Endogenous B-ring oxysterols inhibit the Hedgehog component Smoothened in a manner distinct from cyclopamine or side-chain oxysterols

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Cellular lipids are speculated to act as key intermediates in Hedgehog signal transduction, but their precise identity and function remain enigmatic. In an effort to identify such lipids, we pursued a Hedgehog pathway inhibitory activity that is particularly abundant in flagellar lipids of *Chlamydomonas reinhardtii*, resulting in the purification and identification of ergosterol endoperoxide, a B-ring oxysterol. A mammalian analog of ergosterol, 7-dehydrocholesterol (7-DHC), accumulates in Smith–Lemli–Opitz syndrome, a human genetic disease that phenocopies deficient Hedgehog signaling and is caused by genetic loss of 7-DHC reductase. We found that depleting endogenous 7-DHC with methyl-β-cyclodextrin treatment enhances Hedgehog activation by a pathway agonist. Conversely, exogenous addition of 3β,5α-dihydroxycholest-7-en-6-one, a naturally occurring B-ring oxysterol derived from 7-DHC that also accumulates in Smith–Lemli–Opitz syndrome, blocked Hedgehog signaling by inhibiting activation of the essential transduction component Smoothened, through a mechanism distinct from Smoothened modulation by other lipids.

Hedgehog signaling | SLOS | Smoothened | DHCEO | cyclodextrin

The Hedgehog signaling pathway plays essential roles in embryonic patterning, as indicated by congenital malformations that are associated with mutations in pathway components (1–4). Hedgehog signal transduction is initiated by binding of the Hedgehog signaling protein to its receptor, Patched, which relieves suppression and allows ciliary accumulation of Smoothened, a seven-transmembrane (7TM) protein structurally related to G protein-coupled receptors (GPCRs). Patched is hypothesized to suppress Smoothened activity by controlling the availability of an unknown lipidic Smoothened ligand in the primary cilium (5, 6).

Some of the developmental malformations observed in mice and humans with mutations in *Sonic hedgehog* (*Shh*), especially holoprosencephaly (HPE), are phenocopied by chemical or mutational inactivation of the cholesterol biosynthetic enzyme, 7-dehydrocholesterol reductase (*DHCR7*), which converts 7-dehydrocholesterol (7-DHC) to cholesterol (7, 8). As a result, cholesterol is depleted, whereas 7-DHC accumulates and is converted into abnormal derivatives. One of the underlying reasons for the malformations in Smith–Lemli–Opitz syndrome (SLOS) (*DHCR7* deficiency) is thought to be cholesterol deficiency, and pharmacologic depletion of cellular sterols by methyl-β-cyclodextrin (MCD) inhibits Smoothened activity in wild-type cells (9, 10). It is not clear whether the sterols depleted by MCD directly activate Smoothened or act as permissive factors for Smoothened activation, as noted for sterol regulation of many other GPCR superfamily members (11). As an alternative to a stimulatory/permissive role for sterols that are depleted in SLOS, some evidence suggests a contribution to SLOS pathology by sterols that accumulate to abnormal levels (12–14); how such sterols might affect Smoothened activity is unknown.

Various small-molecule agonists and antagonists that bind to the Smoothened transmembrane domain have been discovered, including the steroidal plant compounds cyclopamine and jervine and synthetic Smoothened antagonists and agonists (SAGs) (15, 16). Several small molecules including cyclopamine derivatives and other synthetic compounds that have been characterized as inhibiting Smoothened by binding to the "cyclopamine pocket" are in clinical trials or have been approved for use as anticancer drugs (17). Recent structural studies define the cyclopamine pocket in atomic detail, but mutations affecting this pocket generally fail to disrupt regulation by Patched (15), suggesting a distinct site of action for an endogenous mediator of Patched regulation.

A distinct class of Smoothened ligand consisting largely of oxygenated cholesterol derivatives and exemplified by 20(*S*)-hydroxycholesterol [20(*S*)-OHC] can activate Smoothened by binding to the cysteine-rich domain (CRD) that is N-terminal to the 7TM helical region (18–20). Although 20(*S*)-OHC is not known to be made naturally, we described two other enzymatically derived sterols that can similarly activate Smoothened via its CRD (18). Mutations in the relevant enzymes fail to exhibit phenotypes related to Hedgehog pathway dysregulation (21), however, and the physiologic relevance of these sterols therefore remains unclear. Furthermore, we demonstrated that Patched

Significance

The Hedgehog protein signal (Hh), covalently modified by cholesterol, functions to coordinate embryonic tissue patterning and postembryonic tissue maintenance. Cholesterol and several of its side-chain oxidized derivatives also function in Hh response by augmenting the activity of Smoothened, an essential seven-transmembrane protein. Here, we show that a distinct class of sterols, oxidized in the B-ring, also affect Hh response, but act by a distinct mechanism to inhibit Smoothened activity. These sterols and their precursor, 7-dehydrocholesterol, accumulate in the human genetic disease Smith–Lemli–Opitz syndrome, providing a rationale for diminished Hedgehog pathway activity in Smith–Lemli–Opitz syndrome and suggesting new candidates as potential modulators of Smoothened activity in normal cells.

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regulation of a Smoothened construct lacking the CRD is intact (18), indicating that any endogenous Smoothened-modulating ligand controlled by Patched must bind elsewhere. The role of endogenous CRD or cyclopamine pocket ligands thus remains unclear, and the putative mediator controlled by Patched, whether an agonist or an antagonist, is unknown.

To identify novel lipids that can activate or inhibit Smoothened activity, we undertook an unbiased, activity-guided fractionation of a *Chlamydomonas* lipid extract that showed inhibitory activity in a mammalian Hedgehog pathway reporter assay. We purified and identified ergosterol endoperoxide (5α,8α-epidioxy-22*E*-ergosta-6,22-dien-3β-ol) as the inhibitory species, which led us to test the involvement of its mammalian analogs derived from 7-DHC in Hedgehog pathway regulation. Using the ability of the synthetic Smoothened agonist SAG to circumvent the requirement for stimulatory/permissive sterols, we show that MCD actually potentiates SAG stimulation of Hedgehog pathway activity and that this effect of MCD correlates with its extraction of 7-DHC. Finally, we show that 7-DHC derivatives such as 3β,5α-dihydroxycholest-7-en-6-one (DHCEO), the most abundant oxysterol in the brain of a SLOS animal model (22), can inhibit Hedgehog signal response.

Results

Ergosterol Endoperoxide Isolated from *Chlamydomonas reinhardtii* Inhibits Hedgehog Signaling. To find novel Smoothened agonists and antagonists, we tested the effect of a total lipid extract prepared from *C. reinhardtii* flagella, which are structurally and functionally related to mammalian primary cilia and amenable to biochemical isolation (23, 24). We also prepared lipid extracts from various other sources, including Hedgehog-responsive NIH 3T3 cells, various mouse tissues, lipoproteins from mammals and *Drosophila* larvae, as well as whole *Drosophila* larval homogenates. Whereas none of these extracts stimulated Hedgehog pathway activity in cultured mammalian cells, in experiments in which the pathway was activated by incubation of cells with the N-terminal signaling domain of murine Sonic hedgehog (ShhN), inhibition was observed, in the following sequence of decreasing relative potency: *Chlamydomonas* flagella > whole *Chlamydomonas* cells ≥ *Drosophila* lipophorin > whole *Drosophila* larvae (Fig. 1*A*). *Chlamydomonas* whole-cell lipid extract antagonized activation by

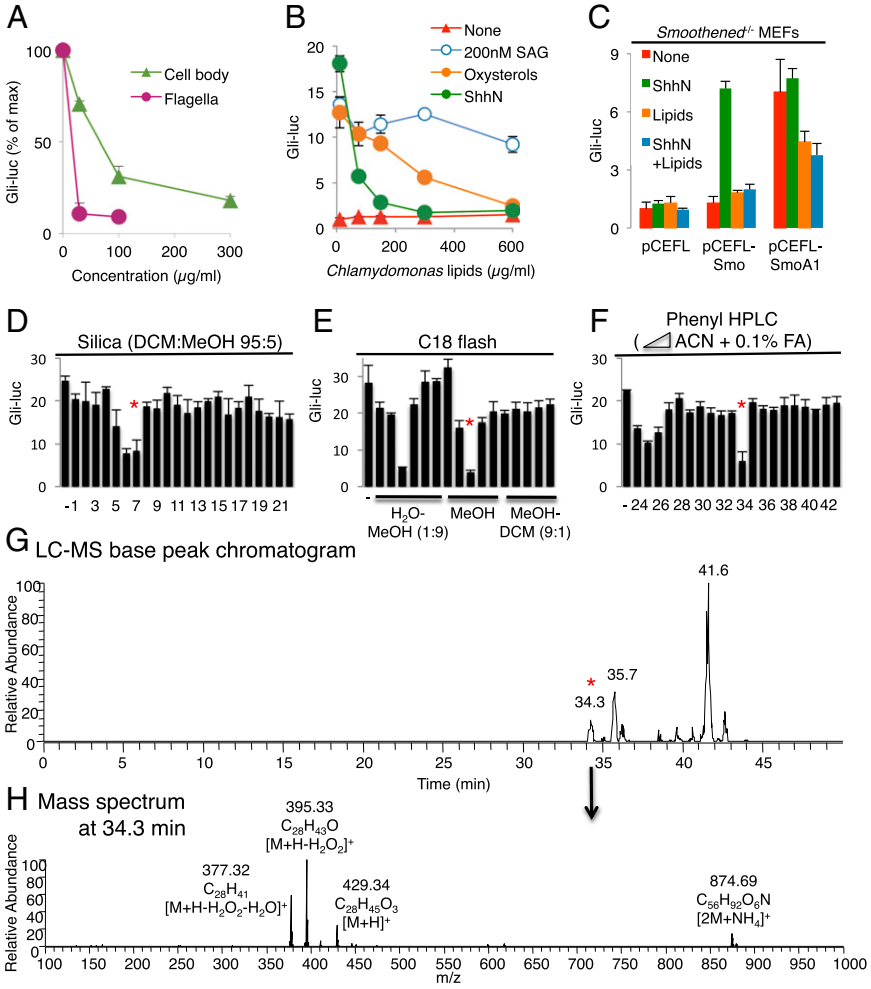

Fig. 1. Identification of ergosterol endoperoxide as a *Chlamydomonas* lipid that inhibits mammalian Hedgehog signaling. (*A*) Gli-luciferase activity in Shh-LIGHT2 cells was measured following treatment with ShhN-conditioned medium in combination with the indicated concentrations of organic extracts from *Chlamydomonas* cell bodies or flagella. (*B*) Dose–response analysis of *Chlamydomonas* whole-cell lipid extract in Shh-LIGHT2 cells with no stimulation (red), stimulation with ShhN-conditioned medium (green), 200 nM SAG (blue) or 5 μM 20(*S*)-OHC and 5 μM 22(*S*)-OHC (oxysterols, orange). (*C*) Gli-luciferase activity in *Smoothened*$^{-/-}$ MEFs transfected with the indicated Smoothened (Smo) constructs and stimulated with ShhN-conditioned medium and *Chlamydomonas* lipids as indicated. (*D–F*) Activity profiles following fractionation of *Chlamydomonas* lipids using the indicated columns and solvents. (*G* and *H*) In-line electrospray ionization–Fourier transform MS analysis, showing masses and formulas of in-source fragmentation products. Red asterisks in *D* and *E* indicate fractions that were used in subsequent chromatography steps. The asterisks in *F* and *G* indicate co-incidence of activity with a chromatographic peak.

ShhN or oxysterols, but only weakly antagonized activation by SAG (Fig. 1*B*). Furthermore, the constitutively active, Patched-resistant SmoothenedA1 mutant conferred significant resistance to the inhibitory activity of *Chlamydomonas* lipids, indicating that these lipids block Hedgehog signaling at the level or upstream of Smoothened (25) (Fig. 1*C*).

We undertook an activity-guided purification of the inhibitory lipid from whole *Chlamydomonas* cells using two steps of flash chromatography (Fig. 1 *D* and *E*) followed by reversed-phase HPLC (Fig. 1*F*) coupled with in-line Orbitrap mass spectrometry (positive-mode electrospray ionization) and parallel automated fraction collection. The activity eluting at 34 min corresponded to a chromatographic peak (Fig. 1*G*) and a collection of related ions that derive from spontaneous in-source fragmentation of a protonated species at *m*/*z* 429.34 (Fig. 1*H*). High mass accuracy permitted elucidation of a neutral molecular formula of $C_{28}H_{44}O_3$. Sequential neutral losses of H_2O_2 (Δm 34) and H_2O (Δm 18) indicated the presence of hydroxyl and peroxide (hydroperoxide or endoperoxide) functional groups (Fig. 1*H*). Interestingly, a partially purified inhibitory fraction obtained from *Drosophila* larval extract contained a species with identical retention time, accurate mass and in-source fragmentation spectrum, as well as a closely related species ($C_{28}H_{42}O_3$) with one more double bond, based on similar retention time, a 2-Da smaller mass, and in-source neutral losses of H_2O and H_2O_2 (Fig. S1).

A search of metabolite and lipid databases with the accurate mass obtained from mass spectrometry revealed 35 sterols or vitamin D derivatives. Chemical analyses indicated a sterol with a single exchangeable hydrogen corresponding most likely to the 3β-OH group of sterols and ruling out the presence of an additional exchangeable hydroperoxide functionality (Fig. S2*A*). Furthermore, the presence of an ester bond was ruled out by resistance of the molecule to base hydrolysis at room temperature (Fig. S2*B*). A scaled-up version of the final HPLC step yielded ∼0.5 mg of purified compound and allowed definitive structure elucidation by NMR. Proton (^{1}H) and ^{13}C chemical shifts matched those previously reported for ergosterol endoperoxide, a natural compound isolated from various fungi (26) (Table S1), with mass and other features that are consistent with the compounds isolated from *Chlamydomonas*.

As this compound was not commercially available, we synthesized it by photooxidation of ergosterol, purified it, and demonstrated its identity with the lipid isolated from *Chlamydomonas* and *Drosophila* by retention time, MS, and NMR analyses (Figs. S3–S5). We confirmed that this synthetic preparation of ergosterol endoperoxide inhibited ShhN signaling with similar potency to that of the compound purified from *Chlamydomonas* (Fig. 2*A*). Pure ergosterol endoperoxide also inhibited the constitutive pathway activity in *Patched1*-deficient mouse embryonic fibroblasts (MEFs) and blocked Smoothened ciliary accumulation, consistent with action on Smoothened in a Patched-independent manner (Fig. 2 *B*–*D*). Lipid extracts from two *Chlamydomonas* mutants with defects at late stages of ergosterol biosynthesis showed similar activity due to formation of sterol endoperoxides with side chains that are distinct from that of ergosterol endoperoxide found in wild-type *Chlamydomonas* (27) (Fig. S6). A mutant defective in formation of the 5,7-diene structure required for endoperoxide formation has not been described, precluding a test of whether this intermediate is critical for the inhibitory activity of the *Chlamydomonas* extract.

An Endogenous Sterol Inhibits Mammalian Hedgehog Signaling. Identification of ergosterol endoperoxide from *Chlamydomonas* and *Drosophila* as a Smoothened antagonist raised the possibility that an endogenous sterol may act in a similar fashion in mammalian cells. One approach to demonstrating the existence of such a negatively acting sterol would be to remove it from cells by preferential extraction with MCD (Fig. 3*A*). We know, however, that MCD treatment impairs response to ShhN stimulation, presumably by removing from cells the essential permissive/activating function of cholesterol (9). We therefore tested whether Smoothened agonists might function in a cholesterol-independent manner, as such activation might then be enhanced by MCD extraction of endogenous negatively acting sterols.

We indeed found that pathway activation by Smoothened agonist SAG1.5 (a more potent SAG analog) (28) could be enhanced by treatment with MCD (Fig. 3*B*), and this enhancement was also manifested by an MCD-induced shift toward greater sensitivity in the dose–response curve of SAG1.5 (Fig. 3*C*). This enhancement was not observed with any other cyclodextrins, suggesting preferential specificity of MCD for the inhibitory sterol. Activation by ShhN, in contrast, was inhibited by all cyclodextrins except α-cyclodextrin (Fig. 3*D*), which preferentially depletes phospholipids (29). All of the ShhN-inhibitory cyclodextrins we tested are known to extract cholesterol (30), and this result is consistent with the previously described critical requirement for cholesterol in ShhN-mediated pathway activation (9).

Endogenous 7-DHC Derivatives Inhibit Hedgehog Response. Ergosterol endoperoxide, the negatively acting sterol identified in *Chlamydomonas* and *Drosophila* (see above) is generated from ergosterol, which is not produced in mammalian cells, but 7-DHC, an important intermediate in cholesterol biosynthesis, has the same ring structure as ergosterol and accumulates in SLOS patients. In fact, a derivative of 7-DHC with a ring structure identical to that of ergosterol endoperoxide, 5α,8α-epidioxycholest-6-en-3β-ol (hereafter referred to as 7-DHC endoperoxide), has been reported in the plasma of SLOS patients and in various tissues of animal models of this disease (31–33). However, although 7-DHC endoperoxide indeed antagonized ShhN-mediated activation with a potency similar to ergosterol endoperoxide (Fig. 4*A*; synthesis and purification described in Figs. S3–S5), most if not all of this compound forms ex vivo by oxidation of 7-DHC (33), thus indicating that this is not the endogenous inhibitory sterol.

Other B-ring oxidized sterols derived from 7-DHC via enzymatic or free-radical–mediated oxidation have been identified as lipids that accumulate in SLOS animal models (33, 34), and we found that two of them antagonize ShhN treatment (Fig. 4*B*). One of these, another endoperoxide compound referred to as **2a** (5α,9α-epidioxycholest-7-en-3β,6α-diol), does not accumulate in

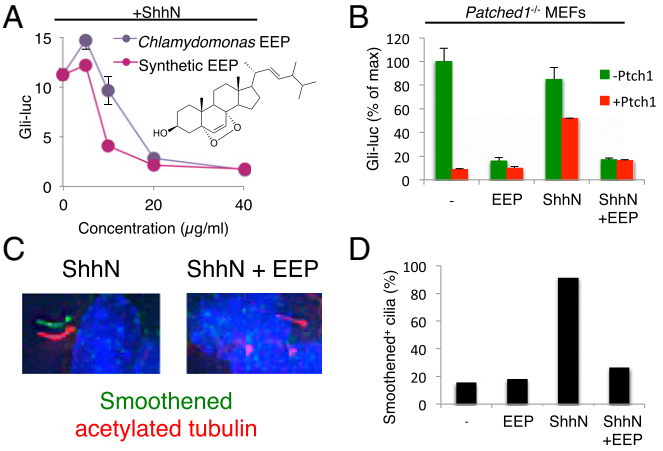

Fig. 2. Ergosterol endoperoxide (EEP) inhibits Hedgehog pathway downstream of Patched1 and at the level of Smoothened. (*A*) Dose–response analysis of EEP purified from *Chlamydomonas* (purple) or synthesized by photooxidation (red). (*B*) Gli-luciferase activity in *Patched1*$^{-/-}$ MEFs transfected with or without *Patched1* and treated with ShhN-conditioned medium and 25 μM EEP as indicated. (*C* and *D*) Ciliary accumulation of endogenous Smoothened in NIH 3T3 cells treated with ShhN-conditioned medium and 25 μM EEP as indicated. Fixed cells were analyzed by immunofluorescence using antibodies against Smoothened (green) and acetylated tubulin (red) with DAPI counterstain (blue). Representative images for selected conditions are displayed as shifted overlays of Smoothened and acetylated tubulin stains. (Magnification: 63×.)

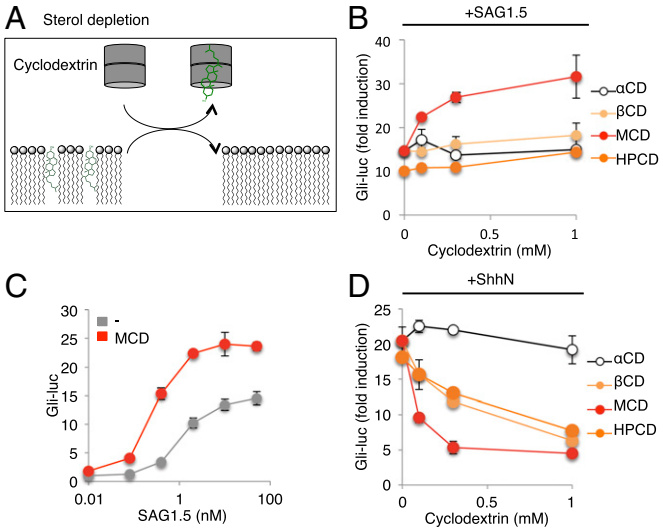

Fig. 3. MCD potentiates SAG. (*A*) Scheme describing use of cyclodextrins to remove cellular sterols. Gli-luciferase activity in Shh-LIGHT2 cells was measured following treatment with (*B*) 10 nM SAG1.5 or (*D*) ShhN-conditioned medium and increasing concentrations of α-cyclodextrin (αCD), β-cyclodextrin (βCD), MCD, or hydroxypropyl-β-cyclodextrin (HPCD). (*C*) Gli-luciferase activity in Shh-LIGHT2 cells was measured following treatment with increasing concentrations of SAG1.5 alone (gray) or together with 1 mM MCD (red).

cells and tissues due to its rapid metabolism (35) and is likely not the endogenous inhibitory sterol. Importantly, compound **2b**, which is the 6β-hydroxy isomer of compound **2a**, did not show inhibitory activity (Fig. 4*B*), indicating a stereospecific mechanism of **2a** action. The other sterol, DHCEO, is more abundant than any other oxysterol, including 24(*S*)-OHC, in *Dhcr7*-deficient cells as well as embryonic and newborn brain tissues (22, 36). The level of DHCEO is even higher in SLOS human fibroblasts (2% of 7-DHC) than in *Dhcr7*-deficient mouse brain (0.05% of 7-DHC) (34). Given the previously described ability of the side-chain oxysterols 20(*S*)-OHC and 22(*S*)-OHC to synergize in activating Smoothened (37), we also tested whether any of the 7-DHC derivatives would synergistically enhance Hedgehog reporter activity, and found that none did (Fig. S7*A*).

Based on the striking specificity of MCD in potentiating SAG1.5-mediated pathway activation (Fig. 3*B*), the endogenous inhibitor or its metabolic precursors should be preferentially extracted by MCD (but not by other cyclodextrins) from ShhN-responsive cells. We were unable to detect DHCEO by targeted liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis in any cyclodextrin extract (method as described in ref. 34). This could be either because of detection limitations, or because MCD does not extract DHCEO itself but, instead, a metabolic precursor essential for its production. Indeed, 7-DHC was efficiently extracted only by MCD and not other cyclodextrins (Fig. 4*C*), correlating with potentiation of SAG1.5 action. To further investigate a possible role for 7-DHC as a precursor of the endogenous sterol inhibitor, we prepared an inclusion complex of MCD with 7-DHC, which effectively exchanges the included sterol with cellular sterols (38) (Fig. 4*D*). We found that the MCD/7-DHC inclusion complex could neither rescue ShhN response nor potentiate SAG1.5 action (Fig. 4*E*), suggesting that 7-DHC exchange mediated by this inclusion complex is able to sustain the endogenous inhibitory sterol, and consistent with the possibility that this inhibitor is a 7-DHC derivative. In contrast, a MCD/cholesterol complex was even more efficacious than empty MCD in potentiating SAG1.5.

The 7-DHC Derivatives Target Smoothened at a Site Distinct from the CRD or the Cyclopamine Pocket. B-ring oxidized sterols appear to act downstream of Patched (Fig. 2*B*), at the level of Smoothened (Fig. 2 *C* and *D*). We further investigated the mechanism of action of these inhibitors in relation to two previously described ligand binding sites on Smoothened: the extracellular CRD and the cyclopamine pocket. We found that DHCEO antagonized activation by the 20(*S*)-OHC-plus-22(*S*)-OHC combination as potently as it antagonized ShhN, consistent with DHCEO action downstream of Patched and at the level of Smoothened (Fig. 5*A*). DHCEO also inhibited two other CRD agonists, 7-keto-27-hydroxycholesterol (7KCh-27OH) (18) and 20(*S*)-yne (39), in an apparently noncompetitive manner (Fig. 5 *B* and *C*). Consistent with the noncompetitive inhibition observed in these functional assays, neither ergosterol endoperoxide nor any of its mammalian analogs blocked Smoothened binding to the 20(*S*)-yne affinity resin, even at concentrations 10 times those sufficient to fully inhibit the functional cell-based reporter assay (Fig. 5*D*). DHCEO and **2a** also failed to compete with BODIPY-cyclopamine for binding to Smoothened (Fig. 5*E*) and failed to antagonize SAG (Fig. 5*F*). Consistently, the dose–response curve of SAG was not altered in the presence of DHCEO (Fig. 5*G*), indicating that DHCEO does not act via the SAG/cyclopamine pocket.

Curiously, despite its inhibition of 20(*S*)-OHC activation, DHCEO does not completely block the synergy between 20(*S*)-OHC and SAG (39). We thus found that addition of 20(*S*)-OHC both increased the potency of SAG (reduced its EC_{50}) and increased the maximum level of pathway activation achieved (Fig. 5*H*, compare green and black tracings); coadministration of DHCEO reversed the effect of 20(*S*)-OHC on the maximum level of pathway activation but not its effect on the increased potency of SAG (Fig. 5*H*, red and green tracings). Altogether, these results support the idea that DHCEO affects Smoothened activity without displacing any of these Smoothened agonists.

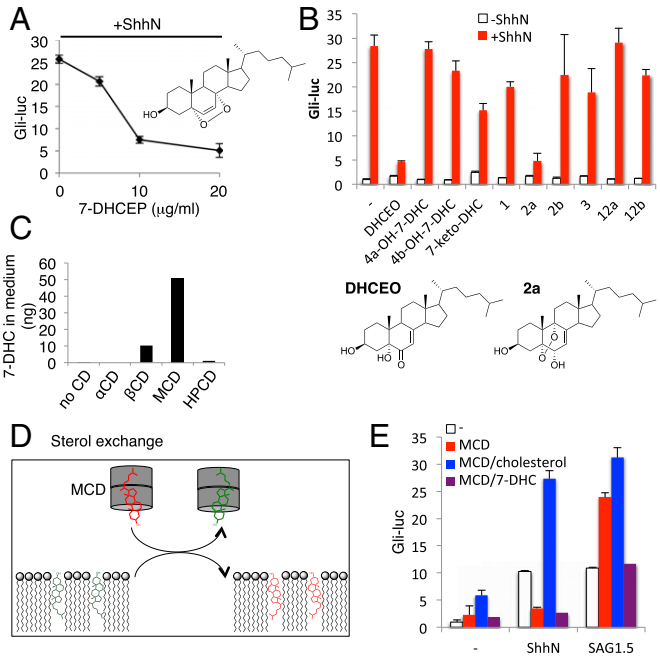

Fig. 4. Endogenous 7-DHC derivatives inhibit Hedgehog signaling. (*A*) Dose–response analysis of 7-DHC endoperoxide (7-DHCEP) in Shh-LIGHT2 cells. (*B*) 7-DHC–derived oxysterols inhibit Hedgehog pathway. Gli-luciferase activity in Shh-LIGHT2 cells was measured following treatment with the indicated sterols either alone (white) or in combination with ShhN-conditioned medium (red). (*C*) 7-DHC levels in the medium after treatment with the indicated cylodextrins were quantified by tandem MS. (*D*) Scheme describing use of MCD to exchange cellular sterols. (*E*) Gli-luciferase activity in Shh-LIGHT2 cells was measured following treatment with ShhN-conditioned medium or 10 nM SAG1.5 and 1 mM MCD complexed with 100 μM cholesterol or 7-DHC.

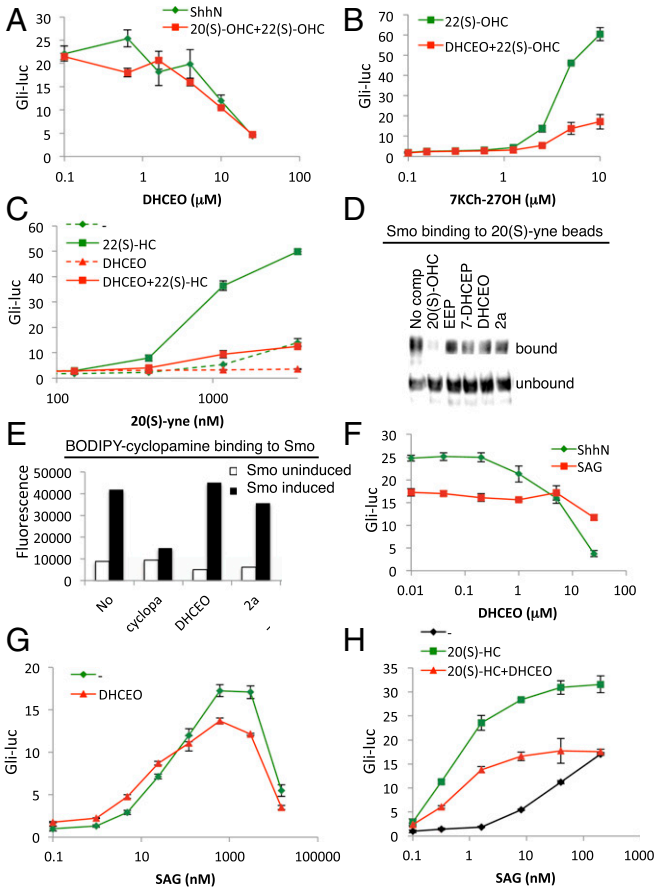

Fig. 5. DHCEO can block the action of CRD agonists but not SAG. (*A–C*) Gli-luciferase activity in Shh-LIGHT2 cells was measured following treatment with: (*A*) increasing concentrations of DHCEO in combination with ShhN-conditioned medium (green) or 5 μM 20(*S*)-OHC plus 5 μM 22(*S*)-OHC (red); (*B*) increasing concentrations of 7KCh-27OH in combination with 5 μM 22(*S*)-OHC (green) or 5 μM 22(*S*)-OHC plus 25 μM DHCEO (red); or (*C*) increasing concentrations of 20(*S*)-yne alone (dashed green) or in combination with 5 μM 22(*S*)-OHC (solid green), 25 μM DHCEO (dashed red), or 5 μM 22(*S*)-OHC plus 25 μM DHCEO (solid red). (*D*) Detergent-solubilized membranes from HEK293S-Smo cells were incubated with 20(*S*)-yne affinity resin in the presence of 50 μM 20(*S*)-OHC, 250 μM ergosterol endoperoxide (EEP), 250 μM 7-DHC endoperoxide (7-DHCEP), 250 μM DHCEO, or 100 μM **2a**. After washing, bound protein was eluted and analyzed by immunoblotting. (*E*) Binding of 5 nM BODIPY-cyclopamine to membranes from tetracycline-inducible HEK293S cells expressing Smoothened was measured in the presence of 3 μM cyclopamine, 25 μM DHCEO, or 10 μM **2a**. (*F–H*) Gli-luciferase activity in Shh-LIGHT2 cells was measured following treatment with increasing concentrations of DHCEO in combination with ShhN-conditioned medium (green) or 200 nM SAG (red); (*G*) increasing concentrations of SAG alone (green) or in combination with 25 μM DHCEO (red); or (*H*) increasing concentrations of SAG alone (black) or in combination with the indicated sterols.

Discussion

The Hedgehog receptor, Patched, is a transporter-like protein thought to act by controlling an endogenous lipid that regulates Smoothened (5). Neither of the two distinct sites on Smo that are known to bind modulatory lipids, however, is likely to respond to Patched regulation. The extracellular CRD domain of Smoothened is not required for Patched action (18), and mutations affecting the cyclopamine pocket generally fail to disrupt regulation by Patched (15), suggesting that the Smoothened site responding to Patched regulation remains unknown, as does the identity of the molecule(s) that mediate this regulation. We therefore undertook an unbiased search for possible mediators of Smoothened regulation, using a cultured cell reporter assay to identify activities from various lipid extracts. By purifying and identifying the active lipid from *Chlamydomonas*, with an enriched source of modulatory activity in flagella, we identified oxidized sterols with modifications within the B-ring as a distinct class of Smoothened antagonists. Mammalian analogs of these B-ring oxysterols derive from the mammalian cholesterol synthesis intermediate 7-DHC, which due to the presence of conjugated double bonds within the B-ring make it subject to several enzymatic or nonenzymatic oxidation reactions that generate the antagonists we have identified.

Of critical importance, our finding that extraction with MCD potentiates the action of the Smoothened agonist SAG1.5 in pathway-responsive cultured cells provides evidence for an endogenous sterol antagonist. Our findings that 7-DHC is extracted specifically by MCD and that incorporation of 7-DHC but not cholesterol in an MCD inclusion complex can negate the potentiating effect of MCD makes 7-DHC or one of its derivatives a likely candidate for such an antagonist. Of note, whereas the potentiating effect of MCD on SAG1.5 action suggests the presence of an endogenous antagonist, SAG1.5 is nevertheless able to activate Smoothened in the presence of this endogenous antagonist, albeit to a more limited extent. This is consistent with the resistance of SAG1.5 to exogenously added B-ring oxysterols that are capable of fully inhibiting pathway activation by the Hedgehog signal.

The current standard of care for SLOS is cholesterol supplementation, which, in addition to increasing cholesterol levels, also provides feedback inhibition of sterol biosynthesis, reducing aberrant accumulation of cholesterol precursors (40). In various cultured cell or animal models of SLOS, both cholesterol deficiency and 7-DHC accumulation have been shown to contribute to the pathobiology of the disease (9, 10, 12–14, 22, 41, 42). Our findings suggest that the accumulation of 7-DHC derivatives in SLOS is likely to be responsible, at least in part, for the apparent reduction of Hedgehog pathway activity seen in this disease. The 7-DHC derivative DHCEO identified here as a Hedgehog pathway antagonist is detectable in normal brain but accumulates to 100-fold higher levels in SLOS—to an overall concentration of ∼3.5 μM—and may contribute to the disease phenotype (22, 36). DHCEO is proposed to form in a three-step process from 7-DHC based on a known biotransformation of cholesterol (34, 43, 44) (Fig. S7*B*). Although formation of the initial epoxide does not require enzyme activity, several known epoxide hydrolase enzymes could carry out the second reaction (45). There is also evidence for an enzymatic basis for the last step, but the specific enzyme has not yet been identified (34).

Other endogenous lipidic molecules proposed to directly modulate Smoothened activity include cholecalciferol (vitamin D_3) (46) and the endocannabinoids (47). We and others have not been able to replicate the pathway inhibition or BODIPY-cyclopamine competition reported for cholecalciferol (48). Furthermore, vitamin D synthesis depends on UV radiation, not oxidation, and cholecalciferol does not accumulate in SLOS, ruling it out as the culprit for SLOS-associated Hedgehog-related phenotypes (49). Regarding endocannabinoids, of the dozen reported to inhibit Hedgehog pathway in mammalian cells, only anandamide (*N*-acylethanolamide 20:4) inhibited BODIPY-cyclopamine binding to Smoothened (47). None of the compounds tested inhibited ciliary accumulation of Smoothened, and most of them including anandamide blocked activation by SAG (46). These observations suggest a site of action for endocannabinoids that is downstream of mammalian Smoothened. A steroidal inhibitory activity was not reported in any extracts examined by these authors. We have found, however, that the high-temperature conditions of conventional saponification used in extract preparation destroys the Hedgehog pathway-inhibitory activity of ergosterol endoperoxide.

Regarding mechanism of B-ring oxysterol action, ergosterol endoperoxide antagonizes Hedgehog signaling downstream of Patched and disrupts Smoothened ciliary accumulation, consistent with a Smoothened-targeting effect. In addition, we find that the mammalian B-ring oxysterol DHCEO is bracketed pharmacologically by distinct classes of Smoothened ligands, as its antagonistic action prevails against pathway activation by CRD agonists but is

surmounted by the cyclopamine pocket agonist, SAG. These observations, coupled with our binding competition studies, together constitute the basis of a model wherein antagonistic B-ring oxysterol derivatives of 7-DHC act directly on Smoothened, but at a site that is distinct from the Smoothened CRD and the cyclopamine pocket. Alternative ligand-binding sites are suggested by the general propensity of most GPCRs to interact with and in some cases be modulated by sterols (50, 51), and by the prediction of additional druggable cavities within the crystallographically determined structure of the Smoothened dimer (17). We have thus identified B-ring oxysterols as endogenous lipidic Smoothened modulators likely to play a role in the pathology of SLOS. Further investigation will be required to determine whether these compounds participate in Patched-mediated regulation of Smoothened in normal cells.

Methods

We carried out Shh signaling assays as described (18) in Shh-LIGHT2 cells, *Smoothened*$^{-/-}$ and *Patched1*$^{-/-}$ MEFs seeded into 96-well plates. Indirect immunofluorescence was performed as described (18) using methanol fixation. Sterol/MCD inclusion complexes (1:10 molar ratio) were prepared by adding 9% (wt/vol) MCD (Sigma; 332615; lot no. STBC2412V; 1.6–2.0 mol CH_3 per unit anhydroglucose) to dried sterols, heating to 80 °C, and sonicating until a clear solution was achieved (52). Purification, chemical syntheses, LC-MS/MS, and binding assays are described in *SI Methods*.

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