Fragment library screening against the Hepatitis C drug target, NS5B 1b, in the search for novel allosteric drug leads

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Biacore™ 8K surface plasmon resonance (SPR) biosensor system was used to screen for fragments binding to NS5B, a viral polymerase that plays a fundamental role in the replication of Hepatitis C virus. The study was performed to prospect for novel allosteric drug leads targeting the thumb site II pocket of the NS5B 1b genotype. The target proved to be experimentally challenging, requiring effective assay development and careful handling. Loss of binding capacity of the protein surface during screening could be avoided thanks to the rapid analysis provided by Biacore 8K. The total run time for screening of 500 fragments in three analysis steps was 20 h. Sixteen hits with low millimolar affinities were identified. Six of these have later been designated as potential allosteric site binders based on competition with a reference ligand.

Introduction

Hepatitis C virus (HCV) causes acute and chronic infections worldwide (1). The intense effort to develop drugs against HCV has been successful, with several direct acting antivirals now being available for many patients. However, the drugs are not effective against all genotypic variants of the virus and resistance against drugs in clinical use will ultimately emerge. As for many other viral diseases (e.g., HIV), the search for new drugs will be a constant mission. Development of such next-generation drugs is facilitated by fragment-based drug discovery (FBDD). It has become a widely established approach to identify starting points for new lead compounds with other scaffolds or modes of action. FBDD focuses on evolving fragments, that is, small compounds with relative molecular mass (M_r) < 300, that typically bind with weak affinity to the target. Once target binding is confirmed, the fragment is expanded by growing, merging, or linking to produce a more optimal binder with higher affinity for the binding site. The fragment-based approach is particularly useful for exploring new or alternative binding sites, like allosteric binding sites. Biophysical assays are most
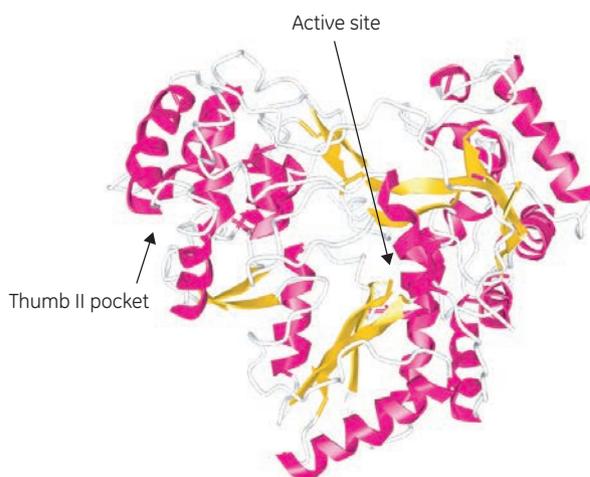
Fig 1. Potential binders for the allosteric thumb II pocket of NS5B 1b were screened to develop new leads that could complement current drugs to improve the efficacy of Hepatitis C virus therapy. (Illustration made in iCn3D using PDB: 1QUV).

commonly employed for fragment screening since they can provide the sensitivity needed to detect weak interactions and the robustness needed for a useful readout. Alongside NMR spectroscopy and X-ray crystallography, SPR biosensor technology belongs to the classical approaches for fragment screening (2).

We have been particularly interested in identifying novel compounds that can be developed into new leads that potentially complement current drugs to improve the efficacy of HCV therapy. We therefore used SPR to perform a fragment screen against NS5B, an RNA polymerase that plays a fundamental role in the replication of HCV. We selected genotype 1b, a globally prevalent variant of HCV that can be allosterically inhibited via one of at least four allosteric sites. Here we used the allosteric inhibitor filibuvir (Sigma) as positive control. It binds specifically to the allosteric thumb II site (Fig 1) and induces a conformational change, thereby indirectly reducing the catalytic activity of the enzyme (3).
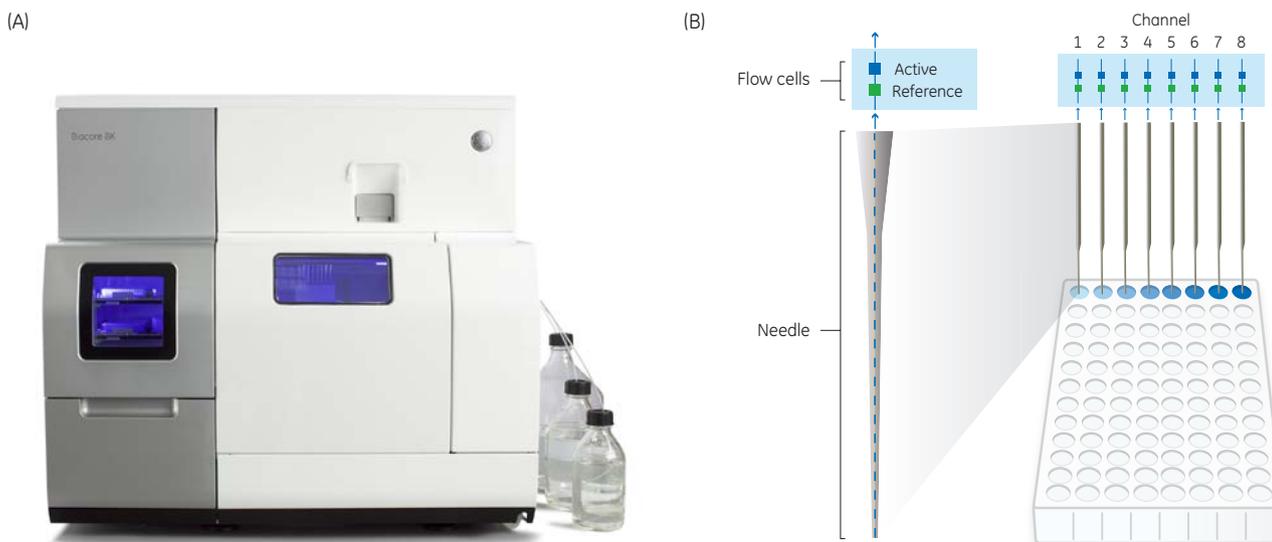
Fig 2. (A) Biacore 8K SPR system for screening and characterization. (B) The system features an eight-needle parallel setup with a microfluidic injection concept that enables each channel to provide high-quality reference-subtracted data. The simple 8 × 2 flow cell-setup makes planning, preparation, and operation straightforward and easy to understand.

This differs from the mode of action of sofosbuvir (4), the clinically used drug that targets the polymerase. Sofosbuvir is a nucleotide analog pro-drug that is activated in the liver in five steps, where after it competes with natural nucleotides thereby terminating RNA replication in the nascent viral genome. It thus acts as a nonobligate chain terminator. Specifically, we wished to identify fragments that potentially bind to the allosteric thumb II pocket site of NS5B 1b and that could be a realistic alternative to filibuvir as a complement to sofosbuvir.

Biacore 8K offers the sensitivity, robustness, and throughput needed for fragment screening, also against challenging targets such as the NS5B polymerase. Biacore 8K is a high-sensitivity, eight-channel parallel SPR system with two flow cells in each channel (Fig 2). The system is designed for screening and characterization of small molecules and discovery of biotherapeutics. It can host up to four microplates in the temperature-controlled sample and reagent compartment.

Materials and methods

Assay development and screening experiments were performed using a Biacore 8K system (GE Healthcare), based on previously published procedures (3). The target, recombinant his-tagged ectodomain NS5B of genotype 1b (M_r 64 000, obtained from Prof. Helena Danielson at Uppsala University), was immobilized using standard amine coupling conditions in the active flow cells (FC2) of all eight channels using a Series S Sensor Chip CM5 (GE Healthcare). The target protein was diluted in 10 mM sodium boric acid, pH 8.0, to obtain immobilization levels in the range of 9000 to 11 000 RU

for the screens. A new immobilization on a new sensor chip was performed prior to each screen, due to significant loss of binding capacity of the target following a day of screening.

A structurally diverse fragment library (Maybridge Ro3 library, Maybridge) consisting of 500 fragments was screened in three separate *Clean Screen*, *Binding Level Screen*, and *Affinity Screen* experiments for the purposes of library curation, identification of binders, and validation of binders, respectively (Table 1).

All experiments were carried out in 20 mM Tris pH 7.4, 130 mM NaCl, 10 mM $MgCl_2$, 0.05% Surfactant P20, 2% dimethyl sulfoxide (DMSO).

Filibuvir was used as positive control, allowing estimation of R_{max} and monitoring of surface binding capacity over time and between experiments. Filibuvir was injected at 250 nM using a 60 s contact time (injection/association time) and a 600 s dissociation time. DMSO, was used as a negative control, prepared in the same way as the fragments to better mimic sample pipetting errors as compared to using running buffer as blank/negative control.

Data was collected at 1 Hz at 30 µL/min with analysis and sample compartment temperature set to 25°C. Solvent correction using a four-point calibration curve was run three times in each of the *Binding Level Screen* and *Affinity Screen* runs. All screens included 10× start-up cycles using buffer only, which was needed to stabilize NS5B 1b.

Table 1. Overview of screening experiments

Experiment	Purpose	Inject type	Contact time (s)	Dissociation time (s)	Including controls, SC*, 50% DMSO wash	Fragment concentration (mM)
Clean Screen	Library curation	Fast injection	10	0	No	1.0
Binding Level Screen	Identification of binders	High performance	30	12	Yes	1.0
Affinity Screen	Validation of binders	High performance	30	30	Yes	0.013 to 1.0

*SC = Solvent correction

For data evaluation, response levels from *Binding Level Screen* and *Affinity Screen* were obtained from the binding early report point (taken 6 s after injection start) because most fragments bind very rapidly and the interaction reaches equilibrium at the start of the injection. The initial response is less sensitive to any potential secondary binding events occurring during the injection.

Results

Parallel immobilization maximizes throughput for sensitive targets

The target protein was immobilized in all eight channels to maximize the throughput of all screening experiments. The need for fast run times was important since it became clear during assay development that the stability of immobilized NS5B 1b limited the number of compounds that could be tested with the same surface. This was experimentally demonstrated by a reduced ability to interact with the positive control following 24 h of immobilization (data not shown). Thus, a new immobilization of NS5B 1b on a new Series S Sensor Chip CM5 was performed prior to each experimental run.

Identification and removal of sticky binders in 1 h

The library was prescreened against the target, immobilized at approximately 10 000 RU (active surface), and an unmodified CM5 surface (reference surface) to detect fragments with unsuitable binding behavior (i.e., fragments that adhere [stick] to the surface and interfere with subsequent injections). This *Clean Screen* was set up in a fast-run mode, without any controls or solvent corrections, using the injection type *Fast Injection* and with short contact times of 10 s and 0 s dissociation time. All 500 fragments were run for approximately 1 h at a concentration of 1 mM, the highest concentration planned to be used in subsequent analyses. *Clean Screen* resulted in the detection of 44 sticky fragments, identified as fragments that cause increased baseline shifts (Fig 3). Of these, 43 fragments were found to be sticky against the NS5B 1b surface solely. Only one fragment was found to be sticky against target protein and the reference surface. All 44 sticky fragments were omitted from subsequent screens.
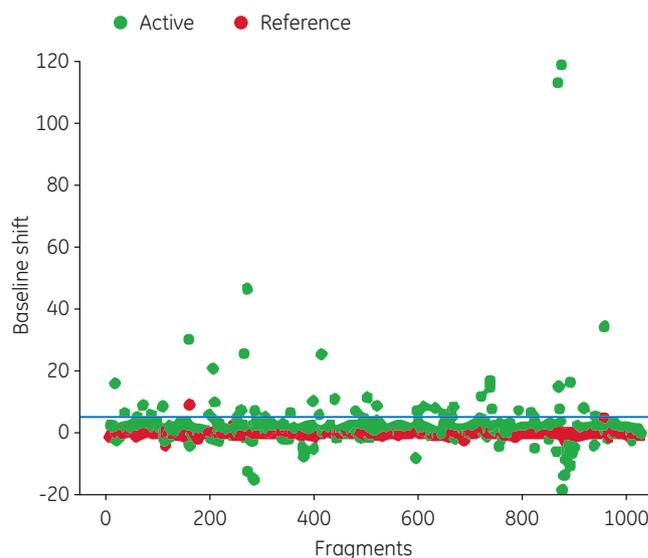
Fig 3. *Clean Screen* results plot showing fragments that cause baseline shifts of more than 5 RU (cutoff = 5 RU). Baseline shifts are defined as *Baseline absolute, (n + 1) - Baseline absolute, n*, where n is fragment cycle number. X-axis displays fragment order number. Data obtained from active flow cells (= amine-coupled NS5B 1b) is colored in green and data from reference surface (= unmodified Series S Sensor Chip CM5 surface) is colored in red.

Screening of 456 fragments for NS5B 1b binders in a working day

The curated fragment library was screened in a *Binding Level Screen* to identify fragments that bind specifically to NS5B 1b and to prioritize these binders for further characterization. In this experiment, fragments were analyzed at a single concentration of 1 mM and using both negative (DMSO) and positive (filibuvir) controls. The controls were prepared in the same way as the samples and distributed across the sample plates in frequent but irregular intervals to minimize the appearance of any systematic bias in the data set. Using the software functionality in Biacore 8K, additional negative controls were introduced into the fragment library plates by replacing sticky fragments with DMSO.
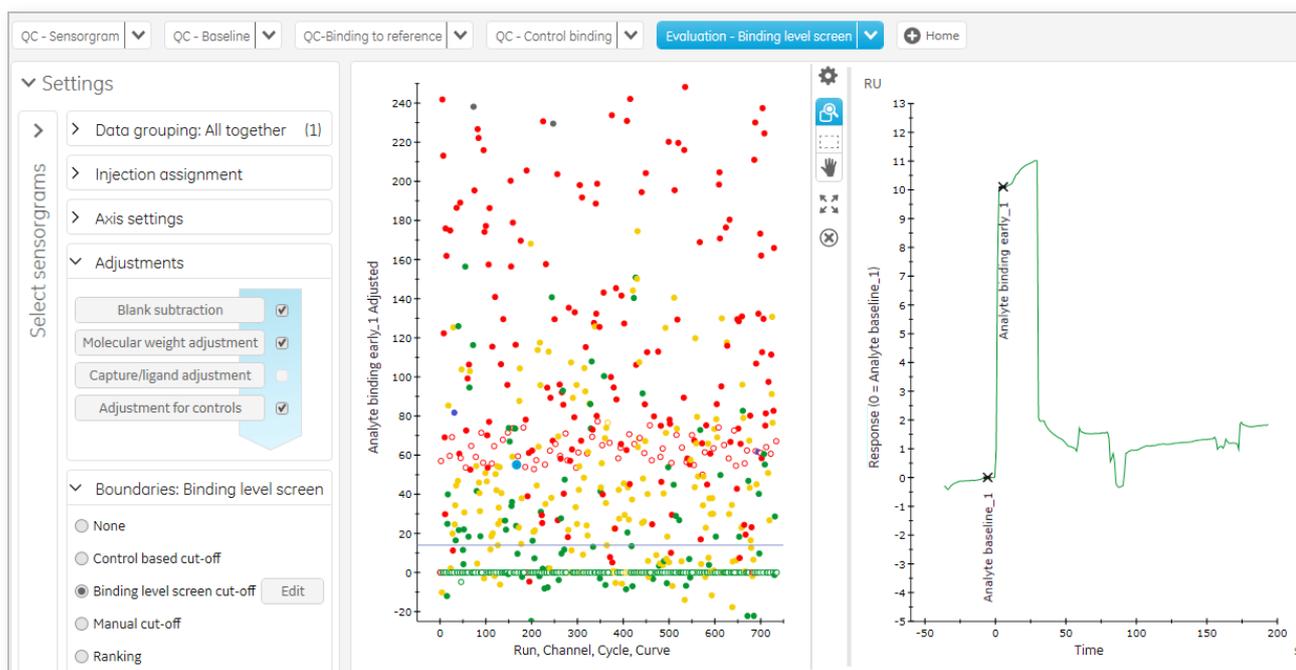
Fig 4. Evaluation of *Binding Level Screen* data using predefined evaluation methods. *Left panel:* Adjustments automatically performed when applying evaluation method to run file. *Middle panel:* Results plot presenting fragments above a predefined cutoff. For this screen, the cutoff was set to *10% of hits without binding behavior markers*, corresponding to 15 RU. Color-coding corresponds to binding behavior markers described in Figure 5. *Right panel:* Easily accessible display of selected fragments in the plot for closer inspection of detailed binding characteristics using the underlying sensorgram.
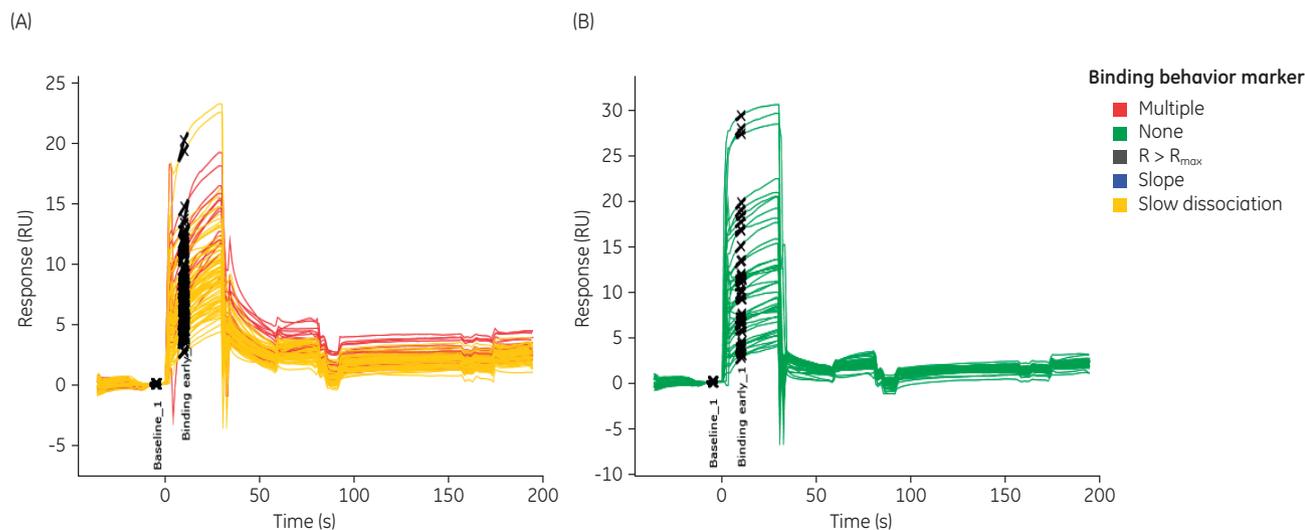
Fig 5. Illustration of the application of *Binding Level Screen* evaluation tools tailored to address challenges associated with fragment screening assays, such as secondary interactions and atypical binding behavior that are reflected in the sensorgram shape. Thus, the shape of the sensorgram is utilized to automatically identify fragments that do not bind in a well-behaved manner (A). $R > R_{max}$ indicates that the maximum response reached during sample injection is higher than expected for the ligand immobilization level. *Slope* indicates a significantly increasing response during sample injection instead of the normally expected rapid binding to a steady-state level. *Slow dissociation:* indicates that the compound does not dissociate immediately after the end of the sample injection. *Multiple* describes fragments with two or more of these binding behavior markers, for example *Slope* and *Slow dissociation*. Fragments above cutoff without binding behavior markers represent the screening hits ([B], denoted *None* in the key).

Binding Level Screen was performed in a single run in 8.5 h. Automatic data evaluation including blank subtraction, molecular weight adjustments, and adjustment for control identified many fragments with binding responses above the cutoff (Fig 4). Thus, NS5B 1b turned out to represent a target protein capable of interacting with many fragments. In this case, sensorgram shape analysis and subsequent color-coding make the Biacore 8K data analysis features particularly advantageous. Automatic tagging of fragments with complex binding behavior or secondary interactions made it possible to prioritize fragments hits (Fig 5). Thus, 48 fragments with well-behaved binding characteristics against NS5B 1b were selected for the following affinity analysis.

Efficient analysis of fragment concentration series using the sample hotel

The 48 fragments selected from the *Binding Level Screen*, were taken forward to steady-state analysis, *Affinity Screen*. Fragment concentration series, made by 1.5× serial dilutions from 1 mM, were performed in 96-well plates. Eight concentration series were prepared per plate. In addition to three sample plates (holding in total 24 fragment concentration series), a fourth plate with solvent correction and control solutions was included in each run, thereby utilizing the full four-plate capacity of the Biacore 8K sample hotel. Thus, the 48 fragments were validated by steady-state analysis in two runs. Filibuvir was used as both positive control, to monitor the binding capacity of the surface during the experiment, and to estimate the maximal fragment binding capacity of the surface for each channel (R_{max}). The molecular weight-adjusted predetermined R_{max} value was then applied during data evaluation for each fragment to improve the reliability of the steady-state analysis (Fig 6). Each *Affinity Screen* run was performed in 5 h. Following combined data analysis, again by utilizing a predefined evaluation method, sixteen fragments were identified. All sixteen displayed a good fit to the 1:1 binding model, revealing interfering secondary

(A) **Accepted**
Fragment 264; NS5B 1b; steady-state affinity (constant R_{max})

(B) **Rejected**
Fragment 304; NS5B 1b; steady-state affinity (constant R_{max})
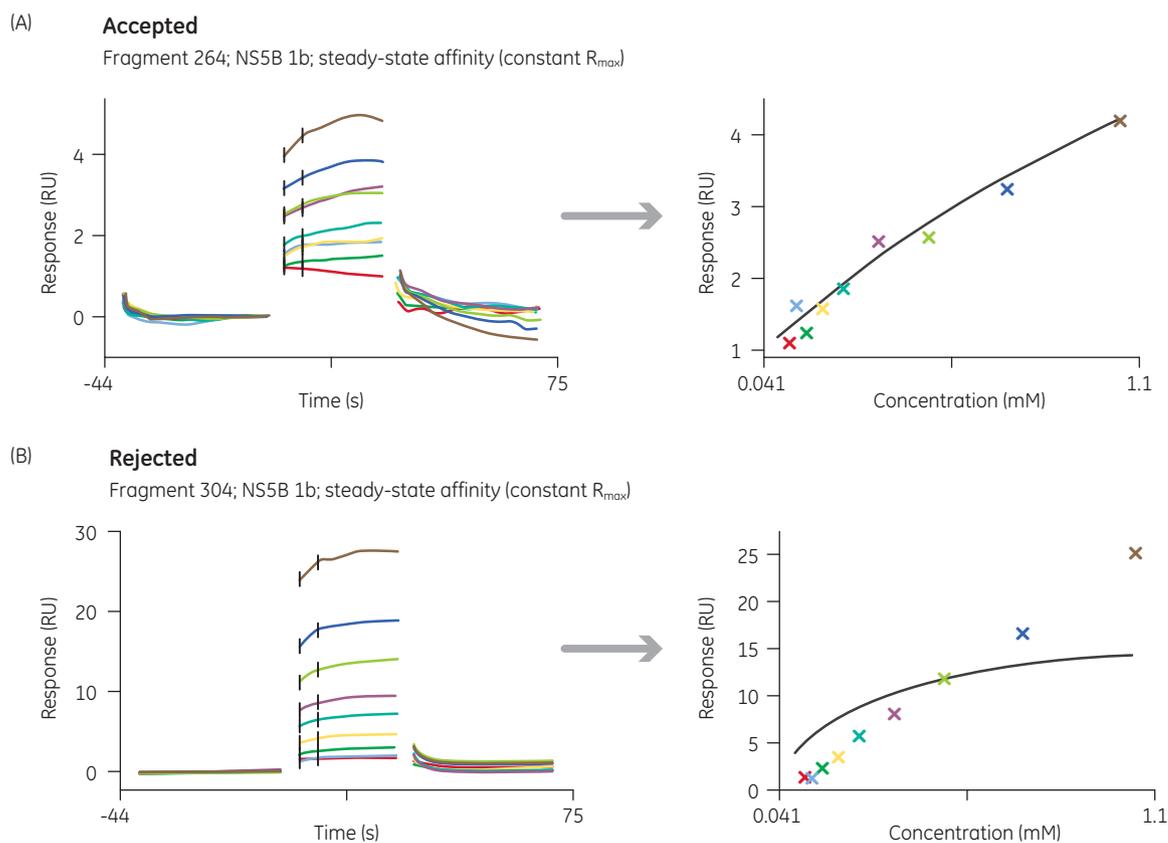
Fig 6. Improved analysis quality using curve fitting with predetermined (constant) R_{max}. This can be achieved when fragments bind weakly, with K_D in the millimolar range, and when steady-state analyses are performed at concentrations below the expected K_D due to solubility limitations. (A) Example of fragment with good fit following steady-state analysis using constant R_{max}. K_D = 2.2 mM. (B) Example of fragment with superstoichiometric data detected by poor fit with constant R_{max}.

interactions and with K_D-values ranging from 0.5 to 5.8 mM.

Conclusions

A complete fragment screen against NS5B 1b, an important Hepatitis C drug target, was performed using Biacore 8K. NS5B 1b proved to be a challenging target, requiring effective assay development and careful handling. The novel features of Biacore 8K enabled fast screening procedures and reduced risk of artifacts resulting from limited protein stability. The total run time for screening of 500 fragments in three steps (*Clean Screen, Binding Level Screen,* and *Affinity Screen*) was 20 h. The high sensitivity and automatic evaluation procedures enabled the identification of sixteen well-behaved fragments with affinities in the low millimolar range. Follow up experiments have demonstrated that six of these fragments compete with filibuvir for binding, indicating that they may be allosteric site binders. They are now the focus of Structure Activity Relationship (SAR) studies using hit analogs.

Acknowledgement

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Ordering information

Description	Product code
Biacore 8K System	29215379
Series S Sensor Chip CM5, pack of 3	BR100530
Amine Coupling Kit	BR100050
96-well Septa	29192561

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