Supplementary Information for

Inhibitors of Hedgehog Acyltransferase Block Sonic Hedgehog Signaling

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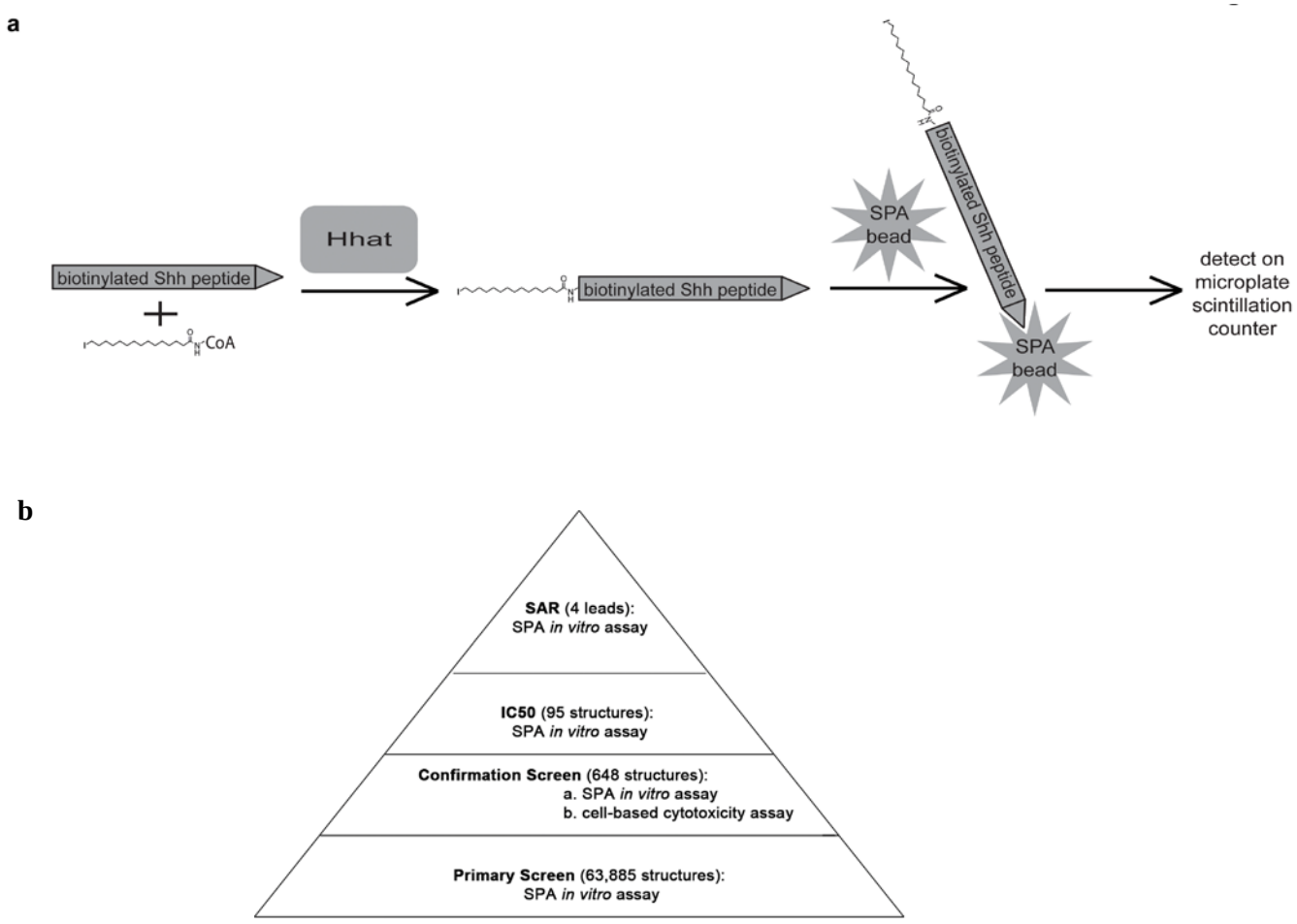
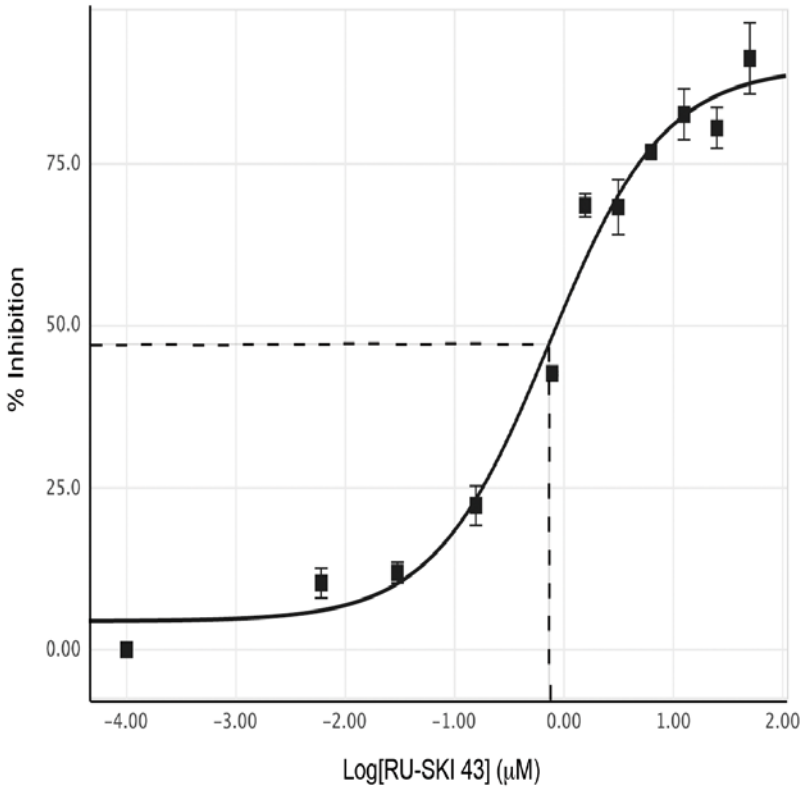
Supplementary Results

Category	Parameter	Description
Assay	Type of Assay	*In vitro* scintillation proximity assay measuring enzyme-dependent incorporation of ^{125}I-iodo-palmitate into a biotinylated substrate peptide
	Target	Hhat
	Primary measurement	Detection of photons by scintillation counting
	Key reagents	P100 membranes from Hhat-transfected cells, ^{125}I-iodo-palmitoylCoA, biotinylated Shh peptide, Streptavidin-coated SPA beads
	Assay protocol	See **Online Methods**
Library	Library size	63,885 unique structures
	Library composition	Known bioactives, drug-like molecules
	Source	See **Online Methods**
Screen	Format	384-well plates
	Concentration tested	12.5μM
	Plate controls	DMSO (high control), 0.125% TFA (low control)
	Reagent/compound dispensing system	Perkin-Elmer Janus nanohead dispenser for compounds; Thermo Multi-Drop, Matrix WellMate for enzyme and co-factors
	Detection instrument/software	Microbeta Trilux
	Assay validation	Z'=0.65, unlabeled peptide and palmitoyl-CoA tested as competitors, signal dependent on enzyme concentration and time
	Correction factors	None
	Normalization	Raw cpm data were converted to % inhibition relative to intra-plate low and high controls
	Additional comments	Screen performed at RU HTSRC, infrastructure described at www.rockefeller.edu/htsrc
Post-HTS analysis	Hit criteria	>60% inhibition, MW <425kDa, no violations of Lipinski's Rule of Five
	Hit rate	1%
	Additional assay(s)	Original *in vitro* assay, Alamar Blue-based cell viability assay, dose-response analysis using the *in vitro* assay
	Confirmation of hit purity and structure	Compounds were repurchased (AMRI, Albany, NY) and purity was verified by mass spectrometry
	Additional comments	See **Online Methods** for additional details on assays

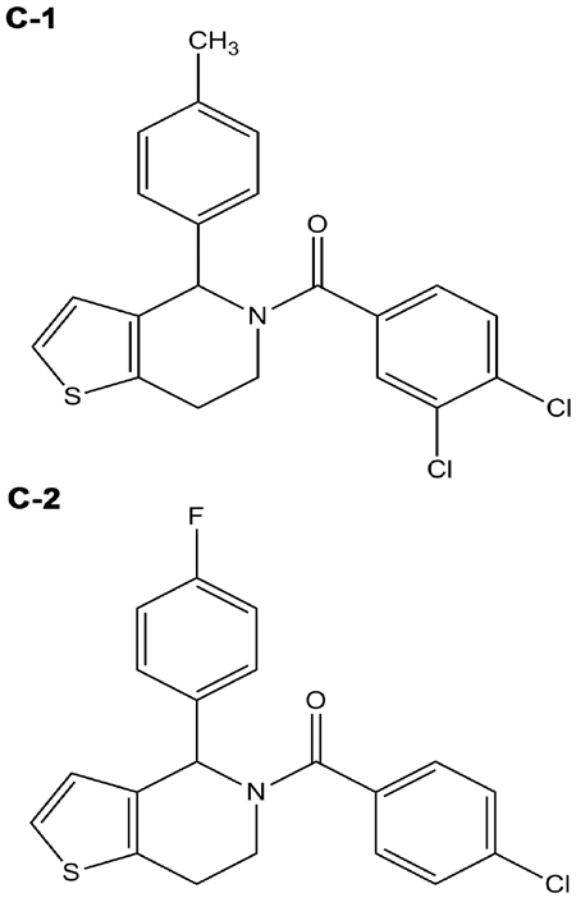
Supplementary Table 1. Small molecule screening data.



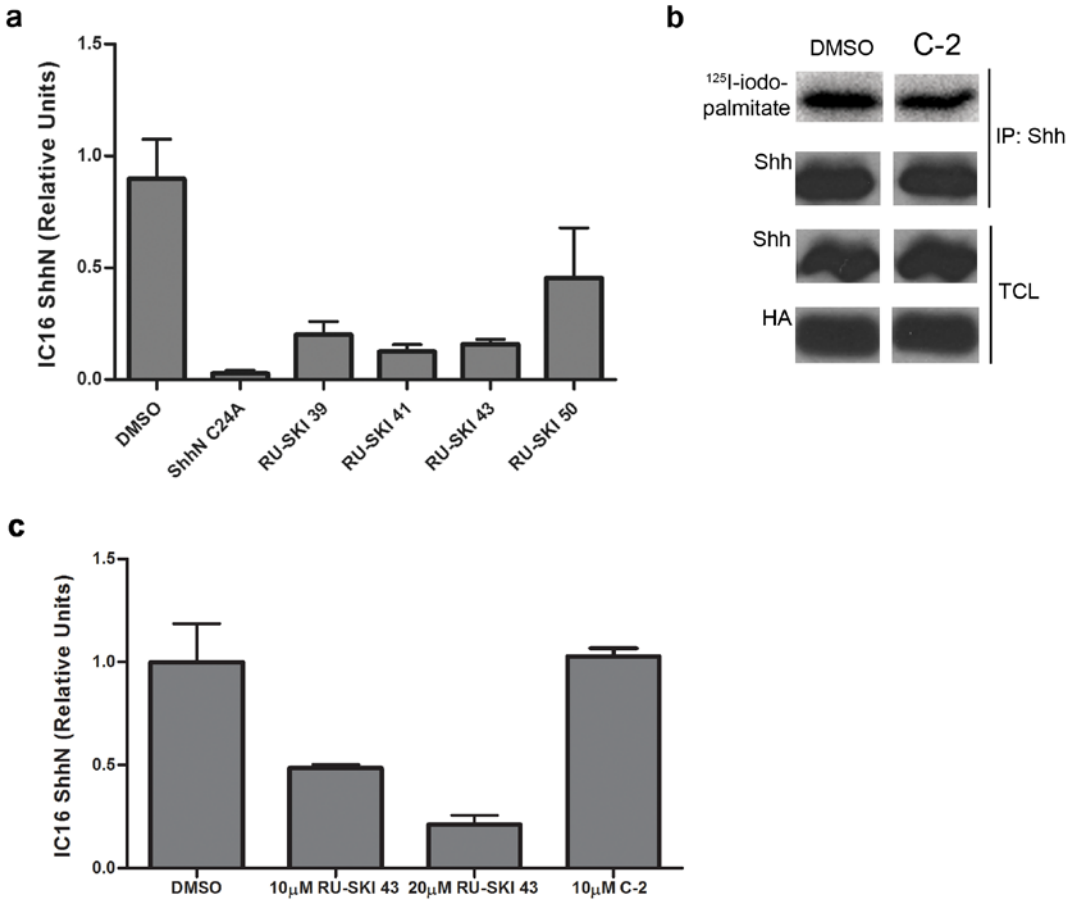
Supplementary Figure 1. High-throughput screen for Hhat inhibitors.
a) Graphical description of the Hhat assay scheme used in the screen. C-terminally biotinylated Shh peptide (3.21μM) was incubated with 5μM ^{125}I-iodo-palmitoylCoA and membranes from Hhat transfected cells. The peptide was pulled down on streptavidin-coated SPA beads, and radiolabel incorporation was detected by scintillation counting.
b) 63,885 unique structures were tested in the primary screen using the assay from a). 648 compounds were rescreened with the SPA-based assay and a cytotoxicity assay (see **Online Methods**). 95 compounds that displayed >60% inhibition in both assays were selected and IC50 values were determined. Based on the potency and chemical structure of the compounds, four hits, with the same core scaffold structure were selected.



Supplementary Figure 2. IC50 curve for RU-SKI 43. 11-point 2-fold serial dilutions in DMSO were prepared to yield final concentrations of RU-SKI 43 ranging from 12.5µM to 0µM. The SPA-based Hhat activity assay, described in **Online Methods**, was performed in duplicate, and the data were used to calculate an IC50 value for RU-SKI 43. Each point represents the Mean±SD.



Supplementary Figure 3. Structures of related compounds C-1 and C-2.



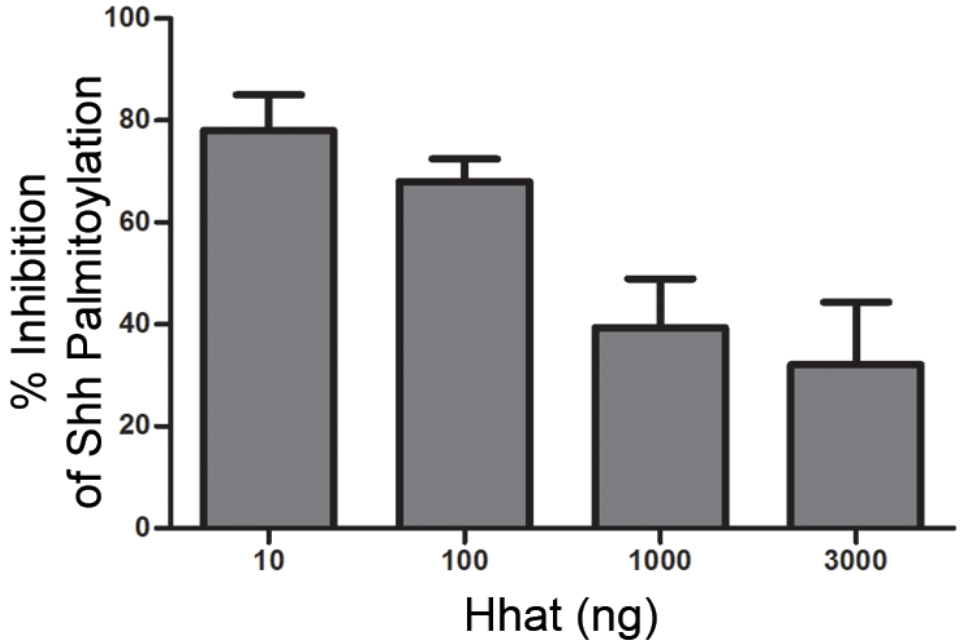
Supplementary Figure 4. Quantification of the effect of RU-SKIs on Shh palmitoylation in cells.

a) Quantification of the results shown in **Figure 1c**.

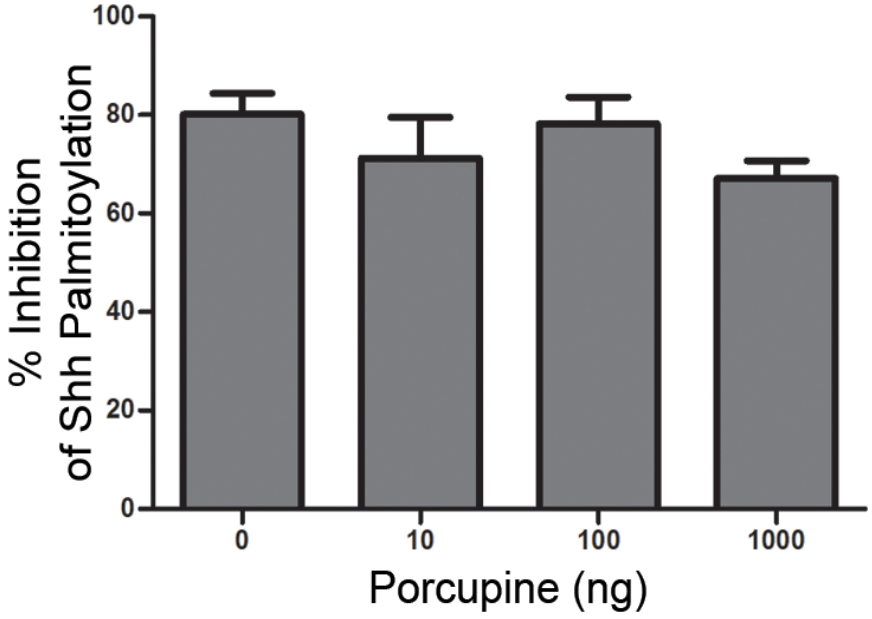
b) Compound C-2 does not inhibit Hhat. Cells were labeled in the presence of DMSO or 10μM compound C-2. Labeling, sample collection and data analysis were performed as described in **Figure 1d**.

c) Quantification of the results shown in **b)** and in **Figure 1d**. Radiolabel incorporation in each Shh band was quantified using ImageGauge software, as described in **Online Methods**, and normalized to samples treated with DMSO. Each bar represents the Mean ±SD of duplicates. Each experiment was repeated three times.

a



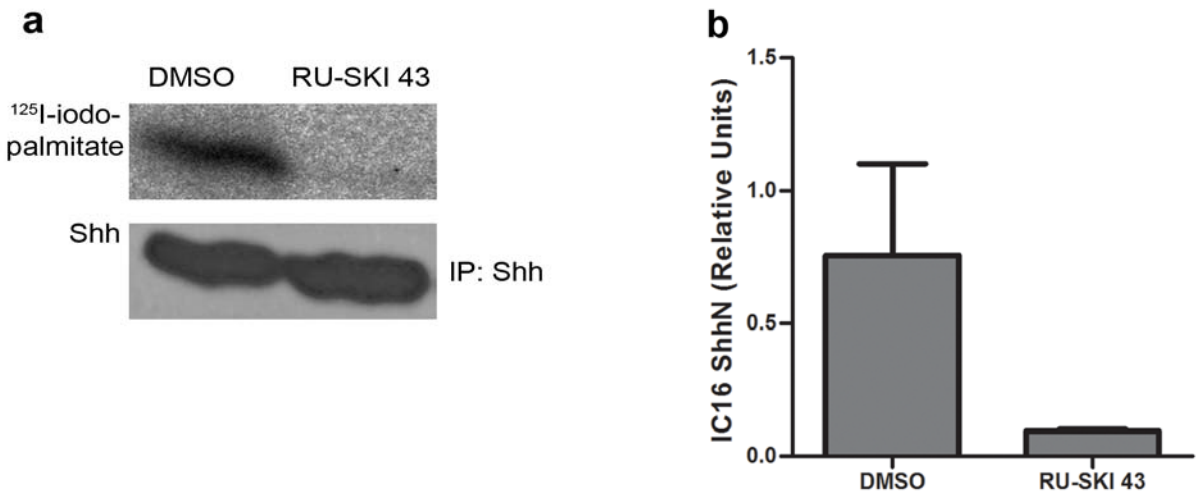
b



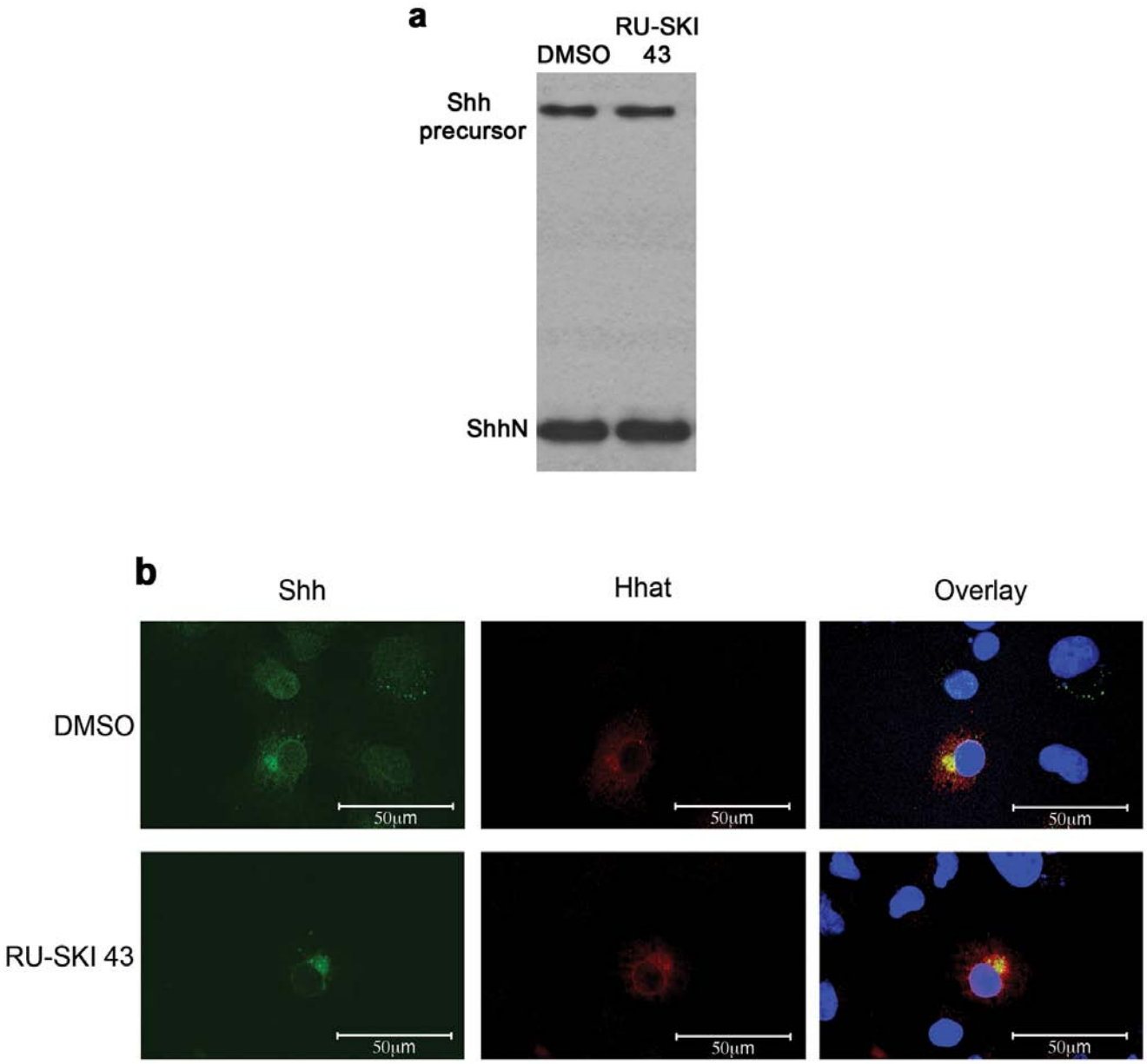
Supplementary Figure 5. Hhat, but not Porcupine overexpression can overcome the inhibitory effect of RU-SKI 43.

a) Hhat can overcome the inhibitory effect of RU-SKI 43. COS-1 cells were transfected with 500ng Shh and increasing amounts of Hhat. The cells were treated with DMSO or 10μM RU-SKI 43, and labeled. Radiolabel incorporation was normalized to samples treated with DMSO; (Mean ± SD, n=2-4).

b) Porcupine over-expression does not affect Hhat inhibition by RU-SKI 43. COS-1 cells were transfected with 500ng Shh, 10ng Hhat, and increasing amounts of Porcupine. The cells were treated and the data was analyzed as described in panel **a**. Each bar represents the Mean ±SD (n=2).



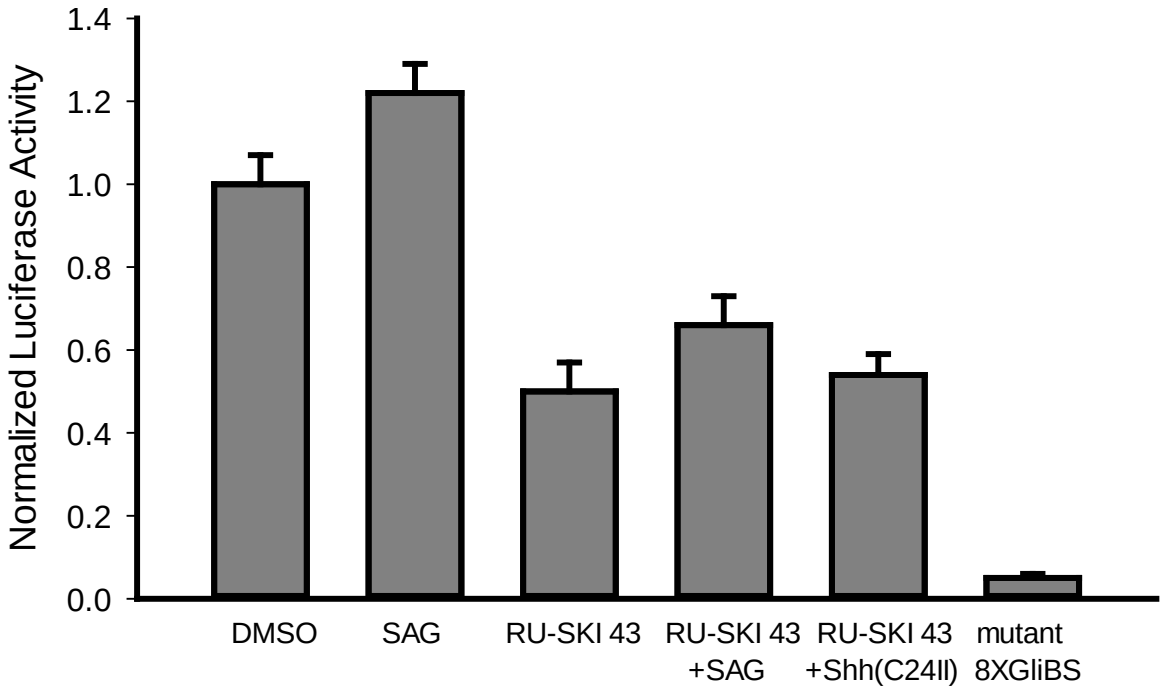
Supplementary Figure 6. RU-SKI 43 inhibits endogenous Hhat in COS-1 cells.
a) COS-1 cells were transfected with 500ng of a plasmid encoding Shh and labeled in the presence of DMSO or 10μM RU-SKI 43. Labeling, sample collection and data analysis were performed as described in **Figure 1c**.
b) Quantification of the results shown in **a)**. Radiolabel incorporation in each Shh band was quantified using ImageGauge software, as described in **Online Methods**. Radiolabel incorporation was normalized to samples treated with DMSO. Each bar represents the Mean ±SD of duplicates.



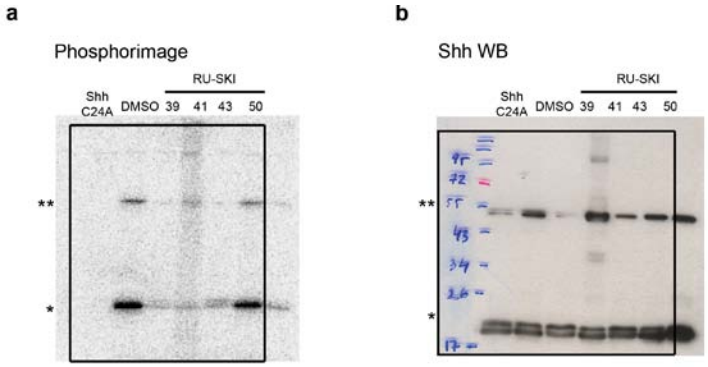
Supplementary Figure 7. RU-SKI 43 does not alter Shh autoprocessing or Shh and Hhat localization.

a) Shh processing is not affected by RU-SKI 43. 48h after transfection with Shh and HA-Hhat, COS-1 cells were treated with DMSO or 10µM RU-SKI 43 for 5h. Cells were lysed and Shh was immunoprecipitated from the samples. Samples were resolved on a 12.5% SDS-PAGE gel and analyzed by Western blot analysis.

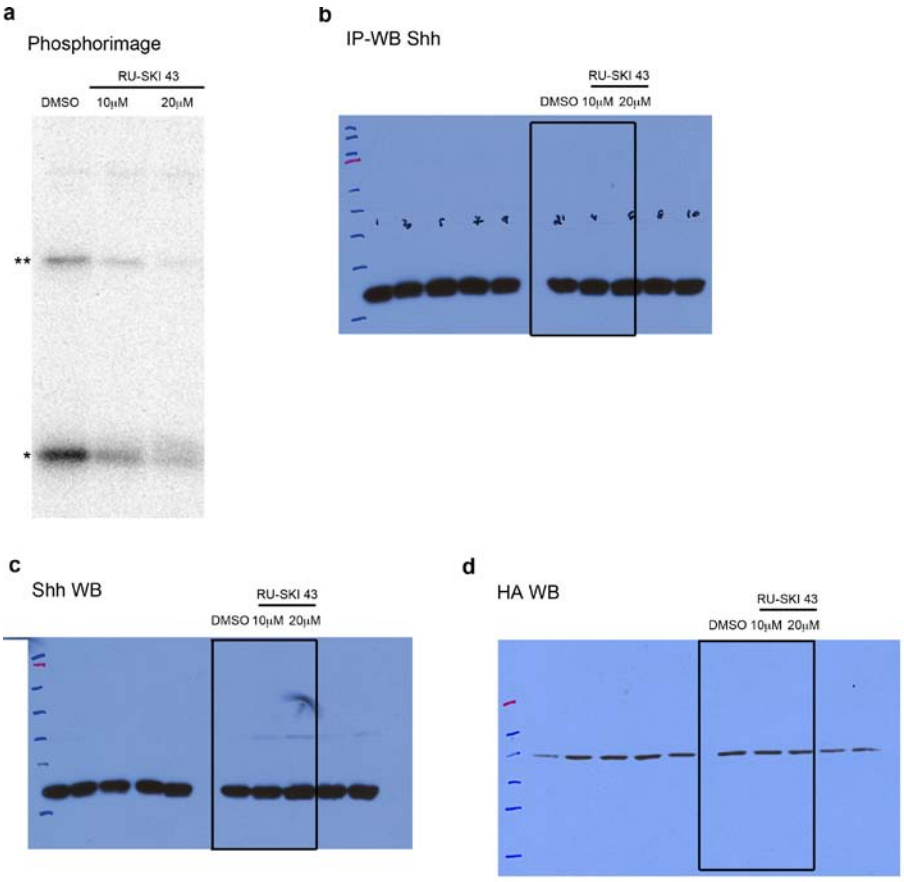
b) RU-SKI does not affect Shh and Hhat localization. 48h after transfection with Shh and HA-Hhat, COS-1 cells were treated with DMSO or 10µM RU-SKI 43 for 5h. The cells were then fixed and processed for indirect immunofluorescence.



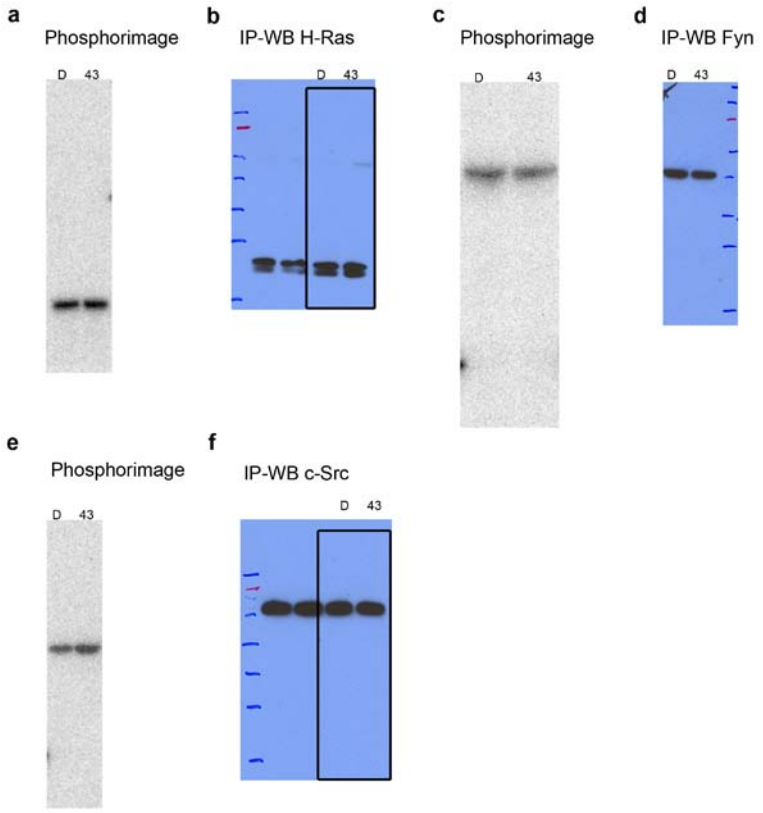
Supplementary Figure 8. Inhibition by RU-SKI 43 is not rescued in Shh-expressing NIH 3T3 cells. NIH 3T3 cells were co-transfected with vectors encoding 8XGliBS-Firefly luciferase (unless indicated otherwise), Renilla luciferase reporter (pRL-TK), and Shh. The following day the cells were split, and upon reaching confluence were treated with DMSO, 100nM SAG, 10µM RU-SKI 43, or 10µM RU-SKI 43 with either 100nM SAG or 1µg/mL Shh (C24II) for 24h. The firefly luciferase (FL)/renilla luciferase (RL) ratio in cell lysates was calculated for each sample, and was normalized to the DMSO-treated samples. Each bar is the average of triplicate readings and represents the Mean±SD (n=2-3).



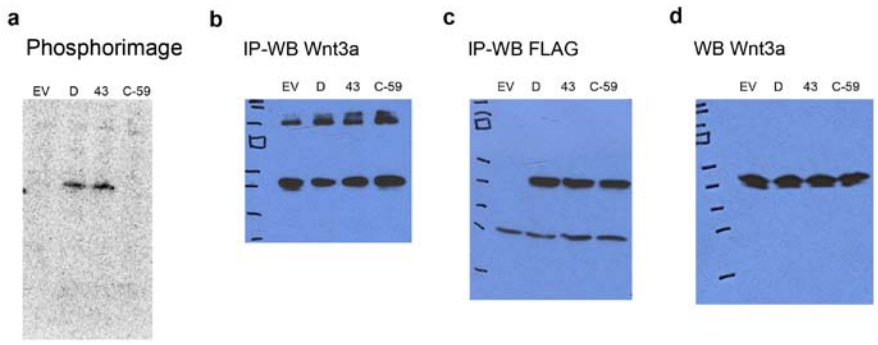
Supplementary Figure 9. Full uncropped gel images for Figure 1c.
a) Phosphorimage of Shh immunoprecipitate.
b) Shh Western blot.
* = ShhN 19 kDa product; ** = 45 kDa Shh precursor



Supplementary Figure 10. Full uncropped gel images for Figure 1d.
a) Phosphorimage of Shh immunoprecipitate.
b) Shh Western blot of of Shh immunoprecipitate.
c) Shh Western blot, total cell lysate.
d) HA Western blot, total cell lysate.



Supplementary Figure 11. Full uncropped gel images for Figure 1e.
a) Phosphorimage of H-Ras immunoprecipitate.
b) Western blot of H-Ras immunoprecipitate.
c) Phorphorimage of Fyn immunoprecipitate.
d) Western blot of Fyn immunoprecipitate.
e) Phosphorimage of c-Src immunoprecipitate.
f) Western blot of c-Src immunoprecipitate.



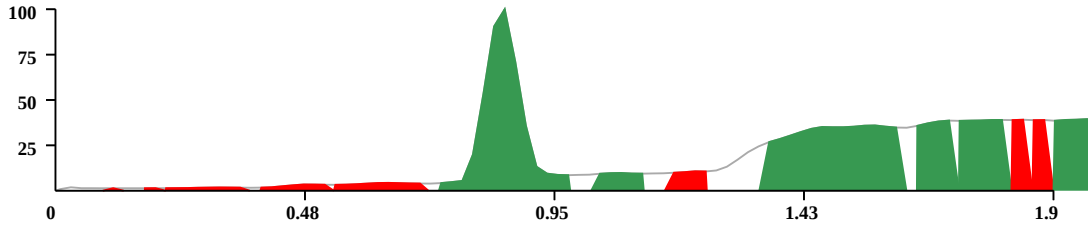
Supplementary Figure 12. Full uncropped gel images for Figure 1f.
a) Phosphorimage of Wnt3a immunoprecipitate.
b) Western blot of Wnt3a immunoprecipitate.
c) Western blot of FLAG immunoprecipitate.
d) Wnt3a Western blot, total cell lysate.

Supplementary Figure 13. MS Data for RU-SKI 43

Sample: 18 Date: 17-Dec-2010 Page 1.
File: Ar37018ac_08 Time: 08:40:57 AMRI code: ALB-H01959680
Vial: H/2 Description: 10749349 Vial label: B156L-087 Column: Acquity (Waters)BEH
C18, 1.7 micron50 x 2.1 mm Solvent: Aqueous 0.05% v/v Trifluoroacetic acid;Acetonitrile 0.05% v/v Trifluoroacetic acid;pH= 2

MS ES+ :TIC

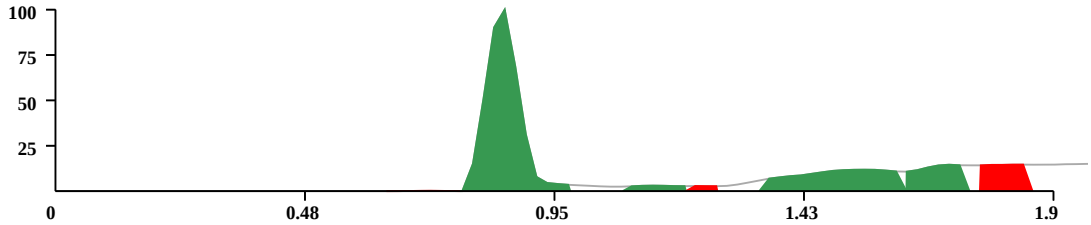
max. intensity: 1.2E7 mAu



Peak_ID	Peak	Area	Area%	Height	Time	Mass Found
1	0.09 0.13	4.E1	0	2.E3	0.11	
2	0.17 0.21	7.E1	0.01	4.E3	0.19	
3	0.21 0.37	2.E3	0.23	3.E4	0.31	
6	0.39 0.53	1.E4	1.12	1.E5	0.47	
8	0.53 0.71	1.E4	1.32	1.E5	0.63	
11	0.73 0.98	8.E5	82.99	1.E7	0.86	386.2
18	1.02 1.12	4.E3	0.44	7.E4	1.08	386.2
20	1.16 1.24	1.E3	0.13	4.E4	1.22	
25	1.34 1.62	1.E5	12.13	4.E5	1.56	386.2
26	1.64 1.72	6.E3	0.63	1.E5	1.70	386.2
27	1.72 1.82	4.E3	0.36	6.E4	1.80	386.2
28	1.82 1.86	2.E3	0.23	7.E4	1.84	
29	1.86 1.90	1.E3	0.14	5.E4	1.88	
31	1.90 2.00	3.E3	0.27	5.E4	1.96	386.2

MS ES+ :387.2

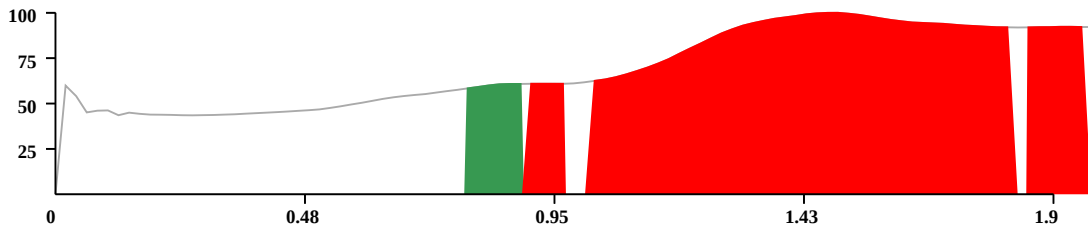
max. intensity: 8E6 mAu



Peak_ID	Peak	Area	Area%	Height	Time	Mass Found
9	0.63 0.77	8.E2	0.13	1.E4	0.71	
11	0.77 0.98	6.E5	91.74	8.E6	0.86	386.2
19	1.08 1.20	2.E3	0.36	4.E4	1.14	386.2
20	1.20 1.26	2.E2	0.03	8.E3	1.22	
24	1.34 1.62	4.E4	6.24	2.E5	1.54	386.2
26	1.62 1.74	8.E3	1.35	1.E5	1.70	386.2
28	1.76 1.86	9.E2	0.15	9.E3	1.84	

MS ES- :TIC

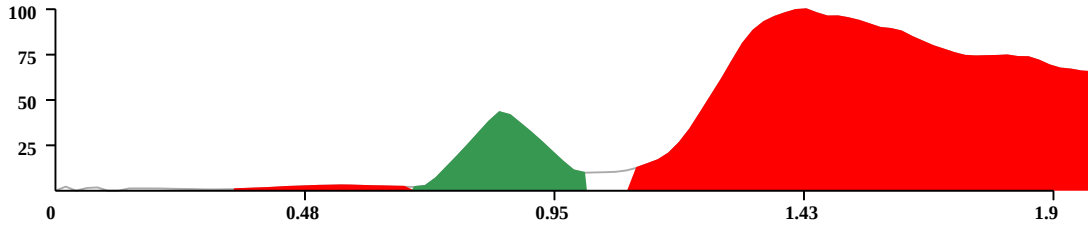
max. intensity: 1.7E7 mAu



Peak_ID	Peak	Area	Area%	Height	Time	Mass Found
12	0.78 0.89	8.E3	0.48	9.E4	0.87	386.2
14	0.89 0.97	6.E2	0.04	1.E4	0.93	
23	1.01 1.83	2.E6	99.35	4.E6	1.49	
30	1.85 1.97	2.E3	0.13	3.E4	1.93	

MS ES- :385.2

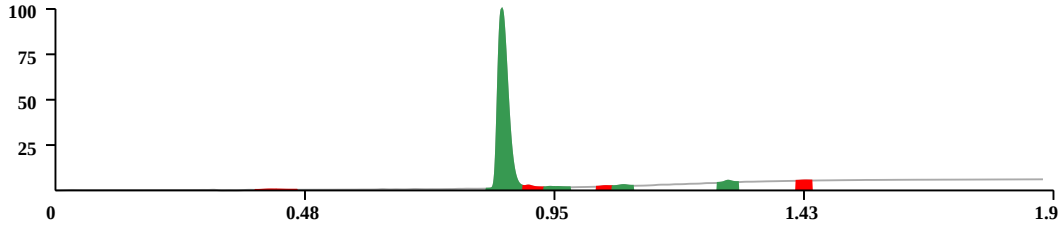
max. intensity: 1.3E5 mAu



Peak_ID	Peak	Area	Area%	Height	Time	Mass Found
7	0.34 0.68	3.E2	0.7	2.E3	0.54	
10	0.68 1.01	7.E3	15.26	5.E4	0.85	386.2
22	1.09 1.99	4.E4	84.04	9.E4	1.43	

DAD: 220

max. intensity: 1.3E6 mAu

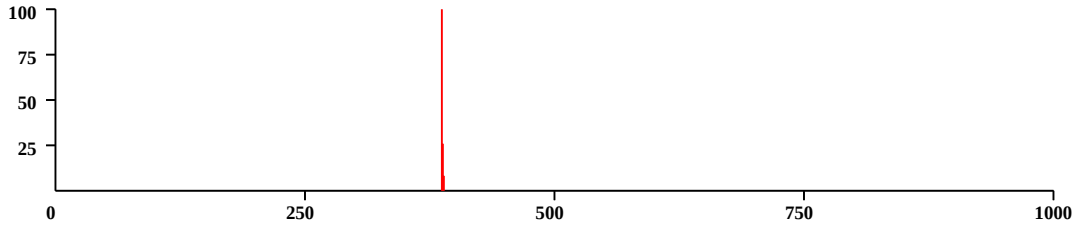


Peak_ID	Peak	Area	Area%	Height	Time	Mass Found
4	0.38 0.42	6.E1	0.21	3.E3	0.41	
5	0.42 0.46	4.E1	0.13	2.E3	0.42	
10	0.82 0.89	3.E4	97.26	1.E6	0.85	386.2
13	0.89 0.93	3.E2	0.91	2.E4	0.90	
15	0.93 0.95	5.E1	0.17	4.E3	0.94	386.2
16	0.95 0.98	3.E1	0.11	2.E3	0.96	386.2
17	1.03 1.06	3.E1	0.11	2.E3	1.05	
18	1.06 1.10	1.E2	0.36	6.E3	1.08	386.2
21	1.26 1.30	2.E2	0.65	1.E4	1.28	386.2
22	1.41 1.44	2.E1	0.08	1.E3	1.43	

ES+

Peak_ID	Time
10	0.85

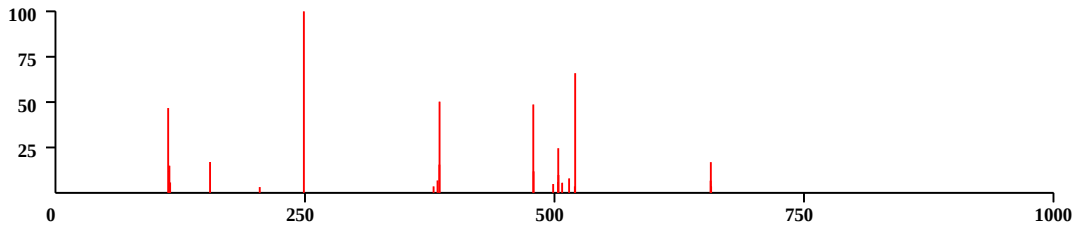
Combine (42:43-(38:39+47:48))



ES-

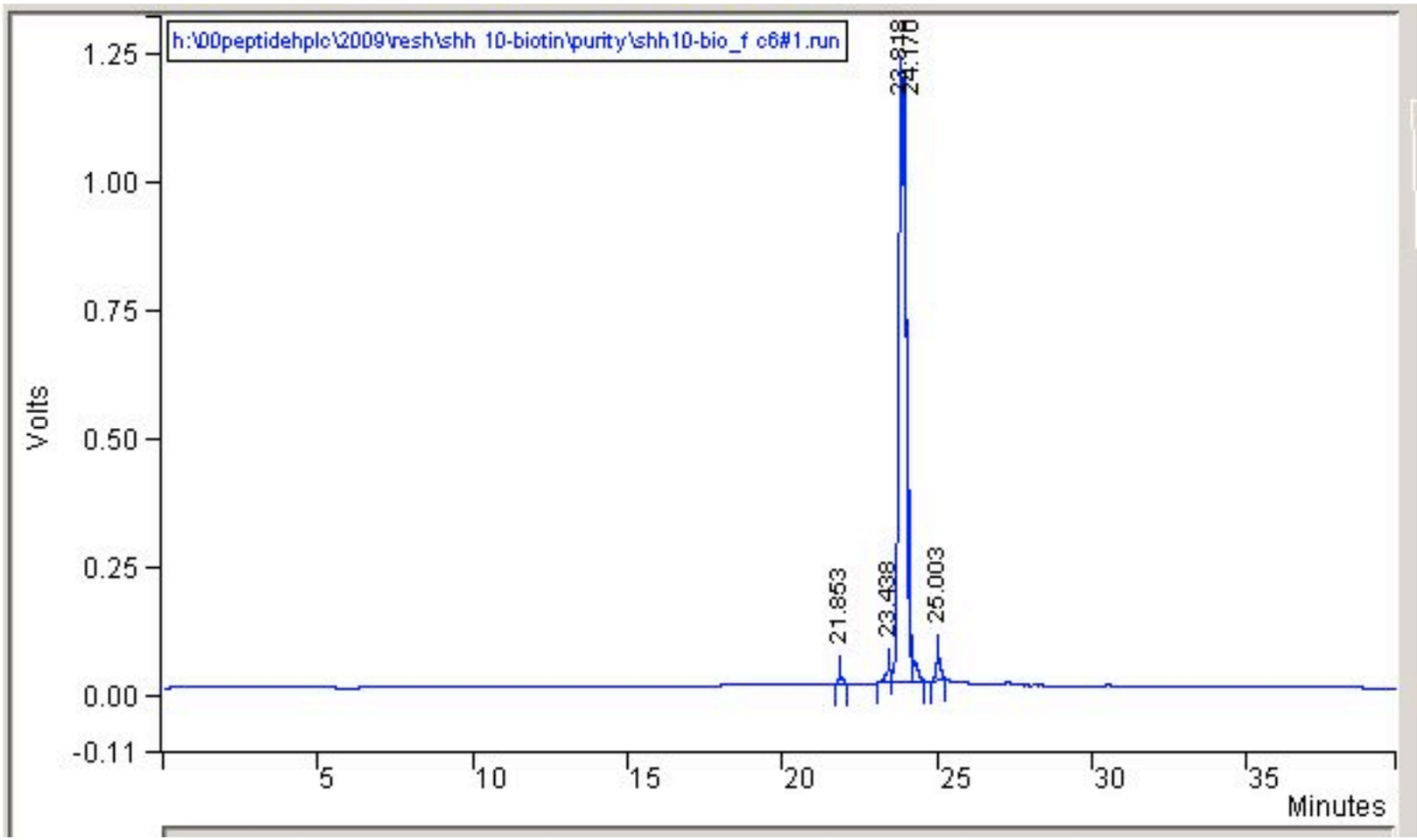
Peak_ID	Time
10	0.85

Combine (41:43-(37:39+46:48))



Supplementary Figure 14

Shh10-Biotin Peptide Reverse Phase HPLC



Supplementary Figure 15
Shh10-Biotin Peptide MALDI MS

H:\00pep mass\2009\Resh\Shh10-Biotin\pure\0_O1\2

Comment 1
Comment 2