A multi-protein panel approach for selectivity-based compound screening using Biacore A100

- Improved efficiency for compound selection in drug discovery
  - selection of compounds based on binding selectivity
  - label-free, real-time screening of virtual hits and fragment library compounds
- Rapid, information-rich analysis of compounds against a complex protein target using a protein array format
  - processing rate equivalent to 3800 interactions/day
  - low target protein consumption compared to HTS assays
- Unique criteria for compound selection from parallel analysis of protein panels
  - simultaneous analysis of binding to wild-type & mutant targets, specific target subunits and reference proteins
  - identifies compounds with highly selective binding to specified target
  - conclusively identifies non-specific protein binders
- Faster, better-informed compound selection with minimized risks for target-dependent artifacts
- Enabled significant progress in a project where HTS had previously failed
  - compounds with agonist activity confirmed in biological assays

Figure 1. Biacore A100 provides label-free analysis of interactions for selectivity-based compound screening.

"The multi-target protein panel approach in Biacore A100 is an excellent way to study complex drug targets. The chance to find biological activity is enhanced when performing these assays, as the information obtained gives a clear picture on the mode of binding"

Dr. Walter Huber, Hoffman La-Roche, Basel, Switzerland

Introduction

Current high throughput screening (HTS) approaches utilize large libraries of low molecular weight (LMW) compounds, with inhibition assays designed to reflect compound affinity for the therapeutic target protein. These assays detect "hits" based on a negative readout and are highly susceptible to artifacts that generate false positives. For example, compounds may interfere directly with the labeling system, or exert a response via non-specific protein binding. Consequently, most hits identified by HTS assays prove to be false positives and do not bind selectively to the target binding site.

The use of structural information for virtual screening and the design of directed libraries can improve the screening process. Screening methods that provide comprehensive, high-quality binding and selectivity data at an early stage would greatly improve the efficiency of selection.

Acknowledgement

We gratefully acknowledge Hoffman La-Roche (Basel) for supplying compounds and proteins and would like to thank Dr. Walter Huber for his invaluable contributions during this collaboration.

Label-free interaction analysis systems are increasingly used in the drug discovery process, providing selection criteria based on direct compound-target protein binding properties. Advantages to this type of assay include unique, high-resolution binding data that provides kinetic characterization of the interactions, low target protein consumption and the inclusion of control proteins for binding specificity[1]. Biacore™ A100 provides high quality interaction data with sufficient throughput to meet the demands of directed compound screening applications. In addition to the number of compounds that can be processed, this interaction array system enables the parallel analysis of compound interactions with a panel of proteins. In practice, up to 16 proteins can be included in the panel for typical LMW applications (allowing for blank reference spots). This opens up new possibilities for more effective compound screening, using a protein panel approach to provide comprehensive selectivity data, improving the quality of compounds selected for further development.

Biacore A100: sample- or target-focused assay formats

Up to 5 different proteins (or other types of biomolecules) can be immobilized in each of four parallel, independent flow cells. For assays requiring maximum sample throughput, identical immobilizations can be performed in all 4 flow cells, allowing 4 different samples to be analyzed in parallel during each analysis cycle (see Figure 3). In assays where information output per sample is prioritized, up to 20 different targets can be immobilized in the 4 flow cells and 1 sample per cycle is injected in parallel over all flow cells (see Figure 6).

Purpose of study

In collaboration with Hoffman-La Roche (Basel, Switzerland), Biacore A100 was used to screen 1280 compounds from a drug discovery program targeted against a complex heterotrimeric protein of significant biomedical importance (identity confidential, referred to here as HTP). The Roche project aims to develop agonistic compounds that bind to an allosteric site in the c-subunit and enhance protein activity. This goal is complicated, because the allosteric site is known to bind similar natural ligands to those associated with the active site in the a-subunit of HTP, thereby creating a selectivity issue. Lack of a suitable protein comprising the c-subunit alone required a two-step approach (outlined in Figure 2). This involved first using the array system to screen all 1280 compounds against full-length and a-subunit targets and so eliminate those compounds exhibiting selectivity for the a-subunit over full-length HTP.

Compounds with promising selectivity properties could then be screened against a panel of seven different proteins in parallel to provide the comprehensive selectivity profile needed for effective compound selection.

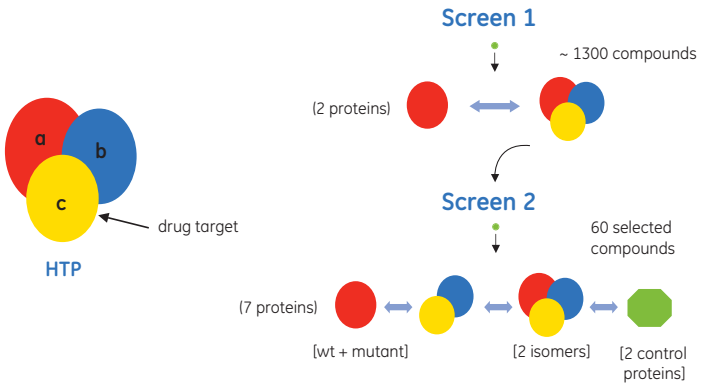


Figure 2. Subunit composition of HTP target and overview of 2-stage screening strategy.

Results

Rapid, sample-focused screening of 1280 compounds against full-length and a-subunit HTP proteins (Screen 1)

All 1280 compounds were analyzed in a sample-focused assay, using full-length HTP (HTP111) and a-subunit (HTPa2) targets. These were immobilized in each of the 4 flow cells, allowing parallel analysis of 4 compounds per analysis cycle. Compounds were derived either from a virtual screening library directed against the functional binding site of the HTP c-subunit (1160 in total, MW range ~200-800 Da), or from a fragment library of polar molecules (320 in total, MW range ~100-400 Da). The basic assay setup is shown in Figure 3.

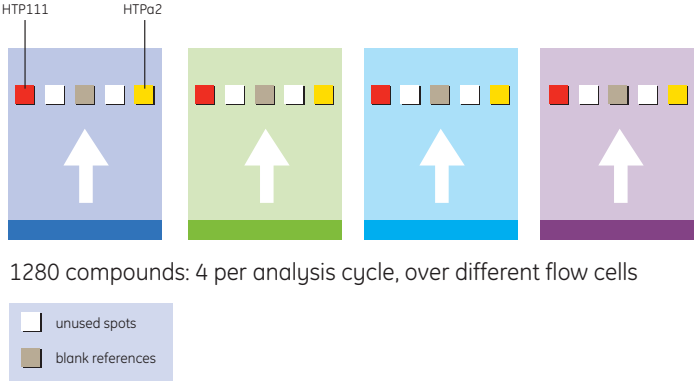


Figure 3. Setup for Screen 1. Sample-focused assay to screen for compounds showing selectivity for full-length HTP over the a-subunit.

Together with appropriate control samples and solvent correction solutions, a total of 240 compounds were screened in each 8 hour run, using one 384-well microplate (maximum capacity is around 1000 compounds in a single 20 hour run). Each compound-target interaction was monitored in real time, producing a plot of binding response (detected using surface plasmon resonance, SPR) against time, known as a *sensorgram* (see *Monitoring Interactions* information box at the end of this note). Analysis of the large volume of data produced during the screening was aided by an automatic quality control (QC) function in Biacore A100 Evaluation Software, which identifies and removes poor quality sensorgrams, thereby eliminating occasional experimental artifacts. Figure 4 shows an example of the sensorgrams from the quad- ruplicate injections of control samples used in one run.

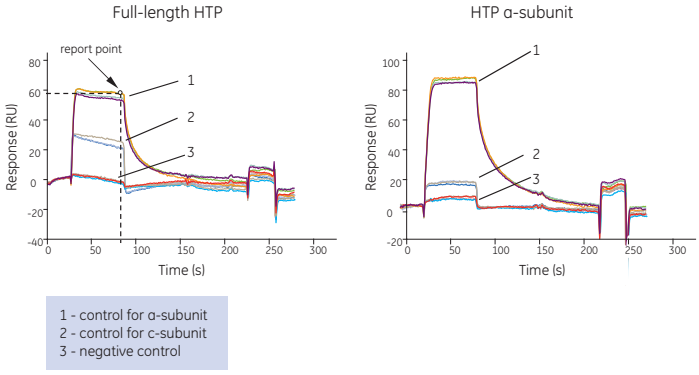


Figure 4. Sensorgrams from positive and negative controls used in Screen 1. Responses for the four sets of controls used in each run are shown separately for the two immobilized targets. The report point value chosen for data evaluation was taken immediately prior to the start of the dissociation phase, as indicated.

Selection of compounds

The binding levels for each compound to the two targets were compared using a scatter plot (Figure 5). This enabled identification of selective binders for full-length HTP over the a-subunit, providing a subset of 60 compounds for a more comprehensive protein panel analysis in the Screen 2 assay. In order to include a broader spectrum of binding properties in the second screen, a few non-selective compounds were also chosen.

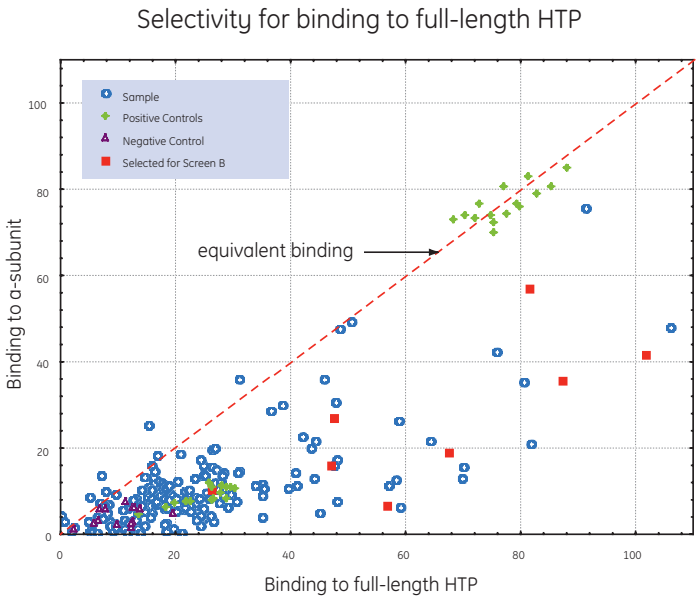


Figure 5. Selection of compounds for full panel screening (results shown for 240 compounds from one run). Scatter plot of late-association phase binding levels to the two targets.

Comprehensive analysis of compound selectivity: Target-focused screening of 60 compounds against a panel of 7 proteins

In Screen 2, the chosen compounds were comprehensively analyzed for binding selectivity, using the array in a target-focused assay employing a panel of seven different proteins. With this setup, one compound is injected over all four flow cells in parallel during each cycle, enabling simultaneous measurements of binding responses to all immobilized targets (Figure 6).

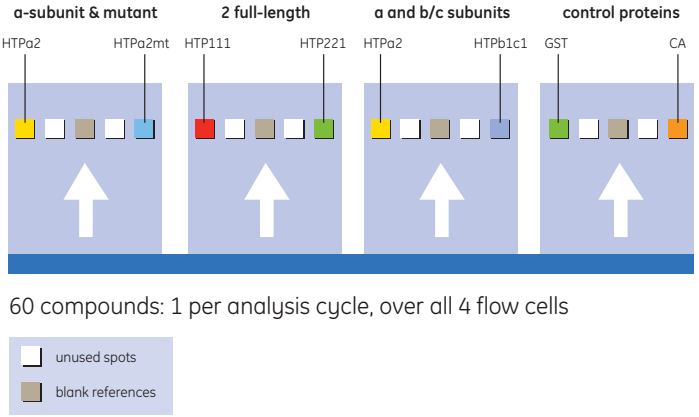


Figure 6. Setup for Screen 2. See Table 1 for a full description of the proteins comprising the panel. Note that the a-subunit target, HTPa2 was immobilized in two positions.

Table 1. Proteins used in the panel for Screen 2.

Protein	Description	Notes
HTP111	full-length trimer ($a_1b_1c_1$) isomer – expressed in *E.coli*	
HTP221	full-length trimer ($a_2b_2c_1$) isomer – expressed in human cell-line	significantly glycosylated compared to HTP111
HTPa2	a-subunit monomer	
HTPa2mt	mutated a-subunit	triple point-mutation of known functional site
HTPb1c1	b/c-subunit dimer	
GST	glutathione-S-transferase (control protein for non-specific binding)	commonly used tag for recombinant proteins
CA	carbonic anhydrase (control protein for non-specific binding)	drug target protein unrelated to HTP

The seven proteins in the panel were chosen to enable well-informed identification of compounds with desired binding selectivity, while minimizing the risk of target-dependent artifacts (leading to false positives/negatives) and conclusively identifying any non-specific protein binding tendencies. The a-subunit target (HTPa2) was immobilized in two flow cells, providing a useful pairing with two of the other targets (an a-subunit mutant and the b/c-subunit protein), as well as an internal reproducibility control. Relevant details concerning the proteins used in the panel are given in Table 1.

Together with control samples, 60 compounds were screened using one 96-well microplate. Before considering the details of the selectivity results, a number of basic observations can be made from Screen 2.

- Control compounds showed the expected patterns of binding to the HTP-derived proteins in the panel
- Controls and samples generally showed a very low level of binding to the two control proteins, CA and GST
- Results from the duplicate immobilizations of the HTPa2 a-subunit target in different flow cells were extremely consistent in all cases, so that all HTPa2 results were taken as the mean of these values

Compounds chosen from Screen 1 show predicted selectivity to protein panel

Comparison of the results for compound binding to the HTP111 and HTPa2 targets confirmed that the majority of compounds were selective for full-length HTP over the a-subunit, although they varied significantly in the degree of selectivity (central panel, Figure 7). This behavior was generally consistent when examining the results for the entire panel, so that compounds showing selectivity for the original full-length HTP111 protein against the a-subunit exhibited a similar preference for the full-length isomer and b/c-subunit proteins (see Figure 7 insets for examples).

Pairwise comparison of protein-binding responses provides unique information on compound properties

Informative pairwise comparisons between specific targets provided an excellent overview of general target-dependent properties and enabled the identification of particular compounds exhibiting anomalous behavior. These observations would not have been possible using standard, single-target strategies to identify binders. Figure 8 shows examples of these pairwise target comparisons and the conclusions that could be drawn from them.

Higher target affinity does not correlate with better binding selectivity

One motivation for the multi-protein panel screening approach was that identifying compounds with a high degree of selective binding might be more important than screening for those with high target-affinity. It was therefore necessary to examine the relationship between these properties. When selectivity (as defined by the ratio of HTP111: HTPa2 binding responses) was compared directly to absolute HTP111 binding level (Figure 9), it was clear 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. These results suggest that screening assays based on binding to a single target are not optimal for the identification of highly selective compounds.

Combined target evaluation: The multi-target panel provides unique criteria for compound selection

Combined analysis of selectivity properties proved to be even more revealing than the pairwise target comparisons described. It could be argued, for example, that the full panel screen was somewhat redundant since the selectivity comparisons of full-length against a-subunit and b/c-subunit against a-subunit should give identical results. When this comparison was made, however, it was clear that compound behavior was more complex and that both of the HTP-derived targets were required to obtain a comprehensive picture of binding selectivity (Figure 10).

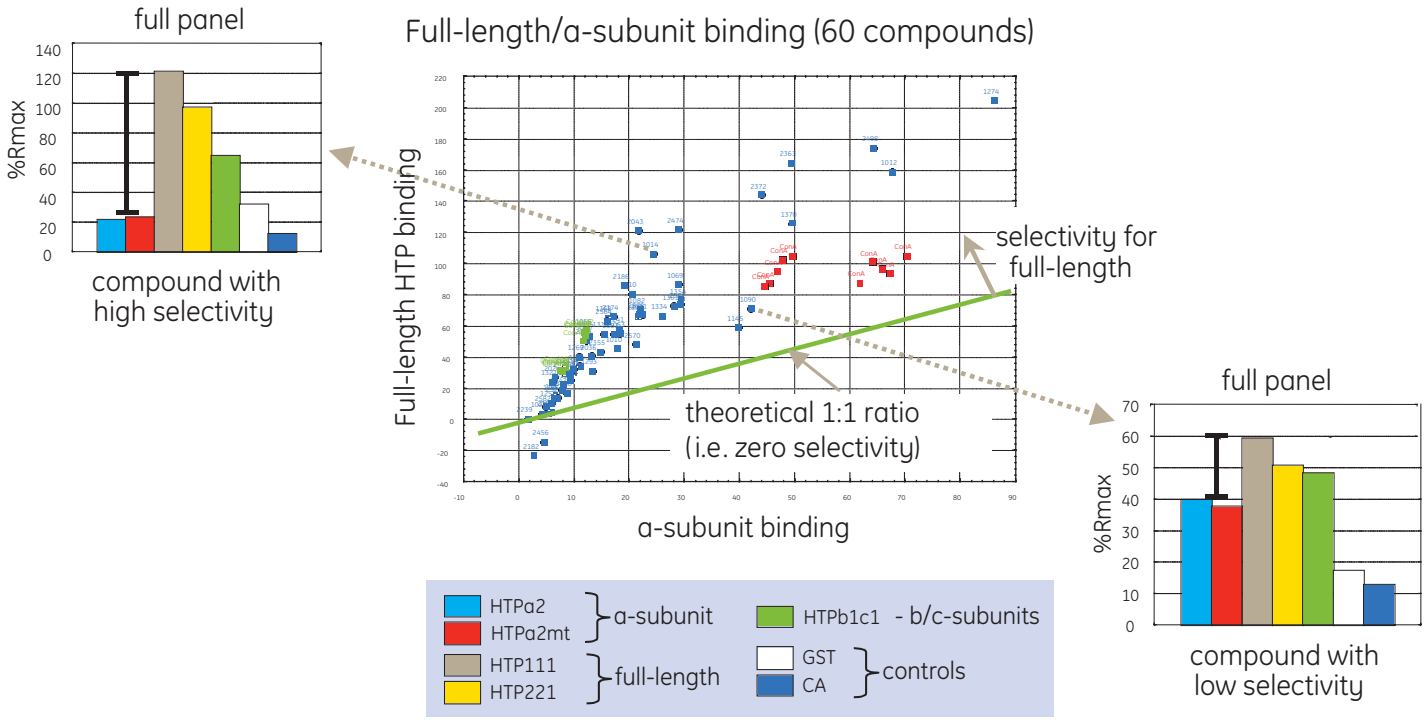


Figure 7. Overview of compound selectivity. Insets show binding patterns to the full panel of 7 immobilized proteins for two specific compounds that exhibited different degrees of selectivity (%Rmax = response as percentage of theoretical maximum binding response to each protein).

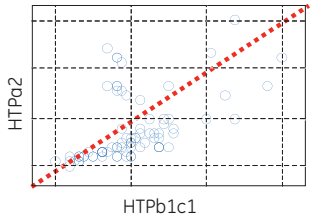
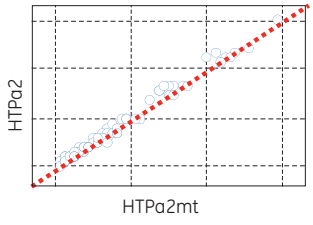
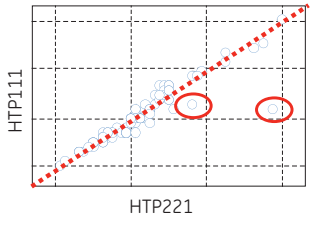
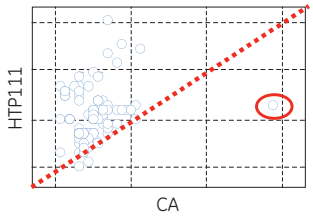
Scatter Plot	Targets compared	Comments/conclusions
	a-subunit and b/c-subunits	• Very little correlation between non-overlapping HTP targets • Indicates little non-specific binding among Screen 2 compounds
	wild-type and mutated a-subunits	• Very close correlation • Indicates any compound binding to a-subunit is not directed specifically against mutated active site
	2 full-length isomers, expressed in bacteria and human cells respectively	• Very close correlation in general - compounds do not discriminate between isomers • Expected observation since targeted c-subunit is common to both forms • Two compounds show distinct preference for HTP221 - may indicate non-specific binding to sugar residues on glycosylated isoform
	full-length HTP target and control protein	• Very little correlation & very low binding to CA • Shows very little general protein binding among compounds • However, one strong CA-binder identified

Figure 8. Scatter plots showing relative binding levels (as % Rmax) of all 60 compounds to different pairwise combinations of targets from protein panel. Red dashed lines show theoretical 1:1 (non-selective) binding levels. Red-ringed data points highlight specific compounds referred to in comments/conclusions column.

The plot of selectivity for the b/c-subunit (HTPb1c1/HTPa2 responses) against selectivity for the full-length protein (HTP111/HTPa2 responses) showed that while many compounds mapped in the vicinity of the diagonal indicating selectivity equivalence, a number of compounds exhibited a distinct preference for either the full-length or b/c-subunit target.

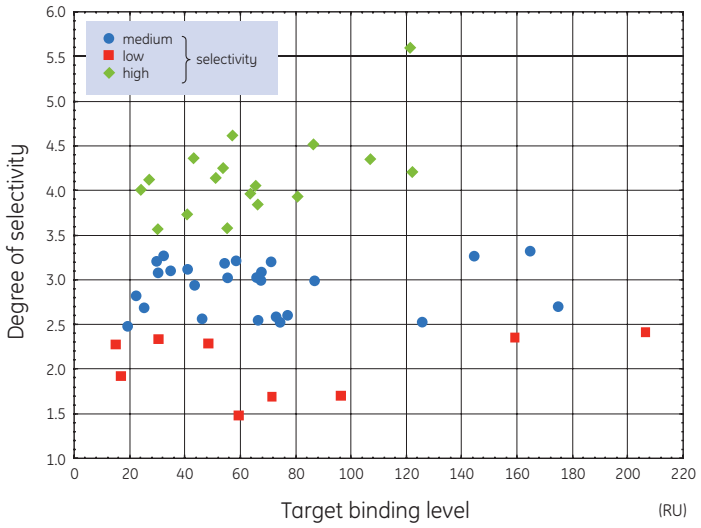


Figure 9. High affinity for the full-length HTP target does not correlate with high selectivity for full-length over a-subunit binding.

Figure 10 shows three groups of compounds with very different combined selectivity profiles:

1. The large majority lying close to the equivalence diagonal show the expected behavior for compounds that bind selectively to the c-subunit of HTP, i.e. are equally selective for the full-length and b/c-subunit targets compared to the a-subunit.
2. A few compounds showed much stronger selectivity for the full-length HTP than for the b/c-subunit, indicating that an intact target is required for optimal binding. Screening based exclusively on subunit-specific targets would therefore risk missing these potential drug candidates.
3. Some compounds showed strong selectivity for the b/c-subunits, but little or no selectivity for full-length HTP. This may reflect binding sites within the b/c-subunits that are inaccessible in the context of the intact therapeutic target. Using a less comprehensive screening panel would therefore risk the identification of good binders that would be of little value against the biological drug target.

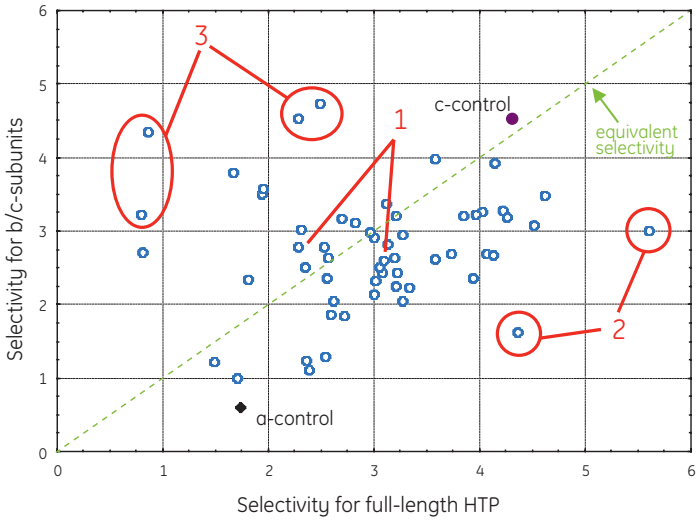


Figure 10. Combined selectivity analysis for 60 compounds. Increasing distance from the equivalent selectivity diagonal indicates a higher degree of selectivity for the b/c-subunit (above the line) or full-length HTP (below the line), in reference to binding to the a-subunit. See main text for discussion of highlighted compound groups 1-3.

Taken together, these results indicate that the identification of highly selective compounds against complex therapeutic targets may require a comprehensive panel approach in order to eliminate potential target-dependent artifacts that may result in false-positive or false-negative leads. Furthermore, the use of multiple control targets to identify non-specific protein binding, binding to recombinant protein tags, and possible expression-system artifacts, should be generally applicable to almost any drug discovery program.

Using the selectivity data generated by Biacore A100, Roche performed confirmatory analyses in-house and took several of the most promising compounds into biological, cell-based assays. Preliminary results from these assays identified several compounds with agonist activity, indicating that high quality binding data from Biacore A100 can contribute to more effective drug discovery by maximizing the chances of finding biologically active compounds.

Summary

- Parallel processing & flexibility make Biacore A100 a powerful tool for selection of selective compounds against a complex protein target
- Assays focused on sample throughput enabled rapid screening of 1300 compounds based on fundamental selectivity criteria
- Target-focused assays employing an extended panel of proteins provide comprehensive selectivity information for identification of best candidates
- Unique information content from parallel analysis of multi-protein panels enables faster, better-informed compound selection to increase the chances of success in drug discovery

References

1. Huber, W. A new strategy for improved secondary screening and lead optimization using high-resolution SPR characterization of compound-target interactions. J. Mol. Recognit. 18, 273-281 (2005)

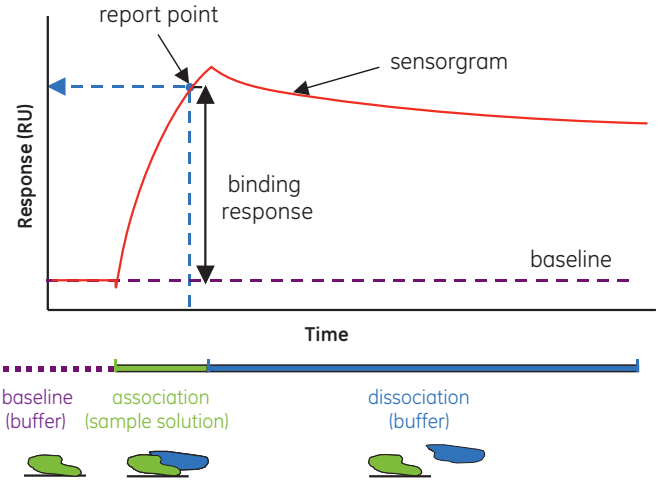
More information about label-free interaction analysis can be found at www.biacore.com

Methods

Assays were run on a Biacore A100 prototype. HTP proteins and all compounds were supplied by Hoffman La-Roche (Basel). Proteins were immobilized onto carboxymethylated sensor chips (Series S Sensor Chip CM5) using standard amine coupling.

Monitoring interactions

Biacore systems monitor molecular interactions in real-time using a label free detection method. One of the interacting molecules is immobilized onto a sensor surface, while the other is injected in solution and flows over the sensor surface. As molecules from the injected sample bind to the immobilized molecules, this results in an alteration in refractive index at the sensor surface that is proportional to the change in mass concentration. Using the phenomenon of surface plasmon resonance (SPR), these changes are detected in real time and data is presented as a sensorgram (SPR response plotted against time). Sensorgrams display the formation and dissociation of complexes over the entire course of an interaction, with the kinetics (association and dissociation rates) revealed by the shape of the binding curve.



The sensorgram provides real-time information about the entire interaction, with binding responses measured in resonance units (RU). Binding responses at specific times during the interaction can also be selected as report points

GE Healthcare
Biacore AB
Rapsgatan 7
754 50 Uppsala
Sweden

www.biacore.com

imagination at work

BR-9004-21 (05/2007)
28-9214-32 AA First published 08/2005