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FRET-based cholesterolysis assay identifies a novel hedgehog inhibitor

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Abstract

Hedgehog (Hh) proteins function in cell/cell signaling processes linked to human embryo development and the progression of several types of cancer. Here we describe an optical assay of hedgehog cholesterolysis, a unique autoprocessing event critical for Hh function. The assay uses a recombinant FRET-active hedgehog precursor whose cholesterolysis can be monitored continuously in multi-well plates (dynamic range, 4; Z', 0.7), offering advantages in throughput over conventional SDS-PAGE assays. Application of the optical assay in a pilot small molecule screen produced a novel cholesterolysis inhibitor (apparent IC_{50}, 5×10^{-6} M) that appears to inactivate hedgehog covalently by a S_NAr mechanism.

Proteins in the hedgehog (Hh) family function as cell-cell signaling factors that help govern embryo development, while also having pathogenic effects in multiple types of cancer [1-4]. Hedgehog's oncogenic activity has galvanized small molecule discovery efforts, resulting in the recent approval of vismodegib (Erivedge) for the treatment of advanced basal cell carcinoma [5]. Vismodegib, like several other Hh inhibitors under clinical study, interferes with transduction of the Hh signal by antagonizing a cell surface receptor called Smoothened [6, 7]. While a major milestone, alternative approaches to combat aberrant Hh activity are worth pursuing for combination therapy and to contend with clinical resistance to vismodegib [8-10]. Here we describe an optical activity assay to monitor Hh cholesterolysis, a crucial yet relatively under-studied autoprocessing reaction characteristic of this important protein family. We demonstrate the assay's utility with a small molecule screen, resulting in the identification of a novel cholesterolysis inhibitor. .

Cholesterolysis of a hedgehog precursor liberates functional hedgehog ligand (HhN) and couples its incipient carboxyl terminus to cholesterol [11-13] (**Figure 1A**). At least two

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chemical steps are involved in the reaction: first, an N-S acyl shift activates the terminal glycine residue of HhN as a thioester; next, the hydroxyl group of substrate cholesterol attacks the thioester, thereby displacing HhN [12]. No cofactors or accessory proteins seem to be required for either step. All catalytic activity resides in the adjacent C-segment of the precursor, referred to as HhC [14][15, 16]. Congenital mutations in the HhC segment of human “sonic” Hh that attenuate cholesterolysis are associated with developmental anomalies of the brain, highlighting the importance of this reaction [17-20].

With a view toward increasing the speed of antagonist/agonist screens, as well as enabling structure-function studies of this unusual lipidation event, we sought a continuous cholesterolysis assay suitable for microplate readers. Current means of assaying cholesterolysis include the separation of precursor and products by SDS-PAGE, and more recently by fluorescent polarization [21]. The latter assay, while attractive in many respects, suffers from a low dynamic range and requires refolding of HhC followed by labeling with arsenic-based reagents [21]. The assay system we describe here involves FRET (Förster resonance energy transfer) between soluble recombinant fluorescent proteins genetically fused to an HhC segment (residues 255-471) from the *Drosophila melanogaster* Hh (**Figure 1B**). It is analogous to the FRET sensor of Amitai *et. al*, used to characterize the kinetics of self-cleaving “inteins” [22]. The key construct, termed **C**-**H**-**Y**, has cyan (**C**) and yellow (**Y**) fluorescent proteins [23] fused to the amino and carboxyl termini of **H**hC. Recently we reported that C-H-Y could be used to detect binding of HhC by an irreversible inhibitor of cholesterolysis [24]. Here we pursue the expectation that cholesterolysis of C-H-Y will yield cholesterol-modified CFP and HhC-YFP, with a relatively large change in the FRET ratio (i.e. loss) as the fluorescent proteins physically separate. We calculate the FRET ratio after excitation at 400 nm by dividing the emission intensity at 540 nm, predominately from YFP, by the emission intensity at 460 nm, predominately from CFP. In *E. coli*, C-H-Y expresses in soluble form, and the precursor remains largely unprocessed owing to the absence of cholesterol. Two control constructs used for the FRET system are a catalytic mutant, C-H($Cys^{A}Ala$)-Y, (residue Cys258Ala) whose cholesterolysis activity is reduced by > 10^4-fold; and, C-Y, which is identical to C-H-Y except that the HhC domain has been replaced by a short peptide linker. The first control construct, C-H($Cys^{A}Ala$)-Y, can be used to mimic 100% inhibition in screens for antagonists of cholesterolysis; C-Y serves as a control to flag compounds that interact with the fluorescent proteins.

Activity assays using C-H-Y were conducted in Bis-Tris buffer (50 mM) at pH 7.1, the prevailing pH of the endoplasmic reticulum where cholesterolysis seems to occur in the cell [25, 26]. Following earlier work, Triton X-100 was added to a final concentration of 0.4% for the purpose of solubilizing cholesterol [11]. Ethylenediaminetetraacetic acid (5 mM, final) and Tris(2-carboxyethyl)phosphine (4 mM, final) were also included in the reaction buffer to suppress oxidation of two catalytically essential cysteines of HhC [26]. After a 10 minute preincubation with C-H-Y (0.2 μM) in assay buffer at 30 °C, reactions were initiated by the addition of cholesterol from an ethanolic stock. As can be seen in **Figure 1C**, added cholesterol led to a gradual attenuation of the FRET ratio from C-H-Y, falling from ~1.6 to 0.4. Full spectra were also recorded at selected intervals, which showed time dependent loss of FRET acceptor signal at 540 nm, and gain of FRET donor signal at 460 nm, consistent

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with donor and acceptor separation. The final FRET value corresponds to that of a 1:1 mixture of C and H-Y (**Figure 1C**, bottom trace), suggesting that cholesterolysis had proceeded to completion. The extent of cholesterolysis was also confirmed by separating the product mixture with SDS-PAGE (**Figure 1C**, inset). In addition, the FRET ratio of the catalytic mutant, C-H(Cys^{A}Ala)-Y, and control C-Y seemed relatively insensitive to added cholesterol (**Figure 1C**, top traces), as expected.

To substantiate the role of cholesterol as a nucleophile in the C-H-Y reaction, control assays were conducted with C-H-Y using a fluorescent sterol derivative, 25-NBD cholesterol. Separation of the product mixture by denaturing SDS-PAGE, followed by UV illumination of the gel, showed that the fluorophore tracked with liberated CFP (**Supporting Figure 2A**). Co-migration of 25-NBD cholesterol with denatured CFP is consistent with covalent interaction. No appreciable activity of C-H-Y was apparent in control experiments lacking cholesterol or with the de-oxy cholesterol analogue, 5-cholestene (**Supporting Figure 2B, C**). The apparent stability of C-H-Y in the absence of added sterol indicates that spontaneous hydrolysis of the internal thioester is slow; our preliminary experiments indicate a rate constant for hydrolysis at 30 °C of ~4×10^{-6} sec^{-1} ($t_{½}$, 2 days).

To further establish that the observed changes in the FRET ratio with C-H-Y were reporting cholesterolysis activity, we confirmed that steady state kinetics exhibited a saturable dependence on cholesterol concentration. With C-H-Y at 0.2 μM, reactions were initiated by adding cholesterol to concentrations ranging from 5 μM to 350 μM. Assays were carried out in triplicate, and wells containing C-H-Y without cholesterol served as control. Michaelis-Menten plots of initial rates as a function of increasing cholesterol concentration showed saturation behavior (**Supporting Figure 3A**). Fits of the data to a hyperbolic function (see Supporting Information) yielded an apparent K_m for cholesterol of 15 μM (+/− 3), in accord with earlier work [21]. To determine a maximum rate of cholesterolysis, we fit reaction progress curves at saturating cholesterol (350 μM) to a first order exponential, yielding an apparent k_{cat} value of 0.001 sec^{-1} (t ½ ~ 11 minutes) (**Supporting Figure 3B**). This value at pH 7.1, the prevailing pH of the ER [25], is remarkably similar to the apparent rate of Hh precursor processing measured by pulse-chase experiments in cultured mammalian cells (0.0006 sec^{-1}) [27]; it is also within range of autocatalytic “protein splicing” reactions brought about by inteins [22, 28-30], which may be ancestral to Hh proteins [14, 31]. Thus, the optical format facilitates kinetic analysis, and affords results that are reproducible and appear physiologically relevant.

To evaluate the assay’s high-throughput compatibility, we carried out a pilot screen aimed at identifying cholesterolysis inhibitors. The compound library consisted of 240 protease inhibitors and their analogues, a selection biased by the target, which acts in a manner analogous to a protease (i.e. peptide bond cleavage). Assays were conducted in 96-well plates with C-H-Y at a final concentration of 0.2 μM in the reaction buffer above. Potential inhibitors were added from DMSO stocks to a final concentration of 40 μM into columns 2-11 of the plate. Control experiments indicated that the final concentration of DMSO (2%, v/v) has a minor effect (<5%) on reaction kinetics (**Supporting Figure 4A, B**). Prior to cholesterol addition, samples were equilibrated at 30 °C in the plate reader. FRET values calculated during this preincubation proved useful in identifying compounds that interfered

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optically with the assay output, as well as compounds that were not optically active but could alter the FRET signal from C-H-Y presumably through direct binding [24]. Reactions were initiated with cholesterol (50 μM, or 250 μM, final) and monitored continuously for a period of 1 to 3 h.

Intraplate controls were located in column 1 (minus cholesterol, minus compound) and in column 12, (plus cholesterol, minus compound) (**Figure 2A**). Data from these wells were used to evaluate assay performance through the Z' calculation; column 1 used as the "max" and column 2 used as the "min", according to formulation of Zhang [32]. The Z' values were consistently above 0.7, indicating a reliable assay. Readings from control wells were also used to flag highly fluorescent compounds whose emission washed out signal from C-H-Y. Anomalously high, stable FRET signals were observed in the presence of compounds that fluoresced at 540 nm, whereas anomalously stable, low FRET signal was apparent in the presence of compounds fluorescing at 460 nm. Compounds in the latter class were more abundant, in accord with similar studies [33]. If emission values at 460 nm were >3 standard deviations from the mean emission value at 460 nm of the control wells, the well data was discarded. A histogram of the 460 nm emission data from compound and control wells is provided in the supporting information (**Supporting Figure 5**).

With optically active compounds flagged, our attention was drawn to compound ST044643 as a potential inhibitor (**Figure 2B**). Kinetic traces in the presence of ST044643 showed minor changes in the initial cholesterolysis rate, however, the end point FRET ratio seemed high compared with controls (**Figure 2C**). That elevated end point signal suggested to us that a fraction of the original C-H-Y remained unprocessed; a hypothesis we subsequently confirmed by SDS-PAGE (**Supporting Figure 6A**). Interestingly, data from the pre-incubation period in the presence of ST044643, but in the absence of cholesterol, indicated that FRET ratio of C-H-Y underwent a noticeable and reproducible quenching (**Figure 2C**, shaded). No evidence for decomposition of C-H-Y in the presence of ST044643 was found by SDS-PAGE analysis (**Supporting Figure 6B**). We have observed similar quenching behavior with C-H-Y in the presence of phenylarsine oxide, an active-site covalent inhibitor of cholesterolysis [24]. To explore the specificity of this effect, ST044643 was added to the control construct, C-Y, in which the HhC segment is replaced with a glycine-serine linker; no significant change in the FRET ratio of C-Y was apparent with added ST044643 ($p = 0.276$, $n = 15$) (**Supporting Figure 6C**). Next, we tested whether added ST044643 could alter the FRET from C-H(Cys^{A}Ala)-Y, which is identical to C-H-Y except that a conserved cysteine residue of HhC (residue 258) is mutated to alanine, rendering it catalytic inert[26]; here, a significant change in the FRET ratio with ST044643 was observed ($p < 0.005$, $n=12$). Together these results are consistent with the notion that the ST044643 binding site on C-H-Y maps to the "H" segment, but does not seem to involve this particular catalytically essential cysteine residue (CysA) of HhC.

To further examine the strength and specificity of inhibition by ST044643, the compound was repurchased as a powder from the supplier and used in dose response experiments in a gel-based activity assay. In place of C-H-Y, we used a chimeric Hh precursor, SHhN-DHhC, where the signaling domain from human sonic hedgehog protein (residues 24–197) is fused to the same autoprocessing C-terminal segment of Hh from *Drosophila melanogaster* [24].

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This construct is overexpressed as a soluble, His-tagged protein in E. coli. ST044643 was tested as an inhibitor of SHhN-DHhC cholesterolysis at concentrations ranging from 0.1 μM to 29 μM. The extent of processing was determined by gel densitometry 3 h and 24 h after adding cholesterol (200 μM, final). A representative gel and corresponding dose response curve, which indicate an apparent IC_{50} value of ~ 5 μM, are shown in **Figure 2D**. Consistent with covalent inhibition, the extent of cholesterolysis after 3 h and after 24 h in the presence of equivalent concentration of ST044643 were comparable. The IC_{50} value with SHhN-DHhC also accords with inhibitory strength toward C-H-Y measured in the optical assay. Collectively, these results serve to validate ST044643 as a cholesterolysis inhibitor and further suggest a covalent inhibitory mechanism.

Covalent modification of an active-site cysteine through S_NAr (substitution nucleophilic aromatic) is a likely mode of action for ST044643. The pair of nitro groups, along with the ester, all in resonance with the carbon bearing the mercapto tetrazole moiety, activate the phenyl group of ST044643 for S_NAr (**Supporting Figure 7A**). Structurally similar molecules have been reported to inhibit other cysteine-dependent biocatalytic transformations by S_NAr [34, 35]. In addition, preliminary mass spectrometry of HhC treated with ST044643 support covalent inactivation by this mechanism (**Supporting Figure 7B**). Notwithstanding, results of the FRET quenching experiments (above), argue against S_NAr, at least to the extent that it requires the CysA residue of HhC. There is a however a second conserved cysteine residue of HhC, termed CysB (residue number 400), that is required for cholesterolysis and located in the active site [14, 26]. To explore the potential involvement of CysB in ST044643 binding, we generated the point mutant, C-H(Cys^BAsp)-Y, and subjected it to the FRET quenching analysis. Attempts to prepare an Ala point mutant at this position resulted in a protein that expressed poorly. Unlike C-H-Y and C-(Cys^AAla)H-Y, the FRET ratio of C-H(Cys^BAsp)-Y mutant seemed unresponsive to added ST044643 ($p = 0.162$, $n=13$) (**Supporting Figure 6C**). The unresponsiveness of this mutant is consistent with the notion that CysB is required for interaction with ST044643. Unambiguous identification of the binding site, and the nature of the adduct await more detailed structural analysis. Meanwhile we have begun using ST044643 as a positive control in small molecule screens using larger, more diverse chemical libraries.

To accelerate discovery of a novel class of Hh-directed small molecules that may ultimately develop into effective drug candidates, we devised a continuous, optical assay to monitor Hh cholesterolysis. Agonists of cholesterolysis are expected to increase the concentration of Hh ligand in circulation, with potential use in rescuing Hh mutations linked to birth defects; antagonists have potential use in treating Hh-driven cancers, where Hh is chronically over produced. The FRET assay's dynamic range, favorable Z' factor, and ease of use encourage its application to these and to other discovery efforts.

Supplementary Material

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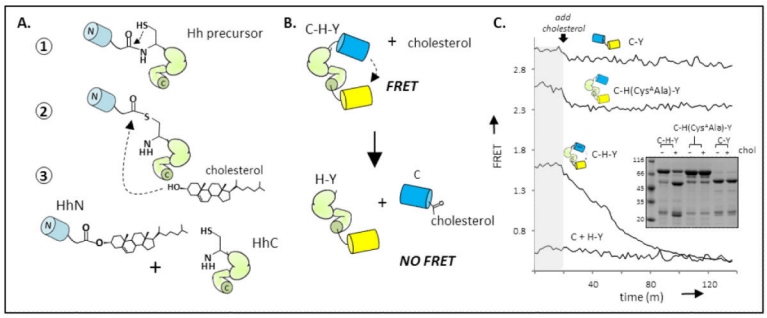

Figure 1.

Mechanism and monitoring of Hh cholesterolysis. (A) Reaction pathway. Cholesterolysis of Hh precursor proteins involves an N-S acyl shift at a conserved glycine-cysteine dipeptide (step 1); transesterification to cholesterol (step 2); and dissociation of cholesteroylated Hh ligand (HhN) from the Hh autoprocessing segment, (HhC) (step 3). (B) FRET reporter of cholesterolysis. Construct, abbreviated C-H-Y, contains **H**hC as an internal fusion to **C**yan fluorescent protein and **Y**ellow fluorescent protein. (C) Cholesterolysis assay. FRET from catalytically active C-H-Y, and inactive control constructs,C-Y and C-(Cys^{A}Ala)H-Y, are monitored before and after cholesterol addition. The kinetic trace of C-H-Y shows FRET loss following cholesterol addition, approaching FRET of a 1:1 mixture of CFP and YFP. *Inset* Characterization of cholesterolysis reactions by SDS-PAGE followed by Coomassie blue staining. Molecular weights: C-H-Y, 80 kDa; CFP, 27 kDa; H-Y, 52 kDa; C-Y, 48 kDa.

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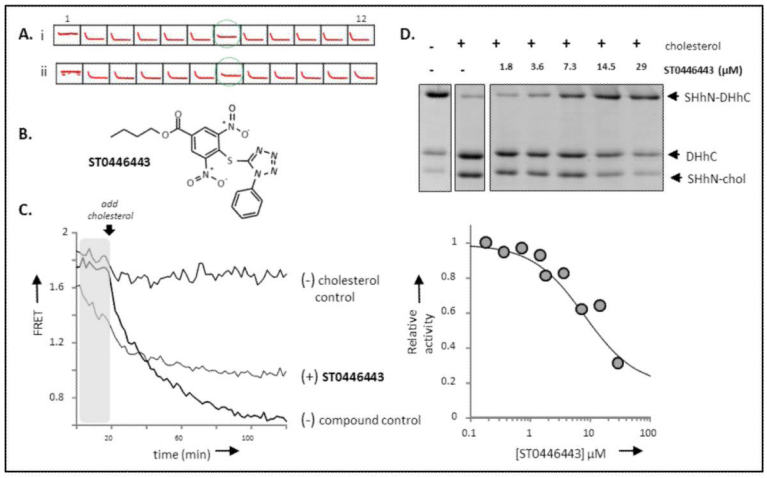

Figure 2.

Hh cholesterolysis inhibitor identified by chemical screening. (A) Screening output. Traces of 12 sample wells from a 96 well plate, showing controls, column (1, 12); and compound wells (2-11). Compound in well 7 (green circle) was identified as an potential inhibitor in screens with cholesterol at 60 μM (upper) and 200 μM (lower). (B) Inhibitor structure. TIMTEC chemical identification code ST044643. (C) Effects of ST044643 on C-H-Y. Added ST044643 diminishes FRET from C-H-Y in the pre-incubation period (gray), and limits the extent of reaction compared to the C-H-Y control. (D) Secondary screen of Hh cholesterolysis inhibition by ST044643. Upper: Cholesterolysis activity of chimeric hedgehog precursor, ShhN-DHhC, in the absence and presence of increasing amounts of ST044643. Precursor and products were separated by SDS-PAGE and stained with Coomassie blue. Lower: Relative cholesterolysis activity plotted as a function of increasing ST044643, using data in upper panel. Dose-response curve (solid line) was calculated using an IC_{50} value of 5 μM.