Rapid Assay Development and Optimization for Small Molecule Drug Discovery

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Introduction

The drug discovery and development process requires assays amenable to high throughput, where large libraries of small molecules are screened to identify those that interact with high affinity to their protein target. Being able to perform this type of analysis with an assay that has a short development time is key. At later stages in the drug development process, the affinity of the small molecule lead compound is evaluated against human serum albumin and other relevant serum proteins to fully understand its absorption, distribution, metabolism, and excretion (ADME). The affinity and specificity of the lead compound must also be tested in several animal models in order to choose the most appropriate one for initial toxicity studies. High sensitivity and reproducibility for these binding assays, while maintaining throughput, is an absolute requirement in this process.

Surface plasmon resonance (SPR) can give detailed information on the binding affinity and kinetics of an interaction, without the need for a molecular tag or label. Labeling adds extra time and cost to assay development and can in some cases interfere with the molecular interaction by occluding a binding site (Cooper 2002). SPR technology can be used to design information-rich assays that provide a quantitative ranking of interaction affinities and the active concentration of protein ligand, which can be extremely valuable in the early stages of drug discovery (Huber and Mueller 2006). However, to be useful to the drug discovery process, interaction studies using SPR must be fast and cost effective to develop and be amenable to medium to high throughput.

The ProteOn™ XPR36 protein interaction array system and the One-shot Kinetics™ approach (Bravman et al. 2006) can provide the rapid assay development and high throughput required in a drug discovery environment. This multiplexed SPR device integrates a 6 x 6 interaction array for the analysis of up to six ligands with panels of up to six analytes, producing 36 data points in a single injection. Multiplexing enables several quantitative binding experiments using multiple conditions to be performed in parallel, so that robust interaction assays can be developed and optimized very quickly. This one-shot approach reduces assay costs and time and will generate a complete kinetic profile for a biomolecular interaction in a single experiment using a single sensor chip, without the need for regeneration.

The ProteOn XPR36 system has been used to rapidly screen and accurately characterize the affinity of small molecule drugs for human serum albumin (Bronner et al. 2008). It is also an effective tool for the rapid screening of monoclonal antibody supernatants to identify high-affinity candidates for potential drug development (Yousef 2007).

In this report, we demonstrate the application of the ProteOn XPR36 interaction array system and the One-shot Kinetics approach to the rapid development of a small molecule screening and characterization assay. The model system described in this study is composed of a putative lead compound (Y) and a protein domain of its target protein X (PDX). The inhibition of PDX is assumed to be efficacious in the treatment of cancer.

Methods

Instrument and Reagents

Experiments were performed using the ProteOn XPR36 system with ProteOn GLM sensor chips. ProteOn PBS/Tween running buffer (phosphate buffered saline, pH 7.4 with 0.005% Tween 20) containing 2% DMSO was used as running buffer throughout, and all experiments were performed at 25°C. The PDX (N-terminal polyhistidine-tagged protein construct of around 240 amino acids; MW 30.9 kD), the small molecule inhibitor, and the putative lead compound Y (439 Da) were obtained from Merck KGaA.

PDX Immobilization Conditions

The PDX was preincubated either in the presence or absence of 50 µM inhibitor and then immobilized in all six vertical channels. Immobilization was performed at three different pH values: 4.0, 4.5, and 5.0 in 10 mM acetate buffer.

Kinetic Binding Analysis

After deactivation, ten different concentrations of compound Y (625 to 1.22 nM in twofold dilutions) were injected five at a

time in the horizontal direction. Running buffer was injected in the sixth channel as a reference. Dissociation was monitored for 10 min. Regeneration of the ProteOn GLM sensor chip between the two injections was not required as Y had a relatively fast dissociation time from PDX.

Data Analysis

The data were analyzed using ProteOn Manager™ software, version 2.0. Binding curves were processed for baseline and start injection alignment, and interspot reference subtraction was used. Excluded volume correction was also performed because DMSO was present in the running buffer. Each set of six reference-subtracted sensorgrams was fitted globally to curves describing a homogeneous 1:1 biomolecular reaction model. Data from the six ligand surfaces were grouped together to fit the k_a, k_d, and R_{max} parameters. The equilibrium dissociation constant, K_D, was calculated using the equation $K_D = k_d/k_a$.

Results and Discussion

Optimization of PDX Binding to the Sensor Chip

Immobilization of PDX to the sensor chip surface was optimized by altering the binding buffer conditions. Three sodium acetate buffers with different pH values were tested. To prevent ligand inactivation as a result of crosslinking to the sensor chip at the binding site, the protein was preincubated with 50 µM inhibitor at each pH value tested. This way, six different immobilization conditions (three different pH values; with or without preincubation with the inhibitor) were tested in parallel on one sensor chip.

Figure 1 shows the immobilization levels for the six different conditions. The final immobilization levels were significantly higher for all three buffer types when the protein was preincubated with the protecting inhibitor molecule. However, the final immobilization level of PDX may be lower as the presence of the inhibitor seems to increase protein stability in the acidic environment. The highest immobilization level was obtained using the pH 4.5 buffer (~9,300 RU) although the two other buffers, pH 4.0 and 5.0, also yielded relatively high immobilization levels. All six binding curves in each vertical channel are superimposed, illustrating identical binding of the ligand across all six spots within the channels.

The activity of the bound PDX ligand was then determined for each immobilization condition. High activity levels are indicative of a more sensitive assay for the analyte, compound Y. The interaction of PDX with 10 different concentrations of compound Y was determined using the One-shot Kinetics approach. Two analyte injections were performed, each containing five channels with different concentrations of the analyte and one channel containing only running buffer

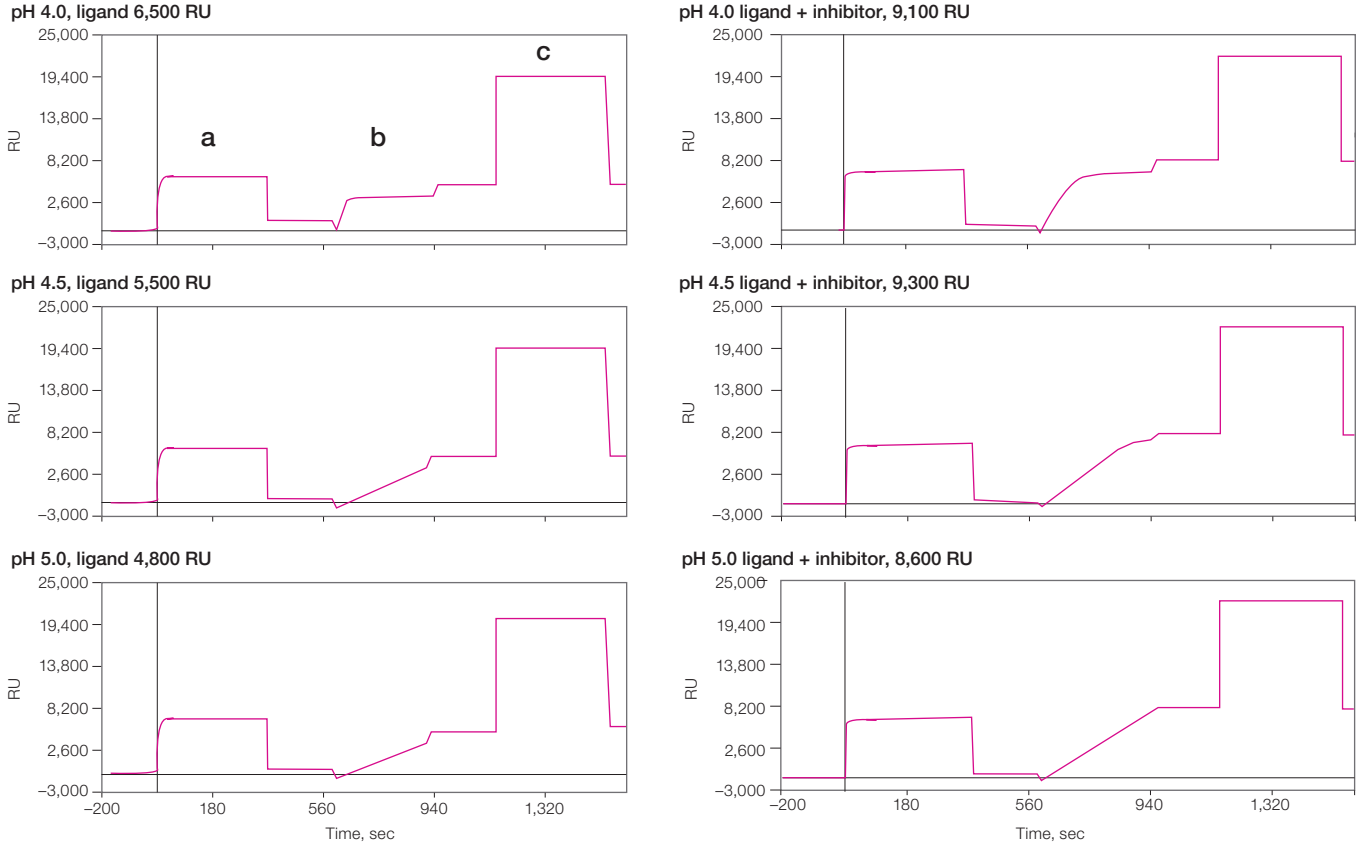

Fig. 1. Immobilization of the target protein PDX on the ProteOn GLM sensor chip. These six panels show the sensorgrams representing the six vertical channels on the sensor chip at a given pH and with or without the PDX inhibitor present. Each panel represents a different binding condition and contains data collected from the six interaction spots present within that channel. In this case, all six binding curves are superimposed due to identical binding of the PDX ligand across all spots within the channel. Areas a, b, and c in each graph represent successive injections of the EDAC/sulfo-NHS activator, the target protein, and the ethanolamine inactivator, respectively. The height of the sensorgram at the end of area c indicates the final amount of target protein PDX bound to the sensor chip.

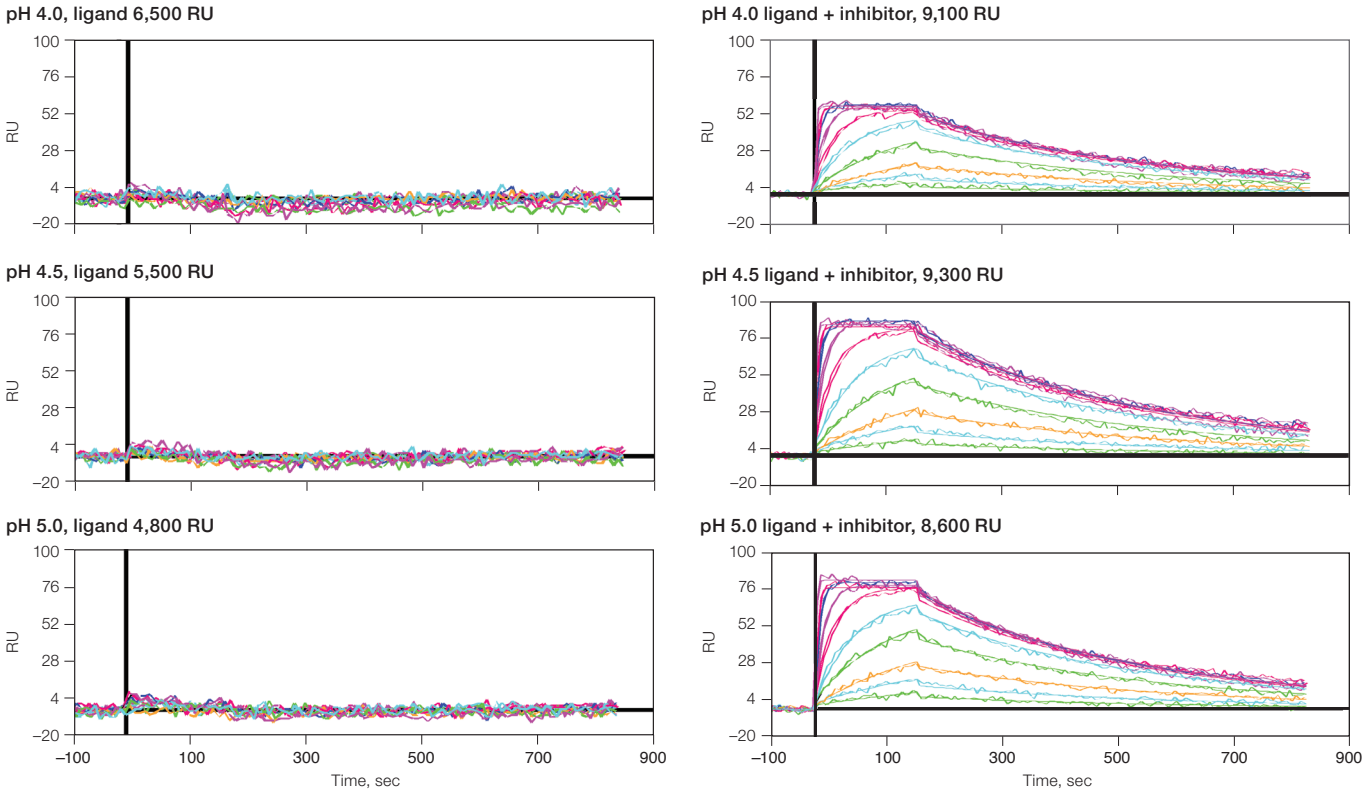

Fig. 2. Activity of the target protein PDX for the putative lead compound Y. PDX was immobilized in all six vertical channels, under six different binding conditions, using three acetate buffers of different pH (4.0, 4.5, and 5.0), and in the presence (left sensorgrams) or absence (right sensorgrams) of 50 µM inhibitor. A flow rate of 25 µl /min was used at 25°C for 5 min. Multiple concentrations of analyte were injected in the horizontal direction at 100 µl/min for 3 min. Dissociation was monitored for 10 min. In the three left sensorgrams, the calculated binding model is visible overlaid on the experimental data for each analyte concentration.

Table 1. Kinetic data* for the interaction of the target protein PDX with the putative lead compound Y.

	Grouped Fitting					Global Fitting				
	k_a, $M^{-1}sec^{-1}$	k_d, sec^{-1}	K_D	R_{max}, RU	Chi^2	k_a, $M^{-1}sec^{-1}$	k_d, sec^{-1}	K_D	R_{max}, RU	Chi^2
pH	Grouped	Grouped	Auto Defined	Grouped		Global	Global	Auto Defined	Grouped	
4.0	2.61×10^4	2.82×10^{-3}	1.08×10^{-7}	53.35	3.76	2.42×10^4	2.69×10^{-3}	1.12×10^{-7}	54.10	4.46
4.5**	2.31×10^4	2.69×10^{-3}	1.17×10^{-7}	75.88	5.42				75.37	
5.0**	2.65×10^4	2.89×10^{-3}	1.09×10^{-7}	73.30	3.73				72.40	

* Data were fitted to a 1:1 binding model using ProteOn Manager 2.0 software. For both methods, the R_{max} value was fitted as a grouped parameter, as it is specifically dependent on the level of immobilization.

** Interaction data at pH 4.5 and 5.0 were not determined for global fitting.

(no analyte), which is used for double referencing. The results shown in Figure 2 clearly demonstrate that PDX ligand immobilized without preincubation with the inhibitor is essentially inactive, regardless of the pH used during immobilization. However, when preincubation with the inhibitor was used, the protein maintained its activity regardless of the pH used during immobilization, suggesting that the binding site on the target protein was protected by the small molecule inhibitor.

Evaluation of the Kinetic Data

The kinetic binding constants data are summarized in Table 1. The experimental data and calculated binding model are almost superimposable, as shown in Figure 2, and all binding curves for the three active ligand surfaces could be fit to a homogeneous 1:1 binding model. The k_a and k_d could be fit globally or grouped, whereby each ligand surface has a separate k_a and k_d.

The kinetic values obtained from either method are almost identical, and when comparing data for the three different surfaces determined independently, there is also a close correlation.

The low chi^2 values obtained for each surface individually, shown in Table 1, are also an indicator of the confidence of the binding model generated in response to the experimental data collected (1 RU is the lowest theoretical chi^2 attainable, since it is at the noise level of the system). The chi^2 values were also very low when the data were fitted globally, giving increased confidence in the quality of the data, as it is an indicator that the data are robust and of the highest quality. They also indicate an absence of systematic errors from the instrumentation, reagents, or experimental design.

Conclusions

The ProteOn XPR36 protein interaction array system and the One-shot Kinetics approach are powerful tools for rapid, high-throughput assay development in the drug discovery environment. Assay development times are reduced from days to hours because up to 36 biomolecular interactions can be assayed simultaneously, yielding valuable kinetic, equilibrium, and concentration data. Multiplexed SPR analysis is rapidly becoming an indispensable asset across the drug discovery workflow.

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