The Utility of Drug Residence Time for the Assessment of Inhibitor Potency

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Introduction

Drug-target residence time is becoming increasingly recognized as a critical parameter for the assessment of drug potency. Inhibition of the target protein's activity can only take place when the drug is bound, which is governed by the affinity of the compound for the target, as well as the dissociative off-rate of the compound from the drug-target complex. Traditionally, drug-target residence time studies have been performed in late stage preclinical studies using radio-labeled compounds, but many programs now desire to probe hits from screens which require technologies that provide greater sample throughput.

Here we describe the ultility of the Tag-lite detection technology for performing high throughput kinetic ligand binding using a microplate reader. This HTS mix-and-ready technology is suitable for assessing many compounds rapidly. The microplate reader requirements are principally governed by the need for high temporal resolution without sacrificing sensitivity for the accurate assessment of drug-target kinetic parameters. A case study using CXCR4 and its natural ligand, CXCL12 was chosen to demonstrate the determination of the residence time for AMD 3465, a known CXCR4 inhibitor. A comparison of both kinetic and thermodynamic ligand and competitor binding was performed.

BioTek Instrumentation

Figure 1. Synergy™ Neo2 Multi-Mode Reader: Synergy Neo2 Multi-Mode Reader is designed for speed and ultra high performance, incorporating BioTek's patented Hybrid Technology™. Independent optical paths accommodate diverse assay requirements with variable bandwidth quadruple monochromators, sensitive filter-based optics, laser-based excitation for Alpha assays and up to 4 PMTs for ultra-fast measurements. Advanced environment controls, including CO_2 /O_2 control, incubation to 65 °C and variable shaking are ideal for live cell assays and cell based detection is optimized with direct bottom illumination. Barcode-labeled filter cubes help streamline workflows and limit errors.

The HTRF® certified reader uses a high performance xenon flash lamp and dual PMTs in the filter-based optics to simultaneously detect the assay's 665 nm and 620 nm fluorescent emissions when the excitation was set to 340 nm. The dual reagent injection capabilities allowed dispensing of cells directly into wells while the assay plate was already in the reading chamber, enabling kinetic analyses of known ligand and competitive binding.

Tag-lite® Receptor Ligand Binding Assay

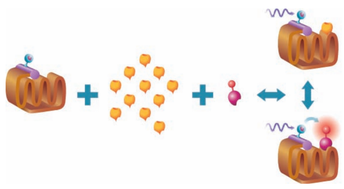

Figure 2. Tag-lite Receptor Ligand Binding Assay Procedure: The Tag-lite CXCR4 assay relies on a fully functional SNAP-tag fused CXCR4 receptor and fluorescent d2 labeled ligand SDF-1α. Being homogeneous, the binding assay allows for binding events to be precisely recorded in time. The assay can be used to derive the kinetic binding parameters of unlabeled compounds by application of the Motulsky and Mahan equations.

Materials

AMD 3465 hexahydrobromide (Catalog No. 4179) was purchased from Tocris Bioscience (Minneapolis, MN). Recombinant human SDF-1α (Catalog No. 350-NS) was purchased from R&D Systems (Minneapolis, MN). 384-well low volume, white, round bottom, non-treated microplates (Catalog No. 3674) were donated by Corning Incorporated (Corning, NY). Tag-lite CXCR4 Stable Cell Line, SDF-1α-d2 and all labeling reagents used for the characterization on the inhibitors' kinetic properties were donated by Cisbio Assays.

Materials

SDF1-α-d2 Association and Dissociation Rate Constants
The association and dissociation rate constants of SDF1-α-d2 ($k_{on\ SDF1\text{-}\alpha\text{-}d2}$ and $k_{off\ SDF1\text{-}\alpha\text{-}d2}$) were determined by placing the CXCR4 expressing cells (5000 cells/well) in the presence of SDF1-α-d2 and measuring specific binding kinetically thereafter. A total of eight [SDF1-α-d2] were tested, thus also allowing for calculation of the half saturation binding constant at equilibrium ($K_{d\ SDF1\text{-}\alpha\text{-}d2}$).

SDF1-α Association and Dissociation Rate Constants
The association and dissociation rate constants of SDF1-α ($k_{on\ SDF1\text{-}\alpha}$ and $k_{off\ SDF1\text{-}\alpha}$) were determined by performing a competitive binding procedure. First, varied concentrations of SDF1-α and a single concentration of SDF1-α-d2 below the K_d value were combined. CXCR4 expressing cells (5000 cells/well) were then injected into the wells and kinetic measurements collected thereafter. A total of seven [SDF1-α] were tested, enabling calculation of the concentration inhibiting binding of the SDF1-α–d2 ligand by half ($IC_{50\ SDF1\text{-}\alpha}$). The K_i is then determined from the IC_{50} value.

Kinetic characterization of AMD3465
The association and dissociation rates of AMD3465 were then generated using same competitive binding procedure previously explained. Here three [AMD3465] were tested with the same [SDF1-α-d2] used to determine the SDF1-α binding rates.

All experimental data were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

SDF1-α-d2 Kinetic Characterization

The association and dissociation rate constant of labeled SDF1-α must be determined with reasonable precision. The values are later used in the equations for the characterization of unlabeled competitors of ligand binding to the receptor. As multiple concentrations of labeled SDF1-α were tested, the k_{on} and k_{off} values for d2 labeled SDF1-α were able to be calculated.

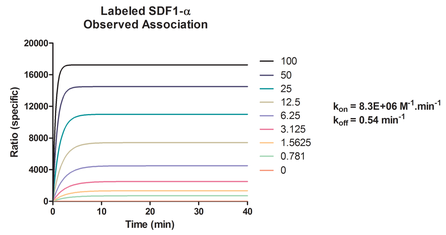

Figure 3. Association binding graph of SDF1-α-d2. Binding increases over time until it plateaus after several minutes. The plateau in an association experiment depends on the concentration of labeled SDF1-α used. Higher plateaus will be obtained with higher concentrations. Fitting of the curves with Graph Pad Prism yields the observed association and dissociation rate values.

The K_d value must also be calculated to determine the concentration of labeled SDF1-α to use when performing competitve binding experiments. Incorporating a concentration greater than the K_d value can right shift results generated from potential inhibitory molecules. The specific HTRF ratios generated at the 40 minute timepoint from the associative binding experiment were plotted vs. [SDF1-α –d2] to calculate the value.

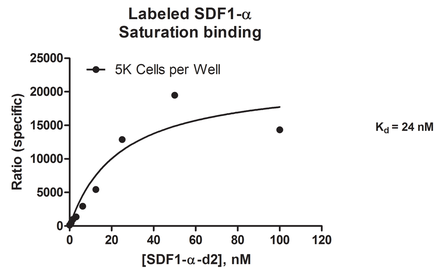

Figure 4. Saturation Binding. In a saturation binding experiment, increasing concentrations of labeled SDF1-α result in increased binding. Saturation is obtained when no further binding can be recorded. The ligand concentration that binds to half the receptor sites at equilibrium represents the K_d value.

The Rate constant values experimentally found for SDF1-α–d2 (Figure 3 and 4) of k_{on}: 8.26E+06 M-1.min-1, k_{off}: 0.54, and K_d: 24 nM were then used as the K1, k2, and L constants, respectively, to properly fit the results generated in subsequent competitive binding experiments.

SDF1-α Kinetic Characterization

The binding kinetics of unlabeled SDF1-α were then ascertained experimentally. Here multiple concentrations of SDF1-α were incubated with a single concentration, 5 nM, of SDF1-α-d2 below the K_d value in a competitive binding experiment. As with the SDF1-α-d2 associative binding experiment described above, the use of multiple concentrations of unlabeled ligand, and kinetic monitoring reaching steady state levels allows k_{on} , k_{off} , and K_d values to be calculated.

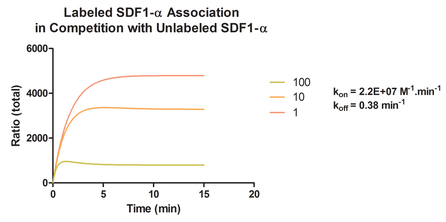

Figure 5. Competitive binding graph of SDF1-α. Use of [SDF1-α] similar to the [SDF1-α-d2] of 5 nM yields binding curves similar to that seen in Figure 3. [SDF1-α] higher than this value illustrate the increasing competition exhibited by SDF1-α to the receptor. Fitting of the curves with Graph Pad Prism yields observed association and dissociation rate values for SDF1-α.

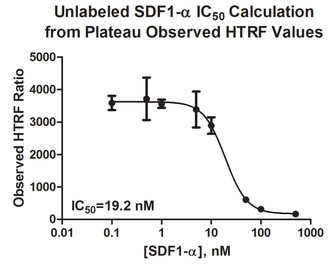

Figure 6. SDF1-α Saturation Binding. The IC_{50} value is calculated by plotting HTRF ratios attained after 15 minutes of binding for each [SDF1-α].

From the k_{on}, k_{off}, and IC_{50} values calculated, thermodynamic and kinetic binding comparisons can then be made between test molecules and SDF1-α.

Kinetic Evaluation of Receptor-Ligand Binding Inhibition

Prior to performing test molecule competitive binding tests, the suitability of using SDF1-α-d2 as a surrogate for SDF1-α was evaluated. By using the formulas $K_i = IC_{50}/1+[X]/K_d$, where X is the concentration of SDF1-α-d2 used, or 5 nM, and $K_d = k_{off}/ k_{on}$, the thermodynamic and kinetic binding constants for SDF1-α can be calculated. When compared to K_i and K_d values for SDF1-α-d2 (Table 1), it is apparent that similar values are attained, verifying the validity of the SDF1-α-d2 molecule for use in competitive binding tests.

	K_i (nM)	K_d (nM)
SDF1-α-d2	24	23.8
SDF1-α	15.8	17.3

Table 1. SDF1-α and SDF1-α thermodynamic and kinetic binding constant comparison.

A competitive binding experiment was then performed between SDF1-α-d2 and the known CXCR4 antagonist AMD3465 (Bodart, *et al.*, 2009). A 5 nM [SDF1-α-d2] was once again incorporated, in addition to variable concentrations of AMD3465 (Figure 7).

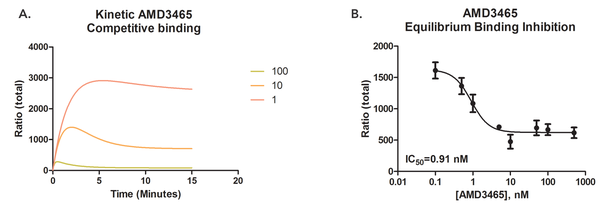

Figure 7. AMD3465 vs. SDF1-α-d2 kinetics of competitive binding. (A) Total HTRF ratios plotted vs. time for 100, 10, and 1 nM [AMD3465] incubated with 5 nM SDF1-α-d2. **(B)** Total HTRF ratios plotted for 500-0.1 nM [AMD3465] following 15 minute incubation.

In the theory developed by Motulsky and Mahan (1984), an unlabeled competitor is co-incubated with a labeled ligand during a kinetic association experiment. From the curve fitting, the kinetic constants of the unlabeled competitor may be derived. From the curve shape, qualitative assumption regarding the constant may be found. In particular, "if the competitor dissociates faster from its target than the ligand, the specific binding of the ligand will slowly and monotonically approach its equilibrium in time. However, when the competitor dissociates slower, the association curve of the ligand will consist of two phases, starting with a typical "overshoot" and then a decline until a new equilibrium is reached. The latter of these two is exhibited in Figure 7.

	K_i (nM)	K_d (nM)
SDF1-α-d2	24	23.8
SDF1-α	15.8	17.3
AMD3465	0.3	2.3

Table 2. Ligand and competitor thermodynamic and kinetic binding constants.

K_i and K_d values for AMD3465 were determined in a manner similar to that used for unlabeled SDF1-α. The K_d was calculated using k_{on} and k_{off} values generated from competitive binding results (Figure 7A). The K_i again took into account the IC_{50} value taken from HTRF ratios seen after steady state binding was reached (Figure 7B).

Upon closer examination of the antagonist curve in Figure 7B and calculated K_i values from Table 2, it is evident that AMD3465 interrupts binding of SDF1-α-d2 to the CXCR4 receptor. However, as the results represent the effect of the molecule at a single point in time, and after steady state binding kinetics have been reached, the lower K_i value does little to explain why the compound has the dramatic observed antagonistic effect. An investigation of the generated binding characteristics of all molecules may provide the answer.

	k_{on} ($M^{-1}.min^{-1}$)	k_{off} (min^{-1})	R (min)
SDF1-α-d2	8.3E+06	0.54	1.9
SDF1-α	2.2E+07	0.38	2.6
AMD3465	5.8E+07	0.13	7.5

Table 3. Kinetic binding constants for ligand and competitor molecules.

When first looking at the k_{on} rate for AMD3465, compared to both SDF1-α and SDF1-α-d2, it can be seen that the value is ~2.5X larger than that for SDF1-α and ~7X larger than SDF1-α-d2, meaning that the small molecule out competes the known ligand for binding to CXCR4. In addition, a lower off rate is also observed for AMD3465. When residence time (R) is calculated by dividing $1/k_{off,}$ it is clear that once the molecule binds to the receptor it also resides for longer periods of time compared to labeled and unlabeled ligand. This is confirmed by the curve shapes generated in Figure 7 and the previously described explanation by Motulsky and Mahan. The combination of these two observations provide a complete explanation for the final antagonistic effect seen by AMD3465.

Conclusions

1. The Tag-lite CXCR4 ligand binding assay affords a simple, yet robust cell-based approach to determine kinetic binding of known receptor ligands, as well as competitive binding of test molecules.
2. Tests performed with the SDF1-α-d2 labeled ligand prove the validity of the molecule for use in receptor-ligand binding experimental procedures.
3. The rapid, simultaneous dual emission capture capabilities of the Synergy Neo2 allow accurate calculations of kinetic association and dissociation rates.
4. Dual injectors allow cells to be dispensed into wells containing test molecules, ensuring capture of initial rate kinetic data.
5. The combination of direct cell-based binding assay chemistry and sensitive HTS microplate reader technology create an ideal method to quickly and easily generate accurate kinetic data, allowing for a more complete understanding of test molecules during the drug discovery process.