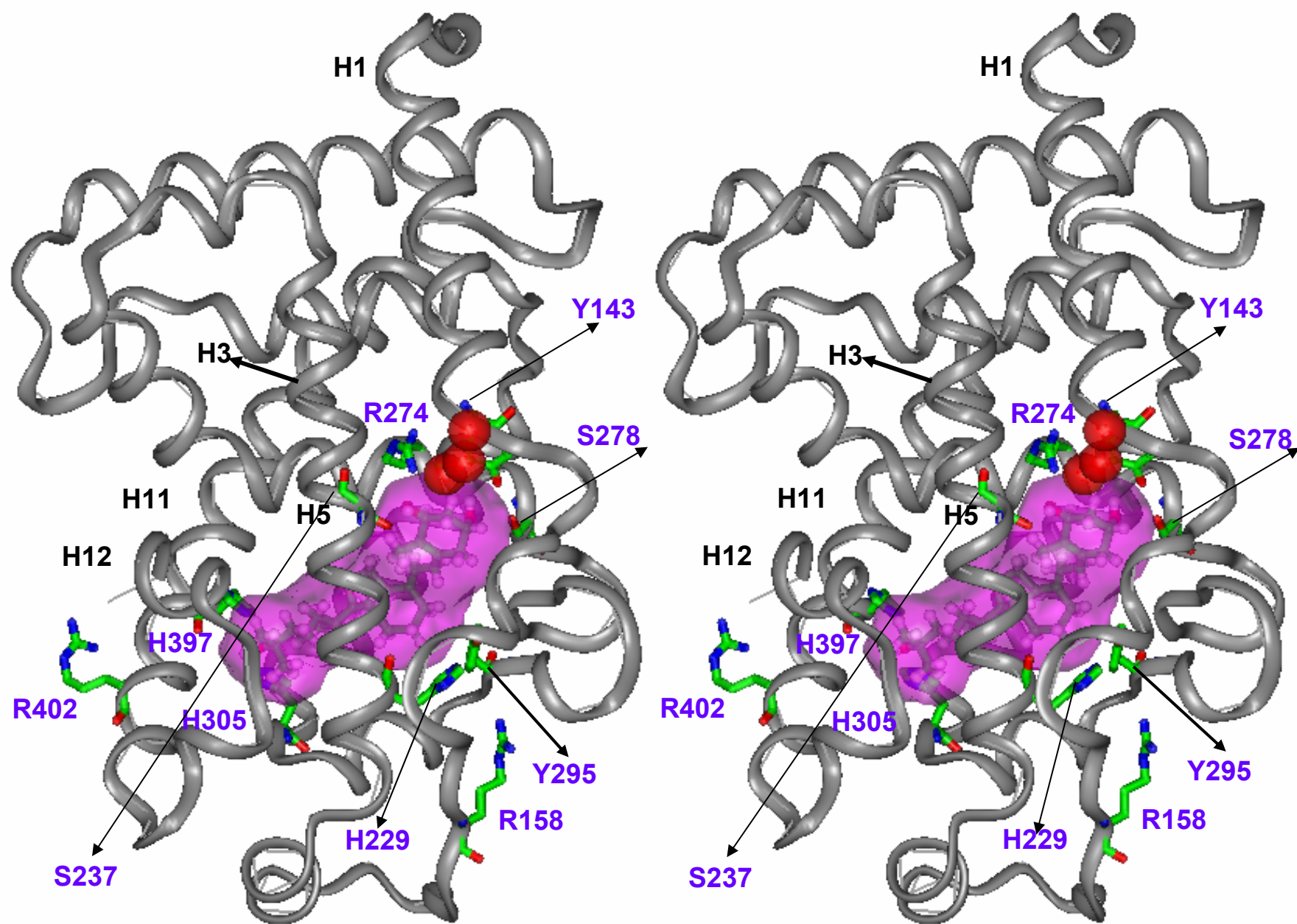


Fig. 3B

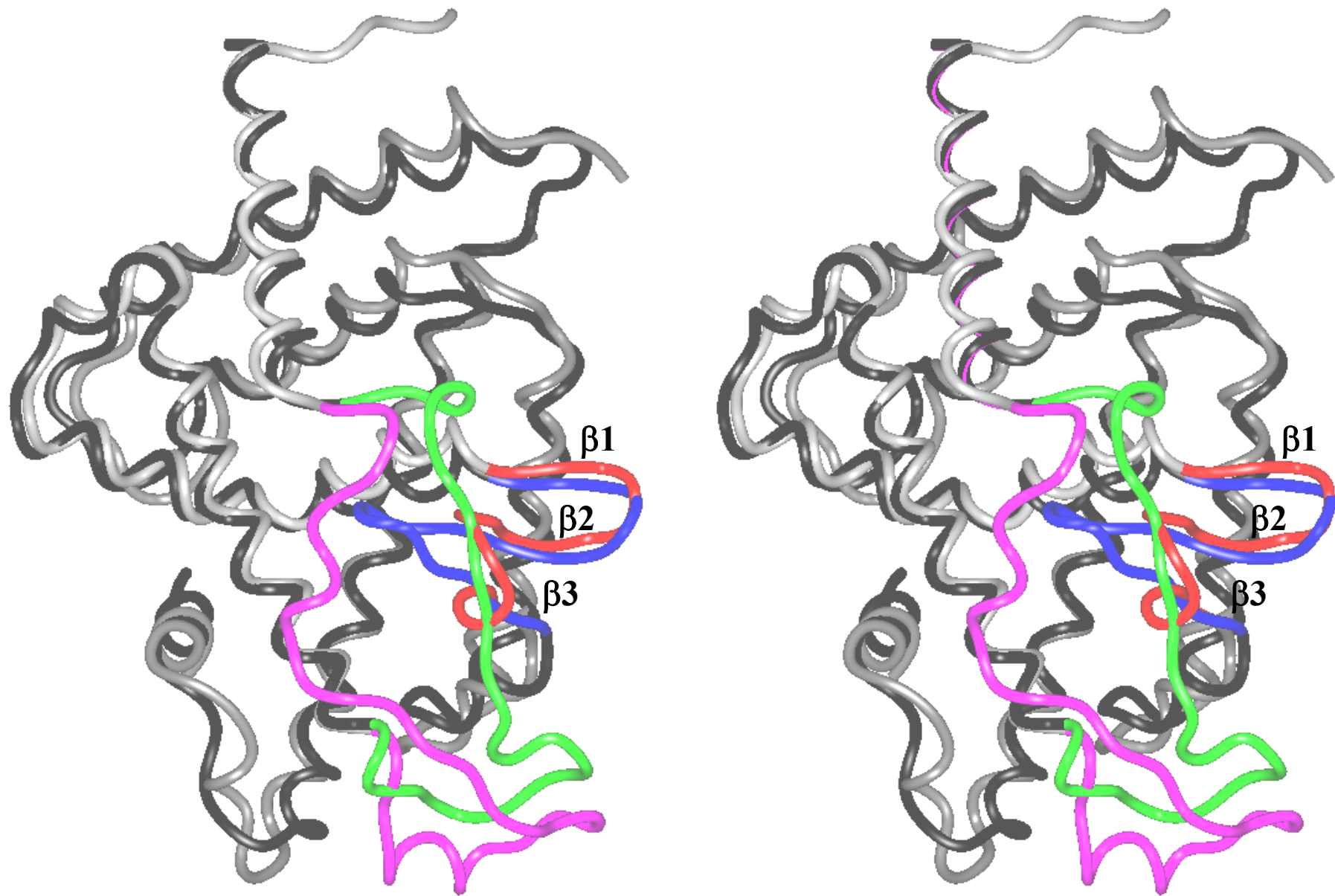
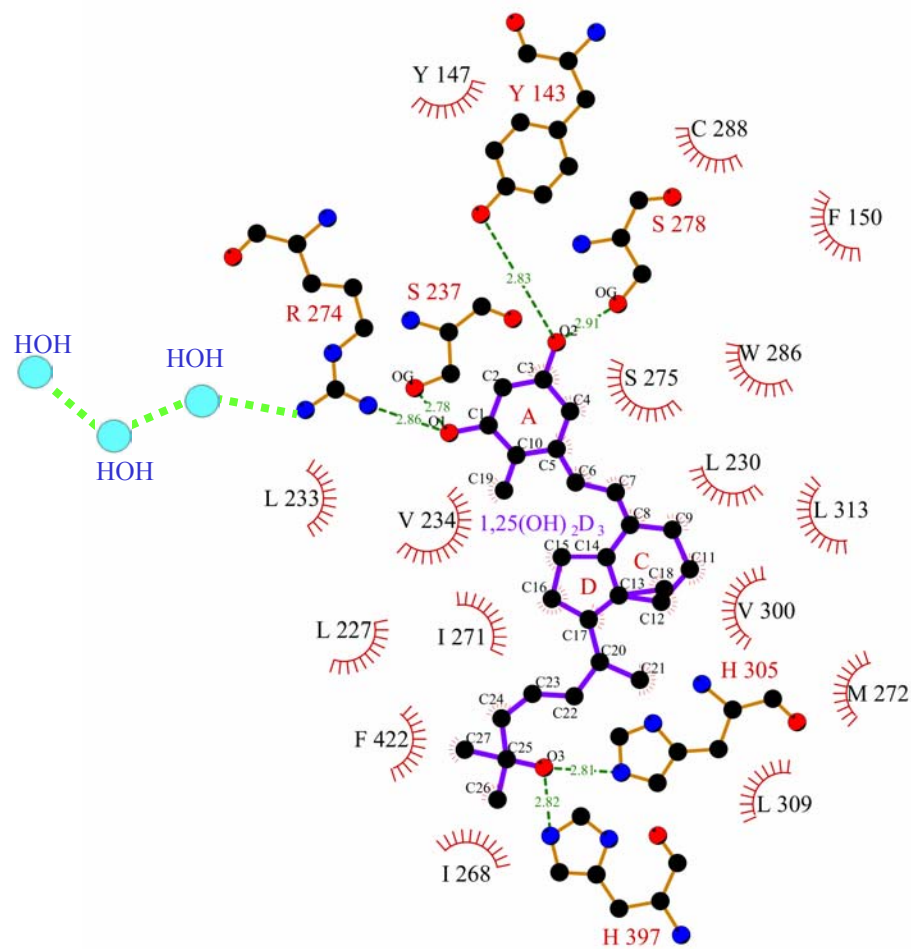


Fig. 4



Key

- | | | | |
|--|------------------------------|--|-------------------------------------------------------------|
| | Ligand bond | | H 53 Non-ligand residues involved in hydrophobic contact(s) |
| | Non-ligand bond | | Corresponding atoms involved in hydrophobic contact(s) |
| | Hydrogen bond and its length | | |

Fig. 5

VDR Regulated Genes in Cancer Cells

+				-			
Prostate	Colon	Breast	Keratino./SCC	Prostate	Colon	Breast	Keratino./SCC
p21	p21	p21	TGase I	CDK 2	c-myc	CDK2	EGF-R
p27	p27	p27	Involucrin	MMP-2	PPAR δ	CDK4	c-myc
IGFBP-3	E-cadherin	TGF- β 1	24-OHase		Tcf-1	Cyclin D1	K16
IGFBP-5	ZO-1	HoxB4	IL-10R		CD44	Cyclin A	SCCA
E-cadherin	ZO-2	IGFBP-3	PLC- β 1		Cyclin D1	Cyclin D3	CRABP II
DUSP-10	24-OHase	IGFBP-5	PLC- γ 1		Cyclin E	c-myc	N-cadherin
TRR1	CAT1	24-OHase	PLC- δ 1		CDK2	MMP-9	
SOD2	CAT2		TGF- β 1		CDK6		
AR	c-Jun		Calcium receptor				
24-OHase	JunB		c-Fos				
Metallo-thioneins	JunD		VDUP1				
	FREAC-1/Fox1		Protease M				
	ZNF-4/KOX7		Cystatin M				
	Plectin		Amphiregulin				
	Filamin		Stromelysin 1				
	K13		Collagenase I				
	Kallikrein 10						
	Protease M						

Fig. 6