Supplementary Information

Chemical Bypass of General Base Catalysis in Hedgehog Protein Cholesterolysis Using a Hyper-Nucleophilic Substrate

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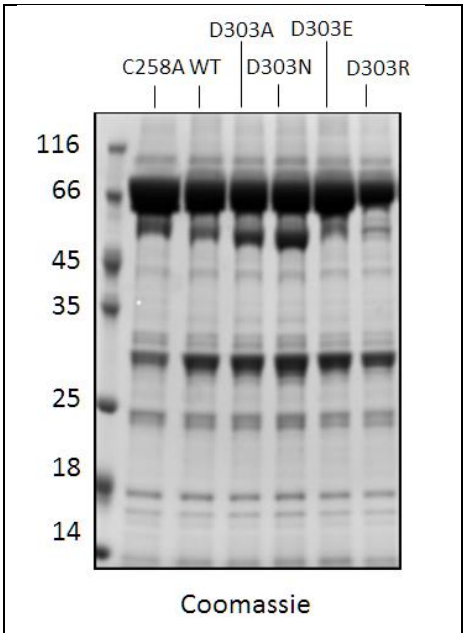
General: NMR spectra were acquired using a Bruker Avance III 600 MHz spectrometer at 30°C. Calibration was by the residual solvent signal ($CDCl_3$: ^1H = 7.26 ppm. ^{13}C = 77.0 ppm). Preparative TLC was performed on glass-backed plates (10 cm in length) coated with a 0.25 mm layer of silica gel 60 F254. HPLC of small molecules was carried out using a Waters 6000A pump, Waters 410 differential refractometer, and two Altex Ultrasphere ODS 5 µm 10×250 mm columns in series using a flow rate of 3 ml/min MeOH.

Protein expression and purification: FRET-active proteins- C-H-Y, C-H(C258A)-Y, C-H(D303A)-Y, C-H(D303N)-Y, C-H(D303E)-Y, C-H(D303R)-Y, each having a C-terminal His_6 sequence, were expressed from pBAD33 plasmids in *Escherichia coli* strain LMG194 using nickel-nitrilotriacetic acid (Ni-NTA) agarose, as described before [1, 2] (right). Briefly, overnight starter cultures (3 ml) in LB broth with chloramphenicol (100 µg/ml) were subcultured to 50 ml of LB with chloramphenicol (100 µg/ml), grown with shaking at 37°C to an OD_{600} 0.5 and then induced with arabinose (0.2% final). Following overnight growth at 16°C, cells were pelleted and frozen at -80°C for 24 hrs. Thawed pellets were lysed by vortexing for 5 min in 3 ml chilled phosphate buffer (pH 7.4) containing Triton X-100 (0.5%), glycerol (10%), potassium chloride (0.1 M), sodium chloride (0.4 M), lysozyme (10 µg/ml), DNase (2 µg/ml) and imidazole (0.01 M). NiNTA affinity chromatography was carried out according to manufacturer's protocol (Thermo Scientific). Proteins were eluted from column using chilled phosphate buffer (pH 7.4) containing glycerol (10%), sodium chloride (0.5 M), and imidazole (0.5 M). Typical elution yielded 300 µl of protein (15-25 µM) with > 80 % purity.



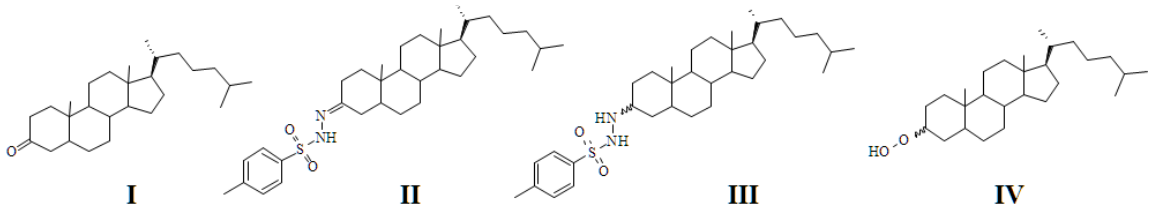
SDS-PAGE showing purified FRET-active Hh precursor, C258 mutant, and general-base mutants. Expected molecular weight of C-H-Y polyproteins is ~ 86 kDa

Hedgehog Activity Assays: For kinetic assays of autoprocessing, FRET-active wild type and mutant HhC proteins were monitored using a BioTek Synergy H1 plate reader [1]. Samples contained protein ($1x10^{-7}$ M) in Bis-Tris buffer (0.02 M, pH 7.1) with ethylenediaminetetraacetic acid (EDTA, 0.005 M), NaCl (0.1 M), TCEP (0.005 M), and Fos-Choline-12 (0.0015 M). Following 10 min preincubation at 30°C, reactions (100 µl) were initiated by addition of the sterol from an ethanol stock to a final concentration ranging 0.1 µM to 50 µM; ethanol concentration in the sample did not exceed 5% (v/v). Activity was measured continuously by the loss of FRET, where FRET = emission 540 nm/emission 460 nm after excitation at 400 nm.

Hedgehog autoprocessing was further analyzed by SDS-PAGE gel. Samples were prepared in the same buffer with a protein concentration of 0.25 µM, final. Following pre-incubation at 30°C, sterol was added

to 50 µM. Aliquots were removed at selected intervals, quenched by SDS-PAGE load dye and heating at 95°C for 5 minutes. Samples resolved on 12% Bolt gels (Thermo Scientific), stained with Coomassie Blue G-250, imaged using BioRad imager and analyzed with ImageJ software [3].

Synthesis*:* The cholestanyl hydroperoxides were synthesized according to the method of Caglioti [4].



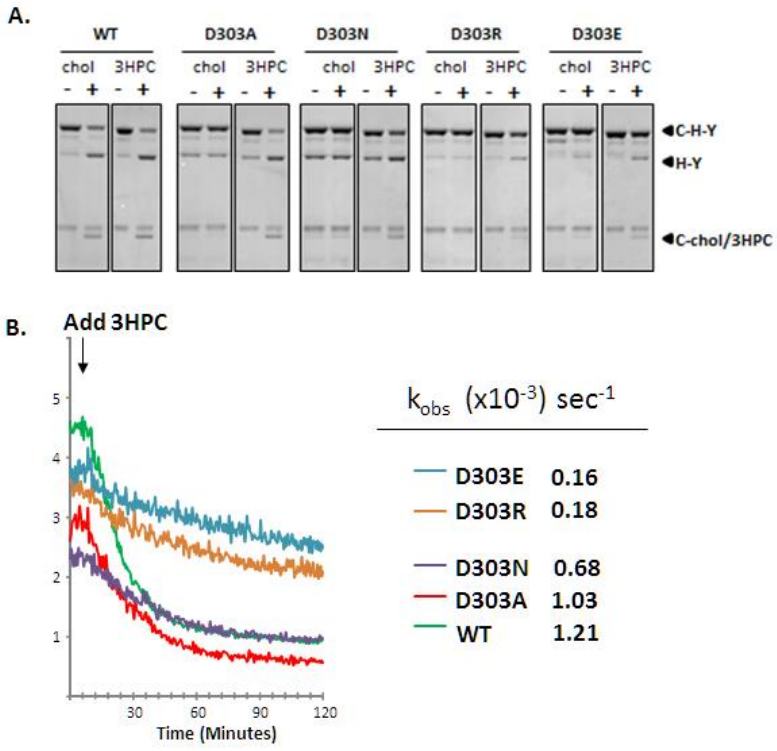
Cholestanone **I** (408 mg) was treated with p-toluenesulfonylhydrazide (221 mg) in 10 ml THF at RT with stirring for 3 days. The mixture was partitioned between water and hexane/ethyl acetate 1:1. The combined organic layers were dried with Na_2SO_4 and the solvent evaporated to give the tosylhydrazone, **II,** (quant.) as a mixture of E and Z isomers as seen by the doubling of the ^{1}H-NMR signals corresponding to the C-2 and C-4 positions (2.6-2.0 ppm), as well as 19-CH3 (0.843 and 0.833 ppm).^{1}H-NMR (600 MHz, $CDCl_3$): 7.835 (1H, d, J=8.2 Hz); 7.306 (1H, d, J=8.2 Hz); 2.428 (3H,s); 0.892 (3H, d, J=6.5 Hz); 0.863 (3H, d, J=6.6 Hz); 0.858 (3H, d, J=6.6 Hz); 0.843 and 0.833 (3H, s); 0.645 (3H, s).

The tosylhydrazone (100 mg) was reduced with $NaBH_4$ (100 mg) in 3.0 ml THF/DMSO 1:1 containing 1% H2O. After stirring 2 hr at RT, the mixture was partitioned between water and hexane/ethyl acetate 1:1. The combined organic layers were dried with Na_2SO_4 and the solvent evaporated. ^{1}H-NMR indicated a 1:2 mixture of starting material and product. The tosylhydrazine product, **III,** was a 2:1 mixture of the α- and ß- isomers. The crude product was used for the next step. α-isomer: ^{1}H-NMR (600 MHz, $CDCl_3$): 7.817 (1H, d, J=8.4 Hz); 7.315 (1H, d, J=8.4 Hz); 5.623 (1H, s); 2.937 (1H, quint, J=2.3 Hz); 2.433 (3H, s); 0.897 (3H, d, J=6.6 Hz); 0.863 (3H, d, J=6.6 Hz); 0.858 (3H, d, J=6.6 Hz); 0.693 (3H, s); 0.621 (3H, s). ß-isomer: ^{1}H-NMR (600 MHz, $CDCl_3$): 7.799 (1H, d, J=8.4 Hz); 7.312 (1H, d, J=8.4 Hz); 5.733 (1H, s); 2.640 (1H, tt, J=11.1, 4.2 Hz); 2.436 (3H, s); 0.888 (3H, d, J=6.6 Hz); 0.863 (3H, d, J=6.6 Hz); 0.858 (3H, d, J=6.6 Hz); 0.702(3H, s); 0.627 (3H, s).

To prepare the hydroperoxides (**IV**), the crude tosylhydrazine (30 mg) was treated with 30% H_2O_2 (0.9 ml) and 6 M NaOH (75 µl) in 3 ml THF. After stirring overnight, the product was extracted with dilute HCl and hexane/ethyl acetate 2:1. The combined organic layers were dried with Na_2SO_4 and the solvent evaporated. The product contained a mixture of cholestan-3α-ol (11%), cholestan-3ß-ol (30%), 3α-hydroperoxycholestane (27%), and 3ß-hydroperoxycholestane (32%). These were separated by preparative TLC (hexane/ethyl acetate 9:1). Further purification was by HPLC. 3α-Hydroperoxycholestane: ^{1}H-NMR (600 MHz, $CDCl_3$): 7.505 (1H, br s); 4.172 (1H, quint, J=2.5 Hz); 0.899 (3H, d, J=6.6 Hz); 0.866 (3H, d, J=6.6 Hz); 0.861 (3H, d, J=6.6 Hz); 0.787 (3H, s); 0.646 (3H, s). 3ß-Hydroperoxycholestane: ^{1}H-NMR (600 MHz, $CDCl_3$): 7.610 (1H, br s); 3.945 (1H, tt, J=11.3, 4.6 Hz); 0.900 (3H, d, J=6.6 Hz); 0.865 (3H, d, J=6.6 Hz); 0.861 (3H, d, J=6.6 Hz); 0.797 (3H, s); 0.650 (3H, s). ^{13}C-NMR (151 MHz, $CHCl_3$): 84.36, 56.49, 56.31, 54.31, 44.59, 42.61, 40.03, 39.52, 36.63, 36.18, 35.84, 35.80, 35.51, 32.60, 32.08, 28.83, 28.24, 28.01, 26.00, 24.22, 23.84, 22.80, 22.55, 21.26, 18.67, 12.22, 12.07.

SUPPORTING FIGURE 1

Activity of the HhC wild-type and D303A mutants toward 3HPC added at 50 µM, 25x the apparent K_M value of 3HPC with D303A. A) Gel analysis. B) Representative kinetic traces of mutant activity toward 3HPC analyzed using FRET assay. *inset*: apparent first order rate constants calculated from each trace.



REFERENCES

1. Owen, T. S.; Ngoje, G.; Lageman, T. J.; Bordeau, B. M.; Belfort, M.; Callahan, B. P. *Anal. Biochem*. **2015**, *488* (1-5).
2. Owen, T. S.; Xie, X. J.; Laraway, B.; Ngoje, G.; Wang, C.; Callahan, B. P. *ChemBioChem* **2015**, *16* (1), 55.
3. Schneider, C.A.; Rasband, W.S.; Eliceiri, K.W. *Nature Methods*, **2012**. *9* (7), 671-5.
4. Caglioti, L.; Gasparrini, F.; Misiti, D.; Palmieri, G. *Tetrahedron,* **1978**. *34 (1),* 135-139.