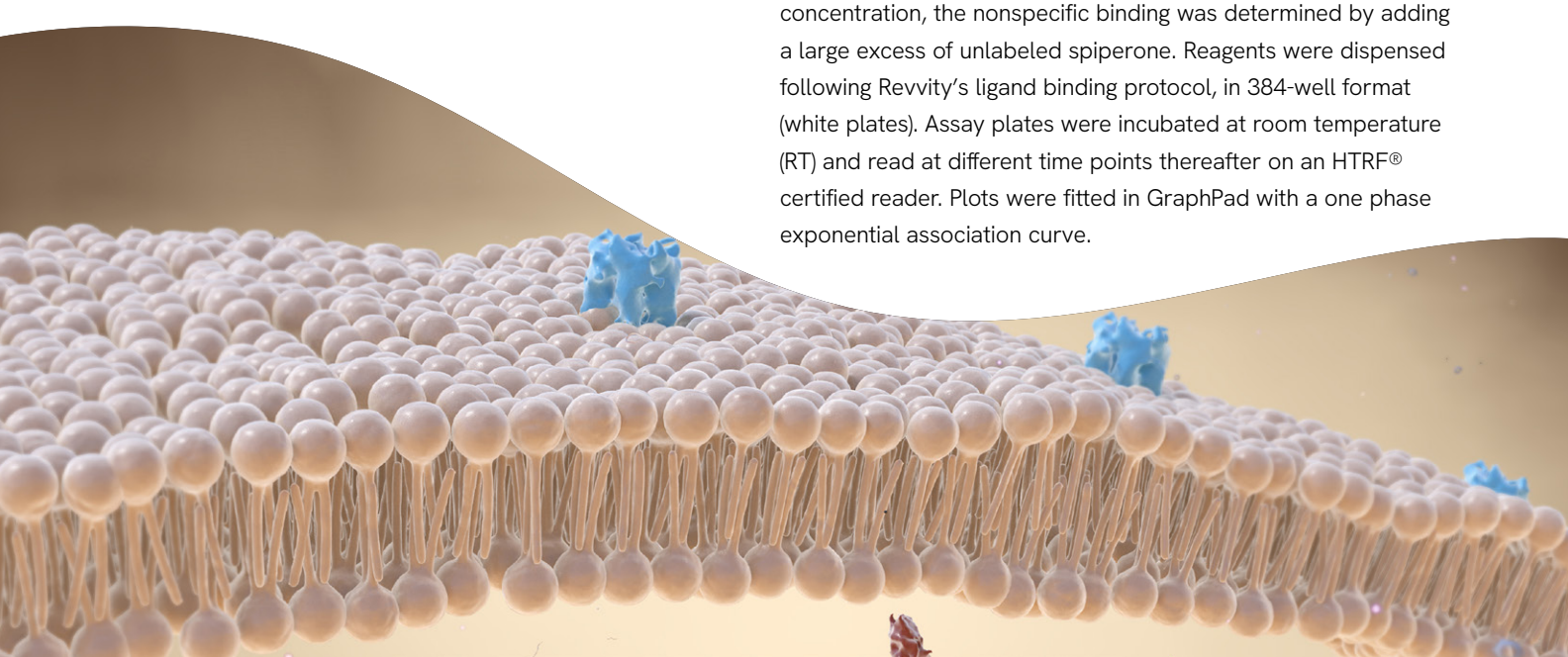
APPLICATION NOTE

Determination of association (K_{on}) and dissociation (K_{off}) rates constants using the Tag-lite® platform.

We have utilized Tag-lite to directly measure the kinetics of Spiperone-d2 binding on the cell surface expressed Dopamine D2 receptor. Tag-lite enables generation of the dissociation and association rates constants for a given labeled molecule in a plate-based homogeneous format.

For research purposes only. Not for use in diagnostic procedures.



Abstract

Discussions around the use of Tag-lite for GPCR applications have revolved solely around the term affinity or potency and the calculation of the K_d output, greatly undermining the kinetic aspect of the binding process. K_d or K_i refer to the equilibrium constant, and thus reflect an equilibrium state. In contrast, K_{off} and K_{on} constants allow the prediction not only of the equilibrium state of a drug, but also of how fast the drug-receptor system responds to changes in the concentration of the drug or to another competitor.

This application note takes advantage of the Tag-lite Dopamine D2 ligand binding kit to calculate the association and dissociation rates of Spiperone-d2. The flexibility and throughput of the Tag-lite platform enables fast and easy calculation of the k_{off} and k_{on} constants for a given receptor.

Materials and methods

Saturation binding and association rate experiments

Labeled Spiperone (Spiperone-d2 - #L002RED) was reconstituted as described in the protocol in Tag-lite labeling medium (#LABMED) and diluted 2 fold from a 100 nM solution to a 1.5 solution. Frozen labeled cells (Tag-lite Dopamine D2 - 1 Million labeled cells, #C1TT1D2) were reconstituted following the protocol and diluted in Tag-lite labeling medium to a 200 cells/µL concentration. Affinities of the fluorescent ligand for the receptor were determined by incubating the cells at RT with increasing concentrations of labeled ligand. For each fluorescent ligand concentration, the nonspecific binding was determined by adding a large excess of unlabeled spiperone. Reagents were dispensed following Revvity's ligand binding protocol, in 384-well format (white plates). Assay plates were incubated at room temperature (RT) and read at different time points thereafter on an HTRF® certified reader. Plots were fitted in GraphPad with a one phase exponential association curve.

Dissociation rate experiment

Dissociation was initiated after the system reached equilibrium (1 hour after ligand addition) by adding 3 µL of a 100 µM Bromocriptine solution in 3% DMSO. The final concentration of Bromocriptine was 13µM, for a final 0.02% DMSO concentration, well in excess of the labeled ligand concentration. Measurements of the plate, incubated at RT, were distributed over a lengthy period of time in order to capture dissociation. In each case, the effect of dilution over time was plotted to obtain the point of a 50% decline in binding (t1/2). The plate was re-read until dissociation was complete.

Plots were fitted in GraphPad with a one phase exponential decay curve.

Results

K_d determination by saturation binding experiment

The HTRF ratio was measured for each labeled ligand concentration. The specific signal was obtained by subtracting the non-specific signal from the total signal. K_d value for the spiperone derivative was calculated after the system reached equilibrium, i.e. after 60 minutes' incubation, and found to be 8 nM (Fig.1).

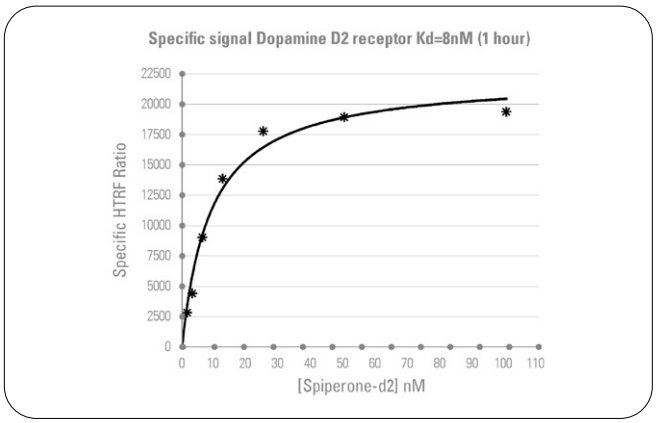


Figure 1: Spiperone-d2 saturation binding experiment

K_{on} and K_{off} determination by association experiments

Association binding experiments were used to determine the association rate constant or K_{on} by addition Spiperone-d2. The specific binding was measured at various times (Fig. 2), and the calculated parameters are presented in table 1.

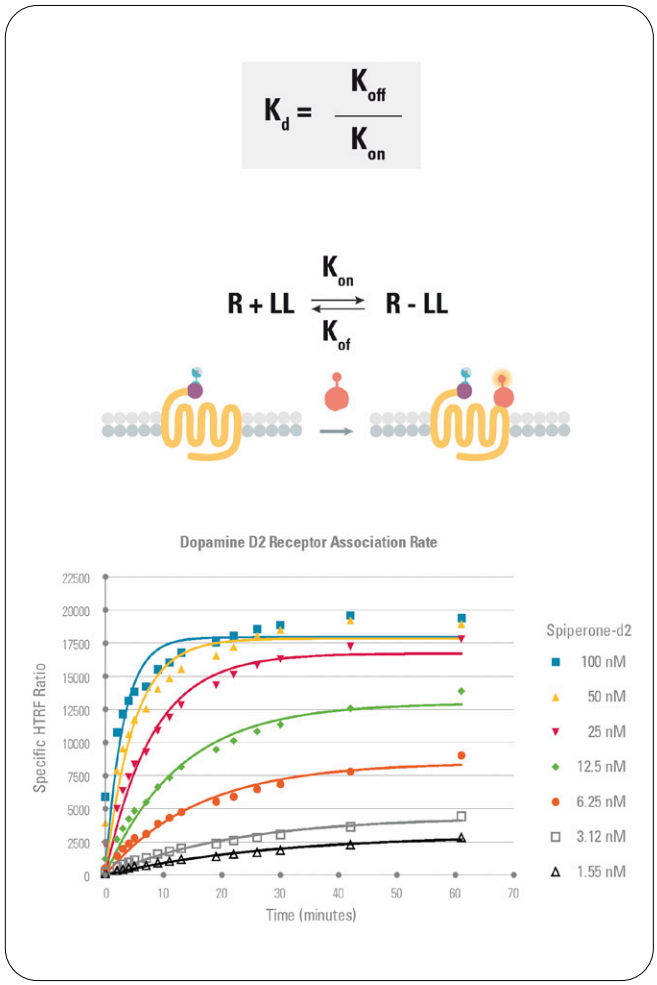


Figure 2: Spiperone-d2 association binding experiment

Binding increases over time, then levels off after 60 minutes' incubation. For each labeled ligand concentration tested, a K_{obs} value was derived, and then later transformed into a K_{on} rate based on the ligand concentration and the K_{off} rate.

Table 1: Association pharmacokinetic values of Spiperone-d2

Spiperone -d2 [LL] (nM)	100	50	25	12.5	6.25	3.12	1.55	0
Ymax	17947	17843	16717	12969	8492	4400	3019	-15.01
k_{obs}	0.3286	0.2072	0.1246	0.0785	0.06292	0.04501	0.03582	2.544
t1/2 association	2.109	3.345	5.562	8.83	11.02	15.4	19.35	0.2725

The observed association rate constant, K_{obs}, is also defined by the following equation:

$$K_{obs} = K_{off} + K_{on} \times [LL]$$

K_{obs} : min^{-1}

K_{on} : $M^{-1}. min^{-1}$

[LL] (labeled ligand): M^{-1}

As the concentration of labeled ligand increases, the observed rate constant or K_{obs} should increase linearly (as opposed to the fixed constant K_{off}). Fig. 3 represents K_{obs} as a function of the labeled ligand concentration for the Dopamine D2 receptor and Spiperone-d2. K_{on} and K_{off} are determined from this figure, and the Kd is then calculated as the K_{off}/K_{on} ratio.

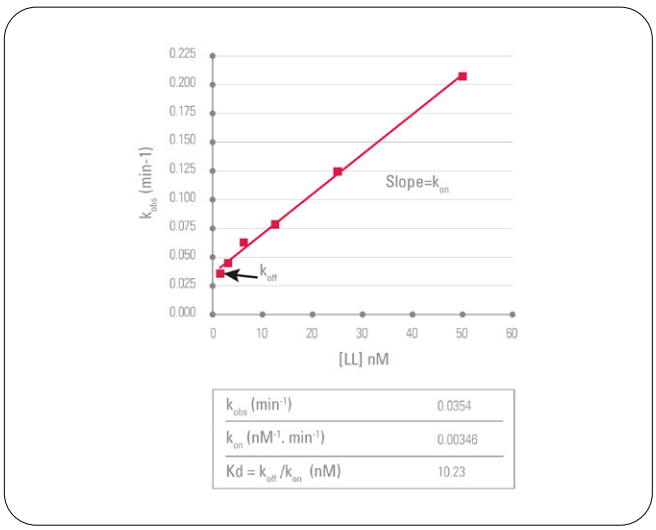


Figure 3: K_{off} and K_{on} determination

Dissociation experiment: another way to calculate K_{off}

A dissociation binding experiment measures the «off rate constant» (K_{off}) for ligand dissociating from the receptor. Initially the ligand and receptor were allowed to bind until equilibrium was reached. At that point, dissociation was initiated by massive introduction of unlabeled Bromocriptine, a full agonist of Dopamine d2 receptor. The experiment was performed at different concentrations of labeled ligand (Fig. 4).

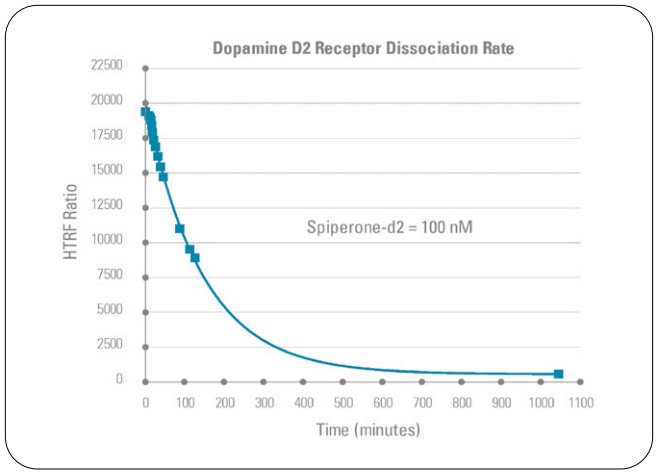


Figure 4: Spiperone-d2 dissociation binding experiment

Binding decreased over time until it leveled off after overnight incubation. K_{off} values were derived, and later used to calculate the K_{on} rate constant.

From the fit, GraphPad also calculates a dissociation half-time (t1/2) in minutes (Table 2). The dissociation half-time represents the time needed for half the ligands to dissociate rom the receptor to which they were initially bound.

Table 2: Dissociation pharmacokinetic values of Spiperone-d2

K_{off} (min^{-1})	0.007
PLATEAU	563.1
T1/2 dissociation (min)	99
$K_{on}=(K_{obs}-K_{off})/[LL]$ ($nM^{-1}.min^{-1}$)	0.00322
$K_D = K_{off}/K_{on}$ (nM)	2.17

Table 2 summarizes the values obtained during the dissociation experiment for Spiperone-d2. K_{off} is obtained directly from the dissociation experiment, while K_{on} and K_d are calculated.

Discussion

The dissociation half-life, using the dilution method, was 99 minutes and the koff rate constant 0.00699 min^{-1}. These results correlate well with the results for unconjugated spiperone, as measured using radioactivity by S Kapur and P Seeman (Antipsychotic agents differ in how fast they come off the dopamine D2 receptors. Implications for atypical antipsychotic action, J Psychiatry Neurosci 2000;25(2):161-6).

Reported spiperone t1/2 was 200 minutes (by excess of Raclopride, an antagonist ligand for the dopamine D2 receptor) and koff rate constant was 0.003 min^{-1}. These results matched the results obtained with the dilution method.

The K_d calculated from the saturation binding (Fig. 1), the association experiment (Fig. 3) and dissociation experiment (table 2) were similar. This confirms that the dopamine D2 binding assays developed with the Tag-lite reagents follow the law of mass action.

Table 3: Comparison of the K_d determination by saturation experiment or pharmacokinetic experiments. K_d calculated after the pharmacokinetic experiments is the ratio K_{off}/K_{on}.

Saturation experiment		Association experiment	Dissociation experiment
k_{off}	-	0.0354 min^{-1}	0.007 min^{-1}
k_{on}	-	0.00347 $nM^{-1}.min^{-1}$	0.00322 $nM^{-1}.min^{-1}$
K_d	8 nM	10.2 nM	2.17 nM

Conclusion

We have utilized Tag-lite to directly measure the kinetics of Spiperone-d2 binding on the cell surface expressed Dopamine D2 receptor. Tag-lite enables generation of the dissociation and association rates constants for a given labeled molecule in a plate-based homogeneous format. The pharmacokinetic values obtained were in agreement with the published literature and matched the equilibrium constant obtained from saturation binding experiments.

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