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Probing the structural requirements for vitamin D3 inhibition of the hedgehog signaling pathway

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ABSTRACT

A structure–activity relationship study to elucidate the structural basis for hedgehog (Hh) signaling inhibition by vitamin D3 (VD3) was performed. Functional and non-functional regions of VD3 and VD2 were obtained through straightforward synthetic means and their biological activity was determined in a variety of cell-based assays. Several of these compounds inhibited Hh signaling at levels comparable to the parent VD3 with no effects on canonical vitamin D signaling. Most notably, compounds **5** and **9**, demonstrated potent inhibition of the Hh pathway, exhibited no binding affinity for the vitamin D receptor (VDR), and did not activate VDR in cell culture. In addition, several compounds exhibited antiproliferative activity against two human cancer cell lines through a mechanism distinct from the Hh or VDR pathways, suggesting a new cellular mechanism of action for this class of compounds.

The hedgehog (Hh) signaling pathway is a developmental pathway which plays a key role in directing growth and tissue patterning during embryonic development. Dysregulation of Hh signaling contributes to the development of a variety of human tumors, including skin, brain, colon, pancreatic, and lung cancers.[1] Constitutive activation of the pathway results in the increased expression of Hh target genes, including several forms of the glioma-associated oncogene (Gli) family of signaling proteins and leads to uncontrolled tumor proliferation. Recent years have seen the development and characterization of numerous small molecule inhibitors of Hh signaling as anti-cancer chemotherapeutics; most notably, cyclopamine and GDC-0449 (Fig. 1).[2] Cyclopamine (Cyc) is a natural product derived from corn lilies that has been used extensively to characterize Hh signaling in vitro and in vivo. An analogue of Cyc (IPI-926) that demonstrates enhanced potency and improved pharmacokinetic properties is currently in clinical development. GDC-0449, a small molecule inhibitor of Hh signaling, recently received fast-track approval by the FDA for the treatment of metastatic basal cell carcinoma. This represents the first Hh inhibitor to receive FDA approval and validates the clinical relevance of this class of anti-cancer compound.

More recently, vitamin D3 (VD3) was identified as an Hh pathway inhibitor that is thought to exert its effects through direct binding to Smoothened (SMO), an integral pathway component that is also the molecular target of Cyc and GDC-0449.[3] VD3 inhibits pathway signaling in Hh-dependent cell culture as well as in murine models of basal cell carcinoma (BCC), a skin cancer widely recognized as Hh-dependent.[4,5] To date, the development of VD3 as an Hh pathway inhibitor with anti-cancer potential has been underexplored, most probably due to its ability to activate canonical vitamin D receptor (VDR) signaling in vitro and in vivo.[5] The VDR pathway plays an essential role in numerous physiological systems and its activation can result in detrimental side effects; therefore, VD3 analogues must potently inhibit Hh signaling while exhibiting minimal or no effects on VDR.[6]

Our initial efforts at identifying structure–activity relationships for VD3 inhibition of Hh signaling began with straightforward modifications at the 3-(*S*)-hydroxyl.[4] Results from the evaluation of these analogues demonstrated that minor changes to the VD3 scaffold, particularly the A-ring, are not sufficient to prevent its cellular activation of VDR. The studies described herein represent a more detailed approach to probing the structural requirements for VD3-mediated inhibition of Hh signaling. We sought to determine which regions of VD3 were essential for Hh inhibition, while also identifying whether these analogues retain the ability to bind and activate VDR.

The first series of compounds prepared for evaluation focused on simple semisynthetic derivatives of VD3 (Scheme 1, 1–4). These compounds were synthesized through known methods to provide insight into how changing the orientation and/or composition of the diene linker and elimination of the 3-hydroxyl affected activity. Triol **1** was prepared through selective conversion of the VD3 C7-C8 olefin to the corresponding diol.[7] The two-step protection/

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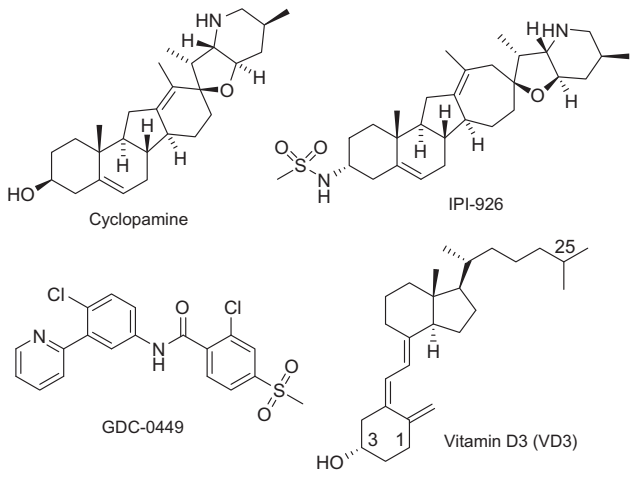
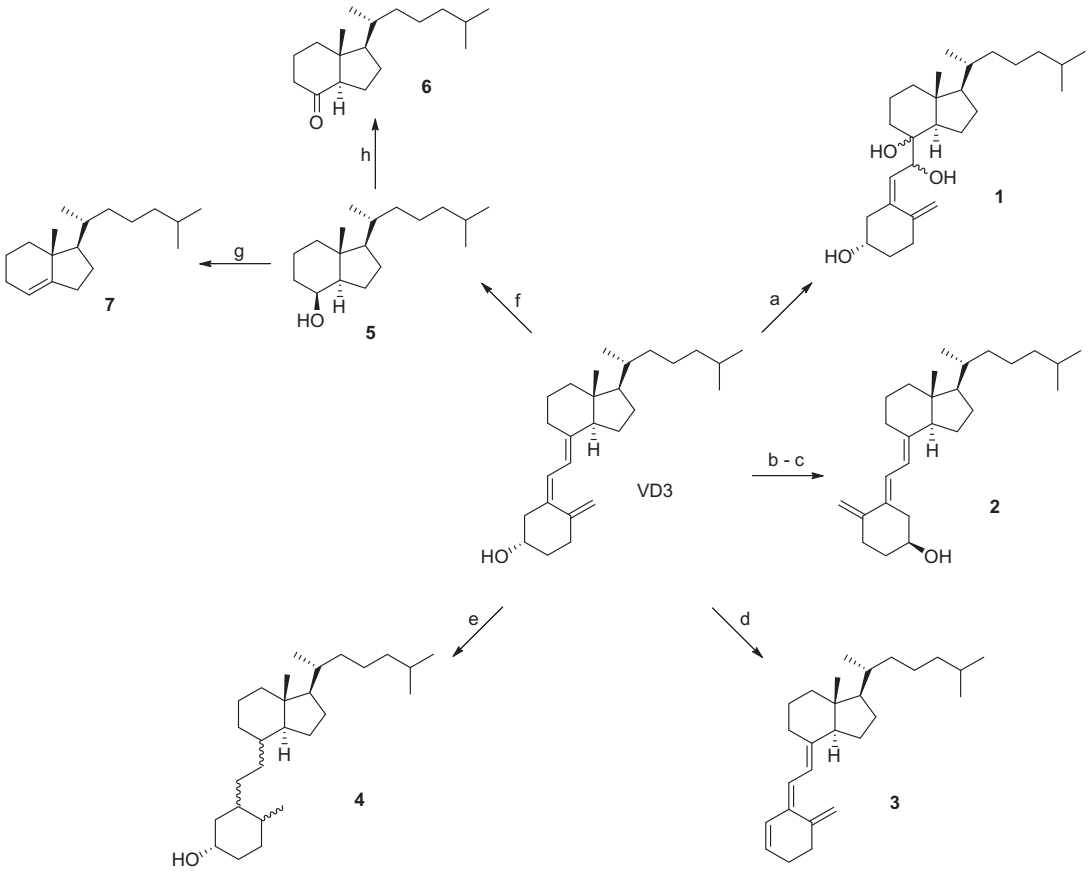


Figure 1. Hh pathway inhibitors.

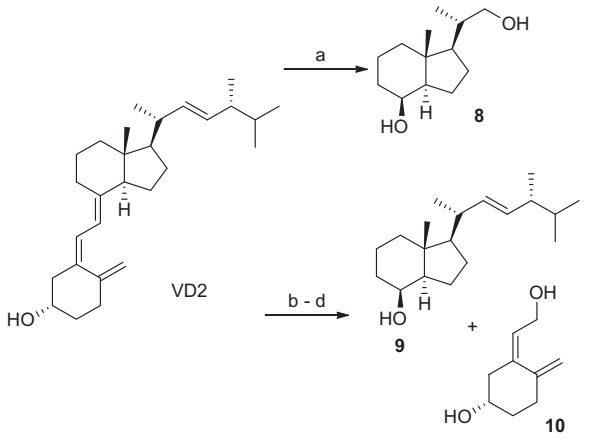
deprotection of the VD3 triene as the sulfur dioxide adduct yielded trans-VD3 **2**.[8] The C-3 hydroxyl of VD3 was found to eliminate under modified Mitsunobu conditions to provide VD3 analogue **3**. Finally, hydrogenation of VD3 provided saturated VD3 isomers, **4**, that were evaluated as a mixture. All compounds were characterized by ^{1}H and ^{13}C NMR, as well as HRMS analysis prior to undergoing biological evaluation.

Our next series of analogues focused on individually evaluating the 'northern' (CD-ring and side chain) and 'southern' (A-ring) regions of VD3 for residual biological activity. The one-pot, two-step global ozonolysis/reductive workup of VD3 provided Grundmann's alcohol **5**, which was oxidized to the corresponding ketone **6** with PDC (Scheme 1).[9] In addition, the hydroxyl of **5** was eliminated directly upon formation of the mesylate to provide olefin **7**. Exhaustive ozonolysis of VD2 afforded Inhoffen-Lythgoe diol **8**, while oxidative cleavage of the VD2 triol provided the 'northern' region of VD2, **9**, and the VD3 A-ring, **10** (Scheme 2).[9] Finally, sulfur dioxide protection of the VD2 triene, followed by selective ozonolysis/reduction of the side chain olefin and removal of the triene protecting group provides the known TBS-protected trans-VD3 alcohol **11** (Scheme 3).[8] Photoisomerism of **11** and deprotection of the 3-hydroxyl affords the VD3 diol **12**; similarly, the trans-VD3 diol **13** was prepared for evaluation.

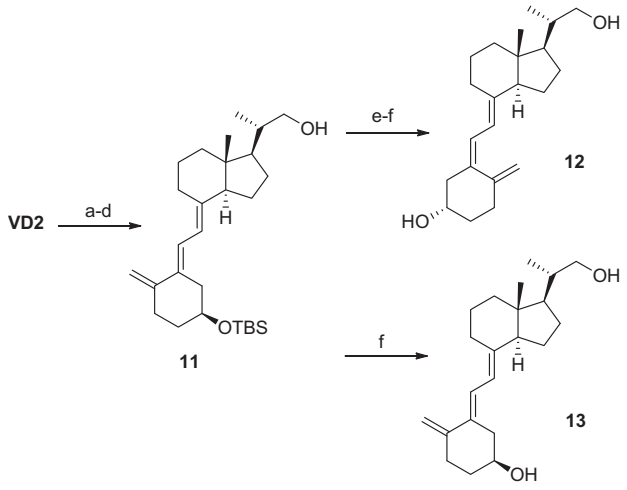
The Hh inhibitory activity of these truncated VD3 analogues was evaluated through their ability to modulate endogenous Gli1 mRNA in the Hh-dependent C3H10T1/2 cell line.[10] Activation of the Hh pathway in these cells (via exogenous addition of Hh ligand or oxysterols) results in the robust and reproducible up-regulation of the Hh target gene Gli1 (~100-fold) and concomitant administration of an Hh pathway inhibitor attenuates this response. Down-regulation of Gli1 levels in this cellular model is a well-established method for determining Hh inhibition. For these studies, analogues were evaluated at 5 μM and Gli1 mRNA expression levels were normalized to oxysterol controls. Several key structure–activity relationships with respect to different regions of the VD3 scaffold were observed from this assay (Table 1). First, with respect to the side chain, VD2 was equipotent at inhibiting the Hh pathway compared to VD3. By contrast, truncation of the side chain (analogue **12**) resulted in a two-fold loss of Hh inhibitory activity. Taken together, these data indicate that this region is amenable to minor modifications that retain the general structure and hydrophobic nature of the natural alkyl side chain. Second, as



Scheme 1. Reagents and conditions: (a) $KMnO_4$, H_2O (65%); (b) H_2SO_3, benzene; (c) Na_2CO_3, EtOH, reflux (80%, two steps); (d) PBu_3, DIAD, DPPA (70%); (e) Pd/C, H_2, ethanol (92%) (f) O_3, pyr, DCM:MeOH, $NaBH_4$ (86%) (g) NEt_3, MsCl, 0 °C (41%); (h) PDC, DCM (75%).



Scheme 2. Reagents and conditions: (a) O_3, pyr, DCM:MeOH, $NaBH_4$ (74%); (b) $KMnO_4$, THF, H_2O (65%); (c) $Pb(OAc)_4$, DCM; (d) Vitride™, DCM (**9**: 69%, **10**: 53%, two steps).



Scheme 3. (a) H_2SO_3, Benzene; (b) TBSCl, imidazole, DCM (95%, 2 steps); (c) O_3, pyr, DCM:MeOH, $NaBaH_4$ (82%); (d) Na_2CO_3, EtOH, reflux (80%); (e) acridine (50 mol%), NEt_3, benzene, 365 nm (95%); (f) TBAF, DCM (75%).

Table 1
Hh and VDR-related activity of VD3 analogues

Analogue	Gli1 mRNA[a] (%)	VDR binding[b]	Cyp24A1mRNA[c]
DMSO	—	—	1.0
Oxy	100	—	—
VD3	35.7 ± 0.3	>100	8336 ± 38
VD2	28.1 ± 7.4	>100	2408 ± 584
1	144 ± 41	>100	6.7 ± 1.9
2	69.7 ± 8.8	>100	43.5 ± 8.3
3	79.4 ± 14.7	>100	3.0 ± 2.4
4	82.1 ± 1.1	>100	1.5 ± 0.3
5	46.4 ± 3.5	>100	3.6 ± 0.5
6	66.1 ± 0.9	>100	3.0 ± 0.4
7	111 ± 21	>100	2.3 ± 0.8
8	87.6 ± 1.2	>100	4.1 ± 0.7
9	32.1 ± 6.3	>100	2.3 ± 1.2
10	80.9 ± 2.1	>100	2.0 ± 0.2
12	80.0 ± 10.4	>100	1354 ± 617
13	66.7 ± 5.7	>100	12.5 ± 2.4

[a] Values represent% Gli mRNA expression relative to oxysterol control (set at 100%). Analogues were evaluated at 5 μM and 24 h.
[b] Analogues were evaluated at 50 and 100 μM in the PolarScreen™ VDR competitor assay, Red (invitrogen) following manufacturer's protocol.[11]
[c] Values represent Cyp24A1 mRNA expression relative to DMSO control (set to 1.0). Analogues were evaluated at 5 μM and 24 h.

evidenced by **5** and **9**, the 'northern' region of the scaffold retains the inhibitory activity of intact VD3. In addition, the comparable level of Gli down-regulation exhibited by both **5** and **9** (46.5 ± 3.5 and 28.1 ± 7.4, respectively) further supports the conclusion that minor changes to the side chain do not affect the overall ability of the compounds to inhibit Hh signaling. Modification (**6**) or removal (**7**) of the C-8 hydroxyl significantly attenuated the Hh inhibition of the 'northern' region, suggesting its presence and orientation is essential for optimal activity. In a similar fashion, the inability of truncated A-ring analogue **10** to inhibit Hh signaling provides further evidence that the CD-ring and side chain maintain the residual activity of the VD3 scaffold. Finally, several analogues that contain the intact 'northern' region of VD3 (**1**–**4**) demonstrate reduced Hh inhibition, suggesting that, while not required for activity, modifications to the seco-B-ring or A-ring can prevent optimal Hh inhibition.

The next series of assays performed for the VD3 analogues was designed to evaluate their ability to bind and activate VDR (Table 1). Using a competitive displacement binding assay for VDR,[11] none of the analogues displayed affinity for VDR at concentrations up to 100 μM (Table 1). This result was not surprising as our previous work with VD3 demonstrated that it and several A-ring analogues do not bindVDR.[4] Conversely, VD3 is able to functionally activate VDR in C3H10T1/2 cell culture (as measured by up-regulation of the well-characterized VDR target gene Cyp24A1) irrespective of its ability to bind purified VDR; therefore, we also determined the ability of these compounds to up-regulate Cyp24A1. The majority of VD3 analogues did not demonstrate VDR activation compared to DMSO control. In addition, all of the compounds that induced Cyp24A1 up-regulation (VD3, VD2, **2**, and **12**) did so at a level significantly less than that observed for VD3. These results provided important insight into the regions of VD3 essential for activating VDR. Primarily, the intact seco-steroid backbone (A-, seco-B-, and fused CD rings) is essential for a VD3 analogue to significantly activate VDR signaling. This is supported by the findings that none of the truncated analogues demonstrated significant Cyp24A1 up-regulation. Taken together, these data provide evidence that truncated analogues such as **5** and **9** will not activate canonical VDR signaling and hold promise as selective Hh pathway inhibitors.

Previous studies have demonstrated that the anti-proliferative activity of Hh pathway inhibitors (including Cyc, GDC-0449, and VD3) in cultured cancer cell lines does not correlate with pathway inhibition in Hh-dependent cells.[12,13] However, each of these compounds demonstrates modest anti-proliferative effects in multiple cell lines, suggesting either Hh signaling does play a role in preventing cancer cell growth in vitro or the anti-proliferative effects of these compounds is mediated through unidentified cellular mechanisms distinct from Hh inhibition. With respect to VD3, cellular effects unrelated to Hh signaling most probably result from activation of VDR signaling; therefore, studying the anti-proliferative activity of the VD3 analogues could potentially provide more evidence as to their ability to selectively inhibit the Hh pathway. For these reasons, we sought to determine the anti-proliferative effects of VD3 and its analogues in two distinct cancer cell lines, U87MG, a glioblastoma cell line that exhibits anti-proliferative and anti-Hh effects following Cyc administration,[14] and HT-29, a colorectal adenocarcinoma that over-expresses VDR and responds to VDR activation with a robust, characteristic response.[15,16]

VD3, VD2 and all of the analogues evaluated were significantly more active in the U87MG cell line (Table 2). The GI_{50} values for VD3 and VD2 (23.2 ± 5.1 and 33.7 ± 0.2 μM, respectively) were comparable, suggesting the mechanisms responsible for their anti-proliferative activity in this cell line are similar. By contrast, VD3 demonstrated significantly reduced anti-proliferative activity against HT-29 cells and VD2 was inactive in these cells. Several

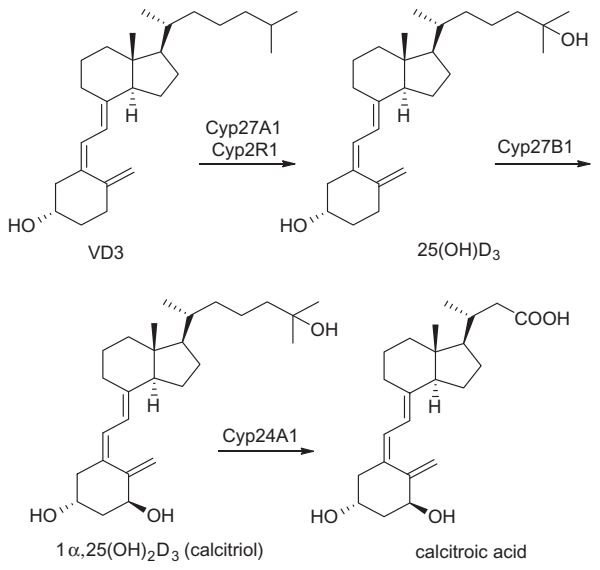
Table 2
Anti-proliferative activity of VD3 analogues[17]

Analogue	U87MG GI_{50}[a] (μM)	HT-29 GI_{50}[b] (μM)
VD3	23.2 ± 5.1[c]	68.5 ± 10.5
VD2	33.7 ± 0.2	>100
1	25.9 ± 4.2	>100
2	46.0 ± 6.8	>100
3	48.5 ± 7.4	>100
4	>100	>100
5	5.7 ± 1.4	66.5 ± 17.9
6	12.1 ± 2.0	>100
7	>100	>100
8	>100	>100
9	19.0 ± 5.0	49.7 ± 13.9
10	>100	>100
12	>100	>100
13	88.8 ± 14	>100

[c]Values represent mean ± SEM for two separate experiments performed in triplicate.
[a] GI_{50} values measured in U87MG cells (MTS assay).
[b] GI_{50} values measured in HT-29 cells (MTS assay).

analogues exhibited modest effects against U87MG cells, with the most active compound being Grundmann's alcohol, **5** (GI_{50} = 5.7 ± 1.4 μM). Of note, the only truncated VD3 analogues that exhibited anti-proliferative activity in both cell lines, **5** and **9**, were those that demonstrated the most robust Gli1 down-regulation in C3H10T1/2 cells, suggesting a correlation between Hh inhibition and anti-proliferative activity in these cells. As our initial hypothesis was that the metabolic conversion of VD3 and analogues to a form that can more readily activate VDR was responsible for its anti-proliferative effects, we sought to further explore the mechanisms that govern the anti-proliferative effects of these analogues. Specifically, we sought to determine whether anti-proliferation correlated with the following: (1) expression levels of vitamin D metabolizing enzymes and (2) modulation of Hh- and VDR-specific target genes.

The major enzymes associated with the metabolism of the vitamin D sterols, 25-hydroxylases (Cyp2R1 and Cyp27A1) and 1α-hydroxylases (Cyp27B1) are predominantly expressed in the liver and kidney, respectively (Scheme 4). However, recent evidence has shown that various types of human cancers, particularly colon cancer, express increased levels of these metabolic enzymes.[15,16] While it remains unclear whether the over-expression



Scheme 4. Vitamin D metabolic pathway.

of these enzymes plays a role in the proliferation of cancer cells, our goal was to examine whether there was a correlation between their expression in U87MG and HT-29 cells and the anti-proliferative activity of the truncated VD3 analogues. U87MG cells demonstrated significantly higher baseline expression levels for each Cyp isoform (10- to 100-fold), supporting the hypothesis that VD3 can be converted to a from that activates VDR in this cell line (Fig. 2). By contrast, baseline expression of VDR was 40-fold higher in HT-29 cells. The increased relative expression of the vitamin D metabolizing enzymes in U87MG cells, coupled with the lower GI_{50} values in this cell line, provided preliminary evidence to support our hypothesis that in vitro metabolism of VD3 results increased anti-proliferation.

Our final series of experiments, designed to correlate VD3 and analogue metabolism to anti-proliferative activity, was to explore the ability of these compounds to regulate downstream target genes of Hh and VDR signaling in both cancer cell lines (Table 3). Similar to our previous findings,[4] treatment of either cell line with VD3 (10 μM) had no effect on Gli mRNA expression levels while resulting in significant up-regulation of Cyp24A1 (Table 3). Effects of VD2 treatment were consistent to VD3, providing further evidence that these two sterols exert their anti-proliferative effects through VDR activation. In addition, up-regulation of Cyp24A1 in HT-29 cells was significantly greater for both VD3 and VD2 compared to U87MG cells (~10,000-fold and ~25-fold, respectively). None of the VD3 analogues modulated Gli expression in either cell line, suggesting that the anti-proliferative activity of these analogues is unrelated to Hh inhibition. Up-regulation of Cyp24A1 for the VD3 analogues in both cell lines correlated well with the results obtained in the C3H10T1/2 cell line, i.e. treatment with analogues **2** and **12** resulted in significant up-regulation of Cyp24A1. Most importantly, the most active analogues in the anti-proliferation assays, **5** and **9**, did not modulate either Hh or VDR signaling, suggesting these effects may be due to other cellular mechanisms.

The results of these studies provide important SAR information as to the structural requirements for VD3 inhibition of Hh signaling. First, the A-ring is not essential for Hh inhibition and may be detrimental, as the only VD3 analogues that activated VDR retained the intact seco-steroid backbone (A-, seco-B-, and fused CD rings). Second, the alkyl side chain of VD3 appears amenable to modification provided the hydrophobic nature of the natural linear alkyl moiety is maintained. Utilizing this knowledge, we are currently exploring several new series of VD3 analogues that incorporate modifications to these regions to further develop improved VD3-based inhibitors of Hh signaling.

Our initial attempts to characterize the mechanisms through which VD3 and its analogues exert their modest anti-proliferative

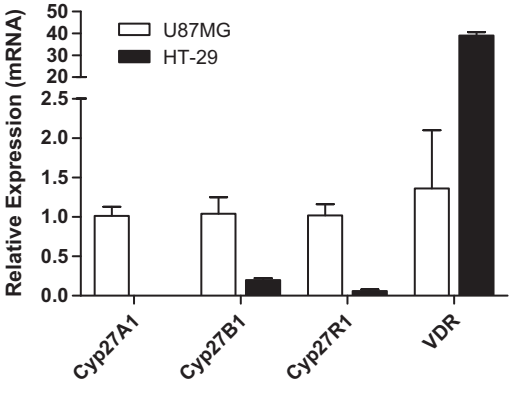


Figure 2. Baseline expression (mRNA) of vitamin D-related metabolic enzymes in cultured cancer cells.

Table 3
Hh- and VDR-modulation in cultured cancer cells

Analogue[a]	U87MG[b]		HT-29[b]	
	Gli1	Cyp24A1	Gli1	Cyp24A1
VD3	0.7 ± 0.02[c]	12.2 ± 4.1	1.3 ± 0.3	14697 ± 1255
VD2	0.9 ± 0.01	49.6 ± 0.8	1.1 ± 0.2	10902 ± 2840
1	1.1 ± 0.09	0.5 ± 0.06	0.9 ± 0.1	1.2 ± 0.2
2	1.1 ± 0.05	33.8 ± 5.3	1.2 ± 0.01	27.5 ± 4.3
3	1.0 ± 0.3	0.9 ± 0.3	1.8 ± 0.3	1.9 ± 0.8
4	0.8 ± 0.2	0.8 ± 0.2	1.4 ± 0.2	1.4 ± 0.5
5	0.7 ± 0.04	0.4 ± 0.01	1.1 ± 0.1	1.1 ± 0.1
6	0.9 ± 0.01	0.5 ± 0.1	1.1 ± 0.4	1.0 ± 0.4
7	1.0 ± 0.2	0.5 ± 0.09	1.1 ± 0.1	1.1 ± 0.3
8	1.0 ± 0.1	0.7 ± 0.04	1.2 ± 0.1	1.0 ± 0.2
9	0.8 ± 0.3	0.3 ± 0.1	0.9 ± 0.02	0.7 ± 0.1
10	0.9 ± 0.08	0.6 ± 0.03	0.9 ± 0.05	0.7 ± 0.01
12	1.0 ± 0.1	4.8 ± 1.0	2.7 ± 0.4	69.1 ± 1.9
13	0.8 ± 0.1	0.8 ± 0.2	1.3 ± 0.4	3.3 ± 1.6

[c]Values represent mean ± SEM of two separate experiments performed in triplicate.
[a] Analogues were evaluated at 10 μM and 24 h.
[b] Values represent Gli1 or Cyp24A1 mRNA expression relative to DMSO (set at 1).

effects have proven contradictory. The increased expression levels of vitamin D metabolizing enzymes in U87MG cells suggested that the enhanced anti-proliferative effects in this cell line may result from the cellular conversion of VD3 to 25(OH)D_3 and/or 1α,25(OH)D_3 and subsequent activation of VDR; however, up-regulation of Cyp24A1 is more robust in HT-29 cells. In addition, VD3 analogues that demonstrated anti-proliferation against these cell lines did not regulate either Hh or VDR signaling, providing initial evidence that these analogues may exert biological effects through a third, as yet unidentified, cellular mechanism. Recent studies have demonstrated that several natural and synthetic cholesterol metabolites, including oxysterols and bile acids, exhibit anti-proliferative effects through a variety of different mechanisms.[18–21] Steroid hormone receptors other than VDR, primarily the estrogen and androgen receptors, have long been studied as anti-cancer targets and several selective estrogen receptor modulators (SERMs) are used clinically for the treatment of breast cancer.[22] The core structural similarities of these steroids and our truncated VD3 analogues, particularly **5** and **9**, suggest that the anti-proliferative activity of these compounds may result from their ability to bind and modulate a myriad of cellular mechanisms. Therefore, in order to fully explore the development of VD3 and its analogues as selective Hh inhibitors, future work must identify and detail all cellular components that bind VD3 and these analogues, not just those related to Hh and VDR signaling.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl. 2012. 05.037.

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10. General RT-PCR protocol: Following treatment and incubation, RNA was extracted with TRIZOL® reagent following the manufacturer's instructions. cDNA synthesis was performed utilizing the high capacity cDNA reverse transcription kit (ABI) per the manufacturer's instructions on a BioRadMyCycler. Quantitative RT-PCR was performed on an ABI 7500 system using the following Taqman gene expression assays: human GLI1, Hs00171790_m1; human Cyp24A1, Hs00167999_m1; human ActB, Hs99999903_ml; mouse GLI1, Mm00494645_m1; mouse Cyp24A1, Mm00487244_m1; mouse ActB, Mm00607939_s1. Relative gene expression levels were computed via the ΔΔCt method.
11. PolarScreen™ VDR competitor assay, red (invitrogen) per the manufacturer's instructions. This assay is a fluorescence polarization-based competition assay that has been optimized for high-throughput applications and contains all the required components (including full length human VDR and a proprietary tight-binding fluorescent VDR ligand).
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