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Method Article

Development and validation of a hedgehog heparin-binding assay for high-throughput screening

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A B S T R A C T

Sonic hedgehog (Shh) is a morphogenic protein with critical roles in embryogenesis and the development of some cancers. Hence, identifying inhibitors of the Shh pathway is of great therapeutic value. Heparin and HSPGs act as crucial modulators of Shh activity. To identify molecules that antagonize Shh binding to heparin we have developed a solid-phase plate-based assay. The N-terminal domain of Shh (ShhN) protein is first coated in 384-well plates and the direct binding of fluorescein-labeled heparin (flu-heparin) assessed by measuring the fluorescence signal after incubation and wash steps. Binding of ShhN protein to the 384-well plates was confirmed and optimized by a standard ELISA using a monoclonal antibody recognizing folded ShhN. The assay was validated using whole plate minimum and maximum signal wells with a Z' of 0.68–0.75 determined. Herein, we describe the development and validation of a high throughput screen to identify small molecule antagonists of Shh heparin binding.

Overall, this method

- Results in an optimized and validated assay for hedgehog heparin binding.
- Delivers a cost effective high-throughput screen format for hedgehog heparin antagonist screening

A R T I C L E I N F O

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Specifications table

Subject Area:	Pharmacology, Toxicology and Pharmaceutical Science
More specific subject area:	*High-throughput binding assay*
Method name:	*Hedgehog heparin HTS assay*
Name and reference of original method:	*NA*
Resource availability:	*NA*

Method details

Assay reagents and chemicals

Fluorescein conjugated heparin (flu-heparin, ~18 kDa) (cat. No. H-7482) was purchased from Life Technologies (Grand Island, NY.) Heparins (3, 5, 13 and 17 kDa) were purchased from Sigma Aldrich. The hybridoma expressing the anti-Shh 5E1 monoclonal antibody (mAb) originally developed by Thomas Jessell [1], was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology (Iowa City, IA, USA). The N-terminal domain of human Shh (ShhN, residues 24–197) was expressed and purified from *E.coli* essentially as previously described [2-4]. Thermo 384-well, flat-bottom, black, MaxiSorp plates (cat. no. 460518) were from Thermo Fisher Scientific. Unless otherwise stated all reagents and compounds were purchased from Thermo Fisher Scientific (Waltham, MA) or Sigma Aldrich (St. Louis, MO) at the highest level of purity possible. Coating buffer/read solution is 50 mM sodium bicarbonate pH 9.5. Binding buffer is 50 mM sodium phosphate pH 6.5, 150 mM NaCl.

Solid phase binding assay for shhN:heparin

We and others have previously demonstrated that Hh binds directly to heparin [2,4-6]. We have developed a 384-well automated solid-phase plate-based assay for identifying small molecule modulators of ShhN/heparin binding. In this assay format, ShhN protein is first coated in 384-well plates and the direct binding of fluorescein-labeled heparin (flu-heparin) assessed by measuring the fluorescence signal after incubation/wash steps. In this assay, compounds may be identified that potentially bind to ShhN or heparin to disrupt ShhN/heparin binding. To confirm first that ShhN protein coats the wells, we used the anti-Shh mAb 5E1 that recognizes folded ShhN protein [7]. Using a standard ELISA format, ShhN protein was incubated for 2 hr in the wells of a 384-well plate at varying protein concentrations in coating buffer at pH 9.5 and after washing, probed with purified 5E1 mAb at different dilutions (Fig. 1A). Bound Ab was detected using an AP-conjugated anti-mouse secondary Ab followed by AP substrate and reading absorbance at 405 nm. The ELISA data confirmed that ShhN did bind to the plate and also that a concentration of 1.25 µg/ml was sufficient to coat the assay plate wells (Fig. 1A).

Because flu-heparin was to be used to indicate relative binding to ShhN, any binding of the flu-heparin by itself to the assay plate would render the assay format unreliable. To assess non-specific binding of flu-heparin to the plates, wells were incubated with coating buffer alone or ShhN (20 µg/ml in 40 µL) and then flu-heparin in binding buffer added at varying concentrations. After the binding steps at pH 6.5, as the fluorescence intensity of fluorescein is dramatically reduced at pH 6.5 and is maximal at around pH 9, binding buffer was replaced by read solution to increase the pH to 9.5 for the fluorescence read. The results showed concentration-dependent increases in fluorescent intensity signal in those wells that were coated with ShhN, but no signal in the wells that received flu-heparin alone, indicating that background flu-heparin binding was not detectable up to 200 nM (Fig. 1B).

Using a broad concentration range, binding of flu-heparin to ShhN on the plates was determined to have EC_{50} values of 418 nM and 431 nM at 5 µg/ml (not shown) and 20 µg/ml ShhN (Fig. 2A) concentrations respectively. To assess the ability of molecules to block Shh/flu-heparin binding in this

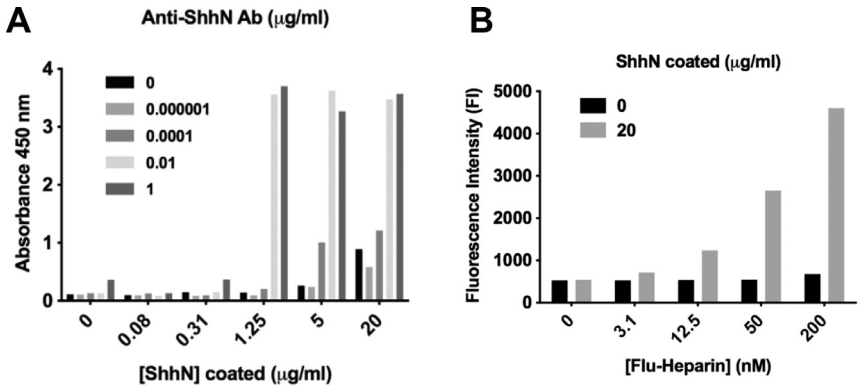


Fig. 1. Optimization of binding conditions for Shh heparin assay. A. Determination of optimal ShhN concentration required to fully coat well surface using ELISA assay format with purified α-ShhN mAb, 5E1. B. To test non-specific binding of flu-heparin to assay plate wells, flu-heparin was added to wells in the absence or presence of ShhN and fluorescence measured after 30 min.

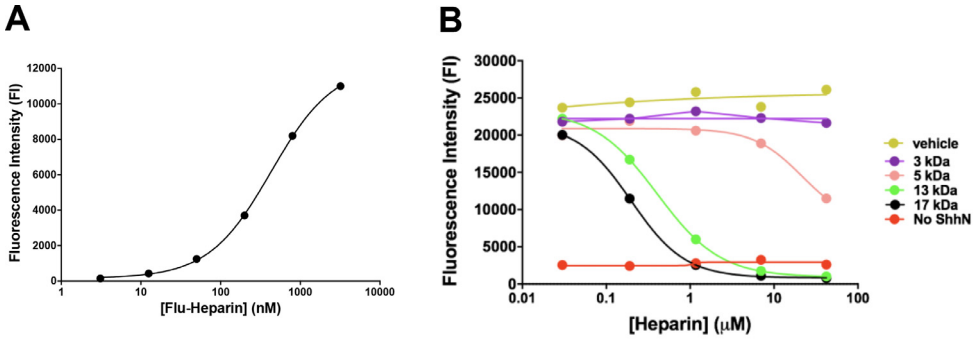


Fig. 2. Determination of concentration values for flu-heparin binding and unlabeled heparin inhibition. A. Binding curve for flu-heparin binding to pre-coated ShhN (20 μg/ml). Flu-heparin was serially diluted in binding buffer and then transferred to assay plates containing pre-coated ShhN. EC_{50} value was determined. B. Four unlabeled heparins (3, 5. 13 and 17 kDa) at six concentrations between 0 and 80 μM concentration were titrated into the ShhN/flu-heparin binding assay and IC_{50} values determined. EC_{50} and IC_{50} values were determined using GraphPad Prism 7 (GraphPad Software, San Diego, CA).

assay, unlabeled heparins were used as control inhibitors. Heparins of varying molecular weights (3, 5, 13 and 17 kDa) were titrated in the binding assay in the range of 0.03–42 μM. Increasing inhibition was observed for the 5, 13 and 17 kDa heparins with IC_{50} values determined as 0.36 μM and 0.18 μM for the 13 and 17 kDa heparins, respectively (Fig. 2B). A comparable effect of increasing heparin size on increased Hh binding was also observed by [8].

As the compounds to be screened were dissolved in DMSO, the tolerance of the binding assay to DMSO was assessed. No effect on ShhN/flu-heparin binding was observed in the presence of 1% DMSO (data not shown). Further, the addition of Tween-20 (0.02%) did not affect the binding characteristics of flu-heparin to the coated ShhN wells (data not shown). A time course was performed to test signal stability in which the same plate was read at 40 min, 75 min and 135 min following addition of the pH 9.5 read solution and no diminishment of signal was observed (data not shown). For HTS, plate additions were staggered every 5 min so that all incubation times were identical.

Table 1
HTS automation protocol for ShhN/heparin solid-phase binding assay.

Step	Event	Parameter	Description
1	Dispense	20 µL	Coat 384-well plates with ShhN in Coating Buffer using Nanoscreen
2	Incubate	2 hr	Room temperature
3	Wash	4 x	Wash with Binding Buffer using Biomek plate washer
4	Dispense	20 µL	20 µM compounds in Binding Buffer using Nanoscreen
5	Incubate	30 min	Room temperature
6	Dispense	20 µL	500 nM flu-heparin in Binding Buffer using Nanoscreen
7	Incubate	30 min	Room temperature
8	Wash	4 x	Wash with Binding Buffer using Biomek plate washer
9	Dispense	20 µL	Read solution
10	Incubate	60 min	Room temperature
11	Read	Ex_{485}/Em_{520}	Read fluorescence signal using Pherastar plate reader

Assay automation and validation

For HTS, a ShhN assay concentration of 10 µg/ml was chosen for coating the plates based on additional ELISA data (data not shown) which showed that 10 µg/ml ShhN provided an adequate assay window. The final automation protocol for HTS is shown in Table 1.

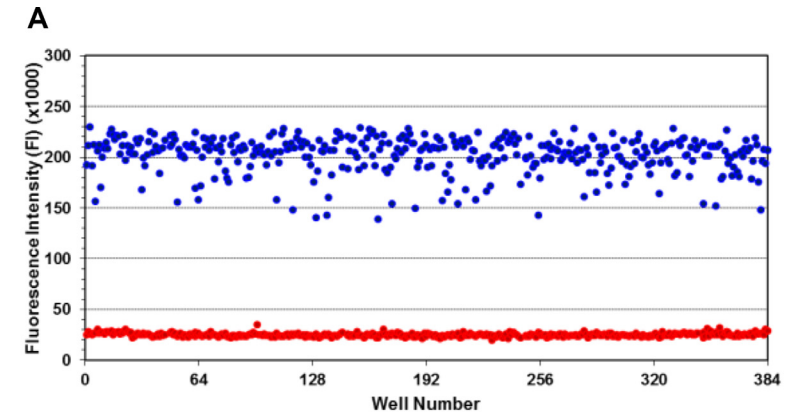
One critical detail to the assay is that immediately following the two wash steps, the plate must be given a good flick to fully empty wells, the plate top blotted dry, and the next addition started so that only 10–20 s elapses between final plate washer aspiration and addition of the next liquid. As configured, the assay can potentially identify compounds that bind ShhN or flu-heparin to disrupt binding.

To assess variability of the ShhN/heparin binding assay with full automation, duplicate 384-well DMSO plates in a typical "Min-Max" experiment [9,10] were analyzed to determine Z'-factor [11] as a measure of assay quality. Two assay plates were coated with ShhN at 10 µg/ml using the Nanoscreen NSX-1536 and following a 2 h incubation, washed to remove excess ShhN. One whole plate then received 25 µl of binding buffer alone (max signal). To generate the min signal plate, 10 µM (~20x IC_{50}) 13 kDa heparin was added to each well using the Nanoscreen. Plates were then processed as described in Table 1, data uploaded and analyzed using Screenable (ScreenAble Solutions, Chapel Hill, NC). A scatter plot for the validation data is shown in Fig. 3A. A Z'-factor was calculated as 0.69, with CV values $<$10% (Fig. 3B) demonstrating a suitable assay window and acceptable variability for HTS [10]. The validation assay was repeated for a second day with comparable results for Z' and CVs (data not shown). To assess signal stability over a typical ten 384-well plate run, duplicate plates were run as the first and last plates using the normal assay protocol. The data yielded calculated Z' for the first and last plate of 0.68 and 0.75, respectively, demonstrating the robustness of the assay over the time frame required to screen ten 384-well compound plates.

As a final validation, a small test set of diverse compounds was screened to determine the performance of the ShhN/heparin binding assay under HTS conditions in the presence of typical small molecule compounds [10]. In this pilot screen, the process and the plates with controls were set-up exactly as for the HTS screen. We used the Prestwick collection (Prestwick Chemical, Washington, DC), a commercial compound library made up of 1120 compounds with established biological activities, 90% of which are FDA-approved drugs. The Prestwick library was screened at single point 14.3 µM concentration in the automated ShhN/heparin assay and four compounds identified with percent inhibition values above a 50% threshold (Fig. 3C), indicating an acceptable hit rate of 0.35%. Compound ID, name and structures are shown in Table 2.

Significance

The development and validation of an automated high throughput screen for identifying antagonists of sonic hedgehog heparin binding should allow the identification of small molecule probes to aid in dissecting the various mechanistic roles of Shh.



B

	Min	Max
Average	24,641	202,053
SD	1,804	17,696
CV	7.3	8.8
Z'	0.69	

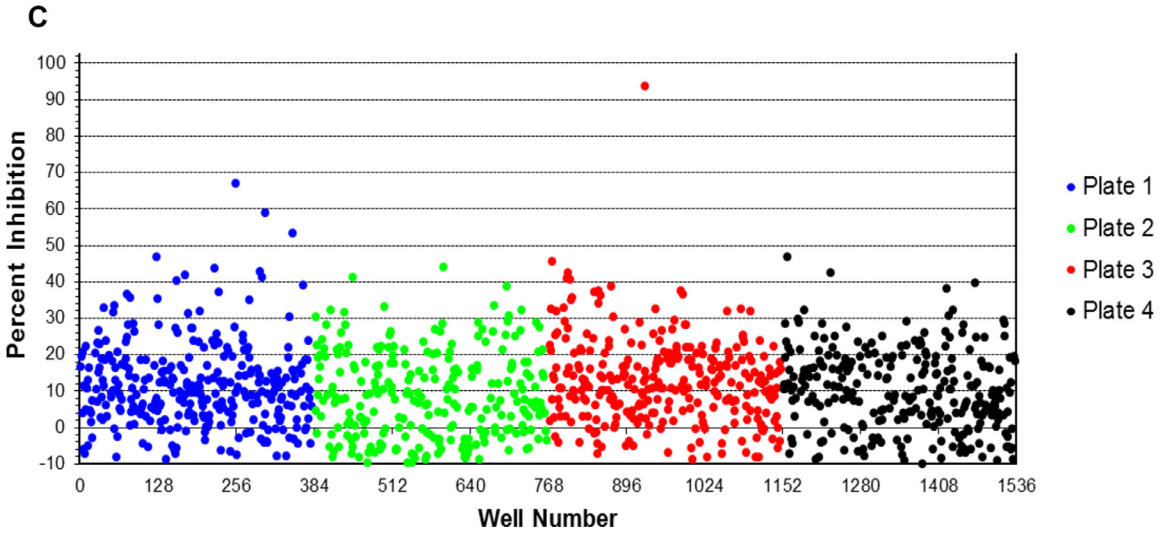
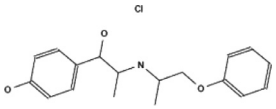
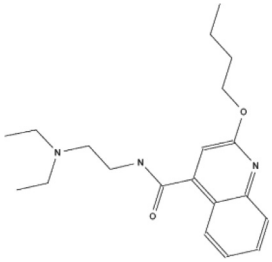
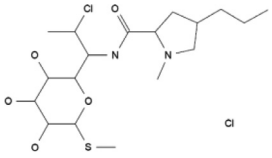
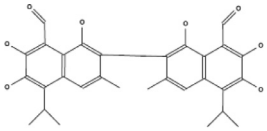


Fig. 3. ShhN heparin binding assay variability assessment and pilot screen validation. A. One plate each was used to determine the maximum signal (blue) and minimum signal (red). The Min and Max plates contained final assay concentration of 0.475 μM ShhN. For the min plate, heparin (13 kDa) in binding buffer was then added to 384-well plates using a Nanoscreen. After a 30 min incubation, flu-heparin was added. The data represent values measured in individual wells, consisting of 320 replicates for each condition. B. The variability for inhibition was determined from the max and min plates. Z'-factors, standard deviations (SD) and coefficient of variance (CV) were calculated in Screenable. C. Scatterplot showing a pilot high throughput screen of the Prestwick compound set screened at a final concentration of 14.3 μM. Each point represents the percent inhibition value for a single compound. Each 384-well plate is represented by a different color.

Table 2
Inhibitors of Hh Heparin binding identified from a pilot screen of the Prestwick set.

Compound ID[a]	Name	Structure	Inhibition in HTS
PWK-432,942	Isoxsuprine hydrochloride		59%
PWK-432,950	Dibucaine		53%
PWK-433,011	Clindamycin hydrochloride		67%
PWK-433,548	Gossypol		95%

[a] Prestwick chemical identifier number.

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Declaration of Competing Interest

The Authors confirm that there are no conflicts of interest.

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