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Design, synthesis, and evaluation of hybrid vitamin D3 side chain analogues as hedgehog pathway inhibitors

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ABSTRACT

Vitamin D3 (VD3) is a moderately potent and non-selective inhibitor of the Hedgehog (Hh) signaling cascade. Previous studies have established that the CD-ring region of VD3 serves as the Hh inhibitory pharmacophore. Subsequently, compound **3**, an ester linked aromatic A-ring and CD-ring derivative was identified as an improved and selective Hh inhibitor. Herein, we report modifications of the CD-ring side chain that afford enhancement of selectivity for Hh modulation thereby diminishing the detrimental effects of concomitant vitamin D receptor activation. In general, linear or moderately branched alkyl chains of five or six carbons were optimal for potent and selective inhibition of Hh signaling. Moreover, hybrid VD3 side chain derivative **20** demonstrated 4-fold improvement in Hh antagonistic activity over VD3(IC_{50} = 1.1–1.6 μM) while gaining greater than a 1000-fold selectivity for Hh signaling over canonical activation of the vitamin D receptor pathway.

1. Introduction

Targeting embryonic signaling pathways is an emerging approach to selectively treat several forms of human malignancy. One such signaling cascade that has been amenable to therapeutic intervention is the Hedgehog (Hh) signaling pathway.[1,2] During the embryonic stages of life, this critical signaling mechanism dictates tissue patterning, proliferation and differentiation; however, it is much less active and primarily responsible for maintaining stem cell populations in skin and the central nervous system among healthy adults. The key upstream mediators of the pathway include a twelve transmembrane protein called Patched (Ptch) receptor which responds to the presence or absence of Hh ligands by correspondingly activating or repressing the action of a seven transmembrane receptor called Smoothened (Smo). Under normal conditions, Smo along with other proteins and protein complexes tightly regulate the production of activator and repressor forms of Hh target genes such as glioma-associated transcription factors (Gli1, Gli2 and Gli3).[1,2] Dysregulation and constitutive activation of the pathway through mutations in either Ptch or Smo has been linked to cancer initiation and progression; most notably, in basal cell carcinoma (BCC) and medulloblastoma (MB).[3,4] In addition, ligand dependent over activation of Hh signaling has been associated with various other forms of cancers such as pancreatic ductal adenocarcinoma, colon, ovarian, prostate, breast, and lung cancer.

Significant efforts to develop cancer chemotherapeutics that act by targeting Hh signaling have been undertaken in the last decade.[1,2] Consequently, GDC-0449 (Vismodegib/Erivedge™), the first small molecule Hh inhibitor was approved for clinical use against advanced/metastatic BCC in early 2012.[5] In recent years, several members of the vitamin D class of *seco*-steroids have been identified as modulators of Hh pathway signaling.[6,7] Previous studies undertaken in our lab revealed that vitamin D3 (cholecalciferol, VD3, Fig. 1) mediated inhibition of Hh signaling in vitro is modest (IC_{50} = 4.1 μM) and non-selective due to its ability to concurrently activate canonical vitamin D receptor (VDR) signaling presumably through metabolic conversion to an active VDR ligand (i.e., calcitriol).[8,9] Thereafter, a medicinal chemistry approach was undertaken to design analogues of VD3 that selectively target Hh signaling.

Initial attempts to improve the potency and selectivity with analogues prepared by facile modification of the 3′-hydroxyl functionality of the A-ring proved inadequate.[8] Next, as a part of our ongoing structure–activity relationship (SAR) studies to elucidate the pharmacophore for selective inhibition of Hh signaling by VD3, we probed the structural requirements of the alkyl side chain appended to the CD-ring.[10] Interestingly, truncation of the side chain as in analogue **2** completely abolished inhibition of Hh signaling, suggesting the aliphatic moiety was essential for Hh inhibition. A parallel series of SAR studies identified analogue **3** as a potent (IC_{50} = 0.74 μM) and selective inhibitor of Hh signaling.[9,11] The structurally related steroidal natural product cyclopamine (Cyc, **4**) is also a well-known inhibitor of pathway signaling.

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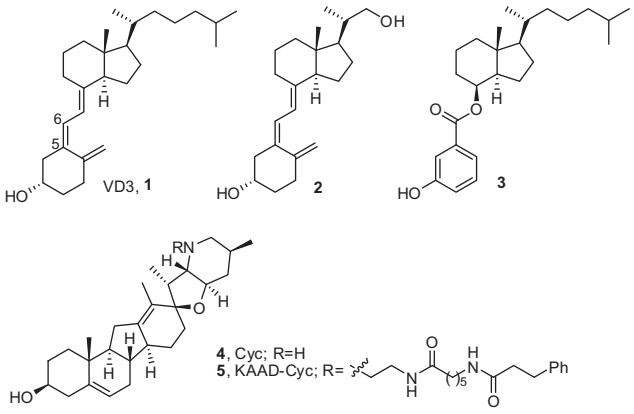


Figure 1. Vitamin D3 and cyclopamine analogues.

Attempts to optimize the potency and drug-like properties of Cyc led to the identification of KAAD-Cyc, which incorporates an extended 'phenyl amino' side chain moiety onto the piperidine ring as an improved inhibitor of pathway signaling (approximate IC_{50} values = 300 and 30 nM, respectively).[12,13] Using each of these compounds as initial leads, we report herein the design, synthesis and evaluation of a series of VD3-based analogues that incorporate modified side chains as potent Hh pathway inhibitors.

2. Results and discussion

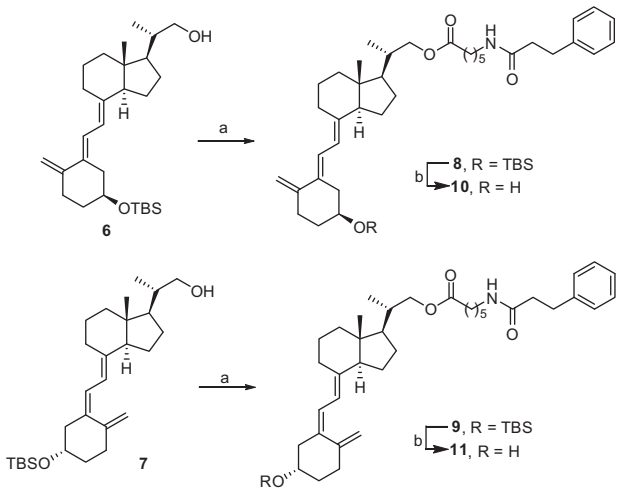
2.1. Chemistry

2.1.1. Synthesis of KAAD-vitamin D3 side chain analogues

Initial analogues prepared to probe the side chain region for optimal Hh pathway inhibition focused on incorporation of the KAAD side chain into the VD3 scaffold. The steroidal nature of Cyc, along with our structural overlays of VD3 and Cyc (Fig. 2, Spartan) and the previous reports that VD3 and Cyc shared a binding site on Smo,[6] suggested that the KAAD and alkyl side chains could potentially bind in a similar location and orientation within Smo. To access the KAAD derivatives of VD3, we coupled the KAAD side chain[14] directly to the primary alcohol of either **6** or **7**[10] via standard esterification conditions to afford both the *trans*- and *cis*-KAAD-VD3 hybrids **10** and **11** (5,6-olefin) (Scheme 1).

2.1.2. Synthesis of hybrid side chain analogues: Route 1

As noted, a concurrent SAR study identified analogue **3** as an improved Hh pathway inhibitor.[9] In addition to its increased potency and selectivity, the synthetic route to access **3** and related analogues is significantly reduced compared to compounds generated from intermediate **7** that incorporate the intact VD3 triene and cyclohexyl A-ring. For these reasons subsequent side chain



Scheme 1. Synthesis of KAAD-VD3 hybrid analogues. Reagents and conditions: (a) KAAD carboxylic acid,[14] DCC, DMAP, anhydrous DCM, 80–89%; (b) TBAF, THF, 45–60%.

analogues described below maintained the ester linkage and aromatic A-ring substituent of **3**.

Complete ozonolysis of vitamin D2 (VD2) and subsequent reduction of the resultant ozonide via a one-pot procedure provided the previously characterized Inhoffen–Lythgoe diol (Scheme 2).[15] Selective tosylation of the primary hydroxyl moiety proceeded in excellent yield to afford the key CD-ring intermediate **12**.[16] For the synthesis of the A-ring aromatic mimic, the phenol of methyl 3-hydroxybenzoate was protected as the methoxymethyl ether, followed by hydrolysis of the methyl ester to provide **14** in excellent yield.[17]

With the necessary precursors in hand, standard esterification protocols afforded the intact ester **15** that we envisioned as a key intermediate that could be directly derivatized through nucleophilic displacement of the tosyl moiety with alkyl and aryl Grignard reagents (Scheme 3).[18] Initial attempts to generate side chain analogues through this method proved difficult. Overall yields of the reaction were poor (<10%) and not reproducible even following extensive modifications to the solvent system, reaction time, temperature, and copper catalyst. Even with these difficulties, a small series of analogues were generated through this method (**19**–**21**, Scheme 3). In addition, the tosyl group of **15** was removed to provide 'des-side chain' analogue **22**.[19]

2.1.3. Synthesis of hybrid side chain analogues: Route 2

Based on the difficulties associated with displacement of the tosyl moiety of **15**, we sought to explore whether improvements in both yield and the number of Grignard reagents amenable to

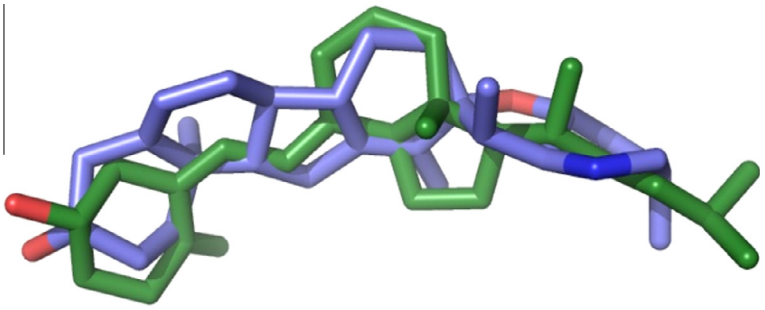
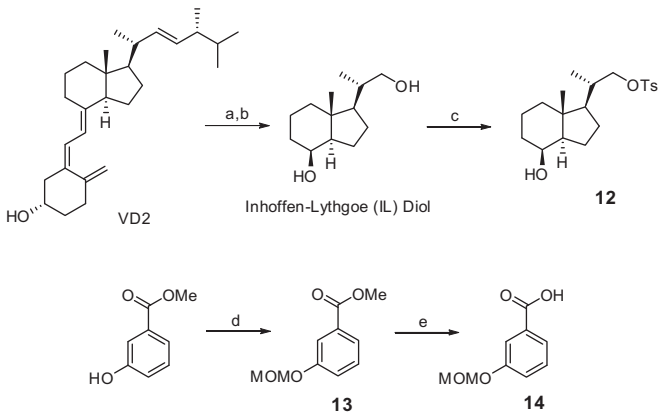
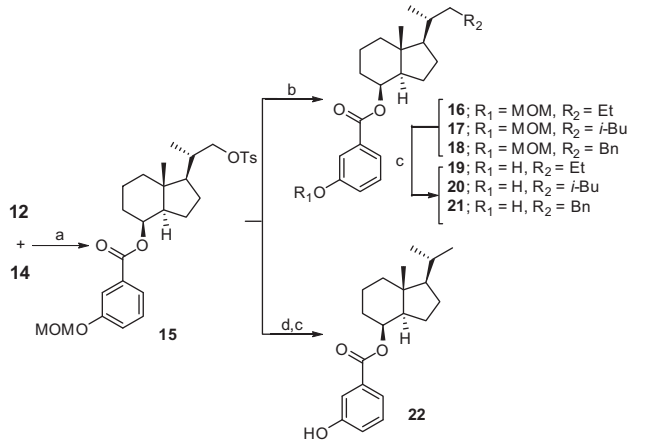


Figure 2. Structural overlay of Cyc (blue) and VD3 (green).

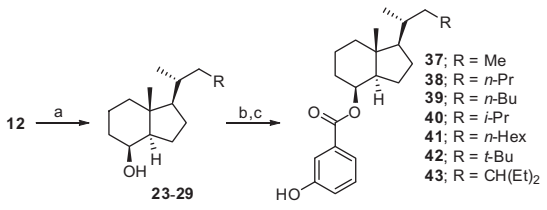


Scheme 2. Synthesis of CD- and A-ring precursors. Reagents and conditions: (a) O_3, anhydrous DCM/MeOH (3:1), (b) $NaBH_4$, 40%; (c) TsCl, DMAP, Et_3N, anhydrous DCM, 85%; (d) MOMCl, DIPEA, anhydrous DCM, 98%; (e) 10% NaOH, MeOH, 76%.



Scheme 3. Initial route to hybrid side chain analogues. Reagents and conditions: (a) DCC, DMAP, anhydrous DCM, 40%; (b) CuI, RMgX, anhydrous THF, 5–10%; (c) TFA, DCM, 80–89%; (d) $NaBH_4$, DMSO, 80 °C, 56%.

coupling could be achieved by carrying out this transformation prior to formation of the ester bond.[20] Our hypothesis was that the steric bulk associated with the ester/aromatic A-ring moiety hinders formation of the ligand–Cu complex, leading to poor reaction progress and low yield. By contrast, the Cu–alkyl complex should more readily displace the tosyl group in the presence of the unsubstituted secondary alcohol of **12**. While using this route increased the number of synthetic steps required to generate each analogue, it resulted in reaction reproducibility and improved yields. Following displacement of the tosyl moiety of **12** with a series of alkyl Grignards, coupling to the aromatic A-ring mimic provided intermediates **30**–**36**. Removal of the methoxymethyl ether groups from **30**–**36** provided corresponding hybrid VD3 analogues **37**–**43** in modest yields (Scheme 4).



Scheme 4. Second route to hybrid side chain analogues. Reagents and conditions: (a) CuI, RMgX, anhydrous THF 40–60%; (b) DCC, DMAP, **14**, anhydrous DCM 60–70%; (c) TFA, DCM, 80–90%.

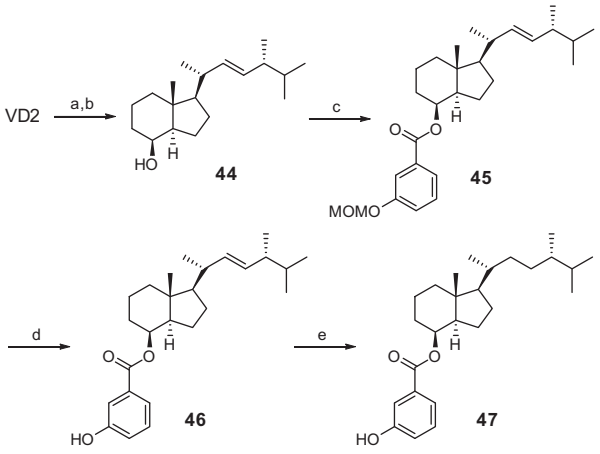
2.1.4. Additional hybrid analogues

The preparation of additional hybrid side chain VD3 analogues proceeded via the selective oxidative cleavage of vitamin D2 (VD2) to afford the unsaturated CD-ring/side chain secondary alcohol **44** (Scheme 5).[21] Coupling of the alcohol with carboxylic acid **14** was followed by removal of the methoxymethyl ether to provide unsaturated side chain analogue **46**. Subsequent reduction of the olefin generated the saturated side chain analogue **47**. Finally, coupling of the TMS-protected Inhoffen–Lythgoe diol **48**[22] to the aromatic A-ring mimic provided analogue **50**, which incorporated a primary hydroxyl in the side chain region (Scheme 6).

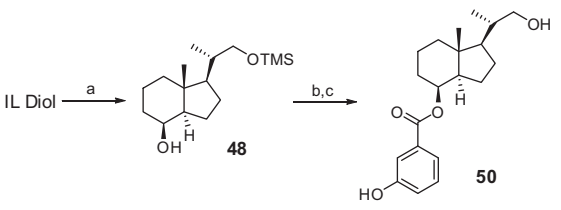
2.2. Biological evaluation

The initial evaluation of all VD3 side chain analogues as Hh pathway inhibitors was performed at a single dose (5 μM) by monitoring endogenous Gli1 mRNA levels in C3H10T1/2 cells following standard protocols.[8–10] At this concentration, neither of the KAAD–VD3 analogues (**10** and **11**) was active against Hh signaling. This data, coupled with our recent findings that VD3 does not displace BODIPY-Cyc from Smo, suggests that these two structures do not share a similar binding orientation and SAR cannot be applied across the two scaffolds.[11] At 5 μM, the majority of hybrid analogues down-regulated Gli1 mRNA by approximately 90%, a level comparable to that of lead **3**. The subsequent evaluation of each analogue at 2.5 μM provided more definitive SAR for this series of analogues (Table 1).

Analogues with linear side chains that were reduced (**19**, **22**, **37**, **38**) or extended (**41**) in length compared to the natural VD3 chain demonstrated reduced activity compared to lead analogue **3**. Not surprisingly, **39**, which retains the 6-carbon chain length, demonstrated activity comparable to **3**. Several interesting effects on the Hh inhibition were observed following introduction of



Scheme 5. Synthesis of VD2 hybrid side chain analogues. Reagents and conditions: (a) $KMnO_4$, THF, H_2O, 65%; (b) $Pb(OAc)_4$, DCM, Vitride™, DCM, 89% (c) DCC, DMAP, anhydrous DCM, **14**, 70%; (d) TFA, DCM, 65%; (e) $H_2/Pd(OH)_2$, MeOH; THF, 75%.



Scheme 6. Synthesis of hydroxyl hybrid side chain. Reagents and conditions: (a) TMSCl, TEA, DMAP, anhydrous DCM, 48%; (b) DCC, DMAP, anhydrous DCM, **14**, 70%; (c) TFA, DCM, 65%.

Table 1
Selective in vitro inhibition of Hh signaling

Compd	Gli1 mRNA[a] (%)		Cyp24A1[b]	VDR[c] (μM)
	5 μM	2.5 μM		
OHC	100		1.0	ND
VD3	31.7 ± 0.7	71.4 ± 2.1	8336 ± 38	>100
2	80.0 ± 10.4	ND	1354 ± 61	>100
3	1.9 ± 0.5	15.0 ± 2.0	10.5 ± 2.1	>100
10	126 ± 6.8	ND	0.7 ± 0.4	>100
11	78.6 ± 1.4	ND	22.7 ± 7.7	>100
19	7.7 ± 5.5	69.8 ± 27.2	4.8 ± 0.9	>100
20	2.9 ± 0.3	23.0 ± 4.3	2.8 ± 0.03	>100
21	7.2 ± 1.8	88.4 ± 55.4	3.8 ± 0.01	>100
22	1.3 ± 0.1	49.8 ± 1.8	0.7 ± 0.2	>100
37	0.7 ± 0.3	33.9 ± 13.1	1.1 ± 0.3	>100
38	38.4 ± 4.2	52.3 ± 13.2	4.9 ± 0.5	>100
39	1.1 ± 0.3	17.3 ± 3.6	4.4 ± 0.03	>100
40	74.4 ± 0.7	90.1 ± 12.5	2.5 ± 1.0	>100
41	29.9 ± 16.1	54.4 ± 9.7	3.8 ± 0.7	>100
42	32.4 ± 28.9	49.1 ± 19.6	7.9 ± 1.4	>100
43	0.5 ± 0.2	17.4 ± 4.5	0.6 ± 0.02	>100
46	2.2 ± 0.5	15.6 ± 0.9	1.1 ± 0.02	>100
47	9.2 ± 6.1	40.4 ± 9.5	6.8 ± 0.02	>100
50	10.2 ± 6.1	56.9 ± 10.2	0.4 ± 0.1	>100

[a] Relative Gli1mRNA levels compared to oxysterol (OHC) control.
[b] Fold up-regulation relative to OHC treated controls.
[c] Calcitriol (IC_{50} = 290 nM) is utilized as a positive control for VDR binding.

branched alkyl chains in this region. Analogues with short, branched chains (**40** and **42**) were less potent; however, medium length branched chains (**20** and **43**) salvaged the loss in potency seen for the corresponding unbranched analogues with similar side chain length. Analogue **47** combined optimal length and non-terminal branching in the same structure; however, instead of a synergistic increase in activity for this compound, overall activity was slightly reduced. Interestingly, slight modification through the addition of an unsaturated olefin (**46**) restored the diminished activity seen for the saturated analogue, resulting in a 2-fold increase in activity for the more rigid side chain. Finally, incorporating an aromatic benzyl side chain (**21**) or a primary hydroxyl (**50**) resulted in modest decreases in activity when compared to the most active analogues.

After establishing the Hh inhibitory effects of modified side chains, the selectivity profile of all the analogues was assessed by measuring their ability to (1) bind recombinant VDR and (2) up-regulate Cyp24A1 mRNA in C3H10T1/2 cells.[9] Unlike calcitriol (IC_{50} 290 nM), none of these analogues or VD3, directly bind to VDR even at high concentrations ($\geqslant$100 μM). As demonstrated previously, C3H10T1/2 cells respond to VDR activation with robust up-regulation of Cyp24A1, a well-characterized target gene of canonical vitamin D signaling.[8] Overall, each of the hybrid side chain analogues maintained the high level of selectivity for Hh inhibition previously demonstrated by analogue **3**. Based on the results, it appears that the changes to the A-ring region of the scaffold are sufficient to mitigate any VDR-mediated effects associated with modification to the side chain region.

Previous studies evaluating Hh pathway inhibition in cultured cancer cells have suggested that the majority of in vitro cancer cell lines fail to appropriately model in vivo Hh signaling.[8] Therefore, we pursued the evaluation of VD3 analogues in a cell line, ASZ001, which has shown initial promise as in vitro model of oncogenic Hh signaling. The ASZ001 cell line was developed from a visible BCC tumor isolated from a *Ptch1*$^{+/-}$ mouse.[23] These cells demonstrate loss of the wildtype Ptch1 allele, high baseline expression of Gli1, and cellular morphology similar to Hh-dependent BCC tumors. In addition, treatment of these cells with either Cyc or VD3 resulting in Gli1 down-regulation has been previously reported.[9,24]

It has been demonstrated previously that while the IC_{50} for VD3 inhibition of Hh signaling correlated well between the C3H10T1/2 and ASZ001 cell lines, lead analogue **3** was less active in the latter.[9,11] Consistent with that observation, correlation of anti-Hh activity for the hybrid side chain analogues between the two cell lines was minimal (Table 1, Fig. 3). Among the potent, selective analogues identified in the C3H10T1/2 cell line, only analogue **20** retained its anti-Hh activity in the BCC cell line. Several analogues (**19**, **21**, **38**, **41**, **50**) that demonstrated modest activity in the C3H10T1/2 cells were active in the BCC cell line, while the inhibitory activity of others (**37**, **40**, **46**, **47**) was completely abolished. It is noteworthy, among the compounds showing improved activity in this cell line, **38** and **41** demonstrated detrimental up-regulation of Cyp24A1. In addition, compounds **21** and **50** demonstrated significant anti-proliferative effects at concentrations of 5 μM. These results indicate that additional non-specific mechanisms of activity/toxicity exist for these scaffolds in the ASZ001 cells and are being investigated further.

Based on our preliminary results at a single concentration, three compounds (**20**, **39**, **43**) that demonstrated selective and enhanced anti-Hh activity consistently were evaluated for their ability to inhibit Hh signaling in a concentration-dependent fashion. Each of these demonstrated low micromolar IC_{50} values for inducing Gli1 down-regulation in both C3H10T1/2 and ASZ cells (Table 2), suggesting modest concentration-dependent inhibition of Hh signaling in these model systems.

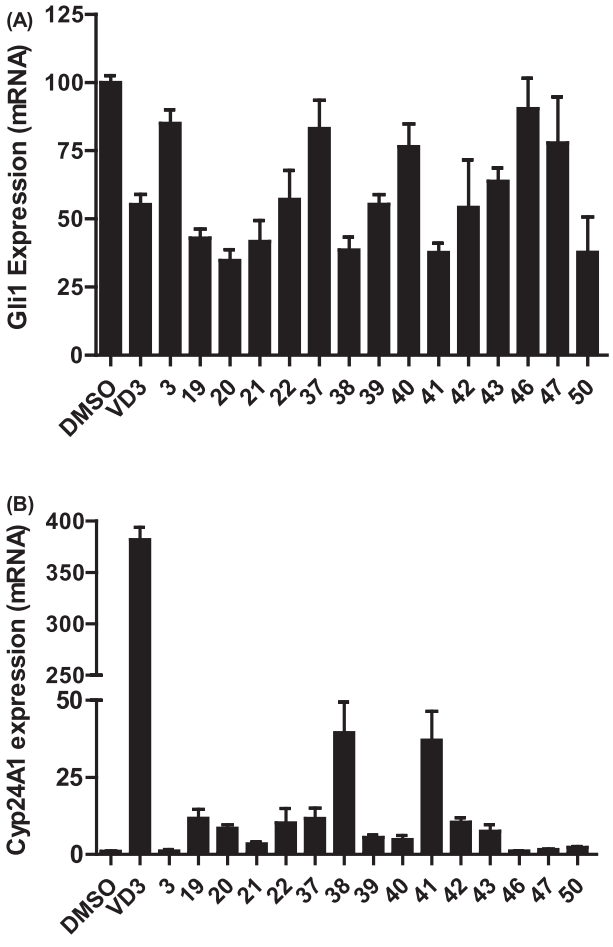


Figure 3. Selective inhibition of Hh signaling in ASZ001 cells. ASZ001 cells were incubated with analogue (2.5 μM, 48 h) or control and down-regulation of endogenous Gli1mRNA (A) or up-regulation of Cyp24A1 (B) was determined relative to DMSO control.

Table 2
IC_{50} values for inhibition of Hh signaling

Compd	IC_{50} value[a] (μM)	
	C3H10T1/2	ASZ001
VD3	4.1 ± 0.3	2.1 ± 0.1
3	0.74 ± 0.1	5.2 ± 0.2
20	1.1 ± 0.6	1.6 ± 0.2
39	1.1 ± 0.4	6.6 ± 2.8
43	1.1 ± 0.5	4.9 ± 2.9

[a] IC_{50} values represent down-regulation of Gli1 mRNA. All values are mean ± SEM for at least two separate experiments performed in triplicate.

3. Conclusion

With the goal of identifying improved Hh pathway inhibitors based on the VD3 scaffold, we have synthesized and evaluated a series of analogues that incorporate modifications to the natural alkyl side chain. During the initial phase of this study, a VD3-based analogue incorporating an aromatic A-ring analogue (**3**) was identified in a parallel SAR study. Subsequently, side chain region analogues focused on the preparation and evaluation of compounds that incorporated this modified and improved VD3 scaffold. Overall, linear or moderately branched alkyl chains of five or six carbons were optimal for potent and selective inhibition of Hh signaling. The most potent analogue, **20**, demonstrated inhibition of Hh signaling in MEFs and BCC cells, comparable to lead compound **3**. The continued exploration of VD3 analogues as improved Hh pathway inhibitors is ongoing.

4. Experimental section

4.1. General

VD2 and VD3 used for chemical synthesis were purchased from HBC Chem, Inc. Methyl ester phenol and other chemicals were purchased from either Sigma–Aldrich or Fisher Scientific. ACS or HPLC grade methanol, acetone, and tetrahydrofuran were purchased from Fisher Scientific. Anhydrous DCM (low water, <50 ppm water) was purchased from BrandNu Laboratories, Inc. (J.T. Baker solvent). Modeling and structural overlays were performed in Spartan '10 Software. NMR data was performed on a Bruker AVANCE 500 MHz spectrometer and analysis was done on MestReNova version 8.0.0. HRMS data was collected on AccuTOF mass spectrometer (JEOL USA, Peabody, MA) with a DART ionization source (IonSense, Saugus, MA) and analyzed at the Mass Spectrometry Facility at the University of Connecticut. Infrared (IR) analysis was performed on a Shimadzu FTIR-8400S spectrophotometer with IR Solution software.

4.2. Synthesis

4.2.1. KAAD–VD3 analogues

4.2.1.1. (*S*)-2-((1*R*,3a*S*,7a*R*,*E*)-4-((*E*)-2-((*S*)-5-Hydroxy-2-methylenecyclohexylidene)ethylidene)-7a-methyloctahydro-1*H*-inden-1-yl)propyl 2-(3-phenylpropanamido)acetate (10). To a solution of **8** (12.6 mg, 0.018 mmol) in anhydrous THF (1 mL) was added tetrabutylammonium fluoride (40 μL, 0.037 mmol). The mixture was stirred at rt for 12 h and subsequently stirred at 60 °C for 4 h. The mixture was diluted with EtOAc (10 mL), washed with H_2O (10 mL), and concentrated. Column chromatography (SiO_2, 5–65% EtOAc in Hex) afforded off-white solid product **10** in modest yield (60%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.31 (m, 2H), 7.22 (m, 3H), 6.56 (m, 1H), 5.90 (m, 1H), 5.39 (br s, 1H), 5.00 (m, 1H), 4.71 (m, 1H), 3.91 (m, 1H), 3.83 (m, 1H), 3.51 (m, 2H), 3.23 (m, 2H), 2.99 (m, 2H), 2.89 (m, 1H), 2.48 (m, 3H), 2.31 (m, 3H), 2.15 (m, 1H), 1.89 (m, 3H), 1.67 (m, 12H), 1.35 (m, 10H), 1.04 (d, J = 6.4 Hz, 3H), 0.59 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.8, 156.7, 149.1, 144.1, 140.9, 135.1, 126.2, 120.8, 116.2, 108.2, 69.3, 68.9, 56.2, 53.2, 49.1, 45.9, 40.3, 39.2, 38.6, 37.1, 36.1, 34.7, 34.1, 33.9, 31.8, 31.1, 29.7, 29.1, 28.9, 27.1, 26.3, 25.6, 24.9, 24.4, 23.4, 22.3, 17.3, 12.1. IR(film) ν_{max} 3325, 2905, 2850, 2352, 2330, 2250, 1729, 1650, 1629, 1555, 1538, 1455, 1446, 1428, 1417, 1088, 1045, 1028, 755, 702, 641. microTOF-HRMS: (a) m/z calculated for $C_{37}H_{54}NO_4$: 576.4053 $[MH]^+$. Found: 576.4035.

4.2.1.2. (*S*)-2-((1*R*,3a*S*,7a*R*,*E*)-4-((*Z*)-2-((*S*)-5-Hydroxy-2-methylenecyclohexylidene)ethylidene)-7a-methyloctahydro-1*H*-inden-1-yl)propyl 2-(3-phenylpropanamido)acetate (11). Analogue **11** was prepared in the same fashion as described above for analogue **10** (45% yield, off-white solid). ^{1}H NMR (500 MHz, $CDCl_3$/MeOD 19:1) δ 7.18 (m, 2H), 7.10 (m, 3H), 6.60 (br s, 1H), 6.13 (m, 1H), 5.94 (m, 1H), 4.95 (m, 1H), 4.72 (m, 1H), 4.00 (m, 2H), 3.72 (m, 2H), 3.37 (m, 1H), 3.06 (m, 2H), 2.83 (m, 2H), 2.74 (m, 1H), 2.55 (m, 1H), 2.51 (m, 1H), 2.48 (m, 1H), 2.37 (m, 3H), 2.29 (m, 1H), 2.21 (m, 3H), 2.04 (m, 1H), 1.80 (m, 8H), 1.45 (m, 15H), 0.93 (d, J = 6.4 Hz, 3H), 0.47 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$/MeOD 19:1) δ 174.1, 172.9, 157.6, 144.8, 141.0, 140.5, 138.4, 135.6, 128.1, 126.2, 125.8, 125.1, 121.5, 117.6, 112.1, 69.2, 68.9, 55.6, 55.1, 52.8, 45.5, 44.8, 40.1, 38.9, 37.9, 35.9, 33.8, 33.4, 28.6, 26.8, 25.9, 25.3, 24.6, 24.2, 23.1, 18.1, 16.9, 11.7. IR(film) ν_{max} 3319, 3028, 2935, 2925, 2853, 2359, 2336, 2243, 1730, 1643, 1642, 1559, 1534, 1471, 1461, 1436, 1375, 1254, 1163, 1089, 909, 836, 774, 733, 698. DART-HRMS: (a) m/z calculated for $C_{37}H_{54}NO_4$: 576.4053 $[MH]^+$. Found: 576.3989; (b) m/z calculated for $C_{37}H_{51}O_3N$: 558.3947 $[M-H_2O]^+$. Found: 558.3906.

4.2.2. Hybrid analogues: Route 1

4.2.2.1. (1*R*,4*S*,7a*R*)-7a-Methyl-1-((*S*)-1-(tosyloxy)propan-2-yl) octahydro-1*H*-inden-4-yl 3-(methoxymethoxy)benzoate (15). Dicyclohexylcarbodiimide (0.34 g, 1.65 mmol), dimethylaminopyridine (0.2 g, 1.64 mmol) and **12** (0.2 g, 0.55 mmol) were dissolved in anhydrous CH_2Cl_2 and cooled to 0 °C. Carboxylic acid **14** (0.3 g, 1.65 mmol) in CH_2Cl_2 was added and the reaction mixture warmed to rt and stirred for 12 h. The reaction mixture was diluted with CH_2Cl_2 (30 mL), washed with 0.1 N HCl (30 mL) and saturated NaCl (30 mL), dried over sodium sulfate and concentrated. Column chromatography (SiO_2, 5–25% EtOAc in Hex) afforded **15** as a clear oil in modest yield (40%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.82 (s, 1H), 7.80 (s, 1H), 7.73 (dd, J = 2.6, 1.5 Hz, 1H), 7.70 (dt, J = 7.7, 1.3 Hz, 1H), 7.40–7.35 (m, 3H), 7.25 (m, 1H), 5.22 (d, J = 0.7 Hz, 2H), 3.99 (dd, J = 9.3, 3.0 Hz, 1H), 3.84 (dd, J = 9.3, 6.2 Hz, 2H), 3.50 (s, 3H), 2.48 (s, 3H), 2.02 (dt, J = 12.5, 3.2 Hz, 2H), 1.85–1.63 (m, 4H), 1.60–1.41 (m, 3H), 1.31–1.11 (m, 4H), 1.04 (s, 3H), 0.99 (d, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.1, 157.2, 144.6, 132.2, 129.8, 129.4, 127.9, 123.0, 120.9, 117.2, 94.5, 75.4, 72.0, 56.1, 52.1, 51.3, 42.0, 39.6, 35.9, 30.4, 26.3, 22.6, 21.7, 17.9, 16.8, 13.5. DART-HRMS: m/z calculated for $C_{29}H_{38}O_7SNH_4$: 548.2682 $[M^+NH_4]$. Found: 548.2686.

4.2.2.2.1. General procedure for tosyl group displacement. Commercially purchased Grignard reagent (1.5 mmol) was added to a solution of CuI (0.5 mmol) in anhydrous THF (2 mL) under argon and cooled to 0 °C. To this mixture was added ester **15** (0.3 mmol) in anhydrous THF and the solution stirred for 1 h at 0 °C and quenched by the addition of H_2O (10 mL). The solution was washed with EtOAc (3 × 10 mL), 0.1 N HCl (3 × 10 mL), dried over sodium sulfate, and concentrated. Column chromatography (SiO_2, 0–20% EtOAc in Hex) afforded coupled products as oils in poor yields (5–10%).

4.2.2.2.2. General procedure for methoxymethyl removal. To a solution of coupled ester (0.1 mmol) in CH_2Cl_2, was added trifluroacetic acid (0.5 mL). The reaction mixture was stirred at rt and pro-

gress monitored by thin layer chromatography. Upon completion, the reaction was neutralized with sodium carbonate (5 mL) and washed with CH_2Cl_2 (2×10 mL). The combined organic layers were dried over sodium sulfate and concentrated. Column chromatography (SiO_2, 0–15% EtOAc in Hex) provided final analogues as clear oils.

4.2.2.2. (1*R*,4*S*,7a*R*)-7a-Methyl-1-((*R*)-pentan-2-yl)octahydro-1*H*-inden-4-yl 3-hydroxybenzoate (19). Clear oil, yield 85%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.65 (m, 1H), 7.59 (d, J = 6.9 Hz, 1H), 7.34 (m, 1H), 7.11–7.05 (m, 1H), 5.51 (s, 1H), 5.45–5.40 (m, 1H), 2.13–2.06 (m, 1H), 1.92–1.77 (m, 2H), 2.01 (m, 1H), 1.69–1.50 (m, 5H), 1.50–1.34 (m, 3H), 1.29 (t, J = 2.3 Hz, 3H), 1.21–1.11 (m, 1H), 1.06 (d, J = 2.7 Hz, 4H), 0.95 (m, 3H), 0.93–0.87 (m, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.6, 155.9, 132.5, 129.8, 122.1, 120.1, 116.5, 72.8, 56.7, 51.8, 42.1, 40.1, 38.3, 35.4, 30.7, 29.9, 27.3, 22.9, 19.4, 18.8, 18.2, 13.8. IR (film) ν_{max} 3400 (br s), 2976, 2947, 2867, 2866, 1684, 1600, 1579, 1296, 1215, 1145, 1110, 946, 756, 680, 659. DART-HRMS: m/z calculated for $C_{22}H_{32}O_3NH_4$: 362.2695 $[M+NH_4]^+$. Found: 362.2714.

4.2.2.3. (1*R*,4*S*,7a*R*)-7a-Methyl-1-((*R*)-5-methylhexan-2-yl)octahydro-1*H*-inden-4-yl 3-hydroxybenzoate (20). Clear oil, yield 89%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.65 (d, J = 7.5 Hz, 1H), 7.59 (d, J = 6.8 Hz, 1H), 7.35 (t, J = 8.0 Hz, 1H), 7.13–7.03 (m, 1H), 5.42 (m, 1H), 2.08 (m, 1H), 2.05–1.97 (m, 1H), 1.93–1.71 (m, 2H), 1.56 (m, 4H), 1.49–1.30 (m, 6H), 1.28–1.09 (m, 4H), 1.05 (s, 3H), 0.96 (d, J = 13.2, 6.2 Hz, 3H), 0.91 (dd, J = 6.6, 1.9 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.3, 155.7, 132.4, 129.7, 122.0, 119.9, 116.3, 72.6, 58.5, 51.6, 41.9, 39.8, 35.5, 31.9, 30.9, 30.7, 30.5, 29.7, 27.3, 23.0, 22.7, 22.4, 18.0, 13.7. IR (film) ν_{max} 3385 (br s), 3019, 2951, 2935, 2868, 1689, 1599, 1589, 1466, 1453, 1366, 1344, 1295, 1215, 1157, 1106, 1075, 1062, 982, 946, 886, 755, 680, 668. DART-HRMS: m/z calculated for $C_{24}H_{36}O_3NH_4$: 391.2848 $[M+NH_4]^+$. Found: 391.2869.

4.2.2.4. (1*R*,4*S*,7a*R*)-7a-Methyl-1-((*R*)-4-phenylbutan-2-yl)octahydro-1*H*-inden-4-yl 3-hydroxybenzoate (21). Clear oil, yield 80%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.64 (d, J = 7.6 Hz, 1H), 7.60–7.57 (m, 1H), 7.33 (dd, J = 21.9, 7.9 Hz, 3H), 7.22–7.17 (m, 3H), 7.08 (dd, J = 8.1, 2.6 Hz, 1H), 5.57 (s, 1H), 5.42 (q, J = 2.7 Hz, 1H), 2.73 (m, 1H), 2.50 (m, 1H), 2.11 (m, 1H), 2.02 (m, 1H), 1.96–1.71 (m, 4H), 1.67 (q, J = 4.1 Hz, 1H), 1.64–1.45 (m, 4H), 1.44–1.33 (m, 1H), 1.29 (s, 2H), 1.24 (d, J = 7.3 Hz, 1H), 1.07 (d, J = 6.6 Hz, 3H), 1.05 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.4, 155.8, 143.3, 132.3, 129.7, 128.4, 128.3, 125.5, 121.9, 120.0, 116.3, 72.6, 56.2, 51.6, 42.0, 40.0, 37.9, 35.3, 32.5, 30.5, 27.0, 22.7, 18.6, 18.0, 13.6. IR (film) ν_{max} 3170, 2949, 2917, 2849, 1692, 1496, 1484, 1453, 1250, 1224, 756. DART-HRMS: m/z calculated for $C_{27}H_{33}O_3$: 405.2430 $[M-H]^+$. Found: 405.2437; calculated for $C_{27}H_{34}O_3NH_4$: 424.2852, $[M+NH4]^+$. Found: 424.2869.

4.2.2.5. (1*R*,4*S*,7a*R*)-1-Isopropyl-7a-methyloctahydro-1*H*-inden-4-yl 3-hydroxybenzoate (22). Clear oil, yield 84%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.65 (m, 1H), 7.58 (m, 1H), 7.35 (m, 1H), 7.07 (m, 1H), 5.42 (m, 1H), 5.36 (s, 1H), 2.13–2.04 (m, 2H), 2.01 (dd, J = 14.5, 3.8 Hz, 1H), 1.84 (m, 3H), 1.56 (m, 7H), 1.50–1.38 (m, 1H), 1.36–1.30 (m, 1H), 1.26–1.18 (m, 1H), 1.06 (d, J = 2.2 Hz, 4H), 0.98 (dd, J = 6.5, 2.2 Hz, 3H), 0.88 (dd, J = 6.6, 2.2 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.5, 155.8, 132.4, 129.7, 122.0, 119.9, 116.3, 72.6, 58.5, 51.6, 41.9, 39.8, 30.7, 30.5, 29.7, 27.3, 23.0, 22.7, 22.4, 18.0, 13.7. IR (film) ν_{max} 3430 (br s), 2956, 2939, 2868, 2858, 1700, 1684, 1601, 1589, 1294, 1217, 1160, 1110, 946, 756, 680, 669. DART-HRMS: m/z calculated for $C_{20}H_{28}O_3NH_4$: 334.2382 $[M+NH_4]^+$. Found: 334.2373.

4.2.3. Hybrid analogues: Route 2

Copper-mediated displacement of the tosyl moiety in **12**, esterification with the aromatic A-ring carboxylic acid, and removal of the methoxymethyl protecting group was as described above for Route 1.

4.2.3.1. (1*R*,4*S*,7a*R*)-1-((*R*)-*sec*-Butyl)-7a-methyloctahydro-1*H*-inden-4-yl 3-hydroxybenzoate (37). Clear oil, yield 87%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.65 (m, 1H), 7.61 (dd, J = 2.6, 1.5 Hz, 1H), 7.38–7.31 (m, 1H), 7.08 (m, 1H), 5.57 (s, 1H), 5.43 (q, J = 2.8 Hz, 1H), 2.12–2.06 (m, 1H), 2.05–1.98 (m, 1H), 1.91–1.77 (m, 2H), 1.64–1.43 (m, 6H), 1.41–1.30 (m, 2H), 1.25 (m, 1H), 1.21–1.07 (m, 2H), 1.06 (s, 3H), 0.95 (d, J = 6.4 Hz, 3H), 0.89–0.83 (m, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.7, 156.0, 132.5, 129.9, 122.1, 120.2, 116.6, 72.9, 56.1, 51.8, 42.1, 40.1, 36.8, 30.7, 29.9, 28.3, 27.1, 22.2, 18.2, 13.8, 10.5. IR (film) ν_{max} 3329 (br s), 3146, 3017, 2950, 2931, 2867, 1671, 1613, 1584, 1485, 1466, 1250, 1208, 1137, 1088, 1061, 1032, 937, 917, 755, 702, 666. DART-HRMS: m/z calculated for $C_{21}H_{30}O_3NH_4$: 348.2539 $[M+NH_4]^+$. Found: 348.2564.

4.2.3.2. (1*R*,4*S*,7a*R*)-1-((*R*)-Hexan-2-yl)-7a-methyloctahydro-1*H*-inden-4-yl 3-hydroxybenzoate (38). Clear oil, yield 85%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.65 (d, J = 7.6 Hz, 1H), 7.58 (d, J = 2.6 Hz, 1H), 7.39–7.31 (m, 1H), 7.12–7.04 (m, 1H), 5.43 (s, 1H), 5.41–5.31 (m, 1H), 2.14–2.05 (m, 1H), 2.05–1.96 (m, 1H), 1.92–1.75 (m, 2H), 1.74–1.51 (m, 5H), 1.51–1.30 (m, 4H), 1.29–1.08 (m, 9H), 1.06 (s, 3H), 1.02–0.96 (m, 3H), 0.89 (t, J = 3.9 Hz, 4H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.4, 155.9, 132.5, 129.9, 122.2, 120.1, 116.5, 72.8, 58.7, 51.8, 42.1, 40.0, 30.9, 30.7, 29.9, 27.5, 23.2, 22.9, 22.6, 22.2, 18.2, 14.2, 13.9. IR (film) ν_{max} 3305 (br s), 3234, 2951, 2931, 2867, 2775, 1610, 1584, 1485, 1466, 1364, 1346, 1250, 1208, 1137, 1086, 1062, 1032, 937, 917, 755, 715, 656. DART-HRMS: m/z calculated for $C_{23}H_{34}O_3NH_4$: 376. 2887 $[M+NH_4]^+$. Found: 376.2891.

4.2.3.3. (1*R*,4*S*,7a*R*)-1-((*R*)-Heptan-2-yl)-7a-methyloctahydro-1*H*-inden-4-yl 3-hydroxybenzoate (39). Clear oil, yield 82%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.65 (dd, J = 7.5, 1.6 Hz, 2H), 7.35 (t, J = 8.0 Hz, 1H), 7.13–7.03 (m, 1H), 5.79 (s, 1H), 5.42 (dd, J = 4.1, 2.2 Hz, 1H), 2.08 (m, 1H), 2.05–1.97 (m, 1H), 1.93–1.71 (m, 2H), 1.56 (m, 4H), 1.49–1.30 (m, 6H), 1.28–1.09 (m, 7H), 1.05 (s, 3H), 0.96 (dd, J = 13.2, 6.2 Hz, 3H), 0.91 (t, J = 7.1 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.7, 156.0, 132.4, 129.9, 122.1, 120.2, 116.6, 72.9, 56.6, 51.8, 42.1, 40.1, 35.8, 35.6, 32.5, 30.7, 27.2, 25.9, 22.9, 22.9, 18.8, 18.2, 14.3, 13.8. IR (film) ν_{max} 3375 (br s), 3138, 3011, 2951, 2931, 2867, 1613, 1584, 1485, 1466, 1364, 1346, 1250, 1208, 1137, 1086, 1062, 1032, 937, 917, 755, 704, 657. DART-HRMS: m/z calculated for $C_{24}H_{36}O_3NH_4$: 390.3008 $[M+NH_4]^+$. Found: 390.3045.

4.2.3.4. (1*R*,4*S*,7a*R*)-7a-Methyl-1-((*R*)-4-methylpentan-2-yl) octahydro-1*H*-inden-4-yl 3-hydroxybenzoate (40). Clear oil, yield 88%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.65 (d, J = 7.5 Hz, 1H), 7.59 (d, J = 6.8 Hz, 1H), 7.35 (q, J = 7.9, 7.3 Hz, 1H), 7.07 (d, J = 7.8 Hz, 1H), 5.42 (s, 2H), 2.17–2.08 (m, 1H), 2.02 (d, J = 14.7 Hz, 1H), 1.94–1.77 (m, 2H), 1.55 (m, 9H), 1.22–1.10 (m, 1H), 1.08 (s, 3H), 1.06–0.97 (m, 1H), 0.93 (d, J = 6.7 Hz, 3H), 0.90 (d, J = 7.3 Hz, 4H), 0.87–0.82 (m, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.5, 155.9, 132.5, 129.8, 122.1, 120.1, 116.5, 72.8, 57.6, 51.8, 45.7, 42.2, 40.2, 33.7, 30.7, 29.9, 27.5, 24.9, 22.9, 21.5, 18.8, 18.2, 13.8. IR (film) ν_{max} 3400 (br s), 3215, 2953, 2941, 2867, 2775, 1610, 1584, 1485, 1466, 1364, 1301, 1250, 1208, 1137, 1086, 1062, 1032, 937, 917, 715. DART-HRMS: m/z calculated for $C_{23}H_{34}O_3NH_4$: 376.2853 $[M+NH_4]^+$. Found: 376.2868.

4.2.3.5. (1*R*,4*S*,7a*R*)-7a-Methyl-1-((*R*)-nonan-2-yl)octahydro-1*H*-inden-4-yl 3-hydroxybenzoate (41). Clear oil, yield 85%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.65 (dd, J = 7.8, 1.3 Hz, 1H), 7.59 (dd, J = 2.6, 1.5 Hz, 1H), 7.34 (t, J = 7.9 Hz, 1H), 7.10–7.04 (m, 1H), 5.45 (s, 2H), 5.43 (s, 1H), 2.12–2.05 (m, 2H), 2.05–1.98 (m, 1H), 1.93–1.76 (m, 3H), 1.65 (s, 2H), 1.62–1.41 (m, 9H), 1.40–1.19 (m, 7H), 1.04 (d, J = 10.2 Hz, 3H), 0.99 (t, J = 6.9 Hz, 3H), 0.89 (dd, J = 6.8, 2.0 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.7, 156.1, 132.5, 129.8, 122.1, 120.2, 116.5, 72.9, 58.7, 51.8, 42.1, 40.0, 33.9, 30.9, 30.7, 30.3, 29.9, 29.8, 29.6, 27.5, 22.9, 22.6, 18.8, 18.2, 13.9, 13.8. IR (film) ν_{max} 3456 (br s), 2992, 2950, 2926, 2907, 1712, 1699, 1485, 1466, 1364, 1346, 1250, 1208, 1137, 1086, 1062, 1032, 937, 917, 715, 656. DART-HRMS: m/z calculated for $C_{26}H_{40}O_3NH_4$: 418.3982 $[M+NH_4]^+$. Found: 418.3301.

4.2.3.6. (1*R*,4*S*,7a*R*)-1-((*R*)-4,4-Dimethylpentan-2-yl)-7a-methyloctahydro-1*H*-inden-4-yl 3-hydroxybenzoate (42). Clear oil, yield 90%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.79–7.54 (m, 2H), 7.44–7.31 (m, 1H), 7.18–6.99 (m, 1H), 5.80 (d, J = 12.1 Hz, 1H), 5.42 (s, 1H), 2.17–2.07 (m, 1H), 2.07–1.98 (m, 1H), 1.97–1.67 (m, 3H), 1.66–1.43 (m, 6H), 1.37 (dd, J = 14.4, 7.0 Hz, 2H), 1.25 (d, J = 10.1 Hz, 1H), 1.20–1.11 (m, 1H), 1.09 (d, J = 1.7 Hz, 1H), 1.06–0.99 (m, 1H), 0.99–0.85 (m, 11H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.5, 155.8, 132.3, 129.7, 121.9, 120.0, 116.4, 72.8, 57.8, 51.7, 49.7, 42.0, 40.0, 32.4, 31.1, 30.5, 30.4, 30.4, 29.7, 27.7, 22.7, 22.6, 18.0, 13.4. IR (film) ν_{max} 3430 (br s), 3146, 3017, 2950, 2931, 2867, 1671, 1613, 1584, 1485, 1466, 1374, 1346, 1250, 1208, 1137, 1088, 1061, 1032, 937, 917, 755, 702, 666. DART-HRMS: m/z calculated for $C_{24}H_{36}O_3NH_4$: 390.3008 $[M+NH_4]^+$. Found: 390.3030.

4.2.3.7. (1*R*,4*S*,7a*R*)-1-((*R*)-4-Ethylhexan-2-yl)-7a-methyloctahydro-1*H*-inden-4-yl 3-hydroxybenzoate (43). Clear oil, yield 86%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.65 (m, 1H), 7.58 (dd, J = 2.7, 1.5 Hz, 1H), 7.35 (m, 1H), 7.07 (m, 1H), 5.42 (q, J = 2.9 Hz, 1H), 5.29 (d, J = 4.4 Hz, 1H), 2.16–1.98 (m, 2H), 1.98–1.76 (m, 2H), 1.64–1.29 (m, 8H), 1.28–1.04 (m, 8H), 1.01–0.79 (m, 10H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.5, 155.8, 132.6, 129.84, 122.2, 120.0, 116.5, 72.8, 57.8, 51.8, 42.2, 39.8, 37.4, 33.6, 30.7, 29.9, 27.6, 26.8, 24.6, 22.9, 18.9, 18.2, 13.9, 11.5, 10.2. IR (film) ν_{max} 3324 (br s), 3215, 2953, 2941, 2867, 2775, 1610, 1584, 1485, 1466, 1364, 1341, 1250, 1208, 1137, 1086, 1062, 1032, 937, 917, 715, 656. DART-HRMS: m/z calculated for $C_{25}H_{38}O_3NH_4$: 404.3208 $[M+NH_4]^+$. Found: 404.3230.

4.2.4. Additional side chain analogues

4.2.4.1. (1*R*,3a*R*,4*S*,7a*R*)-1-((2*R*,5*R*,*E*)-5,6-Dimethylhept-3-en-2-yl)-7a-methyloctahydro-1*H*-inden-4-yl 3-hydroxybenzoate (46). Clear oil, yield 65%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.61 (m, 2H), 7.31 (m, 1H), 7.06 (m, 1H), 5.98 (br s, 1H), 5.39 (m, 1H), 5.18 (m, 2H), 2.01 (m, 3H), 1.81 (m, 2H), 1.68 (m, 1H), 1.49 (m, 6H), 1.24 (m, 4H), 1.15 (m, 1H), 1.04 (m, 3H), 1.02 (d, J = 6.5 Hz, 3H), 0.91 (d, J = 6.8 Hz, 3H), 0.73 (m, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.3, 155.6, 135.4, 132.3, 132.0, 129.6, 122.0, 119.8, 116.2, 72.6, 56.3, 51.7, 42.8, 41.8, 39.9, 39.8, 33.0, 30.5, 27.5, 22.6, 20.8, 19.9, 19.6, 18.0, 17.6, 13.8.IR (film) ν_{max} 3184, 3146, 3017, 2950, 2931, 2867, 1671, 1613, 1584, 1485, 1466, 1374, 1346, 1325, 1301, 1250, 1208, 1137, 1088, 1061, 1032, 937, 917, 883, 800, 755, 702, 666. DART-HRMS: m/z calculated for $C_{26}H_{38}O_3NH_4$: 416.3165 $[M+NH_4]^+$. Found: 416.3171.

4.2.4.2. (1*R*,3a*R*,4*S*,7a*R*)-1-((2*R*,5*S*)-5,6-Dimethylheptan-2-yl)-7a-methyloctahydro-1*H*-inden-4-yl 3-hydroxybenzoate (47)

Clear oil, yield 75%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.62 (m, 1H), 7.58 (m, 1H), 7.32 (m, 1H), 7.05 (m, 1H), 5.58 (m, 1H), 5.39 (m, 1H), 2.05 (m, 1H), 1.98 (m, 1H), 1.81 (m, 2H), 1.65 (m, 1H), 1.53 (m, 5H), 1.40 (m, 4H), 1.29 (m, 8H), 1.02 (s, 3H), 0.93 (d, J = 6.6 Hz, 3H), 0.86 (d, J = 6.9 Hz, 3H), 0.78 (dd, J = 6.8, 3.4 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.4, 155.7, 132.2, 129.6, 121.9, 119.9, 116.3, 72.6, 56.2, 51.5, 41.9, 39.8, 39.0, 35.8, 33.4, 31.4, 30.5, 30.4, 29.7, 27.0, 22.6, 20.5, 18.7, 18.0, 17.5, 15.4, 13.5. IR (film) ν_{max} 3400, 2956, 2939, 2868, 2858, 1700, 1684, 1601, 1589, 1294, 1217, 1160, 1110, 946, 756, 680, 669. DART-HRMS: m/z calculated for $C_{26}H_{40}O_3NH_4$: 418.3321 $[M+NH_4]^+$. Found: 418.3306.

4.2.4.3. (1*R*,4*S*,7a*R*)-1-((*S*)-1-Hydroxypropan-2-yl)-7a-methyloctahydro-1*H*-inden-4-yl 3-hydroxybenzoate (50)

Clear oil, yield 65%, alcohol **48** was esterified with carboxylic acid **15** and the methoxymethyl protecting group removed using the previously described protocols to afford final analogue **50** in excellent yield as a clear oil (65%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.63 (d, J = 7.6 Hz, 1H), 7.56 (d, J = 2.1 Hz, 1H), 7.34 (t, J = 7.8 Hz, 1H), 7.09 (dd, J = 8.2, 2.8 Hz, 1H), 5.66 (s, 1H), 5.39 (d, J = 3.3 Hz, 1H), 4.76 (s, 1H), 4.32 (dd, J = 10.8, 3.6 Hz, 1H), 4.12 (m, 1H), 2.10 (m, 1H), 2.04–1.85 (m, 2H), 1.83–1.74 (m, 3H), 1.61 (m, 4H), 1.44 (m, 2H), 1.14 (d, J = 6.6 Hz, 3H), 0.97 (d, J = 7.8 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.7, 156.1, 132.1, 129.8, 122.0, 120.3, 116.4, 74.8, 70.0, 53.1, 51.0, 42.2, 39.6, 35.8, 30.4, 26.2, 22.6, 17.6, 17.4, 13.0. IR (film) ν_{max} 3621, 3464, 3385 (br s), 3019, 2951, 2868, 1599, 1589, 1466, 1453, 1366, 1295, 1215, 1157, 1106, 1075, 1062, 982, 946, 886, 755, 680, 668. DART-HRMS: m/z calculated for $C_{20}H_{27}O_3$: 315.1960 $[M-OH]^+$, Found: 315.1983.

4.3. Biological evaluation

4.3.1. Cell line maintenance

C3H10T1/2 cells were purchased from American Type Culture Collection (ATCC). ASZ001 cells were a generous gift of Dr. Ervin Epstein (Children's Hospital of Oakland Research Institute). Gibco by Life Technologies culture media was purchased from ABI. C3H10T1/2 cells were cultured in BME (Gibco) supplemented with 10% FBS (Atlanta Biologicals, Premium Select), 1% L-glutamine (Cellgro; 200 nM solution), and 0.5% penicillin/streptomycin (Cellgro; 10,000 I.U./mL penicillin, 10,000 μg/mL). ASZ001 cells were cultured in 154CF media, supplemented with 2% FBS (chelexed, heat-inactivated), 1.0% penicillin/streptomycin, and a final concentration of 0.05 mM $CaCl_2$. Cells were maintained using the media described above (denoted 'growth' media'). Media denoted as 'low FBS' contained 0.5% FBS and the same percentage of other supplements as specified for growth media ('low FBS' media was used for C3H10T1/2 cell assays). Following plating and 24 h growth period, no FBS supplemented media was used for ASZ001 cell assays. All cells were grown in Corning Cell Culture, canted neck T75 or T150 flasks (Fisher Scientific) in an Autoflow IR water-jacketed CO_2 incubator (37 °C, 5% CO_2). Experiments with C3H10T1/2 cells were performed in BD Falcon sterile 60 mm dishes. Experiments with ASZ001 cells were performed in BD Falcon 35 mm dishes or in 6-well, 35 mm plates. DMSO was used as solvent to prepare all drug solutions and the final DMSO concentration did not exceed 0.3%. 20α-Hydroxycholesterol, 22(*S*)-hydroxycholesterol (OHC) and VD3, for biological studies, were purchased from Sigma–Aldrich.

4.3.2. Hh and VDR target gene expression in C3H10T1/2 cells

Cells (500 K, 5 mL) were plated in growth media at approximately 80% confluence. Once cells reached confluence (approximately 24 h post-plating), growth media was removed and replaced with low FBS media (5 mL). This was followed by addition of OHC, OHC and analogue, or DMSO (vehicle control). Cells were incubated (37 °C, 5% CO_2) for 24 h and RNA was isolated and evaluated by qRT-PCR analysis as described previously.[8] Data was analyzed using GraphPad Prism 5 and IC_{50} values computed as mean ± SEM from at least three separate experiments performed in triplicate.

4.3.3. Hh and VDR target gene expression in ASZ cells

Cells (300 K, 2 mL) were plated in growth media at approximately 80% confluence. After 24 h, growth media was removed and replaced with no FBS media (2 mL). Cells were incubated for an additional 24 h. After this time, addition of DMSO (vehicle control) or analogues was performed. Cells were incubated (37 °C, 5% CO_2) for 48 h and RNA was isolated and evaluated by qRT-PCR analysis as described previously.[9,11] Data was analyzed using GraphPad Prism 5 and IC_{50} values computed as mean ± SEM from at least three separate experiments performed in triplicate.

4.3.4. VDR binding

VDR binding was determined using the PolarScreen™ VDR competitor assay kit, Red (Invitrogen), per the manufacturer's instructions.

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Supplementary data

Supplementary data (intermediate synthesis and characterization and NMR spectra for final analogues) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.12.005.

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