Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened

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The steroidal alkaloid cyclopamine has both teratogenic and antitumor activities arising from its ability to specifically block cellular responses to vertebrate Hedgehog signaling. We show here, using photoaffinity and fluorescent derivatives, that this inhibitory effect is mediated by direct binding of cyclopamine to the heptahelical bundle of Smoothened (Smo). Cyclopamine also can reverse the retention of partially misfolded Smo in the endoplasmic reticulum, presumably through binding-mediated effects on protein conformation. These observations reveal the mechanism of cyclopamine’s teratogenic and antitumor activities and further suggest a role for small molecules in the physiological regulation of Smo.

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Plants of the genus Veratrum have a long history of use in the folk remedies of many cultures [Namba 1993, Levetin and McMahon 1996], and the jervine family of alkaloids [Fried and Klingsberg 1953], which constitute a majority of Veratrum secondary metabolites, have been used for the treatment of hypertension and cardiac disease. The association of Veratrum californicum with an epidemic of sheep congenital deformities during the 1950s [Binns et al. 1962] raised the possibility that jervine alkaloids are also potent teratogens. Extensive investigations by the U.S. Department of Agriculture subsequently confirmed that jervine and cyclopamine [11-deoxyjervine] given during gestation can directly induce cephalic defects in lambs, including cyclopia in the most severe cases [Keeler and Binns 1965]. It is now known that the teratogenic effects of jervine and cyclopamine are due to their specific inhibition of vertebrate cellular responses to the Hedgehog (Hh) family of secreted growth factors [Cooper et al. 1998; Incardona et al. 1998], as first suggested by similarities between the Veratrum-induced developmental malformations and holoprosencephaly-like abnormalities associated with loss of Sonic hedgehog (Shh) function [Chiang et al. 1996; Roessler et al. 1996]. In accordance with this general mechanism, cyclopamine also has shown some promise in the treatment of medulloblastoma tumors caused by inappropriate Hh pathway activation [Berman et al. 2002]. How cyclopamine specifically inhibits Hh pathway activation is unclear, but it appears to interfere with the initial events of vertebrate Hh signal reception, which involve the multipass transmembrane (TM) proteins Patched [Ptc] and Smoothened (Smo; Ingham and McMahon 2001). During normal Hh signaling, Hh proteins bind to Ptc [Marigo et al. 1996; Stone et al. 1996; Fuse et al. 1999], thereby alleviating Ptc-mediated suppression of Smo, a distant relative of G-protein-coupled receptors [GCPRs]. Smo activation then triggers a series of intracellular events, culminating in the activation of Gli-dependent transcription [Alexandre et al. 1996; Aza-Blanc et al. 1997].

Cyclopamine appears to interfere with these signaling events by influencing Smo function, as it antagonizes Hh pathway activity in a Ptc-independent manner and exhibits attenuated potency toward an oncogenic, constitutively active form of Smo [W539L, SmoA1, Taipale et al. 2000]. Although these observations suggest that cyclopamine may regulate Smo activity, they reveal neither the biochemical mechanism of Smo activation nor the molecular basis of cyclopamine action. Studies in Drosophila have shown that Hh stimulation is associated with changes in Smo phosphorylation state, subcellular localization, and perhaps protein conformation [De nef et al. 2000; Ingham et al. 2000]. In principle, cyclopamine-mediated inhibition of vertebrate Smo activity could perturb any of these cellular events. How Ptc inhibits Smo function is also unclear, although it appears that Ptc acts catalytically through an indirect mechanism [Taipale et al. 2002].

Here we demonstrate that cyclopamine inhibits Hh pathway activation by binding directly to Smo. This binding interaction is localized to the heptahelical bundle and likely influences the Smo protein conformation. Cyclopamine binding is also sensitive to Ptc function, providing biochemical evidence for an effect of Ptc action on Smo structure. Collectively, these results provide a molecular basis for cyclopamine action and suggest that the regulation of Smo activity by Ptc may involve endogenous small molecules.

Results and Discussion

A photoaffinity derivative of cyclopamine specifically cross-links Smo

To determine whether cyclopamine acts directly on Smo, a photoaffinity reagent (PA-cyclopamine, Fig. 1A) was shown to inhibit Shh signaling in a mouse cultured cell assay [Shh-LIGHT2; Taipale et al. 2000] with an IC50 comparable to that of cyclopamine itself [150 nM versus 300 nM, respectively]. Light activation of 125I-labeled PA-cyclopamine in live NIH-3T3 cells did not detectably label endogenous mouse Smo [mSmo, henceforth referred to as Smo]. As endogenous Smo in these cells is expressed at low levels [Taipale et al. 2002], we tested whether binding could be detected in COS-1 cells transiently transfected with a construct for high-level expression of Smo C-terminally fused to Myc epitopes. Un-
under these conditions, Smo is observed as two distinctly migrating forms, both of which were readily labeled by 125I-labeled PA-cyclopamine upon photoactivation (Fig. 1B). We observed essentially no cross-linking to presumably nonnative, SDS-resistant Smo aggregates, reflecting the requirement for an intact cyclopamine-binding site. Consistent with the resistance of SmoA1 to cyclopamine, PA-cyclopamine also was unable to efficiently cross-link this oncogenic Smo mutant, which is observed as a single form (Fig. 1B). Thus, the W539L mutation either directly disrupts the cyclopamine-binding site or alters the balance between active and inactive Smo states. To investigate the nature of the differently migrating forms of Smo and SmoA1 we characterized them by digestion with endoglycosidase H (endo H), an enzyme capable of hydrolyzing the simpler glycosyl adducts characteristic of the endoplasmic reticulum [ER], but not the more complex adducts associated with post-ER compartments such as the Golgi or the plasma membrane. One form of Smo is endo H-sensitive and presumably localized to the ER, the second form is endo H-resistant and likely represents post-ER protein (Fig. 1C). All of the SmoA1 protein is completely endo H-sensitive (Fig. 1C), suggesting that SmoA1 is trapped in the ER. This localization is confirmed by colocalization of a constitutively active, fluorescent protein-tagged form of SmoA1 [SmoA1–YFP] with an ER-specific marker (Fig. 1D). Accordingly, SmoA1–YFP does not colocalize with a Golgi-specific marker (Fig. 1D).

The specificity of PA-cyclopamine cross-linking of Smo is indicated by its efficient competition by a strongly inhibitory dose of KAAD-cyclopamine, a potent derivative of cyclopamine (IC50 = 20 nM in the Shh-LIGHT2 assay, Taipale et al. 2000, Fig. 1B). Upon titration of this reaction with increasing doses of KAAD-cyclopamine, we found that Smo labeling was competed in a concentration range (Fig. 1E) comparable to that required for inhibition of Shh signaling. The cross-linking competition assay thus appears to faithfully reflect the in vivo properties of cyclopamine derivatives in pathway inhibition.

A fluorescent derivative of cyclopamine specifically binds Smo-expressing cells

The specificity of cyclopamine binding to Smo was further confirmed by assays using BODIPY-cyclopamine (Fig. 2A), a fluorescent derivative that retains potency in Shh signaling inhibition (IC50 = 150 nM). This derivative bound with high capacity to a subpopulation of COS-1 cells transiently transfected for expression of Smo, as determined by fluorescence microscopy and flow cytometry (Fig. 2B,C), but did not bind cells expressing SmoA1, nor to cells expressing the Smo protein from Drosophila (Fig. 2C), in which cyclopamine has no effect on Hh signaling (Taipale et al. 2000). BODIPY-cyclopamine also did not bind cells expressing mouse Frizzled7 protein, the closest structural relative of Smo and a member of the Frizzled family of Wnt receptors, nor to cells expressing mouse Ptc1 (Fig. 2C). BODIPY-cyclopamine binding to cells expressing Smo was blocked by KAAD-cyclopamine in a dose-dependent manner (Fig. 2B,D), with an apparent dissociation constant for KAAD-cyclopamine (Kd = 23 nM) comparable to its biological potency. Similar results were obtained with paraformaldehyde-fixed cells (data not shown), ruling out possible artifacts caused by indirect effects of endocytosis or other trafficking processes. We thus observe in both the covalent PA-cyclopamine cross-linking assay and in the noncovalent BODIPY-cyclopamine-binding assay that cyclopamine interacts specifically with Smo and does so with an affinity that corresponds to its IC50 for pathway inhibition. These results
Cyclopamine/Smo complex (right 3 µM KAAD-cyclopamine; Smoligandsthrough binding competi-
tions (black trace, 0 nM; orangetrace, 80 nM; red trace, left)

D A fluorescent derivative of cyclopamine binds Smo-expressing cells. (A) Chemical
structure of BODIPY-cyclopamine and its inhibitory activity on Shh signaling. (B) BODIPY-
cyclopamine binds to a subpopulation of COS-1 cells transfected with a Smo expression
construct, and KAAD-cyclopamine inhibits this interaction (KAAD-cyclopamine concentra-
tions shown in boldface type). (C) Specific BODIPY-cyclopamine binding to Smo-expressing
COS-1 cells can also be detected by flow cytometry (black trace, left and right panels), as this
subpopulation exhibits high fluorescence intensity (brackets). In contrast, cells expressing
SmoA1 (blue trace; left panel), mouse Ptch (green trace; left panel), mouse Frizzled 7 (red
trace; right panel), or Drosophila Smo (blue trace; right panel) fail to bind BODIPY-cyclopa-
mine in a specific manner. (D) Flow cytometric quantitation of specific BODIPY-cyclopamine
binding to Smo-expressing cells (bracket, left panel) can be used to determine the affinities of
Smo ligands through binding competitions [black trace, 0 nM; orange trace, 80 nM; red trace,
3 µM KAAD-cyclopamine; left panel], yielding an apparent K_D of 23 nM for the KAAD-
cyclopamine/Smo complex (right panel).

strongly support a direct mechanism of cyclopamine ac-
tion on Smo.

Cyclopamine binding is localized to the Smo
heptahelical bundle

Having established Smo as the direct cellular target of
cyclopamine, we investigated the structural determi-
nants of Smo required for its binding. We found that
BODIPY-cyclopamine can bind cells expressing Smo pro-
teins that lack either the N-terminal, extracellular cy-
teine-rich domain [SmoACRD] or the cytoplasmic C-ter-
\[SmoA\]nal domain [SmoA\~CT; Fig. 3A], and that binding to
either protein is sensitive to competition by KAAD-cy-
clopamine (Fig. 3B). The different levels of BODIPY-cyclo-
pamine binding associated with Smo, SmoACRD, and
SmoA\~CT likely reflect variations in protein expression
levels rather than differences in protein–ligand affinities,
as KAAD-cyclopamine inhibited the BODIPY-cyclopa-
mine binding to these different proteins with similar
\[SmoA\]|icities. Thus, despite the importance of the
cytoplasmic C-terminal domain of Smo for Hh signaling [J. Taipale and P.A. Beachy, un-
publ.], and of the homologous CRD of Frizzled
\[SmoA\]ceptors for Wnt-binding and receptor activa-
tion [Bhanot et al. 1996], cyclopamine binding of
Smo appears not to require these domains. In-
stead, the cyclopamine binding site in Smo is
localizated to the heptahelical domain of this integ-
ral membrane protein.

Cyclopamine binding can alter the
conformation of SmoA1

The binding of cyclopamine to the Smo hepta-
helical bundle suggests that Smo inhibition by
this natural product involves a protein conformational shift. The structurally
related GPCR family uses a conformational change to link the binding of extracellu-
lar ligands to the recruitment of intracellular components, in this case G proteins (Christopoulos and
Kenakin 2002). Although G proteins have not been implicated in Smo-me-
diated pathway activation, an effect of
cyclopamine binding on Smo structure is
supported by the ability of KAAD-
cyclopamine to reverse Hh inhibition by
cyclopamine binding to Smo.

Hh inhibition by cyclopamine binding to Smo

Figure 3. Cyclopamine binds to the heptahelical bundle in Smo. (A) COS-1 cells
expressing either SmoACRD (middle panel) or SmoA\~CT (right panel) were treated
with BODIPY-cyclopamine and analyzed by flow cytometry. As with Smo-ex-
pressing cells [left panel], a subpopulation of these cells exhibit specific BODIPY-
cyclopamine binding (see brackets). (B) BODIPY-binding to these cells is inhibited
by 150 nM KAAD-cyclopamine to similar extents.
by the ER quality-control system, and that the binding of small molecules such as cyclopamine or SAG alters SmoA1 structure to resemble a more native state, thus permitting export.

**Ptch activity modulates cyclopamine binding to Smo**

As both cyclopamine and Ptch negatively regulate Smo activity, we next investigated how Ptch activity influences the ability of Smo to bind cyclopamine. We found that increased levels of mouse Ptch expression in COS-1 cells dramatically enhanced the photoaffinity cross-linking of post-ER Smo by $^{125}$I-labeled PA-cyclopamine (Fig. 5A). In contrast, the labeling of ER-localized Smo was not affected, and cellular concentrations of either Smo form were not altered by Ptch expression. Treatment of the Smo- and Ptch-expressing cells with the N-terminal domain of Shh (ShhN) was able to reverse the effect of Ptch expression on PA-cyclopamine/Smo cross-linking, confirming its dependence on Ptch activity (Fig. 5B).

These results provide some insights into the regulation of Smo by Ptch. First, Ptch appears to act only on post-ER Smo, as the PA-cyclopamine cross-linking of ER-localized Smo is independent of Ptch expression levels. This subcellular compartmentalization of Ptch action is consistent with previous observations that Ptch is primarily localized to endosomal/lysosomal vesicles and the plasma membrane (Capdevila et al. 1994; Fuse et al. 1999; Denef et al. 2000). Second, the ability of Ptch expression to significantly increase post-ER Smo labeling by PA-cyclopamine without influencing overall protein levels suggests that the effect of Ptch activity alters Smo conformation and that Ptch and cyclopamine promote inactive Smo states that may be structurally related.

**Endogenous small molecules may regulate Smo activity**

How Ptch influences Smo conformation remains enigmatic, despite extensive genetic analyses of the Hh pathway. Although it was initially proposed that Ptch and Smo form a heteromeric receptor (Stone et al. 1996), it is now believed that Smo activity is modulated by Ptch in an indirect, nonstoichiometric manner (Taipale et al. 2002). In the case of the Frizzled family of seven-TM receptors, which are closely related to Smo in structure, receptor activation involves the binding of Wnt ligands to the Frizzled CRD (Bhanot et al. 1996) and recruitment of an LDL receptor-related protein (Pinson et al. 2000; Wehrli et al. 2000). No analogous protein interactions have been associated with Smo activation, and removal of the Smo CRD does not appear to significantly alter Smo function or its suppression by Ptch (Taipale et al. 2002).

These observations coupled with the susceptibility of Smo to cyclopamine suggest that Smo regulation may involve endogenous small molecules rather than direct protein–protein interactions. Consistent with this model, Ptch is structurally related to the resistance-nodulation-cell division family of prokaryotic permeases (Tseng et al. 1999) and to the Niemann-Pick C1 protein (Davies et al. 2000), which are capable of transporting hydrophobic molecules. Ptch action might similarly affect the subcellular and/or intramembrane distribution of endogenous molecules, thus influencing Smo activity by altering the localization of a Smo ligand. Alternatively, this Ptch activity could influence membrane structure and Smo trafficking (Sprong et al. 2001), a shift in Smo localization might then be accompanied by activity-modulating changes in the molecular composition of specific subcellular compartments (Sprong et al. 2001).

**Pharmacological modulation of Smo activity may be therapeutically useful**

The demonstration of cyclopamine binding to Smo establishes the mechanism of action for this plant-derived teratogen. Our studies show that cyclopamine interacts with the Smo heptahedral bundle, thereby promoting a protein conformation that is structurally similar to that induced by Ptch activity. Equally important, these studies reveal the molecular basis for cyclopamine’s antitumor activity (Berman et al. 2002) and validate Smo as a therapeutic target in the treatment of Hh-related diseases. Aberrant Hh pathway activation has been associated with several cancers, such as medulloblastoma and basal cell carcinoma (Taipale and Beachy 2001; Wicking and McGlinn 2001), and many of these tumors involve mutations in Ptch or Smo. As a specific Smo antagonist, cyclopamine may be generally useful in the treatment of
such cancers, a therapeutic strategy further supported by the absence of observable toxicity in cyclopamine-treated animals (Keeler and Binns 1968, Berman et al. 2002). Additional Smo antagonists might also be discovered through small molecule screens for specific Hh pathway inhibitors, thus comprising a class of pharmacological agents with possible utility in the treatment of Hh-related oncogenesis.

Materials and methods

Preparation of synthetic compounds

Procedures for the chemical synthesis of KAAD-cyclopamine, PA-cyclopamine, and BODIPY-cyclopamine is described elsewhere (Chen et al. 2002).

Cell-based assays for Hh pathway activation

Assays for Hh pathway activation in Shh-LIGHT2 cells, a clonal NIH-3T3 cell line stably incorporating Gli-dependent firefly luciferase and constitutive Renilla luciferase reporters, were conducted as previously described (Taipale et al. 2000).

Preparation of Smo fusion proteins and deletion mutants

Smo–Myc3 and SmoA1–Myc3 contain three consecutive Myc epitopes at the protein C terminus. SmoΔCRD lacks amino acids 68–182, and SmoΔCT lacks amino acids 556–793. Smo-GFP, SmoA1–YFP, and SmoA1–GFP contain fluorescent proteins at the C terminus. All constructs were generated by PCR and verified by DNA sequencing.

Photoaffinity labeling of Smo proteins

COS-1 cells were cultured in 6-well plates and transfected with Smo–Myc3 or SmoA1–Myc3 expression vectors (1 µg/well). Two days after transfection, each well was incubated with 1 µCi of 125I-labeled PA-cyclopamine (0.5 µM) final concentration in phenol red-free DMEM containing 0.5% bovine serum, and analyzed for green fluorescence (FACScan, Beckton Dickinson). A fluorescence intensity range that excludes nontransfected cells was then selected for quantification of specific BODIPY-cyclopamine binding (see brackets in Figs. 2C,D and 3A). Curve-fitting analysis was performed with Kaleidograph (Synergy Software).

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Figure 5. PtcH activity promotes cyclopamine/Smo complexation. (A) PA-cyclopamine cross-linking of post-ER Smo–Myc3 (solid arrowhead) in COS-1 cells is significantly increased upon Ptch expression in a dose-dependent manner (left panel). The labeling of ER-localized Smo–Myc3 (open arrowhead, left panel) is not affected by PtcH expression, and overall Smo–Myc3 expression levels remain constant (right panel). (B) ShhN reverses the effects of PtcH expression on PA-cyclopamine/Smo cross-linking.

Localization studies of Smo and SmoA1 proteins

COS1/10T1/2 cells were transfected with 10% fetal bovine serum, 0.5 µg/mL ZnSO4, and β-mercaptoethanol (3.5 µL/500 mL DMEM) on glass coverslips in 6-cm2 dishes. COSH1/10T1/2 cells were transfected with either Smo–GFP, SmoA1–GFP, or SmoA1–YFP expression constructs, all of which yield functionally active proteins [data not shown]. To assess SmoA1 subcellular localization, either an ER marker (pECFP-ER, Clontech) or a Golgi marker (pECFP-GolgI, Clontech) was cotransfected with the SmoA1–YFP construct. One day after transfection, SmoA1–GFP-expressing cells were treated with either 10 µM KAAD-cyclopamine or 1 µM SAG for 16–20 h. All cells were imaged 2 d after transfection, at 37°C in a closed observation chamber (FCS2, Biotech) with constant laminar flow perfusion of culture medium with or without KAAD-cyclopamine or SAG. Fluorescent protein illumination, detection, and imaging were performed on a Zeiss inverted microscope outfitted with a Xenon light source, single or dual-pass filters, and a cooled CCD camera. Images were acquired with Metamorph software (Universal Imaging).

Endo H digestion of Smo proteins

COS-1 cells were cultured in DMEM containing 10% fetal bovine serum in 6-well plates and transfected with Smo–Myc3 or SmoA1–Myc3 expression vectors (1 µg/well). One day after transfection, 5 µM KAAD-cyclopamine was added to a well of the Smo1–Myc3-expressing cells. Two days after transfection, each well of cells was washed twice with PBS and lysed with 300 µL of RIPA buffer [50 mM Tris-Cl at pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 µg/mL leupeptin, 1 µg/mL aprotinin, 0.2 mM PMSF]. Cell lysates were centrifuged at 20,000g at 4°C for 15 min, and the supernatant was then centrifuged at 100,000g for 30 min, and the supernatant of the second centrifugation was used for glycosidase treatments and/or SDS-PAGE. For glycosidase treatments, 45 µL of cell lysate was denatured in 0.5% SDS, 1% β-mercaptoethanol at room temperature for 10 min and then incubated in 50 mM sodium citrate (pH 5.5) and 50 units of endo H at 37°C overnight. The 9E10 antibody was used for Western blotting, following SDS-PAGE separation and protein transfer to nitrocellulose.

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