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Protein-Induced Long Lifetime Luminescence of Nonmetal Probes

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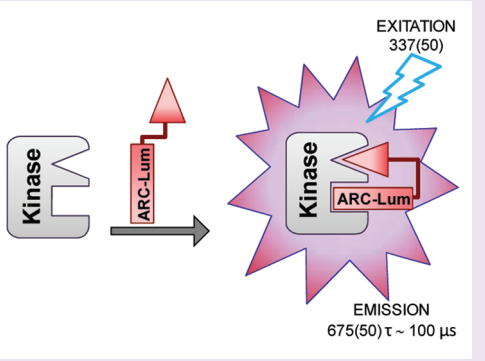
Supporting Information

ABSTRACT: Time-resolved luminometry-based assays have great potential for measurements in complicated biological solutions and living cells as the measured signal can be easily distinguished from nanosecond lifetime background fluorescence of organic compounds and autofluorescence of cells. In the present study we discovered that binding of a thiophene- or a selenophene-containing heteroaromatic moiety (luminescence donor) to the purine-binding pocket of a protein kinase (PK) induces long lifetime photoluminescence signal that is largely intensified through efficient energy transfer to a fluorescent dye present in close proximity to the luminescence donor. The developed ARC-Lum probes possessing 19−266 μs luminescence lifetime when associated with the target kinase can be used for determination of activity of basophilic PKs, characterization of inhibitors of PKs, and as cAMP sensors. An ARC-Lum probe was also used for the determination of kinetic parameters of inhibitor binding to the catalytic subunit of protein kinase A (PKAc). Effective real-time monitoring of the activation of PKA by Forskolin and the displacement of an ARC-Lum probe from its complex with PKA by inhibitor H89 was performed in live cells. The discovered phenomenon, protein-induced long lifetime luminescence of aromatic probes is very likely to occur with all PKs and many other proteins.



Long lifetime photoluminescent probes possessing lifetimes in micro- or millisecond range and specifically binding to the target proteins could be of great value for biological research. Time-resolved luminometry is an excellent measurement regime for homogeneous assays enabling the monitoring of concentration and activity of proteins in complicated biological systems as the measured signal is free from nanosecond-scaled background fluorescence of organic compounds and autofluorescence of cells.

Phosphorescence is a form of photoluminescence in which the exited compound emits light from its triplet state while returning to singlet ground state. This transition is slow resulting in much longer lifetime (usually in μs to ms range) compared to fluorescence (lifetimes in the range of 0.1−10 ns for usual fluorescent dyes).[1] Differently from steady state measurements common in fluorescence spectroscopy the measurement of long lifetime luminescence is usually carried out in cycles (Figure 1a) starting with a light flash excitation, followed by a time delay when the background signal quickly decays and the acquisition of the luminescence that is performed in a time window which duration depends on the luminescence lifetime of the luminophore.[2]

Molecular motions effectively annihilate the triplet state during its long residence time and turn the room-temperature phosphorescence into a rarely detectable phenomenon.[3] This is especially true for small organic substances in homogeneous solution, while several metal−ligand complexes (MLCs), for example, lanthanide chelates and platinum and palladium complexes of porphyrins, show high intensity of long lifetime luminescence and are in use for time-resolved luminescence-based bioassays.[4−7]

Nevertheless, many organic compounds reveal significant room-temperature phosphorescence in micelles, cyclodextrin complexes,[1] polymeric matrixes,[8] and crystals.[3] The phosphorescence of tryptophan residues of proteins has been used to determine the structure and molecular mobility of proteins.[9] Restriction to molecular movements is required to diminish nonradiative relaxations of triplet states to produce a detectable signal of phosphorescence of organic compounds at RT.

The enhancement of luminescence signal of a compound by means of the resonance energy transfer from a long lifetime low quantum yield luminescence donor (D) to a short lifetime high quantum yield fluorescent energy acceptor (A) has been demonstrated in the case of tandem probes incorporating a ruthenium-containing MLC as D and a short-lived fluorescent dye as A.[10−12] The mechanism behind the phenomenon includes the Förster-type radiationless resonance energy transfer (FRET) from the triplet state of D to the singlet state of A. The triplet-to-singlet energy transfer was earlier shown to be in accord with the Förster's theory.[13−15] Although the described probes possess outstanding optical properties (long lifetime, high brightness, long emission wavelength), they have not found wider practical use.[16]

Protein kinases (PKs) catalyze phosphorylation of proteins and play a key role in the regulation of protein functions in cells. The dysregulated activity of PKs may lead to a large variety of

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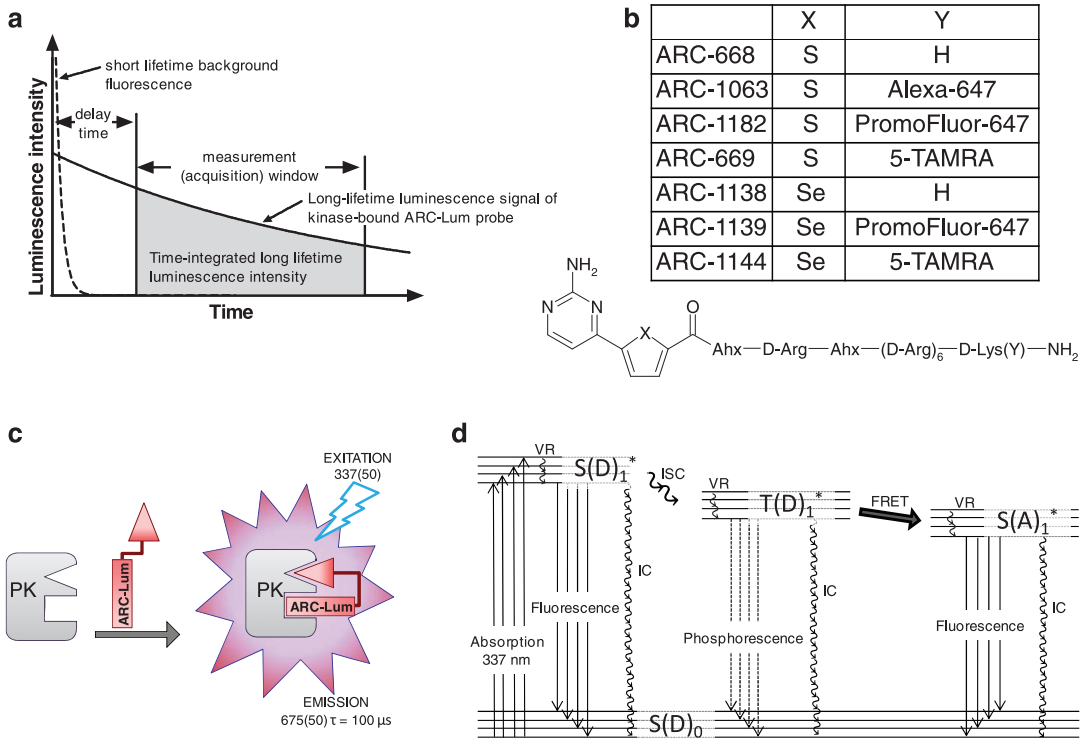

Figure 1. Kinase-induced long lifetime luminescence of ARC-Lum probes. (a) Scheme of time-gated measurement of long lifetime luminescence. Time course of the long lifetime luminescence signal is presented as a solid line and that of the background fluorescence by a dashed line. (b) Structures and ARC codes of ARC-Lum probes. (c) Scheme of the protein-induced long lifetime luminescence. Free ARC-Lum probe gives a negligible signal, whereas its binding to the target kinase (PK) increases the luminescence signal by 50−2500-fold. (d) The Jablonski diagram of long lifetime luminescence of labeled thiophene- and selenophene-containing ARC-Lum probes in the complex with protein kinases. The thiophene- or selenephene-containing fragment (donor, D) is flash-excited at 337 nm to singlet exited state $S(D)_1$*, followed by intersystem crossing (ISC) to the triplet state of the fragment ($T(D)_1$*). Thereafter the energy is passed to the conjugated dye (acceptor, A) that is excited (singlet excited state of the conjugated dye, $S(A)_1$*), followed by the radiation at the emission wavelength of the acceptor dye that is measured. IC, intrnal conversion; VR vibrational relaxation. Theoretical explanations and mathematical equations of this type of optical transitions have been discussed by Lakowicz *et al.*[10−12]

illnesses such as cancers, diabetes, and cardiovascular diseases.[17] Diverse biochemical functions and generally good drugability have made PKs an important class of drug targets and objects of intensive biochemical research. Currently, 10 small-molecule kinase-inhibiting compounds have been approved by FDA as cancer drugs, and about 150 compounds have been in various stages of clinical testing as drug candidates against different diseases.[18] This has created a significant demand for cost-effective and amenable to automation high-throughput methods for analysis of PKs and their inhibitors. Tools for intracellular tracking of kinase functioning are especially valuable.[19]

Most of the disclosed inhibitors of PKs compete with ATP for the purine-binding pocket, but protein substrate-competitive and allosteric inhibitors are also in wider use.[20] Bisubstrate inhibitors are a class of compounds that simultaneously associate with binding areas of both substrates (ATP and the phosphorylatable protein).[21] Therefore bisubstrate inhibitors can give a greater number of interactions with the target protein that is potentially resulting in higher affinity and selectivity of the compounds.

During the past decade conjugates of adenosine analogues and arginine-rich peptides (ARCs) have been developed by us into high-affinity bisubstrate inhibitors of PKs. ARCs of the latest generation possess clear selectivity toward basophilic PKs and high, two-digit picomolar affinity (K_d < 100 pM) toward some kinases (for example, catalytic subunit of protein kinase A, PKA).[22−24] ARCs are cell-permeable and stable in cellular milieu and reveal physiological activity in living cells and tissues.[21]

Here we report on the discovery and application of fluorescently labeled thiophene- and selenophene-containing ARC-type inhibitors (Figure 1b, Supplementary Table 1) as room-temperature luminescent probes (ARC-Lum probes) whose binding to the active site of a PK induces long lifetime (19−266 μs) long wavelength radiation at the acceptor emission wavelength in the case of excitation of the probes with a flash of light at 337 nm (Figure 1c). Free probes gave a negligible signal in nondeoxygenated solution, whereas their binding to the target kinase increased the luminescence in a time-delayed measurement window by 50- to 2500-fold. The discovered phenomenon was used in a simple, sensitive, and homogeneous "mix and read" type binding assay with several PKs of the AGC group. These probes allow the measurement of binding affinities and dissociation rates (residence times) of nonlabeled competitive inhibitors as well as the concentration of the active form of the kinase. Minute amount of both the PK (less than 1 fmol per well) and the probe are needed for the assay, while no antibodies, substrates, and other auxiliary reagents are required. The study also presents examples of the application of ARC-Lum probes for the monitoring activity of the protein kinase A (PKA) in living cells and cell lysate.

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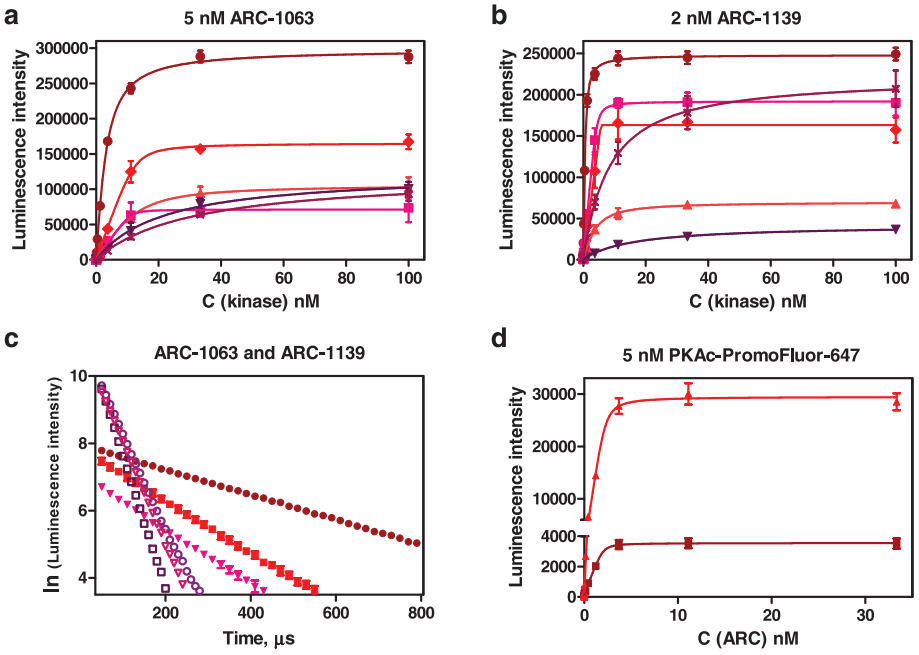

Figure 2. Luminescence characteristics of ARC-Lum−kinase complexes. Titration of luminescent probes ARC-1063 (5 nM) (a) and ARC-1139 (2 nM) (b) with protein kinases MSK1 (●), ROCKII (◆), AKT3 (▲), PKA (■), PKG Iα (▼), or PKC δ (×). (c) Time-dependent decays of luminescence intensities of complexes of ARC-Lum probes [ARC-1139 (open symbols) or ARC-1063 (filled symbols)] with protein kinases MSK1 (●, ○), ROCKII (■, □), or PKAc (▼, ▽). (d) Titration of PromoFluor-647-labeled protein kinase (PKAc-PromoFluor-647) with ARC-668 (■) and ARC-1138 (▲). The mean and SEM of three replicas are plotted.

RESULTS AND DISCUSSION

Discovery of ARC-Lum Probes. Long-lived emission of a thiophene-containing fluorescently labeled ARC was discovered in the course of development of a time-resolved Förster resonance energy transfer (TR-FRET) assay based on the application of lanthanide chelates. In a three-component method an Alexa Fluor 647-labeled ARC-type inhibitor ARC-1063 (Figure 1b) was used in combination with a 6His-tagged recombinant human Akt3 kinase (PKBγ) and an Eu-labeled anti-6His antibody. Energy transfer from the Eu-chelate to the Alexa Fluor 647 dye was awaited as a result of formation of the ternary complex.[4] An intense time-gated luminescence signal was detected (λ_{ex} = 337 nm, λ_{em} = 665 nm, delay time = 50 μs) when ARC-1063 was titrated with Akt3, even without the presence of the Eu-labeled antibody. Closer examination of this phenomenon showed that the measured signal was directly proportional to the concentration of ARC-1063−Akt3 complex. Displacement of ARC-1063 from the complex by competitive inhibitors decreased the luminescence signal in a concentration-dependent manner leading to very low values of the signal at high concentrations of the inhibitors.

Optical Properties of Thiophene- and Selenophene-Containing Peptide Conjugates. The starting compound for making thiophene-based ARC-Lum probes, 5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid and its conjugates with peptides (for example, ARC-668), possess an absorption maximum at 342 nm (molar extinction coefficient ε = 22,000 M^{-1} cm^{-1} at pH 2 and ε = 14,000 M^{-1} cm^{-1} at pH 7; UV spectra in Supporting Information) and emit blue fluorescence. The selenophene-containing compound 5-(2-aminopyrimidin-4-yl)selenophene-2-carboxylic acid and its peptide conjugates (for example, ARC-1138, Figure 1b) show similar absorption in the UV region with a maximum at 349 nm, but their fluorescence is much weaker than that of the thiophene-based compounds, caused by more effective intersystem crossing to the triplet state.[25]

Effect of PK Binding on the Photoluminescence of ARC-Lum Probes. Luminescence measurements with ARC-Lum probes were started with optical setups of the platereader used for time-resolved fluorescence (TRF) assays with Eu and Tb chelates. Twenty-microliter samples on a 384-well microtiter plate were analyzed with a PHERAstar platereader. The samples were excited with a flash of the xenon lamp of the platereader, followed by a long delay time (usually 50−100 μs) before the acquisition of luminescence intensity in a window of 150−400 μs. Later, the TRF optical modules were equipped with wide-pass emission filters [590 (50) for 5-TAMRA or 675 nm (50) for PromoFluor-647 and Alexa Fluor 647] to increase the emitted energy.

The appearance of a long lifetime luminescence signal was detected (Figure 2a,b) when PKAc was added to either the thiophene-containing probes ARC-1063 and ARC-1182 or their selenophene-containing counterpart ARC-1139 (Figure 1b). Although the chemical structure of Alexa Fluor 647 has not been disclosed by Invitrogen Company, the fluorescent dyes Alexa Fluor 647 (the fluorescent label of ARC-1063) and PromoFluor-647 (the fluorescent dye of ARC-1182 and ARC-1139) possess similar optical properties ($\lambda_{ex(max)}$ = 647 nm, ε = 250,000 M^{-1} cm^{-1}) leading to almost identical luminescence characteristics of probes ARC-1063 and ARC-1182 (Supplemenatry Figure 1).

Therefore the different luminescent properties of ARC-1063 and ARC-1139 originate from the five-atom aromatic rings of ARC-Lum compounds containing either a sulfur (ARC-1063) or a selenium (ARC-1139) atom.

In the complex with PKAc, ARC-Lum probes incorporating the fluorescent dye 5-TAMRA (ARC-669 and ARC-1144) gave

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Table 1. Luminescence Intensities and Lifetimes of Complexes of Luminescent Probes ARC-1063 and ARC-1139 with Protein Kinases of the AGC Group

	NLI[a]		τ (μs)[b]		A_0[c]		τA_0[d]	
kinase	ARC-1139	ARC-1063	ARC-1139	ARC-1063	ARC-1139	ARC-1063	ARC-1139	ARC-1063
no kinase (free probe)	~0.015	~0.0005	<7	<10	nd	nd	nd	Nd
MSK1	8.6 ± 0.2	4.2 ± 0.2	34.8 ± 0.1	266 ± 11	~58	~2.4	~17	~5.4
ROCKII	5.8 ± 0.2	2.3 ± 0.1	25.6 ± 0.1	126 ± 4	~88	~2.2	~19	~2.3
AKT3	2.4 ± 0.1	1.5 ± 0.1	19.8 ± 0.3	116 ± 6	~78	~1.3	~13	~1.3
PKAc	6.7 ± 0.1	1.0 ± 0.1	33.9 ± 0.1	118 ± 3	~50	1.0	~14	1.0
PKG Iα	1.5 ± 0.1	1.6 ± 0.1	19.0 ± 1.0	109 ± 9	~34	~1.2	~5.5	~1.1
PKC δ	7.7 ± 0.2	1.6 ± 0.1	41.9 ± 1.6	233 ± 46	~27	~0.6	~9.6	~1.1

[a] Normalized luminescence intensity: integrated luminescence intensity of the ARC-Lum probe−PK complex (50 μs delay time, 150 μs measurement window) normalized to that of ARC-1063−PKAc complex. [b] Luminescence lifetime of the ARC-Lum probe−PK complex. [c] Normalized maximal luminescence intensity, calculated from the time-dependent decay curve by extrapolation of intensity to zero time-point and normalization of the obtained value to that of ARC-1063−PKAc complex. A_0 is proportional to the rate constant of the luminescence emission; [d] Product of τ and A_0, is presumed to be proportional to time-integrated luminescence intensity of the complex.

comparable or slightly weaker luminescence than their Alexa Fluor 647- and PromoFluor-647-containing counterparts (ARC-1063 and ARC-1139, respectively). It is noteworthy that the selenophene-containing probe ARC-1144 with a 5-TAMRA label has significantly longer lifetime than the compound with PromoFluor-647 label (lifetimes 64 and 34 μs, respectively). Time-delayed excitation and emission spectra of protein-induced luminescence of ARC-Lum probes possess large wavelength gaps of the maxima. The luminescence emission spectrum well coincides with the fluorescence emission spectrum of the sensitizing fluorescent dye (acceptor) of the probe (Supplementary Figure 2), and the excitation spectrum overlaps the absorption spectrum of the donor (thiophene- or selenophene-containing fragment). The luminescence intensity of ARC-Lum probes in the absence of a protein kinase was negligible. ARC-Lum probes incorporating thiophene-based fragments other than 5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid revealed weaker luminescence (Supplementary Figure 3). On the other hand PK-bound adenosine-containing ARCs labeled with a fluorescent dye gave no detectable long lifetime luminescence signal.

Protein-induced long lifetime luminescence of the PKAc-bound thiophene-containing conjugate ARC-668, a nonlabeled precursor of ARC-1063 and ARC-669, could be detected only at 100 nM concentration of the probe-kinase complex. Luminescence of ARC-668 was more than 1000-fold weaker compared to the luminescence of its conjugates with 5-TAMRA (detected at 590 nm) and Alexa Fluor 647 (detected at 675 nm) pointing to an immense sensitization of the luminescence signal through intramolecular triplet to singlet energy transfer to the acceptor fluorophore (Figure 1d). All tested fluorescent dyes possessing strong absorption in the wavelength range of 500−650 nm behaved as effective sensitizing acceptors (Supplementary Figures 3, 4a, and 5a). Moreover, the dye could be linked to different positions of ARCs (ARC-688, Supplementary Figure 3b) without losing luminescence-sensitizing properties. 7-Nitrobenzofurazan (NBD) was the only dye whose binding to a thiophene-based ARC caused no detectable sensitization of luminescence (Supplementary Figure 4b). In this case the lack of the effect could be explained with optical properties of NBD, possessing weak absorption below 500 nm and lower brightness than the other used dyes.

ARC-1138, a selenium-containing analogue of ARC-668, in the complex with PKAc gave a 30- to 100-fold stronger luminescence signal [ex 337 (50) nm, em 630 (40) nm; lifetime 121 μs] compared with the latter probe (Supplementary Figure 6), which points to the positive heavy-atom effect of selenium on the transfer to the triplet excitation state.[1] The light emitted by ARC-1138−PKAc complex is strong enough to be used for measurement of the concentration of the kinase bound to the probe, although the compound contains no sensitizing fluorescence acceptors. Still, the intensity of the emitted light is 20- to 100-fold weaker than that of ARC-Lum probes carrying sensitizing dyes.

Luminescence lifetime difference for kinase-bound probes ARC-1138 and ARC-1139 points to ca. 70% efficiency of resonance energy transfer from 5-(2-aminopyrimidin-4-yl)selenophene-2-carboxylic acid moiety to PromoFluor-647 dye as calculated by the equation:

$$E = 1 - \frac{\tau_D}{\tau_D^0}$$

where E is RET efficiency, and τ_D and τ_D^0 are donor lifetimes in the presence and absence of acceptor, respectively.[11]

The appearance of a long lifetime luminescence signal was detected with all tested PKs of the AGC[26] group (Figure 2a,b, Table 1). Interestingly, association of different PKs with ARC-Lum probes leads to substantial differences in the intensity and lifetime of emitted light (Figure 2a,b, Table 1). In Table 1 luminescence intensities for ARC-Lum−PK complexes are normalized in comparison with that of the ARC-1063−PKAc complex. The first column of Table 1 lists time-integrated luminescence intensities (50 μs delay time, 150 μs acquisition time). Signals from complexes with ARC-1139 were substantially bigger than those of ARC-1063 complexes in the case of all tested kinases. MSK1 was the kinase whose binding to the probes induced the highest value of the signal (4.2-fold increase in the case of ARC-1063 and 8.6-fold increase for ARC-1139, both compared with the ARC-1063−PKAc complex). Luminescence lifetimes of the complexes of selenophene-containing probe ARC-1139 ranged from 19 to 42 μs, revealing 3.5- to 7.6-fold shorter lifetimes than those of the complexes with thiophene-based ARC-1063 (lifetimes in the 109−266 μs range, Figure 2c, Table 1). This points to the potential of heavy selenium atom to increase both the probability of transference to the triplet state after photon absorption and the rate of triplet to singlet energy transfer that all together result in both higher intensity of the

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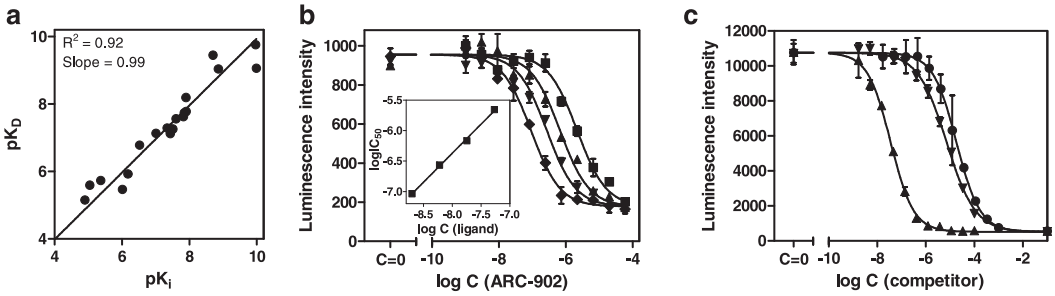

Figure 3. Displacement experiments. (a) Correlation between the values of inhibition constant (K_i) measured according to TLC kinetic inhibition assay[32] and the values of displacement constant (K_d) determined in displacement assay with the probe ARC-1063 for various inhibitors with PKAc. (b) Displacement of fluorescent probe ARC-1063 [at the concentration of 54 nM (■), 18 nM (▲), 6 nM (▼), or 2 nM (◆)] from its complex with PKAc (1 nM) by ARC-902; in the inset, the Schild plot analysis is presented. (c) Displacement of the luminescent probe ARC-1063 (10 nM) from its complex with ROCK II (1 nM) by Fasudil (●), Y27632 (▼), or Staurosporine (▲). The mean and SEM of three replicas are plotted.

emitted light and its shorter lifetime. Differences in lifetime and initial intensity of emitted light are in good agreement with the intensity of the emitted light measured at fixed delay time and acquisition window.

The highest intensity and the longest luminescence lifetime of the signal for complexes of MSK1 may result from higher rigidness of ARC−MSK1 binary complexes and greater protection of the probe from dissolved oxygen. Thus the active center of the PK behaves as a binding, fixing and protecting framework for ARC-Lum probes. Differences in optical properties of the probes bound to different kinases point to the possibility of tuning the optical characteristics of the complexes for different requirements by further optimization of the structure of the probe.

Integrated luminescence intensities[27] were calculated by multiplying initial intensities and lifetimes (data in the last column of Table 1). This value is assumed to be proportional to the relative brightness of the complex (extinction coefficient multiplied with quantum yield). Brightness of ARC-1139 complexes was 3−14-fold higher than those of ARC-1063 complexes.

Table 1 contains data for two ARC-Lum probes whose titration with six PKs resulted in full binding curves. Some other AGC kinases induced a strong long-living luminescence signal, but the binding curves did not reach the plateau either because of low affinity of the PK or low abundance of the active form of the PK in the sample. In the latter cases the determination of exact plateau values of the signal was not possible. Luminescence (measured with a 50 μs delay time using the same TRF module) of unbound ARC-1139 and ARC-1063 was 1,001,000-fold weaker than that of their complexes with the kinases.

Affinity of the probe could be determined either by its titration with the protein (Figure 2a,b) or, *vice versa*, by performing the titration of the protein at fixed concentration with varying concentration of the probe (Supplementary Figure 5a). The titration in the latter mode was possible because of a negligible signal of the unbound probe. Both titration variants resulted in coincident values of the dissociation constants. The heights of plateaus of the binding curves in the case of the titration with kinases were in linear correlation with the concentration of ARC-1063 in the solution (Supplementary Figure 5b).

Intermolecular Luminescence Sensitization. To test whether energy transfer between donor and acceptor luminophores is possible, intermolecularly PKAc was chemically labeled[28] with PromoFluor-647 dye (PKAc- PromoFluor-647), and the kinase was titrated with the thiophene- and selenophene-based conjugates ARC-668 and ARC-1138 (Figure 2d). Indeed, energy transfer could be detected when donor moiety of ARC-s and the PromoFluor-647 fluorescent label of the kinase were in close proximity due to the binding event. The detected intensities of the luminescence signals were comparable to the intensities of the signals obtained for PromoFluor-647-labeled ARC-1182 and ARC-1139 bound to PKAc. Thus the acceptor-mediated sensitization of luminescence also takes place intermolecularly, which enables the usage of unlabeled ARCs in pair with fluorescently labeled kinases. Titration of PromoFluor-647-labeled PKAc (PKAc−PromoFluor-647) with 5-(2-aminopyrimidin-4-yl)selenophene-2-carboxylic acid also evoked detectable long lifetime luminescence signal (K_D value of 15 ± 3 μM could be calculated from the binding curve, Supplementary Figure 7). This result revealed that the phenomen of protein-induced luminescence is characteristic not only for bisubstrate inhibitors but occurs also in the case of small ATP-competitive inhibitors. The used experimental setup points to a possibility for screening inhibitors of PKs for their long lifetime luminescence donor capabilities.

A steady state FRET between fluorophores of PKAc fused with yellow fluorescent protein (YFP) YFP-PKAc and fluorescent dye-labeled ARCs in living cells was described by us recently.[29] We hypostatized that in the case of ARC-668 even greater relative change of the signal intensity might take place if acceptor-sensitized FRET between ARC-668 and the fluorescent protein of YFP-PKAc occurred as the result of the binding event. This effect could help to solve most of the kinase selectivity issues as even in the case of a nonspecific inhibitor a specific signal from the PK fused with YFP emerges. Unfortunately, we failed to get the acceptor-sensitized luminescence of ARC-668 with YFP-PKAc fusion protein (experiments were performed with lysates of cells overexpressing the YFP-PKAc protein). Prospectively, selective chemical labeling of proteins with fluorescent dyes in living cells could be performed by means of FlAsH, ReAsH,[30] or other tagging technologies that enable the positioning of fluorescent dyes with optimal acceptor properties to closer proximity of the purine-binding pocket of the kinase.

Displacement of ARC-Lum Probes from the Kinase Complex by Inhibitors. PK-induced long lifetime luminescence signal of ARC-1063 was abolished by various ATP-competitive, protein substrate-competitive, or bisubstrate inhibitors. The obtained displacement IC_{50} values were in excellent correlation with affinities and inhibitory potencies of the competing compounds as determined in the binding/displacement assay with fluorescence anisotropy readout[31] and/or enzyme inhibition

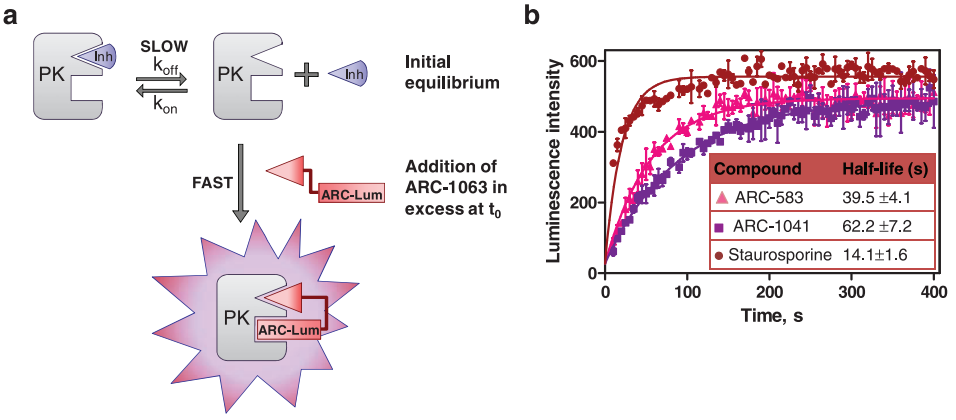

Figure 4. Measurement of dissociation kinetics of PK inhibitors. (a) Scheme of the experiment setup. PK is incubated with an active site-targeted inhibitor until the equilibrium is established. Thereafter an ARC-Lum probe is added in excess, and the increase in the luminescence intensity resulting from binding of ARC-Lum probe to PK dissociating from its complex with the inhibitor is monitored. (b) Time-dependent increase of luminescence intensity resulting from binding of ARC-1063 (100 nM) to PKAc dissociated from its complex with ARC-583(▲), ARC-1041(■), or Staurosporine(●). The mean and SEM of three replicas are plotted.

experiments,[32] respectively (Figure 3a). Lack of the signal from the unbound probe allowed the usage of ARC-1063 at relatively high concentration that supports use of the Cheng−Prusoff equation for simplified calculation of K_d (K_i) values from displacement curves (Figure 3b) and makes the results of measurements almost independent of the concentration of active protein present. This is a clear advantage of ARC-Lum-based luminescence intensity assays compared to fluorescence anisotropy-based measurements where the knowledge of the protein concentration is required.[34] This also enables the determination of affinities for highly potent compounds by shifting their displacement curves away from the tight-binding region. The possibility of application of the reporter ligand at higher concentrations widens the list of kinases that can be analyzed with a single probe as at higher concentration of the probe PKs possessing lower affinity still have an adequate fraction of the kinase bound to the probe. The amount of kinase required for analysis is still minute (starting from 1 fmol per well).

For the assessment of the quality of screening assay the Z′ factor[33] was calculated. In the case of the assay system containing binding partners at very low concentration (0.2 nM ARC-1139, 0.1 nM PKAc) a Z′ value of 0.7 was still achieved. The application of the kinase at higher concentration (for example, 0.5 nM concentration of PKAc) increased the value of Z′ factor to Z′ > 0.8 with all tested ARC-Lum probes (ARC-1139, ARC-1063, ARC-1144, Supplementary Figure 8). Thus low concentration of the enzyme, comparable to the concentration of a PK used in most sensitive kinetic assays, is needed for a sufficiently high value of the Z′ factor characterizing the quality of assay: 2 fmol of both ARC-Lum probe and the PK in 20 μL volume was enough to reach Z′ > 0.7, greater than requested (Z′ > 0.5) for reliable HTS assays.[33] Furthermore, the binding assays based on the use of ARC-Lum probes are simple, quick and need no additional substrates or other reagents (for example, antibodies). ARC-based bisubstrate inhibitors efficiently bind only to active PKs, and thus ARC-Lum probes report on the activity of the kinase (concentration of the active form of the kinase) in the sample. From this aspect ARC-Lum probes are different from other widely used protein-binding probes, fluorescent dye-labeled PK antibodies. The assay has now been used for characterization of a large number of inhibitors for several PKs, like PKAc, Akt3, ROCKII, and others (Figure 3c).

To establish the applicability of ARC-Lum probes for the determination of affinities of strongly fluorescent compounds displacement curves for various fluorescently labeled ARC-type inhibitors were measured in time-gated mode with ARC-1063 as the luminescent probe and PKAc as the target kinase (Supplementary Figure 9). The results clearly showed that fluorescent labels did not interfere with the time-gated measurements and the K_d values calculated from the IC_{50} values obtained from the displacement curves (e.g., K_d = 0.32 nM was determined for ARC-583) were in good agreement with the affinities of the compounds that were determined separately with other fluorescence readout methods, including measurement of affinities of the fluorescent compounds through their direct binding to the kinase (ARC-583, K_D = 0.48 nM).[31]

Selectivity profiling of the thiophene-containing probe ARC-669 with a large panel of PKs indicated that this probe had sufficient affinity and was potentially applicable for most of the kinases of the AGC-group and several other basophilic PKs (PIMs, CAMKs, PAKs) possessing K_i values of <300 nM toward the probe (Supplementary Table 2 and ref 23).

Determination of Dissociation Kinetics of Inhibitor−Kinase Complexes with the Help of ARC-Lum Probes. The strength of a biochemical interaction (affinity) is usually characterized by the value of dissociation constant K_D that is a thermodynamic measure. The kinetics of inhibitor-protein interaction show how quickly the association takes place and the equilibrium is established. Although the importance of kinetic parameters of inhibitor binding for drug development has been acknowledged for some time,[35,36] these data are not widely available. This can be explained by the limited number of methods that enable easy and precise measurement of dissociative off-rates for the complexes.

The drug residence time[35,36] equals the reciprocal of dissociation rate constant $1/k_{off}$ (according to some sources[36] to the half-life of the ligand-protein complex). It has been shown that compounds that possess similar binding constants, but different residence times may have distinct physiological properties.[35,36]

We developed a simple assay for the determination of dissociation rate constants for PK inhibitors based on the application of

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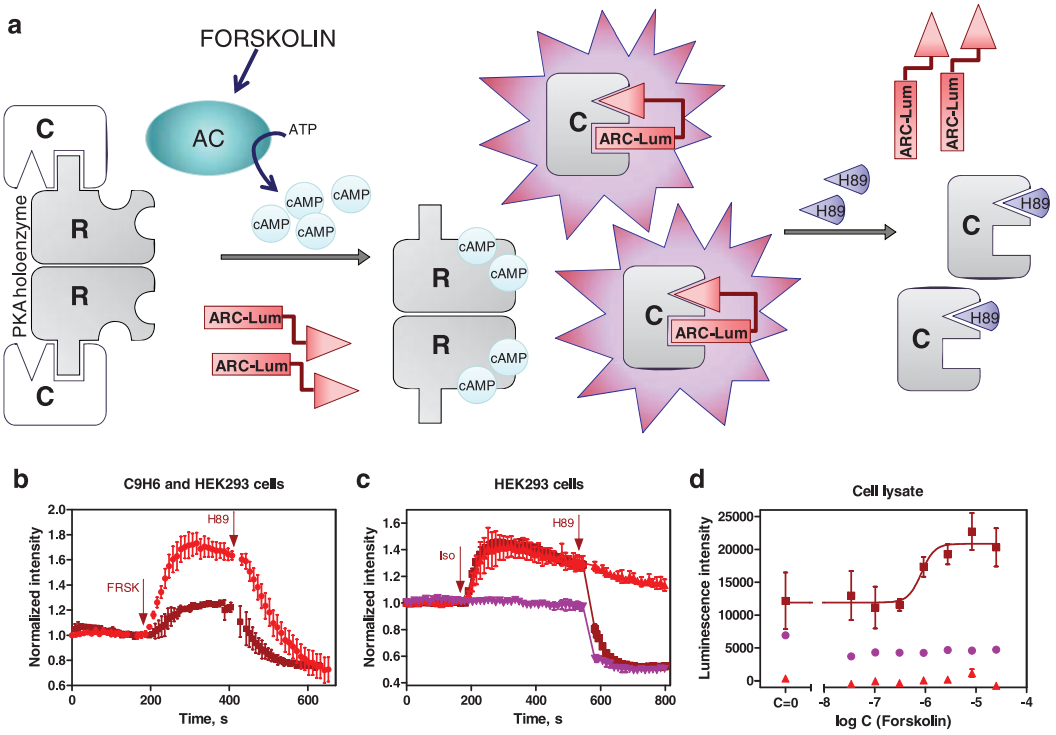

Figure 5. Detection of long lifetime luminescence of ARC-Lum probes in biological systems. (a) Scheme of the application of the ARC-1139 probe for monitoring of PKA activity in cells. C, catalytic subunit of PKA (PKAc); R, regulatory subunit of PKA (PKAr); AC, adenylate cyclase. (b, c) Real-time monitoring of PKA activity in living HEK293 (b: ■, c) and C9H6 (b: ●) cells using intracellular ARC-1139: long lifetime luminescence of cells on the bottom of wells of a microtiter plate was detected with a PHERAstar platereader (λ_{ex} = 337 nm, λ_{em} = 675 nm, delay time = 50 μs), time points of addition of Forskolin (FRSK, 25 μM, b: ■, ●) or Isoproterenol (Iso, 10 μM, c: ■, ▲) and H89 (100 μM, b: ■, ●, c: ■,▼) are marked with the arrows. (d) The application of ARC-1139 (25 nM) for the monitoring of PKA activity in crude lysates of Forskolin-stimulated HEK293 cells (■). The increased luminescence signal was reversed by displacement of the probe from its kinase complex with a PKA-specific inhibitor PKI (1 μM, ●) or a generic PK inhibitor Staurosporine (100 μM, ▲). The mean and SEM of three replicas are plotted.

ARC-Lum probes (Figure 4a). The inhibitor under examination was mixed with the PK at a concentration that resulted in binding of at least 90% of the kinase. The luminescent probe ARC-1063 was added to the solution in great excess (final concentration 100−300 nM), and the increase of long lifetime luminescence signal was monitored. In these conditions the formation of ARC-1063−kinase complex is determined by the rate of release of the kinase from the complex with the inhibitor.[37] The obtained data were fitted to the first order kinetic model (Figure 4b).

Dissociation half-lives for ARC-type inhibitors associated with PKAc ($t_{1/2}$ = 39.5 ± 4.1 s for ARC-583;[31] 62.2 ± 7.2 s for ARC-1041)[24] are well comparable to the values that were previously determined with surface-plasmon resonance[37] and fluorescence polarization[31] methods. In the present format (PHERAstar plate-reader, manual pipetting), the assay failed to work for most of the tested ATP-competitive inhibitors because dissociation kinetics were too fast. The final plateau of the signal was attained before the first measurement was performed (7 s after addition of ARC-1063). Only Staurosporine, a generic inhibitor of PKs, gave an analyzable curve that afforded reliable determination of the half-life ($t_{1/2}$ = 14.1 ± 1.6 s). The assay can be adapted for faster kinetics if the time between mixing and initiation of luminescence measurements is reduced with the help of a quick dispensing/mixing system or a flow equipment.[38]

On the basis of the obtained half-life ($t_{1/2}$) of the inhibitor−PK complex the dissociation rate constant of the first-order reaction can be calculated ($k_{off} = 0.693/t_{1/2}$). Taking into account the k_{off} and K_d values for the ARC-583−PKAc complex (k_{off} = 0.018 s^{-1} and K_d = 0.32 nM at 27 °C) the binding rate constant k_{on} can be easily and reliably calculated for this compound ($K_D = k_{off}/k_{on}$). The obtained value of $k_{on} = 5.5 \times 10^7$ M^{-1} s^{-1} points to very high rate of the association reaction.

Altogether, with the help of an ARC-Lum probe a full and reliable kinetic/thermodynamic characterization of the inhibitor-kinase complex is obtained. Differently from widely used surface plasmon resonance method ARC-Lum-based assays are homogeneous, amenable to automation and can be used for high throughput analysis of drug candidates with the aim of finding inhibitors with suitable residence times. Taking into account the unique bisubstrate character of ARC-Lum probes, in addition to characterization of ATP-competitive inhibitors the probes are also applicable for characterization of compounds binding to the protein substrate binding site of the kinase. In case of PKA it means that the binding efficiency of both the regulatory subunit of PKA (PKAr) and the thermostable inhibitory protein (PKI) with the catalytic subunit (PKAc) is easily measurable.

Monitoring Activity of Protein Kinases in Living Cells. Methods enabling the monitoring of activity of protein kinases in complicated biological solutions such as blood serum, cell lysates, tissue extracts, and living cells have great value for biological research. Such analytical methods have also a good prospective to become novel diagnostic tools.[39,40]

Recently we have described the detection of a steady state FRET signal resulting from association of a cell-penetrative

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ARC-type inhibitor carrying an orange fluorescent dye 5-TAMRA with PKAc-YFP fusion protein in living C9H6 cells (CHO cells overexpressing fusion proteins of the PKA subunits with fluorescent proteins, PKAc-YFP and PKAr-CFP).[29] The intensity of FRET signal was increased as the result of PKA activation and the effect was reversed by a potent PKAc inhibitor H89 that displaced ARC-probe from its complex with PKAc-YFP. This system could be used as an intracellular sensor for monitoring of changes in cAMP concentration and characterization of kinase inhibitors. A great shortcoming of FRET-based assay is the requirement for labeling of the target protein with a fluorophore that in case of cellular assays is usually achieved through generation of recombinant cells expressing fusions of the target protein with a fluorescent protein.

In the present study HEK293 cells (with native expression level of PKA) on the bottom of wells of a microtiter plate or on coverslip were incubated with solutions of thiopehene- or selenophene-containing ARC-Lum probes (10 μM). After removal of excess ARC-Lum from the surface of cells by repeated washing procedures, cellular uptake and intracellular localization of the probes was established with fluorescence microscope (Supplementary Figure 10). Efficient uptake was also established by strong increase of the steady state fluorescence signal of the monolayer of attached cells at emission wavelength of the fluorescent dye (excitation at absorbance maximum of the dye) as detected with fluorescence platereader. For example, after loading the HEK293 cells with ARC-1139 the fluorescence intensity [ex 590 (50) nm, em 675 (50) nm] was increased by a 100-fold.

When the cells were excited with flashes of the xenon lamp at 337(50) nm and the light emission intensity was measured after 50 μs delay in a 150 μs measurement window at 675 (50) nm, a stable baseline signal was obtained (Figure 5a−c). The addition of Forskolin to HEK293 cells led to small increase of the signal in the case of a thiophene-containing ARC-Lum (data not shown) but to a substantial (more than 30%) increase in the intensity of emitted light in the case of the selenophene-containing probe ARC-1139 (Figure 5b). In case of C9H6 cells recombinantly overexpressing both subunits of PKA, an even bigger increase of the intensity of the luminescence signal (more than 50%) was achieved after activation of cells with Forskolin (Figure 5b). As depicted in Figure 5a the addition of Forskolin caused the activation of adenylate cyclases that resulted in increase of the cellular concentration of cAMP. Activation of PKA by cAMP led to an increased level of free catalytic subunit PKAc whose association with ARC-1139 resulted in increased long lifetime luminescence. The same effect was achieved through activation of β-adrenergic receptors natively expressed in HEK293 cells with the agonist Isoproterenol (Figure 5c). A cell-permeable ATP-competitive PKA inhibitor H89 reversed the effect of both compounds (Figure 5b,c). The obtained results point to ARC-Lum probes as promising sensors for real-time monitoring of cAMP concentration in live cells thus supplementing other cellular sensors of cAMP accumulation.[19,41,42]

Lower concentrations of ARC-Lum probes were needed for monitoring the PK activity in cells after their lysis. HEK293 cells at the bottom of 96-well plate were activated with varying concentration of Forskolin (0−100 μM). Cells were lysed, and the solution of ARC-1139 luminescent probe (final concentration 25 nM) was added to the lysate. Figure 5d shows that Forskolin in a concentration-dependent manner increased the luminescence signal that reached the plateau (almost 2-fold increase of the signal compared to nonactivated cells) at 10 μM Forskolin concentration. PKI (a specific PKA inhibitor) reduced the signal in all wells by displacing ARC-1139 from its complex with the catalytic subunit of the kinase, PKAc. The addition of Staurosporine (a potent generic inhibitor of PKs) reduced the luminescence signal to the background level (Figure 5d) by displacing all cellular kinases binding to ARC-1139.

In summary, this work describes the discovery of a novel optical phenomenon and the application of this effect for protein research. Binding of a heteroaromatic fragment of an inhibitor to the purine-binding pocket of a PK induces formation of a luminophore complex with a long lifetime photoluminescence signal that is largely intensified through efficient energy transfer to a fluorescent dye present in close proximity to the luminescence donor. This effect was shown for compounds incorporating a thiophene- or a selenophene-containing heteroaromatic moiety (luminescence donor) binding to the purine-binding pocket of the PK and a fluorescent dye (fluorescence acceptor) whose excitation spectrum overlaps with the long-lifetime emission spectrum of the luminescence donor.

In addition to thermodynamic characterization of inhibitor−PK complexes, ARC-Lum probes were used for determination of kinetic parameters of the complex formation. Altogether, a full and reliable kinetic/thermodynamic characterization of the nonlabeled inhibitor−kinase complex is obtained with ARC-Lum-based assays. ARC-Lum probes were also successfully used for real-time monitoring of activity of PKs in cells possessing native expression level of PKs with no need for recombinant expression of fusions with fluorescent proteins required for other cellular cAMP concentration and PK activity sensors.[19,41,42]

In the present work the cellular experiments with ARC-Lum probes were carried out in a 96-well microtiter plate format using a sensitive fluorescence platereader that measures the averaged signal from a monolayer colony of cells on the bottom of the well. Time-gated luminescence (TGL) microscopy providing means for spatial and temporal resolution and higher contrast for cell studies would make possible the use of ARC-Lum probes for obtaining a closer look at the functioning of protein kinases in living cells.[2,43]

We can prognosticate that, first described in the current work, the phenomena of protein binding-induced room-temperature phosphorescence of heteroaromatic fragments and enhancement of long lifetime luminescence intensity of the organic fragment by energy transfer to fluorescent dyes are much more widespread than the examples with ARC-Lum probes and PKs of the AGC group. Similar cases of protein-induced long lifetime luminescence of aromatic probes are very likely to occur with all PKs, many other proteins of the 3266-member purinome,[44] and even with more distinct proteins. Potential probe should bind into a deep pocket of a protein, absorb light and have tendency for formation of triplet state (inclusion of heavy atoms are favorable). Proximity of a RET acceptor is also important to significantly enhance naturally weak phosphorescence emittion.

Although the novel ARC-Lum-based technology uses no lanthanide chelates (cryptates), the optical setup (wavelengths of excitation and emission filters, delay times) of lanthanide-based TRF methods used by several biotechnology companies (for example, PerkinElmer, Cisbio, Invitrogen) are usable for ARC-Lum-based assays.

METHODS

Synthesis of ARC-Lum Probes. Peptide conjugates of 5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid and 5-(2-aminopyrimidin-

4-yl)selenophene-2-carboxylic acid were synthesized as described earlier.[23] Optimized and simplified synthetic routes are described in Supporting Information.

5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid[23] and 5-(2-aminopyrimidin-4-yl)selenophene-2-carboxylic acid were synthesized from 5-acetylthiophene-2-carboxylic acid and selenophene-2-carboxylic acid, respectively (Supporting Information). All ARC-type inhibitors were synthesized according to conventional Fmoc-strategy of solid-phase peptide synthesis as described earlier[23] and purified by using reverse-phase HPLC. Labeling of ARCs was performed through the side chain of lysine by using *N*-hydroxysuccinimide esters of the fluorescent dyes (triethylamine solution in DMSO, 3 h reaction).[31] High-resolution mass spectra of all new compounds were measured on a Thermo Electron LTQ Orbitrap mass spectrometer using electrospray ionization. Kinase-induced long lifetime luminescence excitation and emission spectra were recorded on a PerkinElmer LS55 luminescence spectrometer with delay time of 50 μs and acquisition time of 500 μs. Excitation spectrum was measured at the emission wavelength 585 nm (slit width 10 nm), and emission spectrum at the excitation wavelength 350 nm (slit width 10 nm).

All biochemical binding/displacement experiments were performed on black low-volume 384-well nonbonding-surface microplates (cat. no. 3676, Corning) on a PHERAstar platereader (BMG Labtech) with TRF optical modules [ex 337 (50) nm, em 675 (50) and 620 (20) nm], [ex 337 (50) nm, em 590 (50) and 545 (10) nm] or [ex 337 (50) nm, em 630 (40) nm] using the time-resolved fluorescence measurement mode. The microplates were incubated for 15 min at 30 °C before each measurement. The ARC-Lum probes were excited with a flash of the xenon lamp (200 flashes per data point) at 337 nm, followed by 50 μs delay time and subsequent acquisition of the luminescence using a constant acquisition time (150 μs) at the wavelength corresponding to the emission maximum of the conjugated fluorescent dye [530 (40) nm, 590 (50) nm, or 675 (50) nm].

The data from the assays were fitted with the aid of Prism (Version 5) statistical analysis program (GraphPad).

Binding Assay. The concentration series of kinases MSK1, ROCK-II, AKT3, PKAc, PKG Iα, or PKC δ (3-fold dilutions) was made in the assay buffer (150 mM NaCl, 50 mM Hepes pH = 7.5, 5 mM DTT, 0.5 mg mL^{-1} BSA), and the fixed concentration of luminescent probe ARC-1063 or ARC-1139 (5 nM or 2 nM, respectively) was added to each well.

Competitive Displacement. The displacement assay was performed by adding fixed concentration of the luminescent probe ARC-1063 in complex with a protein kinase to the concentration series of the competitive compound (3-fold dilutions) in the assay buffer. The displacement curves were fitted to a sigmoidal dose−response model to obtain IC_{50} values. K_d values were calculated using the Cheng-Prusoff equation [$K_d = IC_{50}/(1 + C_{ARC\text{-}Lum}/K_{d\ ARC\text{-}Lum})$].

Measurement of Luminescence Lifetimes. The luminescence lifetimes of the ARC-probes and complexes of ARC-probes with kinases were measured on a PHERAstar platereader using the decay mode. The complex of ARC-Lum probe with kinases MSK1, ROCKII, AKT3, PKAc, PKG Iα, or PKC δ was excited with a flash of the xenon lamp at 337 nm, and the luminescence decay was subsequently recorded. Luminescence lifetime and the initial intensity of the complex was calculated from the decay curves by using exponential decay function with the Prism software.

Intermolecular Sensitization. Labeling of PKAc with PromoFluor-647 was carried out as described previously.[28] The concentration series of ARC-668 and ARC-1138 was made in the assay buffer and thereafter PKAc-PromoFluor-647 (5 nM) was subsequently added. The luminescence signal was acquired by using the optical module [ex 337 (50) nm, em 675 (50) and 620 (20) nm].

Measurements of Dissociation Kinetics of Inhibitor−Kinase Complexes. The examined inhibitor (ARC-583 10 nM, ARC-1041 6 nM, or Staurosporine 7.6 nM) was mixed with PKAc (5 nM) and incubated for 15 min at 27 °C, thereafter ARC-1063 was added at high concentration (final concentration of 100−300 nM), and the luminescence signal was recorded in 5 s time intervals. The collected data were fitted to the first-order kinetic model yielding the dissociation rate constants (k_{off}) of the examined inhibitors.

Luminescence Measurements in Cell Lysate. HEK293 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37 °C in humidified atmosphere containing 5% CO_2. Cells were seeded to 96-well plates and grown to 80% confluency. After washing the cells once with 100 μL of HBSS, different concentrations of Forskolin (0100 μM) in the HBSS buffer containing 100 μM IBMX were added to the cells and incubated for 10 min at 37 °C. Subsequently, 25 μL of 50 nM of ARC-1139 in ice-cold cell lysis buffer [NP40 cell lysis buffer (Invitrogen), protease inhibitor cocktail (10x, Sigma-Aldrich), 0.5 mM PMSF, 1% (w/v) Triton-X] was added to each well. The cells were lysed at 30 °C for 10 min, and the luminescence intensities were measured using the optical module [ex 337 (50) nm, em 675 (50) and 620 (20) nm] in time-resolved fluorescence mode. For the measurement of the displacement of ARC-1139 from its complex with proteins present in the cell lysate, the lysing solutions contained 50 nM ARC-1139 probe and additionally 200 μM Staurosporine or 2 μM PKI (in the presence of 300 μM ATP and 30 mM Mg^{2+}).

Analysis of Activity of Protein Kinases in Living Cells. HEK293 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, and C9H6[29] cells were cultured in F12 Nutrient Mixture supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 300 μg mL^{-1} Zeocine, and 800 μg mL^{-1} Geneticin at 37 °C in humidified atmosphere containing 5% CO_2. Cells were seeded to 96-well plates and grown to 80% confluency. The medium was replaced with fresh serum-free medium containing the luminescent probe ARC-1139 (10 μM) and incubated for 1 h at 37 °C. Subsequently, the cells were washed three times with PBS and analyzed on a PHERAstar plate reader with TRF optic modules [ex 337 (50) nm, em 675 (50) and 620 (20) nm] using the TRF mode. Forskolin (final concentration of 25 μM) or Isoproterenol (final concentration of 10 μM) and H89 (final concentration of 100 μM) were added to the cells during the measurement (after 3 and 7 min from the start of luminescence recording, respectively).

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

Conflict of InterestA.U., E.E., A.V., and M.Ka. are inventers of a patent application filed by the University of Tartu concerning luminesence probes described in the present publication. A.U. is a founder and owner of Kinasera OÜ, a spin-off company of the University of Tartu that develops luminescence-based assays for kinase research.

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