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From Macro to Micro: A Leap in Small Molecule Sensitivity

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Abstract

Small molecule drugs represent ~60% of the total global pharmaceuticals market and, on average, accounted for ~64% of FDA Novel Drug Approvals during the period 2019 - 2024. Traditionally Octet® biolayer interferometry (BLI) has been regarded as not being sensitive enough to detect small molecules and, therefore, surface plasmon resonance (SPR) has been seen as the primary technique to determine real-time kinetics and affinity values for small molecules.

In this application note we analyze the binding interactions of sulfonamide-based small molecules with carbonic anhydrase II using the newly released Octet® R8e. Analysis of the observed kinetics and affinity values shows excellent agreement with previously published SPR data and highlights the feasibility of Octet® R8e BLI as a reliable tool for studying small molecule interactions with macromolecules in various research and development applications.

Introduction

Carbonic anhydrase II (CAII) is an enzyme that plays a crucial role in the regulation of acid-base balance in the body and catalyzes the reversible conversion of carbon dioxide and water into bicarbonate and hydrogen ions. Small molecule inhibitors of CAII are used to treat a wide range of diseases such as glaucoma, epilepsy, altitude sickness and have recently shown promise in transgenic models for Alzheimer's disease[1].

Analysis of small molecule binding using biolayer interferometry (BLI) has traditionally been challenging as the binding of a small molecule to its target produces a wavelength shift that is several orders of magnitude lower than typically observed in protein-protein interactions (PPIs). Therefore, analysis of small molecule interactions has usually fallen under the remit of surface plasmon resonance (SPR). Analyzing a small molecule system necessitates high-quality data, which can be achieved through improved assay design, enhanced experimental setup and accurate data processing. Another significant factor in successful small molecule analysis is the system used to generate the data; here we use the recently released Octet® R8e, powered by enhanced BLI technology that further elevates performance with higher sensitivity, lower baseline noise, and faster data acquisition. Together, these improvements enable confident real-time kinetics and affinity determination even when signals are subtle or targets are particularly challenging, while preserving BLI's fluidics-free, high-throughput workflow.

In order to assess the effectiveness of the Octet® R8e BLI system in measuring the kinetic interactions between small molecules and immobilized macromolecules, a comparative study was performed using a well-characterized system. The objective was to show that data generated by Octet® R8e BLI could match the quality and reliability of values obtained from SPR.

For this purpose, the interaction between sulfonamide-containing compounds (Table 1) and Carbonic Anhydrase II (CAII) was selected as the model system. The molecular masses of the compounds varied from the highest mass of 330.7 Da for furosemide to the lowest of 157.2 Da for benzenesulfonamide. This choice was made because the binding of sulfonamides to CAII is a thoroughly studied interaction, with extensive published data available from SPR experiments. By using this model system, we aimed to validate the Octet R8e's capability to measure binding kinetics and affinities accurately, and to ensure that the results were comparable to those obtained through SPR.

Analyte	Molecular Weight (Da)
Acetazolamide	222.2
4-Carboxybenzenesulfonamide	201.2
Furosemide	330.7
Sulfanilamide	172.2
Dansylamide	250.3
Benzensulfonamide	157.2

Table 1. Sulfonamide-based inhibitors used to assess binding to Carbonic anhydrase II.

Materials and Methods

Materials

Material	Supplier	Product Number
Octet® Super Streptavidin (SSA) Biosensors	Sartorius	18-5057
10X PBS	Gibco	14190-094
384-well, black, tilted bottom microplate	Sartorius	18-5166
DMSO	Sigma-Aldrich	472301
Carbonic Anhydrase Isozyme II from bovine erythrocytes (CAII)	Sigma-Aldrich	C2522
EZ-Link™ Biocytin	Thermo Scientific™	11821445
EZ-Link™ NHS-LC-LC-Biotin	Thermo Scientific™	11841215
Dansylamide	Sigma-Aldrich	218898
Acetazolamide	Sigma-Aldrich	A6011
4-Carboxybenzenesulfonazide	Sigma-Aldrich	C11804
Furosemide	Sigma-Aldrich	F4381
Sulfanilamide	Sigma-Aldrich	S9251
Benzenesulfonamide	Sigma-Aldrich	108146

Table 2. Materials required for sulfonamide compounds binding to CAII assay.

Methods

Octet® SSA Biosensors (18-5057) were prepared by loading either biotinylated Carbonic Anhydrase Isozyme II (CAII) from bovine erythrocytes for assessment of small molecules or biocytin for assessment of non-specific binding. Prior to the assay, all biosensors were hydrated for at least 60 minutes at room temperature in 1X PBS 0.5% DMSO in a 384-well, black, tilted bottom microplate. All assays were performed at 25 °C.

All of the bound complexes dissociated back to baseline within a reasonable time frame; therefore, no regeneration was required.

The Octet® BLI assay followed a general assay flow of:

Assay Step	Step Name	Time (s)	Shake Speed* (RPM)
1	Baseline	30	1,000
2	Association	60	1,000
3	Dissociation	90	1,000

Table 3. General Octet® BLI assay flow for small molecule binding assay.
* Acetazolamide (1500 RPM).

Analyte Preparation

Dansylamide, 4-Carboxybenzenesulfonazide, Furosemide and Benzenesulfonamide were prepared to a final top concentration of 10 µM in 1X PBS 0.5% DMSO and a dose response with a 3-fold dilution series was used.

Acetazolamide and Sulfanilamide were prepared to a top concentration of 250 nM and 50 µM, respectively and dose response with a 2-fold dilution series was used. A reference sample of 1X PBS 0.5% DMSO was used in all assessments to correct for baseline drift. Double reference subtraction was performed using replicate steps with biocytin loaded biosensors.

Data Analysis

Analysis was performed using an Octet® R8e BLI system with Octet® BLI Discovery and Analysis Studio Software version 13.1.0.25. Data was fitted using Octet® Analysis Studio software 13.1.0.38 to a global 1:1 model.

Results & Discussion

To demonstrate the ability of the Octet® R8e to measure kinetic interactions between small molecules and an immobilized ligand, binding of several sulfonamide compounds to CAII was assessed using a 384-well microplate format. In all assays, each concentration of compound was tested in triplicate to assess reproducibility. As shown in Figure 1, the binding responses were exceptionally reproducible with little deviation between the replicates, indicating the immobilized CAII did not lose activity during the assay.

A global fit using a 1:1 interaction model for all compounds is shown in Figure 2. As can be seen, all the data sets are well described by the 1:1 model, and the kinetic rate constants and affinities determined for each compound are shown in Table 4.

As discussed previously, kinetics and affinity assessment of small molecules has traditionally been viewed as best achieved using SPR. As shown in Table 5, the affinity results for sulfonamide compounds binding to CAII measured using Octet® R8e are in excellent agreement with published values for SPR2,[3,4,5].

A visual comparison of the kinetic binding constants is shown in an isoaffinity plot (Figure 3). As discussed by Myszka et al, determining kinetics and affinity using real-time and label-free techniques provides key insights into the binding kinetics of small molecules that would be missed if only affinity-based techniques were used[2]. For example, although Benzensulfonamide and 4-Carboxybenzenesulfonamide both lie near the 1 µM isotherm, comparison of their kinetic rate constants shows that the association and dissociation rate constants are approximately 2-fold slower for 4-Carboxybenzenesulfonamide compared to Benzensulfonamide. Detailed kinetic information such as this is critical when assessing structure-function relationships of small molecules and their targets and can provide deeper levels of understanding than the use of equilibrium assays.

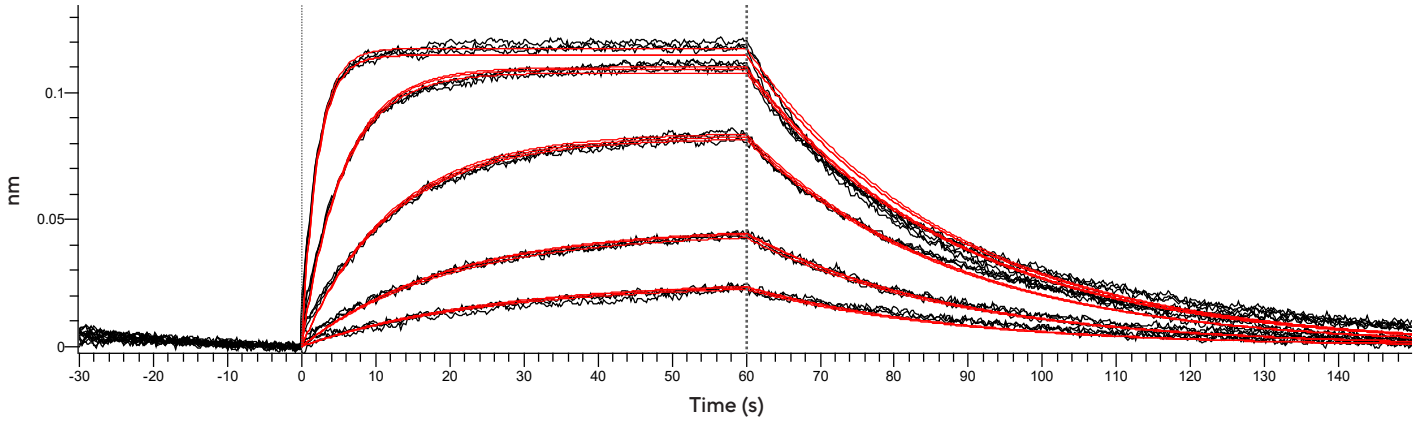

Figure 1. Comparison of triplicate assessment for furosemide binding to carbonic anhydrase II.

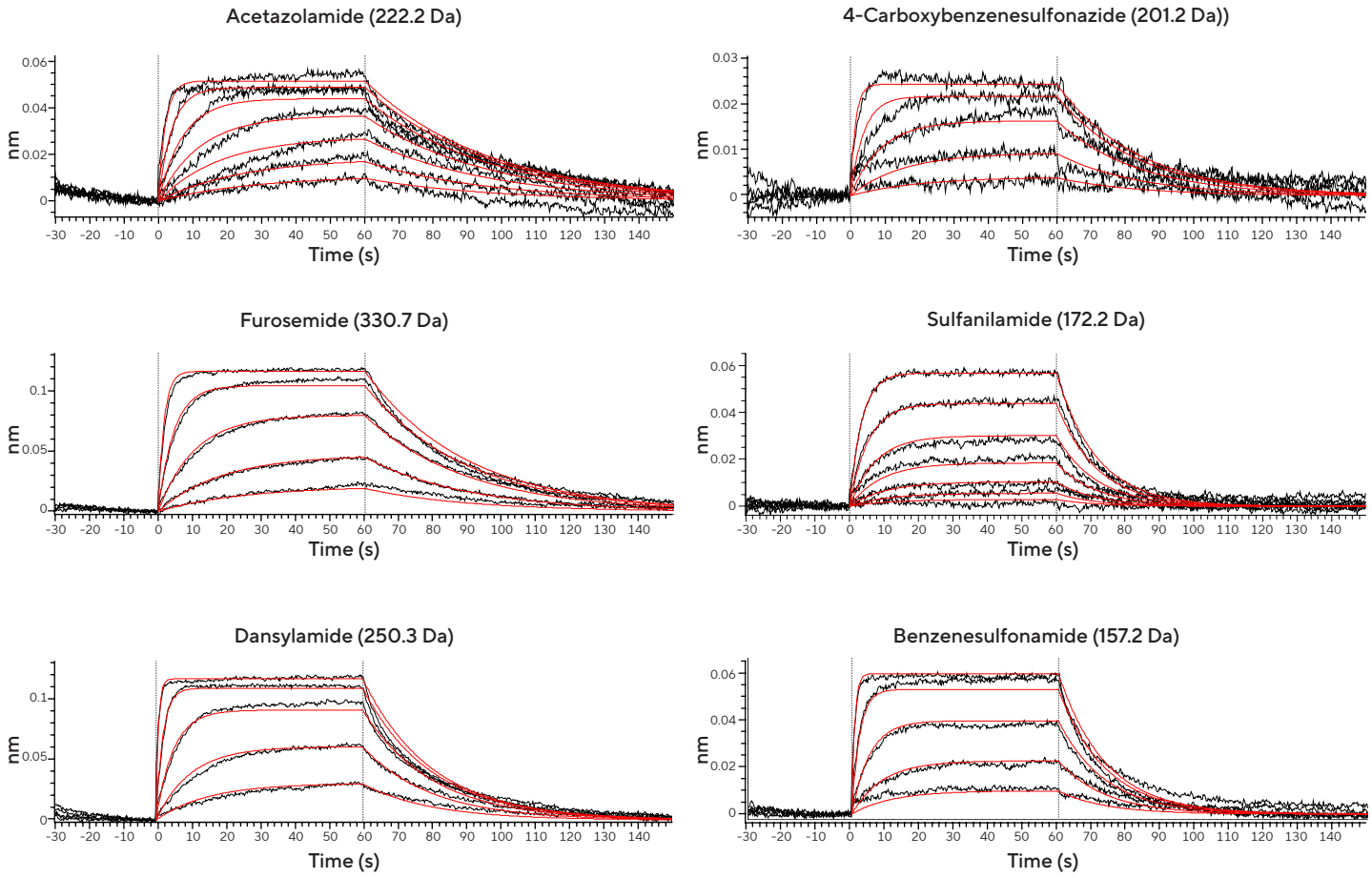

Figure 2. Sensorgrams of sulfonamide compounds binding to CAII. Global fits of the data (black lines) to a 1:1 binding model are shown by the red lines.

Analyte	Molecular Weight (Da)	k_a [$M^{-1}s^{-1}$]	k_a Error	k_d [s^{-1}]	k_d Error	K_D [nM]	K_D Error	R^2
Acetazolamide	222.2	$1.87 * 10^6$	$9.97 * 10^3$	$2.57 * 10^{-2}$	$8.24 * 10^{-5}$	13.7	$8.52 * 10^{-11}$	0.96
4-Carboxybenzenesulfonamide	201.2	$5.30 * 10^4$	$5.48 * 10^2$	$4.48 * 10^{-2}$	$2.90 * 10^{-4}$	845.6	$1.03 * 10^{-8}$	0.93
Furosemide	330.7	$5.57 * 10^4$	$1.65 * 10^2$	$3.42 * 10^{-2}$	$6.06 * 10^{-5}$	613.7	$2.11 * 10^{-9}$	0.99
Sulfanilamide	172.2	$3.47 * 10^3$	$2.24 * 10^1$	$8.06 * 10^{-2}$	$3.20 * 10^{-4}$	23210	$1.76 * 10^{-7}$	0.98
Dansylamide	250.3	$1.41 * 10^5$	$7.45 * 10^2$	$5.19 * 10^{-2}$	$1.71 * 10^{-4}$	368.0	$2.29 * 10^{-9}$	0.98
Benzensulfonamide	157.2	$1.07 * 10^5$	$5.81 * 10^2$	$7.44 * 10^{-2}$	$2.80 * 10^{-4}$	698.1	$4.62 * 10^{-9}$	0.98

Table 4. Kinetic rate and equilibrium constants determined at 25 ° C using an Octet® R8e

Analyte	Molecular Weight (Da)	Myszka (nM)[2]	Papalia (nM)[3]	Octet® R8e
Acetazolamide	222.2	19	31 ± 11	13.7
4-Carboxybenzenesulfonamide	201.2	893	970 ± 170	845.6
Furosemide	330.7	513	1000 ± 200	613.7
Sulfanilamide	172.2	5860	3100 ± 1100	23210
Dansylamide	250.3	760	440 ± 120	368.0
Benzensulfonamide	157.2	848	800 ± 280	698.1

Table 5. Comparison of Octet® R8e equilibrium constants determined in this study and SPR literature values.[2,3]

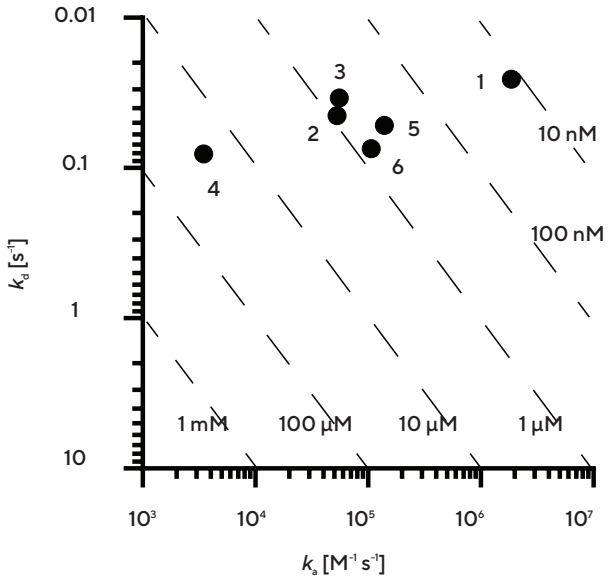

Figure 3. Isoaffinity plot of sulfonamide-based compounds binding to CAII performed on Octet® R8e. 1 – Acetazolamide, 2 - 4-Carboxybenzenesulfonamide, 3 – Furosemide, 4 – Sulfanilamide, 5 – Dansylamide, 6 – Benzensulfonamide.

Conclusions

Advancements in biolayer interferometry, utilizing the Octet® R8e platform, have significantly enhanced the capability to analyze small molecule interactions in a label-free and real-time manner. This evolving technology is now enabling routine application of these advantages to small-molecule systems, which was previously challenging due to sensitivity limitations.

Analysis of sulfonamide inhibitors binding to CAII has demonstrated the detailed level of information that can be obtained regarding small-molecule interactions. By characterizing the kinetics of these interactions, users can gain deeper insights into a compound's activity beyond what equilibrium or inhibition studies alone can provide. The Octet® R8e system has proven capable of detecting interactions of small molecules, characterizing both strong and weak interactions, and interpreting slow and fast kinetic rate constants with high reproducibility. This capability is particularly beneficial when dealing with enzyme preparations that may not be fully active, as it allows for the interpretation of very low signals.

The results of our study validate the use of the Octet® R8e for collecting reliable data on small molecules binding to immobilized macromolecular targets and highlight that the kinetic constants determined align closely with those acquired using the current gold standard of SPR. This confirms the feasibility of using biosensor technology, such as the Octet® R8e, as a powerful tool in drug discovery, supporting secondary screening through precise detection and characterization of small molecule interactions. Overall, the Octet® R8e platform represents a significant advancement in pharmaceutical research and development, offering a robust and reliable method for assessing small-molecule interactions.

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