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Kinetic studies of small molecule interactions with protein kinases using biosensor technology

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Abstract

Protein kinases are among the most commonly targeted groups of molecules in drug discovery today. Despite this, there are few examples of using surface plasmon resonance (SPR) for kinase inhibitor interaction studies, probably reflecting the need for better developed assays for these proteins. In this article, we present a general methodology that uses biosensor technology to study small molecule binding to eight different serine/threonine and tyrosine kinases. Mild immobilization conditions and a carefully composed assay buffer were identified as key success factors. The methodology package consists of direct binding studies of compounds to immobilized kinases, kinase activity assays to confirm inhibitory effects, detailed kinetic analyses of inhibitor binding, and competition assays with ATP for identification of competitive inhibitors. The kinetic assays resolve affinity into the rates of inhibitor binding and dissociation. Therefore, more detailed information on the relation between inhibitor structure and function is obtained. This might be of key importance for the development of effective kinase inhibitors.

Keywords: Surface plasmon resonance; Protein kinase inhibitors; Drug discovery; Methodology; Binding rate constants; Biosensor

Kinases are a large family of proteins that catalyze the transfer of the γ-phosphate group of ATP or GTP to the hydroxyl group of serine, threonine, or tyrosine on a substrate protein, often as a response to membrane receptor activation. Cellular signaling cascades rely on the phosphorylation status of proteins in their pathways. As many as one-third of all human proteins are thought to be substrates for protein kinases [1,2]. Malfunctions in these processes can have pathological consequences such as cancer and inflammatory disease. This makes protein kinases one of the most important target groups for drug discovery [3,4]. The largest group of kinase inhibitors is the ATP competitors [2]. Although many high-affinity compounds have been developed, specificity is often a problem because most kinases have a highly conserved ATP binding site [2].

Biacore's surface plasmon resonance (SPR)-based[1] protein interaction technology provides information-rich data on biomolecular interactions and has been recognized as a powerful tool for drug discovery applications [5]. SPR biosensors enable the interaction between biomolecules to be monitored in real time using label-free assay formats. One of the interacting molecules, the ligand, is immobilized on a biosensor surface. The other molecule, the analyte, is then injected in the mobile phase in contact with the surface. As analytes from the injected sample bind to the ligand attached

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[1] *Abbreviations used:* SPR, surface plasmon resonance; GST, glutathione *S*-transferase; c-Src, cellular sarcoma phosphoprotein; ERK2, extracellular signal-regulated kinase 2; CDK2, cyclin-dependent kinase 2; CamK4, calmodulin-dependent kinase 4; DMSO, dimethyl sulfoxide; MBP, myelin basic protein; DTT, dithiothreitol; SAR, structure–activity relationship; QSAR, quantitative structure–activity relationship; CSK, c-src kinase.

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to the surface, a change in SPR response is detected. The change in response level after the injection is proportional to the change in mass at the surface.

Binding curves corresponding to complex formation and dissociation are recorded in real time. From the shape of the binding curves kinetic rate constants i.e., association rate (k_a) and dissociation rate constants (k_d), can be derived by fitting the data to appropriate interaction models [6]. In drug discovery lead series, the kinetic rate constants can vary by several orders of magnitude even when compounds have similar affinities given that $K_D = k_d/k_a$ [7–9]. This means that determination of affinities alone does not provide an adequate basis for lead selection.

There are very few examples in the literature of analyzing small molecule interactions with kinases using SPR biosensors, and those studies mainly concern the mitogen-activated protein kinase p38 [10,11]. Here we present a methodology that was applied successfully to studies of eight different kinases, representative of this large protein family. Immobilization conditions were found to be critical, and several strategies for preserving the binding capacity for inhibitors and ATP were developed. The composition of the assay buffer was developed for optimized kinase assay performance. The assays presented include kinetic characterization of inhibitor binding to kinases and analysis of binding characteristics in the presence of ATP for identification of ATP-competitive binders. The effect of kinase phosphorylation on the binding of ATP and inhibitors was also investigated, as was the kinase isotype specificity. Furthermore, a complementary activity assay was used to investigate whether there was a correlation between binding to kinase and enzyme inhibition. All of this together suggests that the biosensor methodology that we have developed offers a useful tool in kinase-targeted drug discovery.

Materials and methods

Materials

Biacore S51 and Biacore 3000, Sensor Chip CM5 (classic and Series S), amine coupling kit, HBS-P buffer, glutathione *S*-transferase (GST), and anti-GST were obtained from Biacore. Biacore instruments are based on SPR detection at gold surfaces and a microfluidic system for reagent handling.

All p38 kinases, cellular sarcoma phosphoprotein (c-Src), extracellular signal-regulated kinase 2 (ERK2), calmodulin (bovine brain), and inhibitors SB 203580 and 202190 were obtained from Upstate Biotechnology. Inhibitors PD 169316, 5-iodotubercidin, hypericin, and SU 6656 were purchased from Calbiochem. PP1 and radicicol were purchased from Biomol International. Staurosporin was obtained from Sigma. Anti-penta-His antibody was obtained from Qiagen, and anti-biotin antibody, clone B-E24, was obtained from Diaclone. Kinase inhibitors A, 1A to 1F, Schering kinase 2, Schering kinase 3, and cyclin-dependent kinase 2 (CDK2) were gifts from Schering. Kinases c-Src kinase (CSK) and calmodulin-dependent kinase 4 (CamK4) were gifts from Protometrix.

Immobilization of capture antibodies

Amine coupling of anti-GST, anti-penta-His, and anti-biotin antibodies on Sensor Chip CM5 was performed according to the surface preparation wizard in the Biacore instrument control software, including the following steps: (i) activation with 0.2 M *N*-ethyl-*N*-dimethylaminopropylcarbodiimide and 50 mM *N*-hydroxysuccinimide for 7–10 min; (ii) injection for 15 min (5 μl/min) in 5 mM maleate (pH 6.0) for anti-GST, injection for 10 min (10 μl/min) in 10 mM acetate (pH 5.0) for anti-penta-His antibody, and injection for 10 min in 10 mM acetate (pH 5.5) for anti-biotin antibody, with all three antibodies at 30 μg/ml; and (iii) surface blockage with 1.0 M ethanolamine (pH 8.5) for 7 min.

Immobilization of kinases

Kinases (25–30 μg/ml) were amine coupled under conditions specified in Table 1 according to the same procedure as for antibodies described above.

Capture was performed by injecting the GST or His kinase fusion protein over amine-coupled anti-penta-His or anti-GST antibodies at concentrations of 3–30 μg/ml in buffer (Table 1) for 10–15 min at a flow rate of 10 μl/min using a custom method for surface preparation in the instrument control software. Injections were repeated until saturation or until the desired level was reached. The GST capture resulted in a stable baseline, whereas His-captured kinase was stabilized by surface activation with 0.2 M *N*-ethyl-*N*-dimethylaminopropylcarbodiimide and 50 mM *N*-hydroxysuccinimide for 2.5 min and blocking with 1.0 M ethanolamine (pH 8.5) for 2.5 min (cross-linking).

Binding experiments

Compounds were stored as 10 mM stock solutions in 100% dimethyl sulfoxide (DMSO). All light-sensitive compounds were handled under yellow protective light. After dilution to 1 mM with 100% DMSO, samples were mixed with 1.03- to 1.05-fold concentrated assay buffer and DMSO to yield 10 μM compound in a final buffer composition of 50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 3 or 5% DMSO