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Benzoselenadiazole-based responsive long-lifetime photoluminescence probes for protein kinases

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Benzoselenadiazole-containing inhibitors of protein kinases were constructed and their capability to emit phosphorescence in the kinase-bound state was established. 

Labelling of the inhibitors with a red fluorescent dye led to sensitive responsive photoluminescence probes for protein kinase CK2 that emitted red light with long (microsecond-scale) decay time after excitation of the probes with a pulse of near-UV light. 

Fluorescence-based biosensors have found diverse applications for characterization of the status of biological systems. Small-molecule fluorescent dyes and genetically coded fluorescent proteins both possessing luminescence decay time in low nanosecond range have been used for labelling of bioactive molecules to get fluorescent probes for biochemical and cellular studies. In cells fluorescence signals of such labels are interfered by background autofluorescence of cells and light scattering. Optical probes possessing phosphorescence, a type of photoluminescence with long luminescence decay time (lifetimes τ in μs or ms range), enable time-gated measurements (luminescence intensity is measured after pulse excitation and time delay) that eliminates the interfering signal from short-lifetime fluorescence background. Unfortunately, probes possessing long-lifetime photo-luminescence in water solution at room temperature are not common, being mostly restricted to compounds comprising metal-ligand complexes (e.g., lanthanide chelates, noble metal complexes) and nanoparticles. 

Efficient application of photoluminescent probes for characterization of biochemical interactions requires the capability for discrimination between bound and unbound forms of the probe. This is usually achieved by measurement of a signal that is sensitive to binding status of the probe, e.g., fluorescence polarization (anisotropy), fluorescence lifetime, or intensity of Förster resonance energy transfer (FRET) between the two binding partners (in the latter case the labelling of the binding proteins is also required). Responsive long-lifetime probes would be highly awaited for measurement of protein concentration, but such probes have been described mostly for small molecules (pH, oxygen, metal ions, etc.). 

Recently we have described novel organic photoluminescent probes [ARC-Lum(Fluo)-probes] that in the complex with a protein kinase (PK) emit orange or red luminescence with long (microsecond-scale) decay time upon excitation with near-UV-light, whereas no such signal is inherent to the free probe. ARC-Lum(Fluo)-probes comprised a thiophene- or selenophene-containing aromatic system that bound to the ATP pocket of the PK. Association with kinase resulted in stabilization of the triplet state of the donor luminophore and a weak phosphorescence signal in the 500-700 nm region. Close proximity of a bright fluorescent acceptor dye whose excitation spectrum overlapped with the phosphorescence emission spectrum of the donor led to large amplification of the signal as a result of efficient FRET from the phosphorescence donor to the fluorescent acceptor dye. ARC-Lum(Fluo)-probes have now been used in biochemical binding-displacement assays for characterization of inhibitors of PKs, for analysis of biomarkers in blood plasma samples and for mapping and monitoring PK activity in living cells in time-gated luminescence microscopy.

Scheme 1. Synthesis of conjugates of benzoselenadiazole with peptides.
Here we report on the introduction of a new benzoselenadiazole scaffold for the construction of responsive long-lifetime photoluminescent probes for PKs. If compared to the previously reported probes comprising a selenophene or a thiophene ring linked to another aromatic cycle via a rotatable single bond, the benzoselenadiazole scaffold is more rigid, thus the position of this fragment in a complex with other proteins is better predictable.

To shift the PK affinity of compounds comprising an ATP-competitive benzoselenadiazole fragment into the nanomolar range the bisubstrate approach was applied, conjugating the aromatic structure with peptideogenic fragments through a linker. D-arginine-rich peptides are known to enhance binding of compounds to basophilic PKs (PKA, ROCK, Pim-1) whereas oligoaspartates improve binding to an acidophilic kinase CK2. Conjugates 2,1,3-Benzosenadiazole scaffolds can be easily prepared from aromatic diamines by using the reaction with selenium dioxide (Scheme S1 (†ESI)).

Treating of regio-isomeric diaminobenzoic acids with selenium dioxide gave 2,1,3-benzosenadiazole-5-carboxylic acid 1 and 2,1,3-benzosenadiazole-4-carboxylic acid with a quantitative yield. Coupling of the obtained carboxylic acids with peptides on the Rink-amide resin yielded the peptide conjugates ARC-1601 and ARC-1608, respectively (Scheme 1). Labelling of these conjugates with PromeFluo®-647 (PF647) dye led to ARC-Lum(Fluo)-probes ARC-1602 and ARC-1609 (Scheme 1). Titration of the probes with the catalytic subunit of protein kinase A (PKA) resulted in the formation of highly luminescent complexes (Fig. 1A and Fig. S2 (†ESI)). ARC-1609 gave a slightly weaker signal than ARC-1602. Both probes revealed only moderate affinity towards PKA (K_{d} values of 84 ± 30 nM and 152 ± 46 nM, respectively). The lifetimes of the signals (29 ± 3 ns and 32 ± 3 µs, respectively) were close to the corresponding values for selenophene-containing probes. This shows that 2,1,3-benzosenadiazole is a suitable scaffold for construction of protein-induced phosphorescent probes but the moiety gives only weak interaction with the ATP site of PKA.

The tricyclic benzoselenadiazole fragment is expected to be prone for binding with Pim-1 and CK2 thus the corresponding probes for these PKs were designed. 3,4-Diaminotoluene was converted to compound 3 that was alkylated either with ethyl ester of bromoacetic acid or with isopropyl ester of δ-bromoacetonic acid. Hydrolysis of esters 4 and 5 gave carboxylic acids 6 and 7, respectively that were coupled with peptides on solid phase (Scheme 1 and Scheme S2 (†ESI)). Two conjugates with different peptides were obtained, ARC-3131 with a hexa-(D-arginine) and ARC-3138 with an oligo(aspartic acid). PKs Pim-1 and CK2 possess similar preference for structures of ATP-site binders but have very different consensus sequences of favoured substrate proteins. According to the analogy with our previously constructed compounds we hypothesized that ARC-3131 is a bisubstrate-analogue inhibitor of Pim-1 and ARC-3138 is a bisubstrate-analogue inhibitor of CK2. Both compounds were labelled with PF647 to get the probes ARC-3132 and ARC-3141, respectively. ARC-3132 was titrated with Pim-1 and PKA (Fig. 1B and 1C). Measurements with fluorescence anisotropy readout resulted in K_{d} values of 5.7 ± 3.4 nM for Pim-1 and 45 ± 6 nM for PKA. Excitation of the solution containing ARC-3132/PKAc complex with a pulse of light at 337 nm revealed a strong long-lifetime signal at 675 nm whereas no signal could be detected for ARC-3132/Pim-1 complex or free ARC-3132 probe (Fig. 1B and Fig. S3 (†ESI)). Intensity of ARC-3132/PKAc signal was comparable to that of previously reported thiophene and selenophene containing probes (Fig. S8 (†ESI)). We have shown that among the PKs of the AGC group the association of selenophene-based ARC-Lum(Fluo)-probes with Pim-1 led to a significantly weaker long-lifetime photoluminescent signal. This special behaviour of Pim-1 could originate from the unique structure of the hinge region of PKs of the Pim family, all possessing an amino acid residue proline at the position 123 of the hinge region where other PKs have a valine residue. Proline residue lacks the hydrogen bond donor ability for binding ligands. This bond with the luminescence donor seems to be important for formation or stabilization of the triplet state of the latter. CK2 with Val116 is able to give the required hydrogen bonding interaction.
bond. Indeed, binding of ARC-3141 with CK2 in addition to increase of fluorescence anisotropy value of the PF647 dye ($\lambda_{ex} = 590$ nm, $\lambda_{em} = 675$ nm) also led to a strong luminescence signal ($\lambda_{ex} = 337$ nm, $\lambda_{em} = 675$ nm) with $20 \pm 2$ µs lifetime (Fig. 2D and Fig. S4 (†ESI)). Thereat the binding of the parent conjugate comprising no fluorescent dye (ARC-3138) with CK2 revealed a weak phosphorescence emission signal with long decay time in complex with CK2 but the intensity of this signal ($\lambda_{ex} = 337$ nm, $\lambda_{em} = 675$ nm) was about 400-fold lower than the signal from ARC-3141/CK2 complex (Fig. S5 (†ESI)). Interestingly, ARC-3141 showed higher affinity towards CK2 ($K_D = 25 \pm 9$ nM) than ARC-3138 ($K_D = 82 \pm 22$ nM), revealed both by binding/displacement and inhibition assays (Fig. 1, Fig. S6 and Fig. S7 (†ESI)). This is apparently caused by masking the unfavourable effect of positive charge of lysine in the C-terminus of the conjugate, as the acetylation of the amino group increased the affinity in the same way (Fig. S7 (†ESI)). The high affinity of ARC-3141 to CK2 enables the application of the probe for measurement of concentration of CK2 and characterization of CK2 inhibitors both in buffer solutions and cell lysates. Selectivity profiling of ARC-3138 in the panel of 140 protein kinases revealed its clear CK2 selectivity (Table S3 (†ESI)). At 1 μM concentration it inhibited 80% of CK2 activity while all other kinases were inhibited by less than 50%.

In conclusion, the work demonstrates that benzoselenadiazole is a promising novel scaffold that possesses the property to emit phosphorescence in the complex with the target protein if the lumiphore is excited with a pulse of light in the near-UV region. The microsecond-lifetime signal was drastically amplified by the presence of a fluorescent dye in the proximity of the donor. The amplification of the donor phosphorescence results from efficient FRET to the acceptor dye and high quantum yield of the latter.7 Oxygen- and sulphur-containing counterparts of benzoselenadiazole (benzoxadiazole and benzothiadiazole, respectively) have been widely used as fragments of ligands for several proteins.17 Discovery of an aromatic scaffold with requested optical properties deepens the understanding of principles that have to be considered when constructing new responsive optical probes for PKs and other (non-kinase) proteins.17 Hereto optical properties (excitation and emission wavelengths, fluorescence quantum yields, etc.) of the benzoselenadiazole lumiphore can be easily varied with simple modifications of the core structure the compound,18 thereby further improvement of the protein-responsive probe is apparently possible. Additionally, benzoselenadiazole is a small structural element whose incorporation into various inhibitory compounds is possible without abolishing the binding properties of the inhibitors. On the other hand lack of the long-lifetime luminescence signal of the probes with the PK Pim-1 points to the possibility that binding of a benzoselenadiazole fragment into the ligand-binding pocket of a protein may be not sufficient for the stabilization of the triplet state of the phosphor. A specific interaction of the protein with the benzoselenadiazole fragment is needed to fix the donor lumiphore in a suitable conformation for productive electron transfer and prevent access of oxygen and other quenchers. Thus the development of responsive probes for some of benzoselenadiazole-binding proteins could be not achievable. From the other side, the obtained knowledge confirms the previous understanding that non-specific associations of ARC-Lum(Fluo)-probes with proteins, other biopolymers and small molecules do not result in a microsecond-scale photoluminescence.

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