Journal of Pharmaceutical and Biomedical Analysis 52 (2010) 707–713

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Hedgehog antagonist cyclopamine isomerizes to less potent forms when acidified

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ARTICLE INFO

Article history:
Received 23 October 2009
Received in revised form 8 February 2010
Accepted 12 February 2010
Available online 23 February 2010

Keywords:
Cyclopamine
Hedgehog
Isomerization
NMR
LC–MS

ABSTRACT

The effect of acid treatment of cyclopamine, a natural antagonist of the hedgehog (Hh) signaling pathway and a potential anti-cancer drug, has been studied. Previous reports have shown that under acidic conditions, as in the stomach, cyclopamine is less effective. Also, it has been stated that cyclopamine converts to veratramine, which has side effects such as hemolysis. In this study, we examined in detail the influence of acidification on structure and activity of cyclopamine. We found that of acidified cyclopamine converts to two previously unreported isomers, which we have called cyclopamine (S) and cyclopamine (X). These have likely gone undetected because cyclopamine is often analyzed with fast and hence lower resolving chromatographic methods. Compared to natural cyclopamine, these cyclopamine isomers have a significantly reduced effect on the ciliary transport of the Hh receptor smoothened, and reduced inhibition on the Hedgehog signaling pathway. The side effects of these isomers are unknown. Our findings can partly explain a reduced efficiency of cyclopamine in a gastric environment, and may help with the rational design of more pH independent cyclopamine analogues.

1. Introduction

The natural steroidal alkaloid cyclopamine (11-deoxyjervine) [1] derived from the corn lily *Veratrum californicum* specifically disrupts the Hh signaling pathway [2,3]. Cyclopamine promotes the ciliary translocation of SMO in a mechanism similar to that reported for Hh induced pathway activation and for small-molecule Hh agonists [4,5]. In addition, cyclopamine is thought to lead to a conformational shift of SMO that renders the receptor inactive and blocks effectively further signal transduction [6,7].

Although cyclopamine has shown promise as a potential drug for targeting various solid tumors, there are concerns regarding its practical use due to its limited solubility in water, and its limited potency and instability when in acidic environment; issues that are currently addressed by targeted chemical modifications of the core structure [8,9]. For example, it has been observed that inhibition of the hedgehog pathway by cyclopamine in the stomach requires concomitant acid inhibition [10] and early work stating that acidified cyclopamine converts to veratramine [11] has been used to explain cyclopamine's limited potency at low pH [8]. Veratramine is ineffective in blocking hedgehog signaling [3] and has a different profile of side effects, such as hemolysis [12].

In this present report we describe the conversion of cyclopamine in low pH environment to several previously undetected isomers of cyclopamine, instead of veratramine. The found isomers can easily be mistaken for active natural cyclopamine using standard liquid chromatography-mass spectrometry (LC–MS) analysis conditions as they easily co-elute with cyclopamine, but in contrast to cyclopamine, they do not have a significant in vitro inhibitory effect on Hh signaling. The side effects of these isomers are unknown. The structures of the cyclopamine isomers are presented, along with proposed isomerization reaction mechanisms and energy calculations.

2. Materials and methods

2.1. Chemicals and solutions

Cyclopamine, a known teratogen, was purchased from TCR (Toronto Research Chemicals, Canada) and stock solutions (SS) of 1 mg/mL were made by dilution and vortex mixing in 96% EtOH (AS Arcus, Oslo, Norway). Working solutions of 0.01 mg/mL were made by diluting 100 μL SS in 0.1 mL EtOH and 0.8 mL Type 1 water produced by a Gradient A-10 generator (Millipore, MA). Acidic and basic (10 μg/mL) cyclopamine solutions (0.1% formic acid [measured to be pH 2.7], 1% formic acid [pH 1.5], 0.1 M HCl [pH 1], 10 mM ammonia carbonate [pH 10.0]) were prepared by dilution of the working solutions, and were chromatographed

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doi:10.1016/j.jpba.2010.02.017

1 h after preparation. Analytical formic acid was purchased from Fluka (Sigma–Aldrich Chemie, Steinheim, Germany), while analytical grade HCl and NaOH(s) were purchased from Merck (Darmstadt, Germany). When isolating the isomers prior to NMR, 0.75 mg/mL cyclopamine solutions were prepared by dissolving 3 mg in 1 mL EtOH and slowly adding 3 mL 0.1 M HCl (aq) with vigorous manual shaking. The entire sample volume was chromatographed using eight injections by the preparative liquid chromatography (LC)–UV system described below (within the same day) and the separated compounds were collected manually. Fractions containing the isomers from the eight injections were combined.

2.2. Analyses

Liquid chromatography was performed by using an Agilent 1100 pump (Agilent, Sao Paulo, CA) running a gradient from 5% B to 95% B in 40 min (A, 0.1% formic acid (aq), B, 0.1% formic acid (acetonitrile)). Acetonitrile (ACN) was purchased from Rathburn (Walkerburn, UK). A Zorbax SB-C_{18} (150 mm × 0.3 mm, 5 μm particles) column, an ACE AQ (150 mm × 4.6 mm, 5 μm particles [Advanced Chromatography Techniques, Aberdeen, UK]) and an ACE AQ (250 mm × 10 mm, 5 μm particles) column was used for capillary LC–mass spectrometry (MS) (5 μL/min), conventional LC–UV (1 mL/min) and preparative LC–UV (4 mL/min), respectively. Besides the gradient program described above, a faster solvent described elsewhere used for pharmacokinetic studies of cyclopamine (13) and analogues (8) was used for comparison.

For UV detection, a 486 Tunable Absorbance detector (Waters) was employed, set to 210 nm. For MS detection, an Esquire 3000 plus ion trap mass spectrometer (Bruker Daltonic, Bremen, Germany) and an LCT TOF-MS (Waters, Milford, MA) were used, both operated in positive electrospray ionization (ESI) mode.

2.3. Structure elucidation

Following preparative LC, the ACN in the cyclopamine isomer-containing fractions was evaporated using nitrogen (AGA, Norway). The remaining aqueous solvent was basified to pH 10 with Na_2HPO_4/NaOH (Merck). The cyclopamine isomers were subsequently extracted with HPLC grade dichloromethane (Rathburn). The dichloromethane was then evaporated with nitrogen, and the isomers were re dissolved in 20 μL deuterated dichloromethane (d6) (Cambridge Laboratories, Andover, MA) before NMR analysis. All NMR experimental data were acquired at 304.6 K with a Bruker AVANCEII 600 NMR spectrometer operating at 600.13 MHz (proton frequency) equipped with a triple resonance TCI ($^1H^{13}C^{15}N$) cryo probe. The software used was TopSpin 2.1 pl2

The elucidation of the structures was based on comparison of comprehensive NMR data from pure natural cyclopamine with data from the following NMR experiments run on the sample described above. One dimensional ^1H spectrum; AQ 2.654, PP zg, NS 256, TD 65k, SI 256k, ^1H homodecoupled spectra; AQ 1.363, PP zghd.2, pl24 54 dB, APT (attached proton test); AQ 0.909, PP jmod, NS 30720, TD(F2) 65k, WDW EM, LB 1, COSY; AQ 0.310, PP cosyqf45, NS 64, TD(F2) 2048, TD(F1) 256, SI(F2 and F1) 1024, WDW (F2 and F1) SINE, LB(F2) 1, LB(F1) 0.3, LPfr (NCOF 32, LPBIN 320), TOCSY; AQ 0.310, PP mlevph, NS 128, TD(F2) 2048, TD(F1) 256, SI(F2 and F1) 1024, WDW (F2 and F1) QSINE, LB(F2) 1, LB(F1) 0.3, LPfr (NCOF 32, LPBIN 620), NOESY; AQ 0.310, PP noesyph, NS 192, TD(F2) 2048, TD(F1) 144, SI(F2 and F1) 1024, WDW (F2 and F1) QSINE, LB(F2) 0, LB(F1) 0.3, LPfr (NCOF 32, LPBIN 620), HSQC; AQ 0.090, PP hsqcedetgp, NS 640, TD(F2) 1024, TD(F1) 256, SI(F2 and F1) 1024, WDW (F2 and F1) QSINE, LB(F2) 0, LB(F1) 0, LPfr (NCOF 32, LPBIN 360), HMBC; AQ 0.427, PP hmbcgplpndqf, NS 192, TD(F2) 2048, TD(F1) 144, SI(F2 and F1) 1024, WDW (F2 and F1) SINE, LB(F2) 0, LB(F1) 0.3, LPfr (NCOF 32, LPBIN 320).

Abbreviations: AQ (acquisition time), NS (number of scans), TD(F2)(number of data points in – F2 – observe-dimension), TD(F1) (number of data points in the indirect – F1 – dimension), SI(F2) (number of data points in the Fourier transformed (FT) processed observe – F2 – dimension), SI(F1) (number of data points in the processed indirect – F1 – dimension), WDW (window processing function), EM (exponential multiplication), SINE (sinus window function), QSINE (quadrate sine function), LPfr (linear prediction forward real), in F1 dimension only, NCOF (number of coefficients in LPfr), LPBIN ((number of points in the linear prediction (in LPfr)), PP (pulse program), LB (line broadening in Hz).

2.4. Quantum chemical calculations

All structures were fully optimized at the gradient corrected density-functional theory (DFT) level using the exchange functional of Becke in conjunction with the correlation functional of Perdew (denoted BP) [14,15] making use of the resolution of the identity approximation [16,17]. As basis set the Weigend–Ahlrichs basis set denoted def2-SVP [18] and the corresponding fitting basis [19] were used. Analytic Hessians were used to characterize the nature of the stationary points on the potential energy surface [20]. NMR shieldings were calculated in the GIAO-framework [21]. All calculations were performed using the Turbomole program package (TURBOMOLE V5.10, a development of University of Karlsruhe and Forschungszentrum Karlsruhe GmbH, 1989–2007, TURBOMOLE GmbH; available from http://www.turbomole.com) on the Stallo supercomputer located at the University of Tromsø, Norway.

2.5. In vivo detection of cyclopamine isomers in mouse plasma

Cyclopamine was dissolved in DMSO and was administered to mice (10–12-week old males, of strain CBA/C57Bl6) per oral at a dose of 10 mg/kg in a volume of 200 μL. Four hours after administration the mice were sacrificed and blood was collected from the main artery. The blood was centrifuged and the plasma collected and prepared for LC–MS by precipitation with ACN, as described elsewhere [13,8].

2.6. Biological assays

Shh-Light 2 (Shh-L2) cells (ATCC/LGC Standards AB, Sweden) were cultured in DMEM containing 10% (v/v) heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 0.4 mg/mL G418, 0.15 mg/mL Zeocin (Invitrogen, Carlsbad, CA), and 1% penicillin/streptomycin (BioWhitaker). Shh conditioned medium was harvested from Panc 1 cells (ATCC) stably transfected with a Shh expression vector. Shh-Panc1 cells were grown to confluence and the medium changed to fresh DMEM with 10% FBS and 1% Pen/Strep. The Shh conditioned medium was harvested after 72 h, and used in a 50% dilution for activating of the Hh pathway in Shh-L2 cells. Pathway activity was measured as described previously ([22].

2.7. Immunofluorescence

Immunofluorescence was performed as described previously [23] using pre-coated glass slides (1 h coating with 0.1% Gelatin (G1393) and 0.003% Collagen (C8919) (Sigma–Aldrich, St. Louis, MO) in PBS). When cells reached confluence, medium was switched from DMEM containing 10% FBS to DMEM containing 0.5% FBS, and DMSO, Shh (50% conditioned medium), 1 μM cyclopamine or 1 μM isomers (S/X). After 24 h the slides were fixed with 4% paraformaldehyde (Sigma–Aldrich) in PBS, washed, permeabilized for 10 min in 0.1% (v/v) Triton X-100 (Sigma–Aldrich) in PBS (PBS-T), and blocked with 10% (w/v) bovine serum albumin (BSA)

(Saveen Werner, Sweden) in PBS-T for 1 h. Primary antibodies, anti-acetylated tubulin (T7451, 1:1000, Sigma–Aldrich) and anti-Smo (C17) (sc-6367, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), were diluted in 0.5% (w/v) BSA in PBS-T and left on the slides over night at 4 °C.

Secondary antibodies (1:500, donkey anti-goat IgG-Alexa 594 and donkey anti-mouse IgG-Alexa 488; Invitrogen) were added in 0.5% (w/v) BSA in PBS-T for 1 h. The slides were then washed three times with PBS; with 1 μg/mL 4′,6-diamido-2-phenylindolev (DAPI) present in the second wash.

Confocal images were acquired using an LSM510 microscope (Carl Zeiss MicroImaging, Thornwood, NY), and images were processed and analyzed using ImageJ (http://rsb.info.nih.goc/ij/).

3. Results

3.1. Acidified cyclopamine partially converts to two novel isomers

To investigate chemical alterations of cyclopamine upon exposure to a low pH environment, Selected ion monitoring (SIM) and total ion current (TIC) chromatograms of acidified cyclopamine was generated using capillary LC–TOF-MS, employing a steep LC gradient, similar to one used for pharmacokinetic studies of cyclopamine [13] and analogues [8] and a shallower LC gradient (described in Section 2). With a steep LC gradient the acid treated cyclopamine elutes within a relatively short time (6.5 min), and a single peak appears in the chromatogram (Fig. 1).

However, when using the shallow LC gradient, more partially co-eluting peaks with the same mass-to-charge (m/z) value as cyclopamine appeared (Fig. 2) for the acid treated cyclopamine, and the peak area of these isomers constituted approximately 40% of the total peak area. It was assumed that the compounds had equal ESI-MS response, as it was later revealed that the charged amine group was unaltered by acidification. Hence, the peak area ratio was assumed to be the actual ratio. This ratio was not altered within a period of 24 h, or when the solution was neutralized or basified (results not shown).

Non-acidified cyclopamine appeared as only one peak using the shallower gradient (results not shown). Isomerization and reversion of isomers was not seen in non-aqueous, acidic solutions (results not shown). In aqueous solution slow reversion was observed, and approximately 1/5 of the peak area of the isomers reverted to cyclopamine after 5 days.

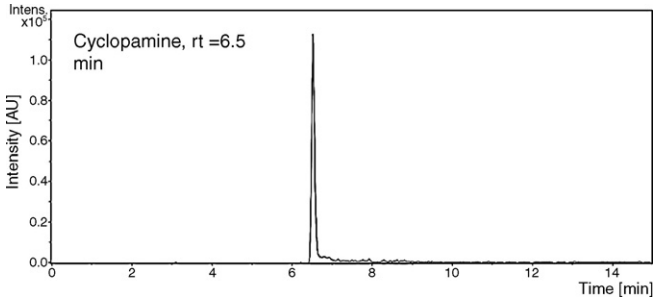

Fig. 1. LC–MS of acidified cyclopamine with steep gradient.

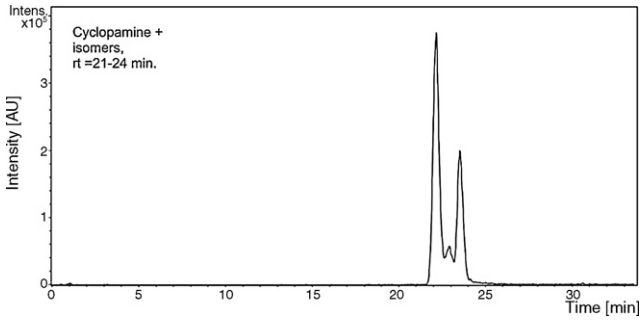

Fig. 2. LC–MS of acidified cyclopamine with a shallow gradient.

3.2. Isomerization also occurs in vivo

To investigate whether similar isomers would be generated in vivo, we analyzed plasma from mice that had received cyclopamine by per oral (po) administration.

A SIM chromatogram of the plasma shows isomerization in vivo (Fig. 3a) 4 h after administration. Cyclopamine isomerization was

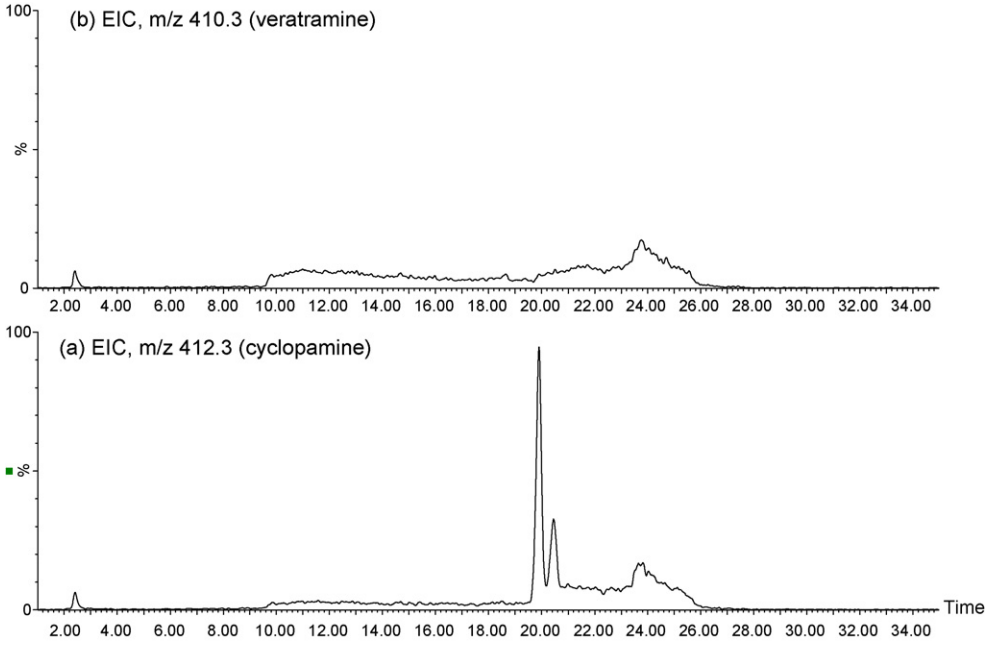

Fig. 3. (a) Selected ion chromatograms (SIM) of cyclopamine and isomers in mouse plasma. (b) SIM of m/z = 410.3 (veratramine).

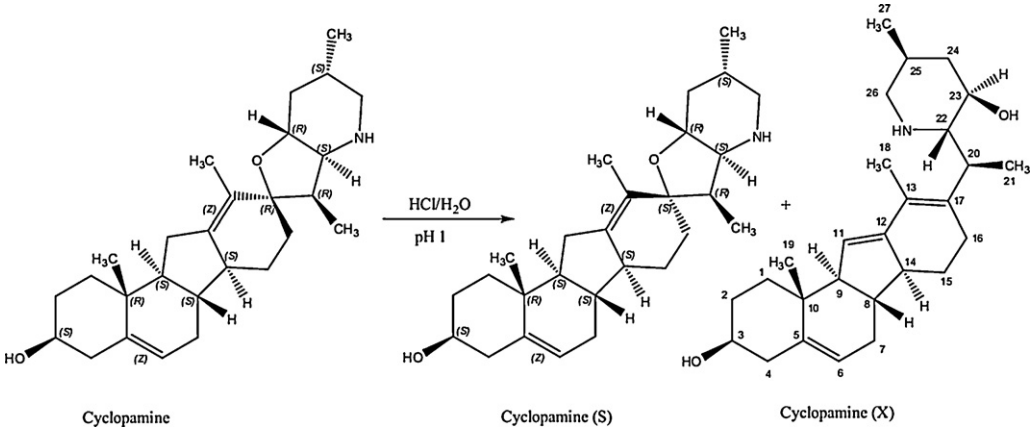

Fig. 4. Structure of cyclopamine and acid induced isomers (cyclopamine (S) and cyclopamine (X).

present (upto 30%, $n = 3$), demonstrating that a per oral application of natural cyclopamine will cause structural alterations. Although it would not be possible to elucidate the structures of the isomers in plasma by NMR, It is likely that they are the same isomers as those detected in vitro. The isomerization was not detected in plasma where the cyclopamine was given intraperitoneally (ip), which supports a hypothesis of stomach acid induced isomerization. However, when considering these results, one should keep in mind that the pharmacokinetics of the isomers is unknown. Since previous reports have suggested that cyclopamine converts to veratramine in an acidic environment the same mouse plasma samples were also examined for the presence of veratramine. Interestingly, Fig. 3b shows that a SIM of m/z 410.3 (veratramine $[M+H]^+$) does not contain a peak that would indicate the presence of veratramine (as we could see with, e.g., LC–MS of veratrum plants [result not shown]).

3.3. Structural elucidation

To further examine the pH-induced structures, the isomers were extracted from an acidic cyclopamine solution using a preparative LC–UV method (see Section 2). With the large injection i.e. lower resolving preparative method, the two isomers ("cyclopamine (S)" and "cyclopamine (X)") co-eluted completely, but the structures of these compounds could still be determined in mixture by NMR and computational techniques (Fig. 4). See Additional information for detail on chemical shifts.

Cyclopamine (X) was less abundant relative to (S) (0.67:1) in the mixture, and no regular cyclopamine was detected in the mixture. Therefore, it is assumed that the smallest peak in the LC–MS Fig. 1 is cyclopamine (X).

Cyclopamine (S) has an inverted configuration at carbon atom 23 as compared to cyclopamine, which has R configuration at this position. The systematic name of cyclopamine (X) is (2S,3R,5S)-2-((S)-1-((3S,6aS,6bS,11aR,11bR)-3-hydroxy-10,11b-dimethyl-2,3,4,6,6a,6b,7,8,11a,11b-decahydro-1H-benzo[a]fluoren-9-yl)-5-methylpiperidin-3-ol, but for simplicity both the numbering of positions and naming follows what has been established for cyclopamine itself (see Fig. 4). Quantum chemical calculations confirmed the NMR signal assignment and helped to rule out other possible isomerization products by comparing the calculated and the experimental ^{13}C chemical shift spectra (see Tables S1 and S2 in the Supplementary material).

3.4. The combination of cyclopamine (S) and cyclopamine (X) show reduced Hh pathway inhibitory activity

Using an Hh pathway reporter cell line, Shh-L2, we investigated whether the acid induced isomers of cyclopamine, cyclopamine (S) and (X), retained an antagonistic activity towards the Hh pathway.

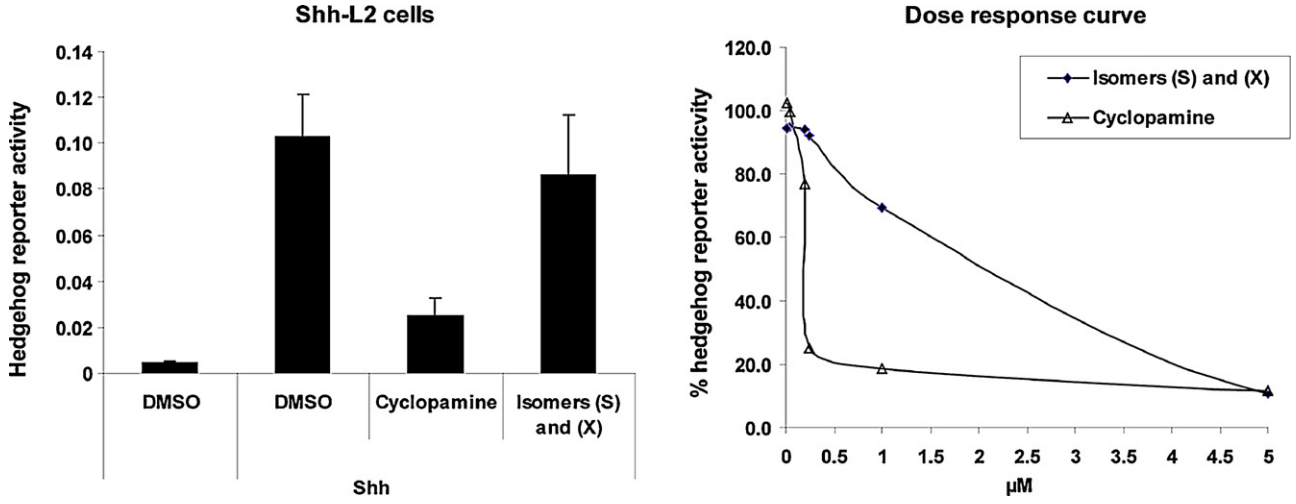

Fig. 5. (A) Shh-Light (L) 2 cells left untreated (DMSO control) or treated with Shh alone or in combination with 1 μM cyclopamine or 1 μM cyclopamine isomers (S/X) for 48 h. Hedgehog reporter activity is plotted as the average ratio of reporter firefly luminescence/control renilla luminescence. (B) Dose response curve for cyclopamine and isomers (S/X) in Shh treated Shh-L2 cells after 48 h. Hedgehog reporter activity is calculated as a % of maximal activation of Hh signaling by Shh conditioned medium.

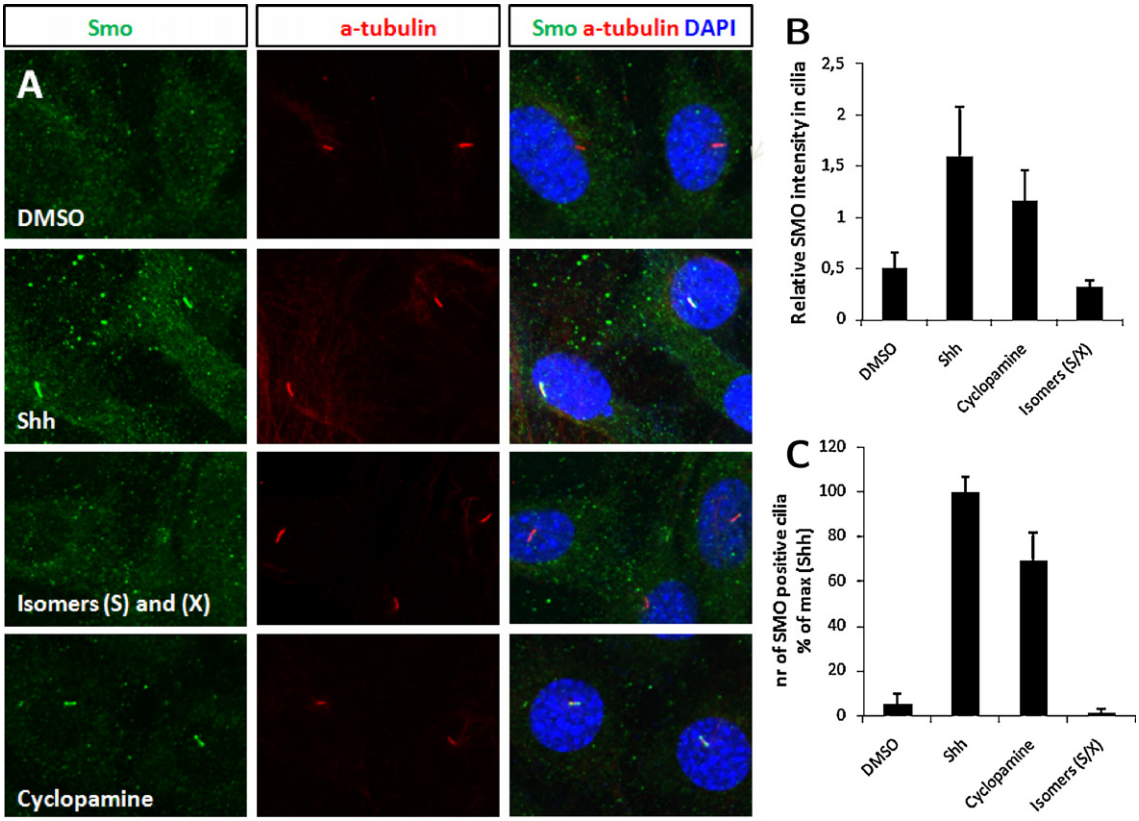

Fig. 6. Enrichment of SMO in primary cilia of Shh-L2 cells treated with DMSO (solvent control), Shh (positive control), 1 μM cyclopamine or 1 μM isomers (S/X) for 24 h. (A) The ciliary marker acetylated tubulin (α-tubulin) (red) and SMO (green) were detected by immunofluorescence. Cellular nuclei were stained with DAPI (blue). (B) Relative SMO intensity in cilia, calculated as the ratio of the measured mean SMO fluorescence intensity divided by mean α-tubulin fluorescence intensity. (C) Counted number of SMO positive cilia as a % of the maximum SMO positive cilia with Shh treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

As seen in Fig. 5, natural cyclopamine antagonizes Shh induced Hh pathway activation by 75% in Shh-L2 cells as expected at a dose of 1 μM. In contrast, the combined isomers cyclopamine (S) and (X) did not display a statistically significant antagonistic effect to Hh signaling at 1 μM (Fig. 5a). This was confirmed in a dose response assay where natural cyclopamine had a classical S shaped drop in activity and a relative IC50 of 250 nM. In contrast, a non-S shaped gradual drop of Hh pathway activity was exhibited by the combined cyclopamine isomers (S) and (X) in Shh-L2 cells with a relative IC50 of 2.25 μM, and thus an order of magnitude lower than seen with natural cyclopamine (Fig. 5b).

3.5. *Cyclopamine (S) and (X) do not induce ciliary transport of SMO*

As cyclopamine (S) and (X) does not block Hh signaling effectively, we investigated at which stage of the Hh pathway the inhibition is lost. Similar to a ligand dependent activation of Hh signaling, natural cyclopamine induces the transport and enrichment of SMO in the primary cilium and thus promotes the initiation of Hh signaling. However, natural cyclopamine subsequently blocks further Hh signal transduction (4). Thus, when Shh-Light-2 cells were treated with either Shh conditioned medium, or 1 μM natural cyclopamine, increased ciliary presence of SMO was observed (Fig. 6) as expected.

A comparable induction of SMO ciliary translocation was not observed upon exposure of cyclopamine (S) and (X), both as measured in absolute numbers of cells with SMO positive cilia, and as percentage of ciliary SMO immunofluorescence intensity normalized for the immunofluorescence intensity of the ciliary marker acetylated tubulin (Fig. 6). This demonstrates an absence of bioactive potential of the acid induced cyclopamine isomers on the Hh pathway at the level of SMO translocation.

In conclusion, pH-induced cyclopamine isomerization abolishes the potential of the molecule to inhibit Hh signaling at the level of SMO, and provides a mechanistic understanding for the reduced efficiency of natural cyclopamine in an acidic environment.

4. Discussion and conclusions

When using LC–MS, especially in pharmaceutical analysis, it is common to use short analysis times, e.g. less than 10 min, often employing a steep solvent gradient. However, only when using a slower, shallower (and hence higher resolving separation) gradient was it possible to reveal the large degree of isomerization of pure cyclopamine as a result of acidification.

Cyclopamine (S) and cyclopamine (X) are energetically destabilized compared to cyclopamine by 0.1 and 3.5 kcal/mol, respectively. The occurrence of both cyclopamine (S) and cyclopamine (X) can be rationalized by invoking the mechanistic scenario as sketched in Fig. 7. Protonation of the ether oxygen with concomitant nucleophilic water attack on carbon 12 can result in ring opening. The cationic intermediate (upper right molecule in Fig. 7) can either eliminate water from carbons 11 and 12 giving cyclopamine X (lower left corner of Fig. 4) or undergo a new cyclization from the opposite side of carbon 17 resulting in the S configuration at this position (cyclopamine S). The calculated three-dimensional structures of cyclopamine, cyclopamine (S) and cyclopamine (X) are given in the Supporting information.

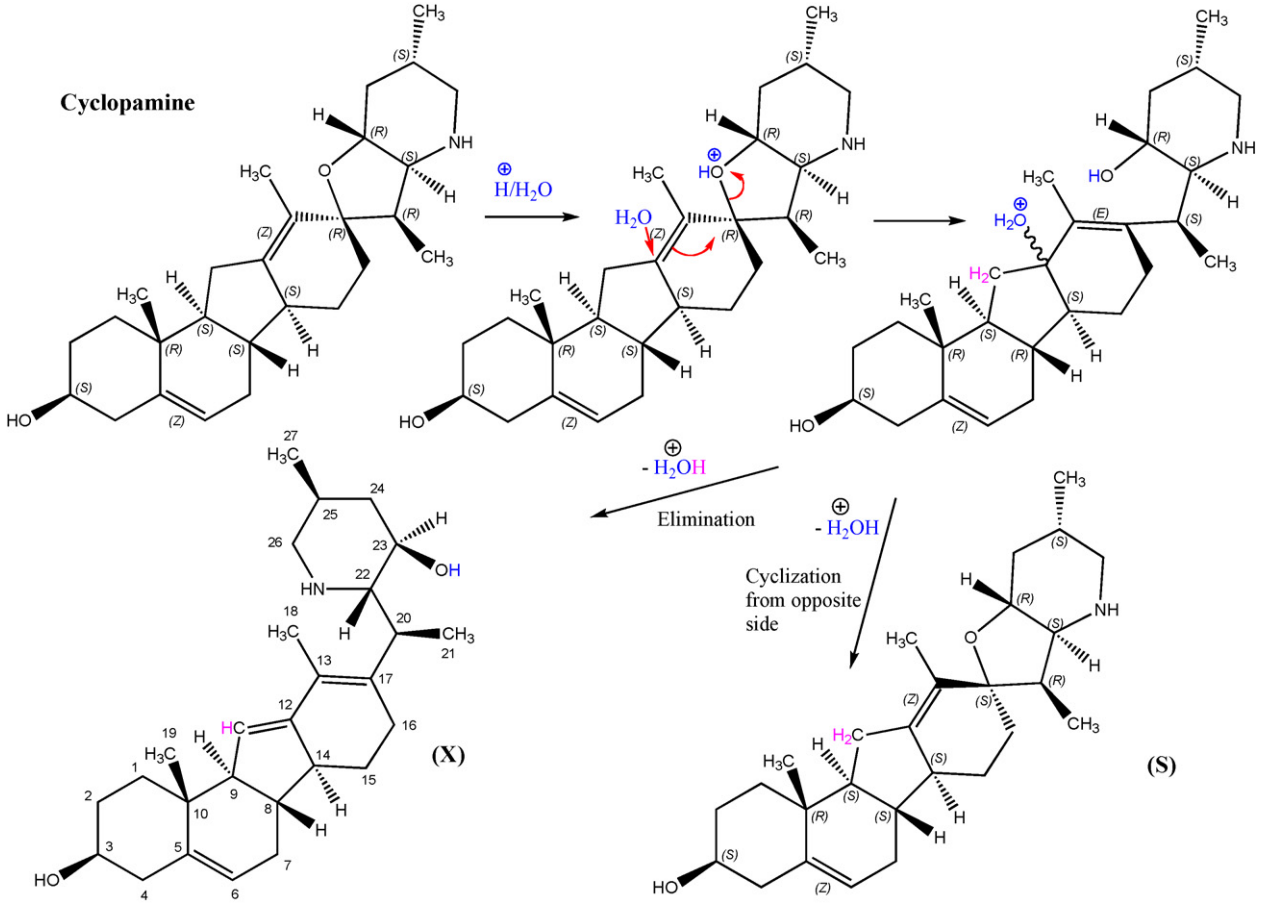

Fig. 7. Proposed reaction mechanisms of natural cyclopamine in to cyclopamine (S) and cyclopamine (X).

Isomerization between cyclopamine and cyclopamine (S), depicted in Fig. 4, is believed to be reversible; the reappearance of original cyclopamine in an aqueous (S/X) solution indicates the existence of a thermodynamic equilibrium between at least two forms. From a chemical point of view the first step in this back reaction—hydratization of one of the double bonds in the conjugated double bond system seems less likely. So our conclusion at this stage is that the R and S forms of cyclopamine do indeed freely isomerize between themselves when in acidic water solution, however the kinetics of this transformation is not fast; chromatography of the isomers the same day after isolation did not show traces of regular cyclopamine.

The reversion to cyclopamine can account for a portion of the activity attributed to the isolated isomers. However, this was difficult to confirm as the cell media in which the activity was tested was difficult to analyze by LC–MS.

During the study, our group detected only marginal/less than detectable amounts of veratramine after acidification of cyclopamine. This agrees with an earlier report [24], but in disagreement with Keeler [11], where the evidence for the conversion to veratramine is based on IR spectroscopy and thin layer chromatography (TLC). We speculate that the IR spectrum of cyclopamine (X) is similar to that of veratramine, since they both have additional hydroxyl groups and double bonds.

The biological activity of cyclopamine against the Hh pathway is markedly reduced in the acid induced isomers. However, since a major portion of cyclopamine does not isomerize, the remaining "regular" cyclopamine will still be able to inhibit the Hh pathway outside the stomach, as has been confirmed by others [25,26]. On the other hand, as mentioned it has been reported that cyclopamine is ineffective regarding the Hh pathway in gastric acid. Since cyclopamine does not isomerize more than 40% or less in acid, we speculate that it is the protonation of cyclopamine in acid rather than isomerization that causes the drop in effect in gastric environment. The importance of protonation on a drug has been previously demonstrated [27].

As these isomers have remained undetected in previously reported analyses of cyclopamine, it may be that this is the case for other pharmaceuticals that have been analyzed with similar resolutions. To our knowledge, this is one of the first reports on sterol drugs showing a decrease in activity because of isomerization due to acidification. Thus we show that high-resolution analysis with shallow gradients is critical for detecting isomers that may be overlooked using a steep gradient.

The awareness of pH-induced alterations of cyclopamine into nonfunctional isomers/states has an impact on, e.g., the understanding of its pharmacokinetics and may provide a further basis for the rational design of bioactive analogues. For both natural cyclopamine, and novel analogues, it will be important to detect possible isomers with altered bioactivity, especially if such isomers escape detection in standard analysis.

Acknowledgements

This work was supported through the Cancer Stem Cell Innovation Centre (CAST) by the Research Council of Norway.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2010.02.017.

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