Analytical Biochemistry 385 (2009) 85–93

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

High-affinity bisubstrate probe for fluorescence anisotropy binding/displacement assays with protein kinases PKA and ROCK

Angela Vaasa [a], Indrek Viil [a,b], Erki Enkvist [a], Kaido Viht [a], Gerda Raidaru [a], Darja Lavogina [a], Asko Uri [a,*]

[a] Institute of Chemistry, University of Tartu, 2 Jakobi St., 51014 Tartu, Estonia
[b] KINASERA OÜ, 50606 Tartu, Estonia

ARTICLE INFO

Article history:
Received 12 August 2008
Available online 31 October 2008

Keywords:
FP assay
Fluorescence anisotropy
cAMP-dependent protein kinase (PKA)
Rho kinase (ROCK)
Bisubstrate inhibitor
Biosensor

ABSTRACT

The bisubstrate fluorescent probe ARC-583 (Adc-Ahx-(D-Arg)$_6$-D-Lys(5-TAMRA)-NH$_2$) and its application for the characterization of both ATP- and protein/peptide substrate-competitive inhibitors of protein kinases PKA (cyclic AMP-dependent protein kinase) and ROCK (rho kinase) in fluorescence polarization-based assay are described. High affinity of the probe (K_D = 0.48 nM toward PKA) enables its application for the characterization of inhibitors with nanomolar and micromolar potency and determination of the active concentration of the kinase in individual experiments as well as in the high-throughput screening format. The probe can be used for the assessment of protein–protein interactions (e.g., between regulatory and catalytic subunits of PKA) and as a cyclic AMP biosensor.

Protein kinases (PKs)[1] play a key role in the regulation of protein functions in living cells. More than 400 human diseases (e.g., cancer, diabetes, HIV, Alzheimer's, malaria, hypertension) have been linked to aberrant PK signaling, making PKs important drug targets [1], especially for the different forms of cancer. More than 70 small-molecule kinase inhibitors are at various stages of clinical trials as anticancer drugs [2].

The importance of the development of PK inhibitors as potential drugs has caused an increasing need for the elaboration and improvement of analytical methods for high-throughput screening (HTS) and characterization of new compounds [3,4]. Currently, the majority of tests are performed in the form of kinetic studies where the potential inhibitors are screened and characterized on the basis of their retarding effect on the rate of substrate (protein or peptide) phosphorylation reaction catalyzed by the kinase. However, to carry out and monitor the phosphorylation reactions, these tests require special substrates and the availability of fluorescence- or radioactivity-based detection methods. In addition, a large number of measurements must be accomplished to characterize the compounds on the level of values of comparable inhibition constants (K_i). The kinetic procedures take time to proceed, and the assays usually comprise several steps that complicate their automation for the HTS format.

Alternatively, the kinase inhibitors may be characterized on the basis of their affinity by measuring, directly or indirectly, binding rather than phosphorylation rate. Some small-molecule inhibitors can be conjugated to a fluorescent dye or some other tag without being deprived of high binding affinity. Because the competitive binding assays possess no intrinsic signal amplification based on high turnover of kinase-catalyzed reactions that is distinctive for kinetic assays, the other ways of signal amplification must be applied. Accordingly, two formats of binding assays are currently on the market from biotech companies that use the amplification of the measured signal either enzymatically (HitHunter, DiscoveRx) [5] or genetically (KINOMEscan, Ambit Biosciences) [6].

Advanced fluorescence detection methods require no biological amplification for the measurement of concentration or binding status of fluorescent probes at low nanomolar or even high picomolar concentrations. The kinase-bound labeled inhibitor (fluorescent

* Corresponding author. Fax: +372 73 75275.
E-mail address: asko.uri@ut.ee (A. Uri).

[1] *Abbreviations used:* PK, protein kinase; HTS, high-throughput screening; MAPKAP K-2, mitogen-activated protein kinase-activated protein kinase 2; TAMRA, carboxytetramethylrhodamine; ASK1, apoptosis signal-regulating kinase 1; FP, fluorescence polarization; cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase; SPR, surface plasmon resonance; ARC, adenosine–oligoarginine conjugate; ARC-583, Adc-Ahx-(d-Arg)$_6$-d-Lys(5-TAMRA)-NH$_2$; Adc, adenosine-4′-dehydroxymethyl-4′-carboxylic acid; Ahx, aminohexanoic acid; PKA C, cAMP-dependent protein kinase catalytic subunit; ROCKII, rho kinase isoform II; ROCK, rho kinase; BSA, bovine serum albumin; NHS, *N*-hydroxysuccinimide; H89, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; RIα/RIIα, regulatory subunit isoforms RIα/RIIα; PKI, protein kinase inhibitor; GST, glutathione *S*-transferase; Fmoc, 9-fluorenylmethoxycarbonyl; RP–HPLC, reversed-phase high-performance liquid chromatograpy; FA, fluorescence anisotropy; NBS, nonbonding surface; ARC-904, Adc-Ahx-(d-Arg)$_6$-d-Lys-NH$_2$; DMSO, dimethyl sulfoxide; DMF, dimethyl formamide; MeCN, methyl cyanide; ESI–HRMS, electrospray ionization–high-resolution mass spectroscopy; UV–Vis, ultraviolet–visible; MALDI–TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; DHB, dihydroxybenzoic acid; TLC, thin-layer chromatography; DTT, dithiothreitol; ARC-902, Adc-Ahx-(d-Arg)$_6$-NH$_2$; ARC-341, Adc-Ahx-(l-Arg)$_6$-NH$_2$.

0003-2697/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2008.10.030

probe, fluorescent tracer) can be displaced by competitive inhibitors that can be registered by measurement of the resulting change in fluorescence properties of the solution. These homogeneous methods require neither substrates nor procedures for monitoring phosphorylation reaction. From the aspect of simplicity, quickness, and possibility for automation, the binding assays applying high-affinity fluorescent probes are superior to kinetic assays, especially if generic fluorescent probes applicable for several kinases are found.

The low popularity of binding assays (if compared with kinetic assays) for HTS of kinase inhibitors has been caused by generally low affinity (submicromolar or micromolar) of fluorescent probes toward kinases, which in turn results in a requirement for high concentration of the kinase. In the case of high-affinity probes (K_d < 10 nM), the concentration of the kinase could be lowered to the subnanomolar or low-nanomolar region that is usual for kinetic assays. Apart from that, it is often not possible to use a single probe for screening and characterization of compounds targeted to either ATP- or protein/peptide substrate-binding sites of the kinase.

Because the development of ATP-competitive PK inhibitors is most supported by interest of pharmaceutical companies, the majority of disclosed fluorescent probes are targeted to the ATP-binding site of the kinase. For example, three recent publications described the application of the generic PK inhibitor staurosporine (K_D value of 38 nM toward MAPKAP K-2 [mitogen-activated protein kinase-activated protein kinase 2]) [7] and its conjugates with fluorescent dyes Alexa 647 [8] and TAMRA (carboxytetramethylrhodamine, K_D = 8 μM toward ASK1 [apoptosis signal-regulating kinase 1]) [9] as fluorescent probes in screening assays detecting the changes in fluorescence intensity, fluorescence lifetime, and diffusion properties. Because of low brightness of the unlabeled staurosporine [7] and its comparatively low affinity toward target ASK1 [9], high concentrations of the kinases (> 100 nM) were required for these binding assays. However, binding experiments with a wide panel of PKs [8] revealed that several kinases possess high affinity ($K_D \leqslant$ 10 nM) toward the staurosporine-based probe; hence, it can be used for the economic HTS assays with these kinases.

None of the fluorescence detection procedures used in the studies mentioned above [7–9] has gained popularity for HTS of inhibitors. Fluorescence polarization (FP) is a sensitive, ratiometric, and robust detection method that has been widely used for testing of compounds toward many biological targets [10], but its application for characterization of inhibitors of PKs has not been frequent. Recently, the application of FP-based binding assay for the characterization of ATP-competitive inhibitors of interleukin-2-inducible T-cell kinase was described [11]. The assay was performed in 15-μl volumes containing 250 nM kinase and 25 nM fluorescent probe in assay buffer. Due to the high dissociation constant (K_D = 45 nM) of the fluorescent probe, a high concentration of the kinase (250 nM) needed to be used for displacement experiments, making the assay expensive to perform. On the other hand, effective fluorescent probes competitive with the protein substrate have been used in FP assays for some special kinases, such as cyclic AMP (cAMP)-dependent protein kinase (PKA) [12,13], but these probes cannot be directly used for the characterization of inhibitors targeted to the ATP-binding site of PKs.

We showed recently with surface plasmon resonance (SPR) biosensor assay [14] that adenosine–oligoarginine conjugates (ARCs) endowed with high affinity toward basophilic PKs [15] associate simultaneously with both ATP- and protein/peptide substrate-binding sites of PKs. Thus, ARCs can be displaced from their complex with the kinase by both ATP- and protein-competitive inhibitors of the kinase. Although SPR-based methods are powerful for kinetic and thermodynamic characterization of inhibitors, the heterogeneous sensor methods are not well suited to HTS studies. Here we describe the application of an ARC-based high-affinity fluorescent probe ARC-583 (Adc-Ahx-(D-Arg)$_6$-D-Lys(5-TAMRA)-NH_2) for competitive displacement assays with cAMP-dependent protein kinase catalytic subunit (PKA C) and rho kinase isoform II (ROCKII) detecting fluorescence anisotropy (polarization) on a fluorescence plate reader. Although the polarization (P) and anisotropy (A) values are interchangeable quantities and differ only in their normalization [$P = 3A/(2 + A)$], the anisotropy is preferred because it is normalized by the total intensity [16].

ARC-583 (K_D values of 0.48 and 3.6 nM toward PKA C and ROCK-II, respectively) can be used for the characterization of inhibitors with the dissociation constants lying in the micromolar and nanomolar range and targeted to either ATP- or protein-binding sites of the basophilic PKs (Scheme 1).

One of the two examined kinases, rho kinase, has been acknowledged as an important drug target, especially for cardiovascular diseases but also for cancer, neuronal degeneration, and other pathological conditions [17]. The new assay method could support the research in the field of developing new potent inhibitors for the regulation of rho kinase (ROCK)-catalyzed phosphorylations.

The role of PKA in the regulation of cellular processes is difficult to overestimate. Thus, it is an important target of biochemical research. The new method makes it possible to screen and characterize in a single binding assay compounds that realize their activity on PKA either through direct binding to the active center of the catalytic subunit or as agonists or antagonists of cAMP-regulated activity of holoenzyme.

Materials and methods

Reagents, materials, and equipment

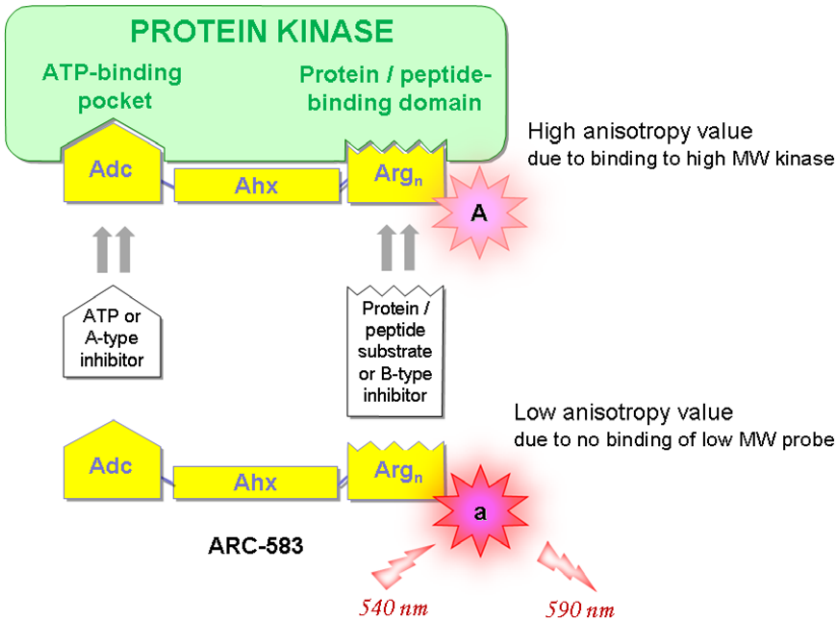
Chemicals from the following commercial sources were used: bovine serum albumin (BSA) from Fluka; peptide synthesis reagents from Neosystem, Novabiochem, Advanced ChemTech, and AnaSpec; 5-TAMRA *N*-hydroxysuccinimide (NHS) ester from Anaspec; 1-aminobutane from Aldrich; organic solvents from Rathburn and Fluka; H89, recombinant RIα (human), RIIα (human), protein kinase inhibitor (PKI)α (r rabbit,) and PKA Cα (murine) from Biaffin; and ROCKII (recombinant human protein, catalytic domain, amino acids 1–552, N-terminal glutathione *S*-transferase [GST] tag) from Invitrogen. The synthetic peptide substrate TAMRA–Kemptide and ARCs were synthesized by Fmoc solid-phase peptide synthesis strategy and purified with reversed-phase high-performance liquid chromatograpy (RP–HPLC) as described previously [14,15,18].

The concentrations of stock solutions of ATP, ARC-type inhibitors, and H89 were determined spectrophotometrically in aqueous buffer (pH 7.5) based on molar extinction coefficients of 15,000 M^{-1} cm^{-1} at 260 nm for adenosine-containing compounds, 80,000 M^{-1} cm^{-1} at 558 nm for 5-TAMRA-containing compounds, and 30,000 M^{-1} cm^{-1} at 260 nm for H89. The absorption spectrum of ARC-583 was taken on a ThermoSpectronic Unicam UV 300 spectrophotometer (ThermoSpectronic). Excitation and emission spectra of ARC-583 were recorded on an LS55 Luminescence Spectrometer (PerkinElmer). Fluorescence anisotropy (FA) readings were taken on a PHERAstar microplate reader (BMG Labtech) with FP optic module [excitation 540(20), emission 590(20)]. Kinase assays were performed in black, low-volume, 384-well, nonbonding surface (NBS) microplates (cat. no. 3676, Corning). GraphPad Prism software (version 4.03, GraphPad Software) was used for data analysis.

Synthesis of fluorescent compounds

Fluorescent probe ARC-583

Solution of ARC-904 (Adc-Ahx-(D-Arg)$_6$-D-Lys-NH_2, 1.0 μmol) in dimethyl sulfoxide (DMSO, 100 μl) and triethylamine (10 μl,



Scheme 1. The fluorescence anisotropy of the dye-labeled small-molecule ARC-type inhibitor (fluorescent probe ARC-583) increases significantly on binding to the active site of the large kinase molecule. The displacement of the bisubstrate fluorescent probe from the complex with PK by either an ATP-competitive or a substrate-competitive inhibitor leads to the decrease of anisotropy of the solution, which can be used for HTS and characterization of inhibitors of PK. MW, molecular weight.

70 μmol, 70 eq) was added to the solution of 5-TAMRA and succinimidyl ester (1.0 μmol, 1 eq) in dimethyl formamide (DMF, 50 μl). After 3 h reaction at room temperature (22 °C), the solvents were removed in vacuo and the product was purified by RP–HPLC (gradient of 10–40% methyl cyanide (MeCN) within 40 min, 0.1% trifluoroacetic acid), R_t = 18.1 min. Electrospray ionization–high-resolution mass spectroscopy (ESI–HRMS): calcd. $M_r(C_{83}H_{127}N_{35}O_{16})$ = 1870.0200, detn. 1870.0123; UV–Vis$_{max}$ 558 nm (aqueous solution, pH 7.0).

TAMRA butylamide

5-TAMRA NHS ester (0.3 μmol) was treated with butylamine (1.8 μmol, 6 eq) in DMF (20 μl). After 4 h reaction at room temperature (22 °C), the solvents were removed in vacuo. The obtained dark red residue was purified by RP–HPLC (gradient of 10–40% MeCN within 40 min, 0.1% trifluoroacetic acid), R_t = 43.2 min. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS, matrix dihydroxybenzoic acid [DHB]): calcd. $M_r(C_{29}H_{31}N_3O_4)$ = 485.2, detn. (m/z) 486.3 ([M+H]$^+$); UV–Vis$_{max}$: 552 nm (aqueous solution, pH 7.0).

PKA C inhibition assay

The activity of PKA C was determined with fluorometric thin-layer chromatography (TLC) assay as described previously [18] in a 40-μl volume in buffer solution containing PKA C (1 nM), TAMRA–Kemptide (30 μM), ATP (100 μM), $MgAc_2$ (10 mM), BSA (7.5 μM), and Hepes (50 mM, pH 7.5) at 30 °C. The phosphorylation reaction was initiated by the addition of the solution of ATP. The inhibitory potencies were determined in the presence of a concentration series of inhibitors (twofold dilutions), and the inhibition curves were fit to a sigmoidal dose–response model to yield IC_{50} values (corresponding to the concentration of the inhibitor decreasing the activity of the enzyme twofold).

FA measurements

The FA experiments were performed in 20- to 30-μl volumes in assay buffer (5 mM dithiothreitol [DTT], 7.5 μM BSA, 150 mM NaCl, and 50 mM Hepes, pH 7.5) in the presence or absence of $MgAc_2$ (10 mM). The microplates were incubated for 15 min at 30 °C before FA measurements.

Direct binding assay: Determination of concentration of active enzyme and K_D of the fluorescent probe ARC-583

The titration of a fixed concentration of ARC-583 was performed with a concentration series of PKA C or ROCKII (twofold dilutions). The binding curves were fitted to Eq. (1) as described previously [10,11]:

$$A = A_f(1 - X_1) + A_b \cdot X_1, \quad (1)$$

Where

$$X_1 = Q \cdot X_2/[1 + X_2(Q - 1)]$$

$$X_2 = \{P + K_D + k \cdot E_0 - \left[(P + K_D + k \cdot E_0)^2 - 4P \cdot k \cdot E_0\right]^{1/2}\}/2P. \quad (2)$$

A is the measured anisotropy, A_f is the anisotropy value for free ARC-583, A_b is the anisotropy value for complex between ARC-583 and the enzyme, P is the total concentration of the fluorescent probe ARC-583, E_0 is the nominal concentration of the enzyme, k is the fraction of active kinase in enzyme preparation (concentration of the active enzyme divided by the nominal concentration of the enzyme), K_D is the dissociation constant of ARC-583 complex with the enzyme, and Q is the ratio of fluorescence quantum yield of bound ARC-583 and the fluorescence quantum yield of unbound ARC-583, determined by measurement of the anisotropies of five wells of unbound ARC-583 (10 nM) and six wells of ARC-583 (10 nM) saturated with PKA C (100 nM).

The fraction of active PKA C (k) in the batch (nominal kinase concentration as defined by the supplier) was determined with direct binding assay at a high concentration of ARC-583 (20 nM), and the binding data were fit to Eq. (1) by fixing K_D below 1 nM. The obtained value of k was used for fitting the binding data obtained with a 10-fold lower concentration of ARC-583 (2 nM) to calculate K_D for the probe–kinase complex. The concentration of active enzyme was calculated as the product of k and the nominal concentration of the enzyme (kE_0).

The fraction of active ROCKII and the K_D of the ROCKII–ARC-583 complex were determined in a similar manner except that the binding data from these two experiments (at 2 and 20 nM ARC-583) were simultaneously fitted to Eq. (1) with shared k and K_D values.

Competition assay

The competition assay was performed with a concentration series of the competitive ligand (threefold dilutions) and fixed concentrations of ARC-583 (1–2 nM) and PKA C (2–3 nM) or ROCK II (15 nM). The displacement curves were fitted to a sigmoidal dose–response model to obtain IC_{50} values and to the exact competitive binding model [10,19] to obtain K_d values:

$$A = A_f(1 - X_3) + A_b \cdot X_3, \quad (3)$$

where

$$X_3 = X_4/(3K_D + X_4) \quad (4)$$

$$X_4 = 2(X_5^2 \cdot 3X_6)^{1/2} \cdot \cos(\theta/3) - X_5$$

$$\theta = \arccos\left\{\left[-2X_5^3 + 9X_5 \cdot X_6 + 27K_D \cdot K_d \cdot E\right] \Big/ \left[2(X_5^2 - 3X_6)^{3/2}\right]\right\}$$

$$X_5 = K_D + K_d + 10^{\log L} + P - E$$

$$X_6 = K_D(10^{\log L} - E) + K_d(P - E) + K_D \cdot K_d$$

K_d is the dissociation constant of the complex between the competitive ligand and enzyme, E is the total concentration of the active enzyme determined prior to each experiment in binding assay as described above, L is the total concentration of the competitive ligand, and other variables are as described above for Eq. (1).

To assess the performance in the competition assay, the Z' value [20] was calculated according to the Eq. (3):

$$Z' = 1 - (3\sigma^+ + 3\sigma^-)/|\mu^+ - \mu^-|, \quad (5)$$

where σ^+ and σ^- are the standard deviation of the positive and negative control wells, respectively, and μ^+ and μ^- are the average values of those wells, respectively. For these calculations, experimental data from the same assay conditions with 24 positive control wells (2 nM ARC-583 and 3 nM PKA C) and 24 negative control wells (free ARC-583, 2 nM) were used.

Dissociation kinetics of ARC-583–PKA C complex

The kinetics of the dissociation of the fluorescent probe ARC-583 from its PKA C complex was measured in three parallel experiments. The mixture of ARC-583 (15 nM) and PKA C (45 nM) in the assay buffer (20 μl) was incubated for 15 min at 30 °C. Thereafter, the solution of ARC-902 (Adc-Ahx-(D-Arg)$_6$-NH$_2$, 10 μl, 3 μM) was added and FA was measured in 5-s time intervals. The collected data were fit to a one-phase exponential decay model yielding the dissociation rate constant (k_d). The association rate constant was calculated according to Eq. (4):

$$k_a = k_d/K_d \quad (6)$$

Specific activity of PKA C

The solution of PKA C (600 nM) was incubated at 40 °C in assay buffer. At fixed time points (0, 5, 10, and 24 h), the aliquots of the solution were analyzed in parallel by both direct binding FA assay for assessing the active concentration of the enzyme and kinetic assay for determining the catalytic activity. The calculated catalytic activities were plotted against concentration of the active enzyme and fit to linear regression.

Effect of cAMP on PKA holoenzyme

The effect of cAMP on the dissociation of PKA holoenzyme was tested on a 384-well microtiter plate. Holoenzyme was prepared by a combination of solutions of PKA C (3 nM final concentration) with regulatory subunit RI or RII (either at a 10-nM monomer concentration). The mixture of ARC-583 (2 nM), PKA C holoenzyme, and cAMP (100 pM–1 μM) was incubated for 15 min at 30 °C in assay buffer, and fluorescence properties of the solution were assessed. The curves were fitted to a sigmoidal dose–response model to obtain EC_{50} values (concentration of cAMP that caused 50% of the maximal increase of the FA).

Results and discussion

Design of the fluorescent probe ARC–TAMRA

We have shown previously that the best of ARCs possess subnanomolar inhibitory potency and binding affinity toward PKA C [14,15]. Based on the structures of these inhibitors, a new fluorescent tracer was designed for binding/displacement assays. 5-TAMRA was attached to the C terminus of ARC-902 via a D-lysine linker to yield the fluorescent probe ARC-583. TAMRA, one of the most popular orange fluorophores applied to label proteins and small molecules, was chosen as the fluorescent dye due to its resistance to photobleaching and wide distribution of appropriate filter sets in fluorescence plate readers. ARC-583 has excitation and emission maxima at 558 and 585 nm, respectively, at neutral pH in water.

The determination of the value of molar extinction coefficient ε for ARC-583 is complicated due to the highly polar nature of the compounds that leads to incorporation of counter ions, salts, and water whose content is difficult to determine. In the current study, the value of ε for ARC-583 was established from the difference of absorption spectra of ARC-583 and butyl amide conjugate of 5-TAMRA (taking into account extra absorbance of ARC-583 resulting from the incorporation of adenosine residue, ε = 15,000 at 260 nm, and equal absorbances at 558 nm, resulting from the TAMRA chromophore) that yielded an ε value of 80,000 M^{-1} cm^{-1} at 558 nm for ARC-583. This molar extinction coefficient of ARC-583 fits well into the range of reported ε values for the TAMRA-labeled compounds.

Characterization of the fluorescent probe ARC-583

To establish the affinity of the fluorescent probe ARC-583, its titration with PKA C was performed. A 2-nM concentration of ARC-583 was chosen, significantly higher than the anticipated value of K_D to achieve a good signal-to-noise ratio of measured fluorescence intensity signals. To determine the equilibrium dissociation constant K_D of the ARC-583–PKA C complex, the FA was measured as a function of kinase concentration (Fig. 1). Fitting the experimentally observed anisotropies according to the Eq. (1) resulted in the binding constant K_D values of 0.48 and 0.66 nM in the absence and presence of magnesium ions, respectively (Table 1). The obtained binding constant for ARC-583 is in good accordance with the inhibitory potency of the compound as determined in the kinetic assay, IC_{50} = 2.9 nM at 100 μM cosubstrate ATP concentration ($K_{m,ATP}$ = 18.7 μM [18]). The subnanomolar affinity of ARC-583 makes it one of the most potent fluorescent probes ever described for PKs and widens the range of inhibitory potencies of compounds that can be characterized with a single probe. Because the lowest inhibitor K_D value that can be resolved in an FP-based binding assay is approximately equal to the K_d value of the tracer [21,22], ARC-583 can be used for characterization of nonlabeled inhibitors with affinities in whole nanomolar and micromolar

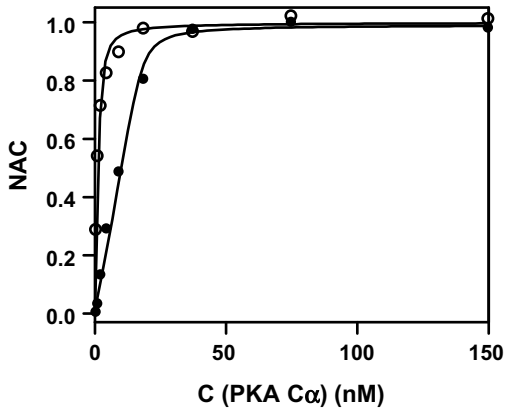


Fig. 1. Titration of the fluorescent probe ARC-583 [2 nM (○) and 20 nM (●); magnesium ions not added] with PKA C. The probe binding constant K_D of 0.48 nM was calculated from these data according to Eq. (1). Normalized anisotropy change (NAC) was calculated as $NA = (A - A_f) / (A_b - A_f)$.

Table 1
Binding constants (K_D) for interaction of ARC-583 with PKA C and ROCK determined with FA method

Protein kinase	K_D (nM)	K_D (nM)
	No magnesium	10 mM $MgAc_2$
PKA C	0.48 (0.06)	0.66 (0.15)
ROCKII	3.6 (0.5)	9.2 (2.2)

Note. Standard errors are in parentheses.

ranges. Furthermore, the application of a probe with high-affinity such as ARC-583 significantly decreases the amount of the kinase needed for binding assays, making HTS cheaper without compromising the assay sensitivity and accuracy [22].

Surprisingly little attention has been paid to an important issue, namely, the origin of concentration value of the interacting protein that is used for calculations according to Eq. (1) [23]. Protein concentrations are usually determined by the method of Bradford [24] or Lowry [25] with BSA or other proteins used as the standard or are measured spectrophotometrically taking into account the protein content of UV-absorbing amino acid residues. These crude and macroscopic methods require more than 1 μg of the protein and estimate the total concentration of all forms of proteins present in the solution, not only the special form of the enzyme that specifically catalyzes the reaction and interacts with the tracer. In commercially available PKs, the content of the active form of the kinase may vary by more than 10-fold, as indicated by different activity values of protein samples per mass expressed as activity units per milligram of the protein sample.

In the current work, the fraction (k) of the active (binding to ARC-583) form of PKA C was first determined with direct binding assay at a higher concentration of ARC-583 (20 nM) (Fig. 1), and the binding data were fit to Eq. (1) with K_D fixed below 1 nM. The obtained value of k was used for fitting the binding data at a 10-fold lower concentration of ARC-583 (2 nM) to calculate K_D for this interaction. The concentration of active enzyme was calculated as the product of k and the nominal concentration of the enzyme (kE_0). Depending on the source of PKA C and its storing conditions, the k values between 0.1 and 1.2 have been determined for different batches of the kinase.

BSA was added to the measurement solutions (0.05%) to reduce the nonspecific binding of ARC-583 and kinase to other assay components and the walls of pipette tips and incubation wells. In the presence of BSA, the fluorescence intensity of ARC-583 solution was higher and retained stability for longer time periods. However, the presence of BSA in the solution of the fluorescent probe reduced the dynamic range (ΔmA = mA of bound ARC-583 – mA of free ARC-583) from 220 mA (solutions without BSA) to 140 mA (solutions with BSA) (Fig. 2). Still, BSA was preserved in the measuring solution to achieve maximal stability and reproducibility of results. No effect of BSA on the K_D value of ARC-583 and K_d values of tested inhibitors was noticed.

Also, for the reduction of adsorbance of assay components to the surface of measurement wells, NBS microtiter plates (Corning) were used.

In the case of another AGC family PK, ROCKII, titration of the fluorescent probe ARC-583 (Fig. 3) led to K_D values of 3.6 and 9.2 nM in the absence and presence of magnesium ions, respectively (Table 1).

Kinetics of the interaction between ARC-583 and PKA C

The rate constant of dissociation of ARC-583 from the complex with PKA C was determined in the presence of competing inhibitor ARC-902 (Fig. 4). ARC-902 (1 μM final concentration) was added to a solution containing ARC-583 (10 nM) preequilibrated with PKA C (30 nM) at 30 °C. The decrease in FA of ARC-583 with time was fit by an exponential function. The dissociation rate constant k_d = 0.0261 s^{-1} ($\tau_{1/2}$ = 26.6 s) was determined for this reaction. Based on the value of dissociation equilibrium constant (K_D = 0.48 nM), the association rate constant $k_a = 5.4\ 10^7\ M^{-1}\ s^{-1}$ was calculated according to Eq. (4). Values of the obtained constants are very similar to the constants for binding of nonlabeled bisubstrate inhibitor ARC-904 to the immobilized PKA C, as determined in SPR biosensor experiments: K_d = 0.48 nM, $k_d = 1.310^{-2}\ s^{-1}$, $k_a = 2.7{\cdot}10^7\ M^{-1}\ s^{-1}$ [14]. The high rate of the association reaction in solution means that short incubation times can be used for binding/displacement experiments.

Application of ARC-583 for determination of activity of PKA C

A good correlation was found between the ARC-583-associating concentration of PKA C and the enzymatic activity of the latter (Fig. 5).

Catalytic activity of 253 ± 16 nM min^{-1} was determined for 1.0 nM PKA C with TAMRA–Kemptide as the substrate, corresponding to the specific activity of 6.3 μmol min^{-1} mg^{-1}. In addition, parallel loss of binding affinity of PKA C to ARC-583 and its catalytic activity were established when the solution of the kinase was stored open to moist air at 40 °C for longer periods. Thus,

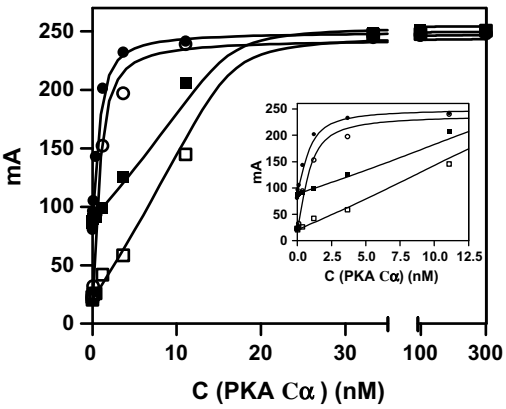


Fig. 2. Effect of BSA for anisotropy measurements: Titration of the fluorescent probe ARC-583 [2 nM (○,●) and 20 nM (□, ■); in the presence (filled symbols) and absence (open symbols) of BSA] with PKA C. The presence of BSA reduces the dynamic range from 220 to 140 mA. In the inset the effect of BSA on the results of anisotropy measurements is represented in the extended scale.

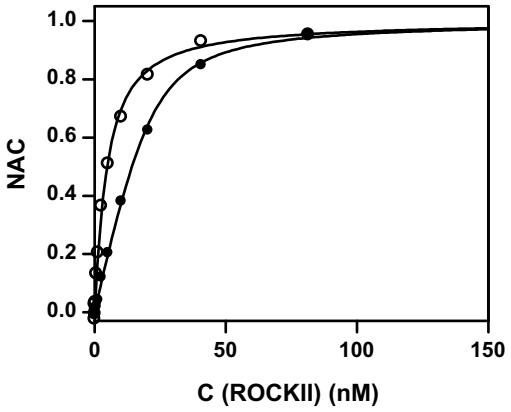


Fig. 3. Titration of the fluorescent probe ARC-583 [2 nM (○) and 20 nM (●); magnesium ions not added] with the kinase ROCKII. The probe binding constant (K_D) of 3.6 nM was calculated from these data according to Eq. (1). NAC, normalized anisotropy change.

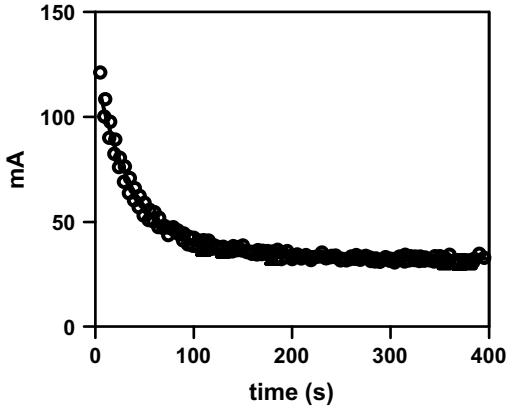


Fig. 4. Dissociation kinetics of the complex of the fluorescent probe ARC-583 and PKA C in the presence of bisubstrate inhibitor ARC-902 (1 μM). Fitting data to a one-phase exponential decay model yielded a dissociation rate constant (k_d) of 0.0261 s^{-1}.

the fluorescent probe associates with the active form of the kinase; hence, it can be used for determination of activity of PKA in solution with no need for any substrates. In a 20-μl well volume at a 1-nM concentration of ARC-583, a 0.1-nM concentration of PKA C (in total 2 fmol or 80 pg of PKA C) could be reliably determined.

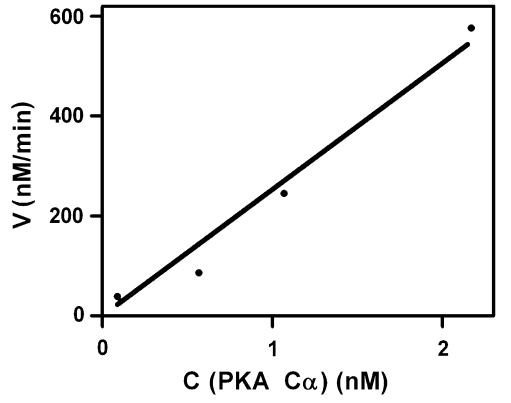


Fig. 5. Correlation between catalytic activity (V, as determined with the TLC kinetic assay) and the active concentration (C, as titrated with the fluorescent probe ARC-583) of the solution of PKA C.

Competitive FP-based kinase binding assay

For the displacement experiments, the 2-nM total concentration of the tracer ARC-583 was chosen to achieve sufficient intensity of fluorescence signals and the stability of the calculated anisotropy values. This concentration is higher than the K_D value for the probe (K_D = 0.48 nM with PKA), leading to the change of the binding states of all participating components (tracer, competitor, and kinase) during the displacement experiment and, hence, requires the application of Eq. (2) for the correct calculation of binding constants from the experimental data. The small decrease of fluorescence signal intensity (Q = 0.8) was attributed to the binding of the tracer to PKA C. This value was taken into account for fitting data of binding and displacement experiments.

The 3-nM concentration of PKA C was used for the displacement assays, resulting in binding of more than 80% of ARC-583 and more than a 100-mA anisotropy window for full displacement of the probe. These assay conditions use a small amount of the protein (2.4 ng of PKA C per 20 μl well volume of a 384-well microtiter plate) and yield a sufficiently large dynamic range and sensitivity to perform HTS experiments.

For these assay conditions with 24 positive control wells (2 nM ARC-583 and 3 nM PKA C) and 24 negative control wells (free ARC-583, 2 nM), the Z' value of 0.74 [20] was obtained for the assay, pointing to its good applicability for the HTS experiments.

The bisubstrate character of the probe was demonstrated by its full displacement from the complex with the kinase PKA C by either the ATP-competitive inhibitor H89 or the substrate peptide/protein-competitive inhibitor RII (unlike RI and PKI, RII does not need the presence of ATP for high-affinity binding) in two separate experiments. This result indicates that the ARC-type inhibitors associate simultaneously with both binding sites of PKA C.

The determined affinity for ATP-competitive inhibitor H89 (K_d = 30 nM) (Table 2 and Fig. 6) fits well into the range of inhibition constants reported for this compound (reported K_i values 48 nM [26] and 15 nM [27]). The cosubstrate ATP and inhibitor ADP displaced ARC-583 in the presence of magnesium ions but not in the absence of these ions. The calculated K_d values of 17 and 9.6 μM for ATP and ADP, respectively (Table 2 and Fig. 6), are close to the previously reported K_d values for these compounds [28–30].

The dissociation constants determined with the binding/displacement assay for PKI and the regulatory subunits RI and RII of PKA (the inhibition of PKA C by all of these compounds is competitive with the protein/peptide substrate) are close to the values of the previously reported inhibition and dissociation constants for these proteins (Table 2 and Fig. 7) [31–35].

Affinities of the two ARC-type bisubstrate inhibitors ARC-902 and ARC-341 (Adc-Ahx-(L-Arg)$_6$-NH_2) toward PKA C were also determined (Table 2 and Fig. 6). ARC-902 is the prototype molecule of ARC-583, incorporating no fluorescent label and no lysine linker. The K_d value of 0.33 nM determined for this compound corresponds to its inhibitory potency (IC_{50} = 8.3 nM measured at 100 μM ATP and 30 μM TAMRA–Kemptide [15]) and to the K_D value of the fluorescent probe ARC-583 (K_D = 0.48 nM) derived from this compound. ARC-341, the L-arginine counterpart of ARC-902, revealed a K_d value of 38 nM that was also close to the inhibition constant reported for this compound [15].

In addition to good fit between the inhibitory potencies and affinities of the reported compounds (Fig. 8), the affinity values of these compounds determined with the current FA assay are very close to the affinities determined previously with the SPR biosensor assay [14].

ARC-583 was also used for the determination of affinities of inhibitors toward another basophilic kinase from the AGC group, namely, ROCKII. H89, long known as a selective PKA inhibitor, displaced the probe with a K_d of 31 nM (Table 2). The found affinity of

Table 2
Comparison of affinities of compounds toward PKA C and ROCKII determined with the FA-based assay and inhibitory potencies of these compounds determined with other methods

Tested compound	Results of FA measurements				Comparison with other methods
	Additives	Kinase	IC_{50} (pIC_{50}, SE)	K_d[a]	
PKIα	10 mM Mg^{2+}, 10 μM ATP	PKA C	2.9 nM (8.54, 0.05)	0.22 (0.06) nM	K_i = 0.22 nM [31], K_i = 0.26 nM [32], IC_{50} = 0.94 nM [33]
RIα		PKA C	> 300 nM		
RIα	10 mM Mg^{2+}, 10 μM ATP	PKA C	2.7 nM (8.57, 0.02)	0.19 (0.03) nM	K_i = 0.15 nM, K_d = 0.23 nM [32], IC_{50} = 3.6 nM [33]
RIIα		PKA C	3.9 nM (8.41, 0.03)	0.32 (0.05) nM	K_d = 0.1 nM [34], K_i = 0.3 nM [35]
RIIα	10 mM Mg^{2+}, 10 μM ATP	PKA C	3.5 nM (8.45, 0.05)	0.24 (0.04) nM	IC_{50} = 0.2 nM [33]
ATP		PKA C	> 1 mM		K_d = 5 mM [14], K_i > 160 μM [28], K_d = 120 μM [29]
ATP	10 mM Mg^{2+}	PKA C	120 μM (3.94, 0.08)	17 (5) μM	K_d = 10 μM [14], K_i = 10 μM [28,30]
ADP		PKA C	> 0.5 mM		
ADP	10 mM Mg^{2+}	PKA C	71 μM (4.15, 0.05)	9.6 (0.7) μM	K_i = 10 μM [30]
H89		PKA C	130 nM (6.89, 0.09)	23 (4) nM	K_d = 9 nM [14]
H89	10 mM Mg^{2+}	PKA C	130 nM (6.88, 0.05)	30 (3) nM	K_i = 48 nM [26], 15 nM [27], IC_{50} = 135 nM [36]
ARC-902		PKA C	4.3 nM (8.36, 0.04)	0.33 (0.07) nM	
ARC-902	10 mM Mg^{2+}	PKA C	5.4 nM (8.27, 0.05)	0.51 (0.1) nM	IC_{50} = 8.3 nM [15]
ARC-341		PKA C	290 nM (6.54, 0.04)	38 (1) nM	
ARC-341	10 mM Mg^{2+}	PKA C	300 nM (6.52, 0.10)	65 (12) nM	IC_{50} = 170 ± 40 [15]
H89		ROCK II	170 nM (6.76, 0.04)	31 (3) nM	IC_{50} = 270 nM [36], K_i = 0.1 μM [38]
Y-27632		ROCK II	160 nM (6.79, 0.08)	27 (5) nM	IC_{50} = 800 nM [36], K_i = 98 nM [37], K_i = 0.09 μM [38]
ARC-902		ROCK II	11 nM (7.96, 0.02)	1.5 (0.7) nM	
ARC-902	10 mM Mg^{2+}	ROCK II	14 nM (7.86, 0.06)	1.4 (0.5) nM	
ARC-341		ROCK II	29 nM (7.54, 0.07)	3.6 (0.9) nM	

Note. SE, standard error.
[a] Standard errors are in parentheses.

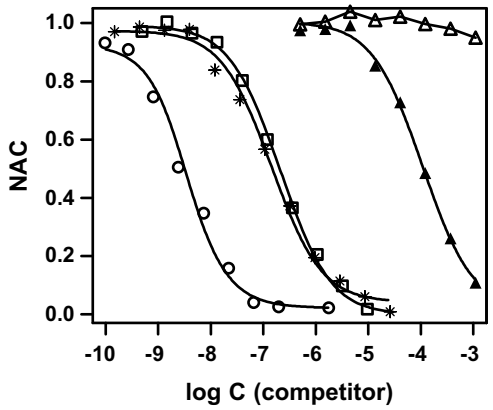


Fig. 6. Displacement of fluorescent probe ARC-583 from its complex with PKA C by ARC-902 (○), H89 (∗), ARC-341 (□), ATP (Δ) (all without Mg^{2+}), and ATP (with 10 mM $MgAc_2$) (▲). The data were fit to Eq. (2). NAC, normalized anisotropy change.

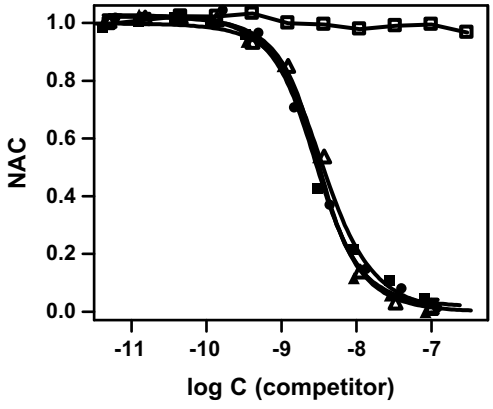


Fig. 7. Displacement of the fluorescent probe ARC-583 from its complex with PKA C by PKIα (●), RIα (□,■), and RIIα (Δ,▲) in the presence of both ATP (10 μM) and $MgAc_2$ (10 mM) (filled symbols) and in the their absence (open symbols). The data were fit to Eq. (2). NAC, normalized anisotropy change.

H89 to this kinase was similar to that toward PKA C (K_d = 23 nM). This result is in good accord with the recent findings claiming similar inhibitory potency of H89 toward both ROCKII and PKA [36]. Y-27632, a selective inhibitor of ROCK, revealed affinity with a K_d of 27 nM that is to some extent better than the reported inhibitory potency of this compound toward ROCKII (K_i = 98 nM [37]). Y-27632 and H89 revealed similar affinities toward ROCKII; close inhibitory potency has been reported for these compounds previously [38]. The unlabeled predecessor ARC-902 had an affinity comparable to that of the corresponding labeled compound ARC-583. The L-arginine-containing ARC-341 had a higher affinity toward ROCKII than toward PKA C (Table 2) [15].

Effect of cAMP on PKA holoenzyme dissociation

We assessed the applicability of the fluorescent probe ARC-583 for monitoring cAMP-induced dissociation of PKA holoenzyme. To avoid the substantial displacement of R from holoenzyme by competing ARC-583 in the absence of cAMP (ARC-583 and regulatory subunits of PKA have comparable affinities toward the catalytic

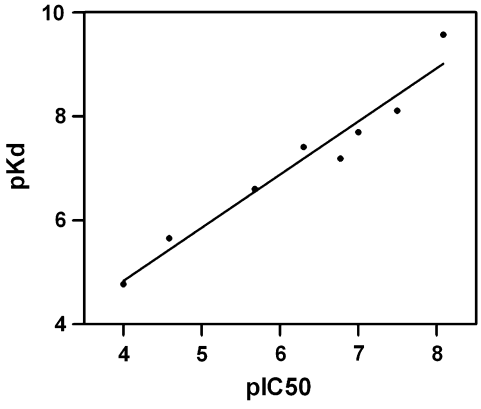


Fig. 8. Correlation between the inhibitory potencies (IC_{50} as measured by TLC kinetic inhibition assay) and the binding constants (K_d as determined in FA assay with fluorescent probe ARC-583) of eight inhibitors of PKA C.

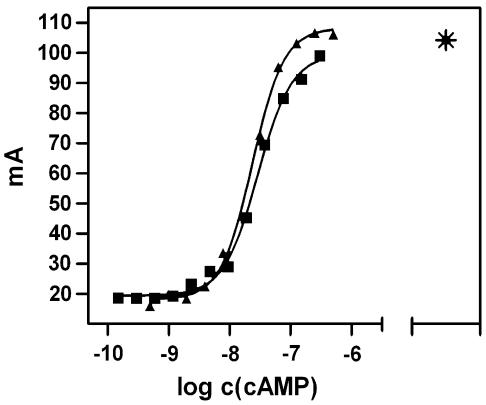


Fig. 9. Effect of cAMP on anisotropy of the solution of ARC-583 (2 nM) and PKA holoenzyme formed from PKA C and either RI (▲) or RII (■). EC_{50} values of 22 and 28 nM for PKA C/RI and PKA C/RII, respectively, were calculated by a sigmoidal dose–response model. (*) Anisotropy value of standard ARC-583/PKA C combination (2 nM ARC-583, 2 nM PKA C).

subunit), a higher excess of R was used; PKA C (3 nM final concentration) was combined with regulatory subunit RI or RII (either at a 10-nM monomer concentration). As illustrated in Fig. 9, the addition of cAMP led to an increase in anisotropy until the value characteristic for the standard ARC-583–PKA C combination (2 nM ARC-583, 3 nM PKA C) was reached, pointing to the full liberation of C at higher cAMP concentrations. The calculated EC_{50} values (22 and 28 nM for PKA C/RI and PKA C/RII, respectively) fit well into the range of reported efficiency values of cAMP-regulated PKA holoenzyme-related processes [39]. Because different factors (e.g., the origin and concentration of R subunit, association with 4 cAMP molecules with a dimer of regulatory subunits, largely different cAMP-binding affinity of free and catalytic subunit-complexed R subunit [40]) influence the EC_{50} value, both lower and higher values have been reported for cAMP effects. Thus, an EC_{50} value of 59 nM was reported recently [13] for the cAMP effect on holoenzyme dissociation (concentrations of C-subunit and RI-subunit were 64 and 77 nM, respectively), as registered with an FP assay when using the fluorescent tracer TR-IP20 at a fixed concentration of 40 nM.

Fig. 9 also demonstrates that ARC-583 in combination with PKA holoenzyme is a sensitive biosensor for the determination of cAMP concentration in the 1- to 200-nM range.

Conclusions

This study has described the high-affinity bisubstrate fluorescent probe ARC-583 generated by the attachment of the fluorescent dye 5-TAMRA to the C terminus of ARC-902. ARC-583 is applicable for the HTS and characterization of active site-targeted inhibitors of PKA C and ROCKII.

The bisubstrate character (simultaneous association with both binding sites of the kinase) of the probe was demonstrated by its displacement from the complex with PKA C by both ATP- and protein substrate-competitive inhibitors. These unique properties of the probe enable its application for characterization of inhibitors targeted to either binding site of the kinase and having affinity in a wide micromolar and nanomolar range. This makes the presented binding/displacement assay a real rival to inhibition assays. The same tracer can be used to characterize compounds that realize their activity on PKA as agonists or antagonists of cAMP-regulated activity of holoenzyme. It can also be used for determination of the concentration of the active form of PKA and as a sensor for cAMP concentration in the nanomolar region.

The other features of the assay, including homogeneity, single step, quickness, no need for special substrates, and capricious antibodies, support the use of the assay for HTS and precise characterization of inhibitors of PK. By virtue of these features, the assay is amenable to automation and can be quickly introduced for inhibitor testing both in small research laboratories and in HTS departments of big pharmaceutical companies.

Acknowledgments

This research was supported by grants from the Estonian Science Foundation (6710) and the Estonian Ministry of Education and Sciences (SF0180121s08).

References

[1] P. Cohen, Protein kinases: the major drug targets of the twenty-first century?, Nat Rev. Drug Discov. 1 (2002) 309–315.
[2] A.L. Gill, M. Verdonk, R.G. Boyle, R. Taylor, A comparison of physicochemical property profiles of marketed oral drugs and orally bioavailable anti-cancer protein kinase inhibitors in clinical development, Curr. Top. Med. Chem. 7 (2007) 1408–1422.
[3] D.M. Olive, Quantitative methods for the analysis of protein phosphorylation in drug development, Expert Rev. Proteomics 1 (2004) 327–341.
[4] Y. Jia, C.M. Quinn, S. Kwak, R.V. Talanian, Current in vitro kinase assay technologies: the quest for a universal format, Curr. Drug Discov. Technol. 5 (2008) 59–69.
[5] I. Vainshtein, S. Silveria, P. Kaul, R. Rouhani, R.M. Eglen, J. Wang, A high-throughput, nonisotopic, competitive binding assay for kinases using nonselective inhibitor probes (ED–NSIP), J. Biomol. Screen. 7 (2002) 507–514.
[6] M.W. Karaman, S. Herrgard, D.K. Treiber, P. Gallant, C.E. Atteridge, B.T. Campbell, K.W. Chan, P. Ciceri, M.I. Davis, P.T. Edeen, R. Faraoni, M. Floyd, J.P. Hunt, D.J. Lockhart, Z.V. Milanov, M.J. Morrison, G. Pallares, H.K. Patel, S. Pritchard, L.M. Wodicka, P.P. Zarrinkar, A quantitative analysis of kinase inhibitor selectivity, Nat. Biotechnol. 26 (2008) 127–132.
[7] G.H. Iyer, P. Taslimi, S. Pazhanisamy, Staurosporine-based binding assay for testing the affinity of compounds to protein kinases, Anal. Biochem. 373 (2008) 197–206.
[8] C.S. Lebakken, H.C. Kang, K.W. Vogel, A fluorescence lifetime based binding assay to characterize kinase inhibitors, J. Biomol. Screen. 12 (2007) 828–841.
[9] M. Kawaguchi, T. Terai, R. Utata, M. Kato, K. Tsuganezawa, A. Tanaka, H. Kojima, T. Okabe, T. Nagano, Development of a novel fluorescent probe for fluorescence correlation spectroscopic detection of kinase inhibitors, Bioorg. Med. Chem. Lett. 18 (2008) 3752–3755.
[10] M.H. Roehrl, J.Y. Wang, G. Wagner, A general framework for development and data analysis of competitive high-throughput screens for small-molecule inhibitors of protein–protein interactions by fluorescence polarization, Biochemistry 43 (2004) 6056–16066.
[11] M.A. Kashem, R.M. Nelson, J.D. Yingling, S.S. Pullen, A.S. Prokopowicz III, J.W. Jones, J.P. Wolak, G.R. Rogers, M.M. Morelock, R.J. Snow, C.A. Homon, S. Jakes, Three mechanistically distinct kinase assays compared: measurement of intrinsic ATPase activity identified the most comprehensive set of ITK inhibitors, J. Biomol. Screen. 12 (2007) 70–83.
[12] T.L. Schneider, R.S. Mathew, K.P. Rice, K. Tamaki, J.L. Wood, A. Schepartz, Increasing the kinase specificity of K252a by protein surface recognition, Org. Lett. 7 (2005) 1695–1698.
[13] S.A. Saldanha, G. Kaler, H.B. Cottam, R. Abagyan, S.S. Taylor, Assay principle for modulators of protein–protein interactions and its application to non-ATP-competitive ligands targeting protein kinase A, Anal. Chem. 78 (2006) 8265–8272.
[14] K. Viht, S. Schweinsberg, M. Lust, A. Vaasa, G. Raidaru, D. Lavogina, A. Uri, F.W. Herberg, Surface-plasmon-resonance-based biosensor with immobilized bisubstrate analog inhibitor for the determination of affinities of ATP- and protein-competitive ligands of cAMP-dependent protein kinase, Anal. Biochem. 362 (2007) 268–277.
[15] E. Enkvist, D. Lavogina, G. Raidaru, A. Vaasa, I. Viil, M. Lust, K. Viht, A. Uri, Conjugation of adenosine and hexa-(D-arginine) leads to a nanomolar bisubstrate–analog inhibitor of basophilic protein kinases, J. Med. Chem. 49 (2006) 7150–7159.
[16] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, 3rd ed., Springer, New York, 2006.
[17] M.F. Olson, Applications for ROCK kinase inhibition, Curr. Opin. Cell Biol. 20 (2008) 242–248.
[18] K. Viht, A. Vaasa, G. Raidaru, E. Enkvist, A. Uri, Fluorometric TLC assay for evaluation of protein kinase inhibitors, Anal. Biochem. 340 (2005) 165–170.
[19] Z.-X. Wang, An exact mathematical expression for describing competitive binding of two different ligands to a protein molecule, FEBS Lett. 360 (1995) 111–114.
[20] J.-H. Zhang, T.D.Y. Chung, K.R. Oldenburg, A simple statistical parameter for use in evaluation and validation of high throughput screening assays, J. Biomol. Screen. 4 (1999) 67–73.
[21] X. Huang, Fluorescence polarization competition assay: the range of resolvable inhibitor potency is limited by the affinity of the fluorescent ligand, J. Biomol. Screen. 8 (2003) 34–38.

[22] Z. Nikolovska-Coleska, R. Wang, X. Fang, H. Pan, Y. Tomita, P. Li, P.P. Roller, K. Krajewski, N.G. Saito, J.A. Stuckey, S. Wang, Development and optimization of a binding assay for the XIAP BIR3 domain using fluorescence polarization, Anal. Biochem. 332 (2004) 261–273.
[23] P. Kuzmic, K.C. Elrod, L.M. Cregar, S. Sideris, R. Rai, J.W. Janc, High-throughput screening of enzyme inhibitors: simultaneous determination of tight-binding inhibition constants and enzyme concentration, Anal. Biochem. 281 (2000) 62–67.
[24] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[25] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[26] T. Chijiwa, A. Mishima, M. Hagiwara, M. Sano, K. Hayashi, T. Inoue, K. Naito, T. Toshioka, H. Hidaka, Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, *N* -[2-(*p*-bromocinnamylamino)-ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells, J. Biol. Chem. 265 (1990) 5267–5272.
[27] J. Coffin, M. Latev, X. Bi, T.T. Nikiforov, Detection of phosphopeptides by fluorescence polarization in the presence of cationic polyamino acids: application to kinase assays, Anal. Biochem. 278 (2000) 206–212.
[28] P.F. Cook, M.E. Neville Jr., K.E. Vrana, F.T. Hartl, R. Roskoski Jr., Adenosine cyclic 3′-5′-monophosphate dependent protein kinase: kinetic mechanism for the bovine skeletal muscle catalytic subunit, Biochemistry 21 (1982) 5794–5799.
[29] R.N. Armstrong, H. Kondo, E.T. Kaiser, Cyclic AMP-dependent ATPase activity bovine heart protein kinase, Proc. Natl. Acad. Sci. USA 76 (1979) 722–725.
[30] D. Bhatnagar, R. Roskoski Jr., M.S. Rosendahl, N.J. Leonard, Adenosine cyclic 3′-5′-monophosphate dependent protein kinase: a new fluorescence displacement titration technique for characterizing the nucleotide binding site on the catalytic subunit, Biochemistry 22 (1983) 6310–6317.
[31] D.M. Gamm, M.D. Uhler, Isoform-specific differences in the potencies of murine protein kinase inhibitors are due to nonconserved amino-terminal residues, J. Biol. Chem. 270 (1995) 7227–7232.
[32] X. Cheng, C. Phelps, S.S. Taylor, Differential binding of cAMP-dependent protein kinase regulatory subunit isoforms Iα and IIβ to the catalytic subunit, J. Biol. Chem. 276 (2001) 4102–4108.
[33] W. Zhang, G.Z. Morris, S.J. Beebe, Characterization of the cAMP-dependent protein kinase catalytic subunit Cγ expressed and purified from sf9 cells, Protein Expr. Purif. 35 (2004) 156–169.
[34] B. Zimmermann, J.A. Chiorini, Y. Ma, R.M. Kotin, F.W. Herberg, PrKX is a novel catalytic subunit of the cAMP-dependent protein kinase regulated by the regulatory subunit type I, J. Biol. Chem. 274 (1999) 5370–5378.
[35] F. Hoffman, Apparent constant for the interaction of regulatory and catalytic subunit of cAMP-dependent protein kinase I and II, J. Biol. Chem (1980) 1559–1564.
[36] S. P Davies, H. Reddy, M. Caivano, P. Cohen, Specificity and mechanism of action of some commonly used protein kinase inhibitors, Biochem. J. 351 (2000) 95–105.
[37] J.W. Trauger, F.F. Lin, M.S. Turner, J. Stephens, P.V. LoGrasso, Kinetic mechanism for human rho-kinase II (ROCK-II), Biochemistry 41 (2002) 8948–8953.
[38] J. Feng, M. Ito, Y. Kureishi, K. Ichikawa, M. Amano, N. Isaka, K. Okawa, A. Iwamatsu, K. Kaibuchi, D.J. Hartshorne, T. Nakano, Rho-associated kinase of chicken gizzard smooth muscle, J. Biol. Chem. 274 (1999) 3744–3752.
[39] S. Schweinsberg, D. Moll, N.C.G. Burghardt, C. Hahnefeld, F. Schwede, B. Zimmermann, S. Drewianka, L. Werner, F. Kleinjung, H.G. Genieser, J. Schuchhardt, F.W. Herberg, Systematic interpretation of cyclic nucleotide binding studies using KinetXBase, Proteomics 8 (2008) 1212–1220.
[40] K.K. Dao, K. Teigen, R. Kopperud, E. Hodneland, F. Schwede, A.E. Christensen, A. Martinez, S.O. Døskeland, Epac1 and cAMP-dependent protein kinase holoenzyme have similar cAMP affinity, but their cAMP domains have distinct structural features and cyclic nucleotide recognition, J. Biol. Chem. 281 (2006) 21500–21511.