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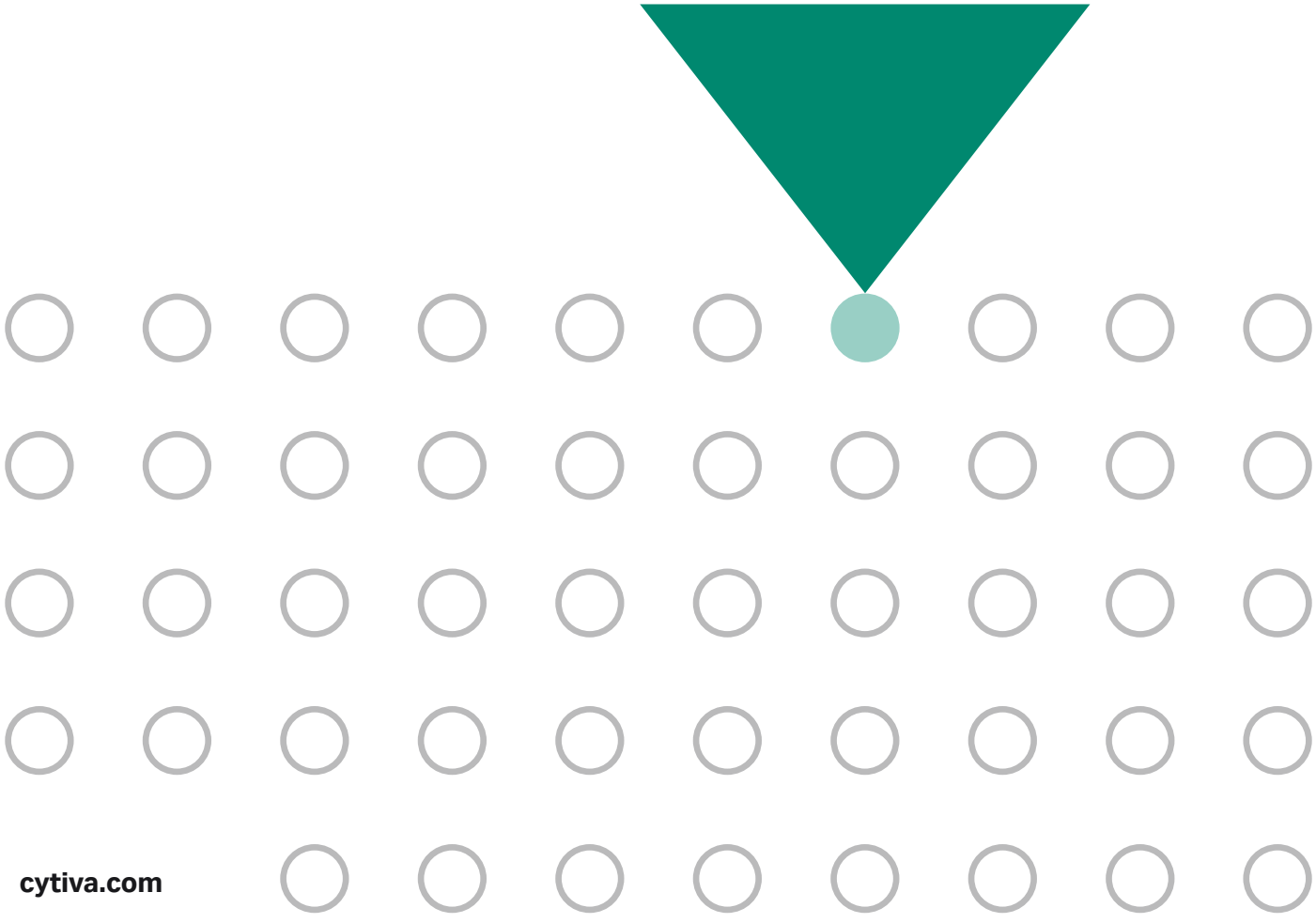
Biacore™ systems in small molecule drug discovery



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Confidently select and optimize drug candidates to improve efficacy

Target based drug discovery requires identification and validation of the target protein. Compounds with a potential to bind the target are then screened for medicinal chemistry optimization programs. Once compounds are identified, their interactions with relevant targets are studied in detail to confirm and characterize the interactions quantitatively and to provide information on the molecular mechanisms.

The trend within drug discovery to work with more difficult and/or complex targets has driven the need for increased sensitivity during assay development, screening, and lead optimization. Surface plasmon resonance (SPR) is a sensitive, label-free, and versatile biophysical method utilized by Cytiva's Biacore™ systems to achieve insights into the interaction process for compound and fragment based drug discovery from assay development to screening and detailed characterization.

Biacore™ SPR analysis in compound discovery and development enables:

- Validation of the activity of the target protein.
- Confident selection of candidates based on direct label-free binding data: selectivity, stoichiometry, affinity, and kinetics.
- Optimization of drug candidates for improved efficacy and safety through increased understanding of molecular mechanism of action.
- Use of affinity and kinetic data as key indicators for lead optimization.

This white paper gives examples of how Biacore™ systems can be used as a powerful tool in fragment screening and lead optimization, particularly where high sensitivity is required to observe low-affinity binding of LMW compounds to target proteins.

Well-characterized targets improve assay performance

A target based drug discovery approach requires the identification of a biological target with a causative activity related to the progression of a disease. This protein needs to be expressed and purified while the quality is controlled, then a therapeutic agent that modulates the activity of this target with limited or no adverse effects must be developed. Target identification is performed using various technologies such as gene expression profiling or functional genomics screens with pathway-specific or whole-genome libraries of siRNA, shRNA, and CRISPR-Cas9 gene knockout reagents (1). In assay development, SPR can be used to answer a range of questions such as whether the protein binds the compounds and/or positive control with the required characteristics as well as identify assay conditions for maximizing target activity *in vitro*.

Deeper insight into hit discovery and optimization

High-throughput screening (HTS) represents the traditional way of finding lead compounds with the potential to become therapeutic agents. However, many hits identified by HTS assays prove to be false positives and do not bind selectively to the target binding site. The need for secondary screening to verify hits is therefore vital and, in the pharmaceutical industry, SPR is heavily used for secondary screening of selected HTS hits to verify elimination of false positives and promiscuous binders (2).

Screening a sample against several proteins allows selectivity data to be obtained at an early stage in the selection process. Biacore™ 8 series systems are particularly well-suited for this by enabling parallel analysis of up to eight samples with the possibility to simultaneously analyze each sample against panels of up to eight proteins with reference.

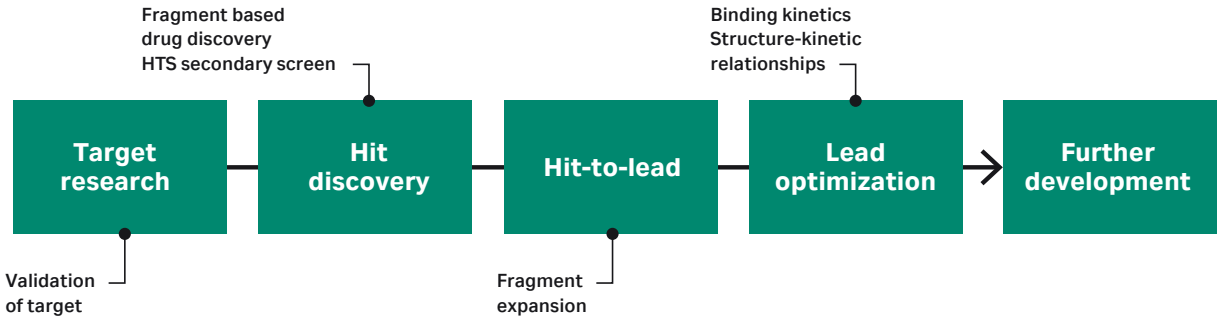

Fig 1. Biacore™ systems are suitable for compound and fragment based drug discovery from target research, throughout hit discovery and hit-to-lead as well as lead optimization and further development.

In a collaboration with Hoffman La-Roche, it was shown that high selectivity of a compound for a certain subunit does not necessarily correlate with high affinity or high binding response to the full-length target (Fig 2), suggesting that screening assays based on binding to a single target are not optimal for the identification of highly selective compounds.

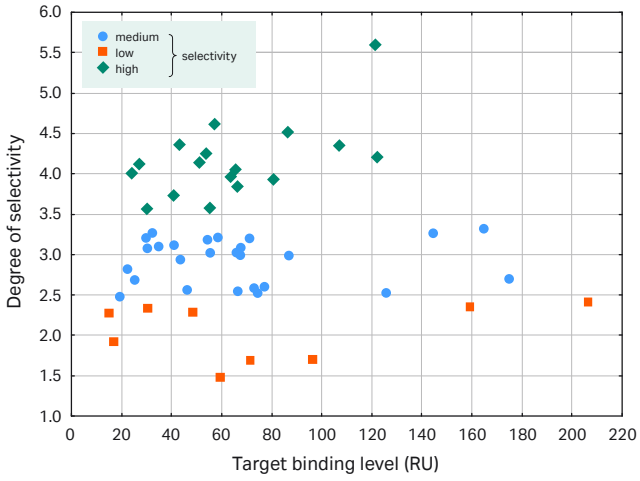

Fig 2. Selectivity (as defined by the ratio of full-length target to a-subunit binding responses) plotted against the binding response for the full-length target. The comparison shows that these properties do not show any significant positive correlation. Thus, the subsets of compounds assigned to low, medium, and high selectivity groups all exhibited a broad range of binding levels to the full-length target. Data courtesy of Dr. Walter Huber, Hoffman La-Roche, Basel, Switzerland.

Structure-based biophysical drug design and virtual ligand screening generate new information and can potentially reduce the number of compounds that need to be evaluated. Focused and directed libraries have thus become popular alternatives to full libraries. Together with structural information and *in silico* methods, SPR is used to improve the precision in hit finding and for characterization of hits identified by structure-activity relationships (SAR) via catalog approaches.

Fragment based drug discovery (FBDD)

This approach has evolved as an alternative to HTS and is now an established method for identifying suitable chemical scaffolds in drug discovery. Similar to HTS, FBDD uses a combination of structural and functional information to screen libraries against a target and identify binders. However, in FBDD the compounds are smaller (120 to 300 Da) reducing the variability, meaning the size of the libraries screened can be much smaller whilst covering a greater percentage of the available chemical space. Both catalytic site and allosteric site binders can be found for active and inactive forms of the target protein because no substrate is needed in biophysical direct binding assays. This versatile method is now being applied to a broad range of target classes (e.g., kinases, proteases, GPCRs, PPIs etc) resulting in high quality drug candidates (3) and an increasing stream of new clinical entities (NCEs) into the clinic.

Fragment screening presents several technical challenges. Biophysical methods such as X-ray crystallography and nuclear magnetic resonance (NMR) consume large amounts of target protein. The low affinities exhibited by fragments, typically in the mM range, require high sample concentrations with associated solubility and nonspecific binding issues. It is also difficult to measure binding affinities with these methods. SPR based biosensors are attractive for this application due to low target consumption, high information content, and high quality interaction data (3, 4). Throughput is also significantly higher than X-ray crystallography and NMR. In screening campaigns targeting the focal adhesion kinase, β1-adregenic receptor, and GPCRs have emphasized the benefits of using SPR for FBDD (5, 6, 7).

Biacore™ 8 series and Biacore™ S200 both have dedicated functionality for fragment screening (Fig 3), although other Biacore™ systems may also be used for analysis of LMW compounds and fragments. Biacore™ 8 series combines high throughput with high data quality and is capable of screening thousands of fragments using only microgram quantities of protein. For more focused screens, Biacore™ S200 provides exceptional sensitivity (Fig 4). A high level of sensitivity is a prerequisite when working with large, multi-domain targets or rare/sensitive targets such as GPCRs, where in some cases only a fraction of the target maintains its biological activity during preparation and analysis. High-sensitivity instruments also have the advantage of providing reliable screening and characterization at lower surface densities, which generally gives fewer secondary interactions and may increase the proportion of target accessible for binding.

(A) (B)

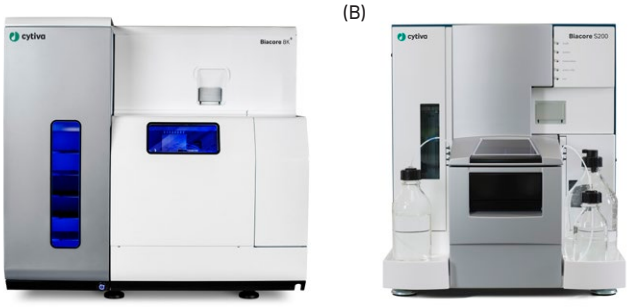

Fig 3. (A) Biacore™ 8K+ and (B) Biacore™ S200 both have dedicated functionality to screen and select fragments and lead compounds. Data evaluation tools designed to meet fragment-related challenges simplify data analysis and shorten time to results.

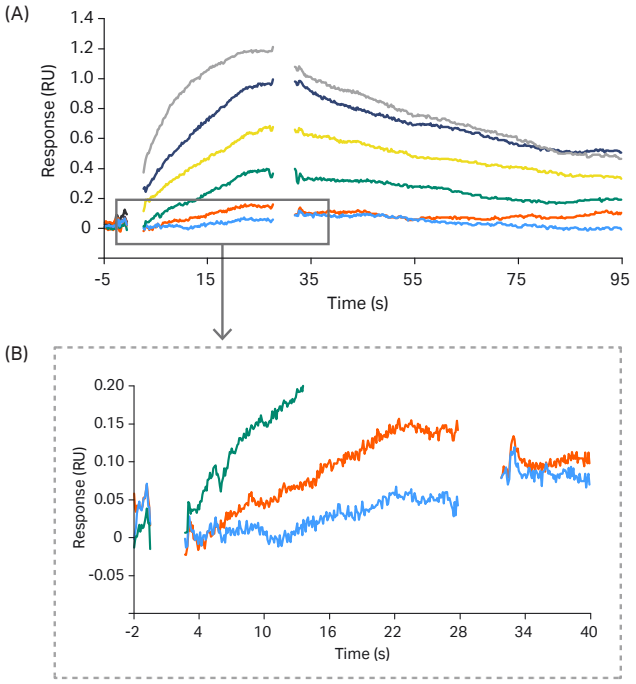

Fig 4. The high sensitivity of Biacore™ S200 enables confident analysis of data even in the sub-resonance units (mRU) response range. This means low density surfaces can also be used for targets with limited activity. (A) Sensorgram from a kinetic experiment between thrombin and melagatran. (B) Zoom-in on the lower concentrations demonstrating the possibility to separate curves at sub-resonance response levels.

A typical fragment screening workflow to prioritize and further characterize the most interesting binders

The experimental approach when using a Biacore™ system for fragment screening is typically divided into different steps (Fig 5) with the overall aim of quickly surveying the library content, rapidly identifying and prioritizing potential binders, and excluding problematic fragments early in the selection process.

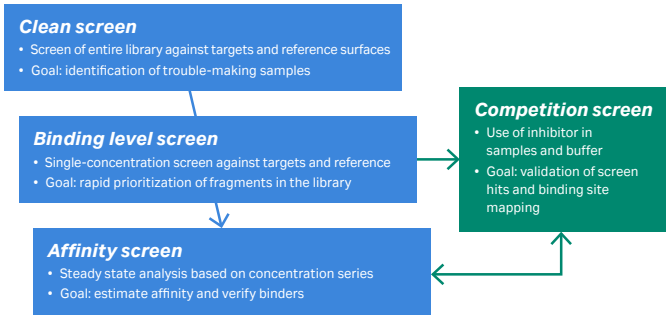

Fig 5. Typical SPR FBDD workflow. Biacore™ 8K, Biacore™ 8K+, and Biacore™ S200 have dedicated functionality for running and evaluating ***Clean screen***, ***Binding level screen***, and ***Affinity screen*** (shown in blue). In addition, Biacore™ S200 provides support for running ***Competition screen*** cost-effectively and rapidly (shown in green).

For new fragment libraries and targets, a ***Clean screen*** analysis is often run first to identify and remove fragments that show persistent binding to the surface thereby disturbing subsequent samples. ***Clean screen*** is run at a single concentration against a blank dextran surface and immobilized target protein and reference protein, if applicable. In order to reduce assay development time, some fragment libraries have been preoptimized for SPR screening (8). In a collaboration with Maybridge, a ***Clean screen*** analysis of the Maybridge Ro3 fragment library against three unimmobilized Biacore™ Sensor Chips (CM5, SA, and CM7) was undertaken. The study showed that only ~ 1% of the fragments were classified as being sticky to these fragment screening surfaces.

After this initial clean-up step, a ***Binding level screen*** is carried out to identify binders that display well-behaved binding characteristics against the target protein. In this assay, the fragment binders are identified by their binding response at a single concentration against the immobilized target protein, related proteins for selectivity, and blank, unimmobilized reference surface. Binding site selectivity can be assessed by using target protein modified either by mutation or by covalent chemical blocking of the active site as an in-line reference together with the wild type protein. In Biacore™ 8 series and Biacore™ S200, the automatic identification of fragments with binding levels above a predefined cut-off point, as well as the tagging of fragments with non-typical binding behavior or secondary interactions, makes it possible to rapidly prioritize fragments for further, more detailed analysis (Fig 6).

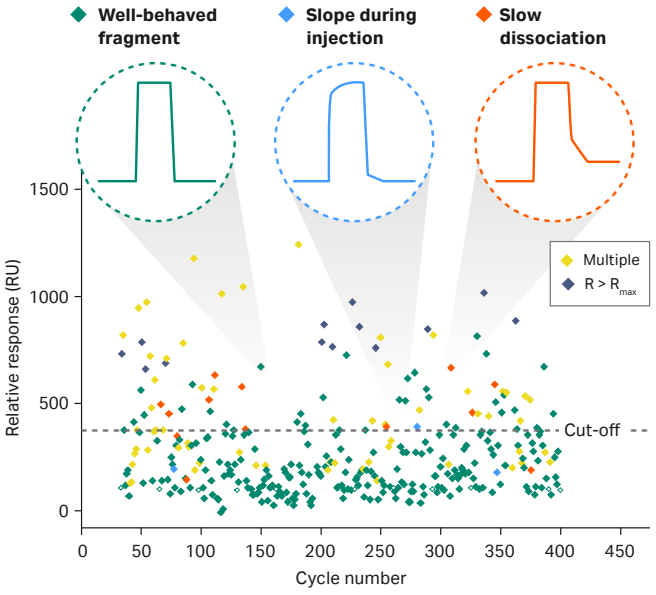

Fig 6. ***Binding level screen*** provides a rapid overview of the library content, identifying LMW fragments and compounds with binding levels above a defined cut-off point and enabling their efficient prioritization for further analysis. ***Binding level screen*** evaluation tools are tailored to address challenges associated with fragment screening assays, such as secondary interactions and atypical binding behavior by utilizing the sensorgram shapes to automatically identify fragments that do not bind in a well-behaved manner.

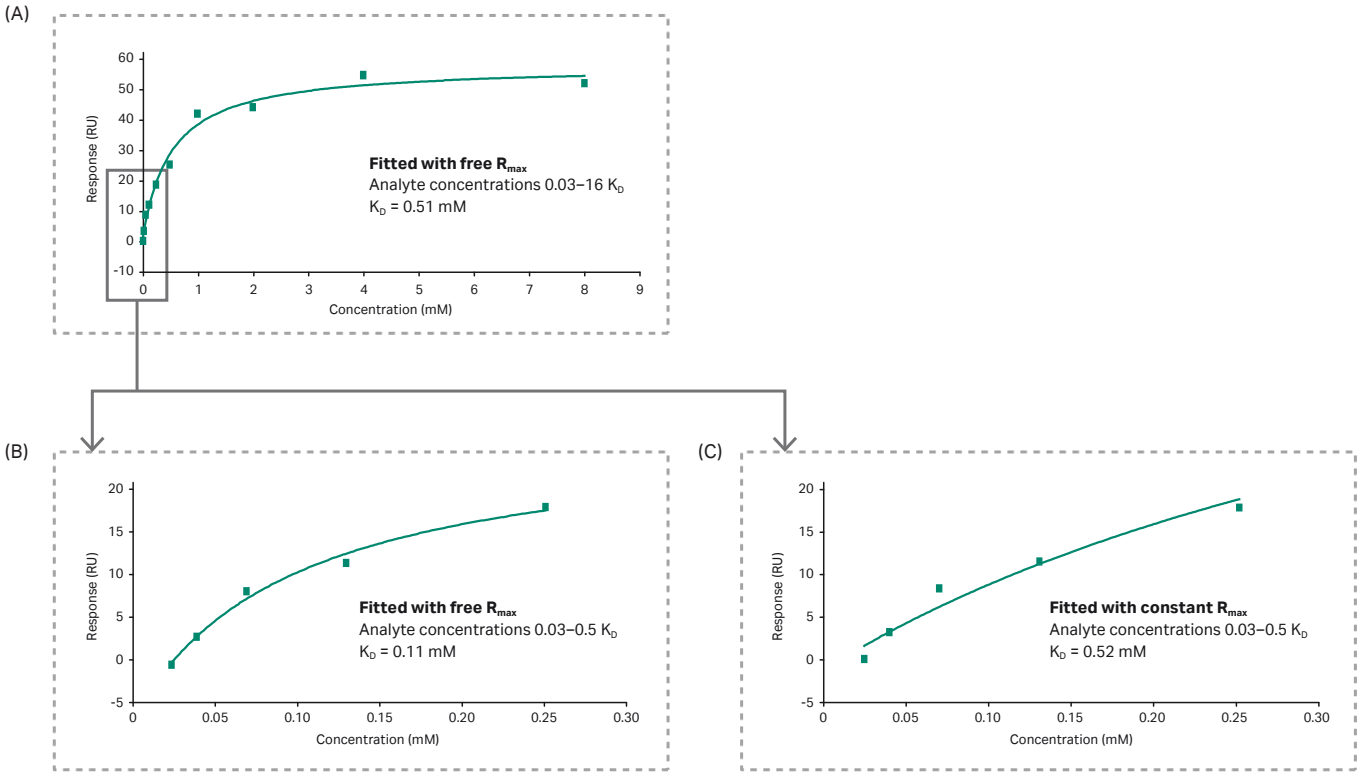

Fig 7. Principles of predetermined R_{max}. (A) Panel shows a fit to an example data set (simulated with R_{max} = 60 RU and K_D = 0.51 mM) with concentrations well above K_D, resulting in a good determination of K_D (0.51 mM), (B) A fit to the first five points in (A), representing a typical situation for fragments. Here, the K_D cannot be properly determined due to the lack of information about R_{max} in the data points. (C) A fit of the first five points in (A) using constant R_{max} (60 RU), resulting in a good determination of K_D, (0.52 mM) close to the simulated value of 0.5 mM. In this case, the information about R_{max}, lacking in the data points, could be provided from another source. In a real experiment, this information can be obtained with a positive control.

Primary hit validation by SPR is performed by an ***Affinity screen*** enabling precise affinity determination of interactions involving the smallest compounds and fragments. Selected fragments are run in a dose-response mode to verify binding and determine an approximate affinity (K_D). In this step, steady-state analysis is possible even for low affinity fragments that are analyzed at suboptimal concentrations in relation to K_D by the use of a positive control to assess the maximum response (R_{max}) of the surface (Fig 7).

A subsequent ***Competition screen*** allows the number of hits from the ***Affinity screen*** to be further narrowed down by studying the binding site specificity. Biacore™ S200 and Biacore™ 8 series enable competition experiments to be run with significantly lower consumption of competitor than in traditional SPR approaches where the competitor is present in the running buffer. By utilizing a dedicated injection type referred to as the ABA injection, fragments are analyzed with and without competitor directly from the microplate (Fig 8). Following the analysis, response levels are compared and fragments capable of binding in the presence of competitor are indicative of non-competition (i.e., binds to a site different to the competitor).

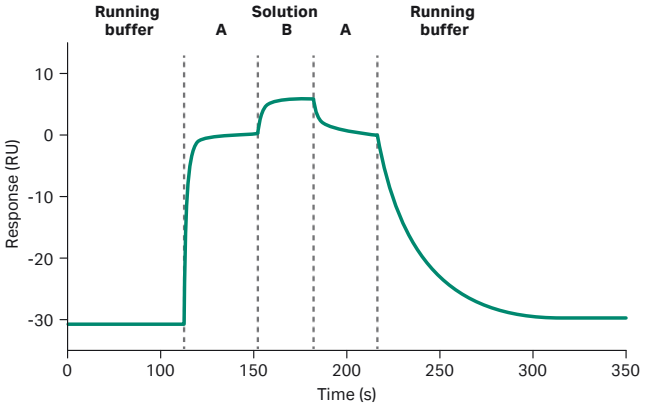

Fig 8. Competition screening experiments have been simplified in Biacore™ S200 and Biacore™ 8 series. The ABA injection enables switching between samples with and without competitor directly from the microplate. This means that binding site mapping can be performed with minimal consumption of competitor/inhibitor. The sensorgram shows the principle of the ABA injection. Solution A (competitor) is first injected over the surface to establish a baseline and block the competitor binding site. Then, Solution B (mix of competitor and fragment) is injected to measure any binding to additional sites. Finally, Solution A is injected to allow the dissociation to be monitored while keeping the competitor binding site blocked. In this example, the ability of the fragment to bind in the presence of the competitor means that it binds to another site on the target.

Off-rate studies during fragment hit-to-lead development

A successful SPR fragment screening workflow results in the identification of a number of hits that are confirmed binders in the mM to µM affinity range with a given stoichiometry as well as some knowledge about the binding site specificity. To further discriminate between the hits, fragments are ranked according to their ligand efficiency that reflects the binding strength of the fragment in relation to its number of non-hydrogen atoms, usually around 7 to 20 (9). Fragments with high ligand efficiencies are desirable since it means that many atoms are involved in the binding to the target and thus, these fragments are deemed as good starting points for further optimization. These initial fragment weak affinity hits are evolved into larger chemical entities which offer more efficient binding to the target via iterative rounds of medicinal chemistry. Once the affinity has been improved down to 10 to 100 µM, and the affinity can be measured kinetically, it could be beneficial to systematically study the dissociation rates (off-rates) (10). In a study (11), off-rate screening using SPR was demonstrated as a novel and efficient method to evaluate newly synthesized fragments. With knowledge of the dissociation rate constants it was possible to identify active compounds straight from crude reaction mixtures. Fragments were simply diluted and injected in Biacore™ system without the need for separation or thorough purification prior to analysis.

Lead optimization for improved prediction of drug efficacy

During lead optimization following either a FBDD or HTS screening approach, the overall goal is to improve the *in vivo* properties of the hits. This is generally approached by optimization for potency (K_d or K_i) on the widely accepted basis that strong drug-target interaction results in better efficacy. However, the drug residence time ($1/k_{off}$) emerged as another important factor for prediction of *in vivo* drugs and

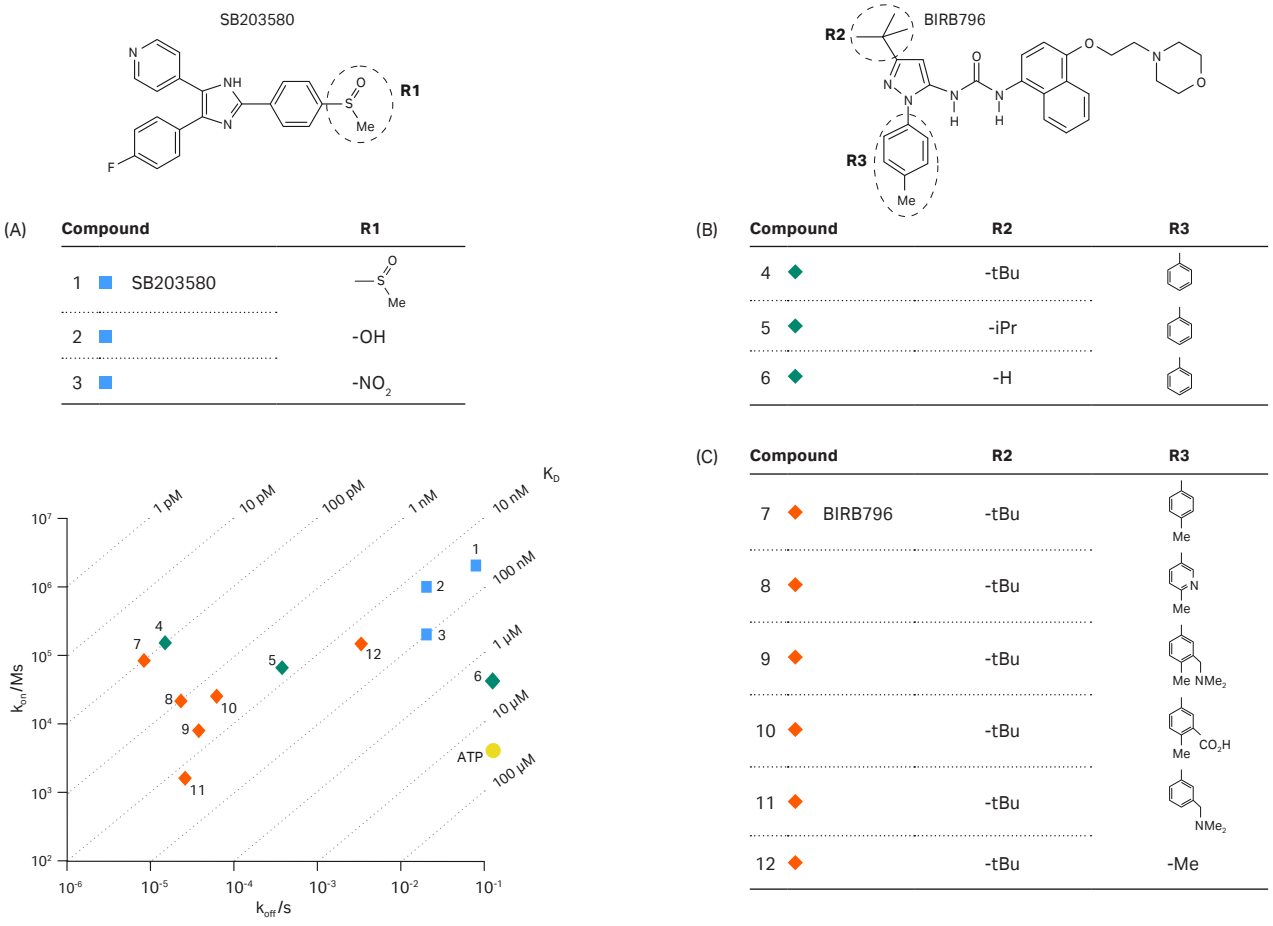

Fig 9. On/off/K_D map of P38 inhibitors. (A) The first generation of inhibitors (SB203580) had affinities in the 10 to 100 nM range with rapid on- and off-rates; (B) the optimization of BIRB796 inhibitors (R3 = Phe, R2 = tBu, iPr, H) showed that forming and filling a new lipofilic pocket mainly improved the affinity, from µM to 100 pM due to four orders of magnitude slower off-rates; (C) when R2 is kept constant (tBu) and more polar substituents are introduced as R3, slower on-rates manifest themselves as interactions losing recognition. ATP and compound 6 are positioned outside the on- and off-rate graph axes, but on the right position on the iso-affinity diagonals. This is because the off-rates for these two are too rapid and outside the measurement range.

there has been an increased focus on binding kinetic studies during lead optimization. Drug residence time relates to the life-time of a drug-target complex and optimization of this parameter can improve drug efficacy *in vivo* as well as reduce off-target mediated toxicity, hence, improving drug safety and tolerability efficacy (12, 13, 14). Binding kinetic studies with a Biacore™ system is possible over a wide dynamic range making it possible to determine the binding kinetic rate constants for all biological interactions. In addition to the dissociation rate constant (k_{off}) and drug residence time, the association rate constant (k_{on}) that relates to target occupancy is also an important parameter for the duration of a given pharmacological effect (12). Optimization of the residence time proved useful for selecting and developing new CCR2 antagonists (15) and the residence time has been correlated to *in vivo* efficacy for kinase inhibitors (16). The relevance of incorporating aspects on the drug-residence time during lead optimization is further supported by the finding that many drugs on the market are characterized by slow dissociation from their targets (17). A model that translates slow-binding kinetics into cellular and *in vivo* effect has now been presented (18).

By comparing how different ligands interact with one target, or to mutants of the target, binding kinetics can provide information that relates to molecular structure. Alongside the well-known concept of structure-activity relationship (SAR), the concept of structure-kinetic relationship (SKR) has provided revealing details into the dependency between binding kinetics and compound structure of small molecules (5, 19, 20). On/off rate plots from SPR kinetic analyses (Fig 9) provide an informative way to demonstrate the resolved scale of affinities and how these affinities can be distributed over a range of on- and off-rates. Thus, considering lead series with the same scaffolds can show large variation in both on- and off-rates (Fig 9), it becomes possible to differentiate compounds with similar affinities to the target molecule *in vitro*. Such differences might reflect differences in binding mechanism, thereby also differences *in vivo*. This was the case for compounds targeting the estrogen receptors when the differences in kinetics made it possible to distinguish agonists form antagonists (21).

Use of capture based assays with Biacore™ systems enables high-affinity interactions in drug-target binding to be studied. In this way, any regeneration issues that might follow from high potency drugs in the nM range are avoided. In addition, capture based assays facilitate interaction studies to difficult targets for which binding activity is dropping off successively. For example, in a SKR study on thermolysin, the determination of the kinetic parameters for nineteen closely related thermolysin inhibitors was enabled by the use of the Biotin CAPture Kit (Cytiva). The approach of capturing biotinylated thermolysin did not only facilitate the assay in respect to regeneration following inhibitor binding but also helped to overcome issues with loss of binding activity for immobilized thermolysin over time. The Biacore™ study revealed that very small modifications in inhibitor structures resulted in a large variation in the kinetic profiles (Fig 10).

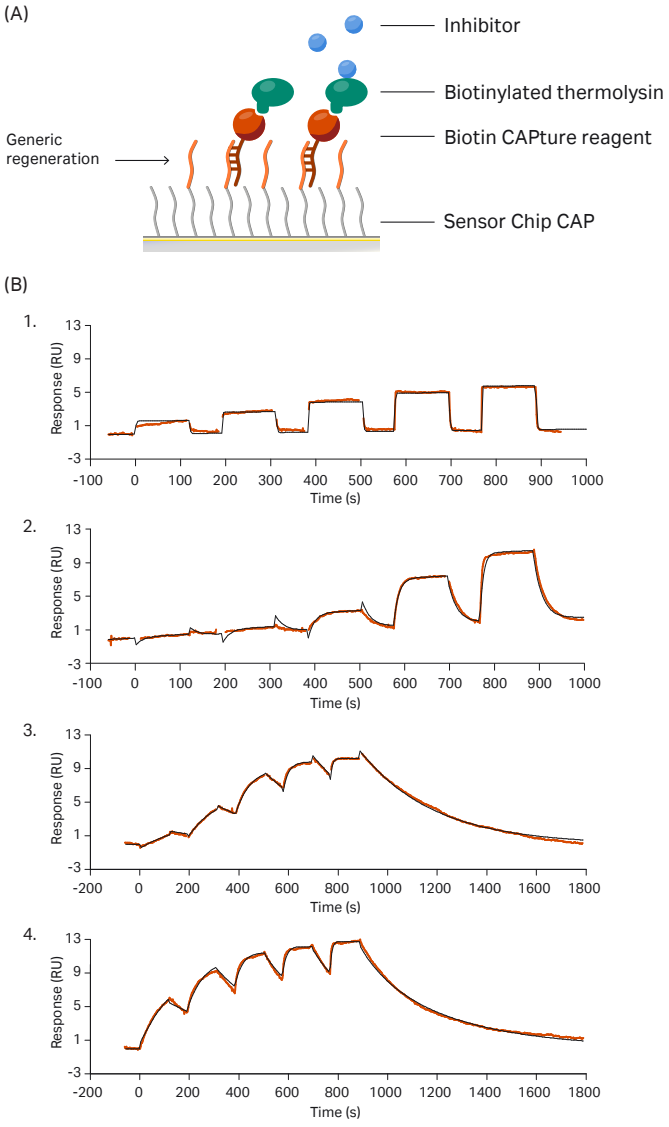

Fig 10. (A) Reversible biotin capture using Sensor Chip CAP in the Biotin CAPture Kit enabled the kinetic study of inhibitor binding to thermolysin. (B) Four example sensorgrams from the structure-kinetic study of thermolysin (38 kDa) showing that small variations in inhibitor structure (different substituents in the same position of the inhibitor) resulted in sensorgrams spanning a broad kinetic range. Inhibitor average MW = 454 Da. (collaboration with Professor Klebe, Philips University, Marburg, Germany).

Save one day for every screen you do with machine learning

Biacore Intelligent Analysis™, part of Cytiva machine learning software, enables the rapid and automated analysis of large data sets by significantly reducing the manual, time-consuming steps of data curation and quality control. This optional add-on extension to Biacore™ Insight Evaluation Software currently offers support for two analysis types — ***Binding level screen*** and ***Affinity screen*** for fragments. Biacore Intelligent Analysis™ comes with evaluation methods including prediction models pretrained by Cytiva's scientists, providing an out-of-the-box solution for analyzing fragment binding level (Fig 11) and affinity screening data sets. The methods have been validated to provide greater than 90% accuracy relative to human expert analysis with excellent sensitivity (> 87%) and specificity (> 90%).

Fig 11. Fragment ***Binding level screen*** analyzed by Biacore Intelligent Analysis™.

Conclusion

Combining high sensitivity and throughput, Biacore™ systems utilize label-free SPR based biosensors to acquire the accurate, highly reproducible, and quantitative binding data required for small molecule and fragment based drug discovery and development. Here, SPR is shown to provide a high level of sensitivity with Biacore™ systems delivering accurate results in the sub-resonance units (mRU) response range making it ideally suited for working with rare or sensitive proteins with low levels of immobilization. Accurate interaction analysis of LMW compounds and fragments binding to targets with low activity levels is also demonstrated providing valuable insights to the interaction process while offering a higher throughput alternative to X-ray crystallography and NMR for secondary screens and characterization of selected hits. Biacore™ systems deliver reliable affinity and kinetics data at the extremes of the kinetic scale and are used to perform binding site-mapping using competition assays to increase screening reliability. They also enable precise affinity determination of interactions involving the smallest compound and fragment plus accurate validation of hits. Biacore™ systems truly incorporate data evaluation tools designed to meet today's fragment-related challenges. Biacore™ Insight software simplifies analysis of all your Biacore™ SPR data in a single software with Biacore Intelligent Analysis™. Our machine learning extension significantly reduces the analysis bottleneck in fragment screening applications.

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