Comparative analysis of 10 small molecules binding to carbonic anhydrase II by different investigators using Biacore technology


Abstract

In this benchmark study, 26 investigators were asked to characterize the kinetics and affinities of 10 sulfonamide inhibitors binding to the enzyme carbonic anhydrase II using Biacore optical biosensors. A majority of the participants collected data that could be fit to a 1:1 interaction model, but a subset of the data sets obtained from some instruments were of poor quality. The experimental errors in the $k_a$, $k_d$, and $K_D$ parameters determined for each of the compounds averaged 34, 24, and 37%, respectively. As expected, the greatest variation in the reported constants was observed for compounds with exceptionally weak affinity and/or fast association rates. The binding constants determined using the biosensor correlated well with solution-based titration calorimetry measurements. The results of this study...
There is growing interest in applying optical biosensor technology, such as surface plasmon resonance (SPR)\(^1\)-based Biacore instruments, in drug discovery. With the wide array of opportunities offered by this technology, however, come some challenges. For example, based on our yearly review of the optical biosensor literature [1–6], we find that there is still a need to train investigators in how to design and perform biosensor experiments as well as in how to properly process and analyze data. And based on comments we receive from manuscript reviewers and participants at scientific meetings, we see that some skepticism remains regarding the validity of the binding constants determined using biosensors. For the past few years, we have been addressing both of these issues by coordinating intra- and inter-technology benchmark studies.

In our first comparative study, 30 SPR biosensor users illustrated that the affinity determined from the biosensor for a small molecule/enzyme interaction matched the value determined in solution using isothermal titration calorimetry (ITC) [7]. These results confirmed that immobilization of an enzyme onto the sensor surface does not necessarily affect the binding constants as is often presumed by critics of the biosensor approach. Then, we organized a study with 36 participants who analyzed the interaction of a small molecule/macromolecular target system having a very fast association rate [8]. The results of this test established experimental and data processing protocols for mass transport-limited reactions. Recently, we coordinated a study with 22 users resolving the binding constants for a high-affinity monoclonal antibody–antigen interaction [9]. This work trained users in how to generate and analyze data for a slowly dissociating system. Together, these benchmark studies educated the users of biosensor technology, as well as the general scientific community, with regard to what we can expect to see in terms of the variability of results within a given experimental system.

The current study builds on this previous work and is modeled on a growing biosensor application in a drug discovery setting: the rapid and reliable determination of kinetic and affinity constants for a small panel of compounds binding to a single protein target. This information is becoming crucial to better rationalizing compound behavior in bioassays and helping to steer compound selection and design strategies. The goal of the current biosensor study was to determine the consistency of the results for small molecule analysis and to discover ways to further improve the application.

The target protein for this work was carbonic anhydrase II, an enzyme responsible for the conversion of carbon dioxide to bicarbonate. Current inhibitors of this enzyme are used to treat diseases such as glaucoma and epilepsy, and future drugs targeting this enzyme may lead to treatments for cancer and obesity [10]. Thus, the choice of carbonic anhydrase as the target represents a realistic model for small molecule drug studies.

All participants in this study were provided the same reagents and were asked to follow the same experimental protocol. We found that a majority of the participants were able to resolve the binding constants for all 10 compounds. We did, however, identify a suboptimal subset of data (sensorgrams that were marred by features such as spikes and drift) and we explored the causes of these features. We also compared the equilibrium dissociation constants obtained for the 10 compounds with those determined by ITC. We found an excellent correlation between the two methods across the wide range in affinities exhibited by these compounds. Our results highlight the strengths of biosensor analysis but also point to some challenges.

Materials and methods

**Instrumentation and reagents**

Interaction analyses were performed using Biacore 1000, 2000, 3000, S51, and T100 instruments (Biacore, Uppsala, Sweden). Sensor chips, N-hydroxysuccinimide (NHS), N-ethyl-N’-(3-dimethylaminopropyl)carbodiimide (EDC), and ethanolamine HCl, as well as sampling vials, caps, and 96-well plates, were obtained from Biacore. Carbonic anhydrase isozyme II (CAII) from bovine erythrocytes, the 10 sulfonamide inhibitors, dimethyl sulfoxide (DMSO), buffer reagents, and general laboratory supplies were purchased from Sigma–Aldrich (St. Louis, MO, USA).

**Instrument cleaning**

Before beginning the experiment, each participant was asked to perform a series of instrument cleaning steps. First, the instrument was primed with water, the previously used chip was undocked, and a maintenance chip was docked. The instrument was then primed five times using 0.5% (w/v) sodium dodecyl sulfate (SDS), once using water, five times using 50 mM glycine (pH 9.5), and once again using water.

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\(^1\) **Abbreviations used:** SPR, surface plasmon resonance; ITC, isothermal titration calorimetry; NHS, N-hydroxysuccinimide; EDC, N-ethyl-N’-(3-dimethylaminopropyl)carbodiimide; CAII, carbonic anhydrase isozyme II; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; RU, response units; CV, coefficient of variation.
**Chip preconditioning**

After docking the CM5 sensor chip and priming the instrument with running buffer, the chip was preconditioned with two 10-µl injections each of 100 mM HCl, 50 mM NaOH, and 0.5% SDS at a flow rate of 100 µl/min.

**Buffer preparation**

Participants were provided with a 10× phosphate-buffered saline (PBS, 1× = 20 mM Na₃HPO₄–NaH₂PO₄, 150 mM NaCl, pH 7.4) stock solution. Then 70 ml of this 10× PBS was added to 630 ml of degassed deionized water to make 1× PBS buffer, and this buffer was filtered. Then 18 ml of DMSO (also provided to the participant) was added to 600 ml of the 1× PBS buffer. DMSO is commonly used as a solvent for small molecule studies. Although the final concentration of DMSO in this buffer via this addition is actually 2.97%, for simplicity this buffer is termed “running buffer with 3% DMSO”. The remaining 100 ml of 1× PBS was divided into two 50-ml portions. One of these portions was set aside for use as “immobilization buffer”, and the other was set aside as “sample preparation buffer” with no DMSO for use in analyte preparation.

**Enzyme immobilization**

Participants were provided with aliquots of 100 µg of lyophilized CA II that were centrifuged and dissolved in 400 µl of 10 mM sodium acetate (pH 4.9) after the biosensor was primed with immobilization buffer and equilibrated to 25°C. Using a flow rate of 20 µl/min, the surface of flow cell 1 was activated for 7 min using a mixture of 0.1 M NHS and 0.4 M EDC. 0.25 mg/ml CAII was injected for 7 min, and residual activated groups on the surface were blocked by a 7-min injection of 1 M ethanolamine (pH 8.5). Users of S51 platforms activated the surfaces via sequential injections of NHS and then EDC for 7 min. This was followed by injection of 0.25 mg/ml CAII for 7 min at a flow rate of 10 µl/min. Surfaces were then blocked via a 7-min injection of ethanolamine at a flow rate of 10 µl/min. On average, 6900 response units (RU) of CAII were immobilized. For this set of studies, we chose to leave flow cell 2 unmodified as a reference surface.

**Analyte preparation**

For simplicity, each of the compounds analyzed in this study was assigned a number (Table 1). Participants were provided with a stock solution of each compound dissolved in DMSO. To prepare the samples for analysis, 30 µl of each compound solution was added to 1 ml of sample preparation buffer with no DMSO and mixed thoroughly. Preparation of analyte in this manner ensures that the concentration of DMSO is matched with that of running buffer with 3% DMSO. Three lower concentrations of each of the 10 compounds were then prepared via fivefold serial dilutions into running buffer with 3% DMSO. For analyses using Biacore 1000, 2000, and 3000, 100 µl of each compound at the highest concentration was added to 400 µl of running buffer with 3% DMSO and this dilution was repeated. Samples and buffer blanks were aliquotted in 7-mm Biacore vials, which were then capped with soft rubber caps (BR-1005-55) that reseal after puncture to minimize evaporation. Using these sample volumes and the resealable caps permitted the three concentrations of each compound to be sampled twice. Biacore S51- and T100-based analyses used 96-well plates for sampling. Dilutions were performed directly in the plates by taking 50 µl from wells containing 250 µl of the highest analyte concentration and diluting successively into wells containing 200 µl of running buffer with 3% DMSO.

To correct for the excluded volume effect, a DMSO calibration series was prepared by adding 15 µl of DMSO and 150 µl of water to separate 1.5-ml aliquots of running buffer with DMSO to obtain “+” and “−” solutions. Then 400, 300, 200, 100, and 0 µl of the “+” solution were dispensed into Eppendorf tubes labeled d1 to d5, and 0, 100, 200, 300, and 400 µl of the “−” solution were added to tubes d1 to d5 so that each tube contained a final volume of 400 µl. The five DMSO calibration solutions were either aliquotted into 7-mm Biacore vials or, for Biacore S51 and T100 analyses, dispensed into the 96-well plate.

**Instrument optimization**

To create a slight mismatch in the refractive index of the running buffer and sample solutions, water (0.4% of the total volume of running buffer) was added to the running buffer with 3% DMSO. By making the start of each inje-

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**Table 1**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Compound</th>
<th>Molecular mass (Da)</th>
<th>Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(±)-Sulpiride</td>
<td>341</td>
<td>2, 10, 50</td>
</tr>
<tr>
<td>2</td>
<td>4-(Aminomethyl)benzenesulfonamide hydrochloride hydrate</td>
<td>223</td>
<td>2, 10, 50</td>
</tr>
<tr>
<td>3</td>
<td>Sulfanilamide</td>
<td>172</td>
<td>0.4, 2, 10</td>
</tr>
<tr>
<td>4</td>
<td>Furosemide</td>
<td>331</td>
<td>0.4, 2, 10</td>
</tr>
<tr>
<td>5</td>
<td>4-Carboxybenzenesulfonamide</td>
<td>201</td>
<td>0.4, 2, 10</td>
</tr>
<tr>
<td>6</td>
<td>Dansylamide</td>
<td>250</td>
<td>0.08, 0.4, 2</td>
</tr>
<tr>
<td>7</td>
<td>1,3-Benzenedisulfonamide</td>
<td>236</td>
<td>0.08, 0.4, 2</td>
</tr>
<tr>
<td>8</td>
<td>Benzenesulfonamide</td>
<td>157</td>
<td>0.4, 2, 10</td>
</tr>
<tr>
<td>9</td>
<td>7-Fluoro-2,1,3-benzoxadiazole-4-sulfonamide</td>
<td>217</td>
<td>0.016, 0.08, 0.4</td>
</tr>
<tr>
<td>10</td>
<td>Acetzolamide</td>
<td>222</td>
<td>0.016, 0.08, 0.4</td>
</tr>
</tbody>
</table>
tion easily identifiable, this mismatch aids in data processing [8]. The instrument was primed three times with this spiked running buffer and then normalized using 40% (v/v) glycerol for Biacore 1000 and 2000 or 70% (v/v) glycerol for Biacore 3000, S51, and T100.

Analyte injection order and analysis method

Data were collected at the highest collection rate possible and at 25 °C. An initial series of buffer blanks was injected first to fully equilibrate the system. The DMSO calibration series was then injected, followed by two additional blank injections. Finally, the individual compound samples were tested (from lowest to highest concentrations within each series) and each compound series was separated by a blank buffer injection. During each binding cycle, analyte was injected for 1 min at a flow rate of 100 μl/min (90 μl/min for Biacore S51) and dissociation was monitored for 140 s. The analyte injection was followed by an Extra-Clean wash command that automatically flushes the sample delivery system with running buffer (for Biacore 2000 and 3000, a 15-μl injection of running buffer was also included as an additional wash step). Analytes were sampled twice using Biacore 1000, 2000, and 3000 and were sampled once using Biacore S51 and T100.

Data processing and kinetic analysis

Data sets were processed and analyzed using Scrubber 2 (BioLogic Software, Campbell, Australia). Double-referenced [11] association and dissociation phase data for compounds 1 to 8 were globally fit to a simple 1:1 interaction model \( (A + B = AB) \). Compounds 9 and 10 were fit to a 1:1 interaction model that included a mass transport term \( (A_o = A + B = AB) \) [12]. Means and standard deviations were computed using Microsoft Excel software. The experimental error or coefficient of variation (CV) was calculated as \( \text{Standard Deviation/Mean} \times 100 \).

Isothermal titration calorimetry

Protein was dissolved in \( 1 \times \) PBS and buffer exchanged to minimize any contaminants from lyophilization. Compound was diluted in the same buffer as protein to minimize heat of

![Image](image-url)

dilution effects from buffer mismatch. Experiments were performed with the compound in the syringe and the protein in the cell. Protein concentration was measured using an extinction coefficient at A280 of 50,070 L mol\(^{-1}\) cm\(^{-1}\). Isothermal titration calorimetry experiments were performed using a Microcal VP-ITC instrument (MicroCal, Northampton, MA, USA)\[13\]. Experimental parameters included: 30 injections of compound, with an initial first injection of 3\(\mu\)l and subsequent 10-\(\mu\)l injections of compound into protein with 240 s between each injection and a stirring speed of approximately 300. Experiments were performed at 25 °C. The heat of dilution was taken either from the points after saturation, control experiment of compound into buffer, or from a minimization of the \(\chi^2\) value. The concentrations of compounds titrated were 960, 666, 600, and 400 \(\mu\)M for compounds 1 to 4, respectively, 350 \(\mu\)M for compounds 5–8, and 300 \(\mu\)M for compounds 9 and 10. Corresponding concentrations of carbonic anhydrase were 44, 65, and 38 \(\mu\)M for titrations of compounds 1–3 and 28 \(\mu\)M for titrations of compounds 4–10. Data were analyzed with a single-site binding model using the Origin software provided by MicroCal. To minimize the dependency of the fitting parameters for compound 1, the weakest inhibitor, the stoichiometry value was set to 1 assuming a 1:1 ratio, which has been well established for this system.

Results

In this benchmark study, participants used Biacore biosensor technology to characterize the interactions of 10 compounds with the enzyme CAII. We provided each participant

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Fig. 3. Data sets obtained using Biacore 1000 (A) and Biacore 2000 (B–I) instruments. Columns 1 through 10 correspond to the numbering of compounds in Table 1. Responses shown in red exhibited one or more of the characteristics described in Fig. 2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
with a detailed experimental protocol as well as stock solutions of the enzyme, compounds, and buffer. CAII was immobilized on one flow cell surface of a CM5 sensor chip using standard amine-coupling chemistry and an unmodified flow cell surface served as a reference. Immobilization densities ranged from 2400 to 13,000 RU; this variation was useful in determining whether there were any systematic trends in the results with surface density. Three concentrations of each compound, prepared in a fivefold dilution series, were analyzed for CAII binding at 25°C. Each concentration series was bracketed by buffer blanks that were included for double referencing [11]. Because each compound dissociated completely from the CAII surface within minutes after the end of the association phase, no regeneration step was required.

Participants using Biacore 1000, 2000, and 3000 platforms did repeat injections of each analyte concentration, whereas the Biacore S51 and T100 users performed a single injection of each.

**Good and bad sensorgrams**

To illustrate how we interpreted the responses generated by the different users, we first highlight examples of good and bad data. An example of a good data set obtained for one compound is shown in Fig. 1. From inspection of this data set, we can tell that the experiment was designed and executed properly, the biosensor was performing optimally, and the obtained responses are reliable. Hallmarks that...
make this data set a good one include responses that are concentration dependent, replicate injections that overlay, and clearly discernible exponential curvature during both the association and dissociation phases. When data sets are of high quality like the one shown in Fig. 1, they often can be described by a 1:1 interaction model. And when data sets are of poorer quality (e.g., when they exhibit unusual profiles or can be described only by a complex model), the anomalies and/or complexity most often can be eliminated by optimizing the assay design.

Although we intentionally chose well-characterized, well-behaved 1:1 interactions for this study, the quality of some of the data sets generated by our study participants was not on par with the example shown in Fig. 1. Six of the common anomalous sensorgram features we observed in some data sets are depicted in Fig. 2.

For example, significant spikes at the both the beginning and end of the association phase apparent after referencing (Fig. 2A) indicate poor-quality injections that may be caused by a fouled integrated fluidic cartridge. Although it is common to have a few data points producing spikes at the transitions between running buffer and the analyte sample, spikes that occur over a larger window of time (e.g., >2 s) can result in the loss of kinetic information when the binding responses are fast. Frequent spikes throughout a sensorgram or sets of sensorgrams (Fig. 2B) often are indicative of a poorly degassed buffer or a dirty injection system. Blanks should have similar profiles and generally overlay, but the not-reproducible responses from the replicate buffer blanks shown in Fig. 2C would make it difficult to choose which blanks to use for double referencing. Also, inconsistencies in buffer injections may indicate sample carryover. The inability to achieve steady state after a fast association phase (Fig. 2D) in a system known to be 1:1 suggests that there may be problems with reagent preparation, the biosensor’s fluidic system, or both. Sensorgrams that, after referencing, dip down during the association phase, and in some cases drop below the baseline during the dissociation phase (Fig. 2E), may result from nonspecific binding to the reference surface. Sustained jumps in refractive index

Fig. 5. Data sets obtained using Biacore S51 (T–Z) and Biacore T100 (AA) instruments. Columns 1 through 10 correspond to the compound numbering given in Table 1. Responses shown in red exhibited one or more of the characteristics described in Fig. 2. Due to a lack of compound, participant Z was unable to collect data for compound 8. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
(Fig. 2F) make obtaining reasonable fits impossible without elaborate data processing. Sensorgrams that displayed any of the characteristics illustrated in Fig. 2 were highlighted in the participants’ data sets.

**Visual inspection of participants’ data**

All of the data sets submitted by the 26 participants are shown in Figs. 3–5. Down each column in these figures, the consistency in the binding profiles demonstrates the similarity between data sets obtained for a single compound from different participants. Across each row, the different binding profiles demonstrate that the 10 compounds bind the target with different affinities. Sensorgrams considered to be acceptable for kinetic analysis are shown in black, whereas those shown in red exhibited one or more problems. Significantly flawed sensorgrams were omitted from the kinetic analysis.

![Figure 6: Kinetic analysis of data sets shown in Figs. 3–5. Binding responses (black lines) are overlaid with the fit of a 1:1 interaction model (red lines). Responses for compounds 1 to 8 were fit to a simple 1:1 model, and responses for compounds 9 and 10 were fit to a 1:1 model that included a mass transport term. Rate constants determined from this analysis are summarized in Table 2. Individual data sets omitted from the kinetic analysis are indicated by red Xs, whereas data panels from participants A, J, K, N, O, P, R, and S were excluded entirely. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)](image-url)

Data set A was obtained using a Biacore 1000 instrument (Fig. 3). Given the challenges in working with this older system, participant A focused on the analysis of the highest affinity compounds: 8, 9, and 10. Due to the absence of an in-line reference flow cell in Biacore 1000, changes in bulk refractive during the course of the injection could not be referenced properly. In some cases, it is possible to model the bulk shift, but other deviations in the sensograms require the ability to double reference the data. It is surprising that it is possible to detect binding responses for small molecules using the Biacore 1000. Although the data might not be as clean as those with more recent instrument platforms, it is interesting to note that even these very early versions of Biacore technology were capable of small molecule detection at the qualitative level.

Data sets B–I were obtained using Biacore 2000 instruments (Fig. 3). Overall, the responses are of excellent quality, and 96% of these sensograms were included in the kinetic analysis. Data sets J–S were obtained using Biacore 3000 instruments (Fig. 4). Much of the data collected from the Biacore 3000 instruments were compromised in quality, and only 18% of these sensograms were included in the kinetic analysis. Common features include large spikes at the beginning and end of the association phase as well as spikes throughout the data sets. Interestingly, data sets Q and S (Fig. 4) were obtained using Biacore 3000 instruments after servicing that included replacing the integrated fluidic cartridge, and yet the data are still of poor quality. These results suggest that the problem might lie elsewhere in these systems. At this time, we do not know the cause of the high number of failed sensograms obtained from these and the other Biacore 3000 instruments.

Data sets T–Z and AA (Fig. 5) were obtained using Biacore S51 and Biacore T100 instruments, respectively, and 90% of these sensograms were included in the kinetic analysis.

Kinetic analysis

Data sets collected for compounds 1–8 were fit to a simple 1:1 model, and data sets for compounds 9 and 10 were fit to a 1:1 model that included a term for mass transport.
The fits of the data sets shown in Figs. 3–5 are displayed in Fig. 6. In general, each compound–target interaction was well described by a 1:1 interaction model. The range of kinetic parameters derived for the 10 compounds is listed in Table 2 and illustrated in the kinetic distribution plot (Fig. 7). The spread of the data points in the horizontal, vertical, and diagonal (perpendicular to the dashed lines) directions shown in Fig. 7 for each compound corresponds to the variability in $k_a$, $k_d$, and $K_D$ reported by the panel of participants. In general, data points are tightly clustered and the CV in each of the binding constants is less than 40%: CV($k_a$) = 34%, CV($k_d$) = 24%, and CV($K_D$) = 37%.

The spread in the constants determined for compounds 2 and 7 (e.g., CV($K_D$) = 47 and 87%) resulted from one and four outlier data sets, respectively; the cause of these discrepancies is not known. An above-average CV (32%) in $k_d$ for compound 1 is not unexpected given that the dissociation rate for this compound is very fast and therefore this set of sensorgrams contains less information than those in other data sets. A larger experimental error in $k_a$ and $k_d$ for compounds 9 and 10 relate to the fact that these compounds have very fast association rates and are influenced by mass transport. However, because under transport limited conditions the $k_a$ and $k_d$ values become coupled, their ratio in fact leads to similar $K_D$ values. The changes in one parameter are compensated for by changes in the other parameter, so the ratio of the two (i.e., $K_D = k_d/k_a$) is not affected during fitting. The result is that the CVs for the $K_D$ of compound 9 (33%) and compound 10 (35%) compare favorably with the average for the 10 compounds (CVavg($K_D$) = 37%).

The 10 compound–target interactions were also studied in solution using ITC. Fig. 8A shows the heat evolved as compounds 2, 4, 6, and 8 were titrated into solutions of CAII. The titrations shown are representative of those observed for all of the compounds. The raw heats from these titrations were integrated to generate plots of kilocalories/mole of injected compound versus the molar ratio of compound and CAII (Fig. 8B). Importantly, all show a molar ratio of 1 at the midpoint of titration, indicating a 1:1 stoichiometry for each interaction.

The $K_D$ values from the ITC analyses were plotted against those obtained using SPR (Fig. 9). The affinities determined from the two methods are highly correlated (99.8%), with the greatest difference in $K_D$ between the two methods being only twofold for compound 6.

**Discussion**

A total of 26 participants contributed to this collaborative study of 10 compounds binding to CAII. To balance sampling throughput and kinetic resolution, the participants examined relatively few (three), but widely diluted (fivefold), concentrations of each compound. Participants performed the analysis using five different Biacore platforms. In our review of the participants’ data sets, we were
surprised by the large number of low-quality sensorgrams obtained using Biacore 3000 instruments (Fig. 4). At this time, we do not know the source of the poorer quality data originating from Biacore 3000 instruments and whether or not it is specific to this particular set of samples. Initially, we thought that this was perhaps attributable to contaminated fluidic cartridges. This explanation seems unlikely, however, given that two data sets were collected from Biacore 3000 instruments before (P and R) and after (Q and S) professional servicing during which the fluidic cartridges were replaced. No improvement in data quality was observed between data sets R and S, and only a partial improvement in sensorgram quality was observed between data sets P and Q. These results suggest that the issue with running this assay using Biacore 3000 instruments may lie elsewhere.

The kinetic distribution plot (Fig. 7) of the compounds provides insight into the variation one could expect to see for this type of analysis. We observed greater experimental error in the binding constants for weak interactions, and this was expected because there is less information in these data sets. We also observed greater experimental error for compounds that were influenced by mass transport. This was not unusual and helps to quantitate the types of variation one would expect to see in the assay. Overall, the experimental error in the reaction parameters for all 10 compounds is small (average ~30%), particularly when one considers the challenges associated with analyzing small molecule interactions using SPR.

The dissociation constants derived from the SPR and ITC studies of the 10 compounds were in excellent agreement (Fig. 9). This observation, along with the high degree of correlation observed for a number of other experimental systems [6,14], deflates the criticism that the surface-based SPR method alters binding constants. This is because these experiments, like the great majority of Biacore-based experiments, involved the immobilization of ligand to a fluid-like dextran layer attached to the gold surface and not to the gold surface directly. Clearly, the affinity of CAII for these small molecules is not significantly altered on immobilization in this manner. Rather than the surface itself producing erroneous binding constants, our experience suggests that sample heterogeneity and inappropriate experimental design and/or data analysis are the most common sources of reported discrepancies between solution- and surface-based methods.

This study also emphasizes the value of kinetic analyses compared with equilibrium-based analyses that yield only affinity information; the CAII affinities of compounds 4, 5, and 8 are statistically identical, but these compounds are distinguishable based on their kinetic profiles. This kinetic profiling provides critical information in a drug development setting, for example, in which an investigator wants to understand in detail how changes in a compound’s structure affect its binding activity.

In general, we are pleased to see that the variation in the binding constants for all 10 compounds averaged approximately 30%. We recognize that, to some degree, this was an idealized study because each participant was provided with reagents and the experimental protocol, and we certainly

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**Fig. 8. ITC analysis of compounds 1–10 binding to CAII.** (A) Representative titrations of compounds 2, 4, 6, and 8. (B) Plots of kilocalories/mole of injectant versus molar ratio for each of the 10 compounds.

**Fig. 9.** Correlation plot of affinities determined for each of the 10 compounds using SPR and ITC. The dashed line depicts a correlation of 1.
would expect the standard errors in the binding parameters to increase as the number of experimental variables increases. This benchmark study, however, serves as an excellent tool for educating users and validating that the biosensor technology does in fact work when it is applied properly. These results and discussions should stimulate further interest and confidence in the use of biosensors throughout the drug discovery process.

References


