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Fragment Screening by Surface Plasmon Resonance

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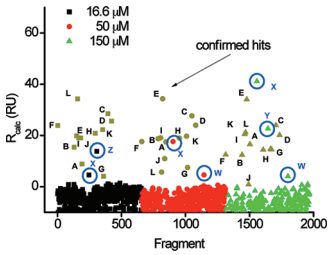
ABSTRACT Fragment-based drug discovery is a validated approach for the discovery of drug candidates. However, the weak affinity of fragment compounds requires highly sensitive biophysical techniques, such as nuclear magnetic resonance (NMR) or X-ray crystallography, to identify hits. Thus the advantages of screening small fragment libraries are partly offset by the high cost of biophysical analyses. Here we present a method for biosensor-based fragment screening using surface plasmon resonance (SPR). In order to reduce the false positive detection rate we present a novel method of data analysis that incorporates multiple referencing with ligand efficiency. By implementing all necessary steps for assay design, data analysis and interpretation, SPR-based fragment screening has potential to eliminate all nonspecific (false positive) binders. Therefore, given the advantages of low protein consumption, rapid assay development and kinetic and thermodynamic validation of hits, SPR can be considered as a primary screening technology for fragment-based drug discovery.

KEYWORDS Fragment screening, surface plasmon resonance, ligand efficiency



Fragment-based drug discovery (FBDD) is now a proven method for designing clinical candidates[1−3] comparable with high-throughput screening (HTS) approaches. However, for fragment-based drug discovery to truly become a disruptive innovation the full economic cost of a hit discovery project from protein production to hit confirmation needs to be not only comparable with HTS but a magnitude faster or cheaper or, ideally, both. Biophysical techniques such as X-ray crystallography and NMR are commonly used to identify low affinity fragment hits. Despite the fewer number of compounds that need to be screened in FBDD, the actual full cost of the project may be comparable with conventional biochemical high-throughput assays, due to the expense of the biophysical analytical methods. The need for high-resolution X-ray crystal structures or NMR protein assignments to determine the mode of action of fragments can often introduce long time lines from project conception to actionable screening data. Indeed, it is not uncommon for medicinal chemists to begin optimizing high-affinity HTS-derived hits for a target before biophysically derived fragment hits are available, thus hindering the adoption of FBDD in organizations with significant HTS investment. Furthermore, the necessity for high protein consumption and, in some NMR experiments, isotope labeled proteins introduces significant costs to a project.

The cost effectiveness of FBDD can be improved by (1) reducing the quantity of protein consumed, (2) reducing the timeline from project initiation to confirmed hits, (3) reducing the number of false positives and (4) identifying ligand efficient hits.

Biosensors, which measure the kinetics of protein−ligand interactions with surface plasmon resonance (SPR), are emerging as a new biophysical technique for fragment screening.[4−10] The sensitivity and throughput of the new generation of SPR instrumentation enables this technology to be used for screening of large libraries of fragments or compounds. The development of SPR-based fragment screening has several advantages over current X-ray and NMR methods. First, the protein consumption for SPR methods is at least 10-fold to 100-fold less than other biochemical and biophysical fragment screening methods. Typically entire SPR fragment screening campaigns can consume as little as 25−50 μg of protein. Second, SPR biophysical interaction assays can be developed far more quickly than other methods. Third, SPR methods provide a rich characterization of each fragment by providing the kinetics and thermodynamics of binding that NMR and X-ray cannot provide. For example, not only can SPR be used to identify which fragments bind but kinetic and van't Hoff thermodynamic analyses[11] can identify fragments with slow offset kinetics[12] or bind with predominately enthalpic energy: properties which may make better starting points for optimization.[13]

A handful of SPR-based fragment screens have been reported to date, include screening campaigns against BACE-1,[6] MMP-12,[7] thrombin[8] and chymase.[10] Here we extend the development of biosensor fragment screening methodology, using carbonic anhydrase II (CAII) as a model

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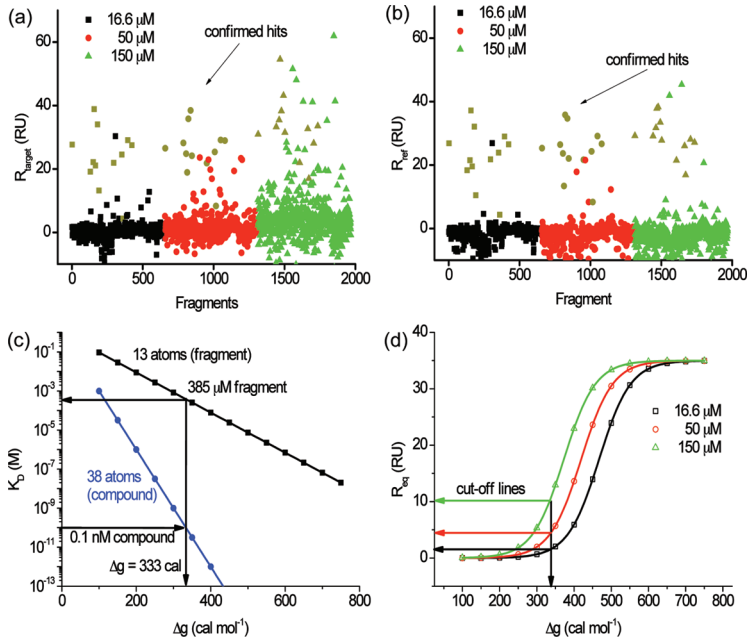

Figure 1. Fragment data analysis: (a) Fragment data overlay for binding referenced for blanks and blank reference flow cell. Fragments were injected at three concentrations: black ■, 16.6 μM; red ●, 50 μM; and green ▲, 150 μM. Dark yellow points represent confirmed hits. (b) Fragment data overlay referenced for blanks, blank surface and reference surface with immobilized SAPk2 protein. (c) Affinity vs ligand efficiency dependence for compound (blue ●, 38 atoms) and fragment (black ■, 13 atoms); arrows represent calculation of ligand efficiency necessary for a fragment to lead to a compound binding to target with affinity 0.1 nM. (d) Cutoff curves calculated from eq 4 based on fragment (13 atoms). Arrows represent R_{eq} cutoff values for each concentration necessary for fragment efficiency 333 cal M^{-1} per heavy atom.

system, to demonstrate a ligand-efficiency-based[14] referencing method to eliminate the detection of false positives and thus improve the overall cost effectiveness of biosensor-based fragment screening.

SPR biosensor technologies are very sensitive, capable of detecting small molecule fragments binding with molecular weights as low as 100 Da to biomolecular targets. However proper assay design and data analysis is required to distinguish actual from nonspecific binding, especially when running a high-throughput screen of large numbers of molecules. In particular, SPR assay sensitivity to DMSO, a solvent with a high refractive index, can be a major source of false positives if the concentration for samples and running buffer is not matched accurately.[15] An advantage that the majority of SPR technologies offer is the availability of multiple biosensor channels that can be used to screen multiple proteins in parallel. The parallel immobilization of the target, reference protein(s) and leaving one channel blank as a reference surface enable the analysis methods to distinguish between actual and nonspecific binding.

In this study we measured binding of 656 fragments at three concentrations (16.6, 50, and 150 μM) to immobilized CAII protein and reference SAP2k protein immobilized on a reference surface (see Supporting Information). The molecular weight (MW) of the fragment library ranged from 94 to 341 Da, with the average MW = 187 Da, equating to 13 non-hydrogen (heavy) atoms. To assess protein stability and reproducibility of results, control compounds furosemide (positive) and SB 220025 (negative) were injected throughout the experiment. The average responses for furosemide and SB220025 were 5.58 (±1.03) and −0.36 (±0.5) with average *Z*-prime for all assays 0.63 (±0.14). The variations of responses throughout the experiment were caused by variations in experimental conditions and immobilization levels, as experiments were split into 8 different assays and total three chips were used for screening. In order to reduce false positives from detergent-sensitive nonspecific aggregation-based binding,[16] 0.005% Tween P-20 was added to the running buffer in all the experiments.[17] Excellent reproducibility has been observed for each assay separately (Figure S1a in the Supporting Information). All experiments were conducted on a Biacore T100. The entire screening campaign from assay development to hit confirmation took a total of 4 weeks consuming 27 μg of each protein.

In total over 1960 sensorgrams were collected in the fragment screen. To improve overall speed and quality of data analysis we introduce a reference/filter method to eliminate noise and nonspecific binders from data collection to reveal specific fragment binders. Response levels for each sensorgram from the association binding phase were extracted at 27 s after injection just priori to dissociations (Figure S1b in the Supporting Information). From plotting the response levels for each fragment at each concentration (Figure 1a) it is evident that the number of nonspecific binders increases with increasing concentration. As SPR analysis is based on detection of mass change on the biosensor surface, knowing the maximum surface capacity of immobilized target allows the determination of the

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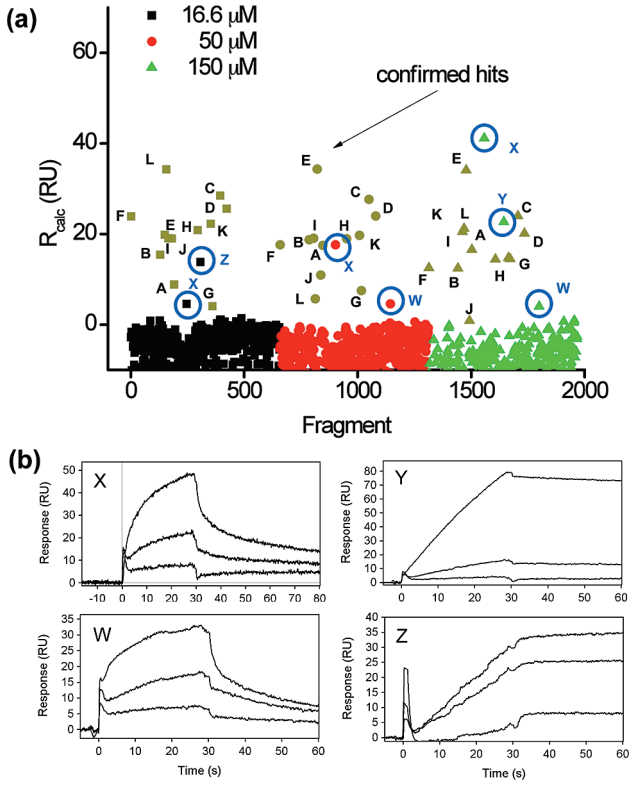

Figure 2. (a) Overlay of fragment responses referenced by using eq 5 for fragments binding to CAII at concentrations 16.6, 50, and 150 μM. Dark yellow labeled points represent confirmed binders. Nonspecific binders X, Y, W and Z are highlighted in circles. (b) Binding sensorgrams for nonspecific binders X, Y, W and Z at concentration series 16.6, 50, and 150 μM. The chemical structures of the specific binders A−L are shown in Figure S2 and the nonspecific binders in Figure S3, both in the Supporting Information.

approximate affinity from the response obtained for each fragment (eq 1).[8] Assuming each binder reaches equilibrium state and based on a Langmuir isotherm (eq 1), where the maximum binding capacity (R_{max}) is 40 RU (average max binding response based on saturation data of real hits), the approximate affinities of each fragment at each concentration to CAII are calculated and the cutoff line is adjusted accordingly.[8]

$$R_{eq} = \frac{R_{max}c}{K_D + c} \quad (1)$$

where c corresponds to a concentration at which fragments were screened.

Therefore for fragments with binding affinities of 1 mM and higher, the cutoff R_{eq} line would be 5 RU for the highest concentration (150 μM). However in addition to identifying 12 confirmed binders 230 false positives for the highest concentration (data not shown) are also present in the resulting data set (Figure 1a). Analysis of the sensorgrams reveals that many nonspecific binders exhibit nonstoichiometric responses[17] to a reference protein immobilized on a reference flow cell. Therefore subtraction of responses collected on the reference protein from all data resulting in Figure 1b significantly reduces the number of false positives

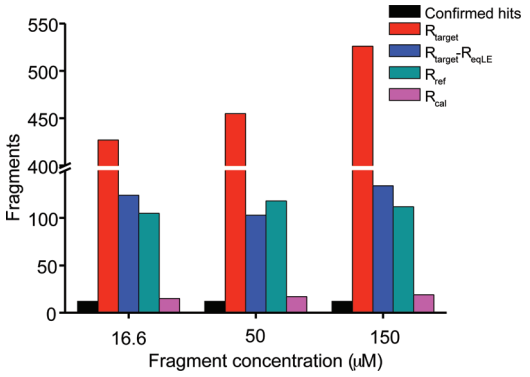

Figure 3. Comparison of four different analysis methods for fragment screening. Numbers of fragments showing responses above 0 RU at three concentrations (16.6, 50, and 150 μM) are plotted vs screening concentrations. Each bar represents individual analysis method. From the left: black bar, number of confirmed hits; red bar, method using responses for raw data (blank and blank surface referenced); blue bar, method using responses for raw data with subtraction for Δg = 333 cal mol^{-1} (Figure 1c); green bar, method using responses for raw data referenced for data collected on reference surface; pink bar, method using responses for data calculated from eq 5.

(eq 2). The correct choice of reference protein(s) is also important as some fragments may genuinely bind to both (target and reference) proteins and therefore could be missed after the subtraction.

$$R_{ref} = R_{target} - R_{reference} \quad (2)$$

By setting the cutoff line to 5 RU, the minimal binding affinity for each fragment showing response above this cutoff at concentration 150 μM is 1 mM. However this type of affinity cutoff analysis may not be suitable, as the efficiency of binding per atom differs for each fragment. By calculating ligand efficiency (LE)[14] values, each fragment can be analyzed separately based on its atomic structure, to determine an affinity value suitable for each individual fragment. Ligand efficiency (LE, Δg) is calculated as a binding energy of the ligand per atom (eq 3).

$$\Delta g = \frac{-RT \ln K_D}{N_{(\text{non-hydrogen atoms})}} \quad (3)$$

where Δg is ligand efficiency (cal mol^{-1}), N the number of non-hydrogen atoms in molecule, R the universal gas constant (1.986 cal K^{-1} mol^{-1}) and T temperature (277.15 K ~ 4 °C or 298.15 ~ 25 °C).

Considering carbonic anhydrase II compounds are characterized by high affinities,[15] a ligand efficiency of Δg = −0.333 kcal mol^{-1} per non-hydrogen atom at 4 °C (LE = 0.333) was chosen as the cutoff limit. Thus the LE cutoff results in minimal affinity required for a fragment with average N = 13 heavy atoms as $K_{D\ min}$ = 0.385 mM (Figure 1c). By implementing ligand efficiency reference in eq 1 we can calculate minimal R_{eqLE} required for each fragment (eq 4).

$$R_{eqLE} = R_{max}c/[e^{-(\Delta gN/RT)} + c] \quad (4)$$

Figure 1d shows the calculation of the cutoff line based solely on the LE for a molecule of 13 heavy atoms but which can be also specifically applied not only to the concentration at

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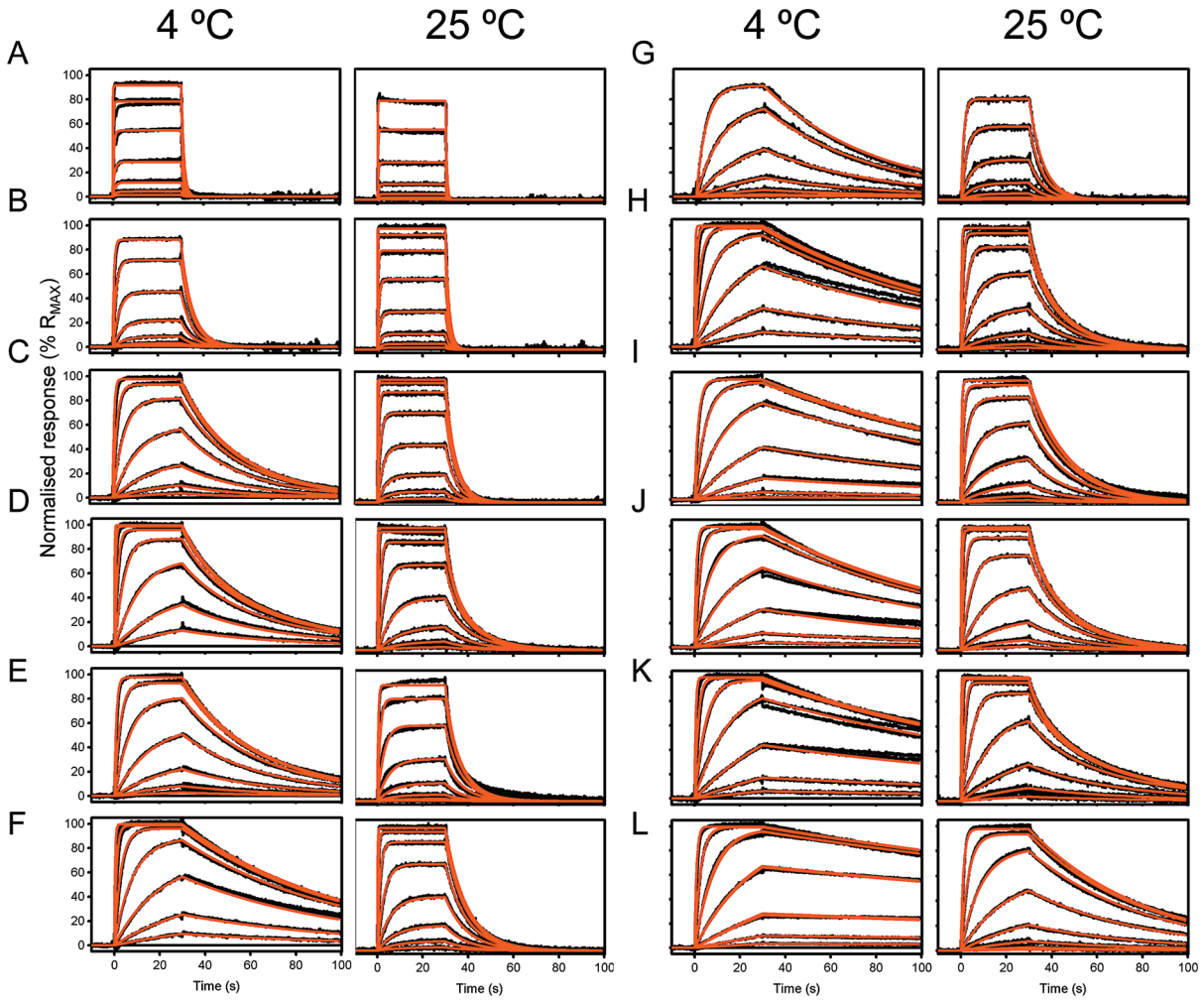

Figure 4. Overlay of sensorgrams for each confirmed hit binding to CAII measured in duplicates at 4 and 25 °C. Black lines represent measured binding curves; red lines represent 1:1 kinetic fit. Each compound is labeled by a letter corresponding to binders in Figure 2a. Kinetic values for each fragment at 4 and 25 °C are summarized in Table S1 in the Supporting Information.

which fragments are screened but also to an atomic value specific to each fragment.

$$R_{\mathrm{calLE}} = R_{\mathrm{ref}} - R_{\mathrm{eqLE}} \quad (5)$$

Combining both methods, where the raw data are referenced for surface with immobilized reference protein and applying LE filtering (eq 5), results in Figure 2a, where each binder is labeled with a letter corresponding to each fragment hit. By filtering data points through the LE filter, the majority of fragments stand out compared to nonbinders that are filtered out of the data set. Only four false positives (fragments X, Y, W, Z) were observed and subsequently identified as nonspecific binders due to nonstoichiometric sensorgram responses (Figure 2b). The complete comparison of four different evaluation methods is summarized in Figure 3. Details of each method are described in the legend and are based on using sole or combined subtractions of raw data sets for values calculated using eqs 1−5. The best results were obtained by combination of protein surface reference method with LE filter as shown in Figure 2. Using this method we identified 12 specific binders to CAII giving assay hit rate 1.8%. Similar hit rates have been observed for other proteins in NMR and X-ray based fragment screens.[18] All binders were confirmed by injecting 3-fold serial dilutions of each fragment ranging from 150 μM to 0.022 μM. CAII demonstrates good stability at 25 °C once immobilized on surface,[15] however the fragment screening is optimally conducted at 4 °C as not all targets are likely to be stable over the lifetime of the assay. This can also be advantageous especially for weak binders, as they can be better identified at lower temperatures due to changes in the curvature of sensorgrams. All binders were then characterized at both 4 and 25 °C and kinetics and affinity were compared. All data were fitted to a 1:1 binding model including mass transport coefficient (Figure 4). The confirmed hits displayed a wide range of affinities (0.13−14 μM). Kinetic analysis identified some fragments showing very slow off rates at 4 °C (e.g., fragment L Figure 2a and Figure 4 with k_d = 0.003 s^{-1}). Figure 5 shows a correlation plot for k_d vs k_a with affinity isotherms. At the higher temperature the points for each fragment shift toward higher kinetic rates, however as the

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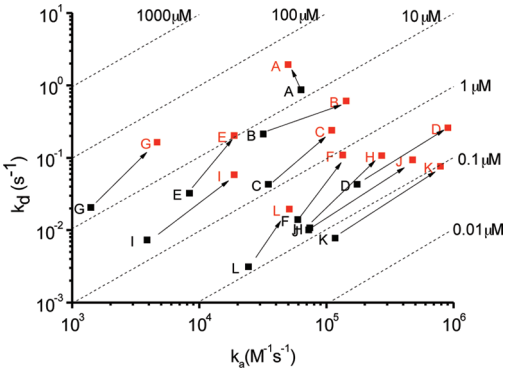

Figure 5. Kinetic values plot of k_d vs k_a. Dashed lines represent affinity isotherms. Black squares represent data collected at 4 °C and red squares data collected at 25 °C. Data points are labeled according to Figure 4 for each fragment. Arrows represent shift in kinetics for each fragment binding at two different temperatures.

affinity is calculated as the ratio between k_d and k_a, most of the fragments maintained approximately the same affinity (K_D) at 4 and 25 °C.[11]

SPR-based biosensor analysis is emerging as an important technique for fragment-based drug discovery. In addition to the advantages of low protein consumption and rapid assay development, SPR technology has the potential to eliminate practically all nonspecific (false positive) binders by implementing all necessary steps for assay design, data analysis and interpretation. Conducting SPR fragment screens priori to X-ray and NMR analysis not only provides fragments for medicinal chemistry optimization but also provides valuable ligands for seeding protein crystallization and quantitative assessment of druggability,[19,20] especially for novel proteins of unsolved structure.

SUPPORTING INFORMATION AVAILABLE Detailed experimental procedures for the fragment screening. This material is available free of charge via the Internet at http://pubs.acs.org.

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