Antagonist binding kinetics at the human D1 dopamine receptor determined by HTRF assay

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Introduction

The binding kinetics of ligands, described by their association (k_{on}) and dissociation (k_{off}) rate constants at the target receptor, significantly influence drug action. For example, recent studies on the D2 dopamine receptor demonstrate links between agonist kinetics and observed signalling bias [1], and correlate antipsychotic side effects with association rates and their potential influence on rebinding [2]. The D1 receptor (D1R) offers an alternate target in ameliorating symptoms of schizophrenia [3], and while functional effects of D1R antagonists have previously been assessed [3], less is known about their binding kinetics. Here we develop a time-resolved fluorescence (HTRF) D1R binding assay, and demonstrate its applicability in measuring antagonist kinetic parameters by the competition association method [4].

Methods

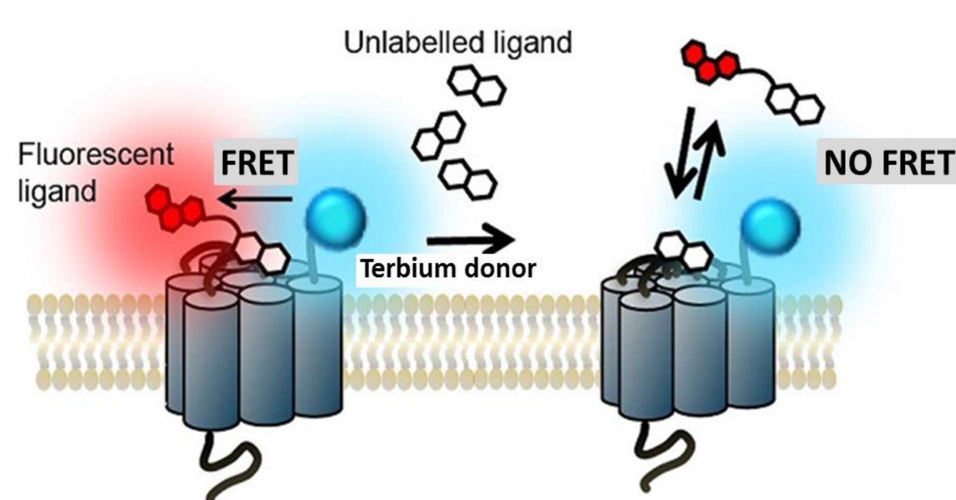

Fig 1. Schematic of the HTRF ligand binding FRET assay principle. Figure from Cisbio, https://uk.cisbio.eu/

Förster resonance energy transfer (FRET) is the transfer of energy between a donor and acceptor fluorophore when in close proximity. Combined with homogenous time resolved fluorescence (HTRF) technology, binding events are able to be robustly and sensitively measured, with interferences reduced due to the time-resolved nature of the dual-wavelength FRET signal measurements.

Cell culture. Chinese hamster ovary (CHO) cells were transfected with cDNA encoding SNAP-tagged human dopamine D1 receptor (D1R) and maintained overnight in Dulbecco's modified Eagle's medium: Ham F12 (DMEM:F12) supplemented with 10% FBS and 2 mM glutamine.

Terbium labelling of SNAP-tagged D1R. Cell culture medium was removed, 100 nM SNAP-Lumi4-Tb was added per flask and incubated for 1h at 37°C, 5% CO_2, before washing 2x with PBS. 5 mL of GIBCO enzyme-free Hank's-based cell dissociation buffer was added to each flask, and cells scraped and centrifuged (1200 rpm for 10 min) to form a pellet.

Membrane preparation. Cell pellets were resuspended in 10 mL ice-cold wash buffer (10 mM HEPES, 10 mM EDTA, pH 7.4), transferred to Lysing Matrix D tubes and fitted to the FastPrep-24 5G homogenizer. Cells were homogenized for 30 s, 6 m/sec, before centrifugation at 22,000 *g*, 4°C for 30 min. Supernatant was discarded, lysates resuspended in 5 mL wash buffer, and re-centrifuged at 22,000 *g*, 4°C for 30 min. Supernatant was again discarded, and the final pellet resuspended in ice-cold 10 mM HEPES and 0.1 mM EDTA, pH 7.4 at a concentration of 2.4 mg/mL. The bicinchonic acid assay kit (Sigma-Aldrich) was used to determined protein concentration. Membranes were aliquoted and frozen at -20°C prior to use.

Fig 2. Mosquito® HTS nanolitre liquid handler (TTP Labtech). Figure from TTP Labtech.

HTRF assays. All experiments were performed in white 384-well Optiplates, in assay binding buffer (Labmed (Cisbio) supplemented with 1% DMSO, 100 µg/mL saponin, 100 µM GTP and 0.02% pluronic acid). Nonspecific binding was determined in the presence of 100 µM SCH-31966. Serial dilutions and stamping of compounds were performed using the Mosquito® HTS nanolitre liquid handler (TTP Labtech).

Kinetic characterisation of SKF83566-green (F-SKF). Association kinetic curves were determined via addition of increasing concentrations of F-SKF (Cisbio) to membranes, with HTRF detection every 20 s over 30 min. Data were globally fitted to the association kinetic model to derive estimates of k_{on} and k_{off}.

Competition binding experiments. The Motulsky-Mahan model requires simultaneous addition of fluorescent ligand and competitor the receptor [4]. 300 nM F-SKF and increasing concentrations of competitor was simultaneously added to membranes containing D1R, and F-SKF binding was determined every 20 s over 30 min via HTRF detection. Nonspecific binding was subtracted from each timepoint, and data globally fitted to the Motulsky-Mahan model [4] to derive k_{on} and k_{off} parameters of unlabeled ligands.

HTRF detection and data analysis. Signal detection was performed using the Pherastar FSX (BMG). The terbium donor was excited with five laser flashes at 337 nm, and kinetic FRET signal from 520 nm and 490 nm received every 20 s for 30 min. HTRF ratios were calculated by dividing acceptor signal (520 nm) by donor signal (450 nm) and multiplying by 10,000.

Results

Characterisation of F-SKF

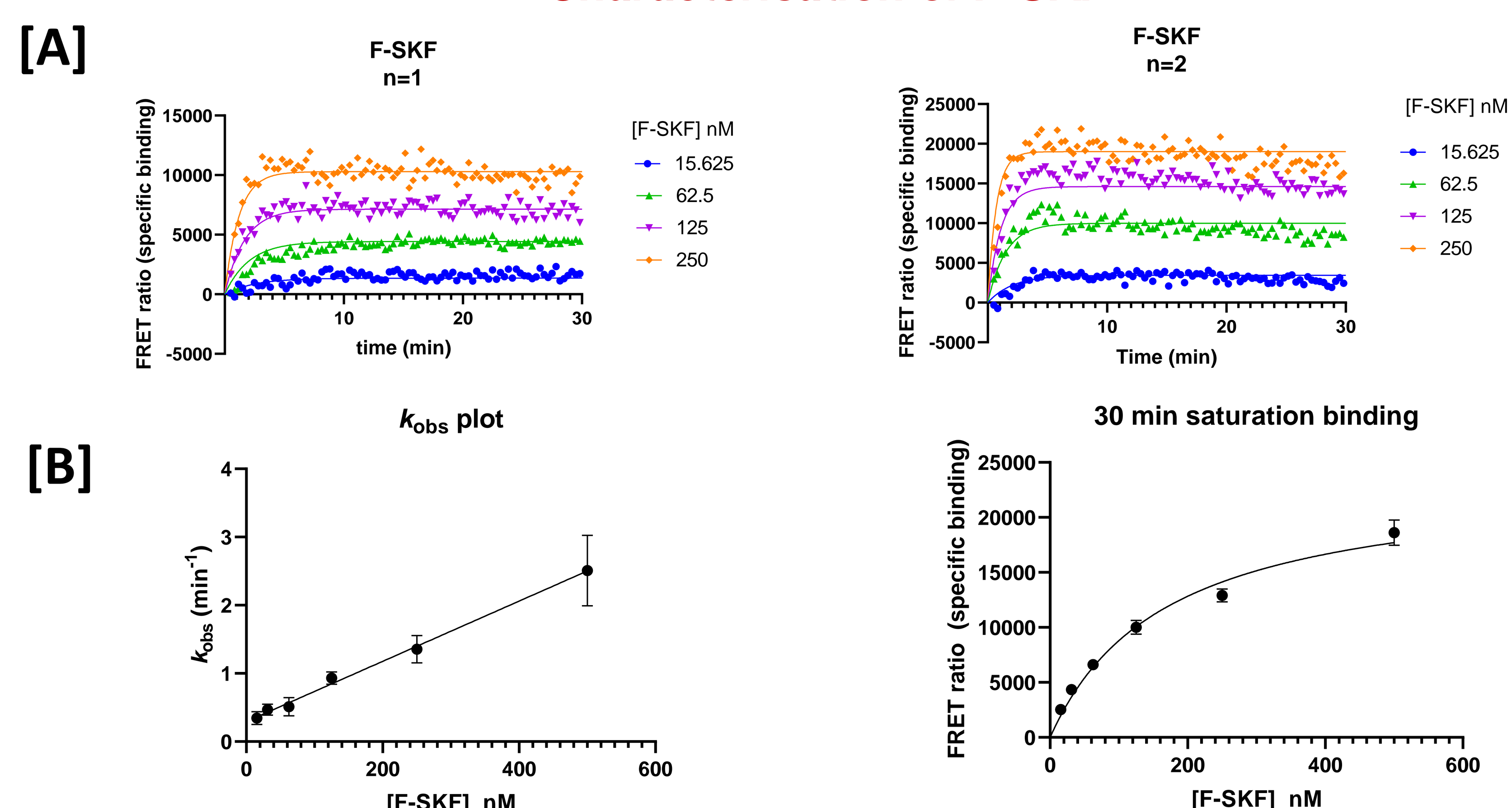

Fig 3. **[A]** Sample association kinetic curves for increasing concentrations of F-SKF from two independent experiments. **[B]** k_{obs} plot and 30 min equilibrium saturation binding of F-SKF from four independent experiments.

Determining kinetic parameters of D1 antagonists

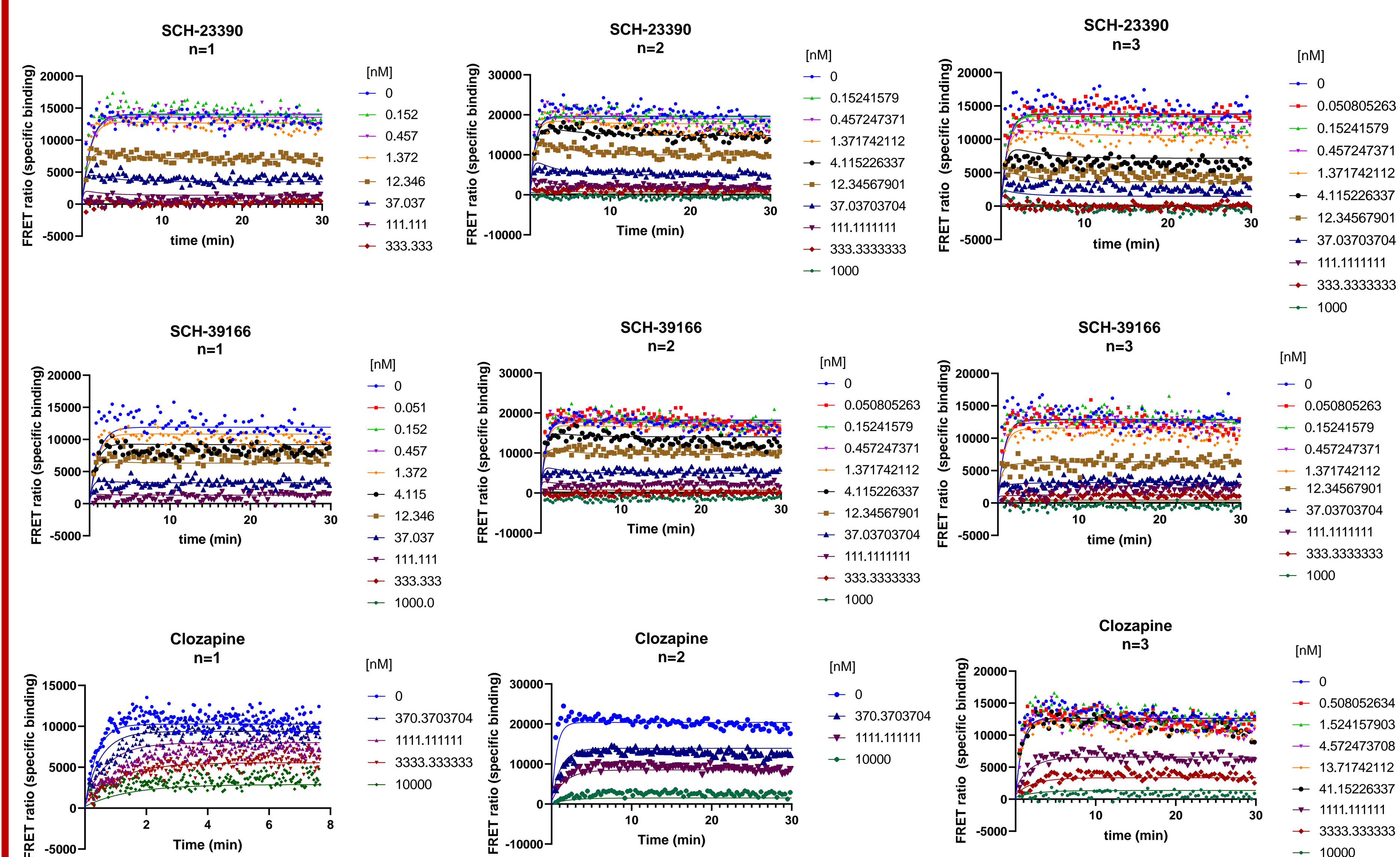

Fig 4. Kinetic analysis of F-SKF binding to D1R in the presence of unlabelled D1 antagonists. Data are from three independent experiments.

Table 1. Summary of kinetic parameters of F-SKF and unlabelled D1 antagonists obtained from 3-4 independent experiments

Compound	k_{on} ($M^{-1}min^{-1}$)	k_{off} (min^{-1})	Kinetic K_D (nM)
F-SKF (n=4)	$3.17 \pm 0.12 \times 10^6$	0.41 ± 0.01	139.1 ± 5.0
SCH-23390 (n=3)	$8.89 \pm 1.01 \times 10^7$	0.21 ± 0.004	3.2 ± 0.3
SCH-39166 (n=3)	$8.82 \pm 0.63 \times 10^7$	0.35 ± 0.02	4.2 ± 0.2
Clozapine (n=3)	$1.68 \pm 0.25 \times 10^7$	5.96 ± 0.61	452.4 ± 51

Conclusion

HTRF screening using F-SKF offers a robust method to assess D1 antagonist binding, with a homogenous, medium throughput format allowing implementation of such studies at an early stage in compound profiling.

References. [1] Klein Herenbrink C et. al., (2016) The role of kinetic context in apparent biased agonism at GPCRs. Nat Commun. 24;7:10842. [2] Sykes DA et. al., (2017) Extrapyramidal side effects of antipsychotics are linked to their association kinetics at dopamine D2 receptors. Nat Commun. 2;8(1):763. [3] Beaulieu JM, Gainetdinov RR (2011) The physiology, signaling and pharmacology of dopamine receptors. Pharmacol Rev, 63(1):182-217. [4] Motulsky HJ, Mahan LC (1984) The kinetics of competitive radioligand binding predicted by the law of mass action. Mol Pharmacol. 25(1):1-9.