Bioorthogonal chemical tagging of protein cholesterylation in living cells

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We report the first chemical probe that enables bioorthogonal chemical tagging of post-translationally cholesterylated proteins with an azide in live cells. Our approach enables rapid fluorescence and affinity labelling of protein cholesterylation, as exemplified by multi-label detection of Sonic hedgehog cholesterylation in multiple cell lines, and multiplexed fluorescence detection of cholesterylation and palmitoylation. These probes are expected to be of great utility for the identification of novel cholesterylated proteins, and for exploring the role of protein cholesterylation in living systems, for example in development and cancer.

Post-translational modification (PTM) of proteins is an essential process in all cells, and plays a key role in signal transduction and protein trafficking.[1] An important example is the mammalian Hedgehog (Hh) protein family; these potent morphogens are essential in the development and patterning of organs and other essential processes,[2] and are subject to a complex series of PTMs, as exemplified by Sonic hedgehog (Shh). During maturation in the endoplasmic reticulum (ER) Shh undergoes intein-like autocleavage of its C-terminal domain (Fig. 1) accompanied by C-terminal cholesterylation,[3] and N-terminal palmitoylation by the enzyme Hedgehog acyl transferase (Hhat).[4] Both PTMs play an important but complex role in subsequent Shh secretion and signalling.[5]

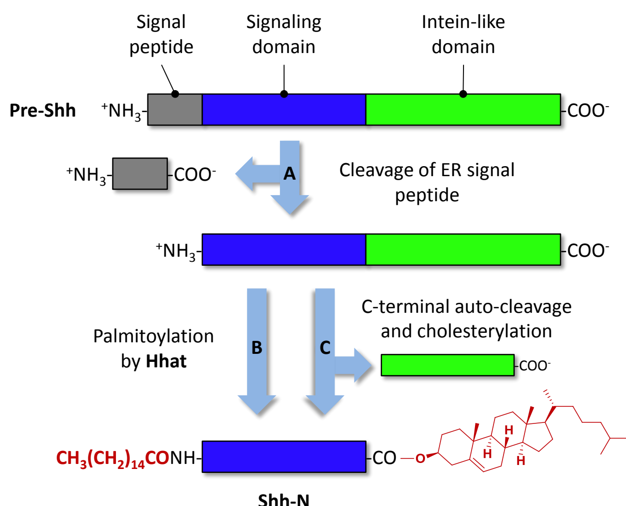
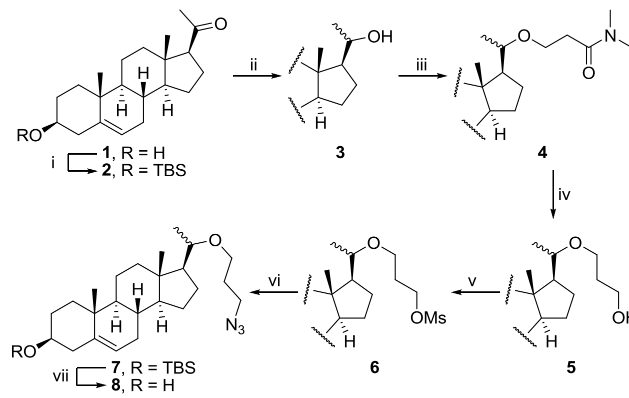


Fig. 1 Post-translational processing of Sonic Hedgehog (Shh) protein in the ER. Cleavage of the signal peptide from Shh precursor protein (**A**) is followed by palmitoylation at the N-terminus (**B**) by the membrane integral acyl transferase Hhat and C-terminal autocleavage-cholesterylation to form a cholesteryl ester at the C-terminal acid (**C**), rendering fully processed N-terminal domain (Shh-N).

Mis-regulated Shh signalling promotes the formation and progression of certain cancers, attracting recent interest in targeting Shh signalling or lipidation for anti-cancer chemotherapy.[6, 7] However, the mechanisms by which the lipid motifs of Shh contribute to signalling remain poorly understood, and appear to vary across different signalling contexts, timescales and distances.[8] It has also been suggested that protein cholesterylation is not restricted to the Hedgehog family,[5] but to date the tools have not been available to explore this hypothesis. Here we report the design and application of the first chemical probe that enables bioorthogonal tagging of cholesterylated proteins in live cells. We also describe simultaneous bioorthogonal tagging of both post-translational lipidations of Shh using a combination of palmitate and cholesterol analogues, and their detection by multiplexed in-gel fluorescence.



Scheme 1 i) Imidazole, TBS-Cl, DMF, 82%; ii) $NaBH_4$, THF, MeOH, 83%; iii) NaH, THF, 0 °C, then *N,N*-Dimethylacrylamide, 54%; iv) $LiBHEt_3$, THF, 0 °C, 89%; v) MsCl, Et_3N, DCM, 0 °C–rt; vi) NaN_3, DMF, 90 °C, 25% over two steps; vii) TBAF, THF, 90%

Despite the importance of palmitoylation and cholesterylation in various signalling activities of Shh, relatively little is known about the tolerance of these processes for modifications in the two lipid donors. Whilst cholesterol analogues are used for labelling cholesterol-rich membrane microdomains and azide functionalized steroids have been used as synthetic intermediates,[9] to date none have been reported as substrates for protein cholesterylation. We reasoned that modifications distal to the 3-OH nucleophile involved in the cholesterylation reaction would prove least disruptive to processing, and envisaged a probe bearing an azide chemical tag in the C22–26 chain, commencing from the prohormone pregnenolone (**1**, Scheme 1).

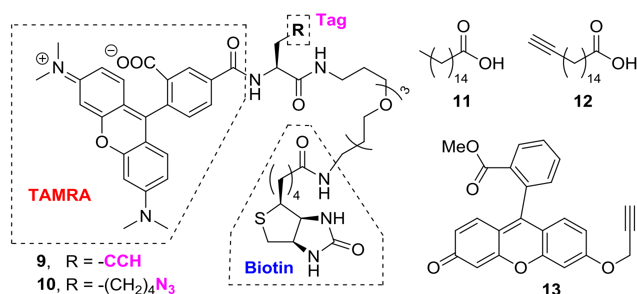


Fig. 2 Trifunctional capture reagents comprising an alkyne (**9**) or azide (**10**) tag, TAMRA fluorophore and biotin labels; palmitic acid (**11**) and alkynyl-palmitate analogue (**12**); bifunctional capture reagent 'alkynyl-fluorescein' (**13**).

The intermediate alcohol **3** proved particularly difficult to alkylate.[10] However, addition to *N*,*N*-dimethylacrylamide under optimised conditions provided the unstable intermediate **4**, which was readily reduced to the alcohol **5** using Super-Hydride® ($LiEt_3BH$) in excellent yield. The high reactivity of this reagent permits reduction to be performed at 0 °C in order to avoid facile *β*-elimination that occurs at higher temperatures. **5** was subsequently converted *via* a short reaction sequence into target probe **8** bearing an azide tag.

To explore the potential of **8** as a substrate for cholesterylation, HEK293a cells transiently transfected to overexpress Shh were treated with different final concentrations of **8** for 16 hours. Cell lysates were then subjected to copper-catalysed azide–alkyne cycloaddition (CuAAC)[11, 12] to trifunctional capture reagent **9** (Fig. 2),[13, 14] introducing dual labels for affinity purification and fluorescence visualisation of ligated targets. These labelled samples were then probed by in-gel fluorescence and α-Shh Western blot. In lysates from cells treated with **8**, labelled Shh-N ('Shh-N*') was readily visualised as a strongly fluorescent band (red arrow, Fig. 3A), with labelling intensity dependent on the concentration of **8** used in the feeding step. α-Shh Western blot (Fig. 3B) revealed a small band shift to higher apparent molecular weight following ligation to **9** (Fig. 3B), consistent with an increase in mass. Interestingly, a range of proteins other than Shh-N are also tagged by **8**, with labelling intensity increasing in proportion to the concentration of the probe (blue arrows, Fig. 3A). This observation is consistent with the presence of multiple cholesterylated proteins,[5] and suggests the potential of **8** as a general tool for exploring cholesterylation.

From these data it appears that **8** is a good substrate for the Shh C-terminal intein-like domain, particularly in view of the fact that **8** has to be transported into cells and into the ER where Shh self-processing occurs,[8] and furthermore is in competition with endogenous cholesterol for binding Pre-Shh inside cells. The degree of Pre-Shh processing also appears to be enhanced with increasing concentration of **8**. At the highest concentration tested (100 μM) **8** caused some rounding of cells whilst lower concentrations had no observable side-effects, and thus 50 μM **8** was selected for further experiments. Taking advantage of the dual label in **9**, pull-down on streptavidin-coated magnetic beads permitted highly specific isolation of Shh-N* from cells fed with **8** (Fig. 3C). Unprocessed Shh (Pre-Shh, Fig. 3) is neither labelled nor affinity purified under these conditions, showing that labelling is dependent on self-processing as for native cholesterylation. This observation is consistent with specific mechanism-based labelling by **8**, and with studies on radiolabeling of Shh-N with tritiated cholesterol.[5]

Shh is known to be upregulated in certain cancers including lung adenocarcinoma[15] and pancreatic carcinoma,[16] and mis-regulated Shh signalling *in vivo* contributes to cancer cell proliferation and maintaining the tumour's physiological niche. To investigate the utility of **8** as a probe for

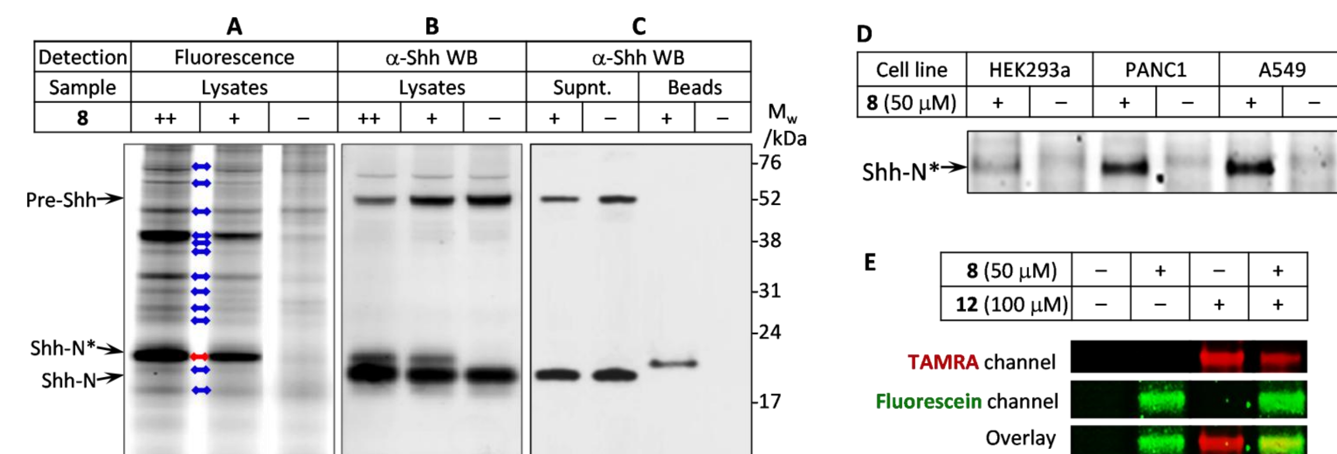


Fig. 3 Tagging cholesterylation in live cells with **8**. **A-C**) HEK293a cells transfected with Shh were fed with **8** (++: 100 μM; +: 50 μM; –: DMSO vehicle only) for 16 h at the concentrations indicated, and lysates captured with **9** (see ESI for further details). Proteins were separated by SDS-PAGE and observed by **A**) in-gel fluorescence or **B**) α-Shh Western blot. Pre-Shh: unprocessed Shh precursor; Shh-N: processed but untagged Shh protein; Shh-N*: Shh tagged with **8** and labelled by **9**. ↔: fluorescent Shh-N* band; ↔: other proteins tagged by **8**. **C**) Labelled lysates affinity purified on streptavidin-coated magnetic beads; protein remaining in supernatant (Supnt.) or retained on beads (Beads) after pull-down, analysed by α-Shh Western blot. Shh-N* is pulled down specifically in cells fed with **8**. **D**) Wild-type (non-transfected) PANC1 and A549 cancer cell lines or HEK293a cells were fed with **8**, captured with **9**, and visualised as in (A). **E**) HEK293a cells transfected with Shh were fed with **8** and/or **12** as indicated; all samples were subjected to CuAAC with **10**, protein precipitation (to remove excess **10**), and then with **13**; in-gel fluorescence imaging at TAMRA and fluorescein wavelengths.

cholesterylation of upregulated Shh, A549 (human lung adenocarcinoma), PANC1 (human pancreatic carcinoma) and wild-type HEK293a (control) cells were fed, and the lysates labelled with **9** and affinity enriched. In-gel fluorescence provides a direct readout for Shh-N* in the cancer cells (Fig. 3D). Notably, fluorescence detection of Shh cholesterylation tagged with **8** is complete in seconds, whereas detection with ^{3}H radiolabeled cholesterol in these lines is orders of magnitude slower, requiring days or even weeks of exposure.

Shh is also palmitoylated *in vivo* by Hhat, and we hypothesised that Shh could be tagged using a chemical probe for palmitoylation, potentially enabling orthogonal multiplexed detection of both lipidation events in the same experiment. Fatty acid probes have been used to label acylated proteins in living systems,[1, 17-20] however this has not previously been reported for Shh and so we determined the suitability of heptadec-16-ynoic acid **12** (Fig. 2), an alkyne-tagged analogue of palmitic acid **11**, as an Hhat substrate in live cells. **12** was fed to HEK293a cells overexpressing Shh and captured from cell lysates using bioorthogonal ligation to azide-TAMRA-biotin capture reagent **10**. **12** was found to be incorporated into Shh-N with reasonable efficiency at 100 μM (ESI, Fig. S1). Encouraged by this result, we investigated simultaneous labelling of Shh cholesterylation and palmitoylation in these cells. HEK293a cells overexpressing Shh were fed with **8**, **12** or **8**+**12** (or DMSO vehicle), and sequential CuAAC chemistry was performed on each cell lysate in turn with **10**, followed by **13**. **10** introduces a dual TAMRA/Biotin label on palmitoylated proteins, whilst **13** labels cholesterylated proteins with fluorescein; protein precipitation between reactions served to remove excess **10**. Shh tagged by **8** and/or **12** was readily observable by two-colour in-gel fluorescence detection (Fig. 3E), with no detectable background labelling of Shh by mismatched capture reagents. No adverse effects on cell proliferation were observed at the probe concentrations used.

We have reported here the design and synthesis of the first chemical probe for bioorthogonal tagging of protein cholesterylation. This tagging system enables multi-label detection of cholesterylated Shh using a multifunctional capture reagent **9**, providing handles suitable for in-gel fluorescence and affinity purification. The combination of **8** and **9** provides very rapid detection of Shh cholesterylation by in-gel fluorescence in a range of cell lines, avoiding time consuming and expensive use of hazardous ionising radiation. Notably, **8** tags a number of protein bands in addition to Shh, suggesting that with further optimisation **8** or similar analogues may permit *de novo* chemical proteomic discovery of new cholesterylated proteins in living systems, by analogy with probes for other PTMs.[19] Further, we have demonstrated multiplexed tagging of Shh cholesterylation and palmitoylation in live cells, one of the first examples of orthogonal tagging of multiple PTMs in a living system.[21] We anticipate that the tools reported here will facilitate exploration of the interdependence and substrate specificity of these modifications, and their roles in cancer and development. Multiplexed fluorescence detection also opens up a range of potential future applications, including two-colour imaging by fluorescence microscopy, and screening for lipidation inhibitors in cells. We will report on our on-going studies in these areas in due course.

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Notes and references

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† Electronic Supplementary Information (ESI) available: full experimental procedures for organic synthesis, cell culture, bioorthogonal ligation; characterisation data for new compounds; supplemental figure. See DOI: 10.1039/b000000x/

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