Application Note

Kinetic Characterization of Small Molecules in 10% DMSO Interacting with GPCR and TNFα Targets

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OVERVIEW

- DMSO is often needed to keep high concentrations of small molecule compounds soluble during early stage drug discovery. This can cause a large amount of background noise on optical kinetic binding platforms such as SPR and BLI, as DMSO negatively impacts optical properties.
- Agile™ R100 leverages a breakthrough orthogonal technology called Field Effect Biosensing (FEB), an electrical technique that is impartial to changes in optical properties. The platform registers zero optical background noise and easily senses in challenging samples such as buffer containing 10% DMSO.
- In this application note, we present Agile R100 kinetic binding data (k_{on}, k_{off}, and K_D) from the interaction of 3 small molecule compounds dissolved in 10% DMSO binding with their respective targets - 2 GPCRs solubilized in additional detergents and a cytokine protein.

ABSTRACT

The ability to test compounds in high concentrations of DMSO is crucial during early stage drug discovery. However, with many common optical kinetic binding platforms, DMSO causes a high level of background noise, making it difficult to accurately characterize compound interactions. In contrast, FEB is an electrical, orthogonal technique that makes it easy to gain accurate real-time data in optically challenging samples such as buffer with high concentrations of DMSO. In this application note, we present kinetic binding data (k_{on}, k_{off}, and K_D) for the interactions of 3 small molecule compounds dissolved in 10% DMSO and their respective targets - 2 GPCRs and 1 cytokine protein. In addition, we show that Agile R100 is also unaffected by buffer additives used to stabilize GPCRs, such as detergents. Agile R100 is a versatile sensing platform capable of testing high concentrations of compounds in high concentrations of solvents and detergents for early stage drug discovery.

INTRODUCTION

During hit-to-lead later in the drug discovery process, selected hits are chemically modified to improve affinities and decrease concentrations. However, in the initial hit identification stage, low-affinity small molecule compounds must be screened in high concentrations (in the micromolar to millimolar range) to gain successful measurements. At such high concentrations, organic solvent such as dimethyl sulfoxide (DMSO) is used to keep the compounds soluble. However, DMSO poses challenges for common optical kinetic binding platforms such as surface plasmon resonance (SPR) and Bio-Layer Interferometry (BLI), which use optics to measure the change in mass as small molecules bind to target. Complex samples impact the optical properties of buffer, increasing background noise and making it difficult to differentiate true binding signal. Controlling for these optical impacts requires time-consuming and error-prone solvent correction processes with SPR and BLI tools.

In contrast, Agile R100 uses an *electrical* technique called Field Effect Biosensing (FEB), not an optical technique, so Agile R100 does not have the problems with complex samples that optical biosensors have. FEB only measures changes in biosensor conductance caused by the energy change in the binding pair when interactions occur.[1,2] Unbound molecules do not change the conductance of the sensor.[1,3] DMSO in the surrounding solution does not alter the Agile R100 biosensor conductance, and thus, small molecule sensing is unaffected, negating the need for solvent correction.

The first study in this application note is 2 drug compounds dissolved in 10% DMSO interacting with their resepctive G-protein coupled receptor (GPCR) targets. GPCRs are membrane-bound proteins that transduce exterior stimuli into intracellular signals, and more than 30% of clinically marketed drugs target the GPCR family.[4,5] Though GPCRs are of great interest as a drug target, purified versions are unstable in non-ideal conditions, and detergents such as n-Dodecyl β-D-maltoside (DDM) and cholesteryl hemisuccinate (CHS) are used to improve stability.[6] Such additives pose challenges to current optical sensing techniques because they alter the optical properties of the buffer or interfere with fluidic systems. Because FEB technology successfully senses in optically challenging fluids, and the sample is brought directly to the sensor surface without fluid flow, Agile R100 also successfully detects in buffers containing detergents such as DDM and CHS for GPCR studies. Specifically, we depict human A_{2A} adenosine receptor, a common GPCR that responds to adenosine in the

central nervous system.[6,7] A_{2A} must be kept on ice to maintain stability, and we took precautions to ensure the A_{2A} was under ideal conditions (i.e. the entire Agile R100 was submerged on an ice bath to match ideal GPCR conditions). We also present chemokine receptor (CR), a purified GPCR from that is stable at room temperature and in buffers with detergents.

In the next study, we show 1 drug compound dissolved in 10% DMSO interacting with the target recombinant tumor necrosis factor alpha (TNFα), a cytokine that regulates inflammation, infection, cellular organization, and anti-tumor responses.[8,9] The cytokine TNF superfamily is a common drug target for anti-inflammatory diseases, and controlling cytokine pathways is a common approach in drug discovery because cytokines are integral components of cell signaling.[8,10] Additionally, compounds targeting cytokines have been found as effective inhibitors.[8–10] TNFα is stable at room temperature and in buffer.[9,11] Agile R100's binding measurements of small molecule compounds in 10% DMSO interacting with GPCRs and a cytokine demonstrates the ability to achieve kinetic binding data for a wide variety of targets in early stage drug discovery when high concentrations of solvents are needed.

MATERIALS AND METHODS

All volumes are 50 µL, and Agile Plus software was used for all measurements and analysis.

Biosensor Chip Immobilization

A_{2A} and CR GPCRs

Both His-tagged GPCR targets were immobilized using NTA biosensor chips and the "Immobilization – NTA" protocol in the Agile Plus software. The immobilization buffer for the A_{2A} GPCR (50 kDa) target was 50 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) pH 7.4, 500 mM NaCl, 0.025% DDM, 0.005% CHS. The A_{2A} experiments were performed with Agile R100 submerged in an ice bath, and measurements were consistently between 5 to 10°C as measured by a temperature sensor integrated into the biosensor chip. The immobilization buffer for the CR GPCR (47.7 kDa) target was 1X phosphate buffered saline (PBS) pH 7.4, 0.05% DDM, 0.01% CHS. CR experiments were performed at room temperature.

To immobilize the GPCRs, deionized (DI) water pH 7.4 was applied to NTA biosensor chips to calibrate a measurement for 5 minutes. Then, 11.3 mM $NiCl_2$ was added to the biosensor chip for 10 minutes, allowing nickel ions to bind to available NTA groups via chelation. DI water pH 7.4 was then added to rinse away remaining nickel for 1 minute. The biosensor chip was rinsed for an additional 2 minutes with fresh immobilization buffer, followed by a 2-minute calibration step in fresh immobilization buffer to acquire a baseline measurement. Target immobilization was achieved using 14.3 µg/mL A_{2A} or 15.0 µg/mL CR. Both targets were incubated for 60 minutes to allow target attachment as well as sufficient detergent equilibration on the biosensor chip, which acts as both a target stabilizer and nonspecific interaction block. The biosensor chips were then rinsed with assay buffer (immobilization buffer plus 10% DMSO) for 5 minutes until the sensor response reached equilibrium.

TNFα Protein

TNFα (17.5 kDa) was immobilized using COOH biosensor chips and the "Immobilization – COOH" protocol in the Agile Plus software. The immobilization buffer for the TNFα protein target was 1X PBS pH 7.4, and immobilization was performed at room temperature. Target immobilization was achieved using 1789 ng/mL TNFα incubated on the biosensor chip for 15 minutes. Quench 1 (PEG-amine) was added to block exposed graphene to nonspecific binding for 15 minutes, followed by Quench 2 (ethanolamine) to deactivate any remaining surface binding sites for 7.5 minutes. The biosensor chip was rinsed with assay buffer (immobilization buffer plus 10% DMSO) for 5 minutes until equilibrium was reached.

Biosensor Chip Measurement

The small molecules interacting with the A_{2A}, CR, and TNFα are theophylline (TH) (180.16 g/mol), Compound A (434.89 g/mol), and SPD304 (620.53 g/mol), respectively. The assay buffer for each interaction was the respective immobilization buffer with 10% DMSO. The A_{2A} experiments were performed with Agile R100 submerged in an ice bath, while the CR and TNFα experiments were performed with the sample at room temperature.

For the measurements, assay buffer was added to the Agile R100 biosensor chips to calibrate a baseline measurement for 5 minutes. A zero-concentration measurement was taken by applying fresh assay buffer to the biosensor chip for 5 minutes. Fresh assay buffer was applied again to calibrate a baseline measurement for 5 minutes. The A_{2A}, CR, and TNFα biosensor chips were then exposed to the respective small molecule at 10 mM TH, 100 µM Compound A, or 30 µM SPD304 in the respective assay buffer for 5 minutes to measure association. Fresh assay buffer was then added to initiate dissociation for 5 minutes.

Analysis

The zero-concentration sensor response was compared to the compound sensor response. Dissociation constant (K_D) values of the target and compound interaction were determined using the kinetic method using Agile Plus software, described here briefly.[12,13] The observed rate (k_{obs}) was calculated as association was recorded between target and compound. The dissociation rate (k_{off}) was then calculated as the dissociation was recorded between the target and compound. The association rate (k_{on}) was calculated as

$$k_{on} = \frac{(k_{obs} - k_{off})}{C} \quad (1)$$

where C is the concentration of compound. Once kinetic binding rates were determined, the K_D was calculated as

$$K_D = \frac{k_{off}}{k_{on}} \quad (2)$$

The values from the Agile Plus software are reported here.

RESULTS AND DISCUSSION

Figure 1 depicts association and dissociation curves for the zero concentration and the compound concentration for the A_{2A} and TH interaction (Figure 1A), the CR and Compound A interaction (Figure 1B), and the TNFα and SPD304 interaction (Figure 1C). The corresponding curve fits are overlaid on each sensor response shown, and the K_D values are reported below each graph (K_D = 1020 µM for TH, K_D = 70.47 µM for Compound A, and K_D = 12.06 µM for SPD304). The concentration of small molecule is shown in the legend as 10 mM, 100 µM, and 30 µM for TH, Compound A, and SPD304, respectively. All interactions resulted in large sensor responses (~150 to 250 BU) that were significantly greater than their corresponding zero-concentration curves, showing that 10% DMSO causes negligible noise on Agile R100. The range of K_D values and concentrations measured show that the platform can detect lower concentrations of compound in DMSO as affinity improves.

Table 1 describes the kinetic binding data for the A_{2A} and TH interaction, CR and Compound A interaction, and TNFα and SPD304 interaction, calculated directly from the sensorgrams in Figure 1. K_D values, as calculated by Agile Plus software, range from low micromolar to low millimolar (K_D = 12.06 to 1020 µM) with fast on-rates (ranging from 1.414 to 120.7 $M^{-1}s^{-1}$) and fast off-rates (ranging from 1.456 to 14.43 ks^{-1}), showing low- to mid-affinity interactions. Thus, low-affinity compounds dissolved in 10% DMSO are easily detected on Agile R100, and the negligible background from high concentrations of DMSO make the binding interaction of compounds easily distinguishable.

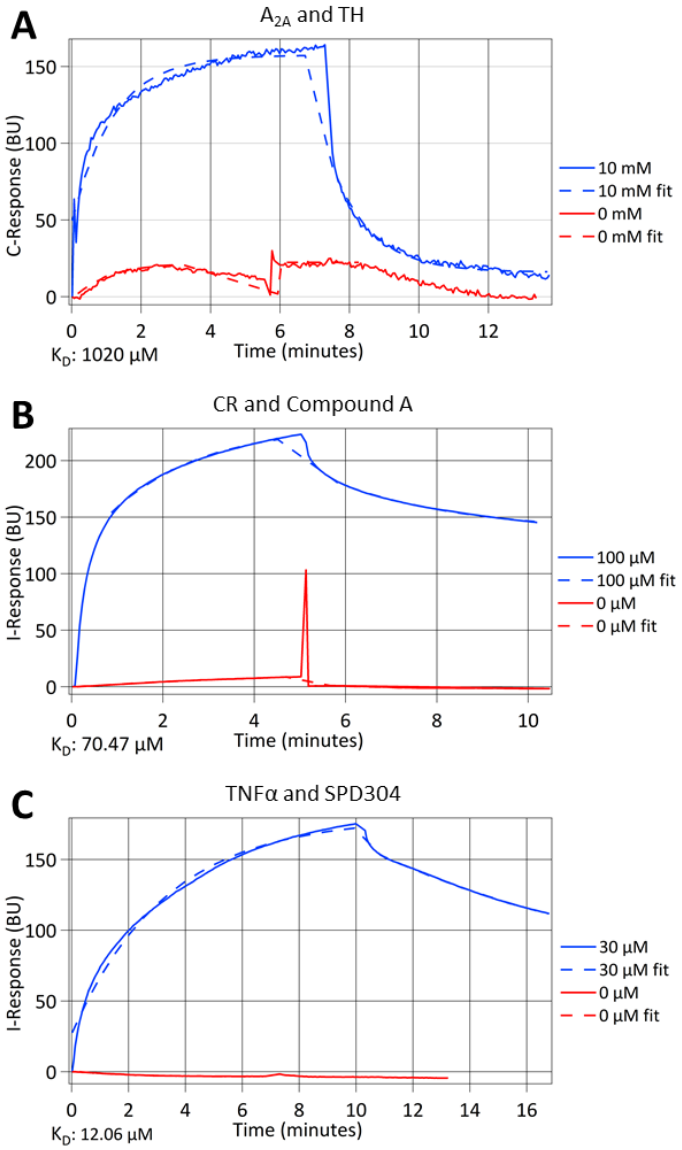

Figure 1: Sensorgrams of interactions of compounds in 10% DMSO. A) 10 mM compound TH is added to target GPCR A_{2A}, and the K_D is 1020 µM. B) 100 µM Compound A is added to immobilized CR, and the K_D is 70.47 µM. C) 30 µM compound SPD304 is added to target TNFα, and the K_D is 12.06 µM.

Table 1: Summary of kinetic binding data for the compounds TH, Compound A, and SPD304 with their respective targets.

Target	Compound	k_a ($M^{-1}s^{-1}$)	k_d (ks^{-1})	K_D (µM)
A_{2A}	**TH**	1.414	14.43	1020
CR	**Compound A**	83.12	5.858	70.47
TNFα	**SPD304**	120.7	1.456	12.06

CONCLUSIONS

Agile R100 uses an electrical technique called Field Effect Biosensing (FEB) that easily senses in optically challenging solutions such as 10% DMSO and detergents. This application note reports kinetic binding data for small molecule compounds in 10% DMSO interacting with their respective targets (2 solubilized GPCRs and the TNFα protein). K_D values range from low micromolar to low millimolar (12.06 to 1020 µM), and fast on-rates (as fast as 120.7 $M^{-1}s^{-1}$) and off-rates (as fast as 14.43 ks^{-1}) inherent of low affinity interactions are easily measured. With the ability shown in this application note to detect micromolar affinities and lower, Agile R100 is a single personal assay platform that can be used throughout the drug discovery process for both low affinity early stage compounds and later stage compounds as affinity improves. Agile R100 characterizes a wide range of crucial drug targets, such as GPCRs and cytokine proteins, in buffers with solvents and detergents, making it a highly versatile label-free kinetic binding platform.

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