Published in final edited form as:

J Cell Biochem. 2017 March ; 118(3): 499–509. doi:10.1002/jcb.25668.

Inhibition of Pancreatic Cancer Cell-Induced Paracrine Hedgehog Signaling by Liver X Receptor Agonists and Oxy16, a Naturally Occurring Oxysterol

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Abstract

The widespread involvement of the Hedgehog (Hh) signaling pathway in human malignancies has driven efforts to develop Hh pathway inhibitors as anti-cancer agents. The majority of these agents antagonize Smoothened (Smo), a plasma membrane-associated signal transducer molecule. However, several such Smo antagonists have failed in clinical trials to benefit patients with cancers that arise from aberrant Hh signaling (which often bypasses Smo). In this study, we report that a naturally occurring oxysterol, 20 α , 22(*R*)-dihydroxycholesterol (Oxy16), a known metabolite in the biosynthesis of steroid hormones, strongly inhibits Hh signaling induced in C3H10T1/2 embryonic fibroblasts and NIH3T3-E1 fibroblasts through a mechanism that is independent of liver X receptor (LXR) activation. We demonstrate that Oxy16 inhibits Hh signaling in Suppressor of Fused (Sufu) null mouse embryonic fibroblast (MEF) cells, indicating that its inhibitory effect on Hh signaling is epistatic to Sufu. We further demonstrate that Oxy16 inhibits Gli1 transcriptional activity in NIH3T3-E1 cells overexpressing Gli1 and a Gli-dependent reporter construct. Altogether, data presented here suggest that Oxy16 may be a suitable starting point for the development of new drugs that inhibit Hh signaling downstream of Smo. By targeting aberrant Hh signaling, such novel Hh pathway inhibitors could significantly broaden the range of clinical applications compared to existing Smo antagonists. Furthermore, the present study adds a new facet to the spectrum of Hh pathway modulation that naturally occurring oxysterol derivatives are capable of, ranging from allosteric activation of the pathway via Smo binding to inhibition of the pathway downstream of Smo. *J. Cell. Biochem.* 118: 499–509, 2017.

Keywords

HEDGEHOG SIGNALING; OXYSTEROLS; LIVER X RECEPTOR; PANCREATIC CANCER; STROMAL CELLS

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Hedgehog (Hh) molecules play key roles in a variety of processes including tissue patterning, mitogenesis, morphogenesis, cellular differentiation, stem cell maintenance, embryonic development, cancer and cardiovascular disease [Ehlen et al., 2006; Robert and Lallemand, 2006; Briscoe and Thérond, 2013]. In mammals, three members of the Hh family of proteins have been identified, namely sonic hedgehog (Shh), indian hedgehog (Ihh) and desert hedgehog (Dhh, known to be present primarily in neural tissues and gonadal cells). In addition to its role in embryonic development, Hh signaling plays a crucial role in postnatal development and maintenance of tissue/organ integrity and function [Petrova and Joyner, 2014]. However, aberrant Hh signaling plays a key role in various cancers including medulloblastoma, basal cell carcinoma, acute lymphoblastic leukemia, multiple myeloma, and pancreatic, prostate, ovarian, breast, colon and lung cancers [Yauch et al., 2008; Hanna and Shevde, 2016].

Hh signaling involves a very complex network of factors that includes plasma membrane proteins, kinases, phosphatases, and factors that facilitate the shuttling and distribution of Hh molecules [Lum and Beachy, 2004; Riobo et al., 2006]. Production of Hh proteins from a subset of producing/signaling cells involves synthesis, auto-processing and lipid modification. In the absence of Hh proteins, Patched (Ptc1), present on the plasma membrane of the responding cells, keeps Hh signaling in a silent mode by inhibiting the activity of another plasma membrane-associated signal transducer molecule, Smo. In the presence of Hh, the inhibition of Smo by Ptc1 is alleviated and Smo transduces the signal that regulates the transcription of Hh target genes. This transcriptional regulation in part involves the Ci/Gli transcription factors that enter the nucleus from the cytoplasm after a very intricate interaction between the members of a complex of accessory molecules that regulate localization and stability of Gli [Kogerman et al., 1999; Kalderon, 2005; Huangfu and Anderson, 2006]. Unregulated stimulation of Hh signaling through various mutations including those that inactivate Ptc1 and Sufu or activate Smo, as well as through production of Hh proteins and autocrine/paracrine signaling has been demonstrated in human cancers [Yauch et al., 2008; Hanna and Shevde, 2016]. Direct Smo antagonists have been under study for inhibition of aberrant Hh signaling for intervention in tumor formation. Two such candidate molecules, vismodegib and sonidegib, have been approved by the FDA for intervention in basal cell carcinoma. Unfortunately, Smo antagonists such as Saridegib and Vismodegib did not provide meaningful clinical benefit in patients with advanced pancreatic cancer [Kim et al., 2014]. The identification of more suitable and effective inhibitors of Hh signaling requires a better understanding of Hh signaling pathway regulators.

Liver X receptors α and β (LXR α and LXR β) are nuclear hormone receptors that upon activation regulate the expression of target genes in various physiological pathways [Edwards et al., 2002a,b]. Perhaps the most commonly examined property of LXR is its ability to regulate intracellular lipid and sterol metabolism by regulating the genes whose products are key members of the cholesterol biosynthetic pathway and lipid homeostasis [Edwards et al., 2002a,b]. LXRs also regulate reverse cholesterol transport from peripheral tissues to the liver mainly by increasing the expression of members of the ATP-binding cassette (ABC) family of membrane transporters that in part mediate cholesterol efflux from cells [Kalaany and Mangelsdorf, 2006]. LXRs were thought to be orphan nuclear receptors until it was found that specific oxysterols, such as 22(*R*)-hydroxycholesterol, 24(*S*)-

hydroxycholesterol, and 20(*S*)-hydroxycholesterol, act as their physiological ligands [Edwards et al., 2002a,b]. In addition, and relevant in the context of the present study, 20 α , 22(*R*)-dihydroxycholesterol (Oxy16) has been shown to display significant LXR activity [Forman et al., 1997]. As alluded to earlier, some oxysterols can profoundly affect Hh signaling and significant structural overlap exists among those oxysterols that also cause LXR activation. We [Dwyer et al., 2007] and others [Nachtergaele et al., 2012] have previously studied Hh activation induced by 20(*S*)-hydroxycholesterol which is also an LXR agonist; however, the activation features of LXR and Hh appear to be mechanistically unrelated. Activation of the Hh pathway is mediated by allosteric modulation of Smo and involves binding of 20(*S*)-hydroxycholesterol to an extracellular domain (CRD) of Smo [Nachtergaele et al., 2012]. We have tested several structural analogs of 20(*S*)-hydroxycholesterol that are devoid of LXR activity but retain Hh-modulation and Smo binding properties [Dwyer et al., 2007]. 20(*S*)-hydroxycholesterol and Oxy16 are both naturally occurring metabolites of cholesterol in the biosynthesis of steroid hormones (Supplementary Material: Scheme S1 and references therein) and bear a close structural resemblance. However, unlike 20(*S*)-hydroxycholesterol, Oxy16 does not bind to Smo [Montgomery et al., 2014] or stimulate Hh signaling but rather displays significant Hh pathway inhibition. This unexpected but potentially valuable finding has prompted us to study the properties of Oxy16 in more detail. We previously reported that activation of the LXR pathway by non-steroidal LXR agonists inhibits Hh signaling in stromal cells through yet unrecognized mechanisms [Kim et al., 2009]. A central question of the present study has been whether such mechanisms could also be operational with oxysterol-based LXR agonists, at least within the subset of oxysterols that, like Oxy16, also display Hh inhibitory properties. Several previous reports have demonstrated the inhibitory effect of LXR activation on clonogenic growth of various human tumors cells including breast, prostate, ovarian, and leukemic lymphocytes [Venteclaf and Ferré, 2014], although it has not been determined whether these reported inhibitory effects of LXR ligands is at least in part responsible for inhibition of Hh signaling. As noted earlier, Hh signaling appears to play an important role in initiation and progression of pancreatic cancer, and inhibition of Hh signaling using small molecule antagonists inhibits pancreatic cancer cells from growing in vitro and in vivo [Thayer et al., 2003; Feldmann et al., 2008; Bailey et al., 2009; Mazumdar et al., 2011]. It has been suggested that Hh proteins expressed by a subset of epithelial cancers, including pancreatic, colon, and ovarian cancer, promote tumor growth indirectly by activating Hh signaling in tumor stromal cells/fibroblasts that are of mesenchymal origin [Yauch et al., 2008; Theunissen and de Sauvage, 2009]. Subsequently, Hh signaling in stromal cells provides a permissive milieu for tumor cells to grow and metastasize [Theunissen and de Sauvage, 2009; Tian et al., 2009]. Therefore, it is conceivable that inhibition of Hh signaling in tumor fibroblasts and in cancer cells that is mechanistically distinct from SMO antagonism, will shed light on developing therapies against Hh pathway-related cancers. Unfortunately, to date SMO antagonists have shown little to no efficacy in clinical trials of human cancers, and even increased tumor aggression in pancreatic cancer; however, it is likely that such failures are attributed to mutations in Smo that disrupt drug binding as well as several other compensatory mechanisms that restore Gli activity [Yauch et al., 2009; Kaye et al., 2012; Berlin et al., 2013]. Gli activity can be induced downstream of Smo by signaling pathways commonly activated in cancer including KRAS, PI3 Kinase,

TGF- β and EGFR [Nolan-Stevaux et al., 2009; Schnidar et al., 2009; Javelaud et al., 2011; Götschel et al., 2013]. Thus, novel Hh pathway inhibitors that act independently of Smo are needed for vismodegib-resistant advanced basal cell carcinoma (aBCC) and other cancers. We propose that oxysterol-based Hh pathway antagonists may act differently based on their different chemical and physical characteristics and mechanism of action that appears to inhibit Hh signaling downstream of Smo. In the present study we begin to examine oxysterols using an in vitro model system in which 1) Hh signaling is induced in C3H10T1/2 embryonic fibroblasts and NIH3T3-E1 fibroblasts by Hh proteins produced in conditioned medium (CM) from CAPAN-1 human pancreatic cancer cells, or 2) in NIH3T3E1 cells and Sufu null mouse embryonic fibroblasts (MEFs) in which Hh signaling is activated by events downstream of Smo. We report that Oxy16, a naturally occurring oxysterol that does not bind Smo, inhibits Hh signaling in these experimental systems and it exerts this inhibitory effect on Hh signaling downstream of Smo and through a mechanism that is independent of LXR activation.

MATERIALS AND METHODS

SYNTHESIS AND MOLECULAR CHARACTERIZATION OF Oxy16

Oxy16 was prepared according to a procedure published by Watanabe et al. [Watanabe et al., 2004] with minor variations, as depicted in Supplemental Material (Scheme S2).

CELL CULTURE AND REAGENTS

C3H10T1/2 mouse embryonic fibroblasts and NIH3T3-E1 fibroblasts were obtained from ATCC (Manassas, VA) and cultured as previously described [Dwyer et al., 2007; Richardson et al., 2007]. CAPAN-1, L3.6pl, and E3LZ10.7 human pancreatic cancer cells were obtained from ATCC and cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT). The Human hepatoma cell line HepG2 was obtained from ATCC and cultured in DMEM containing 10% FBS. Mouse SUFU null (−/−) MEFs were provided by Dr. Philip Beachy at Stanford University and cultured in DMEM containing 10 FBS as previously described. LXR $\alpha\beta$ null (−/−) MEFs were provided by Dr. Peter Tontonoz at UCLA and cultured in DMEM containing 10% FBS. GH3 cells were obtained from ATCC and cultured in Ham's F10 containing 2 mM Glutamine, 15% Horse Serum and 2.5 FBS. TO901317 LXR agonist was purchased from Cayman Chemicals (Ann Arbor, MI). Recombinant human Shh N-terminal peptide and cyclopamine were obtained from R&D Systems, Inc. (Minneapolis, MN), and Shh-neutralizing antibody 5E1 was obtained from Developmental Studies Hybridoma Bank (Iowa City, IA).

CAPAN-1 CONDITIONED MEDIUM

Growth medium in confluent 10 cm² tissue culture plates of CAPAN-1 cells was replaced with 10 ml of fresh growth medium and incubated for a total of 7 days. CM was collected and spun to remove any detached cells and debris, aliquoted, and stored frozen at −80°C.

LXR α AND LXR β siRNA TRANSFECTION

Scrambled, LXR α and LXR β siRNAs (ON-TARGETplus non-targeting pool and ON-TARGETplus SMARTpool; catalog nos. D-001810–10-05, L-040649–01-0005 and

L-042839-00-0005) were purchased from GE Dharmacon (Lafayette, CO). NIH3T3 cells at 80 confluency in 12-well plate were transfected with the siRNAs using Lipofectamine RNAiMAX transfection reagent (Invitrogen) to a final concentration of 10 nM of each siRNA.

QUANTITATIVE RT-PCR

Total RNA was extracted with the RNeasy Plus Mini Kit from Qiagen according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed using an iScript Reverse Transcription Supermix from Bio-Rad, to make single-stranded cDNA. The cDNAs were then mixed with Qi SYBR Green Supermix (Bio-Rad, CA) for quantitative RT-PCR assay using a Bio-Rad I-cycler IQ quantitative thermocycler. All PCR samples were prepared in triplicate wells in a 96-well plate. After 40 cycles of PCR, melt curves were examined in order to ensure primer specificity. Fold changes in gene expression were calculated using the $\Delta\Delta C_t$ method. Primers used were as follows: *GAPDH* (5'-ATGGACTGTGGTCATGAGCC-3' and 5'-AT TGTCAGCAATGCATCCTG-3'), *Rps29* (5'-GTCTGATCCGCAAATA CGGG-3') and (5'-AGCCTATGTCCTTCGCGTACT-3'), *Oaz1* (5'-CCA CTGCTTCGCCAGAGAG-3') and (5'-CCCGGACCCAGGTTACTA-3'), *Gli1* (5'-GAAGCCGAGCCGAGTATC-3' and 5'-GGTGAGTAGACAG AGGTTGG-3'), *Ptch1* (5'-CCATCGGCGACAAGAACC-3' and 5'-CC AGCACAGCAAAGAAATACC-3'), *Hip* (5'-GGCTCTGTGCGAAACGG CTACTAC-3' and 5'-GCACGCTGGCTCACACTTGG-3'), *Sonic Hedgehog ligand (Shh)* (5'-GGTCGGACGTGGTGATGTC-3' and 5'-GAGG ACGGCCATCATTCAGA-3'), *Indian Hedgehog ligand (Ihh)* (5'-CCAC CTTCAGTGATGTGCTTATTT-3' and 5'-CGATGACCTGGAAAGCTCT CA-3').

ALKALINE PHOSPHATASE (ALP) ACTIVITY ASSAY

C3H10T1/2 cells at confluence were treated with control medium or CM in the presence or absence of LXR agonist, oxysterol, or the Hh pathway inhibitor cyclopamine as described. After 96 h, a colorimetric assay for ALP activity was performed on whole cell extracts as we previously described [Johnson et al., 2011].

TRANSIENT TRANSFECTION ASSAY

NIH3T3-E1 and Sufu^{-/-} cells cultured in 24 well-plate at 90% confluence were transiently transfected with 0.1 μ g of Gli response-element reporter (pGL3b-8xGli-Luciferase) plasmid, and 10 ng of pTK-Renilla-Luciferase plasmid with or without co-transfection with 10 ng of Gli1 overexpression vector, pSR α -Gli1 as described [Montgomery et al., 2014] using Lipofectamine LTX Plus transfection reagent (Invitrogen). 24 h after transfection, cells were treated with test agents for 72 h. Then the firefly and *Renilla* luciferase activities were measured using a dual luciferase kit (Promega) and a GloMax-96 Microplate Luminometer (Promega). The firefly luciferase activities was normalized to the *Renilla* luciferase activities. Data are reported as the mean of triplicate determinations $\pm$ SD.

WESTERN BLOT ANALYSIS

After treatments, cells were lysed in lysis buffer, protein concentrations were determined using the Bio-Rad protein assay (Hercules, CA), and SDS-PAGE was performed as previously described [Kim et al., 2009]. The membrane was probed with primary antibody to Shh (Cell signaling, #2207) (1:1000). After washing 3 times with TBST buffer, the membrane was labeled for 1 h at room temperature with Anti-rabbit IgG HRP conjugated secondary antibody (Cell signaling, #7074) (1:1000). The blots were developed with Pierce ECL western blotting substrate (Thermo Scientific) and imaged. The membrane re-blocked by milk again and re-probed with Actin (Santa Cruz Biotechnology Inc. #sc-1616).

STATISTICAL ANALYSIS

Statistical analyses were performed using the StatView 5 program. All *P* values were calculated using ANOVA and Fisher's projected least significant difference (PLSD) significance test. A value of $P < 0.05$ was considered significant.

RESULTS

HUMAN PANCREATIC CANCER CELLS EXPRESS Shh AND Ihh

As noted earlier, aberrant Hh signaling may play a major role in the growth and dissemination of pancreatic as well as other tumors [Yauch et al., 2008; Hanna and Shevde, 2016]. In one scenario, it has been suggested that a subset of pancreatic cancer cells produce Hh proteins that in turn target tumor cells and/or tumor stromal cells. We screened several human pancreatic cancer cell lines for the expression of Shh and Ihh and found that CAPAN-1 cells cultured to confluence in the presence of 10% FBS robustly express the mRNA for these molecules relative to L3.6pl or E3LZ10.7 cells, with CAPAN-1 > L3.6pl > E3LZ10.7 (Fig. 1A and B). Culturing CAPAN-1 cells in 1% versus 10% FBS had no effect on their level of mRNA expression for Ihh and Shh (data not shown), and treatment of CAPAN-1 cells with the Hh pathway inhibitor cyclopamine (4 μ M) or the LXR agonist TO (1–5 μ M) also had no effect on the expression of Ihh or Shh mRNA in these cells (data not shown). Western blot analysis using a specific antibody to Shh confirmed the presence of Shh protein in the CM as well as in the cell lysates obtained from CAPAN-1 cells (Fig. 1C). For studies presented in this paper, instead of using recombinant Hh proteins that are costly, we chose CAPAN-1 CM as a source of Hh proteins to further study regulation of Hh signaling by small molecule oxysterols in responsive cells.

CONDITIONED MEDIUM FROM CAPAN-1 CELLS HAS Hh ACTIVITY

In order to confirm the functional activity of Hh proteins produced by CAPAN-1 cells, we examined the ability of CM to induce Hh target gene expression in C3H10T1/2 embryonic fibroblasts. Treatment of C3H10T1/2 cells for 48 h with CAPAN-1 CM induced the robust expression of Hh target genes, *Ptch1*, *Gli1*, and *Hip* in these cells, which was completely inhibited by the Hh pathway inhibitor, cyclopamine (Fig. 2A) and by the Hh neutralizing antibody 5E1 [Maun et al., 2010] (Fig. 2B). These findings confirmed that the expression of Hh proteins by CAPAN-1 cells translates into production of active Hh proteins. We noticed that the level of expression of *Gli1* and *Ptch1* are different in the two experiments (Fig. 2A

and B). This difference is most likely caused by the use of different batches of CM and the different passage numbers of the cells used in the different experiments. In addition, further proof of functionality of the Hh proteins in CAPAN-1 CM came from treatment of C3H10T1/2 cells with CAPAN-1 CM causing a significant induction of ALP activity, a marker of osteogenic differentiation in these cells [Johnson et al., 2011] (Fig. 2C). Similar to the inhibition of Hh target gene expression, cyclopamine also inhibited CM-induced ALP activity (Fig. 2C). We and others previously reported that activation of Hh signaling by Hh proteins or by specific oxysterols that activate the Hh signaling pathway induce ALP activity and osteogenic differentiation in C3H10T1/2 cells and other multipotent stromal cells [Richardson et al., 2007; Johnson et al., 2011].

Oxy16 AND TO INHIBIT Hh SIGNALING

Next we examined whether LXR activation by LXR agonists inhibits Hh target gene expression in fibroblastic cells using C3H10T1/2 and NIH3T3-E1 cells as a model system [Dwyer et al., 2007; Richardson et al., 2007]. As expected, treatment of NIH3T3-E1 and HepG2 cells with 2 μ M of the non-steroidal LXR agonist, TO901317 (TO), significantly induced the expression of LXR target genes, ABCA1 and SREBP1c after 72 h of treatment (Fig. 3). Similar to the inhibitory effects of cyclopamine, treatment of C3H10T1/2 cells with TO significantly inhibited CAPAN-1 CM-induced expression of Hh target genes (Fig. 2A), as well as ALP activity in these cells (Fig. 2C).

As noted earlier, specific oxysterols are thought to be physiological ligands of LXRs [Edwards et al., 2002a,b] that are classified as partial agonists based on their differential effects on the interaction of LXRs with co-activators and co-repressors compared to those induced by the full LXR agonist TO [Albers et al., 2006; Phelan et al., 2008]. We examined the effects of Oxy16, a naturally occurring oxysterol and LXR agonist, prepared in our laboratory (Supplementary Material, Scheme S2), on Hh signaling in C3H10T1/2 cells (Fig. 2A) and NIH3T3-E1 cells (Fig. 4) treated with CAPAN-1 CM. Unlike naturally occurring oxysterols tested previously in our laboratory, including 20 (*S*)-hydroxycholesterol and 22(*R*)-hydroxycholesterol, which activate various levels of both LXR and Hh signaling, Oxy16 tested up to a final concentration of 10 μ M mainly induced the expression of LXR target genes but not Hh target genes (Figs. 2–4). Activation of LXRs by Oxy16 was confirmed by the induction of ABCA1 in NIH3T3-E1 cells (Fig. 3A). Similar to the effects of TO, Oxy16 also inhibited Hh target gene expression in C3H10T1/2 and NIH3T3-E1 cells treated with CM (Figs. 2A and 4) and ALP activity in C3H10T1/2 cells (Fig. 2C). The inhibitory effects of Oxy16 used at 5 μ M were similar to those of TO at 2 μ M despite an apparently lesser induction of LXR target gene ABCA1 by Oxy16 even at 10 μ M (Figs. 2A and 3A). Most importantly, unlike non-steroidal LXR agonists such as TO, in human hepatocyte cell line, HepG2, Oxy16 at 10 μ M did not induce the expression of SREBP-1c, a master transcriptional regulator of lipogenesis involved in human obesity and type 2 diabetes (Fig. 3B). Since CM may contain other factors such as TGF β 1 and IGF1 that might affect the activity of Hh proteins, those factors might also be the targets for the inhibitory effects of Oxy16 on Hh signaling. In all studies presented, cells were always pre-treated with TO or Oxy16 for 2 h before the addition of CM since we have found that pretreatment (as oppose

to co-treatment) does enhance their inhibitory activities on Hh signaling (unpublished observations).

Oxy16 INHIBITS Hh SIGNALING EPISTATIC TO Sufu

Pathogenic Hh pathway activation may occur in cancer at several levels of the Hh signal transduction cascade, including mutations in Ptch1, Smo, and Sufu, as well as amplification of Gli1 [Hahn et al., 1996; Aszterbaum et al., 1998; Xie et al., 1998; Taylor et al., 2002; Beauchamp et al., 2009; Rajurkar et al., 2012]. Sufu is a negative regulator of Hh signaling required to process the Gli transcription factors and its loss results in Smo-independent Gli activation [Taylor et al., 2002]. To examine whether Oxy16 acts downstream of Sufu, we treated Sufu null MEF (Sufu^{-/-}) cells with Oxy16 and found that Oxy16 significantly inhibited the expression of Hh signaling target genes Gli1 and Hip, suggesting that Oxy16 exerts its inhibitory effect epistatic to Sufu (Fig. 5).

Oxy16 INHIBITS THE TRANSCRIPTIONAL ACTIVITY OF Gli1

The Gli transcription factors are effectors of the Hh signaling pathway. It has been demonstrated that Gli activity in many human malignancies including within pancreatic ductal adenocarcinoma (PDAC) cells is required for tumorigenesis and that both mutant KRAS and TGF β -signaling can induce Gli activity in a Smo-independent manner [Schnidar et al., 2009; Javelaud et al., 2011; Rajurkar et al., 2012]. Accordingly, inhibition of Gli by siRNA or by the inhibitor GANT61 can block pancreatic cancer cell growth and survival [Fu et al., 2013]. Therefore, Gli1 appears to represent a promising therapeutic target in Hh pathway that is downstream of smoothened. We tested whether Oxy16 inhibits Gli1 transcriptional activity in NIH3T3-E1 cells and found that Oxy16, but not Oxy190, a compound with similar structure that did not show inhibitory effect on Hh signaling (data not shown), significantly inhibited the expression of a Gli responsive luciferase reporter that was transactivated by over expressed exogenous Gli1 (Fig. 6A). Furthermore, Oxy16 significantly inhibited the same reporter activity in Sufu^{-/-} cells, in which the reporter was transactivated by endogenous Gli that is constitutively activated due to loss of Sufu (Fig. 6B). We confirmed our data by normalizing the reporter luciferase activities to activities of renilla luciferase expressed by different vectors, such as pRL-SV40 and pRL-CMV to avoid the possibility that the decreased RLU is due to enhanced expression of the renilla luciferase by Oxy16 (data not shown). Our data suggest that Oxy16 inhibits Hh pathway signaling at least in part through inhibiting Gli1.

Oxy16-MEDIATED INHIBITION OF Hh SIGNALING IS INDEPENDENT OF LXR ACTIVATION

We previously reported that the non-steroidal LXR agonist TO901317 inhibits Hh signaling through activating LXR in vitro and in vivo [Kim et al., 2009]. We questioned whether, as a partial LXR agonist [Albers et al., 2006; Phelan et al., 2008], Oxy16 might inhibit Hh signaling through a similar mechanism. In order to examine this possibility, we investigated the effect of LXR elimination on inhibition of Hh signaling by Oxy16. First, we examined the inhibitory effect of Oxy16 in mouse embryonic fibroblasts (MEFs) derived from LXR $\alpha\beta$ null mice. Oxy16 significantly inhibited Gli1 and Ptch1 expression induced by the CAPAN-1 CM not only in MEFs with full functional LXR, but also in LXR null MEFs with no LXR activity (Fig. 7). Introduction of LXR β in LXR null MEFs using a retroviral vector

did not have any effects on the inhibitory effects of Oxy16 on Hh target gene expression (Fig. 7). To further confirm this apparent lack of a role of LXR activation in mediating the inhibitory effect of Oxy16 on Hh signaling, we knocked down both LXR α and LXR β in NIH3T3-E1 cells using small interfering RNA (siRNA). Treatment with siRNA inhibited the expression of mRNAs for both LXRs by more than 80% (Fig. 8A), and significantly inhibited the induction of ABCA1 (an LXR target gene) expression by TO (Fig. 8B). Consistent with the results obtained in LXR null MEFs, knocking down LXRs in NIH3T3-E1 cells did not exert any effect on the inhibition of Hh signaling by Oxy16 (Fig. 8C and D). Altogether, our data suggest that Oxy16 inhibits Hh signaling through a mechanism that is independent of LXR activation.

DISCUSSION

The Hh pathway has been shown to be an active participant in cancer development, progression and metastasis. Although this pathway is activated via autocrine signaling by Hh ligands, it can also initiate paracrine signaling with cells in the tumor microenvironment.

As a result of paracrine signal transmission, the effects of Hh signaling most profoundly influence the stromal cells that constitute the tumor microenvironment. The stromal cells in turn produce factors that may support growth and metastasis of the tumor. Thus, such a resonating cross-talk can amplify Hh signaling and promote tumor progression [Yauch et al., 2008; Hanna and Shevde, 2016].

Pancreatic ductal adenocarcinoma (PDAC) is one of the most stroma-rich cancers. PDAC stroma is very heterogeneous and comprises cellular and acellular components, such as fibroblasts, myofibroblasts, pancreatic stellate cells (PSC), immune cells, blood vessels, extracellular matrix (ECM), and soluble proteins such as cytokines and growth factors [Damhofer et al., 2013]. The fibrotic stroma in PDAC forms an environment that promotes cancer progression by enhancing pancreatic tumor growth as well as regional and distant metastasis [Damhofer et al., 2013]. Furthermore, the stroma has been shown to induce resistance to chemotherapy and radiation therapy [Lunardia et al., 2014] and to constitute a barrier to the delivery of therapeutic agents [Lunardia et al., 2014]. It has been suggested that Hh signaling in pancreatic cancer occurs at least in part in a paracrine mechanism with expression and secretion of Hh ligand by tumor cells and activation of the canonical signaling pathway in the adjacent stromal cells [Damhofer et al., 2013]. Several Hh pathway inhibitors targeting SMO have been developed and evaluated in clinical trials, anticipating that inhibition of ligand-dependent aberrant Hh signaling by such inhibitors should block PDAC. Unfortunately, SMO antagonists such as Saridegib and Vismodegib did not provide meaningful clinical benefit in patients with advanced pancreatic cancer [Kim et al., 2014]. Despite these disappointing clinical results surrounding Smo inhibition, other laboratory studies have demonstrated that Gli activity within tumor cells is required for tumorigenesis and that both mutant KRAS and TGF- β signaling can induce Gli activity in a Smo-independent manner [Schnidar et al., 2009; Javelaud et al., 2011; Rajurkar et al., 2012]. Furthermore, the inhibition of Gli1 by siRNA or the inhibitor GANT61 can block pancreatic cancer cell growth and survival [Fu et al., 2013]. Finally, studies using mice with conditional Gli expression demonstrated that the expression of Gli3T, a dominant repressor of Gli

activity, reduced PDAC whereas overexpression of Gli1 caused accelerated tumor formation in KPC mice [Rajurkar et al., 2012]. Therefore, Smo-independent Gli activity appears to be important in PDAC, and strategies targeting the Hh pathway downstream of Smo may be effective in inhibiting tumor growth.

In the present report, we provide data showing that Hh signaling activated in stromal cells by Hh proteins produced by pancreatic tumor cells was blocked in the presence of an oxysterol, Oxy16. We anticipate that Oxy16 should block the activation of Hh signaling in target cells by Shh-rich CM from other cancer cells. Unlike non-steroidal LXR agonists such as TO, in human hepatocytes Oxy16 did not induce SREBP1c gene expression, which is the main cause of adverse lipogenesis in the liver in response to LXR agonists and has hampered the clinical development of these molecules for use in humans [Chisholm et al., 2003]. We propose that oxysterol-based small molecules, such as Oxy16, as a class, may provide the LXR-dependent and LXR-independent advantages of non-steroidal LXR agonists without causing their adverse effects upon systemic administration, and hence may become drug candidates for treatment of pancreatic cancer as well as other malignancies that arise from aberrant Hh signaling. Such therapies may prove effective when used alone or in combination with other chemotherapeutic agents that are currently used, especially since the inhibition of Hh signaling was found to enhance delivery of chemotherapy in a mouse model of pancreatic cancer by transiently increasing tumor vascularity and concentration of chemotherapeutic agents within the tumor environment [Olive et al., 2009]. Oxy16 significantly inhibited Gli1 and Hip expression in *Sufu*^{-/-} MEF cells, suggesting that Oxy16 acts epistatic to *Sufu*. Most importantly, in a luciferase reporter assay, we found that Oxy16 significantly inhibited the transcriptional activity of Gli1 over expressed in NIH3T3-E1 cells and that of endogenous Gli in *Sufu*^{-/-} MEF cells. Collectively, based on the data presented in the present study, Oxy16 and future analogs with similar or improved characteristics may represent a new class of inhibitors of Gli with promising potential to serve as novel therapeutic candidates for further development in targeting PDAC and other Hh pathway related malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors thank Dr. Anthony Heaney at UCLA for Western blot analysis of CM and CL. This study was funded by MAX BioPharma Inc. Drs. Parhami and Matsui are co-founders of MAX BioPharma and disclose having financial interest in the company. Drs. Wang and Stappenbeck are employed by MAX BioPharma and own stock options in the company.

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Fig. 1. Hedgehog expression by human pancreatic cancer cells. (A and B) Expression of SHH and IHH mRNA in human cultures of pancreatic cancer cells, CAPAN-1, L3.6pl, and E3LZ10.7 were analyzed by Q-RT-PCR and normalized to GAPDH expression. Cells were cultured in DMEM containing 10% FBS and RNA was extracted 3 days after seeding. Data from a representative experiment are reported as the mean of triplicate determinations $\pm$ SD ($P < 0.001$ for CAPAN-1 vs. other two cell types for SHH and IHH mRNA expression). (C) Expression of Shh protein in CAPAN-1 conditioned medium (CM, 20 μ l) and cell lysates (CL) collected from parallel cell cultures to those described in A, B was examined using Western blot analysis. Recombinant human Shh (rhShh) (MW 22Kd) and extracts from GH3 cells were used as positive controls. 110, 40, and 0.004 μ g of proteins were loaded for CL, GH3, and SHH, respectively.

Fig. 2.

Inhibition of pancreatic cancer cell-induced Hedgehog signaling. C3H10T1/2 cells were pretreated for 2 h with control vehicle or the LXR agonists TO901317 (TO, 2 μ M) or Oxy16 (5 μ M), or the Hedgehog pathway inhibitor cyclopamine (Cyc, 4 μ M) (A), or with 5E1 antibody or an isotype matched irrelevant IgG antibody (B). Next, cells were treated with DMEM containing 5% FBS or CAPAN-1 CM in DMEM containing 5% FBS in the presence or absence of TO, Oxy16, Cyc, 5E1, or IgG. After 48 h, RNA was extracted and analyzed by Q-RT-PCR for the expression of Hh target genes Ptch1, Hip, and Gli1 and normalized to GAPDH expression. Data from a representative of 3 separate experiments are reported as the mean of triplicate determinations $\pm$ SD ($P < 0.001$ for Control vs. CM and for CM vs. CM + TO, CM + Cyc, CM + Oxy16, and CM + 5E1 for Ptch1, Hip, and Gli1 expression). (C) C3H10T1/2 cells were treated with with DMEM containing 5% FBS or CAPAN-1 CM in DMEM containing 5% FBS in the presence or absence of TO (2 μ M), Oxy16 (10 μ M) or cyclopamine (4 μ M). After 96 h, a colorimetric assay for ALP activity was performed on whole cell extracts. Data from a representative of 3 separate experiments are reported as the mean of triplicate determinations $\pm$ SD.

Fig. 3.

Induction of LXR target gene expression by LXR agonists. NIH3T3-E1 cells and HepG2 cells were treated with DMEM containing 5% FBS or CAPAN-1 CM in DMEM containing 5% FBS in the presence or absence of TO (2 μ M) or Oxy16 (10 μ M). After 48 h, RNA was extracted and analyzed by Q-RT-PCR for the expression of LXR target genes ABCA1 in NIH3T3-E1 cells (A) and SREBP-1c in HepG2 cells (B) and normalized to Oaz1 and GAPDH expression, respectively. Data from a representative of 3 separate experiments are reported as the mean of triplicate determinations $\pm$ SD.

Fig. 4.

Inhibition of Hedgehog signaling by Oxy16 in NIH3T3 cells. NIH3T3 cells were treated with DMEM containing 5% FBS or CAPAN-1 CM in DMEM containing 5% FBS in the presence or absence of TO (2 μ M) or Oxy16 (10 μ M). After 48 h, RNA was extracted and analyzed by Q-RT-PCR for the expression of Hh target genes Gli1 (A) and Ptch1 (B) and normalized to Rps29 expression. Data from a representative of 3 separate experiments are reported as the mean of triplicate determinations $\pm$ SD.

Fig. 5.

Inhibition of Hedgehog signaling in Sufu null mouse embryonic fibroblasts by Oxy16. Sufu $-/-$ MEF cells were treated with DMEM containing 10% FBS in the presence or absence of Oxy16 (10 μ M). After 72 h, RNA was extracted and analyzed by Q-RT-PCR for the expression of Hh target gene Gli1 (A) or Hip (B), normalized to Oaz1 expression. Data from a representative of 3 separate experiments are reported as the mean of triplicate determinations $\pm$ SD (* $P < 0.05$ vs. control; ** $P < 0.01$ vs. control).

Fig. 6.

Oxy16 inhibits Gli transcriptional activity. (A) NIH3T3 cells cultured in 24 well-plate were transfected with a Gli response-element reporter (pGL3b-8xGli-Luciferase) plasmid, a pTK-Renilla-Luciferase plasmid and a vector expressing Gli1, pSR α -Gli1. 24 h after transfection, cells were treated with the test agents (10 μ M each) as indicated for 72 h. (B) Sufu^{-/-} cells cultured in 24 well-plate were transfected with a Gli response-element reporter (pGL3b-8xGli-Luciferase) plasmid and a pTK-Renilla-Luciferase plasmid. 24 h after transfection, cells were treated as described in (A). Luciferase activity was measured and normalized to the Renilla luciferase activity. Data from a representative of 3 separate experiments are reported as the mean of triplicate determinations $\pm$ SD (** $P < 0.01$ vs. control).

Fig. 7.

Inhibition of Hedgehog signaling by Oxy16 in LXR null mouse embryonic fibroblasts. LXR null MEFs (LXR $\alpha\beta^{-/-}$) and LXR null MEFs expressing exogenous human LXR β (LXR $\alpha\beta^{-/-}$ + LXR β) were treated with DMEM containing 5% FBS or CAPAN-1 CM in DMEM containing 5% FBS in the presence or absence of Oxy16 (10 μ M). After 72 h, RNA was extracted and analyzed by Q-RT-PCR for the expression of Hh target genes Gli1 (A) and Ptch1 (B) and normalized to Oaz1 expression. Data from a representative of 3 separate experiments are reported as the mean of triplicate determinations $\pm$ SD (* $P < 0.05$ vs. control, ** $P < 0.05$ vs. CM).

Fig. 8.

Effect of LXR siRNA on expression of Gli1 in NIH3T3 cells. NIH3T3 cells were transfected with scrambled (SCX) or siRNAs for both LXR α and LXR β as described in section Materials and Methods. (A) After 48 h, RNA was extracted and analyzed by Q-RT-PCR for the expression of LXR α and LXR β and normalized to Oaz1 expression. (B) Cells treated with the SCX and siRNA as described in (A) were cultured in the presence or absence of TO (2 μ M). After 48 h, RNA was extracted and analyzed by Q-RT-PCR for the expression of LXR target gene Abca1. (C and D) Cells transfected with the SCX or siRNA as described in (A) were treated with DMEM containing 5% FBS or CAPAN-1 CM in the presence or absence of Oxy16 (10 μ M). After 48 h, RNA was extracted and analyzed by Q-RT-PCR for the expression of Hh target gene Gli1 and Ptch1 for Oxy16 treated cells and normalized to Oaz1 expression. Data from a representative of 3 separate experiments are reported as the mean of triplicate determinations $\pm$ SD.