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# Design and characterization of a photo-activatable hedgehog probe that mimics the natural lipidated form

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ABSTRACT

We have generated a photoactivatable form of sonic hedgehog protein by modifying the N-terminal cysteine with the heterobifunctional photocrosslinker 4-maleimidobenzophenone (Bzm). The Bzm modification on ShhN imparted a significant increase in activity as assessed in the C3H10T1/2 functional assay with potency comparable to that of the endogenous dual-lipidated form of ShhN (ShhNp). Reversed-phase HPLC analysis indicated that the increase in activity compared to unmodified ShhN may be due in part to the hydrophobic nature of the benzophenone group. In contrast to the fully processed ShhNp, Bzm-ShhN is monomeric as assessed by analytical SEC and does not require detergent to be soluble. Further, we demonstrated that the Bzm-ShhN was able to crosslink *in vitro* in the presence of a known binding partner, heparin. We suggest that Bzm-ShhN can serve as a relatively facile and preferred source of ShhNp for *in vitro* assays and as a probe to identify novel Hh protein interactions.



## Introduction

The Hedgehog (Hh)[3] family of secreted proteins plays fundamental roles in vertebrate and invertebrate embryonic development and continues to control the development of many tissues in adults [1], as well as being a major force in driving Hh-related cancers [2]. Three Hh homologs, Indian, Desert and Sonic have been described in mammals and their activity characterized [3]. Sonic hedgehog (Shh) is the most well characterized of the three, having a central role in limb [4], craniofacial [5,6], and central nervous system [7,8] development. Shh is expressed in the notochord and zone of polarizing activity in the limb bud, and its release as a morphogen gradient specifies patterning of the ventral neural tube [9] and digit formation [10], respectively.

Correct post-translational processing, particularly lipid modifications, are important for proper Hh signaling. The ~45 kDa translated product of *shh* is autocatalytically cleaved to generate a ~25 kDa C-terminal fragment involved in the autoprocessing reaction, and a 20 kDa N-terminal fragment (ShhN) [7] responsible for all known Shh activity [11]. ShhN is doubly lipidated with cholesterol [12] and palmitoyl [13] adducts on the C- and N-termini, respectively. This doubly-lipidated form of ShhN is the fully active form [14]. These lipid modifications are involved in ShhN secretion, its migration to receiving cells, and modulation of ShhN signal intensity. Palmitoylation of Hh is required for processing [15], activity [16] and association with receiving cells [17]. Hh protein lacking palmitoylation is unable to signal or diffuse normally (reviewed in [18]), with the absence of cholesterol and palmitoylation substantially reducing signaling activity [19,20]. Fetal exposure to alcohol disrupts cholesterol modification of Hh during post-translational processing [21] and its trafficking to membranes [22].

Although Patched (Ptc) on responding cells is the primary receptor for Shh [23,24], a number of other proteins, receptors and factors have been shown to participate in modulating its activity either positively or negatively (reviewed in [25]). Membrane proteins Cdo, Boc and Gas1 [26] bind Hh and positively regulate signaling [27–29]. Cdo and Boc are localized to microdomains and actively distribute Shh in filopodia [30]. In contrast, the cell surface Hh-interacting protein (Hhip) acts as a sink to sequester Hh from Ptc and restrict Hh activity (reviewed in [25,31]). Likewise, heparan sulfate proteoglycans (HSPG) have been implicated

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[3] *Abbreviations used*: ShhN, Sonic Hedgehog N-terminal domain; Bzm, 4-maleimidobenzophenone; HSPG, heparan sulfate proteoglycan; Ptc, Patched; NEM, N-ethylmaleimide; Flu, fluorescein; AP, alkaline phosphatase.

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in modulating Hh diffusion and signaling [32,33] either positively or negatively [34,35], and via either their protein [35] or sugar [36] regions, with the latter implied from previous observations that Hh can bind heparin directly [37,38].

How all these components co-operate to fine-tune Hh secretion and signaling is a problem to model. Purified proteins and HSPGs can be used, although it can be a challenge to demonstrate that binding is physiologically relevant. A number of biochemical approaches can be taken to assess interactions including pull-downs, co-crystallization and cross-linking studies. Crystallographic studies of Hh complexed with individual components have provided some clues [38–41], including that the binding sites on Hh for Ptc and Hhip overlap suggesting they compete for binding [42]. Demonstrating direct binding of Hh to the HSPG glypican-3 was not possible with purified components [43] but has been demonstrated for Shh binding to heparin and chondroitin sulfate [38]. Interestingly, recent attempts to recapitulate Shh binding to detergent-solubilized Ptc has proved difficult, suggesting that additional factors may be involved for high affinity binding of Hh to Ptc [44].

Chemical cross-linking can be employed to map protein–protein interactions and to identify specific binding sites. Binding interactions are often transient and short-lived, and as a result difficult to detect. By cross-linking however, the interactions in close proximity to a protein can be studied. Our approach to identify interactions between Shh and potential binding partners and to circumvent the challenges of reconstituting what might be low affinity binding interactions, was to generate a photo-activatable version of Shh using benzophenone [45,46] to target and cross-link these interactions. Benzophenone photophores are used extensively for photoaffinity-labeling studies as they are one of the most stable photoreactive groups and the wavelength for UV crosslinking (~350 nm) does not typically affect proteins [47]. Benzophenone-containing molecules undergo photo-activatable cross-linking to adjacent molecules with high specificity by efficient covalent modification to C—H bonds, even in aqueous buffers. Herein we describe the characterization of benzophenone modified Shh and show that this modified form not only retains activity but has potency comparable to the lipid-modified Shh making it an effective mimetic probe for the natural fully active form of ShhN.

## Materials and methods

### Materials

C3H10T1/2 murine embryonic fibroblast cells were from American Type Culture Collection (Manassas, VA). N-ethylmaleimide was purchased from Thermo Scientific (Rockford, IL) and fluorescein-5-maleimide and benzophenone-4-maleimide from Invitrogen (Carlsbad, CA). Recombinant ShhNp produced in human cells (catalog #SHH-025) was from StemRD (Burlingame, CA). KAAD-cyclopamine was from Calbiochem (La Jolla, CA). Taqman primers and probes for GLI 1 (Assay ID: Mm00494654_m1) and β-actin were from Applied Biosystems (Foster City, CA). Heparin ammonium (H6279) was purchased from Sigma–Aldrich (St. Louis, MO). Primary antibody for ShhN (N-19; sc-1194) and secondary antibodies (rabbit anti-goat IgG-HRP; sc-2768) were from Santa Cruz Biotechnology, Inc. (Dallas, TX).

### Preparation of modified ShhN proteins

The N-terminal domain of human Shh (unmodified ShhN, residues 24-197) was expressed and purified from *Escherichia coli* essentially as previously described [48,49]. Maleimide chemistry was used to form stable thioether bonds between each label and the exposed N-terminal cysteine of ShhN using the following reagents: N-ethylmaleimide (NEM); $M_r$ = 125, 25 mM stock solution in $dH_2O$, fluorescein-5-maleimide (Flu); $M_r$ = 427, 2.5 mM stock solution in dimethylformamide (DMF), and benzophenone-4-maleimide (Bzm); $M_r$ = 277, 25 mM stock solution in DMF).

For modification, maleimide reagent (0.5 mM final concentration) was added to ShhN (100 μM) in 20 mM HEPES, pH 6.5, 200 mM NaCl, 50 μM DTT and the reaction incubated at room temperature for 2 h in the dark. Reactions were quenched by adding 10-fold molar excess of DTT for 1 h at room temperature and then dialyzed into 5 mM sodium phosphate, pH 5.5, 150 mM NaCl, 0.5 mM DTT buffer. Negative controls were generated by incubating ShhN with either $dH_2O$ or DMF alone.

### SDS–PAGE

ShhN proteins were denatured and separated using NuPAGE Novex 4–12% Bis-Tris gels with MES running buffer (Invitrogen) and then visualized by staining with Coomassie Blue.

### Mass spectrometry analysis of modified ShhN proteins

The masses of modified ShhN proteins were determined by electrospray-mass spectrometry (ESI-MS). ShhN samples were diluted in Optima Grade water (Fisher Scientific) to a concentration of 50 ng/μl and desalted using a NanoAcquity UPLC system with a 1.7 μm BEH130 $C_{18}$ column (100 μm × 100 mm, Waters Corporation) connected in-line to a Micromass quadrupole time-of-flight mass spectrometer (Waters). Samples were eluted in a 30 min gradient (1–50% acetonitrile (Optima Grade, Fisher Scientific)) with 0.1% formic acid and a flow rate of 0.4 μl/min. Electrospray mass spectra were collected using MassLynx 4.1 (Waters Corp.) and mass spectra (charge state envelopes) of eluted proteins deconvoluted using the MaxEnt algorithm within MassLynx software (Waters Corporation).

### Assessing ShhN activity in C3H10T1/2 cell-based assay

The concentration-dependent signaling activity of modified forms of ShhN was assessed in Hh-responsive C3H10T1/2 murine embryonic fibroblast cells that induce alkaline phosphatase (AP) expression upon ShhN signaling activation. The assay was performed essentially as described previously [48,49] with cells cultured in high glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum in 96-well plates at 5000 cells/well. After 24 h, cells were incubated with serial dilutions of the different forms of ShhN for 5 days and AP activity assessed after incubating lysed cells with the chromogenic substrate *p*-nitrophenyl phosphate and measuring absorbance at 405 nm. Half maximal effective concentration ($EC_{50}$) constants were determined in prism 6.0 (GraphPad Software Inc., La Jolla, CA) with non-linear regression. Recombinantly generated lipid-modified ShhN (ShhNp) produced in human cells was used for comparison in the assay and was reconstituted as recommended by the vendor with water to give a final concentration of 100 ng/μl in PBS/1% CHAPS.

For inhibition studies, the C3H10T1/2 assay was carried out essentially as above except cells were incubated with a fixed concentration of ShhN protein equivalent to its $EC_{50}$ value in the presence or absence of the Hh pathway inhibitor KAAD-cyclopamine (300 nM).

### RT-PCR for GLI1 in C3H10T1/2 cells

C3H10T1/2 cells were cultured at 60,000 cells/well in 12-well plates and after 24 h incubated with a single concentration of ShhN protein at its $EC_{50}$ value in the presence or absence of the Hh pathway inhibitor KAAD-cyclopamine (1 μM). Total RNA was isolated