A Functional and Putative Physiological Role of Calcitriol in Patched1/Smoothened Interaction*

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Background: Smoothed activity is mediated by a still undiscovered small molecule.

Results: Patched1 is required for calcitriol release and calcitriol inhibits Smoothened independently of the cysteine-rich domain and 7-transmembrane domain.

Conclusion: Calcitriol exhibits excellent characteristics of a signal transduction molecule in the Patched1/Smoothened interaction.

Significance: Understanding how Smoothed activity is regulated for new therapeutic options to fight Hedgehog-associated cancers.

The Patched1 (Ptch)-mediated inhibition of Smoothened (Smo) is still an open question. However, a direct Ptch/Smo interaction has been excluded, Smo modulators were identified, but the endogenous signal transmitting molecule remains undiscovered. Here, we demonstrate that calcitriol, the hormonally active form of vitamin D₃, is an excellent candidate for transmission of Ptch/Smo interaction. Our study reveals that Ptch expression is sufficient to release calcitriol from the cell and that calcitriol inhibits Smo action and ciliary translocation by acting on a site distinct from the 7-transmembrane domain or the cysteine-rich domain. Moreover calcitriol strongly synergizes with itraconazole (ITZ) in Smo inhibition, which did not result from elevated calcitriol bioavailability due to ITZ-mediated 24-hydroxylase inhibition but rather from a direct interaction of the compounds at the level of Smo. Together, we suggest that calcitriol represents a possible endogenous transmitter of Ptch/Smo interaction. Moreover calcitriol or calcitriol derivatives combined with ITZ might be a treatment option of Hedgehog-associated cancers.

The Hedgehog (Hh)² signaling pathway plays an essential role in cell differentiation, organ patterning, cell proliferation, stem cell maintenance, and regenerative responses after injury (1–3). Aberrant Hh signaling during embryogenesis leads to birth defects, whereas overactive Hh signaling in somatic cells of adults results in cancer formation (1). Thus, the exact knowledge about the regulation of the activity of the pathway is highly important for a better understanding of Hh-associated tumorgenesis and the development of new anti-cancer therapies.

Most commonly affected in Hh-associated cancer formation is the interaction between the Hh receptor Patched1 (Ptch) and Smoothened (Smo) (4). In the absence of Hh proteins Ptch inhibits Smo due to an, so far, unknown catalytic mechanism (5–7). The binding of Hh to Ptch terminates the inhibition of Smo followed by the translocation of Smo into the primary cilium and by the activation of the Hh signaling cascade, which is characterized by transcription of Gli target genes (e.g. Gli1) (1, 4).

Ptch consists of 12 hydrophobic transmembrane domains, the intracellular C- and N-terminal regions and two extracellular loops that mediate the binding of the Hh ligand (8, 9). Five of the transmembrane domains form a domain resembling a sterol-sensing domain (SSD) (10, 11), a motif found in proteins, which are involved in intracellular sterol level sensing (12). The SSD is essential for the function of Ptch (13). According to the oligomeric structures of SSD transporters Ptch proteins form stable trimers (14). Thus, it is tempting to speculate that Ptch is a multisubunit transporter whose activity indirectly regulates the function of Smo, e.g. by pumping small sterol-like molecules (15, 16). Experiments from our and other laboratories foster this hypothesis because medium conditioned from wild type (wt) Ptch cells but not from Ptch-mutant (Ptch⁻/⁻) cells has Smo inhibitory properties. This suggests that Ptch⁻/⁻ cells are either defective in the release (16, 17) and/or in the synthesis of Smo inhibitory molecules.

Smo, the signaling partner of Ptch, consists of a 7-transmembrane domain (7TM), an intracellular C-terminal tail and an extracellular N-terminal region containing a cysteine-rich domain (CRD) (18, 19). The 7TM harbors a binding site for Smo inhibitors (e.g. cyclopamine (CP) (20), vismodegib (21))
and activators (Smoothed agonist; SAG (22, 23)). Although no endogenous molecule has been identified that regulates Smo activity upon binding to the 7TM, endogenous oxidized derivatives of cholesterol (oxysterols) (15, 24, 25) such as 20(S)-hydroxycholesterol (20(S)-OHC) can bind and activate Smo via its CRD (18, 19).

Recently we described that the oxysterol calcitriol, a twice hydroxylated vitamin D$_3$ derivative, efficiently inhibits Hh signaling of Ptch-associated tumors and Hh-stimulated wt Ptch and Ptch$^{-/-}$ cells (17, 26). Moreover, we demonstrated that calcitriol inhibits Hh signaling downstream of Ptch but upstream of Gli supposedly at the level of Smo. Thus, calcitriol does not inhibit Hh signaling in Smo$^{-/-}$ cells, but efficiently inhibits Hh signaling after transfection with a SMO expression plasmid. Beyond that calcitriol inhibits Shh-induced Hh signaling activity in vitamin D receptor (Vdr) knock-out cells resembling a Vdr-independent mechanism of calcitriol-mediated Hh signaling inhibition (17).

Due to the facts that (a) Ptc shows similarities to sterol transporters (10, 11), (b) Ptc$^{-/-}$ cells are unable to secrete Hh-inhibitory factors (16, 17), (c) Smo activity is controllable by oxysterols (15, 24, 25) and (d) the natural occurring oxysterol calcitriol efficiently inhibits Hh signaling (17, 26) it is tempting to speculate that calcitriol might be an endogenous mediator of Ptch/Smo interaction and Smo inhibition.

In this study we analyzed the molecular mechanisms of the calcitriol-mediated inhibition of Hh signaling. Mass spectrometric assays and media transfer experiments demonstrate that Ptc is dispensable for the synthesis of calcitriol from its precursor vitamin D$_3$. However, it is essential for calcitriol release into the extracellular space. In addition, functional competition assays, fluorescence-based replacement studies, and analysis of the activity of several Smo variants show that calcitriol does neither bind to the 7TM of Smo nor to its CRD but efficiently inhibits the translocation of Smo into the primary cilia in Ptc$^{-/-}$ and SAG-treated NIH-3T3 cells. Beyond that the antitumoral effects of calcitriol are strongly enhanced byitraconazole (ITZ) which was not induced by an ITZ-mediated elevated bioavailability of calcitriol but by a synergism of both compounds in Hh signaling inhibition.

Taken together, our results strengthened our hypothesis that calcitriol is a physiological regulator of Hh signaling activity. Moreover, this study opens new perspectives for combined calcitriol/ITZ-based therapies against Hh-associated cancers.

**Experimental Procedures**

**Compounds**—Vitamin D$_3$ (Sigma, Germany), 25-(OH)D$_3$ (25-hydroxyvitamin D$_3$; Sigma), calctriol (Sigma), CP (Toronto Research Chemicals Inc., Canada), BODIPY-labeled CP (BODIPY-CP) (Toronto Research Chemicals), and 20(S)-OHC (Torcs Bioscience, UK) were dissolved in ethanol (EtOH). ITZ (Sigma), SAG (Sigma) and vismodegib (GDC-0449) (Selleckchem, USA) were dissolved in dimethyl sulfoxide. Tetracycline and sodium butyrate (Sigma) were dissolved in ddH$_2$O and sterile filtered. Final concentrations of all compounds are indicated in the respective experiments.

**Cell Lines**—The fibroblast cell lines Smo$^{-/-}$, Ptc$^{-/-}$, and the parental wt Ptc$^{+/+}$ cells (see Ref. 17 for generation), the murine BCC cell line ASZ001 as well as, the human keratinocyte cell line HaCaT, are described in Refs. 17 and 27–29. NIH-3T3 cells were purchased from ATCC (CRL-1658). Shh light II cells represent NIH-3T3 cells stably transfected with a Gli-responsive firefly luciferase reporter and a constitutively expressed Renilla luciferase (30). The tetracycline-inducible Smo-overexpressing cell line HEK293S was maintained as described in Ref. 24. Induction of ectopic Smo overexpression was performed according to Ref. 24 and was confirmed by Western blot using anti-c-myc antibody (A-14, Santa Cruz). Shh-N-conditioned medium (Shh-N-CM) or respective control medium were obtained from HEK293-Shh or HEK293 (ATCC; CRL-1537) cells, respectively, as described (22).

**Plasmids**—The plasmids pFR-Luc (Agilent Technologies, Santa Clara, CA), pRL-CMV (Addgene, Cambridge, MA), pGL-TK (Promega GmbH, Germany), and pEGFP-N1 (BD Bioscience, Germany) were purchased. The SMO and SMO-M2 expressing plasmids and the plasmids pCMV-BD-RRα and pCMV-AD-VDR for the calcitriol-sensitive mammalian two-hybrid (M2H) assay have been described previously (30–32).

For generation of wt Smo and Smo$^{ACRD}$ expression plasmids cherry-gene fused mSmo$^{W113Y}$ (mSmo$^{W113Y}$-cherry) was amplified from the plasmid pHAGE mSmo$^{W113Y}$-cherry (19) by an overlap-extension PCR and subcloned into pMSCV (Takara Bio Europe/Clontech, France). Afterward the W113Y mutation was reversed to the wt Smo sequence using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies). To generate the Smo$^{ACRD}$ mutant plasmid the CDR sequence was deleted by an overlap extension PCR. Primer sequences are available upon request. The integrity of the subcloned and modified sequences was verified by Sanger sequencing.

**Generation of Smo$^{wt}$ and Smo$^{ACRD}$ Expressing Cell Lines**—For generation of Smo$^{wt}$ or Smo$^{ACRD}$ expressing cells 50% confluent Platinum E cells (kindly provided by M. Engelke, Institute of Cellular and Molecular Immunology, Goettingen, Germany) were transfected with 2.5 μg of retroviral Smo$^{wt}$ and Smo$^{ACRD}$ expression plasmids in 400 μl of culture medium of the target cell line and 5 μl of Rotinfekt (Carl Roth GmbH Co. KG, Germany). After 48 h the virus-containing supernatants were harvested, sterile-filtered (0.45 μm pore size), and 2.1 diluted with culture medium of the target cell line. After the addition of 3 μg/ml of Polybrene (Sigma) this medium was applied to a 50% confluent 5-cm dish of the target cell line. Next day the medium was changed and after an additional 24 h 2 μg/ml of puromycin was added to select for transduced cells.

**Cell Culture Experiments**—For gene expression analysis or Annexin V/propidium iodide assays (BD Biosciences) cells were seeded at densities of 40,000 or 240,000 cells/well in 24-well or 6-well plates, respectively. For 5-bromo-2′-deoxyuridine (BrdU) incorporation (Roche Diagnostics) or WST-1 (Roche Applied Science) cells were seeded at densities of 8,000 or 7,000 cells/well in 96-well plates, respectively. After 24 h, the cells were washed and incubated for an additional 48 h with the respective growth medium supplemented with the compounds or solvent as indicated in the respective experiments. For ITZ treatment the culture medium was changed after 24 h to medium supplemented with 1.5% BSA (bovine serum albumin).
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BrdU pulse was conducted for the last 22 h of the incubation period. BrdU incorporation, WST-1 and Annexin V/propidium iodide assays were performed according to the manufacturer’s instructions. BrdU incorporation and WST-1 assays were analyzed using a microplate reader (SynergyMX, BioTek Instruments, Inc.). Annexin V/propidium iodide assay was performed as described (33).

For gene expression analyses of NIH-3T3 150,000 cells were seeded per well of a 6-well plate in DMEM containing 10% FCS and 1% PS. The following day the treatment procedure was adopted from the protocol used for immunofluorescent-based detection of Smo accumulation in primary cilia (see below).

For analyzing ciliary localization of Smo NIH-3T3 or Ptch−/− cells were seeded in DMEM containing 10% FCS and 1% PS at a density of 250,000 cells per well of a 4-chamber Culture Slide (Falcon). After 7 h NIH-3T3 cells were starved for additional 24 h in DMEM containing 0.5% FCS and 1% PS followed by 16 h treatment in starvation medium containing the compounds or solvent as indicated in the respective experiments. Ptch−/− cells were treated 7 h after seeding without any additional starvation step for 16 h in starvation medium containing the compounds or solvent as indicated in the respective experiments.

For assaying the concentration-response curves (CRC) Shh light II cells were seeded in 96-well plates at a density of 5,000 cells/well in growth medium. For the determination of CRC for one agonist and one antagonist of Hh signaling Shh light II cells were starved for 24 h in DMEM supplemented with 0.5% FCS and 1% PS 1 day after seeding. Treatments were conducted in DMEM containing 0.5% FCS. For the analysis of two Hh signaling inhibitors the cells were incubated in Shh-N-CM 1 day prior to the treatment. Treatments with the respective compounds or solvents were conducted in Shh-N-CM for 48 h. Afterward dual-luciferase assays (Promega) were performed according to the manufacturer’s instructions using a microplate reader (SynergyMX).

For transient transfection of Smo−/− cells with SMO or SMO-M2 expression plasmids 25,000 cells were seeded per well of a 24-well plate. The next day the Smo−/− cells were transfected with SMO or SMO-M2 expression plasmids using a 3:1 ratio of Rotifect:DNA (µg) (Carl Roth) according to the manufacturer’s instructions. After 24 h the transfected cells were treated with the compounds or solvent as indicated in the respective experiments.

Medium Transfer Experiments—For medium transfer experiments 2,000,000 wt Ptch or Ptch−/− cells were loaded with calcitriol by incubation in 10 ml of pre-warmed culture medium supplemented with 100 ml calcitriol or solvent for 1 h at 37 °C in a 50-ml tube (Sarstedt AG & Co., Germany) rotating in a hybridization oven. After the loading procedure the cells were washed two times with 20 ml of cold 1× PBS and seeded at a density of 200,000 cells/well in 6-well plates in pre-warmed culture medium without calcitriol supplementation for 4, 6, or 8 h. Afterward the conditioned medium was harvested and sterile filtrated. The same loading and conditioning procedure was performed with supplemented medium without cells to control the adherence of calcitriol to the used plastic ware. Conditioned media from wt Ptch and Ptch−/− were stored at 4 °C for up to 1 week (see Fig. 1b for procedure).

To analyze the calcitriol content of the conditioned medium, 200,000 NIH-3T3 cells/well were seeded in 6-well plates. The next day the cells were transfected with components of a mammalian two-hybrid (M2H) system for assaying the heterodimerization of VDR and RXRα (retinoic X receptor α) upon ligand binding (32). The firefly luciferase reporter vector (pFR-Luc), expression vectors encoding RXRα bait (pCMV- BD-RXRα) and VDR prey (pCMV-AD-VDR) fusion constructs, and pRL-TK were transfected in a ratio of 50:5:5:1 using a 3:1 ratio of Rotifect:DNA (µg) (Carl Roth). 6 h after transfection the cells were trypsinized and transferred to 96-well plates (70% confluence). The next day the transfected NIH-3T3 cells were incubated with 100 µl of conditioned medium from wt Ptch cells, Ptch−/− cells or no cell control for 16 h. Finally, dual-luciferase assays (Promega) were performed according to the manufacturer’s instructions using a microplate reader (SynergyMX).

Flow Cytometric-based Replacement Studies—Flow cytometric-based replacement studies using BODIPY-CP were performed in accordance with Chen et al. (20). In brief, ectopic Smo overexpression in HEK293S cells was induced by incubating cells grown to 70% confluence in DMEM/F-12 (Life Technologies GmbH, Germany) supplemented with 10% FCS, 1% PS, 1 µg/ml of tetracycline, and 5 mM sodium butyrate for 48 h. Afterward, the cells were incubated for 4 h with the compounds as indicated in the respective experiments. Subsequently the cells were washed, trypsinized, and centrifuged by 350 × g for 8 min. The cells were washed again and resuspended in 350 to 500 µl of phenol red-free DMEM (Life Technologies) supplemented with 0.5% FCS. Flow cytometric measurement was performed within 2 h on an LSR II flow cytometer (BD Bioscience). Per sample 50,000 cells were counted. Data acquisition and analysis was performed using the BD FacsDiv (BD Biosciences, Pharmingen) and FlowJo (Treestar Ashland) softwares. Experiments were performed in duplicates and repeated at least three times. The data were analyzed from the cumulative distribution function, which reflects the percentage of fluorescence intensity of cells. Each sample was normalized to BODIPY-CP single-treated, tetracycline-induced cells (set to 100%). The respective bar graphs indicate the mean fluorescence intensity of each sample.

Immunofluorescence Staining—Cells were fixed for 10 min with 2% paraformaldehyde at room temperature and for additional 5 min with methanol at −20 °C. After permeabilization with 1× PBS containing 0.5% Triton X-100 unspecific antigens were blocked with 4% BSA in 1× PBS containing 0.1% Tween 20 (PBST) for 1 h. Subsequently the cells were stained for 1 h with anti-acetylated tubulin (Sigma, T6793, 1:500) and anti-Smo (Abcam, ab38686, 1:1000) antibodies. As secondary antibodies FITC-conjugated anti-mouse (Sigma, 1:200) and Cy3-labeled anti-rabbit antibodies (Jackson ImmunoResearch, 1:400) were used. Cells were mounted with ProLong Gold anti-fade reagent with DAPI (Life Technologies) and analyzed by fluorescence microscopy (Olympus BX60, equipped with U-RLF-T). Percentages of Smo positive cilia were calculated by counting all Smo positive and Smo negative primary cilia from
at least six visual fields (×600 magnification) of three independent experiments conducted in duplicates. Fluorescence images at ×1000 magnification were acquired by using a Color View camera (Soft Imaging System) and the software CellSens (Olympus Life Science, Germany). Images were processed with Adobe Photoshop CS5.

Reverse Transcription and Quantitative Real Time-PCR Analyses—Total RNA was extracted using TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. cDNA synthesis and primer combinations used for quantification of 18S rRNA, Gli1, and 25-hydroxyvitamin D3-24-hydroxylase (24-hydroxylase, Cyp24a1) transcripts by quantitative RT-PCR were described previously (17, 34, 35). Gli1 and Cyp24a1 transcript levels were normalized to the transcript levels of 18S rRNA, after determining the respective amounts by the standard curve method. Each sample was measured in triplicates. Graphs represent the mean value of all measurements.

Mass Spectroscopy of Vitamin D3 Derivatives—For the detection of the vitamin D3 metabolites, vitamin D3, 25,26,27-13C3 supplemented with 40 ng/ml of internal standard 25-hydroxyvitamin D3, and calcitriol in cells, cell lysate extracts were analyzed via liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Wt Ptch and Ptch−/− fibroblasts were seeded at a density of 200,000 cells/well in 6-well plates. After starvation of the cells in FCS-free DMEM for 24 h, the cells were treated with FCS-free DMEM supplemented with the respective compounds or solvents for the time points indicated in the respective experiments.

Afterward, the medium was removed, cells were washed with 1× PBS and scraped using a 60% methanol/water (v/v) mixture supplemented with 40 ng/ml of internal standard 25-hydroxyvitamin D3 (25,26,27-13C3) (Cambridge Isotope Laboratories). Scraped cell/solvent suspensions were immediately transferred into a dry ice-cooled reaction tube. The wells were rinsed again with 40% methanol/water (v/v). The cell lysates were stored at −80 °C until analysis.

Prior to LC-MS/MS analysis vitamin D3 metabolites were extracted from the cell/solvent samples via offline solid phase extraction using reversed phase-based cartridges. LC-MS/MS analysis was performed by a 1260 HPLC system (Agilent Technologies) coupled to a Q Trap 5500 mass spectrometer (AB Sciex), which was controlled by Analyst 1.6 software (AB Sciex). The vitamin D3 metabolites were baseline separated on a reversed phase column and detected by multiple reaction monitoring (MRM) after electrospray ionization.

In case of calcitriol, 24,25-(OH)2D3, and 25-(OH)D3 three MRMs were monitored, whereas only one MRM was monitored for vitamin D3. The peaks of the respective vitamin D3 metabolites (the sum of respective MRMs or the single MRM, respectively) were integrated by the Analyst 1.6 software and the areas were normalized by the peak area of the internal standard. The complete procedure will be published in detail elsewhere.3

3 S. Weber, B. Linder, C. Prehn, H. Hahn, A. Uehmann, G. Möller, and J. Adamski, manuscript in preparation.

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Generation of Concentration-Response Curves (CRC) and Fa-CI Plots—CRC were calculated using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). For each individual sample the firefly luciferase activity was normalized to the respective Renilla luciferase value. Samples were normalized to solvent-treated controls, which were set to 100%. Afterward the values were logarithmized, the range of the data were normalized by setting the lowest and highest values to 0 and 100%, respectively, and the curves were fitted by non-linear regression using the log(inhibitor) versus response—variable slope (four parameters) or the respective log (agonist) function. Curve fitting was calculated using 1,000 iterations.

For generation of Fa-CI plots the fractional inhibition (Fa) was calculated by normalizing to the data from 0 (lowest inhibition, i.e. solvent) to 1 (highest inhibition) and the combination index (CI) was calculated by the online software CompuSyn using the non-constant ratio setting (compubiosyn.com) (36). The data were plotted using GraphPad Prism 6.

Statistics—All statistical analyses were performed using GraphPad Prism 6. Statistical differences in ED50 or Fa-CI plots were calculated by the extra sum-of-squares F-test. No constrain or weighing was applied. Results from the M2H assays, assays using Shh light II or Smo−/− cells, and analyses of ciliary Smo accumulation were corrected for outliers using the ROUT-method (Q = 1%). After verification of Gaussian distribution by the D’Agostino and Pearson omnibus normality test one-way analysis of variance using the Holm-Sidak’s multiple comparison test or Dunnett’s multiple comparisons test were conducted. The combination treatments of ASZ001 cells were tested using a non-parametric one-way analysis of variance test (Kruskal-Wallis test). All other results were tested using a non-parametric Mann-Whitney test.

Results

Ptch Is Required for the Extracellular Availability of Calcitriol but Dispensable for the Synthesis from Its Precursors—We first investigated whether calcitriol is produced and released from Ptch-expressing cells. During calcitriol synthesis vitamin D3 is mainly hydroxylated to 25-(OH)D3 by the vitamin D 25-hydroxylase (Cyp27a1). This is followed by hydroxylation of 25-(OH)D3 to calcitriol by the 1α-hydroxylase (Cyp27b1) (37). To examine if Ptch expression is required for the synthesis of calcitriol from its precursor vitamin D3 we quantified the intracellular levels of vitamin D3, 25-(OH)D3, and calcitriol in wt Ptch and Ptch−/− fibroblasts after vitamin D3 supplementation using mass spectrometric analyses (MS) (see “Experimental Procedures”). In both cell lines significantly elevated intracellular vitamin D3 levels were already measured after 0.5 h (Fig. 1a). Intracellular vitamin D3 levels peaked after 1 h, declined thereafter to a plateau, and remained stable until 6 h after its supplementation (Fig. 1a). Intracellular 25-(OH)D3 was first detected after 1 h vitamin D3 supplementation in wt Ptch cells and after 0.5 h in Ptch−/− cells (Fig. 1a). It significantly and continuously increased until the last time point at 6 h (Fig. 1a). Significant hydroxylation of 25-(OH)D3 to calcitriol was detected for the first time 4 h after vitamin D3 treatment start in wt Ptch cells and after 2 h in Ptch−/− cells (Fig. 1a). Both cell lines showed a continuous increase of intracellular calcitriol levels, which
compared with the 0.5 h value, reached significance after 6 h (Fig. 1a). Because the experiments revealed that both wt Ptch and Ptch\(^{-/-}\) cells synthesize calcitriol from its precursor vitamin D\(_3\) and showed comparable vitamin D\(_3\) uptake and catalytic behavior we concluded that Ptch is dispensable for calcitriol synthesis.

We next analyzed if wt Ptch and Ptch\(^{-/-}\) fibroblasts differ in their calcitriol releasing features by analyzing conditioned media from the respective cell lines. Without supplementation of calcitriol or its precursors endogenous calcitriol levels of in vitro cultured cells are very low and not detectable by non-derivatized MS.\(^3\) Therefore, we first loaded both cell lines with calcitriol. After thorough washing steps, the cells were cultured in calcitriol-free media. Thereupon the media were analyzed for its ability to promote recruitment of the VDR co-receptor, RXR\(_a\), in a M2H reporter system (Fig. 1b; see “Experimental Procedures”), which is a very robust and highly sensitive read-out for calcitriol levels (Fig. 1c). Medium conditioned by calcitriol-loaded wt Ptch cells highly induced the activation of VDR/RXR\(_a\) heterodimerization, whereas medium from calcitriol-loaded Ptch\(^{-/-}\) cells was incapable to induce this to a comparable extend (Fig. 1d). Only a minimal activation of the M2H reporter assay by media from Ptch\(^{-/-}\) fibroblasts was observed (Fig. 1d), which might rather reflect unspecific membrane shuttling of calcitriol then active transport through the cell membrane of Ptch\(^{-/-}\) cells.

These data demonstrate that Ptch\(^{-/-}\) cells, despite their ability to synthesize calcitriol from its precursor vitamin D\(_3\), lose their properties to release calcitriol to the extracellular space. Together with the fact that calcitriol efficiently inhibits Hh signaling at the level of Smo in a Vdr-independent manner (17) these data strengthened the hypothesis that calcitriol is a signal transducing molecule, which transfers Hh signaling inhibiting signals from Ptch to Smo.

Calcitriol Efficiently Inhibits the Translocation of Smo into and Facilitates the Removal of Smo from the Primary Cilium—One of the earliest hallmarks of the Hh signaling pathway activation (e.g. by Shh or SAG) is the accumulation of Smo in the primary cilium (38). Because our previous work has shown that calcitriol efficiently decreases Gli1 transcription as a marker of reduced Hh signaling activity (17) (see also Fig. 2a) we analyzed if calcitriol is also capable to prevent ciliary translocation of Smo upon Hh signaling activation.

Hence Smo localization in the primary cilia of solvent-, calcitriol-, SAG-, SAG/calcitriol-, or SAG/vismodegib-treated NIH-3T3 cells were visualized by double immunofluorescence using anti-acetylated tubulin and anti-Smo antibodies. SAG treatment resulted in a strong ciliary localization of Smo, whereas calcitriol and vismodegib significantly reduced the SAG-induced recruitment of Smo into the cilia (Fig. 2, b and c). Similar results were obtained after calcitriol treatment of Shh-induced NIH-3T3 cells (data not shown). Beyond that we also tested the effect of calcitriol on ciliary localization of Smo in Ptch\(^{-/-}\) cells, which show a constitutive ciliary accumulation of Smo (Fig. 2, d and e) and thus a constitutive pathway activation (17). Interestingly, 10 nM calcitriol were as potent as 1 \(\mu M\) vismodegib in reducing the accumulation of Smo in the primary cilium (Fig. 2, d and e).

These data show that calcitriol regulates ciliary translocation of Smo. Moreover, because calcitriol also promotes the removal of Smo from the cilia in Ptch\(^{-/-}\) cells these results confirmed our previously made observations that calcitriol acts at the level of Smo.

Calcitriol Efficiently Inhibits CRD-deleted Smo—The CRD of Smo has been described as an exclusive binding site for oxytetracyclines that are discussed to be physiological regulators of Hh signaling activity (18, 19). To analyze if calcitriol inhibits Hh signaling by binding to the CRD we generated Shh light II or Smo\(^{-/-}\) cell lines stably expressing wt Smo or a CRD-deleted variant (Smo\(^{\Delta\text{CRD}}\)).

In contrast to untransduced cells, wt Smo or Smo\(^{\Delta\text{CRD}}\)-expressing Shh light II cells showed a 5–10-fold higher basal Hh reporter activity, which was barely increased by SAG or Shh treatment (Fig. 3, a and b). Similar observations have been made by Myers et al. (39). Nevertheless the high Hh signaling activation of wt Smo or Smo\(^{\Delta\text{CRD}}\)-expressing cells enabled us to test the Hh signaling inhibiting properties of calcitriol without binding of activating molecules to Smo that may have preoccupied potential calcitriol binding sites. Both, calcitriol and CP treatment resulted in a significant inhibition of Hh signaling in SAG or Shh-treated untransduced Shh light II cells as well as in wt Smo and Smo\(^{\Delta\text{CRD}}\)-expressing Shh light II cells. However, compared with CP, the inhibitory effects of calcitriol were much more efficient in all settings (Fig. 3, c and d). Similar results were obtained by Gli1 expression analyses of calcitriol or CP-treated wt Smo or Smo\(^{\Delta\text{CRD}}\)-expressing Smo\(^{-/-}\) cells, which do not express endogenous Smo (Fig. 3, e and f). Moreover, calcitriol was also capable to inhibit Hh signaling in Smo\(^{-/-}\) cells expressing the constitutively active SMO-M2 variant (Fig. 3g) (31). These data suggest that Hh signaling inhibi-

![FIGURE 1. Loss of Ptch does not influence the vitamin D\(_3\) metabolism but disturbs the calcitriol release from the cell. a, MS-based intracellular quantification of the vitamin D\(_3\) metabolites vitamin D\(_3\) (solid line), 25-(OH)D\(_3\) (dashed line), and calcitriol (dotted line) in wt Ptch (left) and Ptch\(^{-/-}\) (right) murine adult fibroblasts after 0.5, 1, 2, 4, and 6 h incubation with 10 \(\mu M\) vitamin D\(_3\). The data were normalized to the respective solvent-treated controls for each time point. b, schematic representation of the procedure for medium transfer experiments. Freshly trypsinized wt Ptch and Ptch\(^{-/-}\) cells were calcitriol-loaded in a rotating 50-ml reaction tube filled with medium supplemented with 100 nm calcitriol for 1 h (“loading phase”). Solvent-supplemented medium served as control. After washing, the cells were plated in calcitriol-free medium for 4, 6, or 8 h, respectively (“conditioning phase”). The conditioned media were sterile-filtered and transferred to M2H-transfected NIH-3T3 cells. Finally, dual-luciferase-assays were performed (see “Experimental Procedures”). c, sensitivity of the reporter systems used to assess calcitriol-mediated Hh pathway inhibition (gray bars, left y axis) and calcitriol-stimulated VDR/RXR\(_a\) interaction (white bars, right y axis). Data are shown from one representative experiment. For each reporter system values of solvent-treated cells were set to 1 (dashed line). Data are represented as mean \(\pm\) S.E. d, dual-luciferase-based analyses of M2H-transfected NIH-3T3 cells after a 6-h incubation with conditioned media from calcitriol-loaded wt Ptch or Ptch\(^{-/-}\) fibroblasts. The cells were loaded as described in b. As a background control calcitriol loading was carried out using culture media without cells (no cell control). The conditioned medium was prepared in triplicates, the medium from no cell controls in duplicates. The treatment of the transfected cells was conducted in duplicates for each medium. Similar results were carried out after 4 and 8 h incubation of M2H-transfected NIH-3T3 cells with wt Ptch or Ptch\(^{-/-}\) conditioned media (data not shown). Data represent normalized firefly/Renilla luciferase activity. The solvent-loaded control for each cell line and time point was set to 1. All data represent at least 3 independent experiments represented as mean \(\pm\) S.E. *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\); ****, \(p < 0.0001\) compared with solvent control; \(+, p < 0.05\); ++, \(p < 0.01\); ++++, \(p < 0.001\); +++, \(p < 0.0001\) compared with 0.5 h treatment.}
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ITZ Enhances the Antitumoral Potential of Calcitriol—Calcitriol efficiently inhibits Hh signaling and proliferation and stimulates differentiation processes in Ptch-associated cancers in mice (17, 26). Moreover, the inhibitory effect of calcitriol on Hh signaling is still detectable when Hh signaling is constitutively activated due to the expression of the oncogene SMO-M2 (Fig. 3g). These facts suggest that calcitriol might be an attractive candidate for the development of therapies against Hh-associated tumors. Thus we tested if the antitumoral effects of calcitriol could be enhanced by a combination with the potent Smo-inhibitor ITZ (40) or with CP that binds Smo almost exclusively at the 7TM (18).

Single calcitriol, ITZ, or CP treatments of the murine basal cell carcinoma cell line ASZ001 efficiently inhibited Hh signaling activity as revealed by reduced Gli1 mRNA expression (Fig. 4, a, d, and g). Although single calcitriol or ITZ treatments using BSA-supplemented medium did not significantly alter BrdU

FIGURE 2. Calcitriol prevents Smo accumulation in the primary cilia. a, relative quantification of Gli1 expression of NIH-3T3 cells treated for 16 h with solvent 100 nM SAG, 100 nM calcitriol, or 100 nM SAG and 100 nM calcitriol. Data are represented as mean ± S.E. of 2 independent experiments conducted in triplicates. **, p < 0.01; ****, p < 0.0001. b–e, immunofluorescence-based analyses of Smo accumulation in the primary cilia of (b and c) NIH-3T3 cells treated for 16 h with solvent, 100 nM calcitriol, 100 nM SAG, 100 nM SAG, and 1 μM vismodegib (SAG/vismo) or 100 nM SAG and 100 nM calcitriol (SAG/calcitriol) and (d and e) Ptc+/− cells treated for 16 h with solvent, 10 or 100 nM calcitriol, or 1 μM vismodegib (vismo). c, and e, representative images of anti-acetylated tubulin (AT, green) and anti-Smo (red) antibody-stained cells. Nuclei were visualized with DAPI (blue). Top rows, images of entire cells in ×1000 magnification; bottom rows, magnification of boxed regions (white squares) containing cilia. b and d, Percentages of Smo containing primary cilia relative to (b) NIH-3T3 cells treated with 100 nM SAG (100%) or (d) to solvent-treated Ptc+/− cells (100%). Numbers indicate the total numbers of counted Smo positive cilia/all counted cilia. At least all primary cilia in 6 visual fields of three independent experiments conducted in duplicates were counted. Data are represented as mean ± S.E. ****, p < 0.0001 compared with SAG-treated NIH-3T3 (b) or solvent-treated Ptc+/− (d) cells.
incorporation (Fig. 4, c and i), the combined calcitriol/ITZ treatment significantly decreased both proliferation rate and Hh signaling activity (Fig. 4, a and c). This was accompanied by a significant increase in the expression of the Vdr-downstream target Cyp24a1 indicating that ITZ enhances the Vdr-activating properties of calcitriol (Fig. 4b). In contrast, the combined treatments with calcitriol/CP (Fig. 4, d–f) or CP/ITZ (Fig. 4, g–i) did not lead to cooperative effects. Because effectiveness of ITZ-mediated Hh signaling inhibition is quenched by FCS (40) (own observations), ITZ treatment was conducted under serum starvation, whereas calcitriol/CP-treated cells were cultured in media containing 2% FCS (please note the differences in calcitriol-mediated inhibition of proliferation under FCS starved (Fig. 4c) and 2% FCS conditions (Fig. 4f)).

Changes in mitochondrial activity or apoptosis were not seen upon any of the treatments (data not shown). Together these
data suggest that the enhanced effects of the calcitriol/ITZ treatment on cellular proliferation and Hh signaling activity could be either based on a modification of the bioavailability of calcitriol, a direct interplay of the compounds in Hh signaling inhibition, or a combination of both mechanisms.

ITZ Does Not Influence Calcitriol Bioavailability—Azoles have been reported to inhibit the activity of cytochrome P450 enzymes, like the calcitriol-degrading 25-hydroxy vitamin D₃-24-hydroxylase (24-hydroxylase, Cyp24a1) (41, 42). We therefore tested if ITZ might enhance the bioavailability of calcitriol by inhibition of Cyp24a1. If this would be the case, a calcitriol/ITZ treatment should result in increased intracellular calcitriol levels compared with single calcitriol treatment. Unfortunately, it was not possible to measure the calcitriol degradation product 1α,24,25-(OH)₃D₃ by non-derivatized MS-based assays in our hands. However, because Cyp24a1 also catalyzes the degradation of 25-(OH)D₃ to 24,25-dihydroxy vitamin D₃ (24,25-(OH)₂D₃) (37, 43) we assumed that an ITZ-mediated inhibition of Cyp24a1 activity should also lead to a delayed 25-(OH)D₃ degradation and to reduced 24,25-(OH)₂D₃ levels. To follow this line of investigation we incubated ASZ001 cells with 25-(OH)D₃ and ITZ and determined the intracellular 25-(OH)D₃ and 24,25-(OH)₂D₃ levels by LC/MS-MS analyses. This approach revealed that the combination of 25-(OH)D₃/ITZ did neither result in significantly increased 25-(OH)D₃ levels nor in reduced 24,25-(OH)₂D₃ levels when compared with 25-(OH)D₃ treatment (Fig. 5, a and b). In addition, ITZ did not influence the bioavailability of calcitriol in the cells, because the calcitriol levels were comparable after single calcitriol and the combined calcitriol/ITZ treatment (Fig. 5c). Similar results were obtained in HaCaT cells (data not shown) that have an intact vitamin D₃ metabolism (44).

Together, these data indicate that the combined effect of a calcitriol/ITZ treatment was not a result of delayed calcitriol degradation due to an ITZ-mediated Cyp24a1 inhibition.

Calcitriol and ITZ Synergize in Smo Inhibition—Because calcitriol and ITZ are efficient inhibitors of the Hh signaling pathway at the level of Smo (17, 40) we next tested if these compounds might inhibit Hh signaling synergistically. For this purpose we generated CRC of calcitriol in the presence of ITZ or other known Smo modulating molecules using Shh light II cells. Neither increasing amounts of the Hh signaling activators SAG and 20-(S)OH.C or the inhibitors CP and vismodegib altered the IC₅₀ of calcitriol significantly (Fig. 6, a–d; Table 1). Vice versa increasing amounts of calcitriol did not impact the
half-maximal effective dose (ED$_{50}$) of CP, vismodegib, SAG, or 20(S)-OHC (Table 2, CRC not shown). In contrast, increasing amounts of ITZ shifted the half-maximal inhibitory concentration (IC$_{50}$) of calcitriol significantly from $\sim$1.02 to $\sim$0.13 nM (Fig. 6c; Table 1). Similar results were obtained for the IC$_{50}$ of ITZ in the presence of increasing calcitriol concentrations (IC$_{50}$ shift of ITZ from $\sim$0.81 to $\sim$0.59 μM; Table 2). Analysis of the CI (36) furthermore, revealed that the combination of low calcitriol amounts (0.5 to 2.5 nM) with low, moderate, and high ITZ concentrations resulted in a synergistic inhibition of Hh signaling in Shh light II cells (Fig. 6d).

Because none of the exclusive 7TM (vismodegib, SAG) or CRD (20(S)-OHC) binders influenced the IC$_{50}$ of calcitriol and thus did not compete with calcitriol for the binding of Smo at these sites, we assumed that calcitriol occupies an alternative Smo binding site. In addition, the synergistic inhibition of Hh signaling by calcitriol and ITZ indicated that calcitriol and ITZ bind to different Smo sites. To validate this hypothesis that calcitriol binds to Smo outside the 7TM or CRD, we tested if calcitriol or its precursors (vitamin D$_3$ or 25-(OH)D$_3$) may occupy Smo to a hitherto undefined binding site.

**Discussion**

Previous studies implicated the existence of small naturally occurring oxysterols, whose availability is controlled by Ptch-mediated transport and that activate Smo by binding to the CRD (6, 18, 19, 45, 46). However, studies showing that Shh-induced activation of Hh signaling results in internalization of Shh-bound Ptc (14, 47) questions this prediction, because loss of membrane-associated Ptc should also result in loss of secretion of Smo-activating molecules. Therefore, it rather seems feasible that Ptc may secrete a negative regulator of Smo activity. Indeed, vitamin D$_3$ or its derivatives may potentially fulfill the requirements for such a molecule (16, 17, 48, 49). However,
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The direct proof and evidence for those assumptions are still missing (18, 19, 39). In this study we provide evidence that calcitriol, a physiologically existing oxysterol, meets the characteristics of a Ptc+/ Smo signal transmitter molecule that is secreted by Ptc+ and negatively regulates Smo activity. First, we demonstrate that calcitriol is endogenously synthesized from its precursor vitamin D3 and that the exit of Smo from the cilia. Together we conclude that albeit Ptc- deficient cells are able to synthesize calcitriol from its precursor vitamin D3 these cells are incapable of secreting it.

Besides, a Ptc/Smo interacting molecule should also be able to modulate the actions of Smo. Previously we have shown that calcitriol reduces Hh signaling activity measured by Gli1 mRNA reduction (17). Our new results confirm this conclusion because calcitriol inhibits the accumulation of Smo in primary cilia. Together with the fact that calcitriol also suppresses ciliary Smo localization in Ptc-/- cells, our data are in strong favor of a direct interaction of calcitriol and Smo and exclude that calcitriol acts downstream of Smo. Finally, because Ptc-/- cells normally show constitutive ciliary Smo accumulation these results furthermore suggest that calcitriol induces the exit of Smo from the cilia.

Several reports demonstrated the binding of Smo-activating oxysterols to the CRD of Smo (18, 19, 39). This fascinating fact raises the question if this binding pocket might also be a target site for Smo-inhibiting molecules, like calcitriol (17, 26). Nevertheless, a study by Myers et al. suggested that neither the CRD nor the 7TM are the major sites of regulation by Ptc (39). Additionally the authors discussed that oxysterols like 20(S)-OHC or 7-ketocholesterol derivatives are unlikely to subserve the regulatory functions of Ptc (39). In accordance with this hypothesis our novel data provide evidence that neither the CRD nor the 7TM are required for calcitriol binding or calcitriol-mediated Smo inhibition. This conclusion is based on the finding that calcitriol-mediated inhibition of Hh signaling can still be observed in cells expressing SmoCRD. Moreover, we show that calcitriol does not compete with BODIPY-CP, which is known to bind to the 7TM and to a lesser extent to the CRD (18). The assay furthermore revealed that the calcitriol precursors vitamin D3 and 25-(OH)D3 also did not compete with BODIPY-CP for Smo binding. This is in contrast to Bijlsma et al. (16) who showed the contrary. However, because all other compounds (i.e. SAG (22); vismodegib and 20(S)-OHC (18); and ITZ (40)) in our set up showed the described competition with BODIPY-CP we conclude that neither calcitriol nor its precursors bind to the CRD or the 7TM of Smo. Our data furthermore show that calcitriol acts as a non-competitive inhibitor for SAG and 20(S)-OHC-induced Hh signaling activity.

The half-maximal inhibitory concentration (IC50) of calcitriol on Hh signaling activity in combination with CP, vismodegib (vismo), ITZ, SAG, or 20(S)-OHC was calculated from the experiments shown in Fig. 6 as described under “Experimental Procedures.” To detect significant differences of the IC50 of single calcitriol and combined calcitriol/Smo modulator treatments of extra sum-of-squares F tests were conducted.

| IC50 of calcitriol | p value |
|--------------------|---------|
| Without CP         | 0.55    | 0.2014 |
| 0.1 μM CP          | 0.77    |        |
| 0.2 μM CP          | 1.18    |        |
| 0.3 μM CP          | 0.45    |        |
| 0.5 μM CP          | 1.01    |        |
| Without vismo      | 0.46    | 0.0739 |
| 2.5 nM Vismo       | 0.18    |        |
| 10 nM Vismo        | 0.67    |        |
| 40 nM Vismo        | 0.92    |        |
| Without ITZ        | 0.02    |        |
| 0.1 μM ITZ         | 1.02    |        |
| 0.5 μM ITZ         | 0.27    |        |
| 2 μM ITZ           | 0.13    |        |
| Without SAG        | ND*     | 0.7716 |
| 5 nM SAG           | 1.38    |        |
| 50 nM SAG          | 1.50    |        |
| 100 nM SAG         | 1.14    |        |
| Without 20(S)-OHC  | ND      | 0.3725 |
| 2 μM 20(S)-OHC     | 0.13    |        |
| 5 μM 20(S)-OHC     | 1.36    |        |
| 10 μM 20(S)-OHC    | 1.11    |        |

* ND, not determined.

The half-maximal inhibitory concentration (IC50) of CP, vismodegib (vismo), and ITZ and half-maximal effective concentration (EC50) of SAG and 20(S)-OHC in combination with calcitriol on Hh signaling activity were calculated as described under "Experimental Procedures.” To detect significant differences of the IC50 or EC50 of single and combined treatments extra sum-of-squares F tests were conducted.

| IC50 or EC50 | CP | vismo | ITZ | SAG | 20(S)-OHC |
|--------------|----|-------|-----|-----|-----------|
|              | μM | μM    | μM  | μM  | μM        |
| Without calcitriol | 0.25 | 6.00 | 0.81 | 5.40 | 5.16 |
| 0.1 μM Calcitriol | 0.33 | 3.37 | 0.43 | 6.46 | 4.98 |
| 1 μM Calcitriol  | 0.35 | 4.13 | 0.32 | 6.07 | 4.35 |
| 10 μM Calcitriol | 0.55 | 4.20 | 0.59 | 5.03 | 4.66 |
| p value         | 0.0758 | 0.1788 | 0.0075 | 0.6268 | 0.3725 |

The finding that calcitriol-mediated inhibition of Hh signaling can still be observed in cells expressing SmoCRD. Moreover, we show that calcitriol does not compete with BODIPY-CP, which is known to bind to the 7TM and to a lesser extent to the CRD (18). The assay furthermore revealed that the calcitriol precursors vitamin D3 and 25-(OH)D3 also did not compete with BODIPY-CP for Smo binding. This is in contrast to Bijlsma et al. (16) who showed the contrary. However, because all other compounds (i.e. SAG (22); vismodegib and 20(S)-OHC (18); and ITZ (40)) in our set up showed the described competition with BODIPY-CP we conclude that neither calcitriol nor its precursors bind to the CRD or the 7TM of Smo. Our data furthermore show that calcitriol acts as a non-competitive inhibitor for SAG and 20(S)-OHC-induced Hh signaling activity.

FIGURE 6. Calcitriol synergizes with ITZ in inhibition of Hh signaling activity. a-c, e, and f, concentration-response curves of calcitriol in the presence of the Hh inhibitors CP (a), vismodegib (vismo) (b), ITZ (c), or the Hh activators SAG (e) and 20(S)-OHC (f). Data represent normalized firefly/ Renilla luciferase activity. All data represent at least 3 independent experiments measured in triplicates represented as mean ± S.E. d, F2-Cl plot was generated by calculating the CI over the range of the fractional inhibition (F2) of the experimental data shown in c. Shown are the CI values and linear regression of Hh signaling inhibition triggered by 0.5, 2.5, and 5 nM calcitriol (circles, squares, and triangles, respectively) combined with 0.1, 0.5, or 2 μM ITZ plotted against the respective F2. Slopes of 0.5 and 2.5 nM (p = 0.044) as well as 0.5 nM and 5.0 nM calcitriol (p = 0.023) are statistically different. CI > 1, antagonism; C = 1, additive (threshold) CI < 0.7, synergism; CI < 0.3, strong synergism (36).
because calcitriol reduced the maximal degree of Hh pathway activation of both compounds (Fig. 6, e and f) without changing the respective ED_{50} (Table 1; Table 2). This again fosters the assumption that calcitriol binds Smo at a site different from the CRD or 7TM (39, 40). Finally, the observation that calcitriol is capable to inhibit the activity of the mutant SMO-M2, whose mutation site is mapped to the CP-binding pocket (30), strengthened the conclusion that calcitriol acts not at the 7TM.

We also discovered a synergistic inhibition of Hh signaling activity when combining calcitriol with the antifungal azole and Smo inhibitor ITZ (40). Azoles have been reported to inhibit the activity of cytochrome P450 enzymes (41, 42). However, Kim et al. (40) excluded an ITZ-mediated cytochrome P450 regulation, which is implicated in cholesterol biosynthesis. Similarly our data suggest that ITZ does not inhibit the calcitriol-degrading 24-hydroxylase. Indeed combined calcitriol/ITZ treatment led to a significantly higher Cyp24a1 expression levels compared with single calcitriol-treated cells (Fig. 4b). Contrary, our MS-based measurements demonstrated that calcitriol or 24,25-(OH)_{2}D_{3} levels compared with single calcitriol-treated cells and that the level of intracellular 25-(OH)D_{3}, which likewise is degraded by the 24-hydroxylase, was not influenced. However, due to the fact that enhanced Cyp24a1 expression levels rather reflects the general activation of Vdr signaling but does not measure the enzymatic activity of the 24-hydroxylase these results suggest that ITZ might induce Vdr signaling via a separate mechanism but not due to interference of calcitriol catabolizing enzyme activity (e.g. 24-hydroxylase). Thus, we propose that the combined calcitriol/ITZ effects on Smo inhibition are not the result of an ITZ-mediated increase in calcitriol levels.

Besides, ITZ has been suggested to act and inhibit Smo at a site distinct from the CP binding pocket (40). This conclusion is based on the finding that ITZ competes barely with BODIPY-CP for Smo binding (40). However, in our experiments BODIPY-CP, which binds the 7TM as well as the CRD (18), was efficiently replaced by ITZ. Together with the fact that the IC_{50} of ITZ is not affected by the CRD-binder 20(S)-OHC (25) and ITZ act as a non-competitive inhibitor of Hh signaling activity induced by the CRD-binder SAG (40), we propose that ITZ rather binds to the 7TM than to the CRD. This assumption is furthermore strengthened by our findings that ITZ competes with BODIPY-CP binding on Smo.

Calcitriol on the other hand does not compete with BODIPY-CP for Smo binding and represents a non-competi-

FIGURE 7. Calcitriol binds to a site distinct from the CP binding site. Direct competition assays of 10 nM BODIPY-labeled cyclopamine (BD-CP) and the Smo-modulators unlabeled CP (a), ITZ (b), calcitriol (c), and 20(S)-OHC (d) were performed in tetracycline-induced conditional Smo-overexpressing HEK293S cells (induced) as described (24). Solvent-treated, single CP, ITZ, calcitriol, or 20(S)-OHC-treated cells and BODIPY-CP-treated, uninduced HEK293S cells (not induced) served as negative controls. Data acquisition was conducted as described under "Experimental Procedures." BODIPY-CP single treatments were set to 100%. All data represent at least 3 independent experiments measured in duplicates represented as mean ± S.E. *, p < 0.05; **, p < 0.01; ****, p < 0.0001.
itive inhibitor of SAG and 20(S)-OHC-induced Hh signaling activation. Furthermore, it does not synergize with the 7TM binder CP or vismodegib in Smo inhibition. Thus, although we did not demonstrate direct binding of calcitriol to Smo we conclude that calcitriol binds to a Smo site distinct from the 7TM or CRD. This also could explain the synergistic effects with ITZ on Hh signaling inhibition. However, we have to admit that there is still the possibility that calcitriol inhibits the action of Smo and ciliary accumulation by other indirect mechanisms.

Finally, our novel findings that ITZ as well as calcitriol synergistically inhibit Hh signaling may open new perspectives in the treatment of Hh-associated cancers, especially because both compounds are currently being tested in clinical trials for Hh-associated tumors (NCT02120677; NCT01358045). Indeed prospective experiments are needed to optimize the doses of both drugs and to elucidate the functional mechanisms of calcitriol/ITZ synergy in Hh signaling inhibition.

**Author Contributions**—B. L. designed, performed and analyzed the experiments, wrote the paper, and contributed to the preparation of the figures. A. U. conceived and coordinated the study, designed the experiments, analyzed data, wrote the paper, and contributed to the preparation of the figures. S. W. established, performed and analyzed MS-based measurements of vitamin D₃ derivatives. K. D. designed and performed flow cytometric-based replacement studies. J. A. analyzed data and contributed vital reagents and analytical tools and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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