Title
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Permalink
https://escholarship.org/uc/item/4gt2g37h

Journal
ACS omega, 3(9)

ISSN
2470-1343

Authors
Chahal, Kirti Kandhwal
Parle, Milind
Abagyan, Ruben

Publication Date
2018-09-26

DOI
10.1021/acsomega.8b01864

Peer reviewed
Dexamethasone and Fludrocortisone Inhibit Hedgehog Signaling in Embryonic Cells

Kirti Kandhwal Chahal,*†‡ Milind Parle,† and Ruben Abagyan*†‡

*Department of Pharmaceutical Sciences, G. J. University of Science and Technology, Hisar 125001, India
†Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92037, United States

ABSTRACT: The hedgehog (Hh) pathway plays a central role in the development and repair of our bodies. Therefore, dysregulation of the Hh pathway is responsible for many developmental diseases and cancers. Basal cell carcinoma and medulloblastoma have well-established links to the Hh pathway, as well as many other cancers with Hh-dysregulated subtypes. A smoothened (SMO) receptor plays a central role in regulating the Hh signaling in the cells. However, the complexities of the receptor structural mechanism of action and other pathway members make it difficult to find Hh pathway inhibitors efficient in a wide range. Recent crystal structure of SMO with cholesterol indicates that it may be a natural ligand for SMO activation. Structural similarity of fluorinated corticosterone derivatives to cholesterol motivated us to study the effect of dexamethasone, fludrocortisone, and corticosterone on the Hh pathway activity. We identified an inhibitory effect of these three drugs on the Hh pathway using a functional assay in NIH3T3 glioma response element cells. Studies using BODIPY-cyclopamine and 20(S)-hydroxy cholesterol [20(S)-OHC] as competitors for the transmembrane (TM) and extracellular cysteine-rich domain (CRD) binding sites showed a non-competitive effect and suggested an alternative or allosteric binding site for the three drugs. Furthermore, the three steroids showed an additive effect on Hh pathway inhibition when tested in combination with cyclopamine. Our study reports the antagonistic effect of dexamethasone, fludrocortisone, and corticosterone on the Hh pathway using functional assay and confirmed that they do not bind to the CRD or adjacent TM binding cavities of SMO. The study also suggests that dexamethasone could be additionally beneficial as the adjuvant therapy for cancer patients with an established link to the dysregulated Hh pathway.

INTRODUCTION

The hedgehog (Hh) pathway is an important pathway during embryonic development, along with concurrently active ones, such as Wnt or Notch pathways. Dysregulation of the Hh pathway in humans is observed in several developmental diseases and malformations and is found in many cancers. Approximately, one-fourth of all cancers either have a dysregulated Hh pathway or are Hh-dependent for maintenance and growth. The Hh pathway includes several elements. It is activated by Sonic Hedgehog (Shh) protein that is autoprocessed into a shorter and cholesterol-modified bioactive form of the protein (ShhN), that, in turn, binds to the Patched (PTCH) receptor located in or near primary cilium. This binding removes the inhibition of a smoothened (SMO) receptor, a key regulator of the Hh pathway, and induces translocation of the receptor to the plasma membrane of primary cilium. The SMO activation leads to the breakdown of the complex of SUFU and three glioma (Gli)-associated transcriptional regulators. Gli activators move to the nucleus to regulate transcription of various genes. Hh signals induce cell proliferation through the upregulation of N-Myc, cyclin D/E, and FOXM1. Disruption in pathway regulation or feedback mechanisms is associated with the development of cancers including but not limited to basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma. Recent studies have suggested an important agonistic role of cholesterol in Hh pathway regulation. The PTCH sequence is evolutionarily conserved to bacterial transmembrane (TM) transporters. PTCH was proposed to function as a pump for small molecules (such as cholesterol), controlling the activation of SMO. The binding of ShhN to PTCH causes inhibition of cholesterol efflux and increases cholesterol concentration inside the cell, which may be responsible for the induction of SMO translocation from endosomes to the plasma membrane. After translocation of SMO to the primary cilium, an extracellular cysteine-rich domain (CRD) binding site of SMO becomes exposed to its natural ligand. A crystal structure of SMO bound to cholesterol suggested the CRD binding site as the primary binding site for cholesterol.

Corticosteroids and their therapeutic mimics are widely prescribed drugs for many indications, including cancer, because of their anti-inflammatory and immunosuppressive properties. One of their targets is a glucocorticoid receptor (GR) that can modulate the transcription of target genes by...
binding to specific elements of DNA, termed GR elements, and subsequently interacting with the RNA polymerase II. Because of the discovery of cholesterol and other related steroidal compound’s ability to modulate Hh pathway activity, it is vital to study the contribution and mechanism of steroids play in cancer therapy. Corticosteroids are the leading group (comprising of hydrocortisone, prednisone, prednisolone, dexamethasone, and many more) of steroids being used in cancer therapy. They are prescribed to prevent allergic reactions, control chemotherapy-induced nausea and vomiting, increase appetite, reduce pain and swelling in the case of brain cancer, reduce spinal cord compression, or to help in stem cell transplantation. Recently, many steroids have been reported to be SMO agonists along with cholesterol (also crystallized with SMO). Fluorinated glucocorticoids such as halcinonide and fluticasone were shown to bind to SMO and activate the receptor. Because dexamethasone is widely used in cancer therapy to decrease inflammation, swelling, or nausea caused by chemotherapy, its activities against all essential pathways including the Hh one may affect the net therapeutic outcome.

These facts encouraged us to search for other corticosteroids capable of inhibiting the Hh pathway and to explore the SMO binding mode in order to choose a better steroid for combination therapy for the management of Hh-dependent cancers. With this aim, we tested dexamethasone and other corticosteroids having a similar structure (fludrocortisone and corticosterone); shown in Figure 1; for Hh pathway inhibitory activity. The versatile and promising techniques to study receptor–ligand interactions offer robust methods to test various drugs in cell-based enzyme-dependent luminescence methods or fluorescent bio-markers or metal-based complexes for accurate quantification. We chose Gli-1-dependent luciferase production and BODIPY-cyclopamine to test Hh pathway activity and SMO binding, respectively. The cell-based assays confirmed the previously reported results regarding dexamethasone and also showed for the first time that fludrocortisone and corticosterone can also inhibit the Hh pathway. The tested steroids failed to compete with cyclopamine for known binding cavities in SMO, indicating an allosteric mechanism for Hh pathway inhibition, which further can be exploited to design a combination/synergistic therapy for Hh-dependent cancers including SMO inhibitors to reduce the side effects. Our study is the first report to show fludrocortisone as an Hh pathway inhibitor.

Our report warrants more studies focused on steroids and related compounds to find better alternatives for patients with Hh-dependent cancers, immunomodulatory compounds with additional beneficial effects and reduced adverse effects (such as myopathy, neurotoxicity, etc.).

**RESULTS**

SMO is a seven-TM (7-TM) receptor that belongs to the frizzled subfamily (class F) of the GPCR superfamily and plays a vital role in the Hh pathway. The receptor has two primary binding sites, one in the extracellular CRD region and another in the TM region. Most SMO agonists and antagonists bind to the TM binding site. The CRD binding site gained interest recently when the crystal structure of SMO bound to cholesterol was solved. Because of the similar structure of fluorinated corticosteroids and cholesterol, we hypothesized that they also affect the Hh pathway. Different experiments were performed to validate this hypothesis.

Fluorinated Corticosteroids Inhibited Shh-Induced Activation of the Hh Pathway. The three fluorinated steroids selected, dexamethasone, fludrocortisone, and corticosterone, were tested in a functional Hh pathway activity assay using NIH3T3 Gli-RE cells stably expressing firefly luciferase under the control of an 8X Gli response element (8X Gli-RE) and treated with the active soluble form of Shh (further referred to as ShhN). ShhN binding to PTCH induces SMO translocation to PC and results in the activation of the Gli transcription factors. This type of Gli reporter assay is robust and was used to discover SAG, an SMO agonist. Cyclopamine was used as a control and was shown to inhibit the Hh pathway in this functional assay many times. As expected, cyclopamine showed inhibition of Gli-dependent increase in luciferase because of the induction of the Hh pathway by ShhN-conditioned media in NIH3T3 Gli RE cells. Similarly, the compounds tested here also inhibited the functional Gli-luciferase reporter to varying degrees. Among the tested compounds, dexamethasone and fludrocortisone strongly inhibited the Gli-dependent luciferase production. Also, there is evidence that the response to ShhN in NIH3T3 cells is an SMO-mediated pathway. Therefore, Figure 2a shows that the steroids tested in this experiment inhibit Hh pathway activity induced by ShhN.

In addition to ShhN, the SMO receptor and the Hh pathway are also activated by cholesterol and various oxysterols (20(S)-hydroxy cholesterol, OHC, being the most potential). To further confirm the activity of fluorinated steroids under study, we used 20(S)-OHC instead of ShhN-conditioned media as the Hh pathway activator in Gli-luciferase assay. Cyclopamine and tested compounds inhibit the 20(S)-OHC-induced Hh pathway activity (Figure 2b). The Gli-inhibition mechanisms for each of the fluorinated steroids needed to be further studied.

To evaluate the concentration dependence of the Hh inhibition, the NIH3T3 Gli-RE cells were treated with ShhN-conditioned media (10%) with low-serum assay media and serially diluted concentrations of cyclopamine, dexamethasone, fludrocortisone, and corticosterone. The observed IC50 values for cyclopamine, dexamethasone, fludrocortisone, and corticosterone were 72.1, 7.11, 20.1 and 167 nM, respectively. The concentration–response curves for all compounds are presented in Figure 3.

Fluorinated Corticosteroids Do Not Compete with Cyclopamine or 20(S)-OHC for Their SMO Binding Sites.
Cyclopamine directly binds to the 7-TM domain of SMO as demonstrated by crystallography and biochemistry experiments.\textsuperscript{11,30} By doing so, it is believed to influence the activation-associated conformational changes in the receptor, thus inhibiting the downstream Gli signaling. Competition binding assays and functional assays were performed to check the binding of test compounds to SMO at the cyclopamine 7-TM binding site. The competition binding was evaluated in HEK293t cells transiently transfected with mSMO, using BODIPY-cyclopamine (at 5 nM conc.) as the fluorescent probe. Cyclopamine showed a consistent concentration-dependent decrease in fluorescence in the sample indicating the displacement of BODIPY-cyclopamine from its binding site with an IC\textsubscript{50} of 342 nM. However, none of the tested fluorinated steroids (i.e., dexamethasone, fludrocortisone, and corticosterone) displaced BODIPY-cyclopamine (supplemented at 5 nM) from SMO in a dose-dependent manner except for a minimal change in fluorescence at the highest concentration of test compounds (50 μM). The results of the competition binding assay are shown in Figure 4a. The results indicated that the corticosteroids are likely to bind to a different location than the cyclopamine-binding site of SMO.

To further explore the mechanism of inhibition of SMO by corticosteroids, we tested them in a competitive functional assay against 20(S)-OHC, a known small molecule agonist of SMO activation. Figure 3 shows the concentration response profile of SMO activation induced by ShhN-conditioned media measured by the level of production of Gli-dependent luciferase. NIH3T3 Gli-RE cells stably transfected with 8×Gli-RE were used. The cells were then treated with compounds over a range of concentrations for 24 h in low-serum conditions. The data were analyzed by nonlinear regression and sigmoid dose-response using GraphPad Prism (GraphPad Software, Inc.). Data were acquired in triplicates from three independent experiments and are presented as the mean ± SD.
SMO that binds to the CRD pocket. In NIH3T3 Gli-RE cells, 20(S)-OHC causes a robust dose-dependent increase in luminescence with an EC_{50} of 3.5 μM. When the cells were exposed to 20(S)-OHC in the presence of dexamethasone, fluocortisone, and corticosterone at 0.5 μM concentration, the maximum signaling response induced by 20(S)-OHC was affected but its EC_{50} was not shifted indicating noncompetitive inhibition (Figure 4b). The result indicates that the test drugs do not bind to SMO at the 20(S)-OHC CRD binding site.

Cyclopamine and Fluorinated Corticosteroids Combine To Inhibit Hh Pathway Activity. From the data presented, it is clear that the tested corticosteroids (dexamethasone, fluocortisone, and corticosterone) do not compete for the CRD or TMD binding sites of SMO. We hypothesized that the two compounds might inhibit Hh pathway activity. We tested the combination using fixed doses of cyclopamine and fluorinated corticosteroids at 100 nM concentration in NIH3T3 Gli-RE cells using functional Gli-luciferase assay. The combination improves Hh pathway inhibition as compared to individual compounds, but this effect appears additive rather than synergistic. The additive effect suggests that the two categories of compounds tested here work through different sites to inhibit Gli production in the cells, which leads to the inhibition of the Hh pathway in cells. The additive inhibitory effect opens a possibility of using these steroids in combination with other Hh pathway inhibitors (e.g., vismodegib) to increase the anti-Hh treatment efficacy. The results are presented in Figure 5. The level of inhibition was maximal with dexamethasone and minimal with corticosterone, which is in-line with the observed IC_{50} of these compounds.

**Figure 5.** Fluorinated steroids (100 nM) and cyclopamine (100 nM) additively inhibit Hh pathway activity. The Hh pathway activity inhibition was tested by adding steroids (100 nM) with and without cyclopamine (100 nM) in a functional assay using NIH3T3 Gli-RE cells in the presence of ShhN-conditioned media. The two inhibitors have an additive inhibition effect on the Hh pathway, indicating that they act on different pathways resulting in reduced Gli production in cells. Ctr = control, DEX = dexamethasone, FDC = fluocortisone, and CRT = corticosterone. Data were acquired in triplicates from three independent experiments and are presented as the mean ± SD.

**DISCUSSION**

Corticosteroids have been used for brain cancer patients to reduce tumor-associated edema and neurological deficits. When these widely used drugs are used in cancer, the beneficial effects must be carefully evaluated and balanced against numerous side effects, including osteoporosis and cataract formation. Hh signaling pathway, mediated by SMO and Gli proteins, is dysregulated in various cancers including brain cancers. Recent reports on the inhibition of the Hh pathway by steroids and steroid derivatives prompt questions about the scope, scale, and direction of the Hh modulation for each of the drugs in this class. Although several steroids such as cholesterol, halcinonide, fluticasone, and cholesterol derivative 20(S)-hydroxycholesterol are shown to be SMO agonists, others are antagonists. The new SMO crystal structure solved with cholesterol suggested that the steroids could compete with cholesterol at the CRD binding site to impair the Hh signaling. Most of the SMO antagonists (e.g., budesonide and ciclesonide) with a steroidal structure are reported to be inhibitors of SMO-mediated ciliary translocation. Dexamethasone was reported to be the Hh pathway inhibitor, but the mechanism of its inhibitory activity was not fully described. Therefore, we wanted to test if dexamethasone and structurally similar steroids (fluocortisone and corticosterone) can also inhibit the SMO activity and whether they compete with cyclopamine for the same binding site.

The experimental data reported here clearly shows that the tested synthetic halogenated derivatives of cortisol, dexamethasone, and fluocortisone are potential inhibitors of the Hh pathway activity (Figures 2 and 3). The Gli-dependent luciferase reporter assay performed in NIH3T3 Gli-RE cells is a specific assay to see the effect of various molecules on Hh pathway activity. However, these steroids were not binding to SMO CRD and were not competing with cholesterol. Furthermore, none of the tested steroids competed with 20(S)-OHC (SMO agonists binding to CRD cavity) in a functional assay (Figure 4b). The BODIPY-cyclopamine competitive binding assay (Figure 4a) illustrated that the cyclopamine SMO-TM binding site is not affected either. These results lead to a hypothesis that these compounds may be affecting Hh pathway activity through an alternative SMO binding site or an indirect mechanism. The experiment showing an additive effect on inhibition of the Hh pathway by cyclopamine (Figure 5) further supports this hypothesis.

A recent report shows that SMO activation can be mediated by opening a tunnel through the TM part of the receptor (from TM7 to the extracellular opening near CRD). The authors proposed that cholesterol may travel through the tunnel from intracellular to extracellular space of the cells, and then binds to the CRD binding cavity to induce conformational change needed for SMO activation. This report opened up the possibility of explaining the observed effect of fluorinated corticosteroids characterized in our study. Dexamethasone and fluocortisone may compete with cholesterol (because of their structural similarity) and may inhibit the travel of cholesterol through the tunnel such as structure opened in the SMO TM region and allosterically inhibit SMO activation. The tunnel binding may explain the lack of competition with cyclopamine (7TM) or 20(S)-OHC (CRD).

Another plausible mechanism for Hh pathway inhibition by steroids could involve effects on other related pathways. There is evidence that the Hh pathway is influenced by other pathways in the cells and responds to the changing cellular microenvironment. The glucocorticoids (including dexamethasone) have been reported to decrease β-arrestin-2 gene expression in human lung carcinoma cells in a concentration-dependent manner. The Hh pathway inhibitory glucocorticoids may affect the pathway through several mechanisms including but not limited to arrestin-dependent SMO localization in primary cilia or SMO phosphorylation by G protein-coupled receptor kinase 2 (GRK2). It was experi-
��mentally confirmed that before SMO activation, β-arrestin-2 interacts with Kif3a (a subunit of the kinesin-2 motor complex) in cilia. The interaction between β-arrestin and Kif3a is required for localization of SMO in primary cilia and efficient Gli-dependent transcription. \(^{37,39}\) The β-arrestin-2 is also associated with GRK2-mediated phosphorylation of the SMO receptor cytoplasmic domains as demonstrated in HEK-293 cells and zebrafish embryos. \(^{38,40}\) These findings highlight a central role for the GRK-β-arrestin system in SMO signaling in vertebrates. Therefore, it is possible that dexamethasone, fludrocortisone, and corticosterone may exert their Hh pathway inhibitory effects by binding to GR, which leads to the inhibition of β-arrestin-2 gene expression. Glucocorticoids may also affect the Hh pathway by changing the composition of intracellular lipid rafts \(^{41}\) or binding to other possible cholesterol binding sites.

In conclusion, our report presents evidence that Hh/SMO signaling can be efficiently inhibited by at least two steroids which are often prescribed to cancer patients to reduce chemotherapy-related and radiotherapy-related side effects and brain edema. The binding mechanism is likely to be complementary to the SMO inhibitor cyclopamine. This study reveals that dexamethasone and fludrocortisone could be better choices for adjuvant therapy in cancer patients with dysregulated Hh pathway.

**MATERIALS AND METHODS**

**Chemicals and Reagents.** Cyclopamine was purchased from Cayman Chemical Company. BODIPY-cyclopamine was purchased from Biovision. Dexamethasone, fludrocortisone, and corticosterone were purchased from Sigma. 20(S)-hydroxy cholesterol was purchased from Tocris Bioscience.

For the assays, test compounds were prepared at 10-fold the final well concentrations in assay media, across six concentrations, using 1:10 or 1:5 serial dilutions from the highest concentration (stock solution conc. usually 10 mM). All stock solutions were stored at −20 °C.

**Cells and Plasmids.** HEK293t cells were obtained from ATCC, NIH3T3 Gli-RE cells (NIH3T3, mouse embryonic fibroblast cells, stably transfected with firefly luciferase under the transcriptional control of 8x Gli response element) were obtained from BPS Bioscience, San Diego, CA. HEK293t cells were cultured in the Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) at 37 °C in an atmosphere with 5% CO\(_2\), NIH3T3 Gli-RE cells were cultured in the DMEM supplemented with 10% of BCS and Geneticin.

The mSMO and ShhN (Shh N-terminal domain) plasmids were purchased from Addgene. All vectors were propagated in XL10 Gold competent cells, purified with NucleoBond Xtra Midi kit (Clontech), and sequenced (Geneviz).

For the production of ShhN-conditioned media and for the BODIPY-cyclopamine competition binding assay, HEK293t cells were seeded at a density of 1.5 × 10^6 in a 6 cm dish, allowed to grow overnight, and then transfected with either ShhN or mSMO plasmid DNA (6 μg DNA per 6 cm dish) using a TransIT transfection reagent (Mirus Bio LLC) according to the manufacturer’s instructions. Cell culture media was replaced with DMEM + 10% FBS before mSMO transfection and with DMEM + 10% BCS before ShhN transfection. The transfected dish was incubated for approximately 24 h at 37 °C. The ShhN-conditioned medium was produced in ShhN-transfected HEK293t cells. The culture medium from ShhN-transfected HEK293t cells was collected and aliquoted in single-use 1.5 mL Eppendorf tubes. This ShhN-enriched media was either used fresh or stored at −20 °C for later use. For BODIPY-cyclopamine competition binding assay, HEK293t cells transfected with mSMO plasmid were lifted with trypsin (0.25%), re-plated in 96-well adherent plates, and incubated for an additional 24 h before the experiment.

**Gli-Luciferase Functional Assay.** NIH3T3 Gli-RE cells were plated at 1.2 × 10^4 cells per well in 100 μL of DMEM + 10% BCS media in 96-well tissue culture-treated plate (Falcon, 353219). After 24 h, when the cells reached confluency, the culture medium was replaced with 80 μL/well of the assay medium [Optimem ± 10 mM (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid ± 1 mM sodium pyruvate + 1X MEM NEAA]. Serial dilutions of test compounds (10× of final concentrations) were prepared in assay media. 10 μL of diluted compounds (10X the final concentration) or control media were added to the plate after which the plate was incubated for 15–30 min at 37 °C. Then, the cells were stimulated by the addition of either ShhN-conditioned media or control media (DMEM ± 10% BCS) at a final concentration of 10% (1% BCS per well). Following incubation of the plate for at least 28 h at 37 °C, the cells were simultaneously lysed and supplemented with a luciferase substrate by the addition of an equal volume of Steady-Glo reagent (Promega, E2520) directly to the assay media. Plates were mixed by pipetting, centrifuged to eliminate foam, incubated for 10 min, and analyzed using a Perkin Elmer Victor X luminescence plate reader. The results were analyzed using nonlinear regression (Prism 6, GraphPad Software, La Jolla, CA). Data were normalized to the maximal response observed for ShhN-stimulated cells in the same experiment. A sigmoidal-dose response curve was used as a model for data analysis and IC\(_{50}\) value calculation.

**BODIPY-Cyclopamine Competition Binding Assay.** HEK293t cells transiently transfected with mSMO were lifted with trypsin (0.25%) and re-plated at 6 × 10^5 cells in 80 μL DMEM + 10% FBS per well in a 96 well culture-treated plate (Falcon, 353219). The plate was incubated at 37 °C for 24 h. Serial dilutions of test compounds (10× final concentrations) were prepared in culture media. 10 μL of diluted compounds or control media were added to the plate after which the plate was incubated at 37 °C for 10 min. Next, BODIPY-cyclopamine was added to each well at a final concentration of 5 nM, except for control wells that were left unstained. Following at least 1.5 h incubation at 37 °C in 5% CO\(_2\) cells were lifted by vigorous pipetting, transferred to conical bottom 96-well plates, and centrifuged at 400g for 5 min at 4 °C. The supernatant was discarded, cells were resuspended in 300 μL of PBS + 0.5% BSA (freshly prepared), and the plate was analyzed with a Guava benchtop flow cytometer. The results were interpreted with FlowJo software (version v10.1). Dose–response curves were constructed in Prism 6 (GraphPad Software, La Jolla, CA).

**AUTHOR INFORMATION**

**Corresponding Authors**

*E-mail: kkchalal@ucsd.edu (K.K.C.).
E-mail: ruben@ucsd.edu (R.A.).

**ORCID**

Ruben Abagyan: 0000-0001-9309-2976
Mollat, P.; et al. The Hedgehog Receptor Patched Is Involved in leukaemia.

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Chris X Edwards for technical assistance during the study. Handel lab for allowing to use instruments. We also thank the topic and guidance for experimental work. We thank

ACKNOWLEDGMENTS
The authors thank Dr. Irina Kufareva for fruitful discussions on the topic and guidance for experimental work. We thank Handel lab for allowing to use instruments. We also thank Chris X Edwards for technical assistance during the study.

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