ACS Medicinal Chemistry Letters

Letter

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Identification of Vitamin D3-Based Hedgehog Pathway Inhibitors That Incorporate an Aromatic A-Ring Isostere

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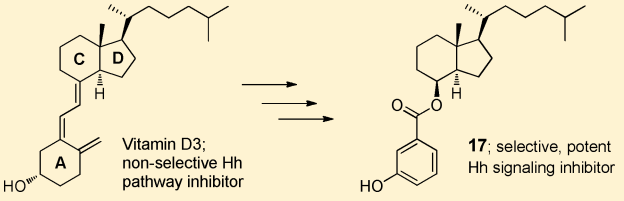
Supporting Information

ABSTRACT: Previous structure−activity relationship studies for vitamin D3 (VD3) inhibition of Hedgehog (Hh) signaling directed the design, synthesis, and evaluation of a series of VD3-based analogues that contain an aromatic A-ring mimic. Characterization of these compounds in a series of cellular assays demonstrated their ability to potently and selectively down-regulate Hh pathway signaling. The most active of these, **17**, inhibited pathway signaling in Hh-dependent mouse fibroblasts (IC_{50} = 0.74 ± 0.1 μM) and cultured cancer cells (IC_{50} values 3.8 ± 0.1 to 5.2 ± 0.2 μM). In addition, **17** demonstrated reduced activation of the vitamin D receptor (VDR) compared to VD3 in these cellular models. These results suggest that VD3-based analogues with an aromatic A-ring are a valid scaffold for the development of more selective and potent Hh pathway inhibitors and identify **17** as an intriguing lead from this class of compounds for further development. In addition, our analysis of Hh pathway inhibitors in cancer cells suggests that the murine basal cell carcinoma cell line ASZ001 and the human medulloblastoma cell line DAOY are appropriate in vitro cancer models for early stage evaluation of pathway inhibition.



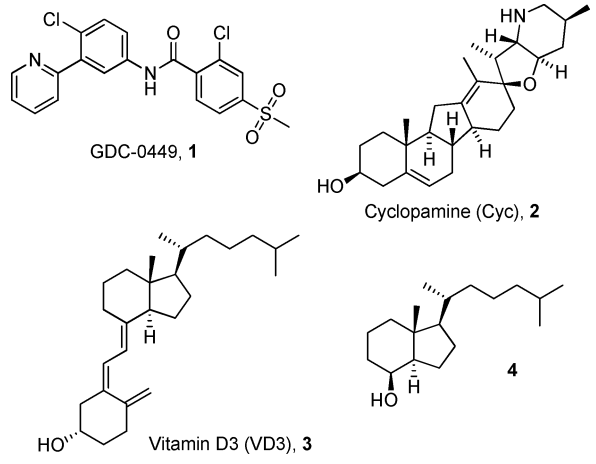
KEYWORDS: *hedgehog signaling pathway, vitamin D3, Gli, basal cell carcinoma, medulloblastoma*

The Hedgehog (Hh) signaling pathway is a developmental pathway that plays a key role in directing growth and tissue patterning during embryonic development. Dysregulation of Hh signaling has been associated with the development of human tumors; most notably, basal cell carcinoma (BCC) and medulloblastoma (MB).[1] Both forms of cancer are widely recognized as Hh-dependent and a significant number of BCCs (70%) and MBs (25%) exhibit detectable genetic mutations in Hh pathway components.[2−4] These mutations result in constitutive activation of the pathway and increased expression of Hh target genes, including several forms of the glioma-associated oncogene (Gli) family of signaling proteins.

Recent years have seen the identification, development, and evaluation of numerous small molecule inhibitors of Hh signaling as anticancer chemotherapeutics; most notably, the natural product cyclopamine (Cyc) and its derivatives, and GDC-0449 (Chart 1).[5] Both of these compounds inhibit the Hh pathway by directly binding Smoothened (SMO), a key regulator of pathway signaling and the most druggable target within the pathway. GDC-0449 recently received fast-track approval by the FDA for the treatment of metastatic BCC, representing the first Hh pathway inhibitor to receive FDA approval and validating the clinical relevance of targeting aberrant Hh signaling.[6] However, a point mutation in SMO that prevents binding and renders the tumor resistant to GDC-0449 highlights the continued need for new and improved Hh signaling inhibitors.[7,8]

Recently, vitamin D3 (VD3) was identified as an Hh pathway inhibitor that was proposed to exert its effects through direct binding to SMO.[9] VD3 has been shown to inhibit pathway signaling in Hh-dependent cell culture[9−11] as well as in murine models of BCC;[12] however, VD3 also activates the vitamin D receptor (VDR),[13] a nuclear receptor that plays an essential role in numerous physiological processes via canonical vitamin D signaling, in these models. This activation of VDR can result in detrimental side effects, including hypercalcemia. For this

Chart 1. Hh Signaling Pathway Antagonists

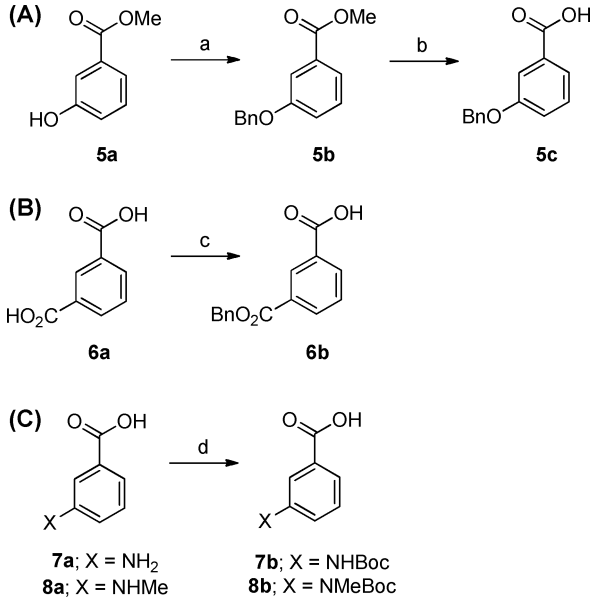


Received: January 10, 2013
Accepted: May 14, 2013
Published: May 14, 2013

   dx.doi.org/10.1021/ml400014t | ACS Med. Chem. Lett. 2013, 4, 590−595

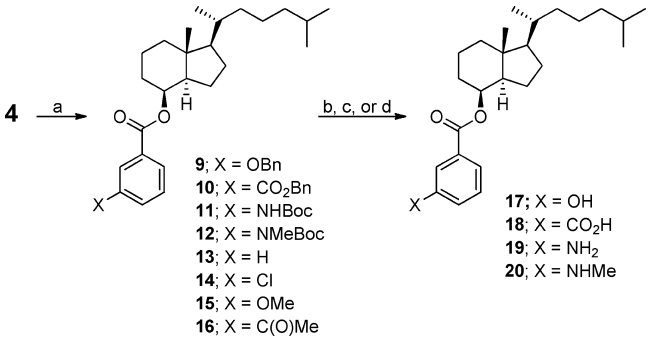
reason, VD3-based Hh pathway inhibitors must exert potent anti-Hh effects while also exhibiting minimal or no effects on canonical vitamin D signaling.

Scheme 1. Preparation of Protected Aromatic Carboxylic Acids[a]



[a]Reagents and conditions: (a) BnBr (3 equiv), K_2CO_3, acetone, 85%. (b) KOH (aq., 20%), THF, 75%. (c) (1) NEt_3, (1 equiv), MeOH:H_2O; (2) BnBr (1.1 equiv), DMF, 40%. (d) $(Boc)_2O$, dioxane, $NaHCO_3$ (aq., 5%), 50−80%.

Scheme 2. Preparation of Aromatic A-Ring VD3 Analogues[a]



[a]Reagents and conditions: (a) DCC, DMAP, DCM, 3-(X)-$ArCO_2H$, 60−95%. (b) **9**, $Pd(OH)_2$ (10%), H_2, MeOH:THF (2:1), 75%. (c) **10**, Pd/C (10%), H_2, ethyl acetate, 95%. (d) **11** or **12**, TFA:DCM (1:1), 55−70%.

A previous SAR study for VD3 inhibition of pathway signaling performed in our lab identified the CD-ring alcohol of VD3 (**4**, Grundmann's alcohol) as an Hh inhibitor with activity equipotent to VD3 (Table 1).[11] In addition, **4** did not bind to purified VDR nor did it activate VDR signaling in cell culture. On the basis of these results, and the fact that **4** is readily available from VD3 in a one pot, two-step reaction sequence, we sought to use this compound as a basis for the development of improved VD3-based Hh pathway inhibitors. Specifically, we report herein the design, synthesis, and evaluation of a series of VD3 analogues that contain an aromatic A-ring mimic with improved potency and selectivity against Hh signaling.

Table 1. Hh Signaling Inhibition in C3H10T1/2 Cells

Analogue[a]	Gli1 mRNA (%)[b]	Cyp24A1 mRNA[c]	VDR binding (μM)[d]
DMSO	1.0	1.0	ND
OHCs	100	ND	ND
VD3	35.7 ± 0.3	8336 ± 38	>100
4	46.4 ± 3.5	3.6 ± 0.5	>100
9	116 ± 34	1.0 ± 0.1	>100
13	105 ± 12	1.2 ± 0.4	>100
14	107 ± 7	1.2 ± 0.2	>100
15	104 ± 9	1.3 ± 0.1	>100
16	77.5 ± 9.0	1.6 ± 0.2	>100
17	1.9 ± 0.5	22.2 ± 4.7	>100
18	40.0 ± 14	6.7 ± 0.5	>100
19	2.4 ± 0.5	24.3 ± 3	>100
20	97.8 ± 10	1.6 ± 0.2	>100
3-HBA	92.6 ± 6.1	1.6 ± 0.6	>100

[a]All analogues tested at 5 μM and 24 h. [b]Values represent % Gli1 mRNA expression normalized to DMSO (vehicle) ActinB levels (set to 1.0). The OHC control Gli1 mRNA levels are set as 100% Hh pathway activity, and analogue modulation is represented as a % relative to this level. [c]Values represent Cyp24A1 mRNA expression normalized to ActinB levels treated with DMSO (vehicle control, set to 1.0). [d]Calcitriol is utilized as a positive control for VDR binding.[17]

The design of this series of analogues focused on the incorporation of aromatic A-ring isosteres that maintain the approximate steric and hydrophobic features of the natural aliphatic A-ring while not being a candidate for metabolism to a form that could more readily activate VDR. An ester linkage between the northern CD-ring region and the aromatic A-ring isostere was chosen due to its ease of formation and because analogue generation could be performed rapidly based on the parallel coupling of **4** with a library of commercially available benzoic acids. Initially, our rationale was to couple 3-hydroxybenzoic acid (3-HBA) with the northern CD-ring region (**4**) for evaluation as a proof-of-concept compound for this class of analogues. Following the synthesis and evaluation of promising lead analogue **17**, we sought to explore this class of compounds by establishing structure−activity relationships (SAR) for the 3-position of the aromatic A-ring.

Aromatic carboxylic acids were either purchased commercially or prepared via standard protection protocols (Scheme 1a−c). Acid **5c** was prepared by benzyl protection of methyl 3-hydroxybenzoate (**5a**) followed by potassium hydroxide-mediated hydrolysis to provide free carboxylic acid **5c**. The monobenzyl ester of isophthalic acid, **6b**, was obtained in moderate yield following a published protocol.[14] Protected carboxylic acids **7b**−**8b** were also generated in modest yield using standard conditions.[15] Acids were coupled to **4** using standard esterification conditions followed by appropriate deprotection (Scheme 2) to afford final analogues in good yields that were subsequently evaluated for their ability to selectively inhibit the Hh signaling pathway.

The Hh inhibitory activity of analogues **9** and **13**−**20** was initially evaluated by determining their ability to down-regulate endogenous Gli1 mRNA levels in the Hh-dependent C3H10T1/2 cell line, a standard cell-based assay used previously to evaluate small molecule inhibition of Hh signaling.[10,11] In addition, we have previously demonstrated that this cell line responds to VDR activation[10,11] with significant up-regulation of Cyp24A1, a well-characterized target gene of canonical vitamin D signaling.[16] For this assay,

dx.doi.org/10.1021/ml400014t | ACS Med. Chem. Lett. 2013, 4, 590−595

Table 2. Inhibition of Hh Signaling in Murine Fibroblasts[a]

	C3H10T1/2		M2-10B4		
Analogue	Gli1	Ptch	Gli1	Ptch	Cyp24A1[b]
VD3	4.1 ± 0.3	2.9 ± 0.3	>10	>10	274 ± 12[c]
4	3.1 ± 0.2	2.8 ± 0.4	ND	ND	ND
17	0.74 ± 0.1	1.1 ± 0.3	1.1 ± 0.6	1.7 ± 0.8	1.2 ± 0.1[c]
19	2.8 ± 0.6	4.5 ± 1.3	ND	ND	ND
GDC-0449	ND	ND	0.2 ± 0.01	0.14 ± 0.03	1.1 ± 0.4[d]
Cyc	ND	ND	0.8 ± 0.1	0.43 ± 0.03	1.5 ± 0.1[d]

[a]Values for Gli1 and Ptch are IC_{50} values in μM and represent at least two separate experiments performed in triplicate. Gli1 and Ptch mRNA expression were determined in the same manner as described for Table 1. ND = not determined. [b]Values represent Cyp24A1 mRNA expression normalized to ActinB levels treated with DMSO (vehicle control, set to 1.0). [c]Evaluated at 5 μM and 24 h. [d]Evaluated at 1 μM and 24 h.

analogues were evaluated at 5 μM and normalized to vehicle control (DMSO). For evaluation of Hh inhibition, cells treated only with the positive control [20(*S*)-hydroxycholeterol:22(*S*)-hydroxycholesterol, 5 μM each] were set as 100% Hh pathway signaling.

The results of this initial study provided important preliminary SAR for the ability of this class of VD3-based analogues to selectively inhibit Hh pathway signaling (Table 1). First, analogue **17** demonstrated potent inhibition of Hh signaling as measured by the ability to completely abolish Gli1 up-regulation at 5 μM. By contrast, masking the free phenol as either a methyl (**15**) or benzyl ether (**9**) eliminated activity from the scaffold. The only other compound that demonstrated Hh inhibition comparable to **17** was analogue **19**, which contains a primary amine at the 3-position of the aromatic ring. Similar to SAR for the phenol and ethers, the 3-NHMe analogue **20** was inactive, further supporting the notion that masking the alcohol or amine with small alkyl groups in this region affects Hh inhibitory activity. Interestingly, analogue **18**, which contains a 3-COOH functionality, demonstrated Hh inhibition similar to VD3, albeit at a level reduced compared to **17**. This reduced activity may result from the carboxylic acid functionality extending its hydrogen bond donating moiety away from the aromatic ring, reducing key interactions at the binding site. On the basis of these results, it was not surprising that neither unsubstituted analogues nor compounds that contained a more lipophilic moiety were active. Finally, as the ester linkage could potentially be cleaved by endogenous esterases, we studied whether the Hh inhibitory activity of **17** could be a result of the individual northern (**4**) and southern (3-hydroxybenzoic acid; 3-HBA) regions following potential cellular cleavage of the ester bond. 3-HBA failed to down-regulate Gli1 mRNA at 5 μM, suggesting that the inhibitory activity of this class of analogues is a result of the intact structure.

Both **17** and **19** displayed modest up-regulation of Cyp24A1; however, on the basis of the analysis that neither of these compounds bound to VDR, it is unclear by what mechanism they up-regulate Cyp24A1. None of the compounds tested displaced a fluorescent VDR ligand at concentrations up to 100 μM, suggesting a lack of affinity for VDR.[17] It is possible that **17** and **19** are metabolized to a form that can bind and activate VDR; however, on the basis of the close correlation between Hh inhibition and Cyp24A1 up-regulation, a more likely explanation is that inhibition of Hh signaling/Gli1 down-regulation triggers an increase in Cyp24A1 mRNA, either through VDR activation or another, as yet unidentified, mechanism. In either scenario, these results clearly indicate that both **17** and **19** are more selective for the Hh pathway compared to VD3.

Both **17** and **19** inhibited Hh signaling in a dose-dependent fashion in C3H10T1/2 cells (Table 2). Interestingly, while the IC_{50} values for Gli1 and Ptch correlated well for VD3, **4**, and **19**, analogue **17** was significantly more active against Gli1 (IC_{50} = 0.74 ± 0.1 μM). To assess whether the potent, selective Hh inhibitory effects of this class of analogues were dependent on cell type, **17** was evaluated in M2-10B4 cells, a murine bone marrow stromal cell line that also responds to Hh pathway stimulation with a characteristic Gli1 and Ptch mRNA up-regulation[18] (Table 2). While VD3 was found to be less active in M2-10B4 cells,[19] down-regulation of Gli1 and Ptch by **17** was comparable to that determined in the C3H10T1/2 line. Evaluation of GDC-0449 and Cyc in this model was also performed to determine whether other Hh pathway inhibitors differentially affected Gli1 and Ptch levels similar to VD3. Interestingly, Ptch knock-down by both of these well-characterized Hh pathway inhibitors appeared to be more potent when compared to Gli1. Treatment of M2-10B4 cells with **17** (5 μM), GDC-0449 (1 μM), or Cyc (1 μM) did not result in increased expression of Cyp24A1 compared to DMSO control, suggesting the enhanced expression seen in the C3H10T1/2 cells may be cell-type dependent. Cyp24a1 levels were increased approximately 270-fold in response to treatment with VD3 (5uM). Finally, **17** did not increase Cyp24A1 levels in HT-29 cells, a human colon cancer cell line that overexpresses VDR and responds to vitamin D (calcitriol) with a robust up-regulation of Cyp24A1[20,21] (data not shown). These results suggest that the enhanced expression of Cyp24A1 seen by **17** in the C3H10T1/2 cells is a cell-type dependent response that is not a general result of Hh pathway inhibition.

Previous studies evaluating Hh pathway inhibition in cultured cancer cells have suggested that the majority of in vitro cancer cell lines do not appropriately model in vivo Hh signaling. With this in mind, we sought to characterize VD3, **17**, and **19** in two cell lines, ASZ001 and DAOY, which have shown initial promise as in vitro models of oncogenic Hh signaling. The ASZ001 cell line was grown from a visible BCC tumor isolated from a *Ptch1*$^{\pm}$ mouse.[22] 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 resulted in Gli1 down-regulation and antiproliferation.[12,22] Several recent lines of evidence suggested that DAOY cells may be a suitable in vitro cancer model of Hh signaling.[23] First, the DNA methylation pattern in DAOY cells is consistent with the pattern observed in mouse models of MB in which a mutation in *PTCH1* is responsible for MB formation.[24,25] Second,

dx.doi.org/10.1021/ml400014t | *ACS Med. Chem. Lett.* 2013, 4, 590−595

expression of REN, a tumor suppressor that negatively regulates Hh signaling and is commonly mutated in human MB, is significantly reduced in DAOY cells.[26,27] Finally, several reports in the patent literature have utilized DAOY cells to evaluate Gli1 down-regulation as a function of Hh inhibition.[28] Previous studies performed in both cell lines demonstrated that Gli1 down-regulation by VD3 (ASZ001)[12] and Cyc (DAOY)[23] was more robust and reproducible following a 48 h compound incubation. Preliminary evaluation in our lab was consistent with a 48 h incubation period resulting in reproducible Gli1 down-regulation induced by GDC-0449 or Cyc. For this reason, all data presented from these two cell lines was obtained at this time point.

Initially, we evaluated the ability of VD3 and several of our analogues to down-regulate Gli1 mRNA expression in ASZ001 cells in a dose-dependent fashion (Table 3 and Figure 1). While

Table 3. Hh Signaling Inhibition in ASZ001 and DAOY Cells

	ASZ001		DAOY	
Analogue	Gli1[a]	Cyp24A1[b]	Gli1[a]	Cyp24a1[b]
VD3	2.1 ± 0.1	764 ± 24[c]	>10	149 ± 90[d]
4	>10	2.4 ± 0.9[d]	>10	0.8 ± 0.1[d]
13	>10	1.1 ± 0.3[d]	>10	0.6 ± 0.1[e]
17	5.2 ± 0.2	1.9 ± 0.1[d]	3.7 ± 0.04	0.4 ± 0.1[d]
19	7.1 ± 1	2.5 ± 1.4[d]	9.2 ± 1.7	0.5 ± 0.1[d]
GDC-0449	0.04 ± 0.01	0.9 ± 0.2[d]	0.086 ± 0.02	1.0 ± 0.1[c]
Cyc	0.66 ± 0.02	0.8 ± 0.1[d]	0.16 ± 0.04	1.0 ± 0.7[c]

[a]All IC_{50} values are in μM and represent at least two separate experiments performed in triplicate. [b]Values represent Cyp24A1 mRNA expression after 48 h incubation with drug relative to DMSO (set as 1.0). [c]Evaluated at 2.5 μM and 48 h. [d]Evaluated at 5 μM and 48 h. [e]Evaluated at 10 μM and 48 h.

the IC_{50} for VD3 down-regulation of Gli1 correlated well with that obtained in the C3H10T1/2 fibroblasts, analogues **17** and **19** were less effective in this model (5.2 and 7.1 μM, respectively). In addition, analogue **4** demonstrated only a modest reduction of Gli1 mRNA levels (65% relative to control) at the highest dose evaluated (10 μM). The inactivity of **4** in ASZ001s provides further evidence that the Hh antagonism of **17** and **19** results from an intact structure. Similar to the results from the murine fibroblasts, VD3 demonstrated significant up-regulation of Cyp24A1, while the analogues had minimal effects. Of note was our finding that, at higher concentrations (10 and 5 μM), VD3 up-regulated Cyp24a1 mRNA levels to a lesser degree than at lower concentrations ($\leq$2.5 μM; Supplementary Figure 1), which may be an indication that VD3 exhibits significant activity through off-target effects or toxicity to the cells at higher doses. To further characterize the ability of ASZ001 cells to function as an early stage in vitro cancer model of aberrant Hh signaling, we evaluated both GDC-0449 and Cyc for their dose-dependent effects on Gli1 expression. Both compounds completely abolished Gli1 expression at high concentrations ($\geq$1 μM) and IC_{50} values correlated well with those previously published in Hh-dependent murine fibroblasts (Table 3). Overall, our results suggest that the ASZ001 cell line is a suitable BCC model for early stage analysis of Hh pathway inhibition.

We next evaluated each of these compounds in the human MB-derived DAOY cell line (Table 3, Figure 1). Interestingly, high concentrations of VD3 (10 μM) exhibited only minimal

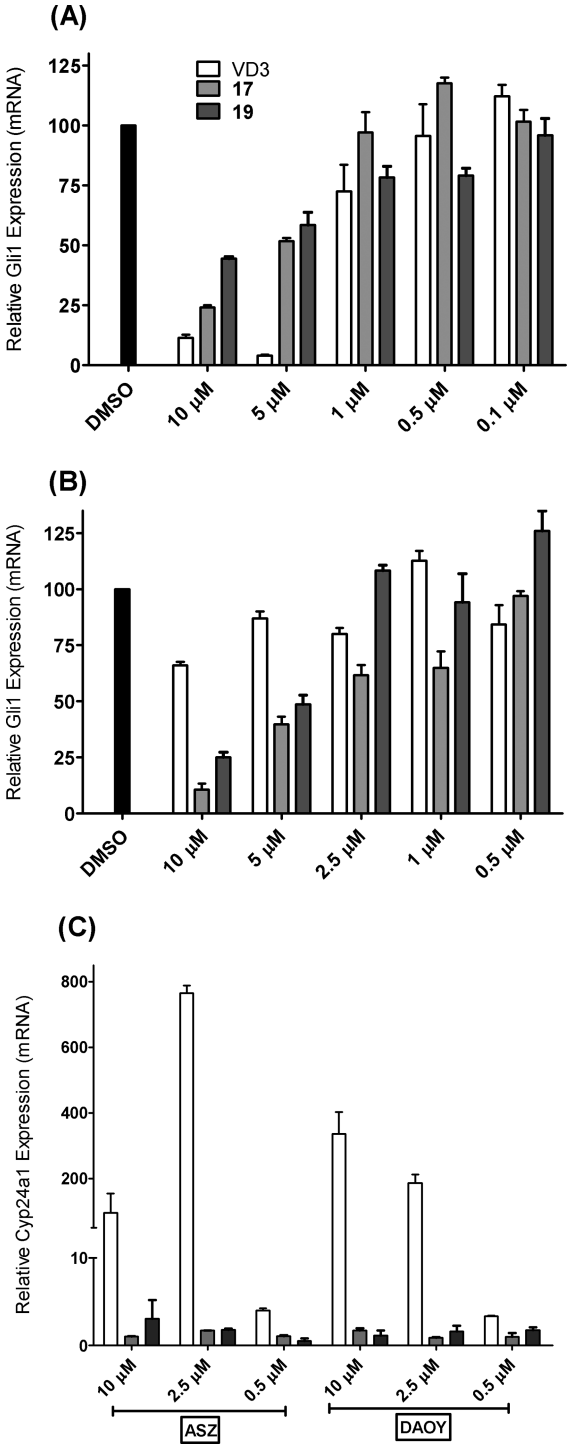


Figure 1. Selective inhibition of Hh signaling by VD3 and analogues in cultured cancer cells. Down-regulation of Gli1 in ASZ001 (A) and DAOY (B) cell lines. Up-regulation of Cyp24A1 by VD3, **17**, and **19** in ASZ001 and DAOY cells (C; DMSO set to 1.0).

down-regulation of Gli1 mRNA expression (Figure 1B).[19] By contrast, VD3 analogues **17** and **19** displayed Hh inhibitory activity similar to that demonstrated in the ASZ001 cells (IC_{50} = 3.7 and 9.2 μM, respectively). In addition, neither **4** nor **13** demonstrated the ability to down-regulate Gli1 mRNA expression in the DAOYs. While the IC_{50} for GDC-0449 was comparable to those previously determined, Cyc was significantly more active in the DAOYs. Finally, only treatment

dx.doi.org/10.1021/ml400014t | ACS Med. Chem. Lett. 2013, 4, 590−595

with VD3 resulted in up-regulation of Cyp24A1 mRNA expression.

To determine whether down-regulation of Gli mRNA in DAOY cells was a function of Hh inhibition or general anticancer activity, we evaluated a series of clinically relevant anticancer agents that work via mechanisms distinct from Hh inhibition for their ability to affect endogenous Gli1 mRNA. None of these, including the HDAC inhibitor Vorinostat (1.51 ± 0.1), the DNA intercalator oxaliplatin (1.16 ± 0.3), the phosphatidylinositol-3-kinase (PI3K) inhibitor wortmannin (0.92 ± 0.2), nor the BCR-ABL kinase inhibitor Gleevec (0.98 ± 0.3) significantly modulated Gli1 mRNA expression compared to DMSO control (1.0) at a concentration of 2.5 μM. Taken together, the results of our studies in DAOYs indicate this cell line is a suitable in vitro MB model for early stage evaluation of Hh signaling inhibition.

In summary, we have identified a lead scaffold suitable for further development as a new class of VD3-based Hh pathway inhibitors. Replacement of the *seco*-B and A-ring of VD3 with an aromatic isostere linked through an ester bond resulted in compounds with enhanced Hh inhibition and improved selectivity compared to VD3. A focused series of analogues provided key preliminary SAR for this class of compounds, resulting in the identification of **17**. As a close mimic to VD3, lead **17** represents a more potent and selective inhibitor of Hh signaling in multiple cellular models. The in vitro results described above suggest the intact analogue structure is required for the modulation of Hh signaling; however, as the presence of the ester linkage poses potential stability concerns for the in vivo evaluation of these compounds, ongoing SAR studies in the lab are focused on further exploration of the aromatic A-ring region for this scaffold as well as the design and synthesis of analogues that incorporate a linker less susceptible to metabolism. Finally, our evaluation of several Hh pathway inhibitors in ASZ001 and DAOY cell lines suggests that both are suitable in vitro cancer models for the early stage analysis of Hh pathway inhibition.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic methods, biological assay protocols, and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

A.M.D. synthesized and characterized all analogues and performed most of the biological assays. U.B. performed VDR binding assays. All authors contributed to the preparation of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We gratefully acknowledge support of this work by the V Foundation for Cancer Research (V Scholar, to M.K.H.), the Charles H. Hood Foundation, and the University of Connecticut Research Foundation. ASZ001 cells were provided by Dr. Ervin Epstein (Children's Hospital Oakland Research Institute).

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