Analyze binding kinetics in a microplate reader

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Introduction

Drugs are characterized by their equilibrium constant K_D which describes the affinity of the compound to its target. It refers to half the ligand concentration of saturated binding. However, this dissociation constant only applies to the equilibrium state and neglects the importance of the speed of association and dissociation. The optimal kinetic binding of a drug differs with the target of interest and related disease. On the one hand, slow dissociation constants can be beneficial to increase the duration of drug action (as for example Tiotropium for COPD treatment - Disse *et al*. Life Science, 1999). On the other hand, it has been hypothesized for specific targets that fast dissociations could decrease on target side effects (Kapur & Seeman, J Psychiatry Neurosci, 2000). Here, we present the determination of the association and dissociation rate of Spiperone-d2 binding to the dopamine receptor D_2 (D2R), a G-protein coupled receptor (GPCR) and target of antipsychotic drugs.

Assay Principle

The binding of Spiperone-d2 to D2R was monitored using cells with terbium cryptate labelled receptor and d2-fluorophore-labelled Spiperone. The terbium cryptate is a fluorophore with a long lifetime acting as a donor. It transfers energy to the Spiperone-associated d2 in case the ligand is bound to the receptor. Thus the interaction of ligand with receptor is described by the ratio of acceptor to donor emission signal.

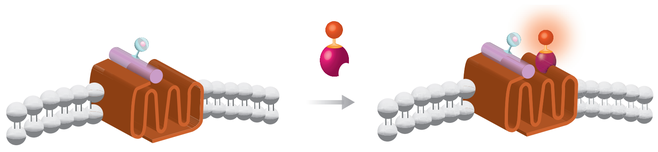

Fig. A: Principle of TR-FRET interaction assay to detemine binding kinetics between receptor and ligand

Materials & Methods

- Tag-lite Dopamine D_2 labelled cells (Cisbio Bioassays, #C1TT1D2)
- Spiperone-d2 (Cisbio Bioassays, #L0002RED)
- Tag-lite labelling medium (Cisbio Bioassays, #LABMED)
- 384 well plate (small volume, Greiner #784075)

The reaction was performed according to the instructions for Cisbio's ligand binding protocol. Briefly, in a total reaction volume of 20 µl, 3.700 donor-labelled cells were incubated with different concentrations of the d2-labelled ligand Spiperone. The binding was monitored in real-time by the PHERAstar *FSX* using the instrument settings outlined in the right-hand column.

Instrument settings

Optic settings	Time resolved fluorescence, plate mode kinetic, simultaneous dual emission	
	Optic module	HTRF
General settings	Number of flashes	40
	Settling time	0 s
	Integration start	60 µs
	Integration time	400 µs
Kinetic settings	Number of cycles k_{on}/ k_{off}	61 / 125
	Cycle time	60 s
Incubation	none	

Conclusion

The TR-FRET-based method to determine the kinetics of drugs binding their targets does not rely on expensive instrumentation, radioactivity or countless reactions for individual timepoints. Rather, a microplate reader with high temporal resolution in TR-FRET measurements is sufficient to resolve binding events and calculate association and dissociation rates. These features increase speed as well as sensitivity and make the PHERAstar *FSX* microplate reader the instrument of choice for measuring binding kinetics.

Key instrument features for kinetic measurements:

- Simultaneous detection of acceptor and donor signal
- Kinetic TR-FRET measurements
- Simultaneously inject and read
- High speed laser for excitation

Results & Discussion

To demonstrate how association kinetics can be measured using a microplate reader and time-resolved FRET, a pair of ligand and cells expressing the target GPCR was used. Spiperone coupled to the acceptor fluorophore d2 served as ligand and the GPCR dopamine receptor D_2 (D2R) expressed in HEK cells and coupled to the donor fluorophore terbium cryptate served as target. Cells were preincubated in 384-well plates and the analysis was started by addition of Spiperone-d2 at increasing concentrations (1.55-100nM). At the same time recording of the TR-FRET signals was started and continued with one measurement each minute.

The PHERAstar *FSX* microplate reader detected the signal coming from donor and acceptor fluorophore simultaneously to guarantee high temporal resolution. The result of FRET measurements is given as a ratio of acceptor signal and donor signal. The ratio accordingly increases with increased binding of the ligand to the receptor. This is shown by the association experiment: at all ligand concentrations, the FRET ratio increases with time. The longer the reaction takes, the more ligand is bound until it reaches the equilibrium. For low ligand concentration the ratio at equilibrium is low whereas it increases along with concentration (Figure B). The association rate as well as the affinity (K_D – equilibrium constant) can be derived from these association curves as detailed on BMG LABTECH's website.

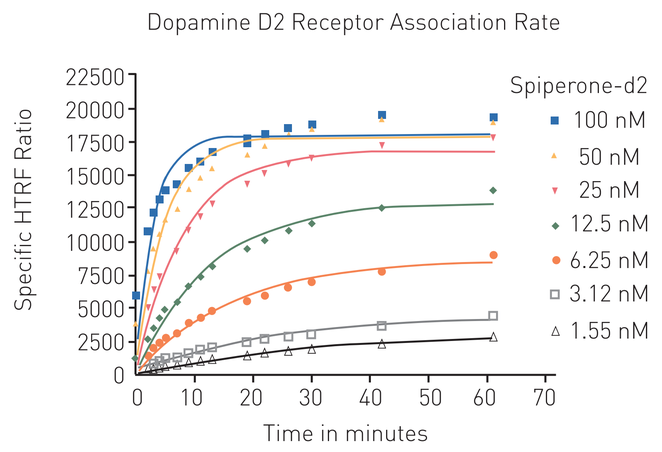

Fig. B: Association experiment: monitoring of Spiperone-d2 binding to dopmaine receptor 2 expressed in HEK cells.

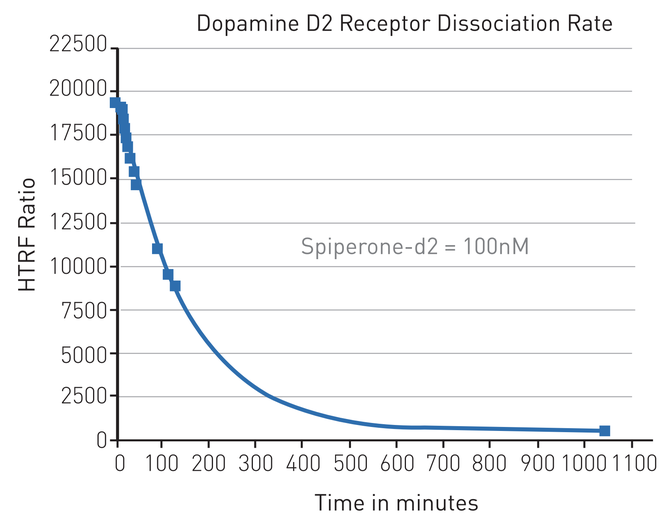

Fig. C: Dissociation experiment: Spiperone-d2 is displaced by excess Bromocriptine.

To determine the dissociation constant, Spiperone-d2 is subsequently displaced by an excess of non-fluorescent agonist of D2R. Dissociation and the related decrease in TR-FRET ratio was monitored in realtime (Figure C) using the PHERAstar *FSX* microplate reader. Subsequent analysis revealed a dissociation rate of 0.007 min-1.

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