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A universal homogeneous assay for high-throughput determination of binding kinetics

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ABSTRACT

There is an increasing demand for assay technologies that enable accurate, cost-effective, and high-throughput measurements of drug–target association and dissociation rates. Here we introduce a universal homogeneous kinetic probe competition assay (kPCA) that meets these requirements. The time-resolved fluorescence energy transfer (TR–FRET) procedure combines the versatility of radioligand binding assays with the advantages of homogeneous nonradioactive techniques while approaching the time resolution of surface plasmon resonance (SPR) and related biosensors. We show application of kPCA for three important target classes: enzymes, protein–protein interactions, and G protein-coupled receptors (GPCRs). This method is capable of supporting early stages of drug discovery with large amounts of kinetic information.

Target-based drug discovery approaches have traditionally used steady-state affinity as the main parameter to assess a compound activity and predict its performance in relevant biological models. Nearly a decade ago, an analysis of the mode of action of marketed drugs highlighted that most of these compounds interact with their molecular targets through mechanisms that are not based on equilibrium binding [1]. Later on, it was suggested that in certain contexts association kinetics (k_a)[1] and dissociation kinetics (k_d) could be at least as important as steady-state affinity for the prediction of cellular potency, in vivo efficacy, and safety [2,3]. For instance, compounds displaying diffusion-controlled association rates are expected to be more efficient inhibitors of rapid onset biological processes such as the coagulation cascade [4]. Transient kinetics can also be of advantage in the field of antipsychotic drugs, where mechanism-based toxicity can occur if the target receptor is inhibited for a long time [5,6]. On the other hand, the selectivity and efficacy of drugs with longer target residence time than their plasma $t_{1/2}$ are likely to be underestimated by classical PK/PD (pharmacokinetic/pharmacodynamic) models [7]. As a consequence, the inclusion of binding kinetic parameters is expected to improve the predictive value of these models. Such considerations have guided the first reported examples of lead compounds and clinical drug candidates selected during early stages on the basis of their target binding kinetics [8,9].

The potential impact of kinetic parameters on drug efficacy and safety has stirred a quest for assay technologies that enable accurate k_a and k_d measurements at early stages of the drug discovery process. "Classical" methods, such as stopped flow, jump dilution, progress curve analysis and radioligand binding/competition assays, are usually restricted to a particular protein class, mostly of limited throughput, and sometimes cumbersome. Higher throughput, biosensor-based techniques, with surface plasmon resonance (SPR) being the most prominent one, require special expertise to be performed and are often not compatible with targets (e.g., membrane proteins) that are not robust enough to endure immobilization on a chip surface [10]. Here we introduce a universal homogeneous and nonradioactive method for high-throughput determination of ligand binding kinetics. This kinetic probe competition assay (kPCA) format combines the versatility of radioligand binding with the advantages of homogeneous nonradioactive assays while approaching the time resolution of SPR experiments.

Materials and methods

Protein expression and purification

His-tagged, biotinylated cyclin-dependent kinase 2 (CDK2) and the first bromodomain of bromodomain-containing protein 4

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[1] *Abbreviations used:* k_a, association kinetics; k_d, dissociation kinetics; SPR, surface plasmon resonance; kPCA, kinetic probe competition assay (kPCA); CDK2, cyclin-dependent kinase 2; BRD4, bromodomain-containing protein 4; BRD4 BD1, first bromodomain of BRD4; H1R, histamine receptor 1; TAMRA, tetramethylrhodamine; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; TR–FRET, time-resolved fluorescence resonance energy transfer; GPCR, G protein-coupled receptor; ePCA, equilibrium probe competition assay; CV, coefficient of variation.

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(BRD4 BD1) were expressed in *Escherichia coli* following established protocols [11]. Proteins were purified with a single IMAC (immobilized metal affinity chromatography) step. For further details, see the online supplementary material.

Preparation of Tb-labeled proteins and cells

Prior to each experiment, the biotinylated CDK2 and BRD4 proteins were mixed with streptavidin–Lumi4-Tb cryptate (Cisbio) in assay buffer (see below) to the final concentrations indicated in Supplementary Table 1 (see supplementary material) and incubated for 30 min at room temperature. Tag-lite cells expressing SNAP-tagged histamine receptor 1 (H1R) and labeled with Lumi4-Tb cryptate were obtained from Cisio. Details on the generation of these cells have been described elsewhere [12]. Vials containing 1×10^6 cells/ml were thawed, diluted 1:20 in ice-cold Tag-lite buffer (Cisbio), and used immediately.

Fluorescent probes

The fluorescent probe used for the characterization of CDK2 inhibitors was Kinase Tracer 236 (Life Technologies), a multikinase ATP-competitive inhibitor labeled with Alexa Fluor 647 [13]. In the case of the histamine H1R, we selected the green-labeled derivative of the antagonist mepyramine available from Cisbio. The tracer employed for BRD4 measurements was a tetramethylrhodamine (TAMRA) derivative of the small molecule antagonist JQ1 recently reported by our laboratory [8]. All fluorescent probes were diluted shortly before use from dimethyl sulfoxide (DMSO) stocks to their working concentrations (Supplementary Table 1) in the corresponding assay buffers (see below).

Compound handling

For inhibitor characterization assays, serial DMSO dilutions (3.5-fold, 11-point for equilibrium competition assays; 10-fold, 5-point for kinetic competition assays) of the 100-fold concentrated test compounds (for final concentrations, see Fig. 2A–F in Results and Discussion) were previously prepared on a Precision microplate pipetting system (BioTek) using clear 384-well normal volume plates (Greiner). In the low- and high-signal control wells, test compounds were replaced by an excess of unlabeled inhibitor (see above) and DMSO, respectively. In the next step, 50 or 100 nl of compound (depending on the final assay volume) was transferred from the stock dilution plates to multiple "assay-ready" plate replicates using a Hummingbird liquid handling system (Digilab). Stock and "ready-to-use" test plates were sealed with adhesive aluminum foil (PerkinElmer) and stored at −20 °C until they were needed.

TR–FRET assays

Measurements were carried out at RT (room temperature) in quadruplicate on black 384-well low-volume plates (Greiner) at a final volume of 5 or 10 µl per well. All CDK2 experiments were performed in HBS–EP buffer (0.01 M Hepes [pH 7.4], 0.15 M NaCl, 3 mM EDTA [ethylenediaminetetraacetic acid], and 0.005% [v/v] Surfactant P20) (GE Healthcare) supplemented with 0.01% bovine serum albumin (BSA) and 1% DMSO. Histamine H1 receptor experiments were conducted in Tag-lite buffer, and BRD4 assays were done in 0.05 M Hepes (pH 7.5), 0.05 M NaCl, 0.01% BSA, 1% DMSO, and 0.005% Tween 20. Concentrations of probes and receptors are given in Supplementary Table 1. Time-resolved fluorescence resonance energy transfer (TR–FRET) signals were acquired in a PHERAstar FS plate reader (BMG Labtech) equipped with syringes for sample injection. Endpoint and kinetic acquisitions at intervals of $\geqslant$120 s were recorded using the standard settings of the reader for HTRF measurements. To perform kinetics reading at higher frequencies, we used the script described in Supplementary Note 2 (see supplementary material). Regardless of which settings were chosen for plate reading, the Tb donor was always excited with 5 laser flashes at a wavelength of 337 nm, and time-resolved recording of the acceptor and donor emission fluorescence channels (A and B, respectively) took place at different wavelengths (depending on the probes used), using the default settings of the instrument. For Kinase Tracer 236 the A- and B-channel filters were set to 665 and 620 nm, respectively, for green mepyramine they were set to 520 and 490 nm, and for TAMRA–JQ1 they were set to 570 and 545 nm. HTRF ratio values were calculated as defined in the instrument software: Ratio = [Acceptor (A) Counts/Donor (B) Counts] * 10,000.

Fluorescent probe characterization

Steady-state affinity of the fluorescent tracers was determined by titrating 5 µl of Tb-labeled receptor (at the concentrations described in Supplementary Table 1) with 5 µl of increasing probe concentrations (Fig. 1A–C). Samples were incubated for 30 min after short centrifugation (2 min, 1200*g*), and finally the endpoint TR–FRET signals were recorded as described above. To measure association kinetics of the probes, the injection device of the plate reader was used to rapidly dispense Tb-labeled proteins or cells (at the same concentration as used for the affinity measurements) to increasing concentrations of probe (Fig. 1D–F). Kinetic TR–FRET signals were obtained as described above in intervals of 10 s (CDK2 and BRD4) and 120 s (H1R). Probe dissociation rates were analyzed by displacement of the tracer with a large excess of an unlabeled ligand known to bind to the same site with similar or higher affinity. To this end, receptor (at the concentrations described in Supplementary Table 1) and probes (see below for final concentrations) were preincubated for 30 min at 2-fold final concentration in a volume of 5 µl, and once equilibrium was reached the unlabeled ligands were quickly added with the injection system to a final volume of 10 µl. TR–FRET signals were read in kinetic mode as described above. For CDK2 400 nM probe was displaced with the ATP-competitive, multikinase inhibitor staurosporine (10 µM), whereas for H1 receptor and BRD4 "cold" mepyramine (100 µM) and JQ1 (100 µM) were used to displace the corresponding tracers at concentrations of 400 and 80 nM, respectively.

Kinetic probe competition assays

Prior to each experiment, 5 µl of fluorescent probe was dispensed to the assay-ready plates containing 100 nl of compound dilutions using a Multidrop Combi liquid dispenser (Thermo Fisher Scientific). Then, the injection system of the PHERAstar FS plate reader (previously washed with NaOH) was primed at least 2 times with 500 µl solution of Tb-labeled proteins or cells. Finally, the assay plates were introduced into the instrument, 5 µl of Tb-labeled target was rapidly dispensed with the syringe to each well, and the TR–FRET signals corresponding to the competitive binding of probe and test compounds were recorded over time as described above.

Equilibrium probe competition assays

Ready-to-use plates containing 50 nl of compound dilutions and controls were filled with 5 µl of a preformed complex of fluorescent probe and Tb-labeled receptor (proteins or cells) using a Multidrop Combi. After short centrifugation, plates were incubated