Identification and Characterization of Several Dietary Alkaloids as Weak Inhibitors of Hedgehog Signaling

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The Hedgehog (Hh) signaling pathway plays an integral role in the patterning and development of diverse structures in the vertebrate embryo. Aberrations in Hh signaling are associated with a range of developmental defects including failure of interhemispheric division of the embryonic forebrain as well as midline facial dysmorphia including cleft lip/palate and cyclopia, collectively termed holoprosencephaly (HPE). Postnatally, Hh signaling has been postulated to play a pivotal role in healing and repair processes and inappropriate Hh pathway activation has been implicated in several types of cancers. The Veratrum alkaloid cyclopamine is a potent inhibitor of Hh signaling and causes HPE-like defects in diverse species including sheep, hamster, mouse, and zebra fish. Using murine cell-based assays, we have determined that a number of dietary alkaloids similar in structure to cyclopamine also inhibit Hh signaling but with significantly lower potency. We found that these dietary compounds act additively through a mechanism similar to cyclopamine, downstream of Ptc1 and upstream of Gli1. Using an embryonic zebra fish developmental assay, we found that while cyclopamine exposure caused HPE-like defects, exposure to one of these dietary compounds, solanidine, did not.

The observed teratogenic activity of cyclopamine prompted concern over structurally related human dietary alkaloids, and subsequent studies suggested a weak teratogenic capacity of these compounds. Gaffield and Keeler (1996) found that solanidine and solasodine induced phenotypes similar to those induced by cyclopamine in hamsters, while Incardona et al. (2000) found that solasodine and tomatidine induced mild HPE-like phenotypic changes in chick embryos. However, the defects induced by the dietary compounds, as compared to cyclopamine, occurred much less frequently in the chick trials and required much higher doses in sheep trials. Because of...
their apparent relatively low potency, these dietary alkaloids have often been implemented as presumptive negative controls in studies using cyclopamine as an Hh antagonist (Cooper et al., 1998; Schnapp et al., 2005).

Given the demonstrated importance of Hh signaling in a broad range of biological processes including embryonic development, tissue repair, stem cell maintenance, and carcinogenesis, the identification of pathway inhibitors with relevant human exposure may have important health implications. Here, we set out to specifically test the capacity of several dietary alkaloids to inhibit Hh signaling in cell-based assays designed to determine their potency, efficacy, and mode of action relative to cyclopamine. We then determined the ability of one dietary alkaloid, solanidine, to induce HPE-like effects in the developing zebrafish embryo. Our results suggest that specific dietary alkaloids inhibit Hh signaling through a mechanism similar to cyclopamine but demonstrate significantly less potency in vitro and in vivo.

MATERIALS AND METHODS

Chemicals. Solanidine, solasodine, tomatidine, and diosgenin were purchased from MP Biomedicals (Solon, OH). Cyclopamine was purchased from Toronto Research Chemicals (North York, Ontario, Canada). Cyclohexanol, 3-methylpiperidine, and nicotine were purchased from Sigma-Aldrich. All compounds were dissolved in 2-hydroxypropyl beta-cyclodextrin (HBC) (45% vol/vol) (Sigma-Aldrich, St Louis, MO) (Incardona et al., 1998) to produce a 5mM stock concentration. For all assays, HBC carrier alone was administered for vehicle control samples.

Gli-luciferase reporter assay. NIH3T3 cells stably expressing Gli-responsive firefly luciferase and TK-renilla luciferase (Shh LIGHTII) were generously provided by Dr Phillip Beachy. Cells were plated in Multwell plates containing 1% FBS media. Cells were allowed to attach overnight, and media were replaced with DMEM containing 1% FBS media. Cells were allowed to attach overnight, and media were replaced with DMEM containing 1% fetal calf serum (FCS) ± 1mM octylated human Shh peptide (Curis/Genentech) and alkaloids or structural constituents at given concentrations.

Target gene transcriptional response assays. Wild-type primary mouse embryonic fibroblasts (MEFs) were harvested and maintained as described (Lipinski et al., 2006). MEFs were plated in DMEM containing 10% fetal bovine serum (FBS) at 2 x 10⁵ cells/well in a Primaria 24-well plate and allowed to attach overnight. Following, media were replaced with DMEM containing 1% FBS media ± octylated Shh peptide at a concentration of 1nM, ± Glil-GFP-expressing adenovirus (Butitta et al., 2003) at 100 ifu/ml, and ± alkaloids at given concentrations. After a 48-h incubation period, RNA was harvested, and gene expression was determined by real-time RT-PCR as described (Lipinski et al., 2006).

β-Galactosidase assays. Immortalized Ptc1ΔC MEFs expressing LacZ inserted into the Ptc1 locus were kindly provided by Dr Phillip Beachy. Cells were plated in DMEM containing 10% FCS at 2 x 10⁶ cells/well in a Primaria multwell 24-well plate and allowed to attach overnight. Following, media were replaced with DMEM containing 1% FBS and alkaloids at given concentrations. After a 48-h incubation period, media were removed, and cell lysate was collected using Reported Lysis Buffer (Promega, Madison, WI). Following, β-galactosidase activity was determined by incubating cells lysates with o-nitrophenyl-beta-D-galactopyranoside substrate and measuring absorbance at 420nm. β-Galactosidase activity was then normalized to total protein concentration as determined by Bradford assays.

Chemical treatment of zebrafish embryos. Cyclopamine or solanidine were dissolved in 45% hydroxypropyl-β-cyclodextrin (HBS). The final concentration of HBC in any dosing solution did not exceed 0.2% as concentrations higher than that are toxic to embryos. Three zebrafish (Danio rerio) embryos at 30–50% epiboly were placed in 1 ml of dosing solution and allowed to develop at 27°C until the desired developmental stage was reached. Embryos were euthanized with Tricaine-S (Aquatic EcoSystems, Apopka, FL) prior to observations.

Zebrafish interocular distance. Interocular distance in zebrafish embryos was measured at 48 h postfertilization (hpf). Dechlorinated euthanized embryos were stabilized in 3% methylcellulose and imaged ventrally with an Optronics MicroFire camera mounted on a Leica MZ16 stereomicroscope. Still images were measured using MetaMorph software (Molecular Devices, Sunnyvale, CA). The distances from the center of one pupil to the center of the other pupil and from the tip of the snout to the center point between the posterior of the eyes were measured in pixels; these distances were then converted into micrometers. To normalize the interocular distance measurement to the relative size of the head, a ratio of pupil-pupil distance divided by the distance from the nose to the posterior of the eyes was calculated. This ratio was designated relative interocular distance (RID). A total of six RIDs were determined for each treatment concentration. An average RID with standard error was then calculated for each condition. Significance of dose dependence was determined using a Kruskal-Wallis one-way ANOVA on ranks, followed by Tukey’s HSD test for multiple comparisons versus control with significance at the p < 0.05 level.

Craniofacial cartilage imaging. Craniofacial cartilage was imaged at 96 hpf. Euthanized embryos were fixed in 4% paraformaldehyde overnight twice, rinsed in PBS, and then dehydrated in 50% ethanol/50% reverse osmosis water for 24 h followed by 24 h in 100% ethanol. Cartilage was then stained with alcian blue according to a protocol obtained from the MD Peterson lab at the University of Washington and modified by the RE Peterson lab at the University of Wisconsin. Briefly, embryos were incubated in cartilage staining solution (70% ethanol, 30% glacial acetic acid, 0.2 mg/ml Alcian Blue) for 24 h with mild agitation. Embryos were then incubated in saturated borate solution (40 mg/ml borax in reverse osmosis water) with mild agitation overnight, followed by bleaching for 20 min with bleaching solution (15% three-percent hydrogen peroxide, 85% one-percent potassium hydroxide) with mild agitation. Embryos were then digested in trypsin digestion/destaining solution (35% saturated sodium borate, 65% milli-Q water, 1 mg/ml trypsin) for 48 h with no agitation, followed by incubation in 30% glycerol/70% one-percent potassium hydroxide for 48 h with mild agitation, then in 60% glycerol/40% one-percent potassium hydroxide for 48 h with mild agitation. Finally, embryos were transferred to 100% glycerol for storage and imaging. Stained craniofacial cartilage was imaged with light microscopy using an Optronics MicroFire camera mounted on a Leica MZ16 stereomicroscope.

RESULTS

We first tested the ability of several alkaloids and their chemical constituents (Fig. 1) to inhibit Hh signaling in NIH3T3 cells stably expressing Gli-responsive firefly luciferase and tyrosine kinase-renilla luciferase (Taipale et al., 2000). Addition of 1nM Shh peptide elicited a 12-fold induction of reporter activity, which was completely blocked by the addition of 5μM cyclopamine (Fig. 2). At equimolar concentrations, the dietary alkaloids solanidine, solasodine, and tomatidine also significantly inhibited reporter activity while nicotine and the nonalkaloid, diosgenin had no significant effect. 3-Methylpiperidine, a constituent of cyclopamine, solasodine, tomatidine, and cyclohexanol also had no effect.
on reporter activity. No treatment directly affected renilla luciferase activity.

To determine the potency and efficacy of solanidine and tomatidine relative to cyclopamine, we performed cell-based dose-response assays. These assays measured inhibition of the endogenous Hh target gene Ptc1 in Shh-stimulated wild-type MEFs (Lipinski et al., 2006). This assay was selected because it affords a more robust response to compare pathway inhibitors and avoids any possible nonspecific effect of the compounds on reporter activity. Shh-stimulation increased Ptc1 expression 70-fold over vehicle treatment. Each of the three alkaloids was able to reduce Ptc1 expression to unstimulated levels (Fig. 3). However, cyclopamine was considerably more potent than either tomatidine or solanidine. The approximate EC50 values presented in Figure 3 suggest that cyclopamine is 100 times more potent than tomatidine and 250 times more potent than solanidine in this cell-based assay. No treatment caused significant cytotoxicity as determined by trypan blue exclusion assays (data not shown).

The dose-response curves for Hh signaling inhibition generated for cyclopamine, solanidine, and tomatidine demonstrated similar slopes, suggesting that a common mechanism of action exists among them. Cyclopamine is known to interfere with Hh signaling by directly binding and preventing the activation of the transmembrane protein Smo (Chen et al., 2002a). We used cells with genetic alterations in Hh pathway components to determine the site of action of the dietary alkaloids. Ptc1−/− MEFs demonstrate ligand-independent constitutive pathway activity. The constitutive activation of Smo in the absence of Ptc1 remains sensitive to inhibition by cyclopamine (Taipale et al., 2000), and we postulated that Smo would also be sensitive to inhibition by the dietary compounds. Ptc1−/− MEFs that express lacZ from the Ptc1 locus were treated with the respective EC50 and EC90 values of cyclopamine, solanidine, and tomatidine, and β-galactosidase activity was assessed as a measure of Hh pathway activity. We found that cyclopamine, solanidine, and tomatidine reduced reporter activity in a dose-dependent fashion, demonstrating that each steroidal alkaloid acts downstream of Ptc1 (Fig. 4). Neither 3-methylpiperidine nor nicotine had an effect at a concentration of 25µM.

Since cyclopamine acts at Smo, direct activation of Hh target gene transcription by Gli overexpression is insensitive to cyclopamine inhibition (Taipale et al., 2000). We tested whether pathway inhibition by the dietary compounds is similarly circumvented by overexpressing Gli1 in wild-type MEFs. Overexpression of Gli1-GFP elicited a 45-fold induction of Ptc1 expression compared to GFP alone. At their
respective EC₉₀ concentrations, none of the tested alkaloids affected Ptc1 induction, suggesting that each acts upstream of target gene activation by Gli1 (Fig. 5).

While each of the compounds studied here has a relatively low potency for Hh signaling inhibition, it is likely that human exposure is not limited to a single compound in isolation, and moreover the human diet may contain other inhibitory compounds of low potency. Toward this end, we investigated the inhibition activity of solanidine, solasodine, and tomatidine, individually or together. We found that each compound significantly inhibited pathway activity and that when added in combination, they acted additively to further reduce pathway activity (Fig. 6).

Cyclopamine teratogenicity is not exclusive to mammals. In fact, Hh signaling has recently been shown to be integral for several developmental processes in the zebra fish and exposure of the developing embryo to cyclopamine causes correlative defects. We used the zebra fish model to test the teratogenic potential of one dietary alkaloid, solandine, relative to cyclopamine. We found that cyclopamine exposure caused a flattening of the normally U-shaped myoseptum, a loss of cartilaginous craniofacial skeleton, as well as reduced interocular distance (Fig. 7). Dose-response assays determined that a 4 lM concentration of cyclopamine was required to significantly reduce interocular distance, a concentration more than 25 times greater than the EC₉₀ for signaling inhibition in cell-based assays. Due to solubility limitations, we were unable to test the activity of tomatidine and were not able to achieve a concentration over 750 lM for solanidine, a concentration at which there was a slight but nonsignificant reduction in interocular distance (Fig. 8).

**DISCUSSION**

The capacity of the plant secondary metabolite cyclopamine to inhibit Hh signaling and instill correlative teratogenic phenotypes is well established and has been exploited as an experimental tool. The potential Hh inhibitory capacity of several structurally related compounds found in dietary plants has been hinted at but not thoroughly studied. Here, using in vitro assays, we found that several of these compounds are weak inhibitors of Hh signaling that, like cyclopamine, act...
downstream of Ptc1 and upstream of Gli1-mediated target gene induction.

Because Hh signaling plays a pivotal role in numerous biological processes, exposure to chemical inhibitors of this pathway could have significant effects on human health and development. In various animal models, cyclopamine has been demonstrated to possess both teratogenic and chemotherapeutic properties related to its potent inhibition of Hh signaling. Like cyclopamine, solanidine and solasodine have been shown to cause congenital craniofacial defects in hamsters (Gaffield and Keeler, 1996; Keeler, 1978; Renwick et al., 1984). Tomatidine and solasodine were found to cause HPE in chicks albeit with much less frequency than cyclopamine. However, each subclass of compounds appears to induce related but distinct patterns of terata (Gaffield and Keeler, 1996). This has been taken to suggest that each compound acts through a different receptor or that there is a considerable and important variation in absorption or clearance rates. The data presented here suggest that each compound acts through the same receptor and that the gradations of teratogenic phenotype induced by these related compounds could be due to differences in potencies as well as differences in distribution and elimination.

Given the relatively low potency of the dietary compounds tested here, it is difficult to speculate about potential human health implications. Studies have investigated the presence of solanidine in human serum and found that among 57 healthy

![Graph showing % Activity (β-gal/total protein) for cyclopamine, tomatidine, solanidine, and nicotine at EC50 and EC90 concentrations.](image)

**FIG. 4.** Dietary alkaloids act downstream of Ptc1. *Ptc1−/−* MEFs were treated with cyclopamine, solanidine, or tomatidine, at their respective EC50 and EC90 concentrations or 3-methylpiperidine or nicotine at a concentration of 25 μM. After 48 h, β-galactosidase activity was measured as a readout of Hh signaling activity and normalized to total protein concentrations. Values indicate the mean ± SEM of three replicate experiments, *indicates *P* < .05 relative to vehicle control.

![Graph showing Ptc1/GAPDH expression for vector, GFP, Gli1-GFP, cyclopamine, tomatidine, solanidine, and nicotine at EC90 concentrations.](image)

**FIG. 5.** Dietary alkaloids act upstream of Gli1. Wild-type MEFs were infected with adenovirus encoding a mouse Gli1-GFP fusion protein or GFP alone and treated with cyclopamine, solanidine, or tomatidine at their respective EC90 concentrations. After 48 h, the expression of *Ptc1* was determined by real-time RT-PCR. Values indicate the mean ± SEM of three replicate experiments.
volunteers, the mean serum concentration was 23.8 nM (high of 56.3) and was correlated with potato consumption (Harvey et al., 1985). While little is known regarding the metabolism of these compounds, Harvey et al. observed that a period of 2–3 weeks was required for serum solanidine concentration to fall below 20% of the initial concentration after volunteers abstained from eating potatoes (1985).

In foods, tomatidine appears to be primarily present in the form of its glycoalkaloid, alpha-tomatine, which in store bought tomato ranges in concentration from 4 to 42 mg/kg.

![FIG. 6.](image)

**FIG. 6.** Dietary alkaloids act additively. Shh LIGHTII NIH3T3 fibroblasts were treated ± 1nM Shh peptide and ± solanidine, solasodine, tomatidine, either individually or together at a concentration of 1µM. After 48 h, cell protein was isolated, and Firefly and Renilla luciferase activity was determined. Values represent the mean ± SEM of three replicate experiments.

![FIG. 7.](image)

**FIG. 7.** Embryos were exposed to vehicle (0.2% 2-hydroxypropyl-β-cyclodextrin) or graded concentrations of cyclopamine continuously beginning at 4 hpf. As cyclopamine concentration increases, somites at 48 hpf become more U shaped and the myoseptum (arrow) disappears (column A), eye placement at 48 hpf shifts inwards and becomes more cyclopic (column B), and the formation of cartilaginous craniofacial skeleton at 96 hpf does not occur (column C).
processed foods such as ketchup and tomato sauce, concentrations were found to be 25 and 64 mg/kg, respectively (Reviewed in Friedman, 2002). Given this, we also assayed the Hh signaling inhibition capacity of alpha-tomatine. Like its aglycone tomatidine, alpha-tomatine also demonstrated dose-dependent inhibition, but a comparison of respective EC50 values showed that the glycoalkaloid is fivefold less potent than the aglycone (data not shown). Unlike solanidine however, human serum concentration of alpha-tomatine and tomatidine have not been reported.

The studies reported here focused on dietary alkaloids with structural similarity to cyclopamine. However, recent small molecule screens for Hh inhibitors have identified several potent antagonists that act at the level of Smo but lack any structural similarity to cyclopamine (Chen et al., 2002b). This suggests that Smo activity is susceptible to interruption by structurally diverse small molecules and increases the likelihood that other small molecules with human exposure may impact Hh signaling. Indeed, screening of a library of currently used pharmaceuticals, natural products, and pesticides has recently identified several other putative antagonists of Hh signaling with disparate chemical structures (Lipinski, R). Studies of these compounds will be the focus of future investigations.

HPE is an uncommon clinical entity, but the public health relevance of perturbations in Hh signaling could be broader. In a study of temporally specific cyclopamine administration in chick embryos, Cordero et al. (2004) observed that Hh signaling blockade prior to Shh expression in the prosencephalon resulted in a single fused telencephalic vesicle as well as severe facial defects. However, signaling blockade following establishment of Shh in the forebrain but prior to its induction in the face resulted in facial defects without detectable effects on the forebrain. These studies suggest that perturbations in Hh signaling during different periods of development may produce a spectrum of midline defects. Therefore, identification of chemical inhibitors of Hh signaling that have relevant human exposure and studies to examine their potential for additive effects or for synergy with genetic mutations in the Hh pathway could offer important insight into the etiology of related birth defects.

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