Novel Mechanisms and Functions for Hedgehog Signaling in Development and Disease

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Inhibition of Hedgehog-Dependent Pancreatic Cancer Cell Growth by Vitamin D₃

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SUMMARY
Besides its roles in prenatal development, it has become clear that defects in the developmental Hedgehog (Hh) pathway can cause certain malignancies in the adult organism. Specifically, tumors of the proximal gastrointestinal tract have been shown to depend on an excessively activated Hh pathway and previous studies have used the plant inhibitor of the Hh pathway cyclopamine to treat Hh-dependent tumor growth. The present study aimed to determine the efficacy and specificity of the recently discovered endogenous inhibitor of the Hh pathway, vitamin D₃, on growth inhibition of pancreatic adenocarcinoma cells. Vitamin D₃ was more effective in inhibiting cell growth of pancreatic adenocarcinoma cells than cyclopamine, and this inhibition was specifically mediated through inactivation of the Hh pathway rather than previously reported effects of activation of the vitamin D receptor. Although the exact mechanism for the observed cell death was not identified, Western blot analysis showed FasR induction by vitamin D₃ but this was shown not to be causative in causing cell death in our model. Analysis of cell cycle progress modulators suggested cell cycle arrest to be responsible for the effect of vitamin D₃ on pancreatic adenocarcinoma cell growth.

INTRODUCTION
The family of Hedgehog (Hh) proteins is involved in a plethora of patterning processes in the developing organism and most of the research effort in the field has so far focused on the roles and mechanisms of Hh proteins in prenatal development (Bijlsma et al., 2004). This effort is at least partly driven by the unusual and complex signal transduction of the Hh proteins (Riobo and Manning, 2007). For instance, the Hh signal is relayed through the interaction between two receptors, Patched-1 (Ptch1 (Ingham et al., 1991; Marigo et al., 1996)) and Smoothened (Smo (Alcedo et al., 1996; van den Heuvel and Ingham, 1996)). In the absence of Hh ligand, Ptch1 is actively inhibiting Smo and the pathway is inactive. Binding of Hh ligand to its receptor Ptch1 alleviates the Ptch1-mediated inhibition of Smo and the pathway becomes active (Murone et al., 1999). Another peculiarity is the synthesis of the Hh ligand itself; after translation, the protein autocatalytically cleaves and a cholesterol group is added to the newly exposed C-terminus (Beachy et al., 1997; Lee et al., 1994; Porter et al., 1996). Following sterolation, a palmitoyl group is added at the other terminus and despite these highly hydrophobic modifications, the protein is secreted.

In addition to being investigated for their interesting signal transduction, the role of Hh proteins in the adult organism is becoming increasingly evident, and research has shown the Hh proteins to be involved for instance in gastrointestinal homeostasis (van den Brink et al., 2001), tissue salvage following ischemia (Kusano et al., 2005; Pola et al., 2003) and T-cell maturation (Uhmann et al., 2007). An important downside of improperly sustained Hh activity in the adult organism is its causative role in carcinogenesis in skin (Fan et al., 1997), prostate (Karhadkar et al., 2004), and the upper gastrointestinal tract (Berman et al., 2003; Watkins and Peacock, 2004). The underlying causes for excessive pathway activity in these diseases vary from activating mutations in Smo rendering it unresponsive to endogenous inhibition by Ptch1, to inactivating mutations in Ptch1 paralyzing its inhibitory action on Smo (Taipale et al., 2000). Mutations in the downstream pathway component suppressor of fused...
(SuFu) have also been implied in tumorigenesis (Tostar et al., 2006). Some tumors, however, do not depend on mutations in pathway components but in disproportionate Hh ligand production, and it is this Hh expression that has been found to cause most tumors of the upper gastrointestinal tract (Berman et al., 2003).

Very few effective treatment strategies are available for tumors of the proximal gastrointestinal tract (esophagus, stomach, duodenum and pancreatic cancer) and the relatively high incidence (10 in every 10000 persons for pancreatic cancer each year in Western countries) necessitates research into novel therapeutic options. As the alkaloid cyclopamine has been found to be a potent inhibitor of Smo and its downstream signaling, it has seen extensive use as a tool in Hh research. Inhibiting the Hh pathway in cancer cells of the upper gastrointestinal tract with cyclopamine has proven to be a successful way of reducing cancer cell viability (Berman et al., 2002; Berman et al., 2003; Feldmann et al., 2007; Taipale et al., 2000). Although cyclopamine thus looks like a promising therapeutic option, and tumor growth could indeed be diminished in mouse models, phase III trials have proven unsuccessful (data not published).

We have recently identified the naturally occurring inhibitor of Smo, vitamin D₃. Using an in vitro model, we were able to show that Ptch1 pumps vitamin D₃ out of the cell, after which it binds to Smo and inhibits Hh pathway activity (Bijlsma et al., 2006). Because of its widespread and long clinical use, the side-effects of administering vitamin D₃ are well known (e.g. hypercalcaemia (Hathcock et al., 2007)), and we predict that treatment of tumors that depend on excessive Hh pathway activity with vitamin D₃ is less likely to cause unexpected side effects compared to the relatively novel compound cyclopamine, as well as show a better efficacy (Bijlsma et al., 2006a). In this study, we estimated the potency of vitamin D₃ in inhibiting growth of tumor cells that are known to be sensitive to cyclopamine, thus confirming the action of vitamin D₃ on Smo and establishing a potential novel therapeutic option for Hh-dependent tumors. Also, we verified that a very significant part of the vitamin D₃ induced cytotoxicity was mediated specifically through inactivation of the Hh pathway.

RESULTS AND DISCUSSION

Cell lines known to be dependent on Hh ligand expression were used as a model system to examine the effect of vitamin D₃ on Hh ligand dependent tumor cell growth. These cell lines included the pancreatic adenocarcinoma cell lines Hs766T, BxPC3, MIA PaCa-2 and PANC-1. These cell lines have previously been shown to have an elevated Hh pathway activity and subsequently increased cell growth, apparently caused by an excessive endogenous production of Hh ligand (Berman et al., 2003; Neureiter et al., 2005). As can be seen in Figure 1A, cell viability was greatly reduced for the studied tumor cell lines when exposed to increasing concentrations of vitamin D₃ for 3d. In comparison, cyclopamine was less effective (Fig. 1B), and at very high doses of cyclopamine, a number of viable cells always remained. Although these data show cytotoxicity to the same cells for both cyclopamine and vitamin D₃, the vitamin D₃ induced cytotoxicity was strongly augmented in the presence of the 7-dehydrocholesterol reductase inhibitor AY-9944 (1 μM AY-9944). This in turn strongly augmented the cytotoxic activity of vitamin D₃, but was also effective in the absence of exogenously added vitamin D₃.

![Figure 1. Vitamin D₃ inhibits pancreatic adenocarcinoma cell growth.](image-url)
vitamin D₃, they do not necessarily prove that vitamin D₃ acts in a similar manner as cyclopamine does.

To assess to what extent endogenously synthesized pro-vitamin D₃ could inhibit cell growth, we used the 7-dehydrocholesterol reductase (7-DHCR) inhibitor AY-9944 (Gaoua et al., 1999). Inhibition of 7-DHCR causes stacking of 7-dehydrocholesterol, which is the precursor to vitamin D₃. The addition of AY-9944 to BxPC3 cells enhanced the inhibitory effect of vitamin D₃ but cell growth was also inhibited by AY-9944 alone (Fig. 1C). Exogenously added as well as endogenously produced pro-vitamin D₃ is thus toxic to cells that depend on an excessively activated Hh pathway.

As we had previously shown vitamin D₃ to bind to Smo and inhibit it, we aimed to confirm this effect of vitamin D₃ on Hh pathway activity in the pancreatic adenocarcinoma cells studied. BxPC3 cells were transiently transfected with a reporter construct sensitive to the Hh pathway transcription glioma associated (Gli) factors (Gli δ51-LucII (Sasaki et al., 1997)), and Hh pathway activity was assessed following vitamin D₃ treatment (Fig. 2A). A strong decrease in pathway activity could be seen following treatment with vitamin D₃, proving the BxPC3 cells to have an active Hh pathway, and vitamin D₃ to be able to counteract this activation. Surprisingly, we could also observe a substantial pathway activation following exogenous stimulation with recombinant N-terminal Shh. This means that although the BxPC3 cells are able to support their own growth by excessive Shh production, this production is not enough to maximally stimulate the pathway.

As the cytotoxic effects of vitamin D₃ on cancer cells are known to be mediated through several mechanisms (nearly all of which are mediated through the vitamin D receptor (Deeb et al., 2007)), we aimed to confirm that (at least part of) the observed reduction of cell viability is mediated through inactivation of Smo and the downstream transcription factors. To this end, BxPC3 cells were transfected with the Ptch1-insensitive Smo mutant SmoM2 (Taipale et al., 2000), or the downstream transcription factor Gli1 (Nguyen et al., 2005). Subsequently, cells were exposed to 10 μM vitamin D₃ and cell viability was assessed by MTT reduction. The BxPC3 cells, known to depend on an activated Hh pathway for their growth, were rendered less sensitive to vitamin D₃ when transfected with SmoM2, and almost completely insensitive when transfected with Gli1, confirming that a very significant part of the cytotoxic action of vitamin D₃ is mediated through inactivation of Smo.

To determine the specificity of vitamin D₃ induced cell-death for cell lines dependent on excessive Hh pathway activation rather than a more general, aspecific cytotoxic effect, we treated HepG2 hepatocarcinoma cells with increasing doses of vitamin D₃. As is apparent, the HepG2 cells are much less sensitive to vitamin D₃ than the BxPC3 cells. Because these hepatocarcinoma cells are not thought to be caused by an excessively activated Hh pathway (Patil et al.,

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**FIGURE 2. VITAMIN D3 SPECIFICALLY INHIBITS PANCREATIC ADENOCARCINOMA CELL GROWTH BY HH PATHWAY INACTIVATION**

(A) BxPC3 cells were transfected with a luciferase reporter construct sensitive to active Hh pathway transcription factors. 16h after transfection, medium was refreshed and cells were stimulated with vitamin D₃ or recombinant N-terminal Shh for another 24h. Following lysis, luciferase activity was determined and data were expressed as percentage in-/decrease over control stimulated cells. Shown is mean ±SEM, n=3; * p>0.01; * p>0.05.

(B) BxPC3 cells were transfected with vector DNA, the insensitive SmoM2 mutant or the downstream Gli1 transcription factor for the Hh pathway. 16h after transfection, medium was refreshed and cells were subsequently stimulated as for Fig. 1. Shown is mean ±SEM, n=8; *** p>0.005; * p>0.05.

(C) HepG2 hepatocarcinoma cells were stimulated as for Fig. 1. For comparison, BxPC3 data is plotted alongside, showing a much-reduced sensitivity to vitamin D₃ for the HepG2 cells.
this was expected and is indicative of the specificity of vitamin D3 on the growth of those cell types known to depend on Hh pathway activity.

To elucidate the mechanism by which vitamin D3 induces cell death, we took clues from mechanisms described for cyclopamine previously. Specifically, we anticipated the cell growth inhibition by vitamin D3 to be mediated through expression of the Fas receptor (FasR (Athar et al., 2004)), ultimately leading to apoptosis, and by the inhibition of cell cycle progress mediators (Chen et al., 2007). When we assessed the induction of FasR by Western blot following treatment of BxPC3 cells with doxorubicin as a positive control, we found a moderate increase in FasR expression (Fig. 3A). Doxorubicin is a DNA intercalating compound that has been shown to cause cell cycle arrest and FasR upregulation (Fulda et al., 1998). When we stimulated with vitamin D3, the FasR expression exceeded that by doxorubicin, suggesting that FasR induction might be responsible for the cell growth inhibition by vitamin D3. To test the actual relevance of the observed FasR upregulation upon vitamin D3 stimulation, we performed a cell viability assay with vitamin D3 in the presence of a Fas-blocking antibody (α-Fas). Surprisingly, the cell growth-inhibitory effect of vitamin D3 could not be diminished. Also, in contrast to what was previously reported, we could not inhibit cyclopamine induced cell death by α-Fas (Fig. 3B).

In addition to FasR upregulation, we could observe induction of p21 and a reduction of cyclin D1 by both doxorubicin and vitamin D3 (Fig. 3A). p21 inhibits cyclin dependent kinase (Cdk) and thus stops cell cycle progression, whereas conversely, cyclin D1 triggers cell cycle progression in conjunction with Cdk (Chen et al., 2007). The observed upregulation of p21 and down-regulation of cyclin D1 could very well explain the cytotoxicity of vitamin D3.

In an experiment similar to that described above for the Fas blocking antibody, we determined if apoptosis was responsible for the observed cell death/growth inhibition by adding the effector caspase inhibitor zVADfmk (abbreviated as zVAD) to the cells with 10 μM vitamin D3 or cyclopamine. Although we were able to diminish the effect of cyclopamine by inhibiting apoptosis as expected, we could not do so for vitamin D3 (Fig. 3B) meaning that vitamin D3 at the dose used does not reduce cell viability by triggering apoptosis. These data might be explained by the possibility that different mechanisms exist downstream of inactive Smo. The latter possibility is supported by the fact that for instance binding to Smo by Smo agonist (SAG) can induce either activation or inhibition of Smo depending on concentration (Chen et al., 2002), meaning that one binding site on Smo can account for different actions downstream. It would be interesting to see how Smo is able to translate signals from one binding site into two different responses, not only in light of the findings presented in this study and the one describing the effects of SAG, but also with regard to the recently discovered ability of Smo to signal in a canonical and a non-canonical fashion (Bijlsma et al., 2007; Bijlsma et al., 2008b).

Concluding, although inhibition of tumor growth by vitamin D3 has previously been attributed to a large number of mechanisms which include for instance inhibition of IL-8 induced angiogenesis, and activation of the Akt/mTOR pathway (nearly all of which are mediated through the vitamin D receptor (VDR), although alternative means of perceiving vitamin D3 are available to a cell (Deeb et al., 2007)), we now report here the action of vitamin D3 on cancer cells through inhibition of the Hh pathway and show that the observed cytotoxic activity of vitamin D3 is mainly mediated through inhibition of Smo rather than activation of the VDR.

In all the experiments described in the present study, we used the inactive form of vitamin D3, also referred to as cholecalciferol. The strong effects of this inactive form of vitamin D3 observed are interesting, as in normal physiology,
this inactive form does not influence mineral homeostasis as active vitamin D₃ does. To attain this activity, it must first be hydroxylated in the liver and the kidneys (Deeb et al., 2007). It should prove valuable to synthesize a non-activatable form of cholecalciferol, to be able to avoid side effects such as hypercalcemia. This compound would still bind to Smo, but not the VDR, thus acting only on the Hh pathway and provide a valuable therapeutic option to treat Hh-dependent tumors. Progress on this matter is eagerly awaited and especially valuable for those compounds that show an inhibitory effect on the Hh pathway should speed up utilization of basic research findings on Hh biology.

**MATERIALS AND METHODS**

**COMPONDS AND CONSTRUCTS**

Cyclopamine was from Biomol (Plymouth Meeting, PA), AY-9944 from Calbiochem (San Diego, CA), vitamin D₃ (cholecalciferol) from Sigma (St Louis, MO). Recombinant N-terminal Shh was obtained from R&D Systems (Minneapolis, MN). SmoM2 in pRK7 was obtained from Genentech (South San Francisco, CA). The Gli1 cDNA in pcDNA1 was a generous gift of Dr. A. Ruiz i Altaba. The Gli-reporter d51-LucII was kindly provided by Dr. H. Sasaki.

**CELL CULTURE AND TRANSFECTIONS**

Hs766T, BxPC3, MIA PaCa-2, PANC-1 and HepG2 cells were cultured in DMEM (Cambrex, East Rutherford, NJ) containing 10% foetal calf serum (FCS (Cambrex)) according to routine cell culture procedures. Transfections were performed using Effectene according to manufacturer’s directions. For reporter assays, cells were transfected in 12-well cell culture plates. Per well, 1 μg DNA was used. For MTT assays, cells were transfected in 6-well plates (2 μg DNA per well) and after 16h, cells were transferred to 96-well plates (see below).

**MTT REDUCTION CELL VIABILITY ASSAY**

Cells were seeded in flat-bottom 96 wells plates in DMEM containing 0.5% FCS and treated with the indicated concentrations of vitamin D₃ for 3d as described perviously (Corcoran and Scott, 2006). During the last 2-4h, 0.5 mg/ml thiazolyl blue tetrazolium bromide (MTT) was added. After incubation, supernatant was discarded; cells were lysed in 100 μL 40 mM HCl in isopropanol and absorbance was measured at 570 nm in a Benchmark Plus Microplate Spectrophotometer (Bio-Rad, Hercules, CA).

**WESTERN BLOTTING**

Following stimulation, cells were lysed in Laemmli buffer and brought onto SDS-PAGE gels. After electrophoresis, protein was transferred onto Immobilon-PVDF membranes (Millipore, Billerica, MA). Membranes were blocked in 3% milk in TBS/0.1% Tween-20 (TBST) for 1h. Mouse monoclonal α-actin I-19 (Santa Cruz Biotechnology, Santa Cruz, CA), α-cyclin D1, and α-p21 (both Transduction Laboratories, Baltimore, MD) were diluted 1:500 in 1% BSA in TBST and membranes were incubated overnight. Goat polyclonal α-β-actin I-19 (Santa Cruz Biotechnology) was diluted to 1:1000 in 3% BSA in TBST. After 1h incubation in 1:1000 of the appropriate HRP-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark), blots were imaged using LumiLight Plus ECL (Roche, Basel, Switzerland) on a LAS-3000 imaging system (Fujifilm).

**REPORTER ASSAY**

Cells grown to 70% confluence in a 12-well plate were transfected as described above with the Gli-reporter construct and a CMV-driven Renilla Luciferase control, and after 16h, stimulated. Subsequently, cells were lysed with passive lysis buffer as provided by Promega and luciferase activity was assayed according to the Promega Dual-Glo Luciferase Assay System (Promega) protocol on a Lumat Berthold LB 9501 Luminometer (Berthold Technologies, Bad Wildbad, Germany). Each Firefly luciferase value was corrected for its co-transfected CMV driven Renilla luciferase standard to correct for transfection efficiency or dilution effects.

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**REFERENCES**


