J Comput Aided Mol Des (2011) 25:669–676
DOI 10.1007/s10822-011-9439-8

Biosensor-based small molecule fragment screening with biolayer interferometry

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Received: 14 April 2011 / Accepted: 18 May 2011 / Published online: 10 June 2011

Abstract Biosensor-based fragment screening is a valuable tool in the drug discovery process. This method is advantageous over many biochemical methods because primary hits can be distinguished from non-specific or non-ideal interactions by examining binding profiles and responses, resulting in reduced false-positive rates. Biolayer interferometry (BLI), a technique that measures changes in an interference pattern generated from visible light reflected from an optical layer and a biolayer containing proteins of interest, is a relatively new method for monitoring small molecule interactions. The BLI format is based on a disposable sensor that is immersed in 96-well or 384-well plates. BLI has been validated for small molecule detection and fragment screening with model systems and well-characterized targets where affinity constants and binding profiles are generally similar to those obtained with surface plasmon resonsance (SPR). Screens with challenging targets involved in protein–protein interactions including BCL-2, JNK1, and eIF4E were performed with a fragment library of 6,500 compounds, and hit rates were compared for these targets. For eIF4E, a protein containing a PPI site and a nucleotide binding site, results from a BLI fragment screen were compared to results obtained in biochemical HTS screens. Overlapping hits were observed for the PPI site, and hits unique to the BLI screen were identified. Hit assessments with SPR and BLI are described.

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Keywords Surface plasmon resonance · Biolayer interferometry · Fragment screening · Protein–protein interactions · Drug discovery · FBDD

Introduction

Biosensor-based fragment screening is an established practice in the drug discovery process. Several screens have been reported with several targets including GPCRs, kinases, a reverse transcriptase, carbonic anhydrase, proteases, and a polymerase [1–8]. This technique measures the interaction of a fragment with a protein target on a biosensor, and kinetic constants are derived from binding data. These methods are advantageous over many biochemical methods because non-specific or non-ideal interactions can be differentiated from ideal binding profiles and responses [9], resulting in reduced false-positive rates.

The identification of small molecule modulators of protein–protein interactions (PPI) represents a particularly difficult challenge relative to enzyme targets, since the binding surfaces are large (750–1,500 square Angstroms), and generally lack well-defined pockets [10]. Nonetheless, PPI targets often contain "hot spots" that comprise significant binding energy, and these regions are targetable with small molecule inhibitors [10]. Fragment screening in particular is an attractive strategy for drug discovery [11] because the physical properties of low molecular weight compounds are generally considerably better than compounds with higher molecular weight [12]. Accordingly, numerous fragments with molecular weights of 170–226 Daltons bind to PPI target Bcl-x_L in two-dimensional NMR studies that unambiguously show binding of the compounds to the binding pocket of this protein [13].

Biolayer interferometry (BLI) is emerging as a viable tool for small molecule drug discovery [14]. This technique has been validated for the detection and characterization of small molecules by comparison of SPR results with carbonic anhydrase, a stable zinc metalloenzyme that it is highly selective for the class of compounds known as sulfonamides [15]. Comparisons of kinetic constants and binding profiles obtained with BLI generally correlate with SPR. Representative examples for the carbonic anhydrase model system include benzenesulfonamide (157 Da), acetazolamide (222 Da), sulpiride (341 Da), and furosemide (330 Da) with equilibrium dissociation constants obtained from BLI data of 2.4, 0.039, 239, and 1.2 μM, respectively [16], and these values correlate well with literature values of 0.8, 0.019–0.039, 48–186, and 0.5–1 μM [15, 17]. Agreement was also established for SPR and BLI results obtained for the interaction of compounds with oncology targets including a kinase and a PPI target [18]. Feasibility for fragment screening with BLI has been demonstrated with the carbonic anhydrase model and the Maybridge RO3 fragment library containing 500 compounds [19]. In this study, a hit rate of 2% was observed, and five of the ten primary hits, including three sulfonamide compounds, were confirmed in a dose-response study.

We now report fragment screening results for BLI with three human PPI targets including BCL-2 and eIF4E, which are important modulators of disease progression in oncology [20, 21], and JNK1, which has been implicated in diabetes and other diseases [22]. The hit rates are compared for BLI screens with these three targets, and for eIF4E we compare the BLI screening results to results from biochemical screens. Hit assessment with both BLI and SPR is also described.

Materials and methods

Proteins and BLI sensor preparation

All proteins were based on published sequences and were prepared at Roche in Nutley, NJ. Target protein is biotinylated in vivo with the biotin ligase recognition sequence GLNDIFEAQKIEWR [23]. Labeling of the target protein can also be achieved with a 1:1 molar ratio of biotin-LCLC-NHSS (Pierce) to protein [24], followed by removal of unreacted biotin derivative with a dialysis cassette with a 3.5 kiloDalton molecular weight cut-off (Pierce). Super Streptavidin Biosensors (SSAv) were obtained from FortéBio Inc and were placed in a solution of 0.5 μM protein and incubated overnight at 4 °C. Sensors with target protein were blocked with biocytin, washed with buffer and sensor activity was verified with a control compound. Reference sensors are typically blocked with biocytin at 1 μg/mL for 2 min. An alternative reference sensor strategy involves loading SSAv sensors with biotinylated streptavidin, which is biotinylated in the presence of biocytin. Small molecule binding experiments with this sensor show reductions in standard deviation of 1–2 pm for analysis of buffer relative to experiments where biocytin-blocked sensors are used. This strategy was used for the eIF4E and JNK1 fragment screens.

Sample plate preparation

Sample plates containing positive and negative controls and "rule of three" fragments with molecular weights less than 300 Daltons, cLogP values less than 3, and less than 3 hydrogen bond donors and acceptors were prepared by adding 6 μL of DMSO solution to a 384-well black polypropylene plate (Greiner). Dilutions with buffer were performed using the Multidrop dispenser (Thermo Scientific) and the final DMSO concentration was 5%.

BLI fragment screening

Automated screens were completed on a FortéBio RED384 instrument interfaced with a Hudson Plate Crane as follows: sample plates containing 6 μL of compound or control in DMSO are transferred to a Multidrop liquid dispensing station. Buffer is added to the 384-well plate, and the plate is transferred to the RED384 instrument for analysis using 16 sensors with target protein and 16 reference sensors, run serially at 25 °C. Typical cycles for analysis include baselines obtained in buffer (30 s), associations in wells containing compound (30 s), and dissociations in buffer (30 s). After analysis, the plate is removed, and the cycle is repeated for a second plate. The run time is 62 min for 168 samples including 140 compounds at 200 μM, 14 negative controls, and 14 positive controls. Analysis of 50 plates containing 6,500 compounds was completed in 10 days. Double reference subtraction was performed with FortéBio data analysis software to remove drift and well-to-well artifacts. The detection limit was established by measuring a plate of buffer, and the cut-off is the average plus three standard deviations (typically 20–30 pm of response). The eIF4E screen required 700 sensors and 1 mg of protein. Control compound 7-methyl guanosine and buffer components were obtained from Sigma.

SPR analysis

Samples were analyzed on a Biacore T100 at 25 °C at a flow rate of 50 μL/min with a streptavidin sensor. Target protein labeled with biotin was loaded to 10 k RU on a streptavidin sensor, and positive control was monitored at

the beginning of the run, and after every 25 injections. Hit confirmation studies were performed using a 7 point 2X dilution series with top concentration of 500 μM. The responses on the PPI target of interest, eIF4E, were compared to a second PPI target, BCL-2, and carbonic anhydrase, a well-behaved protein that has selectivity for sulfondamides.

Biochemical assays

Biochemical assays for eIF4E were europium-based time-resolved fluorescence assays with the appropriate labels on the target protein, a derivative of the nucleotide, and a peptide derived from the eIF4E binding protein 4G.

Results and discussion

Small molecule analysis with optical fiber-based BLI

Optical fiber-based BLI generates an interference pattern by monitoring visible light reflected from two surfaces on the biosensor. The first optical surface is a two-way optical mirror located at the tip of the sensor, and the second surface, located at the sensor/buffer interface, is labeled with a protein target. When molecules bind to the target, the interference pattern shifts to higher wavelength. In a typical experiment, a baseline is obtained in assay buffer in a microtiter plate well. Next, the instrument transfers the biosensor to a well containing compound. If binding occurs, the instrument detects the difference in light reflected from the target surface relative to the response in buffer, and reports response as a change in wavelength shift for the interference pattern. The sensor is transferred back to a well containing buffer, and the dissociation of compound from the sensor is monitored.

For small molecule detection, systematic optical interferences, drift, and variances in composition between the buffer well and the sample well cause interferences that are generally correctable using reference sensors. Drift is measured in parallel to small molecule analysis on a separate sensor run only in buffer wells, and optical interferences are corrected from data obtained in second run on the same plate using a second set of sensors that lack the target of interest. These sensors are typically blocked with biocytin or biotinylated streptavidin that is blocked with biocytin. The raw data and data analysis process are shown in Fig. 1. For small molecule interactions, responses obtained after double-reference subtraction are typically 20–100 pm of shift in the interference pattern, and kinetic constants including k_{on}, k_{off}, and K_D are obtainable from analysis of the data obtained during the association and dissociation of compound with the target of interest.

A typical run in a fragment screen on the FortéBio RED384 instrument occurs at 25 °C in a single 384-well black polypropylene plate that includes analysis with sixteen sensors that contain target, followed by an additional sixteen sensors that are blocked with a biotin derivative such as biocytin, which is a neutral zwitterion. A single one-h run includes analysis of negative controls (N = 14), 140 compounds at 200 μM, and the positive control (N = 14), which is run last so that sensor activity can be verified after

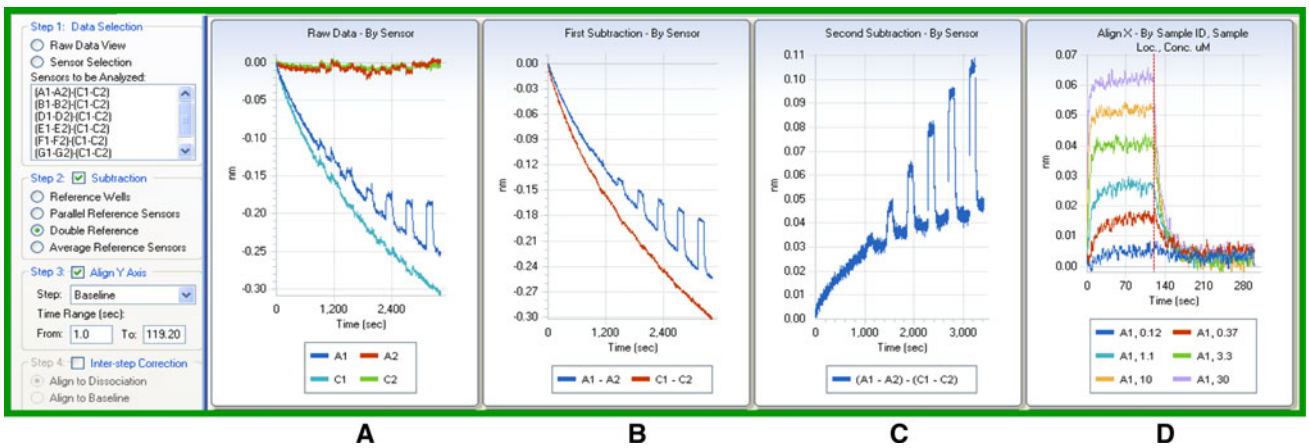

Fig. 1 **A** Raw data for a small molecule binding experiment including (1) streptavidin sensor with biotinylated target protein for analysis of a concentration series of a compound (0.12–30 μM, sensor A1), (2) a second target sensor run in parallel with sensor A1 for analysis of buffer for drift corrections within the run (sensor C1), (3) biocytin-blocked reference sensor run in serial with sensors A1 and A3 for analysis of compound to monitor systematic interferences (sensor A2), and (4) a second biocytin-blocked reference sensor run in parallel with sensor A2 for analysis of buffer for drift corrections during the second run (sensor C2). **B** Removal of optical artifacts by subtraction of reference sensor from target sensor (A1–A2 and C1–C2). **C** Removal of drift by subtraction (A1–A2)–(C1–C2), and **D** Removal of baseline data and alignment of the start of association for the six-point concentration series. The *red line* in **D** at 120 s marks the start of dissociation of the compound from the sensor surface

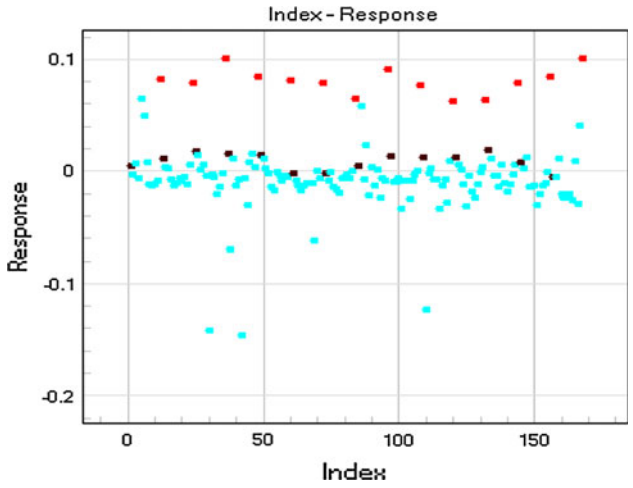

Fig. 2 A plot of BLI responses with eIF4e for 140 samples (*blue*), buffer controls run at the beginning of the run (*black*), and positive control compound run at the end of the run (*red*)

exposure to compound. Responses from a typical run are shown in Fig. 2. If a sensor lacks response due to inactivation by a reactive or denaturing compound, then compounds that were analyzed with the inactive sensor are re-analyzed in a follow-up run. This method is advantageous to other biosensor-based techniques that utilize a single target sensor that can be inactivated during a run, since the FortéBio instruments run up to sixteen sensors in parallel.

Fragment screening with optical fiber-based BLI

Prior to a screen, assay development is performed to establish behavior for a positive control, target stability, hit rates with a test library, and the variability for runs containing buffer. Additionally, sensor lots are qualified with a well-behaved positive control protein and compound, and these results are compared with results from previous lots. The lower limit for detection is the average plus three standard deviations for analysis of a plate containing buffer only, and is typically 20–30 pm of response. A typical fragment screening campaign consists of a primary screen of several thousand compounds analyzed in singleton at 200 μM in buffer containing 1 mM dithiothreitol, 5% DMSO, and 0.005% P20 detergent. Depending on the stability of the target protein on the sensor, a single set of sixteen sensors is used for 2–4 plates containing compounds. Primary hits are confirmed with a second screen in singleton at 200 μM or with a three-point concentration series at 125–500 μM. Follow-up assessments include a 6–8 point concentration series.

To assess reproducibility, a test library consisting of 140 unique fragments was analyzed in triplicate on three plates containing each compound in singleton with targets JNK1 and eIF4E. Results for JNK1 show that correlations (R) are >0.98 for comparisons of the three runs. In these studies, 28/34 hits with signal ≥0.03 nm of response were observed in all three studies. The other six hits were observed in two of the three runs, and nearly all signals are within 0.01 nm of the cut-off of 0.03 nm. For the eIF4E screen, the same comparisons were made with the same test library, and with the same cut-off limit, 8/9 hits were reproducibly observed in all three analyses. Again, the hit that was not reproducible for the eIF4E test library had signals near the cut off (0.031, 0.029, 0.026 nm).

Primary hit rates with BLI are target dependent and were 21, 24, and 3.5% for PPI targets BCL-2, JNK-1, and eIF4E, respectively. The relatively high hit rate for target JNK-1 is likely due to the fact that it contains three binding sites, including an ATP binding site, a nearby PPI site known to bind the JIP peptide, and a third site known to bind small molecules [22]. eIF4E, which had a relatively low hit rate in the BLI screen, has at least two known binding sites including one for 7-methylguanosine phosphates, and a site known to bind protein 4G. eIF4E is also known to bind to other proteins [21], but the binding sites are currently unknown. BCL-2 contains a single hydrophobic groove [25] that binds peptides from the Bad and Bak proteins [26], and a peptide from the BH3 domain of BAX protein [27].

The most comprehensive BLI fragment screening study to date was completed with eIF4E, and results were compared to those from biochemical assays. For the BLI screen, the primary hit rate was 3.5%, and after removal of 0.4% due to high signals, and 0.6% that had unreasonably slow off-rates, the overall hit rate was 2.5%. Some responses were less than zero as a result of preferential non-specific binding to the reference sensor, and most responses were binned at 0 ± 0.01 nm of response. This screen was achieved with ~700 sensors and 1 mg of eIF4E that was biotinylated in vivo. Responses for the complete fragment screen with eIF4E are shown in Fig. 3.

eIF4E was also labeled with biotin-LCLC-NHSS in vitro, and responses obtained with reference compound 7-methyl guanosine were similar. The primary advantage of labeling the protein with biotin in vivo is that the biotin can be placed at either the *N*-terminus or *C*-terminus of the protein sequence that is most remote from the binding pocket, whereas labeling with biotin in vitro results in random attachment to lysine residues. For eIF4E, however, there was no clear advantage to labeling with biotin in vivo, based on the binding of the reference compound and comparisons were not performed for other targets.

Hits from the BLI fragment screen were assessed with a second screen in singleton at 200 μM, and the confirmation rate was 50% with an overall hit rate of 1.3%. This hit rate is similar to the combined hit rate of 1.4% for compounds with IC_{50} values obtained from the

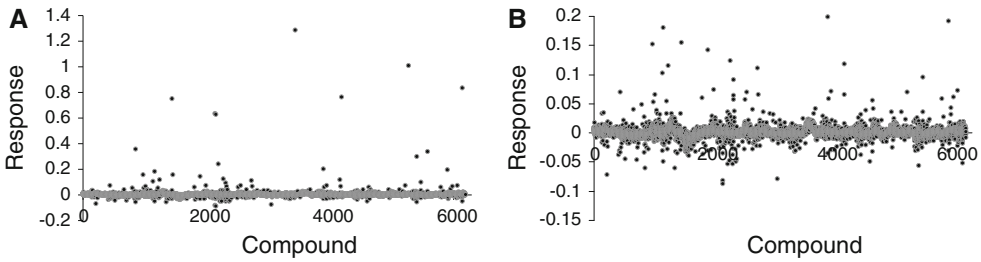

Fig. 3 **A** Responses for a complete BLI fragment screen with eIF4E. **B** Same data as in **A**, but with a limited scale

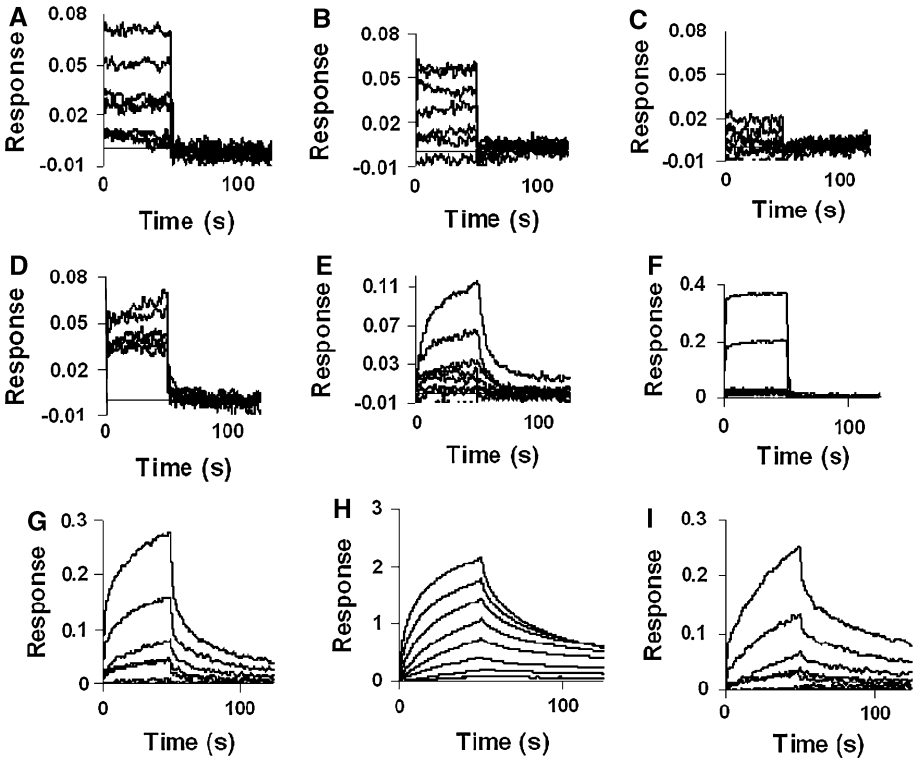

Fig. 4 Representative binding profiles for the interaction of nine compounds with eIF4E obtained with BLI from a dilution series with a top concentration of 1 mM including a positive control 7-methyl guanosine (**A**), preliminary hits (**B**–**E**), and atypical binders (**F**–**I**). Compound **B** shows responses similar to the positive control, and compounds **C**–**E** show a mixture of desirable and undesirable features including a low responses (**C**), narrow response range, possibly due to an affinity constant that is below the lowest concentration examined (**D**), and a doubling of signal with two-fold increases in concentration (**E**). Atypical binders **F**–**I** show non-saturating responses with increasing concentration, signals >3X higher than the positive control, off-rates that are unrealistic for fragments, and incomplete dissociation within the time frame of this experiment (90 s)

biochemical assays, described below. Assessment of the hits was performed with BLI using an 8 point 2× dilution series with a top concentration of 1 mM. For these studies, the K_D for positive control 7-methyl guanosine of 450 μM obtained with human eIF4E was similar to the K_D of 217 μM obtained from fluorescence studies with murine eIF4E, which has affinity constants that are similar to the human form of this protein [28]. The binding profiles for each compound were differentiated into two categories: preliminary hits and atypical binders, and representative examples are shown in Fig. 4. A promising result is the BLI profile for the compound in Fig. 4B, which is very similar to the positive control. Additional preliminary hits contain some desirable features including reasonable signals and binding profiles, but they also contain undesirable features associated with atypical binders. The atypical binders show undesirable characteristics including high signals relative to a positive control, unreasonably slow off-rates, a linear dependency of response with respect to concentration, biphasic binding profiles, or combinations thereof. The high signals observed for some of these compounds may be due to

multiple interactions of a given compound with one or both of the sites, and since the binding pocket for the 4G protein binding site is relatively large, it may accommodate multiple fragments. It is also possible that high signals are due to interactions with small colloidal aggregates [29]. Overall, these results are encouraging, and additional confirmation studies are required for all targets to verify binding behavior and to determine kinetic constants, to rule out interferences from compound aggregation, and to show specificity of binding for either the nucleotide binding site or the 4G PPI site.

Biochemical PPI fragment screens and SPR analysis

Europium-based biochemical screens for the PPI site and the nucleotide binding site were run in parallel to the BLI screen. The primary hit rate for the PPI site biochemical assays with fragments run in quadruplicate at 276 μM was 1.4%, and IC_{50} values were determined for hits with inhibition greater than 45%. Compounds with weak IC_{50} values or no responses were removed, resulting in a hit rate for confirmed binders of 1.0% for the PPI site. With respect to the nucleotide binding site, the primary hit rate for fragments run in triplicate at 247 μM was 1.8%, and values were determined for hits with inhibition greater than 35%. The final hit rate for confirmed binders for the nucleotide binding site was 0.4%. The combined hit rate for confirmed binders for both sites was 1.4%.

Hits from the biochemical screens were assessed first with BLI with a three-point dilution series with a top concentration of 125 μM, and compounds with atypical binding profiles were removed. The remaining fragments were characterized with SPR on a Biacore T100 with eIF4E, BCL-2, and control protein carbonic anhydrase. A small molecule positive control for the 4G PPI binding site was not available, so eIF4E binding was monitored throughout the run with the positive control compound 7-methyl guanosine, which binds the nucleotide binding site. In the SPR studies, this control compound has a K_D of 390 μM, which is similar to that observed with BLI. This compound also has reasonable signals that approach saturation at concentrations near 1 mM, and it has selectivity for eIF4E over BCL-2 and carbonic anhydrase. However, none of the compounds identified in the biochemical screens for the PPI site showed similar characteristics. Some compounds showed reasonable signals at concentrations less than 125 μM, but many responses were linear with respect to concentration up to 500 μM and responses often were 3–5 fold higher than the positive control. Some fragments from the PPI site biochemical screen had SPR responses consistent with those of the positive control, and nearly all compounds had very similar profiles and responses with BCL-2, but these compounds did not bind to carbonic anhydrase. This "non-ideal" behavior was not unique to the SPR studies as similar results were obtained with BLI, and in general BLI binding profiles correlate well with those obtained from SPR. In some instances, "ideal" behavior was observed, including relevant responses that plateau at high concentrations, but compounds with this behavior were obviously precipitating at high concentrations and thus the observed saturation was due to a limit in compound solubility and not due to true saturation of the target protein. For the nucleotide binding site, atypical SPR results were obtained for most compounds, and a few compounds had reasonable responses and the desired selectivity for eIF4E over BCL-2. In general, these compounds do not bind to negative control protein carbonic anhydrase, which suggests that the signals from these compounds are *not* due to non-specific binding of multiple copies of the compound to a target, or due to non-specific interactions with compound aggregates. Stability of eIF4E was monitored throughout all runs, and responses at the end were only 25% lower than those at the beginning of the run. However, the responses for eIF4E at the end became more linear with respect to increases in concentration, indicating that eIF4E loses activity at the 7-methyl guanosine binding site throughout the run (see Fig. 5). Loss of activity of a target during a run is common for SPR assays [2].

Comparisons of BLI and biochemical screens

Comparisons of the hits from the three screens are encouraging, although some discrepancies were noted. The overlap between the hits identified in the biochemical assay for the PPI site with those identified in the BLI screen was 52%. These hits included a common scaffold with derivatives that were identified in both screens. A second scaffold was also identified in both screens, and inspection of the binding profiles in the BLI screen show that these compounds have unreasonably slow off-rates and high signals. All structures containing this scaffold also inhibited binding in the biochemical assay for the nucleotide binding site, and this scaffold represents overlap of 38% of the BLI hits with the hits from the biochemical assay. Solubility measurements with a Becton–Dickinson solubility scanner show that these compounds were soluble up to 1 mM, and purity measurements obtained from LC/MS data also did not correlate well with the unusual behavior observed in BLI studies. In addition to the hits observed in the biochemical screens, 68% of the hits from the BLI screen were unique and were not found in the biochemical screens, and all derivatives corresponding to a novel scaffold were identified.

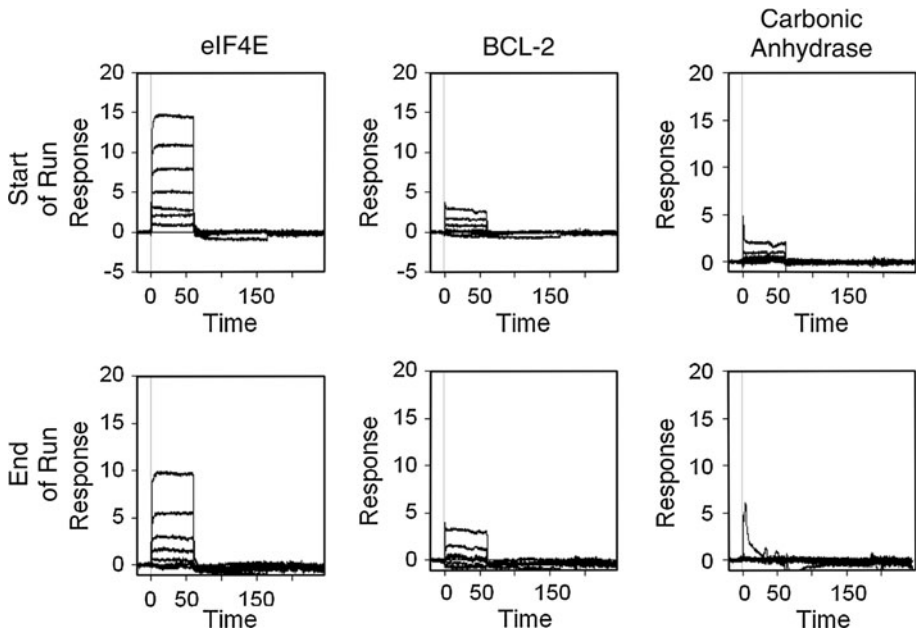

Fig. 5 SPR sensorgrams showing association (0–60 s) and dissociation (60–250 s) of positive control compound 7-methyl guanosine with eIF4E, BCL-2, and negative control protein carbonic anhydrase

Summary

In summary, these fragment screening studies demonstrate that BLI is suitable for small molecule characterization and fragment screening with PPI targets. Results with test libraries show that hits are reproducible. The hit rates from BLI for the targets JNK1, and eIF4E are generally reasonable when the number of binding sites are considered, although the hit rate was higher than expected for BCL2. For eIF4E, the overlap of the hits from the BLI screen with hits from the 4G PPI site biochemical screen is encouraging. The overlap of the eIF4E BLI screen with the biochemical screen for the eIF4E nucleotide binding site was less encouraging, but there were a limited number of hits from the biochemical screen, and additional studies are required to identify the origin of the observed discrepancies. In addition to the overlapping hits, compounds uniquely identified with BLI were observed, including a novel scaffold, and compounds with atypical binding profiles were eliminated. Hit assessment of these PPI targets with BLI and SPR is non-trivial, however, and although numerous hits from the BLI, SPR, and biochemical assays were characterized, most of the BLI and SPR data obtained from the examination of a concentration series in the micromolar range showed linear relationships with respect to concentration, unreasonably high signals, or slow off-rates. As mentioned, these behaviors are not unique to BLI, since similar behavior was also observed in SPR studies. A minority of compounds show signals and profiles suggestive of concentration-dependent binding to a single site, and one compound was identified in the BLI screen with eIF4E that showed concentration-dependent responses similar to those observed for the positive control compound 7-methyl guanosine. SPR analysis of hits from biochemical screens generally showed high signals for hits that were specific to the 4G PPI site and a lack of selectivity for eIF4E over BCL-2. For the biochemical hits that were specific for the eIF4E nucleotide binding site, selectivity for eIF4E over BCL-2 was observed for some compounds in SPR analysis. Additional studies are required to assess the hit confirmation rates for both BLI and SPR, and the origin of these qualitative observations. Overall, we find that these results with BLI hit assessments and screens are encouraging because they help to confirm results from biochemical assays and to identify compounds with atypical binding profiles, and novel hits were identified with BLI that were not observed in biochemical screens.

Acknowledgments We thank Huifeng Niu for guidance in eIF4E biology, the Roche Nutley protein group including Linda Reik, Colin Garvie, and Dave Weber for assistance with proteins and protein chemistry, the structure group and chemistry groups including David Fry and Paul Gillespie for assistance with fragment structure, test libraries, and chemistry, Yang Wen for assistance with solubility studies, Steve Ferguson and Samir Serrano for the LC/MS purity studies, and Janet Diratsaoglu, Peggy Borgese, and Sevan Ibabecki for assistance with compounds.

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