Characterizing lead compounds using label-free interaction analysis: Blocking T-cell activation in rheumatoid arthritis therapy

Blocking T-cell activation is a powerful therapeutic approach to autoimmune diseases such as rheumatoid arthritis (RA). Avidex Ltd. has developed a series of low molecular weight compounds that bind to the CD80 protein found on the surface of antigen-presenting cells (APC), blocking its interaction with CD28 on the T-cell surface and thereby inhibiting T-cell activation. Avidex Ltd. has employed label-free interaction analysis to provide critical binding data during lead optimization studies. A Biacore™ system was used to examine the CD80-binding properties of 259 lead compounds, providing affinity and kinetic rate constant data that enabled characterization of both general lead series properties and individual compounds. A Biacore system was also used in inhibition assays to directly examine the inhibition of CD80-CD28 binding, as well as in specificity and cross-species CD80 binding experiments. Biacore assays thus contributed important information to multiple aspects of the CD80 program. Detailed kinetic rate constant analysis identified a group of compounds of interest for further development, which would have been otherwise disregarded on the basis of standard affinity-based assessments.

Introduction

While the root causes of RA remain elusive, the mechanisms underlying disease progression are well characterized and are the key focus for therapeutic intervention. The earliest clearly definable point in the disease pathway is the aberrant activation of T-cells by healthy cells, leading to cytokine production and an inappropriate immune attack. In the classic symptoms of RA, this results in tissue destruction in the joints, often leading to serious downstream damage and deformation. A number of approaches to RA therapy are in clinical use, but most of these are aimed at late phases of the disease process, or simply aim to reduce the symptoms of the autoimmune attack. Blocking the initial aberrant T-cell activation is an attractive alternative, since this would halt the disease at the earliest possible stage.

T-cell activation is a complex process that requires multiple molecular interactions (Fig 1). Initial recognition of an APC occurs via the T-cell receptor (TCR) on the surface of the T-cell, and the antigenic peptide presented by an HLA (human leukocyte antigen) class II dimer on the surface of the APC. Following this interaction, CD4 molecules on the T-cell surface mediate a stimulatory signal to the T-cell, via interaction with the HLA molecule. This antigen-dependent signal is not sufficient to stimulate T-cell activation, cytokine production and a full immune response, however, and a second, antigen-independent signaling pathway must also come into play. This second signal is generated by the interaction of CD28 transmembrane glycoproteins on the T-cell with CD80 and CD86 on the surface of the APC. Only the combination of both the signals described will activate the T-cell. This phenomenon is known as co-stimulation and appears to be a widely used mechanism in T-cell regulation, with different pairs of molecules involved in multiple stimulatory and inhibitory pathways (1).

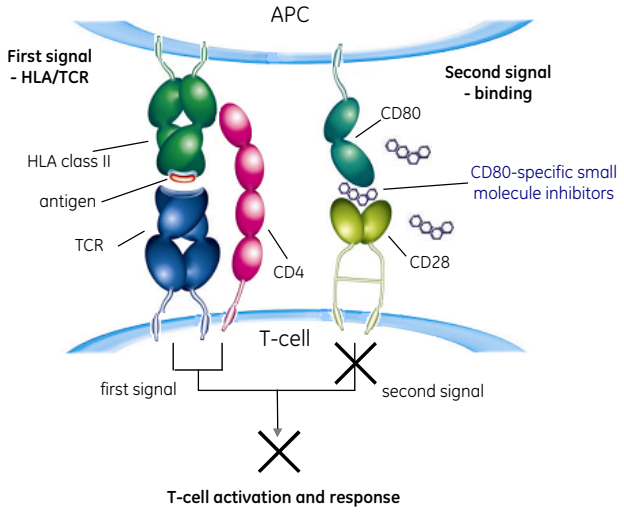


Fig 1. Activation of T-cells by APCs requires co-stimulation by both antigen-dependent and antigen-independent signals. The first signal is triggered by HLA/antigen binding to the T-cell receptor, whereas the second signal depends on the interaction of CD80 (on the surface of the APC) with the T-cell surface glycoprotein, CD28. Both signals are required for full T-cell activation. CD80 inhibitors block the CD80: CD28 interaction, preventing the second signal and thereby blocking T-cell activation (see main text).

Under normal circumstances, T-cells are not activated by self-proteins. In RA, however, the ability to differentiate between self and foreign proteins is lost, leading to uncontrolled T-cell activation and inflammation. In theory, blocking either of the T-cell activation signaling pathways should prevent progression of the clinical symptoms of RA. Blocking of the TCR-HLA/antigen interaction has proven difficult, however, and the CD28/CD80 pathway may be a more promising alternative. Avidex Ltd. has identified inhibitors that bind specifically to CD80, blocking the antigen-independent signal required for T-cell activation and potentially reducing tissue damage seen in RA (Fig 1).

As in most drug development programs, promising lead compounds such as these CD80 inhibitors are subjected to lead optimization in order to try to find improved variants with optimal characteristics for therapeutic application. This process is complex and requires technology capable of delivering high-quality data on the characterization of drug-target binding events. Moreover, a full understanding of drug-target interactions must take both association and dissociation profiles into account. Label-free interaction analysis using Biacore systems provides the type of high-quality binding data required for investigating complex biomolecular interactions, delivering comprehensive information on affinity and kinetic rate constants.

In the application described here, a Biacore system was used to study five lead series of CD80 inhibitors. This work included:

- Characterization and ranking of lead series compounds by affinity and kinetic rate constant determination
- Inhibition studies to show compound-dependent inhibition of CD80: CD28 binding and derive values for EC_{50} drug concentration required to inhibit binding by 50%
- Binding studies using CD80-related molecules to demonstrate the highly specific interaction of lead compounds with CD80

Biacore experiments provided a wealth of important data and identified the most promising compounds for further development, which would otherwise have been disregarded using other analysis methods.

Methods

All assays were performed at an analysis temperature of 25°C. Biotin-labeled recombinant proteins were immobilized by capture on a sensor surface coated with streptavidin (Series S Sensor Chip CM5); 3000 to 3500 RU of CD80 was immobilized on the sensor surface. CD86 was also immobilized and used as a negative control. Running buffer was 10 mM HEPES, pH 7.4, containing 150 mM NaCl, and 0.005% surfactant p20. The buffer was supplemented with 5% DMSO where required.

Hit selection

Hit selection assays were used for initial assessment of compounds and were run as follows: Flow rate was 30 µl/min, with three start-up cycles (buffer) and solvent correction (eight correction points) run every twenty sample cycles. Sample cycles consisted of a 30 to 60 s sample injection, a 30 to 60 s dissociation time and an extra wash with 20% DMSO to eliminate any possible carry-over. Sample compounds were injected at a concentration of 1 µM.

Compound characterization

Compound characterization assays were run as follows: Flow rate was 30 µl/min, with three start-up cycles (buffer) and solvent correction (eight correction points) run after every second sample concentration series. Sample cycles consisted of a 60 to 200 s sample injection, a 400 to 1000 s dissociation time and an extra wash with 50% DMSO. Sample concentration series were typically made as a three-fold dilution series from a maximum concentration of 1 µM, with six different concentrations used (one of which was run in duplicate) and a zero sample.

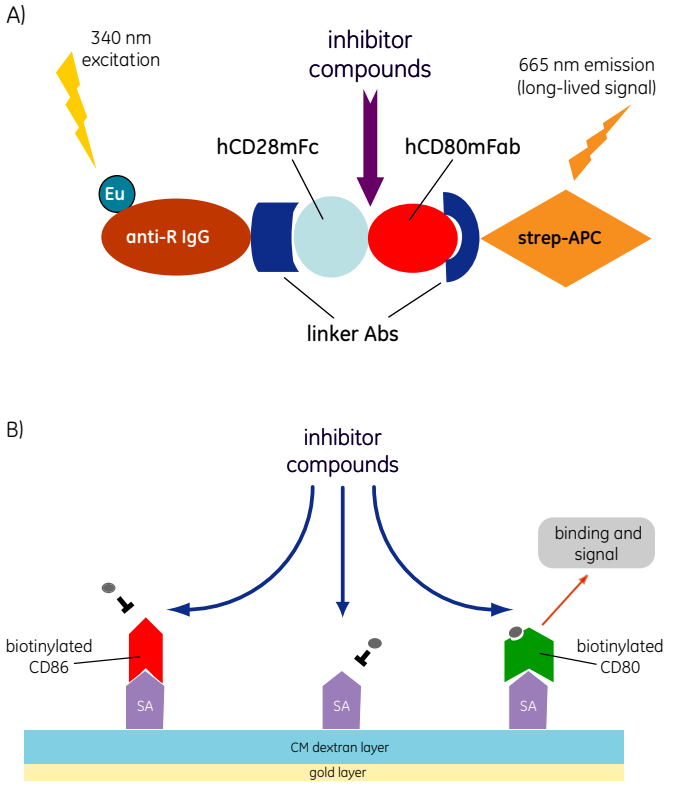


Fig 2. TR-FRET and Biacore assays for CD80 lead compounds. A) TR-FRET assay: Inhibitors are assayed for their ability to block the interaction of a human CD28-mouse Fc fusion protein (hCD28mFc) with a human CD80-mouse Fab fusion protein (hCD80mFab). The recombinant proteins are each coupled via linker antibodies to fluorophores: Anti-rabbit IgG labeled with europium (Eu) and streptavidin-conjugated allophycocyanin (strep-APC). Interaction of recombinant CD28/CD80 proteins results in a long-lived, 665 nm fluorescent signal, due to fluorescence resonance energy transfer between the fluorophores. CD80-binding compounds inhibit the interaction between CD80 and CD28 and block the FRET-dependent signal. B) Biacore assay: A sensor surface is coated with streptavidin (SA) and is used to capture biotinylated CD80 and CD86 (negative control protein). Binding of lead compounds is then detected as a change in mass concentration close to the surface, generating a real-time signal over the entire course of the interaction.

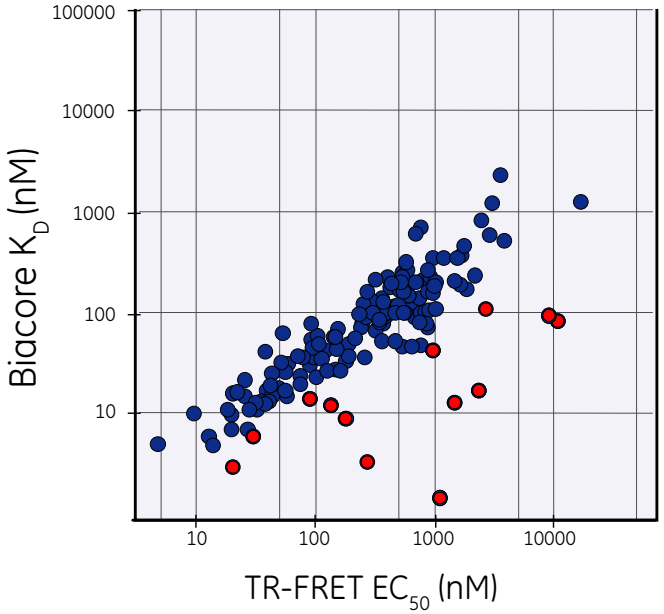


Fig 3. Comparison of Biacore-derived affinity values for compound-CD80 binding and TR-FRET-derived EC_{50} values for inhibition of CD80-CD28 binding. Lead compounds were examined in parallel using Biacore and TR-FRET assays. Affinity constants (K_D) from Biacore direct binding assays are shown on the y-axis and EC_{50} data from the corresponding TR-FRET assays are shown on the x-axis (log scales in both cases). The structure-activity relationships of the majority of compounds (marked in blue) were essentially identical using the two assays. A number of compounds containing terminal benzyl groups (marked in red) had higher affinities when measured using a Biacore assay than predicted by TR-FRET. Interestingly, these compounds were also found to be cytotoxic.

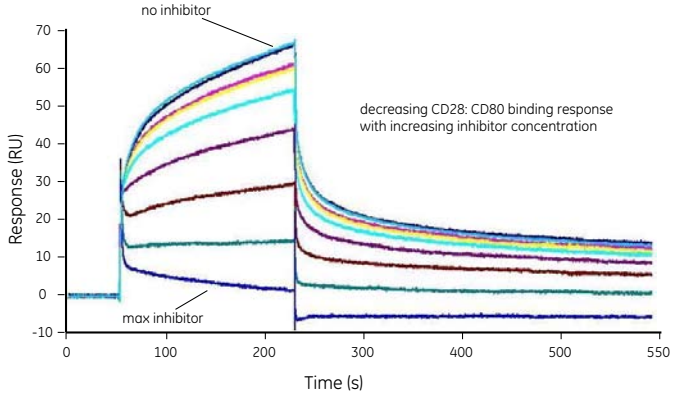


Fig 4. Biacore assay showing inhibition of CD28: CD80 binding by a CD80 program lead compound. CD80 was immobilized on a sensor surface and samples containing 200 nM CD28 in the presence of increasing concentrations of inhibitor (from 4.12 nM to 1 µM) were injected over the prepared surface. The interaction profiles show response in resonance units (RU) plotted against time, with the switch from sample injection to running buffer (marking the transition from association to dissociation phases) seen at around 225 s. Since the inhibitors bind to CD80, response measurements from injections of equivalent concentrations of inhibitor alone were subtracted from the inhibitor + CD28 data to produce the final interaction profiles shown. For the compound shown, EC_{50} was 160 nM.

Results

Analysis of lead compound series using complementary assays

The primary screening was carried out using a time-resolved fluorescence resonance energy transfer (TR-FRET) assay. In this approach, CD80-mouse Fab and CD28-mouse Fc fusion proteins are coupled to fluorescence resonance energy donor and acceptor species, respectively, via linker antibodies. Lead compounds were then screened for their ability to block the interaction of CD80 and CD28 and hence reduce TR-FRET-dependent light emission. This technique measures the functional inhibition properties of the lead compounds and also enables potency to be determined for positive "hits", by varying the compound concentration to establish EC_{50} (the drug concentration required to inhibit CD80-CD28 binding by 50%). The principle of the TR-FRET assay is shown in Figure 2, alongside a schematic design of a Biacore assay.

Although informative, TR-FRET provides no direct information about the characteristics of compound binding to the CD80 target. Using a Biacore assay, however, detailed analysis of compound-target binding can readily be achieved (providing affinity and kinetic rate constant data), complementing lead optimization assays. Since the TR-FRET assays provided EC_{50} data, this enabled a comparison of Biacore and TR-FRET assays used in the CD80 program. For the great majority of compounds, the relationship between structure and activity was essentially identical when assessed by either affinity (Biacore assay) or EC_{50} values (TR-FRET). These comparative data (Fig 3) indicate that the primary inhibition screening was an accurate reflection of compound-CD80 binding characteristics.

Importantly, Biacore analysis of compounds belonging to the five lead series also showed that their affinities for CD80 were at least two orders of magnitude higher than that of CD28 (Table 1).

A Biacore system was also used for inhibition assays. In these experiments, CD80 was immobilized on a sensor surface and a series of samples containing a fixed amount of CD28 in the presence of an increasing concentration of lead compounds was injected over the surface. Using this approach, a concentration-dependent inhibition of CD28 binding to CD80 was readily observed (Fig 4). EC_{50} values obtained using Biacore correlated well with those from the TR-FRET assays (data not shown).

Table 1. CD80-binding characteristics of representative inhibitor lead series compounds and CD28 determined in a Biacore assay. Affinity (equilibrium dissociation constant, K_D) and dissociation rate constant (k_d) data measured using Biacore assays are shown for CD80-binding by one inhibitor compound from each of five lead series (A to E), as well as for the biological CD80 interaction partner, CD28.

Compound	Biacore K_D (nM)	Biacore k_d (s^{-1}) x 10^{-3}
CD28	2000	> 200
A	150	119
B	12	226
C	7	12.2
D	2	7.7
E	30	0.3

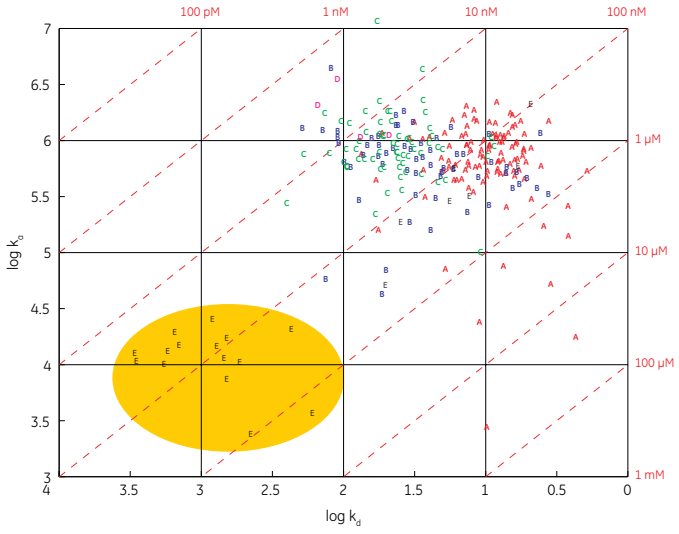


Fig 5. k_a/k_d map of lead inhibitor compound series binding to CD80. A total of 259 lead compounds belonging to five chemically related series (A to E) were analyzed using a Biacore system, to derive k_a and k_d. Log values are shown. The dotted lines indicate isometric affinity diagonals. In this format, "ideal" binding characteristics (most rapid association and slowest dissociation) would appear in the top-left quadrant of the map. The lead series identity of individual compounds is indicated both by letter and color-coding (A = red, B = blue, C = green, D = lilac, and E = black). The yellow-shaded area indicates a group of series E compounds with particularly interesting kinetic properties (see main text).

Kinetic analysis of CD80 binding for five series of lead compounds

As with all drug discovery projects, optimization of the target binding properties of lead inhibitors through development and analysis of chemical variants is an important part of the process. The kinetic rate constants provided by label-free interaction analysis provide an excellent basis for the assessment of lead compounds, enabling a deeper understanding of binding properties than can be achieved from affinity data alone (2). Compound characterization assays were therefore used to examine the CD80-binding properties of 259 lead compounds, belonging to five chemical series, designated A to E. Individual association and dissociation rate constants (k_a and k_d, respectively) were calculated for all of the lead compounds and used to generate a map of log k_a against log k_d, producing diagonals that display the affinities corresponding to the various k_a and k_d coordinates (Fig 5). These maps illustrate the importance of kinetic data, since compounds with the same affinity can have radically different combinations of association and dissociation rates.

As shown in Figure 5, although binding characteristics varied within all five series of lead compounds, some general series-specific properties were apparent. For the three largest series (A to C), target affinity generally increased from series A to C. Moreover, a general trend emerged showing that these increases in affinity were principally due to increases in binding stability (i.e., slower dissociation rates). Although series D contained relatively few compounds, these tended to have higher affinities for the target compared to compounds in series A to C.

The resolution of affinity values into individual rate constants using a Biacore assay identified a particularly interesting subset of the series E compounds, see yellow-shaded area in Figure 5. These compounds were of only moderate-to-low potency, showing affinities with little or no improvement compared to the original series A compounds. Under normal circumstances, this series would have been excluded from further development due to their relatively poor potency. Analysis using Biacore, however, revealed that although this low potency was due to much slower association rates than the other compounds, the series E compounds also exhibited by far the slowest dissociation rate of all the compounds examined. A slow dissociation rate is an extremely desirable binding characteristic for an inhibitory drug, making these series E compounds considerably more interesting than their potency alone would suggest. Since chemical modifications could be engineered to improve the association rates of these compounds while maintaining their slow dissociation rates, they were retained by Avidex Ltd. as a highly promising series for further development.

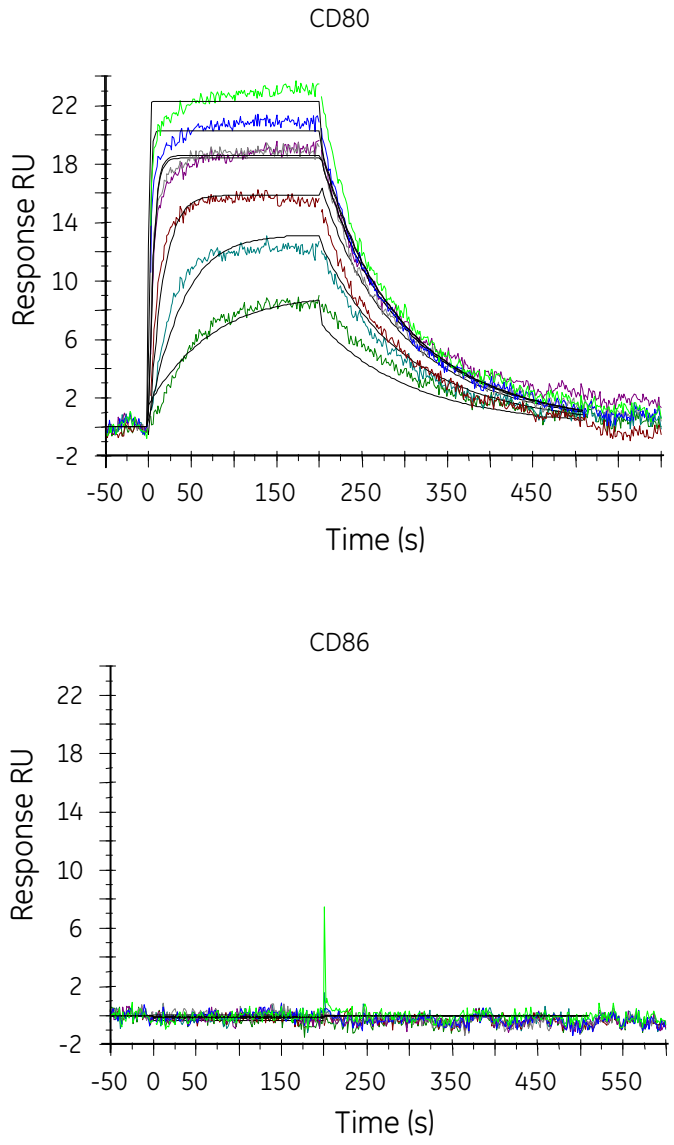


Fig 6. Specificity of lead inhibitors for CD80. The binding responses from a concentration series of one lead compound are shown for immobilized CD80 (top) and CD86 (bottom).

Specificity of binding to human CD80

While target-binding characteristics are the principal focus in the early stages of drug development strategies, specificity is also important. It was desirable, therefore, to show that lead compounds bound specifically to the intended target. Figure 6 shows an example of specificity data for a lead compound binding to target and control proteins. In the example shown, a concentration series of lead compound was analyzed for binding to CD80 and to the closely related CD86 protein. The lead compound was shown to bind CD80 (seen in the interaction profile as a concentration-dependent increase in binding response), whereas no response was seen with CD86 (Fig 6).

Given that later stages of drug development are likely to involve cell-based and animal model experiments, species-specificity of CD80-binding was also of interest. Soluble extra-cellular fragments of mouse, rat, and dog CD80 were cloned and used in Biacore-based specificity assays. The viabilities of these CD80 species variants were checked with CD28 from the appropriate species. A series of 96 compounds was then selected for analysis from a CD80 inhibitor lead compound library. Although the selected compounds included all the most potent examples, and also encompassed the diversity present in the library, no binding was observed to CD80 from mouse, rat, or dog (data not shown). These results are important, since they indicate that there would be little point in performing studies on animal models of human autoimmune disease.

Conclusions

Based on the data presented here, and on additional studies carried out at Avidex Ltd., the CD80 program lead compounds have excellent potential for therapeutic use against RA and other autoimmune diseases:

- High-affinity (nM) binding to CD80 (100-fold higher than affinity of CD28 for CD80)
- Efficient blocking of CD80-CD28 binding in inhibition assays
- Extremely specific for CD80
- Favorable characteristics for oral administration

Data from Biacore assays significantly contributed to the lead optimization process, demonstrating the value of label-free, real-time interaction analysis in drug discovery. In terms of affinity measurements, results from Biacore assays correlated well with TR-FRET screening and also provided a useful complement to the inhibition studies.

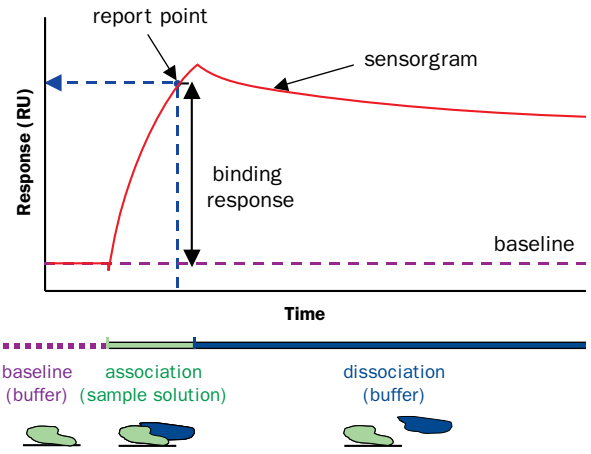
More importantly, interaction analysis using Biacore systems provided crucial kinetic information that enabled a much more informed assessment of the lead series compounds than would have otherwise been possible using alternative technologies. Examining the k_a and k_d values of all the tested compounds provided an excellent overview of lead series characteristics, while at the same time pinpointing individual compounds of particular interest. The key findings from this approach included:

- A clear trend for improved target affinity going through lead series A to D, with the specific contributions of association and dissociation rates readily apparent
- The identification of the low-affinity series E compounds as a promising group for further development, due to their very slow dissociation rates

In summary, the studies described here show that the CD80 inhibitor lead compounds may provide the basis for an effective new therapy for RA. Moreover, the data demonstrate the great value of Biacore assays in multiple aspects of drug discovery and highlight the invaluable contribution of high-quality kinetic information in making such studies possible.

Monitoring protein interactions with Biacore systems

Biacore systems monitor protein 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 are 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. More information can be found at **www.gelifesciences.com/biacore**.

References

1. Chambers, C. A. The expanding world of co-stimulation: the two-signal model revisited. *Trends Immunol.* **22**, 217–223 (2001).

2. Proof Statement. Quantitative structure activity relationship (QSAR) studies using Biacore, Biacore AB, BR-9003-15 (2003).

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For local office contact information, visit
www.gelifesciences.com/contact

GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

www.gelifesciences.com/biacore

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