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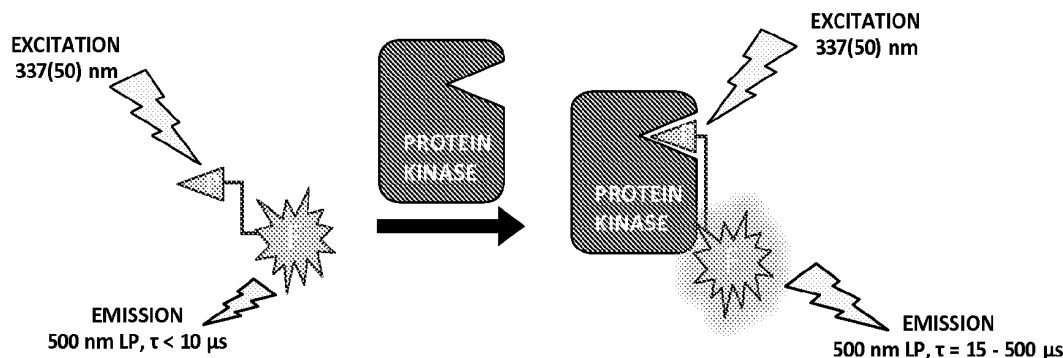
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50606, Tartu (EE)(54) **A photoluminescent probe**

(57) The invention relates to organic long lifetime photoluminescent probes that contain no metal complexes and whose specific association with purine-binding proteins leads to increased emission of long wavelength luminescence with long lifetime (in microsecond region). This enables the time-gated discrimination of the signal over fluorescence present in the biological samples, ei-

ther autofluorescence of cells or emission from other fluorophores. The invention concerns also the use of the improved method for monitoring activity of protein kinases in living cells, characterization of inhibitors of protein kinases, analysis of protein kinase-based disease biomarkers and other tasks of biological and medical importance.

**Figure 1**

Description

FIELD OF THE INVENTION

[0001] The invention concerns novel photoluminescent probes whose specific association with purine-binding proteins leads to increased emission of long lifetime luminescence, and the application of the probes for monitoring activity of protein kinases (PKs) and other purine-binding proteins, screening of compounds as inhibitors of PKs and characterization of inhibitors targeted to the kinase, and methods of manufacturing of such probes.

[0002] The invention also relates to the application of the luminescent probes in biochemical and cellular screening assays for identifying compounds that bind to and modulate the activity of a wide variety of members of PK super-family. The invention relates to the assay for identification and evaluation of PK inhibitors and determination of the concentration of PKs.

[0003] Probes of the invention can be used for, for example, monitoring the responsiveness of cells to receptor activation, high throughput screening of inhibitors of PKs and agonists and antagonists of metabotropic and ionotropic receptors, medical diagnosis, tissue imaging.

BACKGROUND TO THE INVENTION

[0004] Altogether ca 3000 proteins (protein kinases, small G proteins, dehydrogenases, ATPases, helicases, non-conventional purine-utilizing proteins, synthetases, purinergic receptors, etc.) are known to possess a purine-binding site, forming the humane purinome. Thus, approximately 13% of protein-coding genes of the human genome are devoted to coding for purine-binding proteins. [Knapp et al., Curr. Top. Med. Chem. 6 (2006) 1129] PKs are the most prevalent of proteins binding a purine nucleotide, ATP. The human genome codes 518 different PKs. [Manning et al., Science 298 (2002) 1912]

Protein Kinases and Protein Kinase Inhibitors

[0005] PKs play a key role in the regulation of protein functions in living cells. The activity of one third of proteins is regulated through phosphorylation of one or more of serine, threonine and tyrosine residues of the protein catalyzed by a PK. More than 400 human diseases (incl. cancer) have been linked to aberrant PK signaling. This has made PK the second largest drug target (and fastest growing category of drugs in development) after G protein-coupled receptors. [Cohen, Nat. Rev. Drug Discov. 1 (2002) 309; Fischer, Curr. Med. Chem. 11 (2004) 1563; Zhang et al., Nat Rev Cancer. 9 (2009) 28] Almost 150 inhibitors targeted to 40 different PKs have reached clinical trials. [Fedorov, Nature Chem. Biol. 6 (2010) 166]

[0006] PKs follow ternary complex kinetic mechanism in which direct transfer of the phosphoryl group from ATP to the protein substrate occurs in the active site. [Adams, Chem. Rev. 101 (2001) 2271]

[0007] The majority of known inhibitors realize their inhibitory potency via competitive with ATP binding to the purine-binding pocket of PKs. Despite serious selectivity problems (all 518 PKs and more than 2500 other proteins possess a purine-binding site) the main efforts of drug companies have been directed to the development of small-molecule ATP-competitive inhibitors.

[0008] Bisubstrate-analog (biligand) inhibitors of PKs also compete with ATP for binding to the PK. These inhibitors additionally associate with the protein binding domain of the enzyme. [Lavogina et al., ChemMedChem 5 (2010) 23]

Existing binding assays for PKs

[0009] Development of new assay methods for monitoring the activity of PKs and evaluation of inhibitors of PKs has run in parallel with the increase of importance of effective inhibitors for drug industry. [Zaman et al., Comb Chem High Throughput Screen, 6 (2003) 313]

[0010] Photoluminescence methods can be spatially and temporally more focused than radioactive methods, and as such, they are more suitable for application in high-throughput screening (HTS) assays. [Olive, Expert Rev Proteomics. 1 (2004) 327]

[0011] Some small-molecule inhibitors can be conjugated to fluorescent dyes leading to luminescent probes (indicators) that bind to PKs. The term "luminescent probe" as used herein is a compound whose optical (luminescent) properties are changed on binding with a target protein. Usually, the emission intensity, anisotropy and/or lifetime are altered when the analyte (PK) is bound to the probe. The probe can then be displaced by a competitive kinase inhibitor and the change in luminescence characteristics measured to establish the affinity of the non-labeled inhibitor. This interaction therefore forms the basis for a competitive binding assay of kinase inhibitors where neither a substrate nor an antibody to the phosphorylated substrate is required. Several papers and patent applications describe the use of fluorescent probes

for determination of the binding characteristics of PK inhibitors. [e.g., Chen et al., 268 (1993) 15812, WO2005/033330].
[0012] The importance of binding assays for PKs is quickly increasing [Uri et al., Biochimica et Biophysica Acta 1804 (2010) 541] as small-molecular fluorescent probes can be used for both biochemical assays in kinase solutions and for measurements in living cells.

Limitations of Current Binding Assays

[0013] Both binding and optical properties of luminescent probes have limited their use in binding assays of PKs. Fluorescent probes with micromolar affinity towards PKC were disclosed by Chen et al. [Chen et al., 268 (1993) 15812]. Low (micromolar) affinity and complex fluorescence spectrum make it difficult to use these fluorescent conjugates as probes for binding assays. [WO9906590] Another fluorescent probe described in the patent application WO2005/033330 has affinity in high nanomolar range ($K_d = 161$ nM) which leads to the need for high concentration of the kinase in the assay format (200 nM STK12 kinase was used in the disclosed Example). The requested kinase concentration, arising from the high K_d value of the fluorescent probe, is a hundred-fold higher than the kinase concentration usually applied for kinetic measurements (ca 1 nM concentration of the enzyme is often used in kinetic assays). This leads to substantial increase of the cost of the assay. Due to sub-micromolar dissociation constant of the probe it cannot be used for determination of binding constants of inhibitors with nanomolar affinity [Fluorescence Polarization Technical Resource Guide THIRD EDITION 2004, Invitrogen Corporation. Chapter 7]

[0014] Bisubstrate inhibitor-based fluorescent probes for PK binding assays with steady-state fluorescence polarization/anisotropy, fluorescence intensity and FRET detection have also been described previously. [WO2008019696; Vaasa et al., Anal Biochem 385 (2009) 85; Uri et al., Biochimica et Biophysica Acta 1804 (2010) 541]

[0015] The application of long lifetime luminescent labels enables the use of time-gated measurements where the signal is measured with certain delay time after the excitation pulse. This leads to the increase of signal/background ratio. Time-resolved (TR) luminometry (time resolution in micro- or millisecond range) is an excellent measuring regime for homogenous assays because it can free the measured signal from nanosecond-lifetime background fluorescence caused by organic compounds and light scattering. Several recent publications have described the application TR-FRET phenomenon for detection of binding of fluorescent probes to PKs. [Zhang et al., Analytical Biochemistry 343 (2005) 76; Lebakken et al., J Biomol Screen 14 (2009) 924; Kwan et al., Analytical Biochemistry 395 (2009) 256; WO 2010/127980] All these applications use antibodies or other recognition proteins (e.g., avidine, streptavidine) labeled with lanthanide cryptates (or chelates) in combination with fluorescently labeled small-molecule PK inhibitors to get a measurable TR-FRET signal for the complex. In addition to the complexity (both fluorescent probes and antibodies labelled with lanthanide complexes are needed, slow binding of antibodies to the antigen takes time to complete, etc.) and high price of such detection systems, problems with stability of lanthanide complexes in cellular milieu and inability of antibodies to penetrate cell plasma membranes and their instability in cellular milieu make the use of such systems non-applicable for measurements in living cells.

Room-temperature long lifetime luminescence of small organic molecules

[0016] Room-temperature phosphorescence of aromatic compounds in water solution occurs in special conditions. Cyclodextrin-enhanced room-temperature phosphorescence is known for many compounds. [Scypinski et al., Anal. Chem. 56 (1984) 331] Tryptophan phosphorescence of proteins at room temperature has been used as a tool to study protein structure and dynamics [Papp et al., Photochem. Photobiol. 49 (1989) 775] Room temperature phosphorescence of some aromatic compounds in the liquid state is also a powerful tool in analytical chemistry. [Kuijt et al., Anal. Chim. Acta 488 (2003) 135] Förster-type of non-radiative dipole-dipole energy transfer [Förster, Ann. Physik. 6 (1948) 55] takes place between two molecules in condition where their energies (emission of donor D with absorption of acceptor A) overlap and they are located at a suitable distance from each other. In addition to the more usual transfer from short lifetime singlet state, radiationless intermolecular resonance energy transfer from long lifetime triplet state of the donor to the singlet state of the acceptor is also in agreement with the Förster dipole-dipole interaction and this phenomenon has also been described. [Bennett et al., J. Chem. Phys. 41 (1964) 3040] These data point to the possibility of application of long lifetime luminescent labels as donors for FRET measurements in combination with short lifetime fluorescence acceptors. The substantial enhancement of emission induced by RET from a long-lifetime, low quantum yield donor based on metal-ligand complexes to a long-wavelength, high quantum yield acceptor, has been described and explained. [WO2002/007779 Kang et al., J. Fluoresc. 12 (2002) 97] This phenomenon was visible both inter- and intramolecularly when a ruthenium complex (metal-ligand complex, MLC) with microsecond luminescence lifetime was used as a donor in this system, and a fluorescent red dye BO-PRO-3 was used as the fluorescence acceptor excited by resonance energy transfer. [Maliwal et al., Anal. Chem. 73 (2001) 4277]

[0017] All previously described methods based on TR-FRET phenomenon use binding proteins labeled with lanthanide cryptates (or chelates) in combination with fluorescently labeled small-molecule PK inhibitors to get a TR-FRET signal

for the complex. This makes these assays expensive and binding of antibodies requires long incubation times. Problems with the stability of metal-ligand complexes in cellular milieu and the inability of antibodies to penetrate cell plasma membranes make the use of such expensive systems non-applicable for measurements in living cells.

5 Benefits of the current invention

[0018] The current invention concerns small-molecule optical probes whose specific binding to purine-binding site of the target protein leads to substantial change of parameters (e.g., intensity and lifetime) of long lifetime (1-1000 ns) luminescence.

10 **[0019]** The photoluminescent probes of the current invention are applicable in binding assays for determination of the concentration (activity) of PKs and identification and characterization of inhibitors of PKs. If compared to the probes from previous inventions, the luminescence probes of the present invention are small organic molecules that in complex with a purine-binding protein emit long wavelength light with long lifetime (in microsecond region) that enables the time-gated discrimination over fluorescence present in the sample, either autofluorescence of cells or emission from other fluorophores. A single small-molecule synthetic compound is needed to perform the analysis. The luminescence of the probes in a measurement window after longer delay time (more than 10 ns from the excitation pulse) is substantially increased upon specific binding of the luminescence donor into the purine-binding pocket of the target protein. The novel luminescent probes contain no voluminous and degradable metal-ligand complexes that have been previously used as luminescence donors for TR-FRET measurements. The endpoint assays based on the use of luminescent probes of the current invention are very quick, sensitive, and require the use of only one additional chemical (luminescent probe). This makes the assays amenable to automatization and very cost-effective.

SUMMARY OF THE INVENTION

25 **[0020]** The invention relates to small-molecule photoluminescent probes comprising a long lifetime luminescence energy donor and a fluorescence energy acceptor for use in binding assays with purine-binding proteins. The non-radiative energy transfer based emission of the acceptor is detected in a suitable time window after delay from donor excitation pulse. The specific binding of the purine-mimicking fragment of the probe to a PK leads to substantial change in the intensity and lifetime of the emitted light. The concentration of the probe bound to a PK in the sample is determined based on the change of the intensity of the emitted light. The probes possess a great gap between the excitation and measurement wavelengths.

30 **[0021]** The invention also concerns biochemical and cellular bioaffinity assays and tissue imaging with time-resolved luminescence readout based on the use of the probe.

35 BRIEF DESCRIPTION OF THE DRAWINGS

[0022]

40 Figure 1. Scheme of the kinase induced long lifetime luminescence. LP - long-pass filter, □ - luminescence lifetime.

Figure 2. Scheme of the kinase induced long lifetime luminescence in the case of intermolecular resonance energy transfer. LP - long-pass filter □ - luminescence lifetime.

45 Figure 3. General structure of a long-lifetime luminescent probe.

Figure 4. Titration of the luminescent probe ARC-1063 (5 nM) with PKs MSK1 (●), ROCKII (◆), AKT3 (▲), PKA (■), PKG (▼), or PKC (×).

50 Figure 5. Titration of the luminescent probe ARC-1139 (2 nM) with PKs MSK1 (●), ROCKII (◆), AKT3 (▲), PKA (◆), PKG (▼), or PKC (×).

55 Figure 6. Displacement of the luminescent probe ARC-1 063 [at the concentration of 54 nM (■), 18 nM (▲), 6 nM (▼), or 2 nM (◆)] from its complex with PKAc by ARC-902. In the inset the Schild plot analysis of the data is presented (■).

Figure 7. Displacement of fluorescent the luminescent probe ARC-1 063 (10 nM) from its complex with ROCK (1 nM) by Fasudil (●), Y27632 (▼), or Staurosporine (▲).

Figure 8. Correlation between the values of inhibition constants (K_i) measured in TLC kinetic inhibition assay and displacement constants (K_d) determined in binding assay using the probe ARC-1 063.

Figure 9. Titration of the labeled kinase TAMRA-PKAc with ARC-668. An example of enhancement of luminescence by intermolecular RET.

Figure 10. Increase of long lifetime luminescence intensity that results from the binding of ARC-1 063 to the kinase dissociated from complex with ARC-583(Δ), ARC-1 041 ($\blacksquare$), or Staurosporine($\bullet$). Examples of the measurement of the kinetics of dissociation of the inhibitor from its complex with the kinase.

Figure 11. Normalized emission intensity at 675 nm in living C9H6 cells in the presence ($\blacksquare$) or absence ($\square$) of ARC-1139. Treatment of the cells with Forskolin (FRSK) leads to dissociation of PKAc from the holoenzyme resulting in the interaction of PKAc with ARC-1139 that leads to increased emission at 675 nm (excitation at 337 nm). The latter effect was reversed by a cell-permeable PKA inhibitor H89.

Figure 12. Activation of PKA by adrenergic agonist isoproterenol in live HEK293 cells as measured through the change of luminescence from ARC-1139.

Figure 13. Activation of PKA by Forskolin in C9H6 cells as measured with ARC-1139 (25 nM) in the cell lysate..

Figure 14. Measurement of low concentrations of the kinase PKAc in human plasma. The luminescence signal of 2 nM ARC-1063 in human blood plasma spiked with concentration range of recombinant PKAc (0-333 pM) in the absence (filled symbols) and presence (empty symbols) of both ATP (100 μ M) and PKA selective inhibitor PKI (100 μ M). The luminescence signal is directly proportional to the amount of PKA present in the sample. Moreover, the concentration of the endogeneous PKA in blood plasma can be determined from the difference of the results obtained in the presence and absence of PKI. Extracellular PKAc in blood plasma is an acknowledged cancer biomarker. [WO/2000/079281]

DETAILED DESCRIPTION OF INVENTION

[0023] This invention relates to long-lifetime photoluminescence probes whose binding to the target protein induces substantial alteration of the signal intensity emitted by the probe (Fig. 1).

[0024] We established and improved molecular structures of luminescent probes whose specific association with the purine-binding site of the target protein leads to substantial change in long lifetime photoluminescence. Our attempt to develop small-molecule long lifetime photoluminescent probes for PKs was based on the following previous knowledge.

1. Phosphorescence of flexible aromatic molecules which are restricted in their excited state reorientation by fixation into a complex with a binding molecule (e.g., cyclodextrin) is largely increased, such binding also reduces collisional quenching of luminescence by oxygen and other impurities.
2. Heavy atoms in aromatic systems have been shown to increase phosphorescence of organic compounds.
3. The possibility of substantial enhancement of emission induced by RET from a long-lifetime, low quantum yield donor to a long-wavelength, high quantum yield acceptor has been previously been shown.

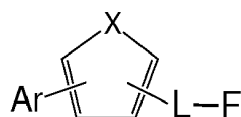
[0025] Some organic compounds have shown significant room temperature phosphorescence in micelles, cyclodextrin complexes, polymeric matrixes and crystals. Room temperature phosphorescence originating from the tryptophan residues of a protein has been used to probe structure and molecular mobility of proteins. Restriction of molecular movements is required to diminish nonradiative relaxations of triplet states in all cases of room temperature phosphorescence of pure organic luminophors to produce a detectable signal. Enhancement of luminescence through resonance energy transfer from emitters with low quantum yield to bright energy acceptors has been described. [WO2002/007779]

[0026] The current invention provides compounds of the formula P-L-F, wherein P contains a fragment incorporating a long lifetime luminescence donor that binds specifically to a purine-binding pocket of the target protein leading to substantial alteration of the lifetime and intensity of the emitted light when the donor is excited, L is a linker system, and F is a fluorescent dye with good light absorbance and high quantum yield. The most important roles that the linker L has to play in the structure of the luminescent probe P-L-F is the increase of binding affinity of the structural fragment P, tuning of the kinase selectivity of P towards specific PKs, the positioning of the fluorescent label F in a location where it causes minimal hindrance to the binding of the luminescent probe to the kinase, at the same time F should be positioned close to P to achieve maximal energy transfer from the luminescence donor P to the acceptor F. In the case of ATP-competitive inhibitors L may possess a fragment of acknowledged inhibitors whose role in the starting inhibitor is to

influence the affinity and selectivity profile of the inhibitor towards PK-s and other purine-binding proteins. P incorporates a fragment of the inhibitor whose optical properties are strongly influenced by its binding to the purine-binding site of the target protein.

[0027] The first embodiment of the invention is a luminescent probe comprising a long-lifetime luminescence donor and a fluorescence acceptor.

[0028] More particularly, the probes of the present invention have the structure



Ar = aromatic system

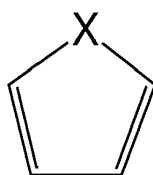
X = S, Se, Te

L = linking structure

F = fluorescent dye

where the aromatic system Ar contains differently substituted (hetero)aromatic rings (FIG.3).

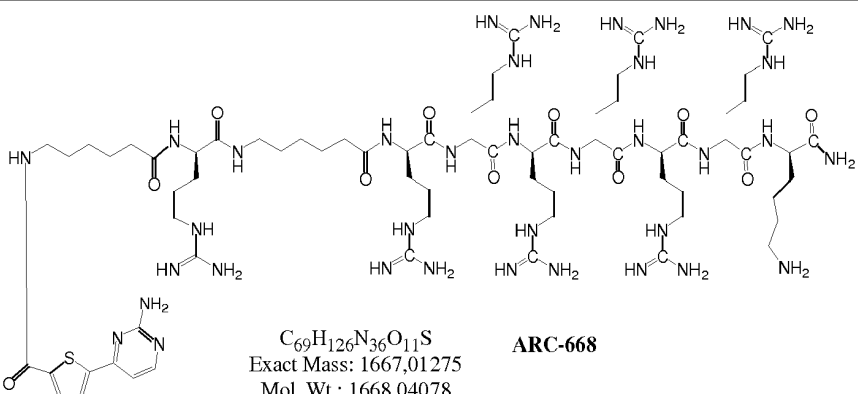
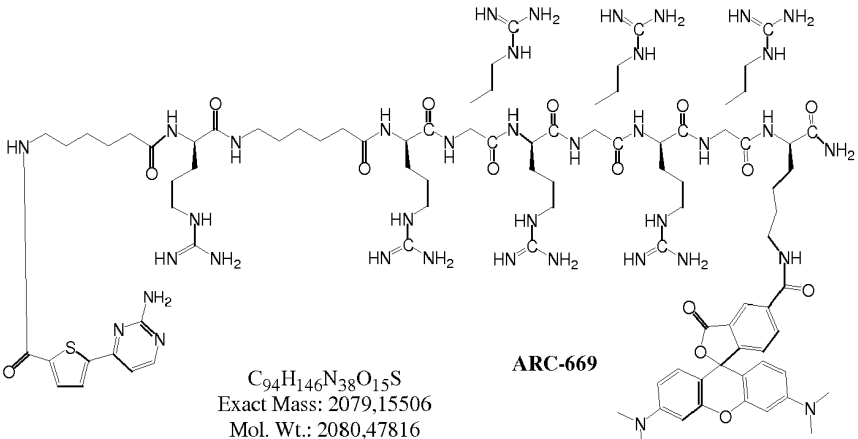
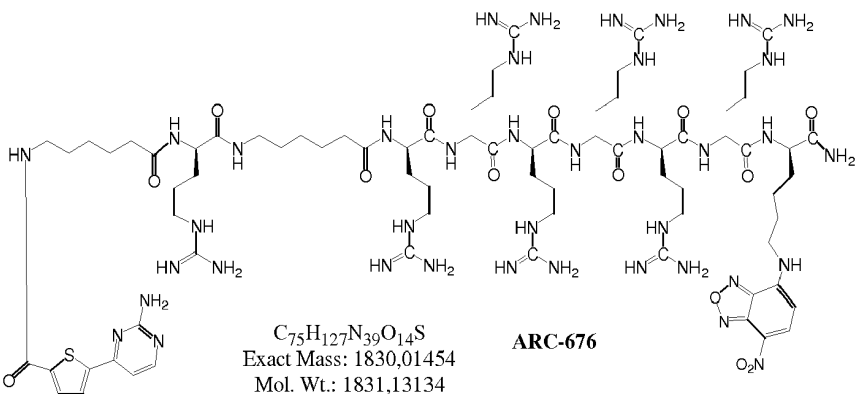
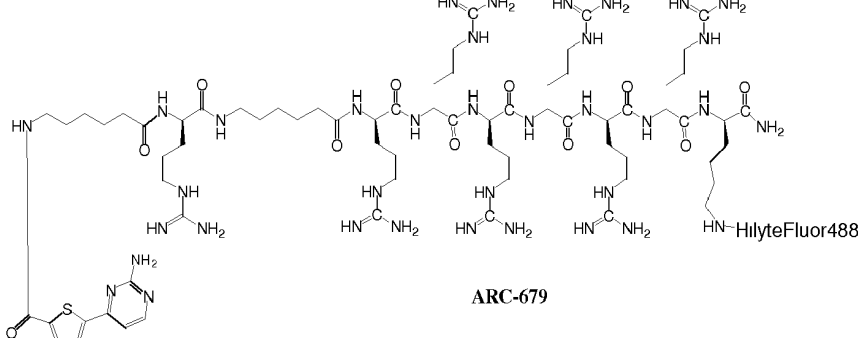
[0029] An important unit of the luminescent probes is the heteroaromatic moiety



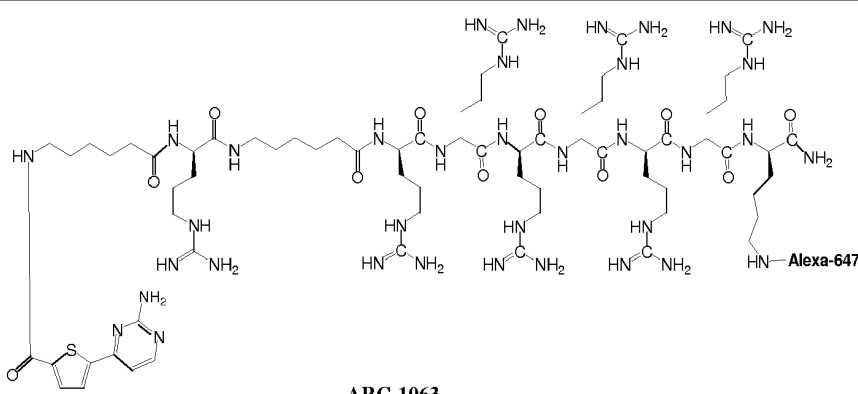
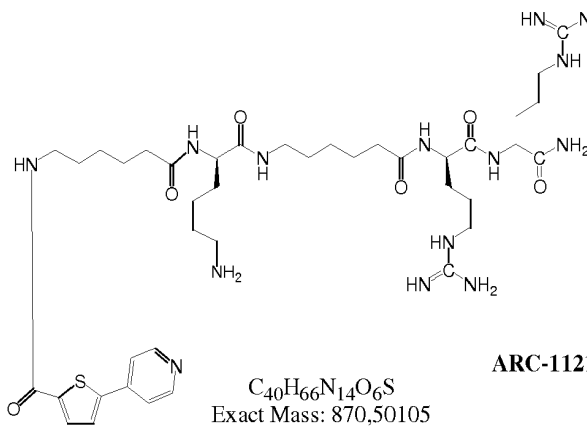
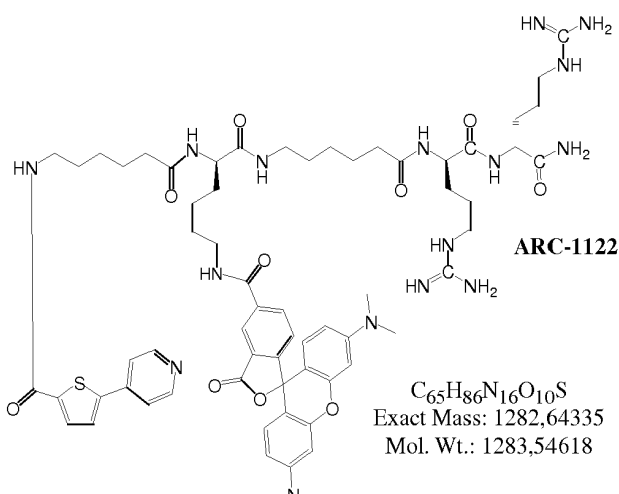
containing as the atom X a sulphur (S, in the case of thiophene), selenium (Se, in the case of selenophene) or tellurium (Te, in the case of tellurophene) atom. Other 5-membered S-, Se- or Te-containing heterocycles, e.g., these that are additionally incorporating 1-4 nitrogen atoms, also possess optical characteristics supporting their use as optical donor moieties of luminescent probes of the present invention. These 5-membered S-, Se- or Te-containing heterocycles may be also substituted with halogeno groups and other functional groups.

[0030] The aromatic system Ar incorporates structural units whose role is the probe is the enhancement of binding affinity of the probe to the target protein and the tuning of the binding selectivity towards purine-binding proteins. Ar also tunes the optical properties of the probe making P a long life-time luminescence donor of the probe and rendering the luminescence of the probe sensitive to its binding to the protein. The Ar moiety may be connected to a single atom of the the thiophene (selenophene, tellurophene) ring or simultaneously fused to two atoms of the ring while retaining the conjugation between Ar moiety and the thiophene (selenophene, tellurophene) ring. Aromatic systems tested include six-membered heteroaromatic rings and purine-type fused heteroaromatic systems (Table 1) but are not limited to these disclosed in the Table 1 as inhibitory potency of many other thiophene-containing aromatic systems towards different PKs has been previously reported.

Table 1. ARC-codes, structures and HRMS data of the compounds (deconvoluted monoisotopic mass is presented)

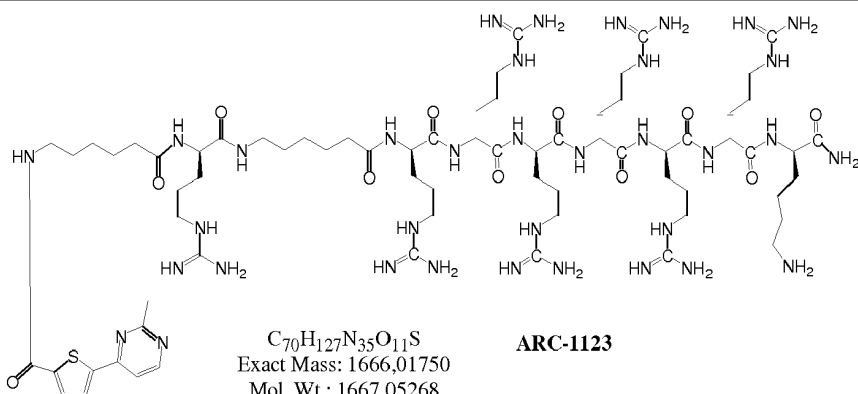
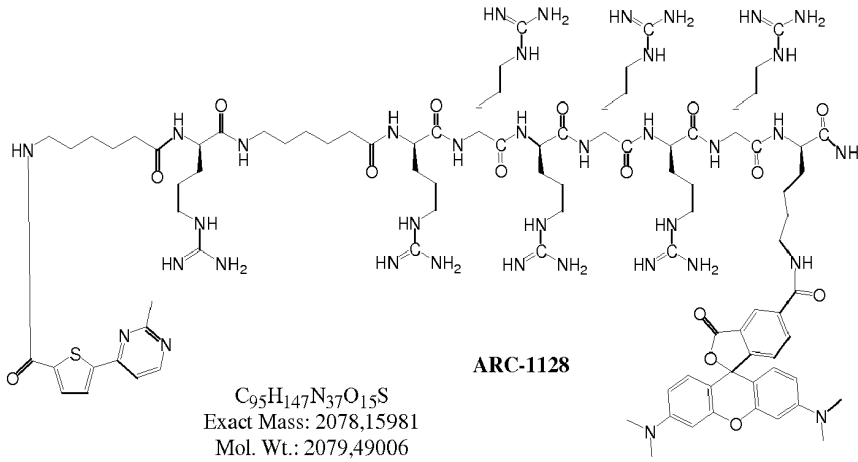
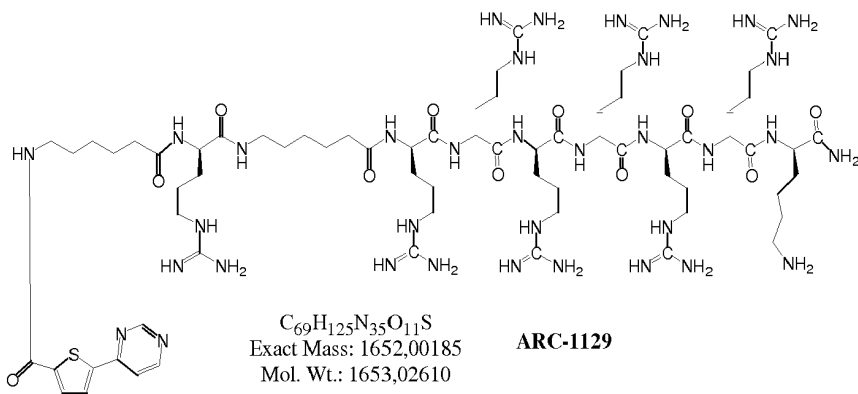
ARC-code	Structure	HRMS Found
ARC-668	 $C_{69}H_{126}N_{36}O_{11}S$ Exact Mass: 1667,01275 Mol. Wt.: 1668,04078 ARC-668	1667.01219
ARC-669	 $C_{94}H_{146}N_{38}O_{15}S$ Exact Mass: 2079,15506 Mol. Wt.: 2080,47816 ARC-669	2079.15284
ARC-676	 $C_{75}H_{127}N_{39}O_{14}S$ Exact Mass: 1830,01454 Mol. Wt.: 1831,13134 ARC-676	1830.01281
ARC-679	 $C_{75}H_{127}N_{39}O_{14}S$ Exact Mass: 1830,01454 Mol. Wt.: 1831,13134 ARC-679	2136.1739*

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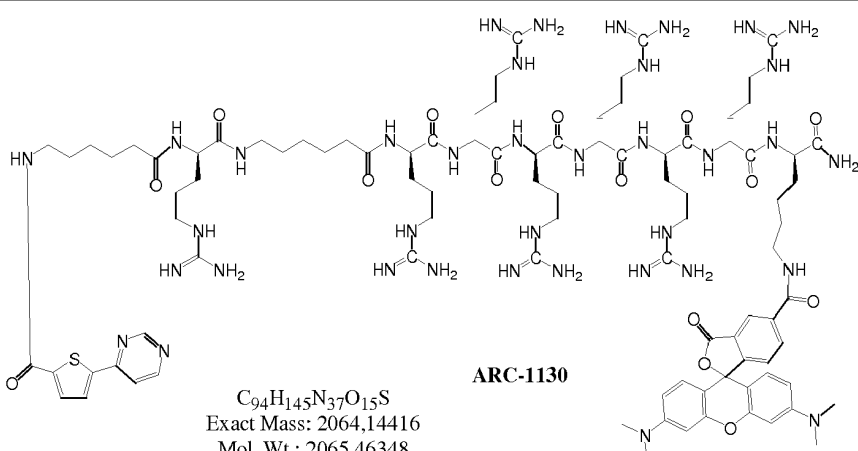
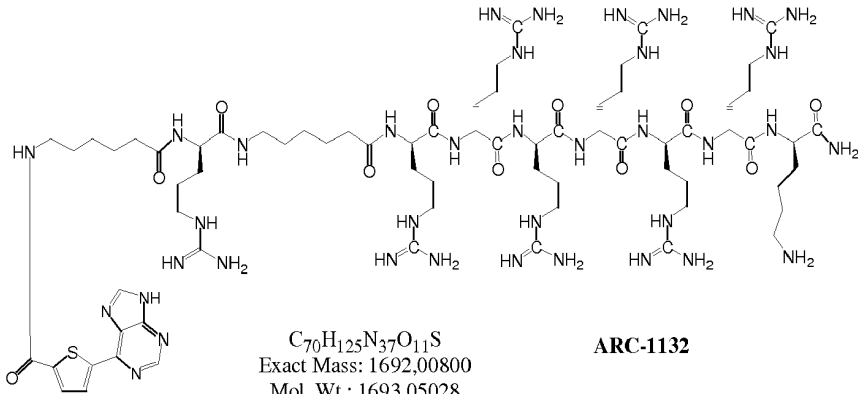
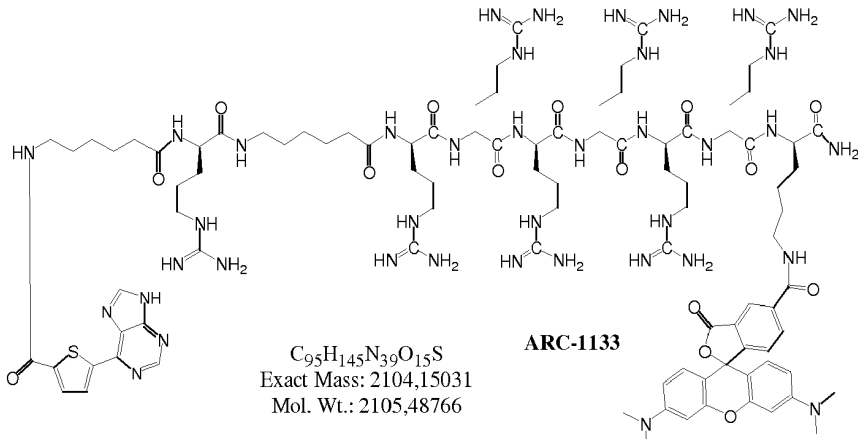
ARC-code	Structure	HRMS Found
ARC-1063	 ARC-1063	2507.1846*
ARC-1121	 ARC-1121 $C_{40}H_{66}N_{14}O_6S$ Exact Mass: 870,50105 Mol. Wt.: 871,10880	870.49849
ARC-1122	 ARC-1122 $C_{65}H_{86}N_{16}O_{10}S$ Exact Mass: 1282,64335 Mol. Wt.: 1283,54618	1282.64083

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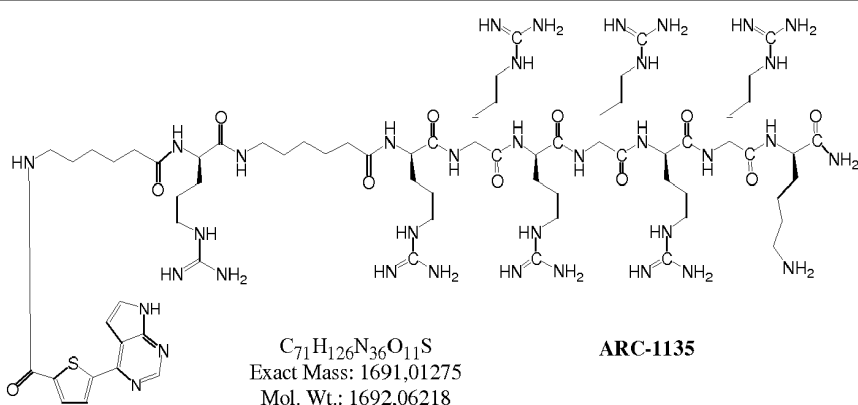
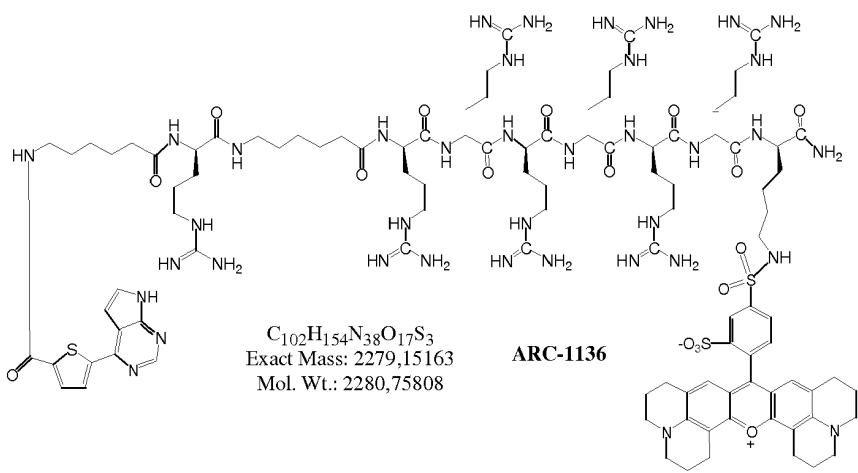
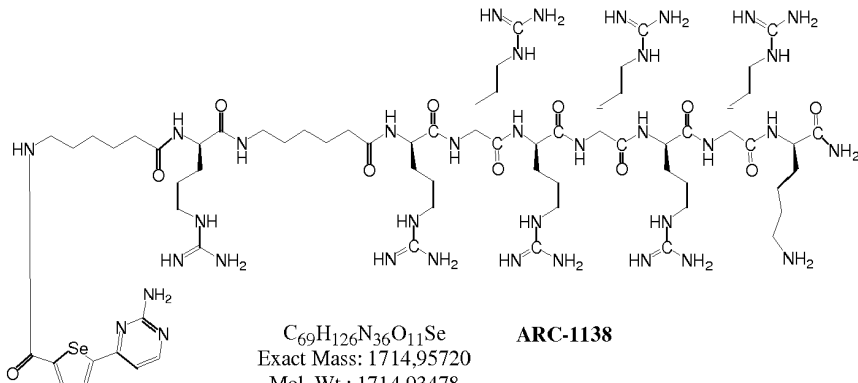
(continued)

ARC-code	Structure	HRMS Found
ARC-1123	 $C_{70}H_{127}N_{35}O_{11}S$ Exact Mass: 1666,01750 Mol. Wt.: 1667,05268 ARC-1123	1666.01168
ARC-1128	 $C_{95}H_{147}N_{37}O_{15}S$ Exact Mass: 2078,15981 Mol. Wt.: 2079,49006 ARC-1128	2078.16026
ARC-1129	 $C_{69}H_{125}N_{35}O_{11}S$ Exact Mass: 1652,00185 Mol. Wt.: 1653,02610 ARC-1129	1651.99576

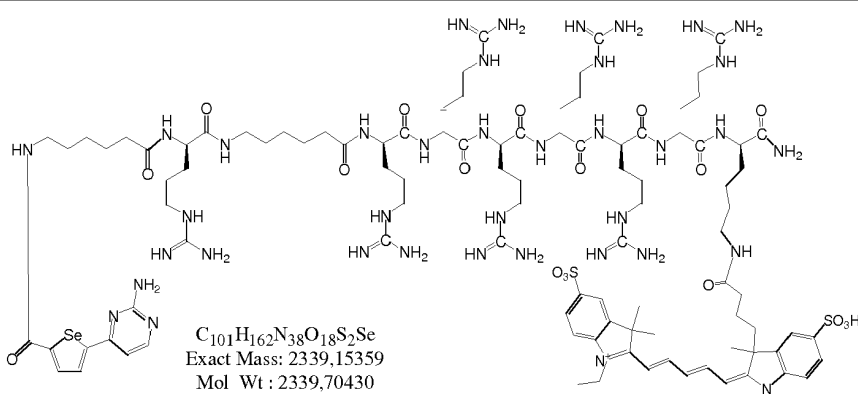
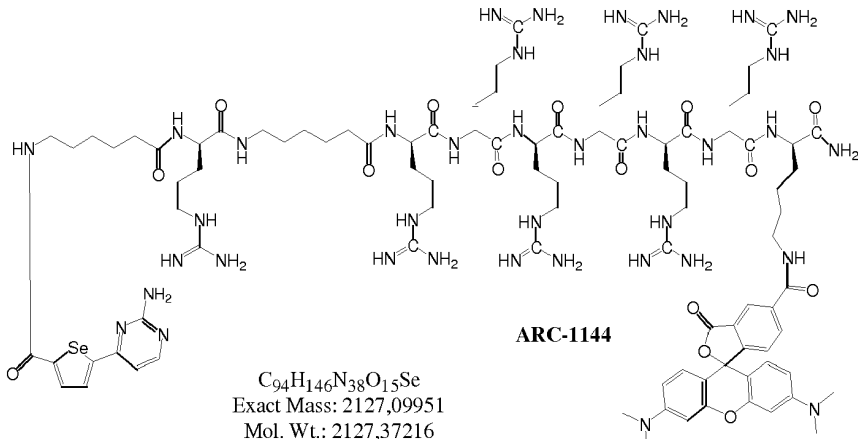
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ARC-code	Structure	HRMS Found
ARC-1130	 $C_{94}H_{145}N_{37}O_{15}S$ Exact Mass: 2064,14416 Mol. Wt.: 2065,46348 ARC-1130	2064.14487
ARC-1132	 $C_{70}H_{125}N_{37}O_{11}S$ Exact Mass: 1692,00800 Mol. Wt.: 1693,05028 ARC-1132	1692.01165
ARC-1133	 $C_{95}H_{145}N_{39}O_{15}S$ Exact Mass: 2104,15031 Mol. Wt.: 2105,48766 ARC-1133	2104.15625

(continued)

ARC-code	Structure	HRMS Found
ARC-1135	 $C_{71}H_{126}N_{36}O_{11}S$ Exact Mass: 1691,01275 Mol. Wt.: 1692,06218 ARC-1135	1691
ARC-1136	 $C_{102}H_{154}N_{38}O_{17}S_3$ Exact Mass: 2279,15163 Mol. Wt.: 2280,75808 ARC-1136	2279
ARC-1138	 $C_{69}H_{126}N_{36}O_{11}Se$ Exact Mass: 1714,95720 Mol. Wt.: 1714,93478 ARC-1138	1714.95391

(continued)

ARC-code	Structure	HRMS Found
ARC-1139	 $C_{101}H_{162}N_{38}O_{18}S_2Se$ Exact Mass: 2339,15359 Mol. Wt.: 2339,70430 ARC-1139	2339.14580
ARC-1144	 $C_{94}H_{146}N_{38}O_{15}Se$ Exact Mass: 2127,09951 Mol. Wt.: 2127,37216 ARC-1144	2127.09949
*Structures of HilyteFluor448 and Alexa-647 have not been disclosed. Monoisotopic masses for selenium containing compounds are calculated for most abundant isotope ^{80}Se .		

[0031] Structures of several compounds incorporating P and L moieties but lacking the fluorescent label F are also presented in Table 1 (compounds ARC-668, ARC-676, ARC-1121, ARC-1123, ARC-1129, ARC-1132, ARC-1135, ARC-1138). These compounds with high affinity (subnanomolar or low nanomolar) bind to the catalytic subunit of protein kinase A (PKAc) and some other PKs of the AGC group. They strongly absorb light between 280 - 360 nm (molar absorption coefficients 10000 - 25000 $M^{-1}cm^{-1}$) and when excited at 337(50) nm show week long lifetime luminescence (phosphorescence) in the 450 - 600 nm region in bound to the kinases form (fluorescence spectrometer LS 55 from Perkin Elmer and luminescence platereader PHERAStar fom BMG were used were measurements throughout this work).

[0032] The fluorescent acceptor F of the luminescent probe of the current invention should exhibit a large extinction coefficient and a good quantum yield, and its excitation spectrum should overlap with the emission spectrum of the long lifetime luminescence donor P. Fluorescence labels whose excitation spectrum better overlaps with the emission spectrum of the luminescence donor, who have longer emission wavelengths, and greater photostability are preferred, but the choice of the label also depends on the origin of the analytical instrument used for the assay and special analytical situation. Fluorescent dyes that may be useful in the present invention include inter alia fluorescein and fluorescein derivatives, rhodamine and rhodamine derivatives, cyanine dyes and many other fluorescent labels that are produced and sold by different companies (e.g., Invitrogen, PromoCell, ATTO-TEC GmbH, GE Healthcare, Sigma-Aldrich). Other fluorescent labels that absorb light in the 400-900 nm range and emit light in the 450-1000 nm range are suitable for the application as the fluorescent acceptor moiety F of the luminescence probes of the present invention. Well-known fluorescence dyes FITC, TAMRA Cy3, Cy3B, Cy5, Bodipy 564/570, Texas Red, Alexa Fluor 647, PromoFluor 647 and others have shown suitable optical characteristics when used in combination with thiophene and selenophene-based fragments P. Fluorescent probes incorporating mineral fluorescent labels, e.g., quantum dots, may be useful for special applications. Luminescent probes P-L-F (e.g., compounds ARC-669, ARC-676, ARC-679, ARC-1063, ARC-1122, ARC-

1128, ARC-1130, ARC-1133, ARC-1136, ARC-1139, and ARC-1144 in Table 1) are excited at wavelengths below 400 nm with single-photon excitation and at wavelengths over 600 nm when multiphoton excitation is used. The emitted radiation is measured at wavelengths 450 - 1000 nm

[0033] 5-carboxytetramethylrhodamine (5-TAMRA) is a good fluorescent label to be used as a fluorescence acceptor of a luminescence probe of the current invention, as the dye is a single-position isomer, the dye is excited at a suitable wavelength, it has high brightness and great photostability.

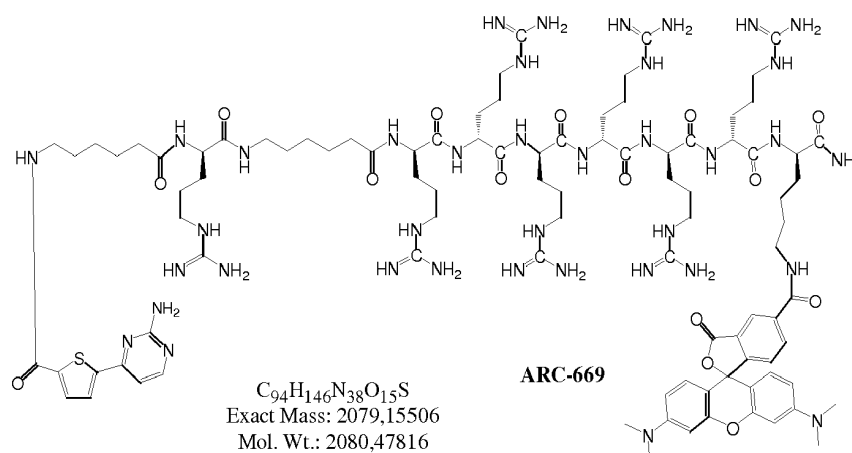
[0034] Many other fluorescent labels are suitable for the application as the acceptor moiety F of the luminescence probes. These labels are described in the Handbook: [The Handbook - A Guide to Fluorescent Probes and Labeling Technologies Web Edition of The Handbook, Tenth Edition 2006 Invitrogen Corporation]

[0035] In the case of other luminescent probes the linker unit L may be a C₁-C₂₀ alkylene group, a C₁-C₂₀ alkenylene group, a C₁-C₂₀ alkynylene group, wherein one or more of the CH₂ groups present in the C₁-C₂₀ alkylene group, C₁-C₂₀ alkenylene group, C₁-C₂₀ alkynylene group is optionally replaced with -O-, -C(O)-, -C(O)N-, -S-, -S(O)-, -SO₂-, -N(R)-; R is H or C₁₋₆ alkyl. In the case of some probes L may be omitted.

[0036] The novel fluorescently labelled thiophene- or selenophene-based luminescent probes possess 15 - 500 ns lifetimes when these probes are bound to the purine-binding site of a PK (FIG. 1). Binding of the probes to different kinases leads to a luminescence signal whose magnitude is dependent on the origin of the probe and that of the kinase (FIG. 5). When longer delay times (e.g., > 50 ns) after the excitation pulse are used for the measurement of the emission signal, the binding of the probes to the target PKs leads to 50 - 2500-fold increase of the luminescence signal. Microsecond lifetimes of the probes enable the performance of time gated measurements with the application of commercial plate-readers and microscopes.

[0037] Another embodiment of the present invention is the use a PK inhibitor possessing a long lifetime luminescence donor properties (P-L) but lacking a fluorescent dye, e.g., ARC-668 (Table 1), in combination with a PK that is labeled with an organic dye, fused with a fluorescence protein or bound to specific fluorescently labelled antibody. Other tagging methods (e.g., FIAsH, HaloTag, etc.) can be used for labelling of the target proteins. The binding of the probe P-L to the labelled kinase that leads to intermolecular resonance energy transfer from the bound luminescence donor to the labelled protein (FIG. 2) that can be detected measuring the signal emitted by the fluorescence acceptor.

[0038] A special embodiment of the invention is the luminescent probe ARC-669 with the structure:



[0039] The probe, ARC-669, has high affinity ($K_d = 0.1$ nM) towards the catalytic subunit of cAMP-dependent protein kinase (PKAc). It was previously described as a fluorescent probe for fluorescence polarization/anisotropy and fluorescence intensity measurements in steady state conditions [Lavogina et al., Biochim Biophys Acta. 1804 (2010) 1857]. It is displaced from its complex with the kinase by ATP-competitive inhibitor H89, protein substrate competitive inhibitor protein PKI, and bisubstrate inhibitor AdcAhx(D-Arg)₆-NH₂ (ARC-902). Now the compound ARC-669 was developed into a long life-time luminescence probe that binds to several PKs with high affinity (Table 2). The probe can be used as a high-affinity luminescent probe for time-resolved luminescence assays.

[0040] When the solution of ARC-669 was illuminated at 337 (50) nm its binding to a PK induced 50 - 2500-fold increase of the luminescence signal when 50 ns delay after the excitation pulse and 100 ns measuring window was used for the detection by the intensity of the signal at 590 (40) nm. The luminescence lifetime of the signal was dependent on the origin of the binding PK and it was in the range of 50-300 ns.

[0041] According to the general structure P-L-F, in the case of the probe ARC-669 P is represented by a aminopyri-

midine moiety and a thiophene residue, linker L incorporates a carboxylate group, a 6-aminohexanoic acid residue, a D-arginine residue, a second 6-aminohexanoic acid residue, six D-arginine residues and a D-lysine residue with amidated carboxylate group, F is a 5-carboxytetramethylrhodamine dye connected to the side chain of the lysine residue.

[0042] The potential of ARC-669 to inhibit different PKs was tested in a panel of 115 kinases. It was established that ARC-669 binds with high affinity to many PKs (Table 2) and thus it can be used as a luminescent probe with several PKs.

[0043] Authors of the present invention have recently shown that highly potent ATP-competitive conjugates of adenosine analogs and arginine-rich peptides are cell membrane permeable [Uri et al., *Bioorg. Med. Chem. Lett.* 12 (2002) 2117; Enkvist et al., *Bioorg. Med. Chem. Lett.* 19 (2009) 6098; Vaasa et al., *Biochem Biophys Res Commun.* 397 (2010) 750], therefore some of the fluorescent probes of the present invention are applicable in experiments with live cells, tissues and organisms.

[0044] TR-FRET assays are homogeneous and as such they do not require separation steps like chromatography, filtration, centrifugation, precipitation or electrophoresis. Due to the high sensitivity of TR luminescence assay it is well suited for the assay miniaturization, and the assays can be performed with equal success in a cuvette of a fluorescence spectrometer and in low $\square$ volumes in wells of 384-well and 1536-well microtiter plates with a fluorescence plate reader with delayed fluorescence measurement possibilities.

[0045] The present invention embodies an assay in which the binding characteristics of the test compounds are determined on the basis of their ability to displace the fluorescent probe from its complex with the kinase. This is described in Example 3 and shown on FIG. 6-8.

[0046] The high affinity of the fluorescent probes of the present invention ($K_d < 1.0$ nM) makes it possible to characterize the high-affinity inhibitors with sub-nanomolar and nanomolar affinity. K_d -values of the inhibitors could be calculated according to the Cheng-Prusoff equation.

[0047] The application of the luminescent probes for the determination of the concentration of the active form of the kinase is another embodiment of the current invention. Determination of the concentration of the special form of a kinase is of paramount importance for many enzyme applications. In kinetic assays the kinase amount in the assay volume is characterized on the activity basis and the molar concentration of the kinase is often not known. Majority of the methods in use for determination of enzyme concentration (Bradford, Lowry, SDS electrophoresis) give the total concentration of the protein (not the concentration of its active form), are inaccurate and need unacceptably large amounts of the protein for analysis.

[0048] The application of the photo-luminescent probe of the current invention enables a simple procedure for the determination of the concentration of the active (binding to the fluorescent probe) form of the kinase in the sample (Example 2).

[0049] It was established that there was a good proportionality between the concentration of the binding protein (as established with the photo-luminescent probe) and the phosphorylation activity of the solution of the PK.

[0050] The luminescent probes of the current invention can be used for measurements with several PKs. Titration of probes ARC-1063 and ARC-1139 with many kinases (PKA, MSK1, ROCKII, AKT3, PKG, PKC) resulted in substantial increase of luminescence emission (FIG. 4 and 5).

[0051] A selectivity testing performed (Example 7) for the probe ARC-669 established the inhibitory potency of the compound towards PKs of different groups. Profiling with the kinase panel (Example 7) reveals strong tendency of the compound to inhibit basophilic PKs that points to the active participation of both functionary moieties in the formation of the binary complex with the kinase. In addition to the majority of AGC kinases, many kinases of CAMK and STE groups and some kinases not falling into major groups (classification by [Manning et al., *Science* 298 (2002) 1912]) were strongly inhibited by the probe. All the inhibited kinases are targets of fluorescent probes of the present invention and the probes can be used in analytical methods concerning these kinases. Probes targeted to PKs of other groups can be developed using luminescence donors and acceptors described in the present invention by adding structural elements that support binding of the probes to these kinases. Additionally, the potential targets of the fluorescent probes of the present invention are different mutated forms of PKs, truncated forms of kinases inactive states of PKs, and pseudokinases.

[0052] In addition to their use in biochemical assays with purified PKs and PKs in complicated biological solutions (cell lysates, tissue extracts, body fluids) the probes of the present invention can be used for monitoring the activity of PKs in living cells (FIG. 11 - 13). This enables the application of the probes for monitoring the physiological response of the cells to activation of membrane receptors. Both agonists and antagonists of receptors can be screened and characterized with the application of the probes as far as these receptors activate intracellular signalling cascades involving PKs. Regulation of PKA, e.g., mediated via G_s and G_i proteins (FIG. 11 - 13), but also isoforms of PKC and Rho-kinase can be monitored with luminescent probes of the present invention in living cells and cell lysates. Thus these probes can be used for screening and characterization of ligands leads to intracellular activation PKs.

[0053] PKs are acknowledged disease biomarkers and changes in the level of their activity in body fluids and tissues signal about diseases like cancer, diabetes, atherosclerosis, etc. The luminescent probes of the current invention can be used for diagnosis of these diseases, for monitoring of the efficiency of the cure of patients, and for supporting drug discovery and development in pharmaceutical industry. Cancer cells leak out PKAc and the higher level of its activity in

blood plasma and serum of cancer patients has been reported. [WO/2000/079281] The luminescent probes of the present invention can be used for high throughput analysis of plasma samples for establishment of the level of PKAc activity (FIG. 14 and Example 9). The assay based on the use of luminescent probes of the current invention has very high sensitivity and affords the determination of the concentration of PKA with very low limit of quantification (LoQ = 15 pM, 20 μ l volume in a well of 384-well microtiter plate). The use of plasma samples makes the diagnosis of cancer minimally invasive for patients and low price and high throughput format of analysis of the samples support the development of analysis methods based on luminescent probes of the present invention for massive analysis of blood samples at hospital laboratories and blood banks for early diagnosis of cancer.

EXPERIMENTAL PART

Abbreviations and definitions

[0054]

Adc - 5'-adenosine carboxylic acid or 1-(6-amino-9H-purin-9-yl)-1-deoxy- β -D-ribofuranuronic acid (CAS 3415-09-6);
 Ahx - 6-aminohexanoic acid;
 Arg - arginine;
 ARC - adenosine-arginine conjugate;
 ATP - adenosine-5'-triphosphate;
 Boc - tert-butoxycarbonyl;
 cAMP - cyclic adenosine 3',5'-monophosphate;
 DIPEA - N,N-diisopropylethylamine;
 DMF - dimethylformamide;
 DMSO - dimethyl sulfoxide;
 DTT - dithiothreitol
 Fmoc - 9-fluorenylmethoxycarbonyl;
 HOBt - 1-hydroxybenzotriazole;
 HPLC - high performance liquid chromatography;
 Ip: 2',3'-O-isopropylidene;
 MALDI TOF MS, matrix assisted laser desorption ionisation time-of-flight mass spectrometry;
 NMR - nuclear magnetic resonance;
 Pbf - 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl;
 PK - protein kinase;
 PKA - cAMP-dependent protein kinase;
 PKAc - catalytic subunit of PKA
 RP - reverse phase;
 TFA - trifluoroacetic acid;
 TLC - thin layer chromatography.

ARCs - conjugates of adenosine analogues and arginine-rich peptides are bisubstrate inhibitors of basophilic protein kinases

Bisubstrate inhibitors simultaneously associate with two substrate-binding sites of PK and thus combine two inhibitory domains. [Lavogina et al., ChemMedChem 5 (2010) 23] The principle advantage of bisubstrate inhibitors is their ability to generate more interactions with the target enzyme that could result in improved affinity and selectivity of the conjugates, when compared with single-site inhibitors.

Oligopeptide refers a short polymer of amino acids (2-20 amino acids) which are joined together by peptide bonds. *The following terms are defined according to the book Lakowicz Principles of fluorescence spectroscopy, 3rd edition, 2006.*

FRET - Förster-type non-radiative dipole-dipole energy transfer [Förster, Ann. Physik. 6 (1948) 55] takes place between two molecules in condition where their energies (emission of donor D with absorption of acceptor A) overlap and they are located at a suitable distance from each other. Typically, a FRET occurs at distance less than 100 Å between the donor and acceptor.

TRF - time-resolved fluorescence. Fluorescence measurements can be broadly classified into two types of measurements: steady-state and time-resolved (TR). Steady-state measurements, the most common type, are those performed with constant illumination and observation. TR measurements are performed after short-pulse excitation of the fluorophore and the emission signal is detected after delay for a certain time period (measurement window). Although measurement of the time-resolved emission requires special instruments, such technology is widely used in biological research because of the increased information available from the data as compared with stationary or

steady-state measurements. **Photoluminescence** is the emission of light from a substance as a result of absorption of electromagnetic radiation, the emitted light usually has a longer wavelength than the incident radiation. Photoluminescence is formally divided into two categories-fluorescence and phosphorescence-depending on the nature of the excited state. In excited singlet states, the electron in the excited orbital is paired (by opposite spin) to the second electron in the ground-state orbital. Consequently, return to the ground state is spin allowed and occurs rapidly by emission of a photon. The emission rates of fluorescence are typically 10^8 s^{-1} , so that a typical fluorescence lifetime is in the range of 1-10 ns.

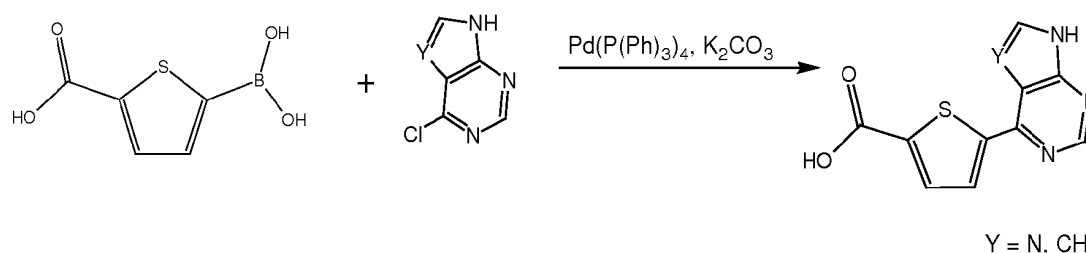
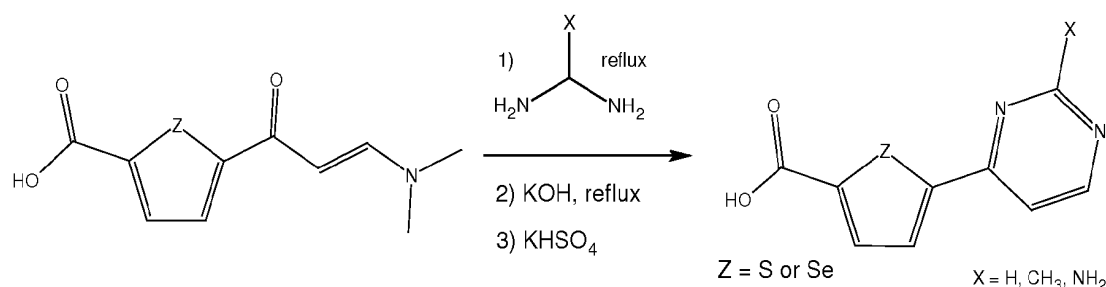
Phosphorescence is emission of light from a triplet excited states, in which the electron in the excited orbital has the same spin orientation as the ground-state electron. Transitions to the ground state are forbidden and the emission rates are slow (10^{-3} to 100 s^{-1}), so that phosphorescence lifetimes are typically milliseconds to seconds.

The luminescence **lifetime** refers the average amount of time a fluorophore remains in the excited state (following excitation) prior to return to the ground state. Typically, fluorescence lifetimes are near 1-10 ns and a phosphorescence lifetimes are in the range of microseconds to seconds.

EXAMPLES

EXAMPLE 1. SYNTHETIC PROCEDURES

[0055]



Synthesis of adenosine mimetics

5-(3-Dimethylaminoacryloyl)thiophene-2-carboxylic acid methyl ester

[0056] 2 ml (15 mmol) of N,N-dimethylformamide dimethyl acetal was added to the suspension of 5-acetyl-2-thiophenecarboxylic acid (1013 mg, 5.95 mmol) in DMF (5 ml) and the reaction mixture was refluxed overnight. 100 ml of hexane/tBuOMe (1:1) was added to the cooled reaction mixture and the precipitate was separated by filtration to yield 1140 mg (80%) of the product.

5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid

[0057] 1 (466 mg, 1.95 mmol), guanidine hydrochloride (468 mg, 4.9 mmol) and K₂CO₃ (888 mg, 6.4 mmol) were refluxed in DMF (15 ml) for 3 days. The solvents were removed and the residue was suspended in solution of KOH (0.5

M, 20 ml) and refluxed overnight until clear solution was formed. The product was precipitated with 20% solution of KHSO_4 , filtrated, washed and tried to yield the product (360 mg, 83.5 %). TLC (DCM/MeOH/AcOH, 9:1:1) $R_f = 0.47$.

[0058] ^1H NMR (CD_3SOCD_3) δ 6.85 (2H, br, NH_2), 7.17 (1H, d, $J = 5.2$ Hz), 7.76 (1H, d, $J = 4$ Hz), 7.92 (1H, d, $J = 4$ Hz), 8.34 (1H, d, $J = 5.2$ Hz), 13.33 (1H, br, COOH). ^{13}C NMR (CD_3SOCD_3) δ 104.6, 127.7, 133.7, 136.6, 148.6, 158.0, 159.3, 162.7, 163.5.

5-(2-methylpyrimidin-4-yl)thiophene-2-carboxylic acid

[0059] The compound was synthesized as 5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid except acetamidine was used instead of guanidine. Yield 85 %.

[0060] ^1H NMR (CD_3SOCD_3) δ 2.63 (3H, s, CH_3), 7.78 (1H, d, $J = 4.0$ Hz), 7.89 (1H, d, $J = 5.4$ Hz), 8.07 (1H, d, $J = 4.0$ Hz), 8.76 (1H, d, $J = 5.4$ Hz), 14.40 (1H, br, COOH). ^{13}C NMR (CD_3SOCD_3) δ 25.3, 112.7, 128.2, 133.5, 137.6, 147.2, 157.2, 158.0, 162.2, 167.5.

5-(pyrimidin-4-yl)thiophene-2-carboxylic acid

[0061] The compound was synthesized as 5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid except formamidine was used instead of guanidine. Yield 74 %

[0062] ^1H NMR (CD_3SOCD_3) δ 7.79 (1H, d, $J = 4.0$ Hz), 8.05 - 8.15 (2H, m), 8.88 (1H, d, $J = 5.4$ Hz), 9.17 (1H, d, $J = 1.2$ Hz).

[0063] ^{13}C NMR (CD_3SOCD_3) δ 115.7, 128.5, 133.5, 138.1, 146.5, 157.1, 157.8, 158.5, 162.2.

5-(2-aminopyrimidin-4-yl)selenophene-2-carboxylic acid

[0064] Selenophene-2-carboxylic acid was refluxed overnight in acetic anhydride in the following synthesis was carried out as described for 5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid (except 5-(3-dimethylaminoacryloyl)selenophene-2-carboxylic acid methyl ester did not precipitate from ether/alkane mixture and the solvents were removed before next step). The yield of 5-(2-aminopyrimidin-4-yl)selenophene-2-carboxylic acid from selenophene-2-carboxylic acid was 17%. ^1H NMR (CD_3SOCD_3) δ 6.80 (2H, br, NH_2), 7.16 (1H, d, $J = 5.1$ Hz), 7.97 (1H, d, $J = 4.2$ Hz), 8.12 (1H, d, $J = 4.2$ Hz), 8.32 (1H, d, $J = 5.1$ Hz).

[0065] ^{13}C NMR (CD_3SOCD_3) δ 103.7, 129.6, 136.3, 142.2, 156.0, 159.2, 159.4, 163.6, 164.0.

5-(9H-purin-6-yl)thiophene-2-carboxylic acid and 5-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)thiophene-2-carboxylic acid

[0066] 6-Chloropurine (108 mg, 0.70 mmol) or 6-chloro-7-deazapurine (105 mg, 0.68 mmol), 5-carboxythiophene-2-boronic acid (145 mg, 0.84 mmol), K_2CO_3 (315 mg, 2.3 mmol) and tetrakis(triphenylphosphine)-palladium (120 mg, 0.1 mmol) were stirred overnight at 100 °C under argon. The solvents were removed under vacuum. The residues were dispersed in water and filtrated. The filtrates were acidified with KHSO_4 and the precipitates were collected. The products were used in the solid phase synthesis without further purification.

Solid-phase synthesis of peptide conjugates

[0067] Peptide fragments were prepared by using traditional Fmoc solid-phase peptide synthesis methods on Rink amide MBHA resin. Protected amino acids (3 equivalents) were dissolved in DMF and activated with HBTU/HOBt (2.94 eq each) in DMF/N-methylmorpholine. Coupling solutions were added to the resin and shaken 40-60 min. The completeness of each step was monitored with Kaiser-test, which was followed by deprotection of Fmoc-group by 20% piperidine solution in DMF (20 min). Fmoc-protected linkers were attached to the peptide part following the same protocol.

[0068] 1.5 eq of a thiophene or selenophene containing precursor was activated with HOBt/HBTU (1.47 eq each) in DMF/N-methylmorpholine and added to the resin and shaken at least 3h. Finally the resins were washed 5 times with each solvent (DMF, isopropanol, DCE) and dried. Treatment with TFA/ H_2O /triisopropylsilane (90/5/5 by volume) for 2-3 h was used as standard cleavage procedure. The products were purified by reversed-phase HPLC and lyophilized.

Labelling of the ARC-s with fluorescent dyes (a typical example of the procedure)

[0069] 1 μmol of a lysine-containing ARC-type inhibitor and 1.3 μmol *N*-hydroxysuccinimide esters of the fluorescent dye were dissolved in 50 μl of DMSO and 10 μl triethylamine. The solution was kept 3 h at room temperature and then the solvent were removed in high vacuum. The residue was purified by reversed-phase HPLC and lyophilized to give

the desired products in 40 - 70% of yields.

EXAMPLE 2. TITRATION OF LUMINESCENT PROBES WITH PROTEIN KINASES

[0070] Biochemical binding experiments were performed in black, low-volume, 384-well nonbonding surface NBS microplates (Corning) on a PHERAstar plate reader (BMG Labtech) with TRF optic modules [ex. 337(50) nm, em. 675(50) and 620(20) nm] or [ex. 337(50) nm, em. 590(50) and 545(10) nm] using the time-resolved fluorescence mode. The microplates were incubated for 15 min at 30°C before each measurement. The luminescent probe was excited with a short pulse of flashes (200 flashes per datapoint) at 337 nm, followed by 50 μ s delay time and subsequent measurement of the luminescence using a constant acquisition window (150 μ s) at the wavelength corresponding to the emission maximum of the conjugated fluorescent dye [590(50)nm or 675(50) nm].

[0071] The concentration series of kinases MSK1, ROCKII, AKT3, PKAc, PKG I α , or PKC δ (3-fold dilutions) was made in the assay buffer (150 mM NaCl, 50 mM Hepes pH=7.5, 5 mM DTT, 7.5 μ M BSA) and the fixed concentration of the luminescent probe ARC-1063 or ARC-1139 (5 nM or 2nM, respectively) was added to each well, incubated and measured as described above. Structures of luminescent probes ARC-1 063 and ARC-1139 are listed in Table 1.

[0072] Selenophene-based probes (e.g., ARC-1139) gave generally several-fold higher intensity of the emitted signal than the thiophene-based counterparts (e.g., ARC-1063). Minding to the kinase MSK1 led to the strongest signal with both of the luminescent probes, ARC-1 063 and ARC-1139. All the tested PKs of the AGC group produced strong TR luminescence signal when bound to the luminescent probes of the current invention (FIG. 4 and 5).

EXAMPLE 3. COMPETITIVE DISPLACEMENT OF A LUMINESCENT PROBE BY PK INHIBITORS

[0073] Displacement assay was performed with a concentration series of the competitive compound (3-fold dilutions) in assay buffer (150 mM NaCl, 50 mM Hepes pH=7.5, 5 mM DTT, 7.5 μ M BSA) and the fixed concentration of luminescent probe ARC-1063 in complex with a PK was added. The displacement curves were fitted to a sigmoidal dose-response model to obtain IC₅₀ values. K_d values were calculated using the Cheng-Prusoff equation ($K_d = IC_{50}/(1 + C_{ARC-Lum}/K_d ARC-Lum)$).

[0074] ARC-Lum probes were successfully displaced from kinase complexes by various ATP-competitive, protein substrate competitive and bisubstrate inhibitors (FIG. 6 and 7). IC₅₀-values of competitive inhibitors were dependent on the concentration of ARC-1063 proving the validity of the Cheng-Prusoff equation in this assay (FIG. 6). Calculated K_d-values were in good correlation with K_i-values from enzyme kinetic measurements (FIG. 8). Reliable signals for displacement measurements were obtained even at low, subnanomolar concentration of the PKs tested.

EXAMPLE 4. ENHANCEMENT OF LUMINESCENCE BY INTERMOLECULAR FRET (FIG. 2)

[0075] A sample of the recombinant kinase PKAc protein was labeled with 5-TAMRA-NHS ester and the labelled protein PKAc-TAMRA was purified by using a Sephadex column. The concentration series of ARC-668 (structure in Table 1) was made in the assay buffer and fixed concentration of PKAc-TAMRA (25 nM) was subsequently added. The luminescence signal was acquired by using the optic module [ex. 337(50) nm, em. 590(50)]. Intermolecular enhancement of kinase induced long lifetime luminescence by the acceptor was shown to be effective when PKAc was chemically labeled with 5-TAMRA (FIG. 9).

EXAMPLE 5. MEASUREMENT OF DISSOCIATION KINETICS

[0076] Measurements of dissociation kinetics of non-labeled inhibitors from the inhibitor-kinase complexes (residence time) were carried out by mixing the solution of the examined inhibitor (5-10 nM) with PKAc (10 nM), and incubation of the solution for 15 min at 27 °C, followed by addition of a high concentration of the luminescent probe ARC-1 063 (final concentration of 100 - 300 nM), and measurement of the luminescence signal at 5 s time intervals (FIG. 10). The collected data were fitted to the first-order kinetic model yielding the dissociation rate constants for the examined inhibitors.

[0077] Dissociation half-lives of PKAc complexes with the tested inhibitors were the following: ARC-1041, 63 s; ARC-583, 43 s; and staurosporine, 12 s (FIG. 10).

EXAMPLE 6. MONITORING OF KINASE ACTIVITY IN LIVING CELLS

[0078] CHO, C9H6 and HEK cells were cultured in F12 Nutrient Mixture supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in humidified atmosphere containing 5% CO₂. In case of C9H6 cells, 300 μ g/ml of Zeocine™ and 800 μ g/ml of Geneticin® were additionally introduced to the nutrient mixture. Cells were seeded to 96-well plates (for plate reader experiments) and grown to 60%-80% confluency.

The medium was replaced with fresh serum-free medium containing the luminescent probe ARC-1139 (10 μ M) and incubated for 1 hour at 37°C. Subsequently, the cells were washed three times with PBS and analyzed on a PheraStar platereader with TRF optic modules [ex. 337(50) nm, em. 675(50) and 620(20) nm] using the time-resolved fluorescence measurement mode. Forskolin (final concentration of 25 μ M) and H89 (final concentration of 100 μ M) were added to the cells during the measurement (after 3 min and 7 min from the start of data acquisition, respectively)

[0079] Forskolin caused up to two fold increase of the luminescence from live cells loaded with ARC-1139. The signal decreased after addition of 100 μ M H89 that displaces ARC-1139 from its complex with PKAc (FIG. 11). This proves that the luminescent probes can be used for monitoring of kinase activities and effect of the agonists of G_s protein-coupled receptors in living cells (FIG. 12).

EXAMPLE 7. PK SELECTIVITY PROFILING OF A LUMINESCENT PROBE

[0080] Selectivity testing (Table 2) was performed on the commercial basis at the Division of Signal Transduction Therapy, University of Dundee. ATP concentrations that were used in testing were close to the K_m value of the kinase.

Table. 2. Percentages of residual activities of the kinase reaction in the presence of 1 μ M ARC-669

MKK1	69	CHK1	29	PAK6	35
MKK2	68	CHK2	28	MST2	94
MKK6	107	GSK3b	77	MST4	45
ERK1	114	CDK2-Cyclin A	78	GCK	10
ERK2	80	PLK1	101	MINK1	51
JNK1	91	Aurora A	86	MEKK1	19
JNK2	85	Aurora B	81	MLK1	60
JNK3	77	LKB1	101	MLK3	70
p38a MAPK	55	AMPK	36	TA01	94
p38b MAPK	71	MARK1	65	ASK1	74
p38g MAPK	104	MARK2	77	TAK1	13
p38d MAPK	104	MARK3	20	IRAK4	82
ERK8	23	MARK4	86	RIPK2	47
RSK1	2	BRSK1	44	TTK	66
RSK2	4	BRSK2	77	MPSK1	97
PDK1	88	MELK	31	Src	106
PKBa	1	NUAK1	12	Lck	11
PKBb	4	CK1	72	CSK	51
SGK1	8	CK2	7	YES1	64
S6K1	4	DYRK1 A	27	ABL	88
PKA	1	DYRK2	65	BTK	36
ROCK 2	10	DYRK3	18	JAK2	80
PRK2	4	NEK2a	55	SYK	73
PKCa	15	NEK6	71	ZAP70	82
PKC γ	13	IKKb	50	TIE2	86

(continued)

PKCz	10		IKKe	37		BRK	100
PKD1	37		TBK1	27		EPH-A2	98
STK33	76		PIM1	3		EPH-A4	76
MSK1	3		PIM2	15		EPH-B1	90
MNK1	74		PIM3	7		EPH-B2	96
MNK2	76		SRPK1	66		EPH-B3	75
MAPKAP-K2	13		EF2K	43		EPH-B4	98
MAPKAP-K3	31		HIPK1	94		FGF-R1	54
PRAK	63		HIPK2	63		HER4	82
CAMKKb	41		HIPK3	97		IGF-1R	38
CAMK1	3		CLK2	1		IR	52
SmMLCK	46		PAK2	43		IRR	32
PHK	32		PAK4	32		TrkA	101
DAPK1	20		PAK5	18		VEG-FR	25

[0081] Data of selectivity profiling indicates that the luminescent probe ARC-669 has adequate affinity towards most of the AGC kinases and several PKs from other groups to be used in binding experiments with detection of kinase-induced long lifetime luminescence. Thus the probe can be used for high throughput characterization of inhibitors of many PKs.

EXAMPLE 8. MEASUREMENT OF CELLULAR ACTIVATION OF PKA VIA LYSATE ANALYSIS

[0082] CHO cells stably over-expressing PKAc were grown to 80% confluence on black 96-well micro plates (Corning). Cells were washed once with 300 μ L HBSS (Gibco). Thereafter 25 μ L of 100 μ M IBMX in HBSS containing different concentrations of Forskolin (0-100 μ M) were added to the cells and incubated for 10 minutes at 30°C. The cells were lysed by adding 25 μ L of cell lysis buffer (Invitrogen NP40 cell lysis buffer) containing additionally 1% Triton X, 1 x Protease inhibitor cocktail (Sigma-Aldrich Protease inhibitor cocktail for general use), 10 μ M PMSF and 0 or 50 nM ARC-1139. The cells were lysed at 30°C for 10 minutes and the luminescence intensities were measured using a PheraStar plate reader (BMG) with HTRF module TRF excitation filter [(BMG 337), emission at 675(50) nm; 200 flashes, delay 50 μ s, integration time 150 μ s]. The results were fitted to sigmoidal dose-response model using GraphPad Prism 5 software.

[0083] The obtained luminescence intensities were significantly higher when 50 nM ARC-1139 (final concentration in the cell lysate 25 nM) was included in the lysis buffer. The increase of the luminescence signal arises from the formation of the complexes of ARC-1139 and different kinases present in the cell lysate. The luminescence intensity is significantly increased (FIG. 13) as the concentration of Forskolin a cell permeable activator of adenylate cyclase is increased. Adenylate cyclase catalyses the formation of cAMP from ATP and cAMP in turn activates PKA. Thus, luminescent probes of the current invention are cAMP sensors and as such can be used for monitoring the activity of receptor systems. They can be used for screening agonists and antagonists of metabotropic receptors related via G-proteins (e.g., G_s and G_i) to the intracellular activation of adenylate cyclases

EXAMPLE 9. MEASUREMENTS OF KINASE ACTIVITY IN BLOOD SERUM

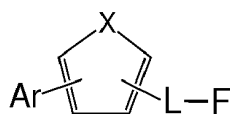
[0084] To human plasma from the local blood bank a concentration series of recombinant PKA was added to the

plasma. 20 μ L of each plasma sample was added to 384-well micro plate (Corning) already containing 5 μ L of 10 nM ARC-1063 (final concentration 2 nM) or 5 μ L of 10 nM ARC-1063, 1 μ M PKI, 1 mM ATP and 100 mM MgAcetate (final concentrations 5 nM, 200 nM, 200 μ M and 20 mM respectively). The samples were incubated at 30°C for 15 minutes and the luminescence intensities were measured using a PheraStar plate reader (BMG) with HTRF module [TRF excitation filter (BMG 337), emission at 675(50) nm; 200 flashes, delay 100 μ s, integration time 100 μ s]. All samples were analysed as three replicate points and plotted against the concentration of PKA added to each samples (FIG. 14). The results were fitted to linear regression using GraphPad Prism 5 software.

[0085] The obtained luminescence signal is directly proportional to the amount of recombinant PKAc added to the plasma (FIG. 14). Moreover, if PKI, a specific competitive inhibitor of PKAc, is added to the binding assay in the presence of ATP and Mg^{2+} ions the complex of ARC-1 063 and PKAc is not formed and only background luminescence (originating from blood components) is detected. Therefore, from the difference of the luminescence signal obtained from the plasma samples in the absence and presence of PKI, the concentration of the endogenous PKAc in plasma can be determined. This sensitive assay has very low limit of quantification (LoQ = 15 pM, 20 μ L volume in a well of 384-well microtiter plate) that enables the monitoring of PKAc activity in blood samples.

Claims

1. A photoluminescent probe having structure P-L-F, where P contains a fragment that has luminescence donor properties and that specifically associates with the purine-binding pocket of the target protein, L is a linker system and F is a fluorescence acceptor dye, whereas the binding of the purine binding protein to said probe induces long lifetime in the range of 1 microsecond to 1000 microsecond luminescence signal.
2. A photoluminescent probe according to the claim 1 containing a heteroaromatic fragment based on a thiophene, selenophene or tellurophene moiety linked to an aromatic system Ar and connected via a linker system L with a fluorescent dye F.



Ar = aromatic system
 X = S, Se, Te
 L = linking structure
 F = fluorescent dye

3. A photoluminescent probe according to claims 1-2 where the target protein is a protein kinase.
4. A photoluminescent probe according to claim 3 where the fragment P-L of the probe is a bisubstrate inhibitor of protein kinases.
5. A photoluminescent probe P-L for use in combination with a purine-binding protein that is labelled with a fluorescent dye F.
6. A photoluminescent probe of claims 1-5, wherein said probe is excited in the wavelength range 280 - 450 nm in the case of single photon excitation and the probe emits radiation in the wavelength range 450 -1000 nm.
7. A photoluminescent probe of claims 1-5, wherein said probe is excited at wavelengths above 600 nm in the case of multiphoton excitation, and emits radiation in the wavelength range 450 -1000 nm.
8. A photoluminescent probe of the claims 1-7, wherein the emission lifetime of the probe is 1 microsecond to 1000 microsecond when the probe is bound to a purine-binding site of the target protein.

9. The binding assay for protein kinases applying probes of claims 1-8 wherein the protein kinase-induced luminescence signal is measured in time delayed (time-resolved) mode.
10. The method based on binding assay of claim 9 for quantification of protein kinases.
11. A method based on binding assay of claim 9 for determination of affinities of protein kinase inhibitors by using the displacement of probe of claim 1-8 from the complex by competitive inhibitors.
12. A method based on binding assay of claim 9 for characterization of dissociation kinetics (residence time) of complexes of inhibitors with protein kinases.
13. A binding assay for monitoring of activity of protein kinases in living cells wherein the luminescence of protein kinase complexes with probes of claim 1-8 are detected by using a fluorescence microscope, a fluorescence spectrometer or a fluorescence platereader with pulse excitation and time delayed (time-resolved) luminescence detection possibilities.
14. A method based on the binding assay of claim 13 for characterization of inhibitors of protein kinases in living cells.
15. A method based on binding assay of claim 13 for characterization of agonists and antagonists of G_s and G_i protein-coupled receptors through measurement of the change in luminescence intensity of probes of claims 1-8 caused by activation of PKA by the second messenger cAMP in living cells.
16. A method based on binding of claim 13 for characterization of agonists and antagonists of metabotropic and ionotropic receptors through measurement of the change in luminescence of probes of claims 1-8 caused by activation of protein kinases other than PKA in living cells.
17. A method based on binding assay of claim 9 for quantification of activity of protein kinases in body fluids, cell lysates and tissue extracts.
18. A method based on binding assay of claim 9 for monitoring of receptor activation and cellular concentration of cAMP by analysis of cell lysates.
19. A method for monitoring of kinase activity in living cells that uses a luminescent probe according to claim 5 and the target protein is a protein kinase fused with a fluorescent protein or tagged with a fluorescent dye.
20. A method based on binding assay of claim 9 for quantification of protein kinases as biomarkers of diseases in body fluids, blood cells, and tissue extracts.

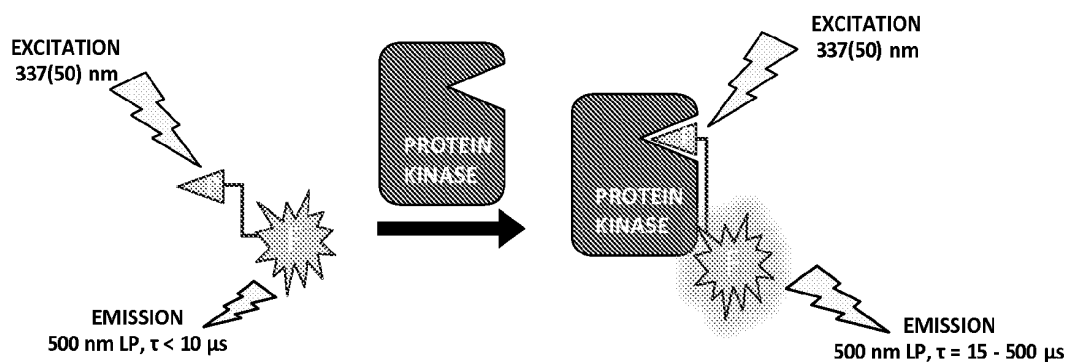


Figure 1

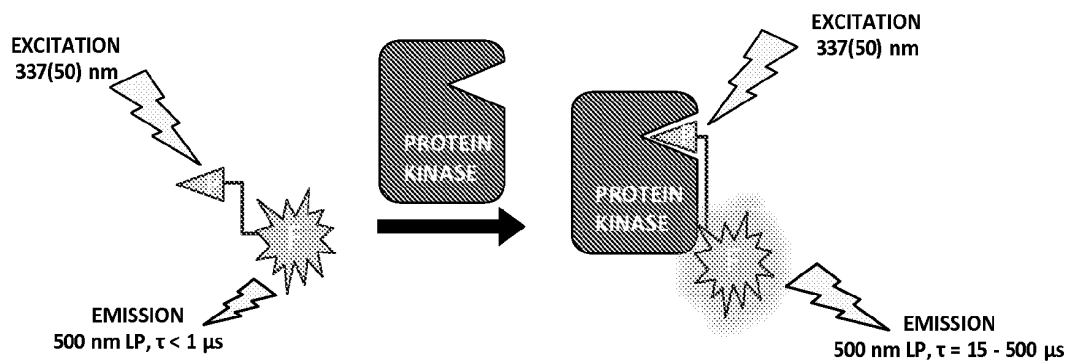


Figure 2

Ar = aromatic system

X = S, Se, Te

L = linking structure

F = fluorescent dye

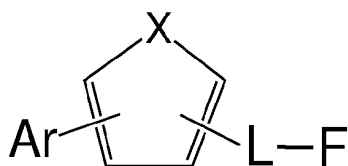


Figure 3

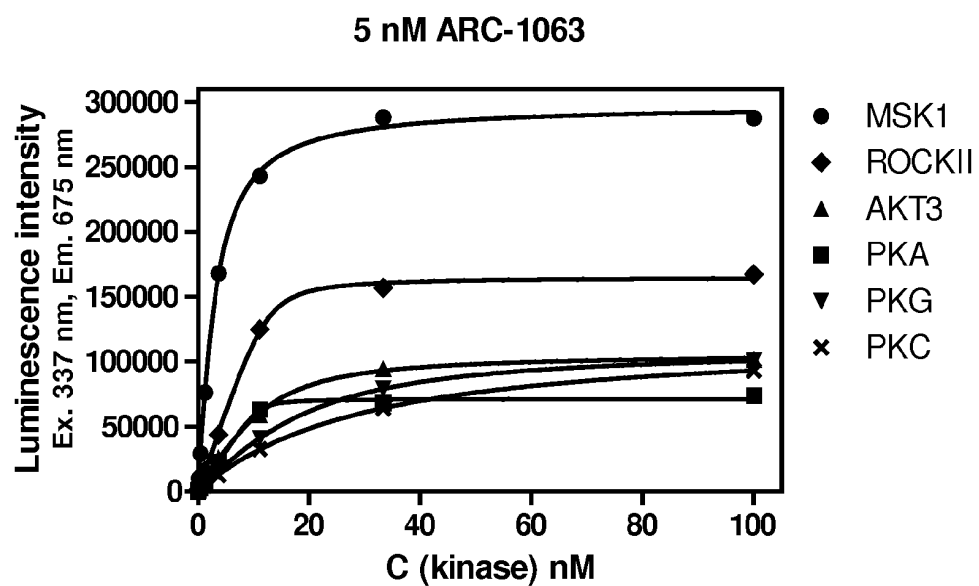


Figure 4

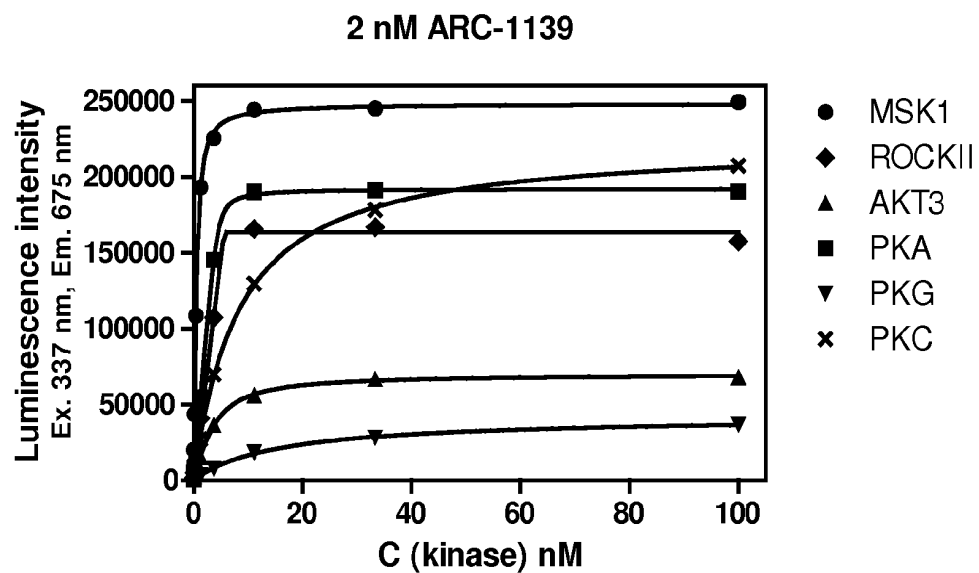


Figure 5

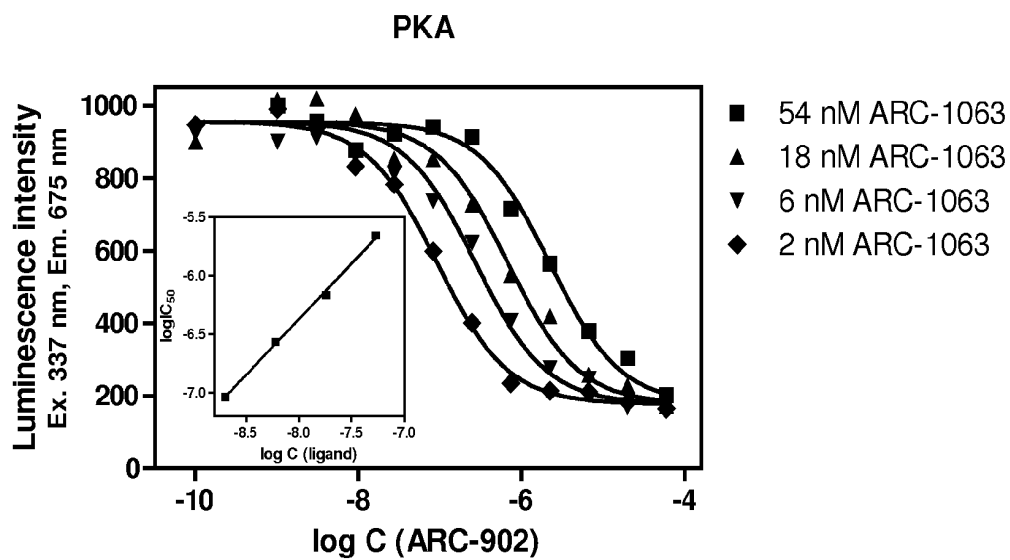


Figure 6

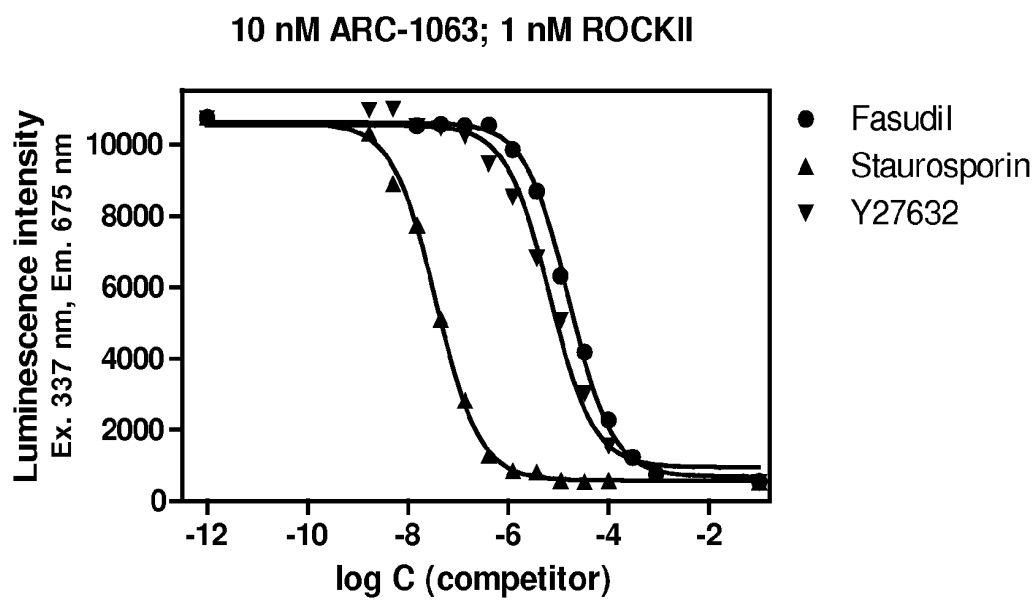


Figure 7

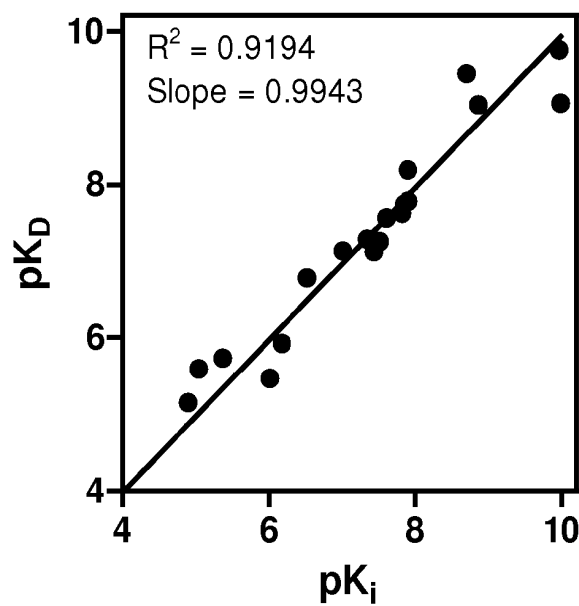


Figure 8

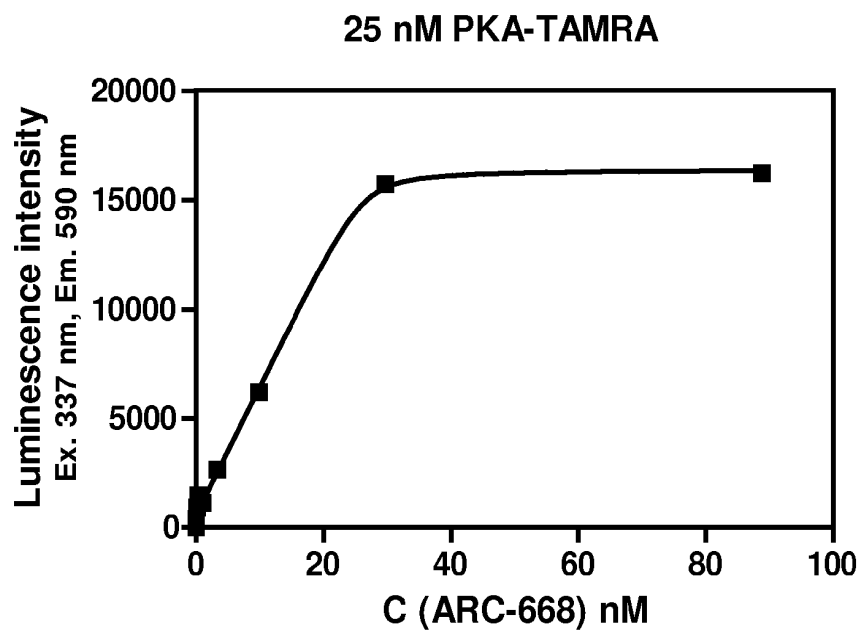


Figure 9

ARC-1063; PKA

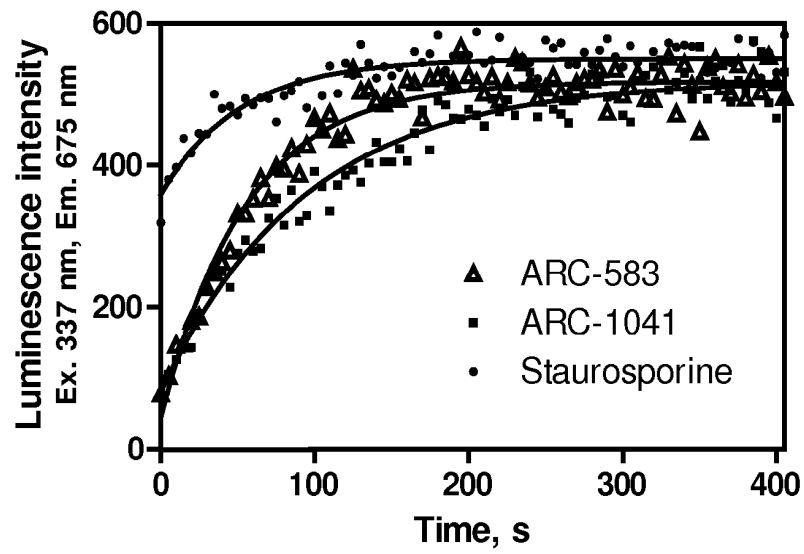


Figure 10

ARC-1139; C9H6 cells

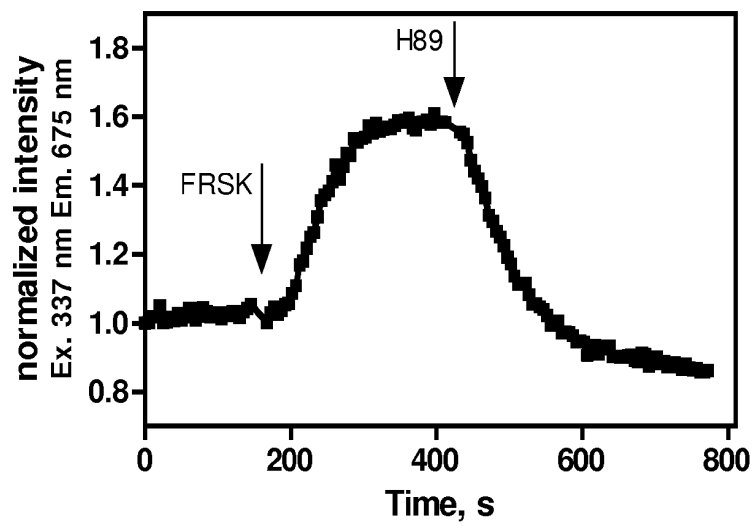


Figure 11

ARC-1139; HEK293 cells

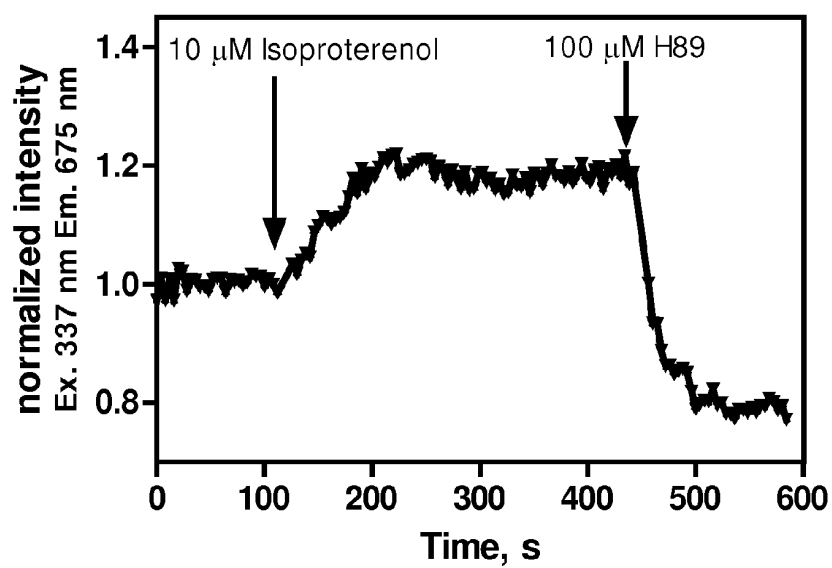


Figure 12

C9H6 cells

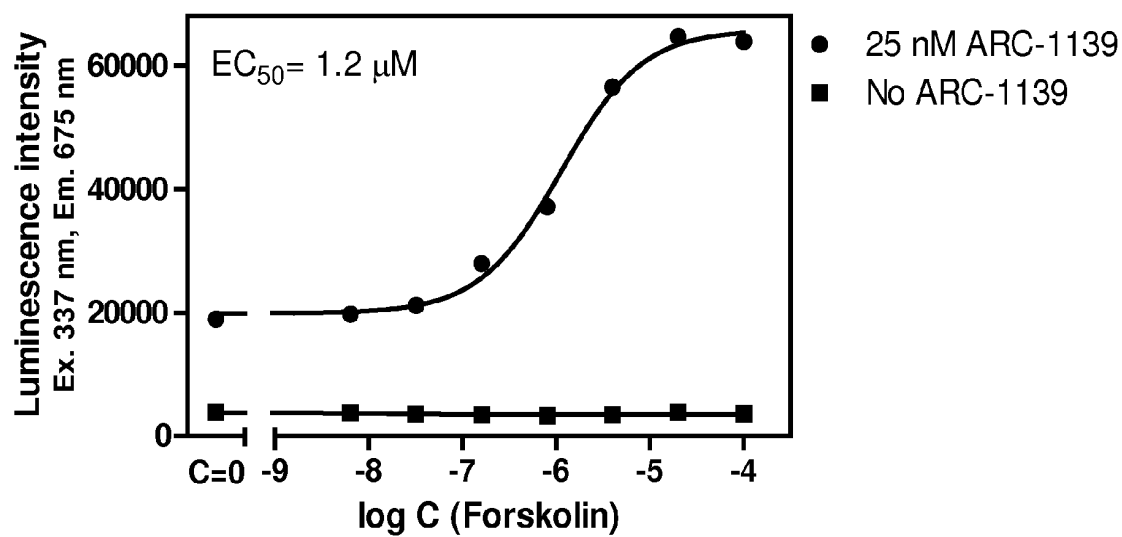


Figure 13

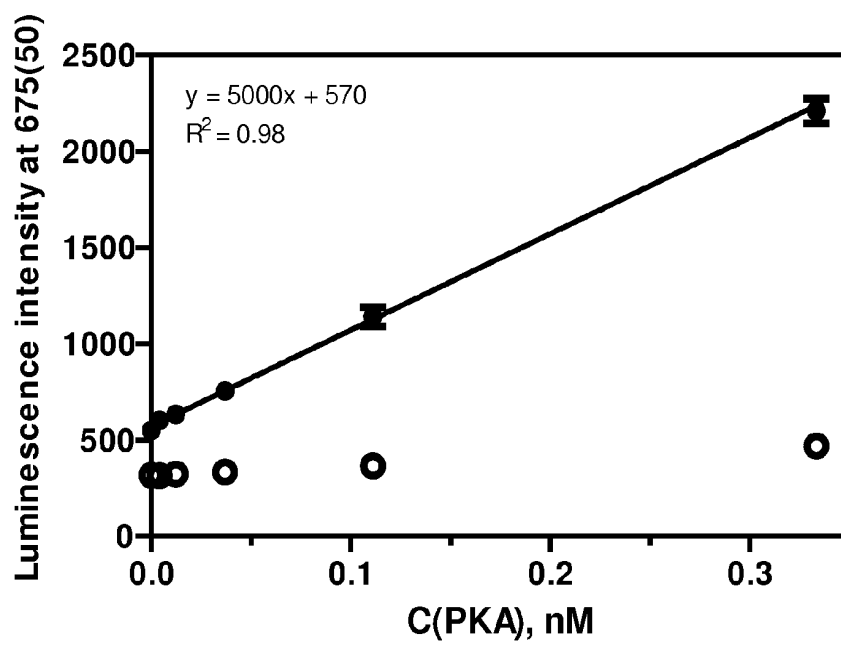


Figure 14



EUROPEAN SEARCH REPORT

Application Number
EP 11 15 2289

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
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X	LAVOGINA D ET AL: "Adenosine analogue-oligo-arginine conjugates (ARCs) serve as high-affinity inhibitors and fluorescence probes of type I cGMP-dependent protein kinase (PKG1alpha)", BIOCHIMICA ET BIOPHYSICA ACTA (BBA) - PROTEINS & PROTEOMICS, ELSEVIER, NETHERLANDS, vol. 1804, no. 9, 18 April 2010 (2010-04-18), pages 1857-1868, XP027170139, ISSN: 1570-9639, DOI: DOI:10.1016/J.BBAPAP.2010.04.007 [retrieved on 2010-07-22] * See also: supplemental info;compounds arc-669 *	1-20	TECHNICAL FIELDS SEARCHED (IPC) G01N C07D
The present search report has been drawn up for all claims			
Place of search Munich		Date of completion of the search 13 July 2011	Examiner Vogt, Titus
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

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EUROPEAN SEARCH REPORT

Application Number
EP 11 15 2289

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A	ENKVIST E ET AL: "Effect of the structure of adenosine mimic of bisubstrate-analog inhibitors on their activity towards basophilic protein kinases", BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, PERGAMON, ELSEVIER SCIENCE, GB, vol. 19, no. 21, 1 November 2009 (2009-11-01), pages 6098-6101, XP026673787, ISSN: 0960-894X, DOI: DOI:10.1016/J.BMCL.2009.09.026 [retrieved on 2009-09-12] * tables 1, 2; compounds pre6, 6a, 6b, 6c *	1-20	
X	----- URI A ET AL: "Bisubstrate fluorescent probes and biosensors in binding assays for HTS of protein kinase inhibitors", BIOCHIMICA ET BIOPHYSICA ACTA (BBA) - PROTEINS & PROTEOMICS, ELSEVIER, NETHERLANDS, vol. 1804, no. 3, 29 October 2009 (2009-10-29), pages 541-546, XP026886287, ISSN: 1570-9639, DOI: DOI:10.1016/J.BBAPAP.2009.10.019 [retrieved on 2009-10-29] * abstract; figures 2,3 * ----- -/--	1,3-20	TECHNICAL FIELDS SEARCHED (IPC)
The present search report has been drawn up for all claims			
Place of search Munich		Date of completion of the search 13 July 2011	Examiner Vogt, Titus
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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EPO FORM 1503 03.82 (P04C01)



EUROPEAN SEARCH REPORT

Application Number
EP 11 15 2289

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
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X	WO 2008/019696 A2 (UNIV TARTU [EE]; URI ASKO [EE]; ENKVIST ERKI [EE]; VIIL INDREK [EE]; L) 21 February 2008 (2008-02-21) * claims *	1,3-20	
			TECHNICAL FIELDS SEARCHED (IPC)
The present search report has been drawn up for all claims			
Place of search Munich		Date of completion of the search 13 July 2011	Examiner Vogt, Titus
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13-07-2011

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		JP 2010500580 A	07-01-2010
		US 2010233743 A1	16-09-2010

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专利名称(译)	光致发光探针		
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申请(专利权)人(译)	塔尔图大学		
当前申请(专利权)人(译)	塔尔图大学		
[标]发明人	URI ASKO ENKVIST ERKI VAASA ANGELA KASARI MARJE		
发明人	URI, ASKO ENKVIST, ERKI VAASA, ANGELA KASARI, MARJE		
IPC分类号	G01N33/533 G01N33/573 G01N33/58 C07D233/00 C07D333/00 C07D473/00		
CPC分类号	C12Q1/485 C07D409/04 C07D413/14 C07D493/10 G01N33/533 G01N33/542 G01N33/581 G01N33/582		
审查员(译)	福格特TITUS		
其他公开文献	EP2482072B1		
外部链接	Espacenet		

摘要(译)

本发明涉及不含金属络合物的有机长寿命光致发光探针，其与嘌呤结合蛋白的特异性结合导致长波长发光的发射增加，具有长寿命（微秒区域）。这使得能够对生物样品中存在的荧光进行时间门控区分，细胞的自发荧光或来自其他荧光团的发射。本发明还涉及改进的方法用于监测活细胞中蛋白激酶活性，蛋白激酶抑制剂的表征，基于蛋白激酶的疾病生物标志物的分析以及具有生物学和医学重要性的其他任务的用途。

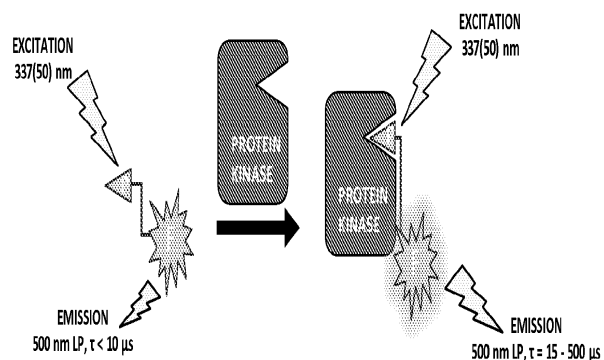


Figure 1