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FRET-based screening assay using small-molecule photoluminescent probes in lysate of cells overexpressing RFP-fused protein kinases

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ABSTRACT

An assay was developed for the characterization of protein kinase inhibitors in lysates of mammalian cells based on the measurement of FRET between overexpressed red fluorescent protein (TagRFP)-fused protein kinases (PKs) and luminophore-labeled small-molecule inhibitors (ARC-Photo probes). Two types of the assay, one using TagRFP as the photoluminescence donor together with ARC-Photo probes containing a red fluorophore dye as acceptor, and the other using TagRFP as the acceptor fluorophore in combination with a terbium cryptate-based long-lifetime photoluminescence donor, were used for FRET-based measurements in lysates of the cells overexpressing TagRFP-fused PKs. The second variant of the assay enabled the performance of the measurements under time-resolved conditions that led to substantially higher values of the signal/background ratio and further improved the reliability of the assay.

Protein kinases (PKs)[3] play a crucial role in the regulation of protein functions in cells. Aberrant activity of PKs may be an indication or a cause of multiple complex diseases such as cancer, diabetes, cardiovascular diseases, and others [1]. Since the beginning of this century 28 small-molecule inhibitors with reported activity toward one or several PKs have been approved by the U.S. Food and Drug Administration for clinical use. Inspired by this progress, researchers have taken approximately 400 new inhibitors of PKs to various stages of clinical trials. Nevertheless, the majority of the compounds that have recently reached the trial stage are not targeting new kinases but are focused on new subindications using already known PK targets [2]. To increase the influx of new compounds targeting a wide variety of PKs into the drug development pipeline, cheap and flexible analytical methods are needed for screening and characterization of inhibitors of PKs. New analytical tools should be easily adaptable for specific kinases and usable in small academic medicinal chemistry labs, which perform a large part of the work of carrying new compounds to preclinical development.

PK inhibitors can be characterized in both inhibition assays and binding assays. In widely used kinetic inhibition assays the potential inhibitors are characterized on the basis of their retarding effect on rate of the PK-catalyzed peptide/protein phosphorylation reaction. On the other hand, recently several academic research groups [3] and biotechnology companies [4,5] (DiscoveRx, Ambit Biosciences, Kinasera, Invitrogen/Life Technologies, etc.) have come out with different platforms for binding assays. This has increased the affinity-based characterization of PK inhibitors. However, the usability of many of these assays is restricted by a limited number of available reporter probes possessing sufficiently high affinity toward the target PK, as the range of resolvable binding affinities is constrained by the affinity of the reporter probe [6].

Both the classical enzyme inhibition assays and binding assays require the use of active PKs. Prices of purified recombinant PKs from the commercial sources are high; on the other hand, the

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[3] *Abbreviations used:* ARC, conjugate of adenosine analogue and a peptide; BRET, bioluminescence resonance energy transfer; CK2α, catalytic subunit of protein kinase CK2; FA, fluorescence anisotropy/polarization; FI, fluorescence intensity; FP, fluorescent protein; FRET, Förster resonance energy transfer; GFP, green fluorescent protein; LI, luminescence intensity; PK, protein kinase; PKA, protein kinase A; PKAc, catalytic subunit of protein kinase A; PKAr, regulatory subunit of protein kinase A; QY, quantum yield; RFP, red fluorescent protein; TBBi, 4,5,6,7-tetrabromo-1*H*-benzimidazole moiety; TR, time-resolved; TRF, time-resolved fluorescence; YFP, yellow fluorescent protein.

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production of PKs in-house in bacteria is problematic because of difficulties in ensuring the correct posttranslational modifications of mammalian PKs in bacteria and because of tedious production, purification, and quality control procedures.

Therefore assay formats that afford the performance of binding assays in lysates of mammalian cells that overexpress target recombinant PKs could be a good alternative to biochemical assays with purified PKs for inhibitor screening. Although fluorescence anisotropy/polarization (FA) readout has been widely used for biochemical binding/displacement assays with purified PKs in buffer solutions, the applicability of FA-based measurements in biological solutions (bodily fluids, cell lysates, tissue extracts) is limited because of the sensitivity of this assay format to nonspecific interference by nontarget proteins and other components of the samples.

Förster resonance energy transfer (FRET)-based measurements show less interference from nontarget interactions and thus could be better suited to such measurements [7]. The applicability of FRET detection for protein kinase A (PKA) activity measurement was demonstrated in two conceptual papers a long time ago. First a sensor was designed for PKA in which the catalytic (PKAc) and regulatory (PKAr) subunits were each labeled with a different fluorescent dye, fluorescein and rhodamine, respectively, capable of FRET in the holoenzyme complex [8]. Thereafter, a cAMP sensor was engineered by fusing a blue-emitting fluorescent protein (FP) to the PKAr subunit and a green-emitting FP to the PKAc subunit [9]. Thus, when cAMP is low, the majority of the two fluorophores are in close proximity and generate FRET, whereas increasing amounts of cAMP determine a progressive reduction of FRET as the two fluorophores diffuse farther apart. Both FRET-based assays worked specifically with PKA and they were not adaptable for other PKs.

For PK inhibitor screening purposes assays using the time-resolved (TR) format of FRET measurements have turned out to be especially suitable [4,10–12]. A recombinant tagged PK that associates with a lanthanide (europium or terbium)-labeled anti-tag antibody (or lanthanide-labeled streptavidin in the case of biotin-tagged PKs) is used in combination with a small-molecule fluorescent probe that binds to the active site of the PK. In this triple complex donor and acceptor luminophores are positioned close to each other, leading to efficient FRET between the luminophores resulting in sensitization of the emission signal of the acceptor fluorophore. Displacement of the fluorescent probe from the complex with the PK by an inhibitor leads to a decrease in energy transfer between the luminophores that can be measured with a luminescence plate reader. The reported variants of TR FRET assay presume the application of tagged PKs and lanthanide-labeled antibodies.

In the present study, methods of genetic engineering to produce fusions of PKs with FPs were combined with methods of chemical construction of photoluminescent probes (ARC-Photo probes) based on conjugates of adenosine analogues and peptides (ARCs), high-affinity inhibitors of PKs [13], to set up an assay format for characterization of inhibitors of PKs in lysates of mammalian cells. We had previously shown that ARC-Photo probes attached to an orange 5-carboxytetramethylrhodamine (TAMRA) dye (ARC-TAMRA) could be used together with PKAc tagged with fluorescein isothiocyanate dye or yellow fluorescent protein (YFP) to monitor the activation of PKA in living cells or cell lysates via measurement of FRET-sensitized fluorescence of the TAMRA fluorophore [14]. Now the applicability TagRFP-fused PK as a FRET partner for the ARC-Photo probes labeled either with PF647 red fluorophore (ARC-PF647) or with Lumi4 terbium cryptate [15] luminophore (ARC-Lumi4Tb) was tested in a screening assay of inhibitors of PKs in two formats. In the steady-state fluorescence FRET-based assay TagRFP-fused PK acted as a donor for the fluorophore of the ARC-PF647 probe (Fig. 1A). In the TR version of the FRET assay TagRFP served as the luminescence acceptor for the ARC-Lumi4Tb probe with long emission lifetime (Fig. 1B).

TagRFP [16] was used as a PK-fused fluorophore because it is a bright, monomeric, pH-stable, and fast-maturing orange FP [17] whose emission spectrum overlaps well with absorption spectra of red dyes (e.g., PF647) and whose absorption spectrum (maximum at 555 nm) overlaps with main luminescence emission peaks of terbium cryptates (e.g., Lumi4Tb) at 490 and 545 nm. Both photoluminescent probes gave strong FRET-sensitized emission signal of the acceptor fluorophore in the complex of TagRFP-fused PK with ARC-Photo and therefore could be used for characterization of inhibitors of PKs in lysates of mammalian cells. The application of the ARC-Lumi4Tb probe enabled TR measurement of sensitized TagRFP emission that further improved the signal/noise ratio of the assay.

Materials and methods

Equipment

Fluorescence intensity (FI) and TR luminescence intensity (LI) measurements were performed on a PHERAstar microplate reader (BMG Labtech). For FI measurements, a simultaneous dual-emission fluorescence intensity module (λ_{ex} 540(20) nm, λ_{em} 590(20) and 675(50) nm) was used. TR LI measurements were performed using LanthaScreen and HTRF modules of the PHERAstar instrument possessing an excitation filter of 320(30) and various customized emission filters that enabled the measurement at the requested wavelengths (490(10), 545(10), 570(10), and 610(10) nm). A predefined gating (delay) and acquisition times were set for each TR measurement according to the luminescent properties of the probe used. To avoid the disturbing effect of afterglow of the xenon flash lamp in the plate reader, a delay time of at least 50 μs was used for TR measurements.

The concentration of stock solutions of ARC-probes and inhibitors was determined with a Nanodrop 2000c spectrophotometer (ThermoScientific). The concentration of PF647-labeled compounds was determined in 50 mM Hepes buffer (pH 7.5) using its molar extinction coefficient of 250,000 M^{-1} cm^{-1} at 653 nm.

Assays were performed in black, low-volume, 384-well, nonbonding surface microplates (code 3676, Corning). Microplates were incubated for 20 min at 30 °C before the measurements. In the case of the high-affinity probe ARC-1450 longer (60–120 min) incubation times were used because of slower dissociation kinetics.

Data analysis was performed using GraphPad Prism software (version 5.01, GraphPad Software, San Diego, CA, USA).

Synthesis of ARCs

Structures of ARCs used in this study and corresponding reference to synthetic procedures and characterization details are given in Supplementary Table S2.1.

Synthesis of ARC-1450

ARC-1411 (150 nmol) was dissolved in 20 μl of dimethyl sulfoxide and 1 μl of triethylamine. The solution was added to 5 nmol of Lumi4-Tb-NHS cryptate (Cisbio Bioassays, product reference 62TBSPEA). After 20 h the solvents were removed under reduced pressure and the residue was purified by HPLC (Supplementary Fig. S1). All peaks that absorbed light at 340 nm were collected and freeze-dried after the addition of terbium nitrate (50 nmol). Of five fractions collected, three fractions emitted long-lifetime luminescence that corresponded to the Lumi-4 Tb cryptate but only one of them gave TR FRET signal with TagRFP-PKAc lysate.