High-Throughput Profiling of Kinase Inhibitors Selectivity Using the ProteOn™ XPR36 Protein Interaction Array System

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Introduction

Protein kinases are pharmaceutically one of the most important classes of proteins. They are involved in a number of essential cellular events such as cell division or cytokine signaling and provide a number of points where these cellular events may be controlled by inhibiting their activities. A number of kinase inhibitors such as Imatinib, Gefitinib, and Erlotinib, are now on the market and have revolutionized how cancer is treated (Janne 2009). Some protein kinases are also well positioned to prove themselves as effective for the treatment of chronic diseases such as rheumatoid arthritis or type II diabetes (Budhiraja and Singh 2008; West 2009).

More than 500 protein kinases are encoded in the human genome. They all share the ability to accommodate an ATP molecule in their active sites as a substrate (Manning et al. 2002). When developing kinase inhibitors it is essential to control their selectivity so that they interact only with specific targets and exert the desired pharmacological activities. The potency of protein kinase inhibitors is evaluated by a comparison of IC50 values measured in enzymatic assays. However, IC50 values are not a reliable characterization of the selectivity profile of a kinase inhibitor because the sensitivity of the assay differs depending on the assay conditions such as the concentration of ATP and the kinase. Comparison of equilibrium constants (K_D) of kinase inhibitors is a better metric to evaluate the selectivity of an inhibitor since it is unique at a given temperature. It is possible for inhibitors to have different IC50 values against two kinases while their K_D are comparable, indicating that the compounds are actually not selective.

Surface plasmon resonance (SPR) is a label-free technology that can be used for determining the K_D values for two interacting molecules. However, in the past this technology was hampered by throughput issues and lack of flexibility. Bio-Rad's ProteOn XPR36 system and its One-shot Kinetics™ approach is the perfect tool to generate robust K_D values to assess the selectivity profiles of kinase inhibitors with a single analyte injection. In this application note, we highlight data that show how researchers at Chugai Pharmaceuticals utilized the ProteOn XPR36 system and the One-shot Kinetics approach to screen kinase inhibitors against important kinase targets to generate K_D values. These K_D values were then used to rank the inhibitors for their activity against the targets. Also discussed is the quality of the assay data generated by the ProteOn XPR36 system as evaluated by the Z'-factor (Zhang et al. 1999).

Materials and Methods

All biosensor experiments were performed on the ProteOn XPR36 protein interaction array system (Bio-Rad Laboratories). Kinase inhibitors A and B were synthesized in-house and their chemical structures were confirmed by mass spectrometry (MS) and nuclear magnetic resonance (NMR). Staurosporine and other chemical substances were purchased from commercial sources. The ATPase was expressed in *E.coli* in its native form and GST fusion-tagged kinases (kinase A, B, C, D, and E) were expressed in Sf9 cells. The expressed proteins were purified to ≥95% purity using column chromatography, as verified by SDS-PAGE. The GST tag was cleaved during the purification process by proteolytic digestion using PreScission protease (GE Healthcare). The purified protein was then minimally biotinylated by incubating with sulfo-NHS-LC-LC biotin (Thermo Scientific) on ice for 2 hr at a molar ratio of protein/reagent of 0.8/1.

Ligand Immobilization

NeutrAvidin (Thermo Scientific) was immobilized to a high density on all six vertical channels of a ProteOn GLH sensor chip by the standard amine coupling method. Briefly, phosphate buffered saline with 0.005% Tween20 (Bio-Rad) was used as a running buffer and the temperature was set to 25°C. NeutrAvidin (200 µg/ml) was injected for 5 min at a flow rate of 30 µl/min over the surface after preactivation with a mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and 0.05 M N-hydroxysulfosuccinimide (sulfo-NHS). After the injection of the NeutrAvidin solution, the surface was deactivated with 150 µl of 1 M ethanolamine HCl, pH 8.5, giving an immobilization level for NeutrAvidin of approximately 20,000 RU.

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The six biotinylated proteins, one ATPase and five kinases were immobilized, one per vertical channel for 7 min at a flow rate of 30 μl/min. Concentrations of the protein solutions were 100, 35, 40, 20, 70, and 20 μg/ml for ATPase and Kinase A, B, C, D, and E, respectively. For Kinase B, C, and E, another 7-min injection was made to increase the immobilization level sufficiently to detect binding of small molecules. The final immobilization levels were 3,200, 1,700, 3,000, 2,900, 3,100, and 2,400 RU for ATPase, and Kinase A, B, C, D, and E, respectively. Running buffer was changed to TBS buffer (50 mM Tris-HCl, pH7.6, 150 mM NaCl, 1 mM dithiothreitol (DTT), 10 mM MgCl2, 0.005% Tween20) and the temperature was set to 15°C.

One-shot Kinetics Approach

Interaction analysis between the small molecule test compounds and the proteins was carried out using the One-shot Kinetics approach at 15°C. The following compounds were examined: ATP, ADP, staurosporine and two proprietary kinase inhibitors, inhibitor A and inhibitor B. For each test compound, a series of solutions at five different concentrations, and a blank sample for double-referencing, were prepared and injected simultaneously into the six horizontal analyte channels for a period of 1 minute at a flow rate of 100 μl/min. No regeneration was applied. The TBS buffer containing 1% (v/v) DMSO was used as a running buffer to facilitate dissolution of the test compounds. To calibrate responses between reference and ligand channels that are caused by a small difference in the DMSO concentration of analyte samples, six samples containing different concentrations of DMSO ranging from 0.5–1.5%, were also injected.

The obtained sensorgrams were processed and fitted to determine the dissociation constants using ProteOn Manager™ software (Bio-Rad). Interspot reference spots were used and double referencing was applied to all sensorgrams by subtracting the blank injection signal. The excluded volume correction analysis was also employed to calibrate bulk responses caused by DMSO. For ATP and ADP, for which fast association/dissociation to the immobilized proteins was observed, a steady state analysis was performed to derive the equilibrium constants. For the interactions of the inhibitors, the obtained sensorgrams were globally fitted to the 1:1 bimolecular binding model to calculate kinetic rate constants (k_a, k_d).

Calculation of the Z'-factor

To evaluate and validate the quality of the binding data, the Z'-factor was calculated according to the following equation:

$$Z' = 1 - \frac{(3\sigma_p + 3\sigma_n)}{\mu_p - \mu_n}$$

where σ is the standard deviation and μ the mean of the SPR signals. The subscripts p and n represent the positive

and the negative control, respectively (Zhang et al. 1999). As a positive control sample, a series of ADP solutions at six different concentrations including the blank solution was used. The solutions were injected into the six channels eight times consecutively for periods of 60 s at a flow rate of 100 μl/min at 15°C.

An assay system with a Z'-factor larger than 0.8 is considered an excellent assay. The Z'-factor should be ≥0.5 to deliver statistically meaningful data.

Results

Surface Preparation

It is essential to obtain active and stable surfaces of the captured protein of interest for the successful measurement of molecular interactions using SPR technology. In this study, we exploited the highly stable biotin-streptavidin coupling method for capturing proteins. It has been reported that kinases often lose their activity when amine coupled to a sensor chip due to the acidic conditions required during capture of the protein to create an efficient tethering. Biotin-streptavidin coupling is a milder method as the capture can be done at a neutral pH. The ATPase and five protein kinases were minimally biotinylated prior to capture and injected into the channels where a high density of NeutrAvidin was pre-immobilized by amine coupling on a GLH sensor chip. The biotinylated proteins were coupled at densities of 2,000 to 3,000 RU, which was sufficient for detecting the binding of these small molecules.

Confirmation of Surface Activity

To assess the activities of the six immobilized proteins, ADP and ATP were injected. Because the affinities of these two molecules to the tested proteins vary significantly, injections were made twice, one at a lower concentration range and the other at a higher concentration range. All the immobilized proteins except for Kinase C, showed binding to both ADP and ATP with fast association and dissociation, as is commonly observed for kinases (Figure 1). Equilibrium constants were determined by steady state analysis and are summarized in Table 1. Unexpectedly, neither ADP nor ATP binding was observed for Kinase C, but as described below, inhibitors such as staurosporine did show binding to this kinase. This would indicate that the captured protein was folded properly, but may have adopted an inactive form or required Mn^{2+} instead of Mg^{2+} for binding of ADP/ATP.

Table 1. Dissociation constants of ADP and ATP for ATPase, and Kinase A, B, C, D, and E.

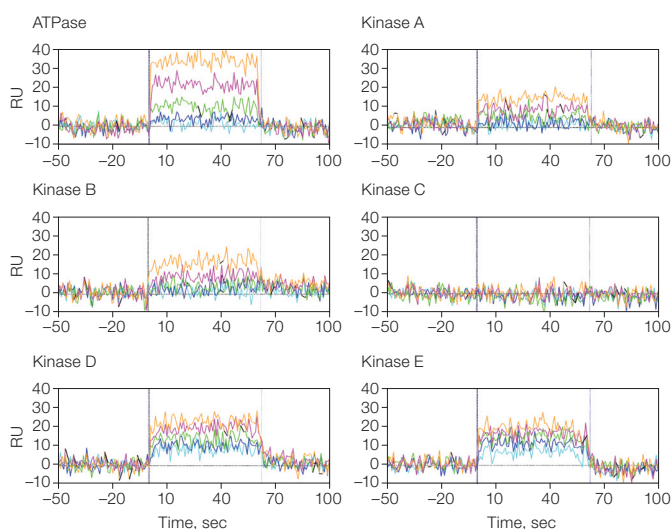| Protein	$K_{D, ADP}$, μM	$K_{D, ATP}$, μM
ATPase	8.2	1.5×10^2
Kinase A	59	1.3×10^2
Kinase B	85	2.1×10^2
Kinase C	—	—
Kinase D	9.5	9.1
Kinase E	45	9.3

A. ADP
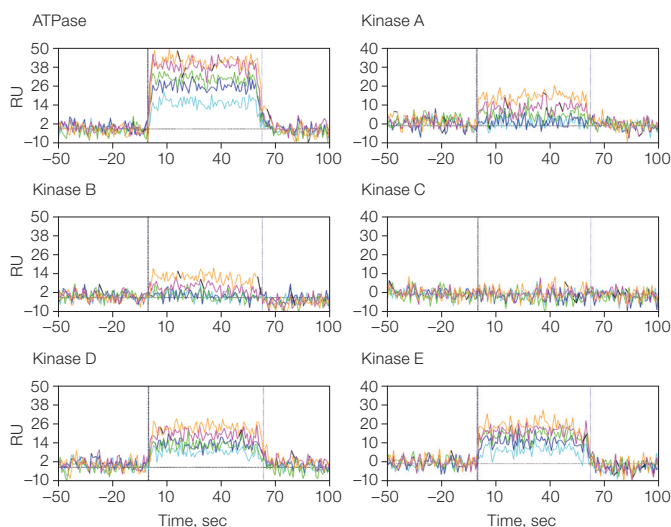
B. ATP

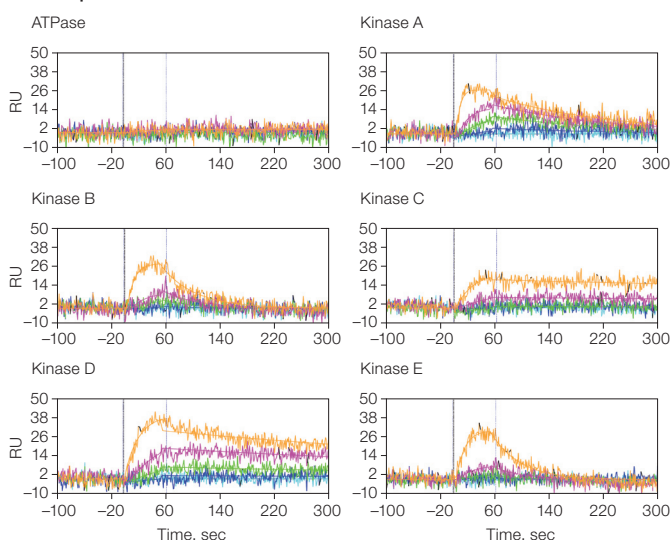**Fig. 1. ADP and ATP binding kinetics to the six different ligands.**
The concentrations of ADP and ATP are 400 (—), 133 (—), 44 (—), 15 (—), 4.9 (—) µM.

Kinase Panel Assay

Since stable and active protein surfaces were obtained, a kinase panel assay was conducted using the One-shot Kinetics approach. Three inhibitors were tested in this study: first, a well-known kinase inhibitor, staurosporine, was injected across all six surfaces at a range of 5 concentrations plus a buffer blank. As expected, staurosporine showed interaction with all five immobilized kinases and no binding to the ATPase (Figure 2). Interestingly, although staurosporine bound to all the five kinases, it is evident from the sensorgrams that the binding kinetics to these kinases were significantly different. Next, two inhibitors, inhibitor A and B, identified by Chugai's in-house kinase program, were tested. The results clearly indicated that inhibitor A was selective to Kinase B with a K_D of 8 nM, with some binding to Kinase C, K_D 34 nM. Inhibitor B was selective only to Kinase D with a K_D of 8.4 nM (Figures 3 and 4). For these two inhibitors, the

K_D results generated using the ProteOn XPR36 system were consistent with the in-house enzymatic kinase panel assay and therefore very useful for ensuring that the developed inhibitors possessed the desired selectivity profile.
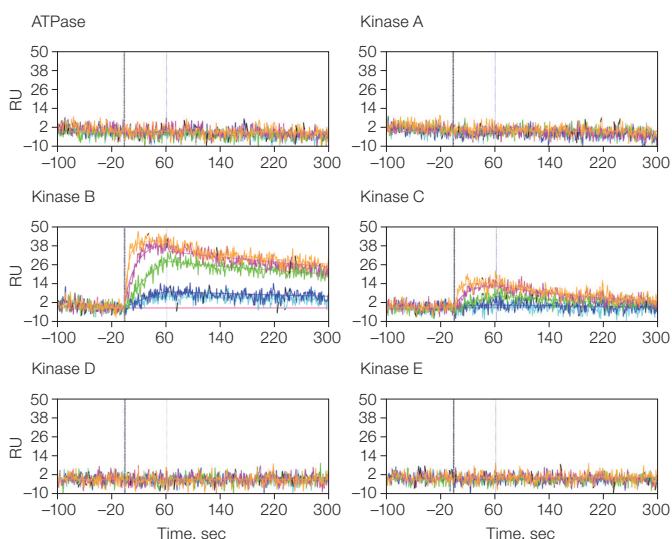
Staurosporine

	K_D, nM	k_a, M^{-1}s^{-1}	k_d, s^{-1}
Kinase A	8.3	6.9 x 10^5	5.7 x 10^{-3}
Kinase B	65	3.1 x 10^5	2.0 x 10^{-2}
Kinase C	4.0	1.6 x 10^5	6.3 x 10^{-4}
Kinase D	7.2	1.9 x 10^5	1.4 x 10^{-3}
Kinase E	100	2.3 x 10^5	3.2 x 10^{-3}

Fig. 2. Staurosporine binding kinetics to the six different ligands.
Staurosporine concentrations are 250 (—), 83 (—), 28 (—), 9.3 (—), 3.1 (—) µM.

Inhibitor A

	K_D, nM	k_a, M^{-1}s^{-1}	k_d, s^{-1}
Kinase B	8	1.75 x 10^5	1.4 x 10^{-3}
Kinase C	34	1.60 x 10^5	5.5 x 10^{-3}

Fig. 3. Inhibitor A binding kinetics to the six different ligands. Inhibitor A concentrations are 1,000 (—), 333 (—), 111 (—), 37 (—), 12 (—) µM.
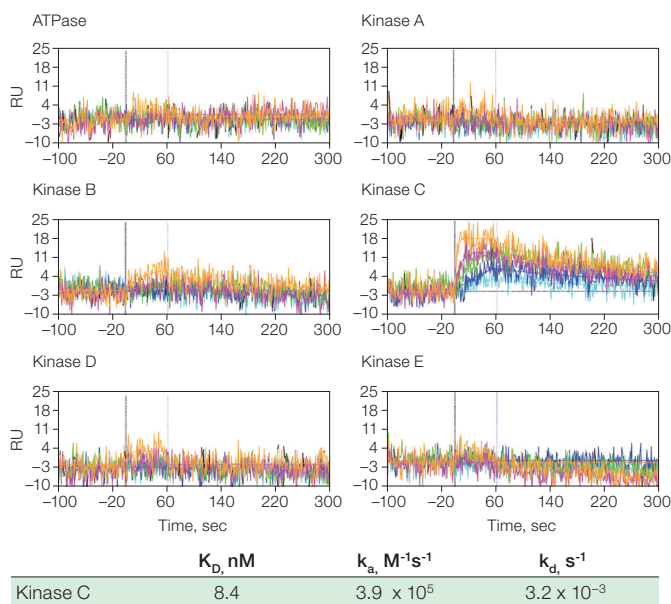
Inhibitor B

	K$_D$, nM	k$_a$, M^{-1}s^{-1}	k$_d$, s^{-1}
Kinase C	8.4	3.9 x 10^5	3.2 x 10^{-3}

Fig. 4. Inhibitor B binding kinetics to the six different ligands. Inhibitor B concentrations are 1,000 (—), 333 (—), 111 (—), 37 (—), 12 (—) µM.

Robustness of Binding Responses

The magnitude of the SPR signal arising from the binding of small molecules to captured proteins is small since it is proportional to the molecular weight of the analyte. Therefore, it is very important to know how much protein needs to be captured in order to obtain reliable and reproducible binding data. One way to estimate the required level is to calculate a so-called Z'-factor of the system. The Z'-factor was first introduced to validate robustness of the assay system for high-throughput screening and then for the SPR assay (Zhang et al. 1999; Perspicace et al. 2009). If the Z'-factor is larger than 0.5, the system is considered capable of delivering reliable data. To determine how much density is required to satisfy this condition, the Z'-factor must be determined for protein surfaces with different binding densities or alternatively for several analytes with different molecular weights. In this study, the Z'-factor for surfaces with different densities could be determined conveniently with the Bio-Rad ProteOn XPR36 system, since the capture levels were different in six channels, covering a range of values. Thus, one analyte, ADP, at five different concentrations was injected repeatedly eight times and the Z'-factors were calculated for each channel (Figure 5). The sensorgrams of the eight injections overlaid nicely, indicating superb reproducibility of the binding responses. From the calculated Z'-factor for different binding densities, it was estimated that an R$_{max}$ of as little as 17 RU would correspond to the Z'-factor of 0.5. The value for the
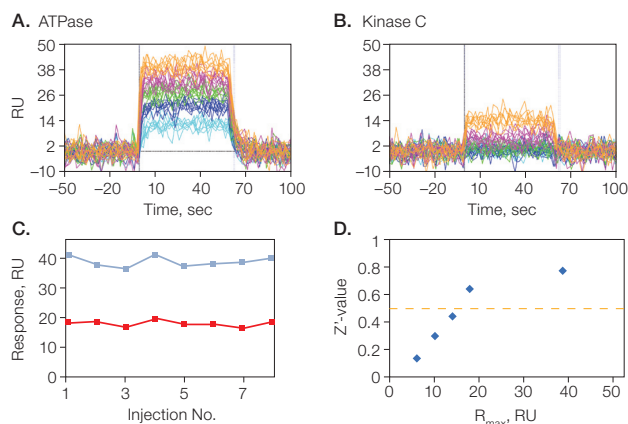

Fig. 5. Overlaid sensorgrams of eight individual injections of ADP solutions obtained for ATPase and Kinase C. The experimental R$_{max}$ values are 38.6 and 17.8 RU for ATPase and Kinase C, respectively. The concentrations of ADP are 400 (—), 133 (—), 44 (—), 15 (—), 4.9 (—) µM. **C,** Response values to eight injections of 400 µM ADP solution. The values for ATPase and Kinase A are shown in blue and red, respectively. **D,** The Z'-values calculated for different R$_{max}$ values. The orange dashed line corresponds to a Z'-value of 0.5.

Biacore S51 system is estimated to be approximately 10 RU (Perspicace et al. 2009). Although the number is somewhat higher with the ProteOn XPR36 system, the difference is well within acceptance levels. Considering the much higher throughput attainable with the ProteOn system and the small difference in Z'-factor we would recommend the Bio-Rad ProteOn XPR system for future studies.

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