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Active Site Targeting of Hedgehog Precursor Protein with Phenylarsine Oxide

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Abstract

Hedgehog proteins, signaling molecules implicated in human embryo development and cancer, can be inhibited at the stage of autoprocessing by the trivalent arsenical phenyl arsine oxide (PhAs^{III}). The interaction (apparent K_i , $4 \times 10^{-7} \text{M}$) is characterized by an optical binding assay and by NMR spectroscopy. PhAs^{III} appears to be the first validated inhibitor of hedgehog autoprocessing, which is unique to hedgehog proteins and essential for biological activity.

Keywords

active-site probe; arsenic; autoprocessing; cholesterol; hedgehog

Hedgehog (Hh) proteins represent a family of cell signaling ligands that influence human embryo development as well as multiple types of cancer.^[1] In this work, we focus on the covalent modification of Hh proteins with cholesterol, a relatively unstudied event in Hh protein biosynthesis.^[2] As shown in Scheme 1, cholesterol modification takes place autocatalytically in two chemical steps. First, a Hh precursor protein rearranges an internal peptide bond into a thioester; second, this intermediate is displaced by cholesterol. Activity for this “cholesterolysis” reaction^[3] resides entirely in a C-terminal segment of the Hh precursor, referred to as HhC. Cholesterolysis is imperative for bioactivity, and appears to be specific to Hh proteins;^[4] therefore, small molecules that can modulate this reaction are of fundamental and practical interest.^[5]

Hedgehog (Hh) precursor proteins across the animal kingdom contain three functional regions: an amino terminal domain with signaling activity (HhN) to which cholesterol is attached; a central autocatalytic “HINT” domain (Hedgehog/Intein) resembling self-splicing

intains; and a carboxy terminal subdomain with affinity for cholesterol (Scheme 1, white, gray, black, respectively).^[4] The latter two segments comprise HhC and are essential for cholesterololysis. Structural studies thus far have focused on HhN signaling domains; only one 3D structure of the HINT domain has been obtained at atomic resolution,^[6] and no structural data is available for the cholesterol-binding subdomain. Accordingly, compounds that target precursor Hh are scarce.^[7]

Here, we report that the trivalent arsenical compound phenylarsine oxide (PhAs^{III}) is a potent active-site probe of precursor Hh (apparent K_i , $\sim 4 \times 10^{-7}$ M). Binding of PhAs^{III} by Hh irreversibly blocks cholesterololysis. We probed this interaction using a novel optical assay that detects bimolecular association of PhAs^{III} and Hh by FRET. We also applied complementary NMR methods to map the PhAs^{III} binding site on Hh. Together, these techniques provide a general platform for identifying and characterizing inhibitors of Hh cholesterololysis. The inhibitory activity of PhAs^{III} reported here might also offer insight into arsenic's teratogenic activity, as well as its anticancer properties.^[8]

We were drawn to trivalent arsenic (As^{III}) as a potential cholesterololysis inhibitor on the basis of this metalloid's thiophilicity. PhAs^{III} and its organoarsenical analogues condense rapidly and covalently with dimercaptans and with closely spaced cysteines in peptides and proteins.^[9] The products are presumed to be a cyclic S-As^{III}(R)-S adduct and water. In the HINT domain of precursor Hh, there are two conserved cysteine residues, termed CysA and B. Although separated by >100 residues in primary sequence, CysA and B are brought together upon folding.^[6a] The two residues are catalytically essential; single alanine point mutation at either position abolishes cholesterololysis.^[6a, 10] CysA serves as the nucleophile in the first step of cholesterololysis (Scheme 1); recent studies suggest that the second cysteine can engage CysA in a disulfide bond, thereby imparting redox control over cholesterololysis.^[10] If two cysteine residues are sufficiently close to engage in a disulfide bond, it seems conceivable that in the absence of steric or electrostatic constraints they would also prove reactive to As^{III}.

Our initial experiments to test As^{III} inhibition used a conventional gel-based cholesterololysis assay.^[2a] Along with three As^{III} compounds (*meta*-arsenite, arsenic trioxide, and PhAs^{III}), we also investigated representative As^V compounds (phenylarsonic acid and dimethylarsenic acid). Inhibitory activity was assessed against an engineered Hh precursor. The construct, ShhN-DHhC, contains the HhN of human sonic hedgehog protein fused to the HhC of *Drosophila melanogaster* Hh. This chimeric precursor shows robust expression in *Escherichia coli* and is catalytically competent. Expression and purification of native Hh precursors has proved exceedingly difficult. Cholesterololysis assays on ShhN-DHhC were performed under reducing conditions in Bis-Tris buffer (pH 7.1) with added TritonX-100 (0.4 %, v/v, final). Reactions were initiated by adding cholesterol to 500 μ M. At selected intervals, aliquots of the reaction were quenched by boiling with added SDS-PAGE loading buffer. The extent of precursor cholesterololysis was then assessed by densitometry after resolving precursor and products by SDS-PAGE.

As shown in Figure 1A, PhAs^{III} produced marked inhibition of Hh precursor cholesterololysis, blocking production of the two processed fragments: cholesteroylated ShhN and DhhC to a

level comparable to a control reaction lacking cholesterol (compare first and last lanes). Neither of the As^V compounds was capable of measurably inactivating cholesterololysis at a concentration of 80 μM. It is interesting to note that, under the present conditions, arsenic trioxide and (meta)arsenite also failed to inactivate cholesterololysis. These two As^{III} compounds have been used interchangeably with PhAs^{III} as dithiol-modifying agents.^[11] As arsenite, arsenic trioxide, and PhAs^{III} contain the same reactive As^{III} atom, this apparent preference for the uncharged organoarsenical indicates a degree of specificity in the inactivation mechanism.

To quantify inhibition, the Hh precursor (4×10^{-6} M) was titrated with PhAs^{III} from 0.5 to 500 μM. The data fit reasonably well to a general dose–response equation (Figure 1B, solid line, and Methods section in the Supporting Information), with a calculated IC₅₀ value of 2.2 μM. The correspondence of the IC₅₀ value with 50% of the initial precursor concentration indicated that PhAs^{III} was bound stoichiometrically by the precursor. In view of the apparent tight binding, the data were analyzed with a quadratic equation for high-affinity inhibitors (Figure 1B, dotted line), yielding a better fit and an apparent K_i value of 0.4 μM. In two follow-up experiments, we found that 1) inhibition of cholesterololysis by PhAs^{III} remained unchanged after overnight incubation with a saturating amount of cholesterol, and 2) inhibition could not be reversed by extended dialysis (Figure S1 in the Supporting Information). Collectively, these observations, together with direct detection of a PhAs^{III}-HhC adduct by mass spectrometry, are consistent with covalent inhibition (Figure 1C, D).

To monitor the kinetics of the association of HhC with PhAs^{III}, we prepared a FRET-active Hh precursor. The construct, C-H-Y, has fluorescent proteins CFP and YFP fused to the amino and carboxy termini of the *Drosophila melanogaster* HhC, respectively (Figure 2A, inset). A similar construct was used to study the kinetics of self-cleaving inteins.^[12] We tested C-H-Y on the speculation that HhC would undergo a structural change upon binding PhAs^{III} which could be detected by FRET. The spectra in Figure 2A are consistent with this hypothesis, as the addition of one equivalent of PhAs^{III} to a buffered solution of C-H-Y resulted in a substantial FRET change. Analysis of the PhAs^{III}-treated precursor by SDS-PAGE ruled out decomposition as a possible explanation for the observed FRET loss (Figure S2 A, inset). To assess specificity, two control constructs were used: C-Y, in which HhC was replaced with a peptide linker,^[12] and, C-H^{CysA/A}-Y, a mutant in which CysA was replaced with alanine. Neither of the control constructs exhibited an appreciable change in FRET in the presence of PhAs^{III} (Figure S2B and C). Thus, the altered FRET induced by added PhAs^{III} was consistent with a physical interaction with HhC that required its catalytically essential CysA residue.

Next, we initiated kinetic studies with C-H-Y to determine the second-order rate constant for PhAs^{III} binding, as measured by FRET loss (Figure 2 B). Pseudo-first-order rate constants from experiments over a range of PhAs^{III} concentrations are plotted in Figure 2C. The curvature apparent in the plot is consistent with a two-step kinetic model in which a noncovalent encounter complex between PhAs^{III} and C-H-Y forms first, and then transitions irreversibly to a covalent adduct (Supporting Information). Accordingly, the data conform to a hyperbolic function yielding best fit values of 52 μM and $7 \times 10^{-3} \text{ s}^{-1}$ for the apparent dissociation constant of the encounter complex (K_d) and the rate of covalent modification

(k_{inact}), respectively. Expressed as a bimolecular rate constant ($k_{\text{inact}}/K_{\text{d}}$), $\sim 10^2 \text{M}^{-1} \text{s}^{-1}$, the value is within the range of other As^{III} -protein interactions.^[9b] The apparent affinity of the stable PhAs^{III} -C-H-Y complex, obtained by plotting the final FRET change as a function of increasing PhAs^{III} concentration (Figure 2D), is nearly identical to the value obtained with the ShhN-HhC precursor described above. Thus, appending the fluorescent proteins to HhC did not seem to disrupt its structure or the interaction with PhAs^{III} .

As a final step aimed at mapping the site of interaction, we examined the HINT domain of Hh in its complex with PhAs^{III} by using two complementary NMR techniques. First, we titrated [U- ^{15}N]-labeled HINT domain with PhAs^{III} at molar ratios of 0.5:1, 1:1, and 4:1, respectively. The results of the titration were monitored by $^1\text{H}\{^{15}\text{N}\}$ HSQC. A cutoff of 80% signal reduction was used to identify residues close to PhAs^{III} in 3D structure (Figure S3). By using that filter with the 4:1 data set, ten residues were identified as comprising the PhAs^{III} binding site, with the majority forming a contiguous surface (Figure 3A) flanked by CysA and B. Many of the residues implicated in PhAs^{III} binding are conserved in the HINT domains of human Hh proteins (Figure S4), raising the possibility that the biogenesis of human Hh proteins would also be blocked by PhAs^{III} .

The effect of PhAs^{III} on CysA and B, not resolved in the $^1\text{H}\{^{15}\text{N}\}$ HSQC spectra, were evaluated next by using an $^1\text{H}\{^{13}\text{C}\}$ HSQC experiment. CysA and CysB of the HINT domain were enriched with ^{13}C by recombinant expression by using the cysteine auxotroph strain of *E. coli*, CysE(DE3).^[13] In the presence of PhAs^{III} , along with a large chemical shift perturbation (Table S1), the peak intensity of CysA was markedly suppressed, consistent with conformational dynamics in the bound state (Figure 3B, compare blue and red). The chemical shift of CysB, however, appeared less sensitive to added PhAs^{III} . These NMR findings might be reconciled by proposing that an S- As^{III} (R)-S complex requires minimal change in the conformation of CysB. Alternatively, the putative S- As^{III} (R)-S complex could represent an intermediate state only, with CysB ultimately displaced by another nucleophilic amino acid side chain in the HINT domain. Structural studies to evaluate these possibilities are ongoing.

In summary, we have identified and characterized, to our knowledge, the first active-site directed inhibitor targeting cholesterolysis, a specialized autoprocessing event required for biological signaling by Hh proteins. In addition, the results could be relevant to the teratogenicity and medicinal properties of As^{III} in the context of Hh. Inhibition of cholesterolysis is expected to suppress Hh function with effects in early life that could include severe developmental anomalies.^[14] In the adult, however, Hh inhibitors can be therapeutic, as chronic overproduction of mature Hh can promote tumor growth and metastasis.^[15] Recently, As^{III} , in the form of arsenic trioxide, was reported to interfere indirectly with Hh through binding of Gli proteins, the Hh-responsive transcription factors.^[11, 16] This discovery by Beachy et al. accords with the teratogenic activity of As^{III} and has prompted efforts to explore arsenic trioxide as a therapeutic agent to treat Hh-driven cancers.^[17] Our results expand the potential therapeutic applications of As^{III} by suggesting PhAs^{III} as a means for direct antagonism of Hh itself.

Supplementary Material

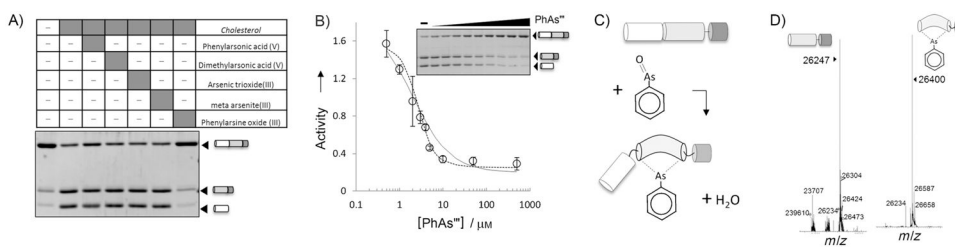
Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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**Figure 1.**

Identification of PhAs^{III} as a tight-binding inhibitor of Hh cholesterololysis. A) Screen of arsenical compounds as potential inhibitors of Hh cholesterololysis activity. Hh precursor (4 μM) was preincubated with indicated arsenical compounds (60 μM , final) before initiating reaction with cholesterol (500 μM). Samples were electrophoresed on 15% SDS-polyacrylamide gels, stained with Coomassie Brilliant Blue, and digitized with a BioRad imager. B) Titration of Hh activity by PhAs^{III}. Activity of Hh precursor (2 μM) was determined by the extent of processing as in (A). Data were fit to a standard dose-response curve (—) by using an IC₅₀ value of 2 μM , as well as to a quadratic binding equation (⋯) by using a K_i value of 0.4 μM . Error bars indicate the data range ($n=2$). C) Proposed mechanism for covalent interaction of PhAs^{III} and Hh precursor. D) Detection of Hh-PhAs^{III} adduct by mass spectrometry. Deconvoluted mass of autocatalytic HhC (10 μM) without (left) and with (right) 1 equiv of Hh-PhAs^{III}. Additional mass is consistent with a condensation mechanism (expected mass change: 154; observed mass change: 153).

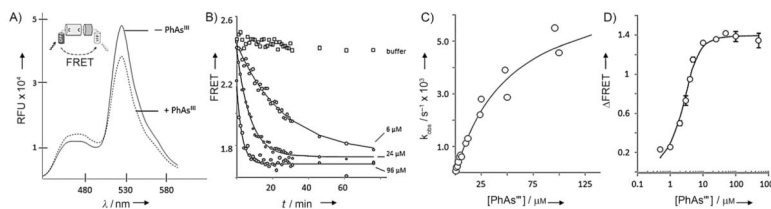


Figure 2. Real-time detection of PhAs^{III} binding by Hh using an optical reporter. A) Effect of PhAs^{III} on the fluorescence of FRET-active Hh precursor protein. Spectra of precursor, C-H-Y, in aqueous buffered solution (pH 7.1) with and without 1 equiv of added PhAs^{III} ($\lambda_{\text{ex}}=400$ nm, $\lambda_{\text{em}}=420-600$ nm). Inset: Schematic of FRET-active construct with cyan fluorescent protein fused to the N terminus of the HhC domain and yellow fluorescent protein fused to the C terminus. B) Kinetics of PhAs^{III} binding by C-H-Y measured by FRET change. Time traces showing loss of FRET signal from C-H-Y after addition of the indicated concentration of PhAs^{III}. Solid lines show first-order exponential decay (k_{obs}), calculated by using best-fit values. C) Bimolecular kinetics of PhAs^{III} binding by C-H-Y is saturable. Rate constants (k_{obs} [s^{-1}]) for FRET loss plotted as a function of increasing PhAs^{III} concentration. Solid line shows the expected behavior, assuming reversible formation of a noncovalent encounter complex, followed by irreversible formation of the PhAs^{III}/C-H-Y complex. D) Fluorometric titration of binding of PhAs^{III} to C-H-Y. Plot shows the overall FRET change ($\text{FRET}_0 - \text{FRET}_t$) as a function of PhAs^{III} concentration. Solid line shows the expected behavior for an inhibitor with a EC_{50} value of $0.4 \mu\text{M}$. Error bars indicate data range ($n=3$)

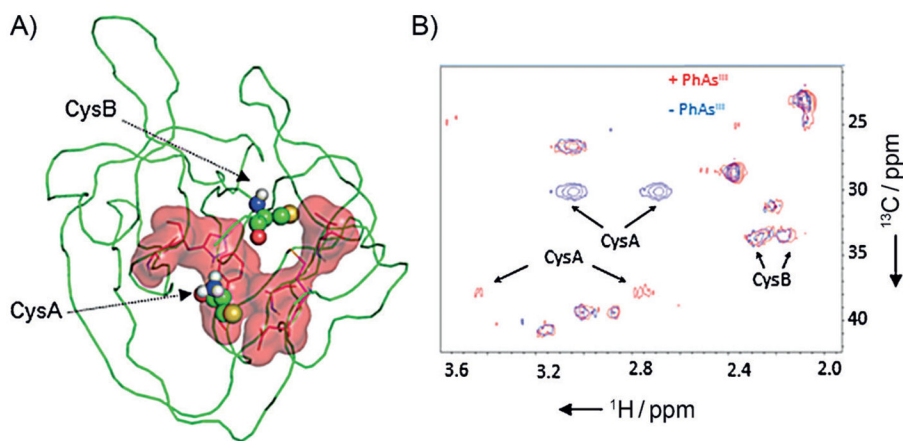
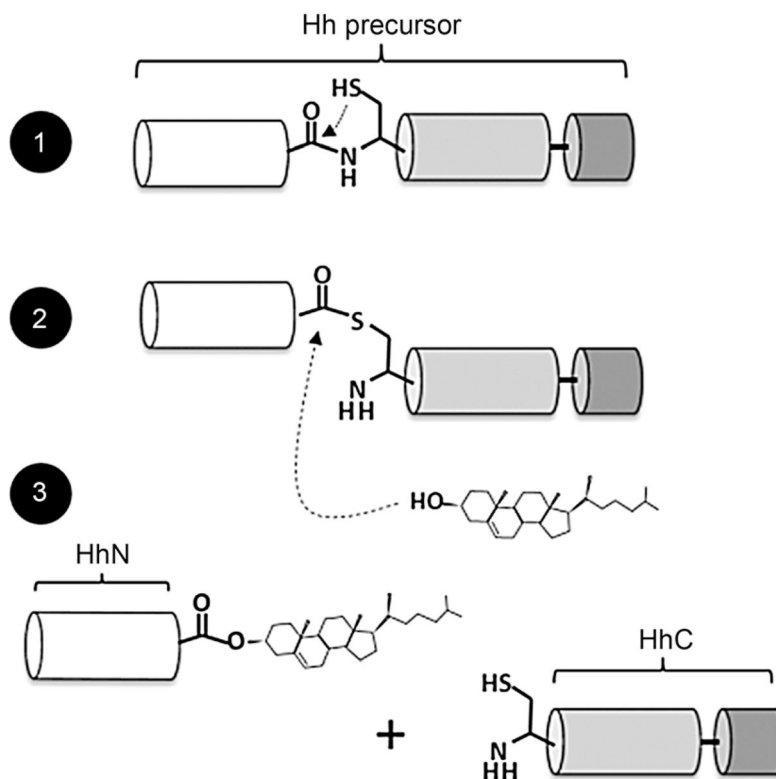


Figure 3. PhAs^{III} binding site on Hh located by NMR chemical shift mapping. A) Proposed binding site. Residues in $^1\text{H}/^{15}\text{N}$ -labeled HINT domain of Hh whose resonances were suppressed by >80% following the addition of 4 equiv of PhAs^{III} are displayed in red (sticks), along with the calculated surface area. Catalytic cysteine residues (CysA and B) are shown as ball-and-stick. This figure was generated from PDB ID: 1ATO by using PyMOL (<http://www.pymol.org>). B) Chemical shift perturbation of CysA in the PhAs^{III}-bound HINT domain. $^{13}\text{C}/^1\text{H}$ spectra of the Hh HINT domain enriched with Cys- $^{13}\text{C}_3$ at CysA and B, acquired without (blue) and with (red) added PhAs^{III}.

**Scheme 1.**

Hedgehog precursor protein cholesterolysis. Autoprocessing begins with the central “HINT” domain’s first residue, cysteine, rearranging the adjacent amide bond to form a thioester. In step two, the C-terminal subdomain (dark gray) activates bound cholesterol for attack at the activated acyl group. Finally, the N-terminal domain, which possesses cell signaling activity, dissociates with its newly formed carboxy terminus esterified to cholesterol.