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Potent 19-norvitamin D analogs for prostate and liver cancer therapy

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Abstract

The active form of vitamin D₃, 1 α ,25(OH)₂D₃ or calcitriol, is known to inhibit the proliferation and invasiveness of many types of cancer cells, including prostate and liver cancer cells. These findings support the use of 1 α ,25(OH)₂D₃ for prostate and liver cancer therapy. However, 1 α ,25(OH)₂D₃ can cause hypercalcemia, thus, analogs of 1 α ,25(OH)₂D₃ that are less calcemic but exhibit potent antiproliferative activity would be attractive as therapeutic agents. We have developed 2 α -functional group substituted 19-norvitamin D₃ analogs with and without 14-epimerization. Among them, 2 α - and 2 β -(3-hydroxypropyl)-1 α ,25-dihydroxy-19-norvitamin D₃ (MART-10 and -11, respectively) and 14-*epi*-2 α - and 14-*epi*-2 β -(3-hydroxypropyl)-1 α ,25-dihydroxy-19-norvitamin D₃ (14-*epi*-MART-10 and 14-*epi*-MART-11, respectively) were found to be the most promising. In this review, we discuss the synthesis of this unique class of vitamin D analogs, the molecular mechanism of anticancer actions of vitamin D, and the biological evaluation of these analogs for potential application to the prevention and treatment of prostate and liver cancer.

The need to synthesize less calcemic analogs of vitamin D with potent anticancer activities

The hormonally active form of vitamin D₃, 1 α ,25-dihydroxyvitamin D₃ (1 α ,25(OH)₂D₃, **1**), is recognized as a potent regulator of calcium homeostasis and bone growth, and a potent cellular modulator [1]. 1 α ,25(OH)₂D₃ is synthesized from 25-hydroxyvitamin D₃ (25(OH)D₃), the circulating form of vitamin D and the index of vitamin D nutritional status, by CYP27B1 in the kidneys and in specific target organs including the prostate.

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Epidemiological studies have linked low plasma 25(OH)D₃ levels with the increased risk of many forms of cancer, including prostate [2]. More importantly, the potent cellular modulatory properties of 1 α ,25(OH)₂D₃, including antiproliferation, anti-invasion, antiangiogenesis, anti-inflammation, pro-apoptosis and pro-differentiation, suggest that the active form of vitamin D₃ can be used as a therapeutic agent for cancer treatment [1]. However, 1 α ,25(OH)₂D₃ can cause hypercalcemia, analogs of 1 α ,25(OH)₂D₃ that are less calcemic but exhibit potent antiproliferative activity would be more attractive as therapeutic agents.

At the present time, several thousand vitamin D analogs have been synthesized. Among those analogs, several have been demonstrated to exert promising anticancer effects with reduced calcemic effect and even with greater antiproliferative activity than 1 α ,25(OH)₂D₃ [3]. For example, seocalcitol (EB1089, Leo Pharmaceutical products), one of the most studied synthetic analogs, has been shown to be more potent than 1 α ,25(OH)₂D₃ with respect to the anticancer effects in a rat model of mammary gland carcinoma without inducing hypercalcemia [4,5], in an *in vivo* prostate cancer study where EB1089 inhibited prostate cancer cell proliferation and reduced tumorigenesis as well as tumor metastases [6] in liver cancer cells *in vitro* [7] and in a xenograft animal model [8]. EB1089 has also been studied in a Phase II clinical trial in liver cancer patients [9], however, it failed to show any significant benefits on the survival of advanced patients.

Phase I and II clinical trials conducted in the advanced prostate cancer patients using hectorol (1 α -hydroxyvitamin D₂) showed that the drug was well tolerated with the main toxicities being hypercalcemia and renal insufficiency that were reversible with drug discontinuation [10,11]. The studies also showed improved disease stability (>6 months) in 30% of the patients and the median survival of 21 months, which is higher than the 17.7 months predicted by the survival nomogram for that patient group. In spite of these less-than-conclusive results, the findings do warrant further studies with less calcemic vitamin D analogs while having much greater anticancer potency.

Development of 19-nor vitamin D analogs

A vitamin D analog with 19-nor structure in which the methylidene group on C19 is replaced with two hydrogen atoms was first described in 1983 from a study describing the isolation and identification of 19-nor-10-ketovitamin D from a mixture of vitamin D or 25(OH)D₃ solution incubated with bovine rumen microbes [12]. Later, Perlman *et al.* synthesized 19-nor-1 α ,25(OH)₂D₃ primarily to study the SAR of 1 α ,25(OH)₂D₃ and reported that the analog induced the differentiation of human leukemia HL-60 cells *in vitro* with potency comparable to 1 α ,25(OH)₂D₃ while it caused very little or no calcemic effect in animal [13]. The findings led to the synthesis of 19-nor-1 α ,25(OH)₂D₂ and the studies of its biological activities [14]. Similar to its D₃ counterpart, 19-nor-1 α ,25(OH)₂D₂ was shown to induce CYP24A1 promoter activity. Furthermore, it was shown to suppress PTH secretion in hemodialysis patients with secondary hyperparathyroidism without inducing hypercalcemia or hyperphosphatemia [15]. This has led to the approval of 19-nor-1 α ,25(OH)₂D₂ by the US FDA as a drug called ZemplarTM or Paricalcitol to treat secondary hyperparathyroidism. Subsequent modifications at the A-ring generated a variety of C2-modified 19-norvitamin D analogs [15–23]. Studies on the antiproliferative activity of 19-nor-1 α ,25(OH)₂D₂ as compared to 1 α ,25(OH)₂D₃ have been conducted in prostate and pancreatic cancer cells *in vitro* [24,25]. In these two studies, 19-nor-1 α ,25(OH)₂D₂ showed comparable potency as 1 α ,25(OH)₂D₃. In a xenograft nude mouse model, 1 α ,25(OH)₂D₃, given three-times per week, inhibited the growth of AsPC-1 pancreatic tumor cell at a dose that did not cause hypercalcemia. The inhibition of tumor growth was accompanied by *in vivo* upregulation of p21 and p27 expression [25]. A Phase I/II clinical trial with paricalcitol

has been conducted in a small group of advanced, androgen-insensitive prostate cancer patients, and showed no objective responses in the drop of prostate-specific antigen [26]. The results may suggest that a more potent non- or less-calcemic vitamin D analog may be needed to be effective in treating this group of patients.

Rational for the synthesis of 2 α -(ω -hydroxyalkyl)-19-norvitamin D₃ & 14-*epi*-2 α -(ω -hydroxyalkyl)-19-norvitamin D₃ & their 2- β stereoisomers

The authors have previously reported several 2 α -(ω -hydroxy)alkylated vitamin D₃ analogs of **1** that exhibit stronger vitamin D receptor (VDR) binding affinity than that of the natural hormone **1** [27,28]. x-ray crystallographic analysis of the VDR–[2 α -(3-hydroxypropyl)-1 α , 25-dihydroxyvitamin D₃] complex, for example, has clearly demonstrated that the terminal hydroxyl of its 2 α -(3-hydroxypropyl) group forms a direct hydrogen bond with Arg274 and replaces one of the water molecules in the ligand-binding domain (LBD) of the VDR to stabilize the complex to show three-times greater binding affinity for the VDR than **1** [29,30]. It is noteworthy that demethylenated analog **2c**, 1 α ,25-dihydroxy-19-norvitamin D₃, possesses a selective active profile that is noncalcemic while maintaining the other potent cellular activities [13,16]. However, the removal of the 10(19)-methylidene group from the natural hormone **1** reduces the VDR binding affinity due to its loss of hydrophobic interaction with the LBD of the VDR. The binding affinity of **2c** has been reported to be only 30% and 17% of **1** for the porcine VDR [31] and for the calf thymus VDR [17], respectively. Researchers are now interested in a vitamin D skeleton having a combined structure of '2 α -(ω -hydroxy)alkylated' and '19-nor' in **1**. Furthermore, Bouillon's group reported that 14-*epi*-19-norvitamin D analogs, TX522 and TX527, possessed a much enhanced antiproliferative action on breast cancer cells *in vitro* and *in vivo*, with much lower calcemic effects compared to **1** [32]. Therefore, the authors synthesized 2 α -(ω -hydroxyalkyl)-19-norvitamin D₃, MART-10 (**2a**), and its 2 β -stereoisomer, MART-11 (**2b**), and their C14 epimers, 14-*epi*-MART-10 (**2a**) and 14-*epi*-MART-11 (**2b**) as shown in Figure 1, and studied their biological activities, especially their anticancer actions.

The chemistry of novel synthetic schemes for the C2-substituted vitamin D analogs

The active compounds were synthesized as described briefly below and retrosynthetic analysis of the target molecule **2** is shown in Figure 2. The key coupling reaction of an A-ring ketone and a CD-ring for the C5–C6 double bond was performed by a **modified Julia olefination** method. This is a more efficient and convergent route than the classical linear coupling route at the C7–C8 double bond for the synthesis of 19-norvitamin D₃ analogs using Grundmann's ketone as the starting material of the CD rings moiety. The CD ring synthon **3** was converted from 25-hydroxy Grundmann's ketone (**4**) via *E*-selective Horner–Wadsworth–Emmons carboxyolefination followed by reduction. On the other hand, the A-ring synthon **5** was synthesized based on chiral pool synthesis. Thus, (–)-quinic acid (**7**) seems fitting as a starting chiral material because of its appropriate stereochemistry of the (3*R*,5*R*)-dihydroxy groups to the natural (1 α ,3 β)-dihydroxy groups of vitamin D₃. It appeared that the most serious problem of the authors' synthetic plan was a C–C bond-forming reaction to introduce the C2-alkyl chain (three-carbon unit), which was solved by trialkylallyltin(IV)-mediated radical allylation of the corresponding thioester derived from the 4-hydroxy group of the starting substrate **7**.

The A-ring synthon **5** was synthesized as shown in Figure 3. Both hydroxy groups at the less hindered C3 and C5 positions of methyl ester of (–)-quinic acid were selectively protected by bulky *tert*-butyldimethylsilyl groups followed by thioesterification of the C4-hydroxy

group to give a substrate **6** for radical allylation. The allylation reaction was carried out with allyltributyltin (IV) and 2, 2'-azobis(isobutyronitrile) in refluxing benzene. A 0.2 M benzene solution of 2, 2'-azobis(isobutyronitrile) was added dropwise to the reaction mixture followed by stirring for several hours under the refluxing conditions to give the best results. The allylated product **9** was obtained as an approximately 1:1 epimeric mixture based on C4-allylated carbon atom. It should be noted that the conversion to **10** will turn a stereogenic allylated carbon to nonstereogenic one.

Allylated compound **9** was converted to the 3-hydroxypropyl moiety by the hydroboration-oxidation sequence. The reaction product was treated by sodium borohydride for reduction of the ester moiety to give the glycol whose oxidative cleavage with sodium periodate afforded C₂-symmetric ketone **10** as a sole stereoisomer. The free hydroxy group of ketone **10** was protected by the TBS group to give the A-ring synthon **5**.

The CD-ring synthon **3** for MART-10/11 was synthesized from 25-hydroxy Grundmann's ketone **4** as shown in Figure 4. The *O*-methoxymethyl-protected ketone **11** was converted to α,β -unsaturated ester **12** by Horner-Wadsworth-Emmons reaction in high chemical yield with high geometrical (*E*) selectivity. The ester **12** was reduced by diisobutylaluminum hydride to the allylic alcohol in quite high yield and regioselectivity, which was treated under the Mitsunobu reaction conditions with benzothiazole-2-thiol followed by *in situ* Mo(VI)-catalyzed oxidation to yield the desired arylsulfone **3**.

The CD-ring synthon **3'** for 14-*epi*-MART-10/11 was synthesized from the 14-epimerized ketone **4'** in a similar manner described above. The ketone **4'** was prepared from 25-hydroxy Grundmann's ketone (**4**) by treatment of sodium methoxide in methanol. The C14, the α -position of ketone, was readily epimerized under the basic conditions, and the thermodynamically stable **4'** was obtained preferentially (**4'**/**4** = 6:1).

The carboxyolefination of the ketone **5** with arylsulfone **3/3'** was carried out using lithium hexamethyldisilazide (LiHMDS) in THF at -78°C. These coupling reactions, so-called 'one-pot' Julia olefination, proceeded to give the desired coupling adducts **13/13'**, respectively, in moderate yield. The diastereoselectivity based on the C2-C5-C6 axis was approximately 1:1 for **13a/13b** and 2.4:1 for **13a'/13b'**. The axially epimeric mixture **13** was deprotected by 10-camphorsulfonic acid (CSA) in methanol to give the mixture of MART-10 (**2a**) and MART-11 (**2b**), which was separated by a reversed phase HPLC [33]. The other diastereo mixture **13'** was deprotected and followed by pivaloylation of the primary hydroxy group to afford the epimeric mixture **14'**, which was separated into **14a'** and **14b'** with a reverse-phase HPLC. Isolated **14a'** and **14b'** were deprotected to afford desired 14-*epi*-MART-10 (**2a'**) and 14-*epi*-MART-11 (**2b'**), respectively (Figure 5)[34].

Stereochemistry of 2 α - and 2 β -isomers was determined by ¹H NMR and NOE analysis in CDCl₃ solution (14-*epi* series in Figure 6) (**17**). Determination of the stereochemistry of **14**, for example, was described as follows. For compound **14a'**, H₃ can be assigned by observations of NOE between H₉-H₆ and H₆-H_{4ex}, and the small coupling constant between H_{4ex}-H₃. The large coupling constant between H₃-H₂ (9.9 Hz) indicates that the 3-hydroxy group occupies the equatorial orientation because of H₃ and H₂ at the axial position. Thus, it was determined that both 2-substituent and 3-hydroxy group occupy the equatorial orientation, and the 1-hydroxy group occupies the axial orientation. For compound **14b'**, H₃ can be assigned in a similar manner. The small coupling constant of H₃-H₂ and the large coupling constant of H₁-H₂ (10.5 Hz) indicate that the 1-hydroxy group occupies the equatorial orientation. Thus, it was determined that both 1-hydroxy group and 2-substituent occupy the equatorial orientation, and the 3-hydroxy group occupies the axial orientation. It is important to note that these conformations of the two ligands in the

solution are forced by the C2 equatorial substitution (3-hydroxypropyl group), since the bulky group tends to take an equatorial position.

x-ray crystallographic analysis of the VDR– $1\alpha,25(\text{OH})_2\text{D}_3$ complex reveals that the 1α -OH group of the active vitamin D_3 has an equatorial orientation and the 3-OH group occupies an axial position in the ligand-binding domain of hVDR [29]. Similar orientation for MART-10 and 14-*epi*-MART are expected when they are bound to hVDR, that reflects a stable crystalline structure of ligands in the receptor ligand binding pocket resulting from the conformational change of the A-ring of MART-10 and 14-*epi*-MART-10.

The molecular mechanism of the genomic anticancer actions of vitamin D and its analogs

It is now well established that the active form of vitamin D_3 , $1\alpha,25(\text{OH})_2\text{D}_3$, regulates more than 200 genes in cells of the hematopoietic system [35,36]. The genomic actions of $1\alpha,25(\text{OH})_2\text{D}_3$ are dependent upon VDR as demonstrated through the experiments using cells stably transfected with VDR cDNA or antisense VDR cDNA [37], and cells derived from VDR-knockout mice [38]. The genes responsible for the anticancer actions of vitamin D include those involved in regulating cellular proliferation, differentiation, inflammation, apoptosis, and **angiogenesis** in a tissue- and cell-specific manner [39–45].

Antiproliferative effects of vitamin D

A central part of the antiproliferative effects of vitamin D depends on its ability to arrest **cell cycle** progression at the G_0/G_1 phase. Cell cycle progression through four phases (G_1 , S, G_2 and M) is regulated by multiple molecular pathways and checkpoints, including the expression of cyclin-dependent kinases (CDKs) to phosphorylate retinoblastoma proteins (Rb)/E2F complex, and CDK inhibitors (CKIs) in a coordinated manner [46]. The phosphorylation of Rb/ E2F leads to the release of E2F transcriptional factors from the complex, that in turn induce the expression of S phase genes required for DNA replication [46]. The activity of CDKs required for G_1/S transition is regulated by endogenous CKIs, including tumor repressors such as p21 and p27. In various cancer cells, multiple **VDR response elements** (VDREs) have been identified within the $\text{p21}^{\text{waf1/cip1}}$ promoter region [47,48]. Induction of $\text{p21}^{\text{waf1/cip1}}$ mRNA occurs within 2 h of the $1\alpha,25(\text{OH})_2\text{D}_3$ addition and is a direct effect of liganded VDR. The expression of other CKIs, such as p27^{kip1} and the Ink4 family member p15, p16, and p18, were also found to be induced by $1\alpha,25(\text{OH})_2\text{D}_3$ [24]. However, data from studies using LNCaP prostate cancer cells and HepG2 liver cancer cells indicate that the upregulation of p27^{kip1} proteins induced by $1\alpha,25(\text{OH})_2\text{D}_3$ or its analogs may not involve new p27^{kip1} mRNA synthesis [49–51], and is more likely the consequence rather than the cause of $1\alpha,25(\text{OH})_2\text{D}_3$ -induced growth inhibition [52].

In addition to the direct effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on $\text{p21}^{\text{waf1/cip1}}$ and other cell cycle-related genes [53], the hormone may act through other mechanisms to inhibit cellular proliferation. For example, $1\alpha,25(\text{OH})_2\text{D}_3$ has been shown to up-regulate the insulin-like growth factor binding protein-3 (IGFBP3) and transforming growth factor- β (TGF- β) signaling pathways and down-regulate the EGFR signaling cascade [54–56]. In prostate cells, it has been proposed that the modulation of prostaglandin concentration by $1\alpha,25(\text{OH})_2\text{D}_3$ may be one mechanism responsible for the growth inhibitory action of $1\alpha,25(\text{OH})_2\text{D}_3$ [57].

Apoptotic effects of vitamin D

Apoptosis, a regulated process, is fundamental to the advantage of the organism. In tumor cells, activation of the apoptotic pathways is a key mechanism by which chemotherapeutic

or cytotoxic drugs eliminate those cells [58]. Extensive research has revealed that Bcl-2 proteins are the 'master regulator' of the intrinsic apoptotic pathway that is crucial to the apoptotic response from DNA-damage and other carcinogenic challenges [59]. The Bcl-2 family of proteins has two subclasses that either promote (e.g., Bcl-X_s, Bax) or suppress (e.g., Bcl-2, Bcl-X_L, Mcl-1) apoptosis [60,61]. The ratio of apoptotic promoters and suppressors is one determinant of cellular response. 1 α ,25(OH)₂D₃ induces apoptosis in a variety of cancer cells to exert anti-tumor effects by repressing the expression of the anti-apoptotic proteins Bcl-2 and Bcl-X_L, or inducing the expression of pro-apoptotic proteins, such as BAX, BAK and BAD [35,43,61–63]. In a study using LNCaP prostate cancer cells, Blutt *et al.* found a downregulation of Bcl-2 and Bcl-X_L proteins without affecting other proteins important in apoptotic control after 1 α ,25(OH)₂D₃ treatment [64]. In addition, 1 α ,25(OH)₂D₃ may directly activate caspase effector molecules, especially caspase-3, to induce apoptosis in some cancer cells [65–67]. In the authors' studies with HepG2 cells, however, no expression of active caspase-3 protein was found either in the control or the group treated with 1 α ,25(OH)₂D₃ [68]. In addition, flow cytometry study with Annexin V-FITC and PI staining to analyze apoptotic and necrotic cell populations of HepG2 cells after 1 α ,25(OH)₂D₃ treatment also showed similar apoptotic and necrotic cell populations between the control and the treated groups, suggesting that the decrease in cell number after 1 α ,25(OH)₂D₃ treatment in HepG2 cells does not involve apoptosis. The 1 α ,25(OH)₂D₃-induced apoptotic response may also be mediated by the destabilization of telomerase reverse transcriptase mRNA, which will lead to the down-regulation of telomerase activity [69]. A report by Brosseau *et al.* demonstrated that, by combining with immunomodulatory drug lenalidomide, 1 α ,25(OH)₂D₃ was able to cause apoptosis of MDA-MB-231 cells, a triple-negative and vitamin D-resistant cell line, through the Bcl-2 inhibition mechanism, whereas the combination did not affect Bcl-2 in two other vitamin D-resistant breast cancer cell lines MCF7VDR and HBL-100 [70]. Therefore, 1 α ,25(OH)₂D₃ induces apoptosis in a cell-specific manner [71,72] and may involve different mechanisms in different cells.

Anti-angiogenesis

Angiogenesis, the formation of new blood microvessels from existing vessels, is a process that is regulated by a range of endogenous angiogenic factors and inhibitors [73]. It plays an important role in reproduction, development and wound healing. Normal physiological angiogenesis is focal and self-limited in time. On the contrary, pathological angiogenesis can last for years, and is necessary for tumors and their metastases to grow beyond a microscopic size [74]. Tumor cells induce angiogenesis through a multistep process, called the 'angiogenic switch', which, ultimately, tips the balance toward proangiogenic factors [75], including VEGF, PAI-1, angiopoietins, PDGF β and MMP-2 and -9. [76]. There is considerable evidence that VEGF is a major tumor angiogenesis factor [77–81]. Increasing evidence has indicated the anti-angiogenic action of vitamin D, including the expression of VDR in venular and capillary endothelial cells of human skin biopsies, inhibition of bovine aortic endothelial cells proliferation and anti-angiogenesis in animal models [82–85]. It has been proposed that the inhibitory effect of 1 α ,25(OH)₂D₃ on metastasis observed in the prostate and lung murine models may partially depend on its anti-angiogenic property [86,87].

Prodifferentiation

The observation by Abe *et al.* that 1 α ,25(OH)₂D₃ was capable of inducing mouse myeloid leukemia cells to differentiate into multinucleated macrophages initiated a new era of vitamin D research [88]. Subsequently, 1 α ,25(OH)₂D₃ and its analogs were shown to inhibit proliferation and increase the expression of a variety of differentiation markers for keratinocyte transformation into cornified envelopes, including involucrin, transglutaminase, loricrin and filaggrin, and the expression of *E*-cadherin, occludin and vinculin, adhesion

glycoproteins that play an important role during cell migration and the cell–cell tight junction formation, thus inhibiting the processes critical for tumor growth and metastases in a variety of cancer cells [41,89–92]. Hsu and colleagues demonstrated that $1\alpha,25(\text{OH})_2\text{D}_3$ promoted prostate cancer cell aggregation by upregulating E-cadherin expression and, therefore, interfering their adhesion to microvascular endothelial cells and reducing their metastatic potential [93]. Thus, $1\alpha,25(\text{OH})_2\text{D}_3$ -induced differentiation may be one mechanism responsible for inhibiting tumor growth and metastases.

Anti-inflammation

Feldman and co-workers performed cDNA microarray analyses of normal and cancer-derived primary prostate epithelial cells [94] and LNCaP cells [95]. They revealed that $1\alpha,25(\text{OH})_2\text{D}_3$ regulated a wide array of genes, including those involved in the synthesis and catabolism of prostaglandins, which are well-established inflammatory mediators. They showed that $1\alpha,25(\text{OH})_2\text{D}_3$ up-regulated the expression of NAD^+ -dependent 15-PGDH gene and downregulated COX-2 expression. Since prostaglandins are known to play a role in the development and progression of many cancers [96], the ability of $1\alpha,25(\text{OH})_2\text{D}_3$ to decrease prostaglandin concentration strongly suggests that one mechanism of anticancer effect of vitamin D may be mediated through its anti-inflammatory action.

Nongenomic anticancer actions of vitamin D

The structural flexibility of $1\alpha,25(\text{OH})_2\text{D}_3$ and many of its analogs, which allows the formation of a continuum of potential ligand shapes extending from 6-*s-cis* to the 6-*s-trans*, has been proposed as a base of rapid and non-genomic biological responses induced by $1\alpha,25(\text{OH})_2\text{D}_3$ and some of its analogs. The nongenomic rapid response has been shown in several biological systems [97], which may be mediated through a functional VDR in some systems [98,99] or may not in another systems [100]. Furthermore, it has been known for some time that $1\alpha,25(\text{OH})_2\text{D}_3$ reduces UV-induced DNA damage [101] in the form of cyclobutane pyrimidine dimers in human keratinocytes in cultures, and in mouse and human skin [102,103]. The photoprotection by $1\alpha,25(\text{OH})_2\text{D}_3$ against oxidative insults [101,103,104] is thought to be mediated by a nongenomic signaling mechanism because $1\alpha,25(\text{OH})_2$ lumisterol₃, which has almost no transactivating activity, reduces UV-induced DNA damage, apoptosis and immunosuppression with similar potency as $1\alpha,25(\text{OH})_2\text{D}_3$ [105].

The antiproliferative activity of C2 substituted 19-norvitamin D analogs in prostate & liver cells

The antiproliferative activity of a series of nine 19-nor- $1\alpha,25(\text{OH})_2\text{D}_3$ analogs modified at C2 position with different hydrocarbon moieties was studied and compared with that of $1\alpha,25(\text{OH})_2\text{D}_3$ in PZ-HPV-7 prostate cells, a cell line derived from the epithelial zone of a normal prostate. Among them, 19-nor-2 α -(3-hydroxypropyl)- $1\alpha,25(\text{OH})_2\text{D}_3$ (MART-10) and 19-nor-2 β -(3-hydroxypropyl)- $1\alpha,25(\text{OH})_2\text{D}_3$ (MART-11) were 500- to 1000-fold more active than $1\alpha,25(\text{OH})_2\text{D}_3$, respectively [106]. The antiproliferative potency of the C-14 epimers of MART-10 and MART-11, 14-*epi*-MART-10 and 14-*epi*-MART-11, was also studied in PZ-HPV-7 cells [34]. 14-*epi*-MART-10 was found to be approximately 100-fold more potent than $1\alpha,25(\text{OH})_2\text{D}_3$, whereas 14-*epi*-MART-11 showed similar activity as $1\alpha,25(\text{OH})_2\text{D}_3$. Since MART-11 and 14-*epi*-MART-11 have the same A-ring conformation, MART-11 has about the same activity as MART-10; whereas, 14-*epi*-MART-11 has only 1% of the activity of 14-*epi*-MART-10. The difference in activity displayed by these two MART-11 isomers as compared to their MART-10 counterparts in PZ-HPV-7 cells may reflect yet unelucidated molecular dynamics associated with VDR–ligand recognition/

ligand-dependent activation of VDR transactivation as described in a previous section related to NMR and x-ray crystal structure measurements. Further comparisons between MART-10 and $1\alpha,25(\text{OH})_2\text{D}_3$ were performed in two prostate cancer cell lines, androgen-dependent LNCaP and androgen-independent PC-3 cells, and HepG2 liver cancer cells by hemocytometer cell counting. Similar to the findings using PZ-HPV-7 prostate cells, MART-10 is approximately 1000-fold more active than $1\alpha,25(\text{OH})_2\text{D}_3$ in inhibiting prostate cancer cell proliferation [107,108], while MART-10 is at least 100-fold more potent than $1\alpha,25(\text{OH})_2\text{D}_3$ in inhibiting HepG2 liver cancer cell growth (Figure 7)[68].

Since a major mechanism of the vitamin D-dependent cell growth inhibition is its ability to upregulate endogenous CKIs, such as p21 and p27 to induce cell cycle arrest at the G_0/G_1 phase [46–48]. The authors, therefore, performed western blot analysis to examine the effects of $1\alpha,25(\text{OH})_2\text{D}_3$ and MART-10 on the expression of p21 and p27, in HepG2 cells (Figure 8). The data show that MART-10 is much more potent than $1\alpha,25(\text{OH})_2\text{D}_3$ in the upregulation of p21 and p27 in HepG2 cells in a dose-dependent manner. Cell cycle distribution analysis by flow cytometry using propidium iodide staining of the cellular DNA also shows that treatment with 10^{-7} M MART-10 increased the fraction of HepG2 cells in G_1/G_0 phase from 42.7 to 63.58% and decrease the S phases from 33.24 to 16.45%, which is much greater than an increase from 42.7 to 53.2% of the fraction of HepG2 cells in G_1/G_0 phase and a decrease from 33.24 to 24.65% in S phases after exposure to 10^{-6} M $1\alpha,25(\text{OH})_2\text{D}_3$. From these results, it was concluded that 10^{-7} M of MART-10 is even more potent than 10^{-6} M $1\alpha,25(\text{OH})_2\text{D}_3$ in arresting the HepG2 cell cycle progression at the G_1 phase, in accordance with the p21 and p27 expression data (Figure 8).

Effects of $1\alpha,25(\text{OH})_2\text{D}_3$ & MART-10 on PC-3 cell invasion

In addition to the proliferation assay, the authors also compared the ability of MART-10 and $1\alpha,25(\text{OH})_2\text{D}_3$ to inhibit PC-3 cell invasion using Matrigel-coated inserts as described previously by Bao *et al.* [109]. Briefly, PC-3 cells (5×10^4 cells) pretreated with ethanol vehicle, or different concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$, or MART-10 for 72 h in FBS-supplemented medium were applied to the upper chambers of Matrigel coated inserts (Becton Dickinson Labware, MA, USA). The upper chambers were then added with serum-free media containing ethanol vehicle or different concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$ or MART-10 as indicated, whereas the lower chambers were filled with 10% FBS medium containing either ethanol vehicle or the same concentration of $1\alpha,25(\text{OH})_2\text{D}_3$ or MART-10 as the upper chambers. The chambers were incubated for 22 h at 37°C . The cells that had invaded to the lower surface of the membranes were fixed and stained with 1% Toluidine Blue and the total invading cell number in five random fields was counted under a light microscope. Using this method, the authors found that MART-10 was about tenfold more active than $1\alpha,25(\text{OH})_2\text{D}_3$ in inhibiting PC-3 cell invasion (Figure 9). Similar results were obtained with a diastereoisomer of MART-10, 19-nor-2 β -(3-hydroxypropyl)- $1\alpha,25(\text{OH})_2\text{D}_3$ (MART-11).

MART-10 can induce VDR trans-activation at a lower concentration than $1\alpha,25(\text{OH})_2\text{D}_3$

To compare the ability of $1\alpha,25(\text{OH})_2\text{D}_3$ and MART-10 to induce VDR activation, $1\alpha,25(\text{OH})_2\text{D}_3$ -responsive luciferase (Luc) reporter plasmid pVDRELuc was transfected into LNCaP cells with ethanol control, or increasing concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$ or MART-10 for 24 h [110]. Cells were then harvested and the normalized promoter activity was determined as a read-out for VDR mediated transcriptional activation. As shown in Figure 10, MART-10 stimulated VDR reporter transactivation activity by 30% over the control at 10^{-10} M ($p < 0.05$), and the stimulation increased to 70 and 270% over the control

at 10^{-9} and 10^{-8} M, respectively. No stimulation by $1\alpha,25(\text{OH})_2\text{D}_3$ was observed at 10^{-10} and 10^{-9} M. However, a 210% stimulation was observed at 10^{-8} M $1\alpha,25(\text{OH})_2\text{D}_3$. Thus, the data indicate that MART-10 is able to stimulate VDR transactivation at much lower concentration than $1\alpha,25(\text{OH})_2\text{D}_3$ and is at least tenfold more active than $1\alpha,25(\text{OH})_2\text{D}_3$ in stimulating VDR transactivation in LNCaP cells. Similarly, the transactivation activity of osteocalcin promoter by 14-*epi*-MART-10 and 14-*epi*-MART-11 was evaluated in human osteosarcoma cells (HOS). Kittaka *et al.* found that 14-*epi*-MART-10 and 14-*epi*-MART-11 were approximately 388 and 54% as active as $1\alpha,25(\text{OH})_2\text{D}_3$ in activating the osteocalcin promoter, respectively [34]. The authors also compared the VDR binding affinity and HL-60 cell differentiation-inducing ability of 14-*epi*-MART-10 and 14-*epi*-MART-11 to those of the natural hormone, $1\alpha,25(\text{OH})_2\text{D}_3$. In spite of their much lower binding affinity to VDR as compared to $1\alpha,25(\text{OH})_2\text{D}_3$, 14-*epi*-MART-10 was approximately eightfold as potent as $1\alpha,25(\text{OH})_2\text{D}_3$ and 14-*epi*-MART-11 was only slightly less active than $1\alpha,25(\text{OH})_2\text{D}_3$ in inducing HL-60 differentiation [34].

Induction of *CYP24A1* gene expression in prostate cancer cells by MART-10

The *CYP24A1* gene promoter contains VDRE [111] and is highly inducible by VDR-mediated $1\alpha,25(\text{OH})_2\text{D}_3$ transactivation [111,112]. Therefore, the induction of *CYP24A1* gene expression has been widely used to monitor the biological potency of vitamin D analogs as compared to $1\alpha,25(\text{OH})_2\text{D}_3$ [107]. Using this approach, the authors performed the dose responses of $1\alpha,25(\text{OH})_2\text{D}_3$ and MART-10 induction of *CYP24A1* gene expression in LNCaP and PC-3 cells using quantitative (q)-PCR (Figure 11). As shown in Figure 11A & 11B, $1\alpha,25(\text{OH})_2\text{D}_3$ and MART-10 caused a dose-dependent increase in the expression of *CYP24A1* in LNCaP and PC-3 cells. MART-10 was significantly more potent than $1\alpha,25(\text{OH})_2\text{D}_3$ in inducing *CYP24A1* gene expression at 10^{-9} , and 10^{-8} M ($p < 0.05$) in either cell type. Interestingly, the time course study in PC-3 cells (Figure 11C) demonstrated a bell shape upregulation of *CYP24A1* gene expression in response to $1\alpha,25(\text{OH})_2\text{D}_3$ at 10^{-8} M with a peak response at 24 h, whereas MART-10 at the same concentration continued to stimulate *CYP24A1* gene expression reaching more than 800-fold increase at 48 h. The drastic decrease in the expression at 48 h by $1\alpha,25(\text{OH})_2\text{D}_3$ suggests that significant amounts of $1\alpha,25(\text{OH})_2\text{D}_3$ were degraded by CYP24A1 resulting from its upregulation by $1\alpha,25(\text{OH})_2\text{D}_3$. Therefore, the prolonged stimulation by MART-10 suggests that MART-10 may be more resistant to the CYP24A1-mediated degradative pathway in agreement with the data obtained from a cell-free reconstituted enzyme system and the molecular model docking studies [107,108]. Overall, these data indicate that MART-10 has enhanced biological activity and remains bioavailable for a longer period of time as compared to $1\alpha,25(\text{OH})_2\text{D}_3$.

MART-10 is more potent than $1\alpha,25(\text{OH})_2\text{D}_3$ in downregulating matrix metalloproteinase activities in PC-3 prostate cancer cells

As it has been shown previously, MART-10 inhibited PC-3 cell invasion at lower concentrations compared to $1\alpha,25(\text{OH})_2\text{D}_3$ [106]. To further investigate the potential mechanisms responsible for the more potent anti-invasive effect of MART-10, the authors investigated the expression of a key enzyme involved in the cell invasion pathway, MMP-9, which has been shown to be regulated by $1\alpha,25(\text{OH})_2\text{D}_3$ [108]. First, the authors measured the mRNA level of MMP-9 by q-PCR in PC-3 cells treated with $1\alpha,25(\text{OH})_2\text{D}_3$ or MART-10 at different concentrations for 24 h. As shown in Figure 12A, the MMP-9 transcript expression was inhibited by MART-10 by approximately 40% at 10^{-9} M, whereas no inhibition was observed with $1\alpha,25(\text{OH})_2\text{D}_3$ at this concentration. At 10^{-8} M, $1\alpha,$

25(OH)₂D₃ exhibited approximately 35% inhibition, which is similar to that induced by MART-10 at 10⁻⁹ M. Interestingly, no further enhancement of inhibition by MART-10 at 10⁻⁸ M was observed. On the contrary, the protein expression of MMP-9 as determined by western blot analysis demonstrated a dose-dependent decrease from 10⁻¹⁰ to 10⁻⁸ M of MART-10 (Figure 12B & 12C). A dose-dependent inhibition of MMP-9 protein expression by 1α,25(OH)₂D₃ was also observed between 10⁻⁹ and 10⁻⁶ M. The discrepancy between the mRNA and protein expression suggests that MART-10 may exert additional regulation by an unknown post-transcriptional mechanism at the higher concentrations. These results do indicate that the greater inhibition of MMP-9 activity by MART-10 compared with 1α,25(OH)₂D₃ at both the mRNA and protein levels may be responsible for the more potent anti-invasive effect observed in the presence of MART-10.

MART-10 & 14-*epi*-MART-10 are active & less calcemic *in vivo* in animal models

As mentioned previously, 14-*epi*-MART-10 is a potent osteocalcin transactivator in HOS cells. We therefore investigated whether 14-*epi*-MART-10 would have effects on bone mineral density and calcemic activity in ovariectomized adult female Sprague-Dawley rats [34]. 14-*epi*-MART-10 administered to ovariectomized rats at 0.01 μg/kg/day dose for 4 weeks (five-times/week) induced a marked increase in bone mineral density without causing significant calcemic and calciuric side-effects. However, both calcemic and calciuric effects became evident at 1.0 μg/kg/day dose of 14-*epi*-MART-10. To evaluate the *in vivo* activity of MART-10, we studied the upregulation of renal CYP24A1 mRNA as a biomarker in response to MART-10 administration to male Sprague-Dawley rats [108]. The authors observed that MART-10 at a dose of 5 μg/kg for 24 h induced renal *CYP24A1* expression fourfold over the base line without any increase in serum calcium, whereas 1α,25(OH)₂D₃ at the same dose caused a significant elevation in plasma calcium over the control animals. Overall, MART-10 and 14-*epi*-MART-10 are active *in vivo* and are less calcemic than 1α,25(OH)₂D₃, suggesting that both analogs have potential as anticancer agents.

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Key Terms

Apoptosis	Regulated process to eliminate unwanted cells for the advantage of the organism.
Modified Julia olefination	One-pot synthesis for preparing alkenes from aldehydes or ketones using benzothiazol-2-yl sulfones instead of phenyl sulfones, which are used in the original Julia olefination to produce alkenes. In the original method, isolation of the initial carbon-carbon bond-forming products as the acyl isomer such as acetate is generally needed before the next reductive elimination reaction in order to get higher yields, whereas in the modified method, no separation of the initial products is required.

Angiogenesis	Formation of new blood microvessels from pre-existing vessels that is a process regulated by a range of endogeneous stimulators and inhibitors.
Cell cycle	Process of cell propagation through G1, S, G2 and M phases and is regulated by multiple molecular pathways and checkpoints in a coordinated fashion.
VDR response element	Short DNA sequence responsible for the binding of vitamin D receptor to initiate a gene transactivation.

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Future perspective

Since $1\alpha,25(\text{OH})_2\text{D}_3$ induces anticancer actions in a cell-specific manner and may involve different mechanisms in different cells [70–72], there is a likelihood that different $1\alpha,25(\text{OH})_2\text{D}_3$ analogs may exert different potency in different cells mediated by either genomic or non-genomic mechanism. Therefore, understanding the modes of anticancer actions of vitamin D at the molecular level in different systems will be crucial in designing ‘custom-made’ analogs for a mechanism-based cancer treatment for a particular type of cancer.

Executive summary

- The chemical synthesis of a new class of 19-norvitamin D analogs based on the x-ray crystallographic analysis of putative agonists – VDR/LBD complex interaction are described.
- The chemical synthesis was accomplished using a more efficient synthetic route with higher yields.
- That MART-10 is more active than $1\alpha,25(\text{OH})_2\text{D}_3$ in many *in vitro* systems that have examined is demonstrated.
- The mechanisms behind the more potent anti-proliferative and anti-invasive nature of MART-10 in prostate and liver cancer cells are discussed.
- In addition, MART-10 is much more resistant to the 24-hydroxylation catalyzed by CYP24A1, the first step of the degradative pathway of $1\alpha,25(\text{OH})_2\text{D}_3$ and its analogs. Therefore, MART-10 will have a longer half-life in the cells.
- Another unique property of MART-10 is that it has a lower binding affinity for vitamin D binding protein than $1\alpha,25(\text{OH})_2\text{D}_3$. The lower binding affinity for MART-10 will result in a higher concentration of free bioavailable MART-10 in the circulation for its subsequent translocation to various target tissues, such as the prostate and liver, indicating a potential role DBP may play in the effectiveness and pharmacodynamics of vitamin D analogs *in vivo*.
- Both MART-10 and 14-*epi*-MART-10 are either non-calcemic or less calcemic than $1\alpha,25(\text{OH})_2\text{D}_3$ *in vivo*.
- Overall, the unique properties of MART-10 and 14-*epi*-MART-10 suggest that these analogs and their structurally related analogs may be good candidates for the treatment of prostate and liver cancer.

Figure 1. Natural hormone **1** and its 19-*nor* analogs, MART-10, MART-11, 14-*epi*-MART-10 and 14-*epi*-MART-11

Figure 2. Retrosynthetic analysis of 2-alkylated 1 α ,25-dihydroxy-19-norvitamin D₃

Figure 3. Synthesis of A-ring part 5

Figure 4. Synthesis of CD-ring part 3 and 3' (14-*epi*-3)

Figure 5. Coupling reaction between CD-ring sulfone (3 or 3') and A-ring ketone 5 via modified Julia olefination

HPLC separation of C2-epimers (**2**) gave MART-10 and MART-11, and in the case of **2'**, the pivaloylation step was necessary for isolating each isomer of **14'** by HPLC separation followed by deprotection of each epimer to afford 14-*epi*-MART-10 and 14-*epi*-MART-11, respectively.

Figure 6. Stereochemical assignment of protected 14-*epi*-MART-10 and 14-*epi*-MART-11

Figure 7. Comparisons of the antiproliferative activity of MART-10 against 1 α ,25(OH) $_2$ D $_3$ in four cell lines

(A) PZ-HPV-7 nontumorous prostate cells [106]; (B) LNCaP prostate cancer cells [107]; (C) PC-3 prostate cancer cells [108]; and (D) HepG2 liver cancer cells [68]. Cells were plated at 5000 cells per cm 2 in 35 mm dishes. 2 days after the initial plating, the cells were treated with ethanol vehicle (control group) or different concentrations of 1 α ,25(OH) $_2$ D $_3$ or MART-10 dissolved in ethanol as indicated in the figures. The treatment was repeated on day 4 and day 6 after the initial plating. Cells were trypsinized and harvested 2 days after the final dosing. Triplicate aliquots were applied to a hemocytometer for cell counting on four different fields under a light microscope and then averaged. The results are expressed as percent of the control. The data of each group were compared to their respective control by the Student's *t*-test using Microsoft Excel 2007.

p* < 0.05; *p* < 0.01 versus control.

Figure 8. Dose-dependent upregulation of p21 and p27 expression by 1α,25(OH)₂D₃ and MART-10 in HepG2 liver cancer cells by western blot analysis

HepG2 cells were treated for 2 days with various concentrations of 1α,25(OH)₂D₃ or MART-10. Cell lysates containing 30 μg of protein were analyzed by polyacrylamide gel electrophoresis to measure protein expression after transferring the protein bands onto Immobilon-P^{sq} membranes and then incubated with mouse monoclonal antibodies against p21 or p27. Lysates were also analyzed for β-actin expression with mouse anti-β-actin for loading control. Expression of p21 or p27 relative to actin was calculated. The data of each group were compared by the Student's *t*-test using Microsoft Excel 2007. (A) Typical dose-dependent upregulation of p21 and (C) p27 protein expression in response to the treatment.

(B & D) expression relative to actin from three independent experiments. Each value is a mean $\pm$ standard deviation of three determinations.

*p < 0.05 versus control.

Data taken from [68].

Figure 9. Effect of 1 α ,25(OH) $_2$ D $_3$ and MART-10 on the invasion of PC-3 cells

The results are presented as the means $\pm$ standard deviation of three determinations. The data of each group were compared by the Student's *t*-test using Microsoft Excel 2007.

p* < 0.05; *p* < 0.05.

[†]Comparison between control and 1 α ,25(OH) $_2$ D $_3$ or MART-10 (M-10).

[‡]Comparison between 1 α ,25(OH) $_2$ D $_3$ and M-10.

Data taken from [106].

Figure 10. The effects of MART-10 and 1 α ,25(OH) $_2$ D $_3$ on vitamin D receptor transactivation in LNCaP cells

LNCaP cells transfected with pVDRELuc reporter construct were treated with ethanol or the indicated concentrations of 1 α ,25(OH) $_2$ D $_3$ or MART-10 for 24 h, then cells were harvested and assayed for luciferase activity. Luciferase activity is expressed as percentage of ethanol-treated control cells after normalization for protein concentration. The data are means $\pm$ standard deviation of three determinations from a representative experiment. Similar results were obtained from two other experiments. The data of each group were compared by the Student's *t*-test using Microsoft Excel 2007.

**p* < 0.05 compared with control.

Data taken from [107].

pVDRELuc was a gift from Carsten Carlberg.

Figure 11. The dose–response and time-dependent effects of 1 α ,25(OH) $_2$ D $_3$ and MART-10 treatment on endogenous CYP24A1 mRNA expression in LNCaP cells and PC-3 cells

For the dose–response studies, (A) LNCaP cells or (B) PC-3 cells were treated with ethanol vehicle, or indicated concentrations of 1 α ,25(OH) $_2$ D $_3$, or MART-10 for 24 h before cells were harvested for total RNA extraction and mRNA analysis by relative real-time quantitative PCR. For the time course experiments, (C) PC-3 cells were treated with 10 $^{-8}$ M of either 1 α ,25(OH) $_2$ D $_3$, or MART-10 for a time period as indicated. Total RNA was prepared and mRNA expression was analyzed by relative real-time quantitative PCR. Data are expressed as the mean of triplicate determinations. The data were calculated according to the DDCT method and standardized to an endogenous control, S18. Values represent the percentage change in gene expression relative to the ethanol vehicle-treated control cells. The experiment was repeated three times. Data taken from [107,108].

Figure 12. Relative MMP-9 mRNA expression measured by real-time PCR and relative MMP-9 protein expression determined by western blotting in PC-3 cells treated with MART-10 and $1\alpha,25(\text{OH})_2\text{D}_3$

PC-3 cells were treated with ethanol vehicle or the indicated concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$ and MART-10 for 24 h. (A) Total RNAs were extracted and analyzed for MMP-9 mRNA expression by real-time PCR, or (B) whole cell extracts were prepared and analyzed for MMP-9 by western blot using specific antibodies. (B) Tubulin expression was used as a loading control. Western blot results obtained in (B) were quantified using Quantity One software (Bio-rad) and normalized by the values of the control (C). Data are expressed as the mean $\pm$ standard deviation of triplicate determinations. For RT-PCR, the

data were calculated according to the DDCT method and standardized to an endogenous control, S18. Values represent the fold changes in gene expression relative to ethanol vehicle treated control PC-3 cells. The experiment for either RT-PCR mRNA or western blot protein expression was repeated three times.