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MARIE KRIISA

Development of protein kinase-responsive photoluminescent probes and cellular regulators of protein phosphorylation

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Development of protein kinase-responsive photoluminescent probes and cellular regulators of protein phosphorylation

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Press

Institute of Chemistry, Faculty of Science and Technology, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

I Enkvist, E., **Kriisa, M.**, Roben, M., Kadak, G., Raidaru, G., Uri, A. (2009) Effect of the structure of adenosine mimic of bisubstrate-analog inhibitors on their activity towards basophilic protein kinases. Bioorganic and Medicinal Chemistry Letters, 19(21), 6098–6101.

II Enkvist, E., Vaasa, A., Kasari, M., **Kriisa, M.**, Ivan, T., Ligi, K., Raidaru, G., Uri, A. (2011) Protein-induced long lifetime luminescence of non-metal probes. ACS Chemical Biology, 6(10), 1052–1062.

III **Kriisa, M.**, Sinijärv, H., Vaasa, A., Enkvist, E., Kostenko, S., Moens, U., Uri, A. (2015) Inhibition of CREB phosphorylation by conjugates of adenosine analogues and arginine-rich peptides, inhibitors of PKA catalytic subunit. ChemBioChem, 16(2), 312–319.

Author's contribution:

Paper I: The author participated in the planning of the experiments, characterised fluorometric TLC-based protein kinase activity assay for PKBγ, determined inhibitory potencies of the compounds towards PKAc and PKBγ, and contributed to the writing of the manuscript.

Paper II: The author participated in the planning of the experiments, characterised the photoluminescent probes in biochemical and cellular assays, analysed the data, and contributed to the writing of the manuscript.

Paper III: The author planned and performed most of the experiments for biochemical and biological testing of the compounds and was responsible for writing of the manuscript.

ABBREVIATIONS

6His-tag	hexahistidine tag
AC	adenylate cyclase
Adc	adenosine 4'-dehydroxymethyl-4'-carboxylic acid moiety
Ahx	6-aminohexanoic acid moiety
AKAP	A-kinase anchoring protein
AKAR	A-kinase activity reporter
AMSE	5-(2-aminopyrimidin-4-yl)selenophene-2-carboxylic acid moiety
AMTH	5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid moiety
AP	alkaline phosphatase
ARC	adenosine analogue-oligoarginine conjugate
ARC-663	AMTH-Ahx-DAla-Ahx-$(DArg)_6$-DLys-NH_2
ARC-664	AMTH-Ahx-DLys-Ahx-$(DArg)_6$-NH_2
ARC-668	AMTH-Ahx-DArg-Ahx-$(DArg)_6$-DLys-NH_2
ARC-681	AMTH-Ahx-DArg-Ahx-$(DArg)_6$-DLys(Myr)-NH_2
ARC-902	Adc-Ahx-$(DArg)_6$-NH_2
ARC-904	Adc-Ahx-$(DArg)_6$-DLys-NH_2
ARC-1012	Adc-Ahx-DLys-Ahx-$(DArg)_2$-NH_2
ARC-1028	Adc-Ahx-DLys-Ahx-$(DArg)_6$-NH_2
ARC-1063	AMTH-Ahx-DArg-Ahx-$(DArg)_6$-DLys(Alexa Fluor 647)-NH_2
ARC-1102	AMTH-Ahx-DLys-Ahx-$(DArg)_2$-NH_2
ARC-1139	AMSE-Ahx-DArg-Ahx-$(DArg)_6$-DLys(PromoFluor-647)-NH_2
ARC-1143	AMTH-Ahx-Ala-$(DArg)_6$-DLys(Myr)-Gly
ARC-1171	Adc-Ahx-$(DArg)_6$-DLys(Hex)-NH_2
ARC-1172	Adc-Ahx-$(DArg)_6$-DLys(C(O)$(CH_2)_2$-$P^+(Ph)_3$)-NH_2
ARC-1222	Adc-Ahx-$(DArg)_6$-DLys(Myr)-NH_2
ARC-1412	dPurp-C(O)$(CH_2)_7$C(O)-$(DArg)_6$-DLys(Myr)-NH_2
ARC-Fluo	ARC labelled with a fluorescent dye
ARC-inhibitor	ARC-based bisubstrate inhibitor
ARC-Lum	ARC-based probe possessing protein-induced luminescence signal with microsecond-scale lifetime
ARC-Lum(-)	ARC-Lum probe lacking a fluorescent dye
ARC-Lum(Fluo)	ARC-Lum probe incorporating a fluorescent dye
ARC-probe	ARC-based bisubstrate inhibitor possessing photoluminescence properties
AT13148	(S)-1-(4-(1H-pyrazol-4-yl)phenyl)-2-amino-1-(4-chlorophenyl)ethanol
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
C9H6	CHO cells recombinantly overexpressing both subunits of PKA
CaMK	calcium/calmodulin dependent protein kinase

cAMP	cyclic adenosine 3',5'-monophosphate
CBP	CREB binding protein
CHO-K1	Chinese hamster ovary cell line K1
CK	casein kinase
CPP	cell-penetrating peptide
CRE	cAMP-response element
CREB	cAMP-response element-binding protein
dPurp	7-deazapurine-6-piperazine moiety
DTT	dithiothreitol
FA/FP	fluorescence anisotropy/fluorescence polarization
FI	fluorescence intensity
FRET	Förster-type resonant energy transfer/Förster resonance energy transfer
GPCR	G protein-coupled receptor
GSK	glycogen synthase kinase
GSK690693	4-[2-(4-amino-1,2,5-oxadiazol-3-yl)-1-ethyl-7-[(3*S*)-3-piperidinylmethoxy)-1*H*-imidazo[4,5-c]pyridin-4-yl]-2-methyl-3-butyn-2-ol
H89	N-[2-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide
HA1077	1-(5-isoquinolinesulfonyl)-homopiperazine
HEK293	human embryonic kidney cell line 293
Hex	hexanoic acid moiety
HRP	horseradish peroxidase
HTS	high-throughput screening
IC_{50}	half-maximal inhibitory concentration
ICAP	indicator of CREB activation due to phosphorylation
K_D	equilibrium dissociation constant determined from a direct binding assay
K_d	equilibrium dissociation constant determined from a displacement assay
K_i	equilibrium dissociation constant determined from an inhibition assay
KID	kinase-inducible domain
KIX	KID interaction domain
K_m	Michaelis constant
MAPK	mitogen-activated protein kinase
MSK	nuclear mitogen- and stress-activated protein kinase
MW	molecular weight
Myr	myristic acid moiety
PIM	Provirus integration site for Moloney murine leukemia virus kinase
PK	protein kinase
PKA	cAMP-dependent protein kinase, protein kinase A
PKAc	PKA catalytic subunit

PKAr	PKA regulatory subunit
PKB	protein kinase B (Akt)
PKC	protein kinase C
PKG	protein kinase G
PKI	heat-stable protein kinase inhibitor
PRKX	the human X chromosome-encoded protein kinase X
PRKY	Y-homolog of protein kinase PRKX
ROCK	Rho-associated protein kinase
RSK	ribosomal protein S6 kinase
SDS	sodium dodecyl sulfate/sodium lauryl sulfate
TAMRA	carboxytetramethylrhodamine
TGL	time-gated luminescence
TLC	thin layer chromatography
TR	time-resolved
TR-FRET	time-resolved Förster-type resonant energy transfer
Y-27632	*trans*-4-[(1*R*)-1-aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide

INTRODUCTION

Protein kinases (PKs) are important cellular signalling molecules whose aberrance from normal activity can lead to several complex diseases, including cancer, cardiovascular diseases, or neurological disorders. This has made PKs important therapeutic targets (Cohen 2002; Knight *et al.* 2010; Roskoski 2015). More than 30 small-molecule PK inhibitors have reached the drug market during recent 15 years and a large number of new inhibitors of PKs are on various stages of clinical trials (Rask-Andersen *et al.* 2014; Wu *et al.* 2015; Fabbro *et al.* 2015). Additionally, inhibitors of PKs are useful reagents for studying and understanding of both the physiological role of PKs in normally functioning cells and biological mechanisms behind human diseases. Therefore, considerable effort has been put into development of reliable biochemical and cell-based assays to monitor the activity of PKs and screen their inhibitors.

Fluorescence anisotropy (FA) and Förster-type resonant energy transfer (FRET) are common photoluminescence techniques that are used for characterising the activity of PKs and the association of PKs with inhibitors in biochemical as well as in biological assays. However, in complicated biological samples these methods may possess some drawbacks due to the background fluorescence of organic compounds or autofluorescence of cells that cannot be distinguished from the fluorescence signal related to the biological target under inspection. Hence, the application of photoluminescent probes that emit light with long decay time and enable the performance of measurements in time-resolved (TR) mode has caused great progress with luminometry-based assays, which has led to improved assay formats to obtain more accurate and detailed information about the sample.

In this thesis, the research on the conjugates of adenosine analogues and arginine-rich peptides (ARCs) led us from bisubstrate inhibitors to fluorescent probes for biochemical assays, PK binding-responsive long-lifetime photoluminescent probes, and regulators of protein phosphorylation balances in living cells. New structural fragments were introduced into ARCs and the compounds were characterised and reconstructed in order to further improve their affinity and selectivity towards different basophilic PKs, and increase resistance of the compounds to enzymatic degradation to widen their biological applications. Thereafter, the newly constructed and characterised compounds were applied for developing effective assay systems for the investigation of PKs and their inhibitors in biological solutions with the aid of the TR measurement techniques. In the course of this study binding-responsive long-lifetime photoluminescent probes were discovered and developed into unique research tools. Moreover, for the first time, inhibitory potency of ARCs in the living cells was measured. The results of these measurements reveal the potential of ARCs for the regulation of activity of PKs in cells and point to the structural changes that should be performed to improve the potency and selectivity of the inhibitors for regulating protein phosphorylation balances in living cells.

1. LITERATURE OVERVIEW

1.1. Protein kinases

Protein phosphorylation has essential role in cell life. It allows cells to be responsive to their external environment, regulating signalling pathways and cellular processes that mediate metabolism, growth, division, differentiation, membrane transport, apoptosis, intracellular communication, *etc*. (Manning *et al.* 2002a; Johnson 2009). PKs, belonging to the transferase class of enzymes, catalyse the phosphorylation of proteins. The phosphorylation event is preceded by binding of a nucleotide [usually adenosine 5'-triphosphate (ATP)] and the target protein to the active site of the enzyme. Thereafter, the γ-phosphoryl group of ATP is transferred to a serine, threonine, or tyrosine residue of the substrate protein, which causes changes in its activity, localisation, or interactions with other proteins (Schwartz and Murray 2011). Protein phosphatases, on the other hand, catalyse protein dephosphorylation, removal of phosphoryl groups from phosphoproteins via hydrolysis.

PKs form a large enzyme superfamily that carries a huge role in cell signal transduction. There are more than 500 PK genes identified in the human genome, representing about 2.7 % of all human protein-coding genes (Hunter 2000; Manning *et al.* 2002a; Schwartz and Murray 2011). The human kinases are classified into a hierarchy of groups, families, and subfamilies based on the comparison of the amino-acid sequences of the catalytic domains, biological functions, and substrate specificities. According to that, it is possible to distinguish nine main groups of PKs (Hanks and Hunter 1995; Manning *et al.* 2002b; Hanks 2003), which are briefly introduced in the subsequent section.

The TK (tyrosine kinase) group constitutes of a large number of enzymes that specifically phosphorylate proteins at tyrosine residues. PKs of the AGC group, on the other hand, are basic amino acid directed enzymes (PKA, PKG, PKC) phosphorylating substrates at serine or threonine residues that possess arginine and lysine residues in close proximity. The CaMK (calcium/calmodulin dependent protein kinase) group incorporates the family of PKs regulated by calcium/calmodulin. These PKs also favour substrates that are rich in basic amino acid residues. The CMGC group includes enzymes that are proline-directed, phosphorylating substrates at the sites lying in proline-rich environments, *e.g.*, CDK (cyclin-dependent kinase), MAPK (mitogen-activated protein kinase), GSK (glycogen synthase kinase), and CLK (CDC2-like kinase) families. The STE group is named after homologs of yeast Sterile 7, Sterile 11, and Sterile 20 kinases, containing PKs of MAPK cascade families. The CK1 group is named after casein kinase 1 and consists of CK1, TTBK (tau tubulin kinase), and VRK (vaccinia-related kinase) families, which preferably phosphorylate motifs rich in acidic residues. The TKL (tyrosine kinase-like) group constitutes various families that resemble both tyrosine and serine-threonine kinases, like MLK (mixed-lineage kinase) and IRAK (interleukin-1 receptor-associated kinase). The members of RGC (receptor guanylate cyclase) group are

pseudokinases with similar domain sequence to tyrosine kinases. Lastly, there exists a group of atypical kinases in human genome, which contains proteins that have been reported to have biochemical kinase activity, without having sequence similarity to the above-mentioned PK domains and their close homologs.

1.1.1. AGC protein kinases

The AGC group was named after three representative families of PKs, cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG) and protein kinase C (PKC) (Hanks and Hunter 1995). This group contains more than 60 PKs that are divided into 14 families and 21 subfamilies according to their different functional domains, which are important for the activity and localisation of the PK (Manning *et al.* 2002b; Pearce *et al.* 2010; Arencibia *et al.* 2013). More complexity to this group is added by multiple isoforms and splice variants.

The activity of the AGC kinases is regulated by the local concentrations of cytoplasmic second messengers, such as cyclic adenosine 3',5'-monophosphate (cAMP) and lipids, and by the phosphorylation of two highly conserved regulatory motifs (T- or activation loop in the catalytic domain and hydrophobic motif in a non-catalytic region following the kinase domain) (Pearce *et al.* 2010). For some PKs the phosphorylation at turn motif may be crucial for their stability and integrity (Pearce *et al.* 2010; Arencibia *et al.* 2013).

The AGC group includes some widely studied PK families (PKA, PKG, PKC, PKB/Akt, MSK, RSK, ROCK, *etc.*), while several of them may catalyse phosphorylation of the same proteins. This is due to their similar substrate consensus sequence preferences as these PKs tend to be basic amino acid directed enzymes, phosphorylating substrates at serine or threonine residues flanking by arginine and lysine residues (Pearce *et al.* 2010). PKs of AGC group carry a key role in several important intracellular signalling pathways and are therefore potential targets for the treatment of variety of diseases, like cancer, diabetes, neurological disorders, cardiovascular diseases, inflammation, and viral infections (Pearce *et al.* 2010; Arencibia *et al.* 2013).

Fasudil, an inhibitor of ROCK kinase, was the first clinically applied PK inhibitor, used for the treatment of subarachnoid haemorrhage-induced cerebral vasospasm in Japan since 1995 (Asano *et al.* 1998; Tamura *et al.* 2005). Recent evidence indicate that fasudil could also display therapeutic effect on controlling central nervous system disorders, such as Alzheimer's disease (Chen *et al.* 2013). Currently, the first generic inhibitor of AGC kinases, AT13148, is in clinical trials. It is a potent inhibitor of ROCK, PKA, and PKB/Akt kinases (Yap *et al.* 2012). The effectiveness of AT13148 has been demonstrated in case of ROCK-mediated functions in melanoma cells and in case of gastric cancer where PKB/Akt and other AGC family kinases are dysregulated (Sadok *et al.* 2015; Xi *et al.* 2016). Moreover, AT13148 has also the potential to reduce the abnormal activity of PKA. Recent studies have revealed the connection between

the adrenal Cushing's syndrome and the activity of PKA, where the L205R (Lysine205 → Arginine) mutation in the catalytic subunit promotes constitutive activation of the kinase (Cao *et al.* 2014). Therefore, inhibition of multiple AGC kinases may lead to even better anti-tumour activity and minimizes clinical resistance to the drug (Xi *et al.* 2016; Yap *et al.* 2012).

1.1.2. cAMP-dependent protein kinase

The cAMP-dependent protein kinase (PKA) was discovered and first characterised in 1968 (Walsh *et al.* 1968) and it has been thoroughly studied since. PKA is the best characterised PK, mainly due to its simplicity of production and relatively simple dissociative mechanism of activation, being therefore as a prototype for other PKs (Taylor *et al.* 2012). In addition, the catalytic subunit of PKA (PKAc) was also the first PK, whose crystal structure was solved, explaining the role of the highly conserved regulatory motifs and revealing the organization of PKs (Knighton *et al.* 1991a).

The activity of PKA is mainly controlled by an intracellular second messenger, cAMP. In the absence of cAMP, PKA exists as an inactive tetrameric holoenzyme complex, composing of two regulatory (PKAr) and two catalytic (PKAc) subunits. The binding of 4 cAMP molecules to the dimer of PKAr subunits leads to the reduction in affinity between PKAr and PKAc subunits and the holoenzyme dissociates into the dimer of the PKAr subunits and two catalytically active monomers of PKAc (Skålhegg and Taskén 2000).

The subunits of PKAc and PKAr are relatively small proteins (MW-s of 40 kDa and 50-57 kDa, respectively) and in the human genome, there are 5 genes encoding the PKAc subunits (PKAcα, PKAcβ, PKAcγ, PRKX, and PRKY) and four genes that encode the PKAr subunits (PKArIα, PKArIβ, PKArIIα, and PKArIIβ) (Cheng *et al.* 2001; Zhang *et al.* 2004; Hanks 2003; Tasken 2004; Turnham and Scott 2016). Both isoforms, PKArI and PKArII comprise a dimerization/docking domain, a region responsible for interaction with the PKAc subunit at the amino terminus, and two tandem cAMP binding sites at the carboxyl terminus (Taylor *et al.* 2004; Kim *et al.* 2006). To form a holoenzyme complex, the type II subunits are autophosphorylated by catalytic subunits and act as true substrates, whereas type I subunits are not phosphorylated and they act as pseudosubstrates by inhibiting the activity of PKA (Johnson *et al.* 2001; Taylor *et al.* 2012). The PKAc subunit is a globular protein with two lobes that are common for all AGC kinases. The smaller, amino terminal lobe is dominated by β-strands and is associated mostly with the binding of ATP. The larger, carboxyl terminal lobe mainly consists of α-helices and is involved in substrate/peptide binding and phosphoryl transfer (Taylor *et al.* 1999). PKAc has open and closed conformations and in order to be fully catalytically active, it needs to be phosphorylated at Thr197 and autophosphorylated at Ser338 (Johnson *et al.* 2001). Thereafter, active PKAc mediates cellular responses in the cytoplasm or translocates into the nucleus to phosphorylate nuclear proteins.

1.1.3. The signalling through PKA pathway and its regulation

PKA is a widely examined kinase responsible for phosphorylating a broad array of downstream substrates. It is considered as an essential regulator in many cell signalling events (*e.g.,* regulation of intracellular calcium concentration or gene transcription) (Tasken 2004; Turnham and Scott 2016). The PKA-based signalling pathway is known to be activated by a number of different hormones, neurotransmitters, and other signalling substances that bind to G-protein coupled cell-membrane receptors (GPCR) (Skålhegg and Taskén 2000). These extracellular stimuli activate the G-proteins, which then regulate the activation of adenylate cyclase (AC). The subsequent production of cAMP, which acts as the major activator of PKA, leads to the dissociation of regulatory and catalytic subunits. Thereafter, the activated catalytic subunits can catalyse the phosphorylation of a variety of cytosolic and nuclear substrates, including GSK, glycogen phosphorylase kinase, nuclear cAMP-response element-binding protein (CREB), cAMP-responsive modulator (CREM), and cAMP-dependent transcription factor ATF1 (Smith *et al.* 1999; Mayr *et al.* 2001; Johannessen *et al.* 2004a; Sassone-Corsi 2012).

CREB was one of the first transcription factors whose activity was shown to be regulated by phosphorylation (Shaywitz and Greenberg 1999; Mayr *et al.* 2001). It is a member of the closely related CREB/ATF1/CREM family of transcription factors, sharing high similarity in their amino acid sequences and functional domains (Mayr *et al.* 2001). CREB binds as a dimer to the cAMP-response elements (CREs) with the conserved TGACGTCA sequence, which is present in the promoter of many cAMP-responsive genes (Johannessen *et al.* 2004b; Carlezon *et al.* 2005). Phosphorylation of CREB allows association with the general transcriptional co-activators: CREB binding protein (CBP) and its paralogue p300. The formed complex interacts with RNA polymerase II, stimulating the transcription of DNA and production of protein (Mayr *et al.* 2001; Johannessen *et al.* 2004b). CREB (as well CREM and ATF1) can be phosphorylated by many kinases. It has potential phosphorylatable sites for several kinases, like Ser133 residue for PKA, CaMK, MAPKAPK (mitogen-activated protein kinase-activated protein kinase), and PKB/Akt; Ser142 for CaMKII; Ser98 for CaMKIV; and Ser129 for GSK3 kinase. Overall, more than 20 different PKs that have been shown to phosphorylate CREB (Johannessen *et al.* 2004a; Johannessen and Moens 2007).

Additionally, the signalling through PKA pathway can be regulated by the A-kinase anchoring proteins (AKAPs). AKAPs belong to a large family of proteins that influence subcellular localisation of PKA by interacting with the regulatory subunits. They bring specificity into cAMP-mediated signal transduction by placing PKA close to specific effectors and substrates. Specific AKAPs for both PKArI and PKArII subunits have been described, whereas PKArII subunits show considerably higher affinity than PKArI subunits (Tasken 2004). AKAPs can regulate PKA activation by bringing PKA near to the stimulation region or co-localise it with enzymes that are responsible for cAMP degradation

(*e.g.,* phosphodiesterases) or for the proteolysis of PKAr subunits (*e.g.,* calpain) (Shell and Lawrence 2012).

Natural inhibitors of PKA are important regulators of the activity of PKAc. These inhibitors that bind with high affinity and are specific to the PKAc belong to two protein families: the heat-stable protein kinase inhibitors (PKIs) and the PKAr-s (Johnson *et al.* 2001). Type I PKAr and PKI proteins comprise pseudo-substrate consensus sequences where the phosphoacceptor site is an alanine residue rather than serine or threonine. Also, both pseudosubstrates require two magnesium ions and ATP to form a tight complex with PKAc (Knighton *et al.* 1991b; Cheng *et al.* 2001). PKI inhibits PKAc in the presence of cAMP, while it mediates nuclear export of active PKAc, preventing its association with other substrates in signal transduction pathways (Fantozzi *et al.* 1992). PKAr subunits control PKAc activity depending on the levels of cAMP and supress the activity of catalytic subunit in the absence of secondary messengers (Johnson *et al.* 2001; Taylor *et al.* 2012).

1.2. Inhibitors of protein kinases

PKs have critical role in cell signalling pathways, whereby aberrant PK activity may lead to tumour formation. Therefore, PKs are important targets for cancer drug development by pharmaceutical companies (Cohen 2002; Knight *et al.* 2010). Intense efforts have been made to develop specific PK inhibitors as therapeutic agents or as biological tools for diagnosis and monitoring PK-related diseases in their early stages (Wang *et al.* 2007; Kasari *et al.* 2012). Drug development efforts have resulted in a remarkable success: more than 30 small-molecule PK inhibitors have reached the drug market (Rask-Andersen *et al.* 2014; Wu *et al.* 2015; Fabbro *et al.* 2015; Sharma *et al.* 2016). Encouraged by the progress, a large number of new inhibitors of PKs have been taken to various stages of clinical development (Gonzalez de Castro *et al.* 2013). The majority of kinase inhibitors are targeted towards tyrosine kinases (*e.g.,* Gleevec® - the first approved small-molecule tyrosine-kinase inhibitor) (Savage and Antman 2002), however serine/threonine kinases belonging to the AGC group are also of interest for pharmaceutical companies.

In the active site PKs possess specific binding sites for the substrate protein and ATP. Hence, the following active site-directed reversible inhibitors of PKs can be distinguished: ATP-site directed inhibitors, peptide/protein substrate-site directed inhibitors, and bisubstrate-analogue inhibitors. Additionally, allosteric inhibitors regulate kinase activity by binding to an allosteric site of the PK (Schwartz and Murray 2011).

A great majority of reported PK inhibitors are directed to the ATP-binding site. The first series of potent ATP-competitive inhibitors, isoquinolinesulfonamide derivatives (known as H-series inhibitors or Hidaka's inhibitors), was developed in early 1980s (Hidaka *et al.* 1984). Earlier compounds were relatively non-selective inhibitors, but further structural modifications of the com-

pounds led to more active and selective inhibitors (Ono-Saito *et al.* 1999). One of the compounds, H89, has been widely used as a PKAc-selective compound, while HA1077 (fasudil) is known as a selective inhibitor of ROCK kinase. Fasudil was also the first clinically applied PK inhibitor for the treatment of subarachnoid haemorrhage-induced cerebral vasospasm in Japan in 1995 (Asano *et al.* 1998; Tamura *et al.* 2005). In addition, several successful ATP-competitive inhibitors based on pyridine derivatives (*e.g.,* ROCK inhibitor Y-27632), aminofurazan derivatives (*e.g.,* PKB/Akt inhibitor GSK690693) and phenylpyrazole derivatives (*e.g.,* multi-AGC inhibitor AT13148) have been developed and taken to clinical trials (Uehata *et al.* 1997; Rhodes *et al.* 2008; Okumura *et al.* 2015; Xi *et al.* 2016; Nitulescu *et al.* 2016).

However, ATP-site targeted drugs are known to have selectivity problems as all PKs, together with other proteins of the purinome (3266 purine-binding proteins in human) bind purine (a structural constituent of ATP) and its derivatives (Haystead 2006; Knapp *et al.* 2006). Moreover, a serious disadvantage of ATP-competitive inhibitors is that they have to compete with high intracellular concentration of ATP (1–5 mM) (Beis and Newsholme 1975; Ando *et al.* 2012).

As the structure of peptide/protein substrate-binding domain of kinases is more variable than that of the ATP site, relatively selective peptide-based inhibitors have been described (Bogoyevitch *et al.* 2005; Harrison *et al.* 2008). However, in order to achieve high inhibitory potency, longer peptidic structures are needed. This is due to the nature of peptide/protein substrate-binding site where interactions appear to involve larger and less well-defined contact area. Resulting compounds possessing higher molecular weight lead to the problems with cellular transport and proteolytic stability (Bogoyevitch *et al.* 2005; Breen *et al.* 2014).

In recent years the bisubstrate approach has gained popularity for the construction of potent and selective inhibitors of PKs (Parang and Cole 2002; Lavogina *et al.* 2010a; Stebbins *et al.* 2011; Lamba and Ghosh 2012). Bisubstrate inhibitors (also known as bisubstrate analogue inhibitors) consist of two conjugated fragments, each targeting one binding site of a particular substrate. This approach enables the incorporation of additional interaction points into the inhibitor to afford contacts to a less-conserved peptide/protein substrate-binding site of the kinase (Lavogina *et al.* 2010a). Thus, the two important biochemical characteristics of a good inhibitor, affinity and selectivity, can be easily improved. However, the cellular transport of those compounds is still an obstacle to overcome.

1.2.1. ARC-based bisubstrate inhibitors

ARCs are conjugates of hydrophobic heteroaromatic fragments (binding to the ATP site of the PK) and peptide analogues (binding to the peptide/protein substrate site of the PK), connected via a linker chain with optimized structure (Figure 1) (Loog *et al.* 1999; Enkvist *et al.* 2006; Lavogina *et al.* 2009). The

acronym ARC is derived from the first generation of ARC-based bisubstrate inhibitors, called *Adenosine analogue (**A**) and arginine (**R**)-rich peptide conjugates (**C**)* (Enkvist *et al.* 2006). Although, far-reaching changes have been introduced to the nucleoside-mimetic and peptidic moieties, the acronym has been retained for the historical perspective.

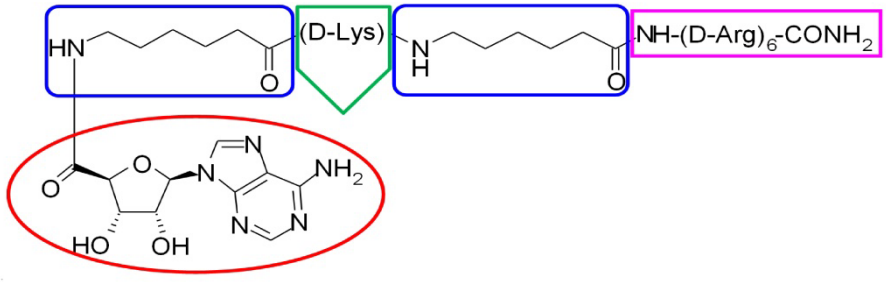


Figure 1. Example of a third generation ARC-based bisubstrate inhibitor (ARC-1028). The nucleosidic part of the inhibitor is surrounded with the red ellipse, the linkers are rounded by the blue rectangle, the chiral spacer is placed in the green polygon, and the peptidic part in the magenta rectangle (Lavogina *et al.* 2010a).

Extensive structure–affinity studies and X-ray analysis of ARC/PK co-crystals have been used to develop highly potent ARC-inhibitors towards several basophilic and acidophilic PKs (K_i values in low nanomolar or subnanomolar range have been obtained towards PKA, PKB/Akt, ROCK, PIM, and CK2 kinases) (Enkvist *et al.* 2006; Lavogina *et al.* 2009; Lavogina *et al.* 2012; Enkvist *et al.* 2012; Ekambaram *et al.* 2013). The first substantial structural change in the structures of ARC-based inhibitors, which significantly increased their affinity towards different basophilic PKs was the replacement of the L-amino acid residues with D-amino acids in the linker and peptidic moiety. This structural modification was also contributing to the stability of the conjugates towards proteolysis (Enkvist *et al.* 2006). Subsequently, better interactions with the target kinases was obtained by the introduction of a chiral spacer that tethered two flexible linkers (Lavogina *et al.* 2009; Pflug *et al.* 2010).

So far, the affinity and selectivity of ARC-inhibitors has been improved by varying the nucleoside-mimicking moiety and/or linkers with or without chiral spacers, while the oligoarginine peptide has served as a recognition fragment for basophilic PKs. Still, some important modifications have been introduced into the peptidic moiety. Recently, a conjugate for mitotic kinase Haspin was reported, that was comprising an aromatic fragment targeted to the ATP-site and a peptide mimicking the N-terminus of histone H3 targeted to the substrate protein site (Kestav *et al.* 2015). Moreover, the bisubstrate approach has been successfully used for the development of inhibitors of an acidophilic kinase CK2, where ATP-competitive fragment has been conjugated with peptides

comprising several aspartic acid residues or negatively charged peptoids (Enkvist *et al.* 2012; Viht *et al.* 2015).

ARC-inhibitors have found applicability in variety of assays, mainly due to their high inhibitory potency and affinity, whereas the later property is preserved after labelling of ARCs with fluorescent dyes or immobilizing to the chip surface of a biosensor (Viht *et al.* 2007; Vaasa *et al.* 2009). The arginine-rich transport peptides in the structure of ARCs give them cell plasma membrane-penetrative properties (Uri *et al.* 2002; Räägel *et al.* 2008). Additionally, intensive cellular uptake has been demonstrated for ARCs labelled with fluorescent dyes (ARC-Fluo probes), which supports the application of ARC-derived fluorescent probes for mapping and monitoring kinase activity in living cells (Vaasa *et al.* 2010).

1.2.2. Cellular transport of protein kinase inhibitors

In addition to the inhibitory potency, two other critical factors that affect the success of a compound as a PK-inhibitor-based cancer drug are its effective cellular accumulation and the appropriate intracellular localisation. Additionally, cell plasma membrane-permeable chemical inhibitors could be valuable tools for studying cellular functioning of PKs.

Small-molecule hydrophobic PK inhibitors have usually good cell plasma membrane permeability. For instance, the success of the cancer drug imatinib (Gleevec®) might be explained by its intense intracellular accumulation (Widmer *et al.* 2006; Lipka *et al.* 2012; Berglund *et al.* 2014), thereby the mechanism responsible for its intense uptake is still not clear (Nies *et al.* 2014). Because of unfavourable physico-chemical properties (negatively charged and polar) and low proteolytic stability of initial peptide-derived and phosphate-comprising bisubstrate inhibitors, their effect on living cells and cell plasma membrane permeability has been shown only in a limited number of publications in recent years (Räägel *et al.* 2008; Stebbins *et al.* 2011; Lamba and Ghosh 2012; van Wandelen *et al.* 2013).

One of the possibilities to overcome the issue of limited cellular transport of polar compounds is the application of cell-penetrating peptides (CPPs). CPPs are relatively short (< 30) amino acid sequences, capable for penetration of cell plasma membrane and transporting bioactive cargos (plasmid DNAs, oligonucleotides, siRNAs, therapeutic proteins/peptides, nanoparticles, *etc.*) into cells either in a covalent or non-covalent manner (Heitz *et al.* 2009; Koren and Torchilin 2012; Ramsey and Flynn 2015). Various protein derived (penetratin, Tat-peptide), chimeric (transportan), and synthetic (oligoarginines) transport peptide sequences have been described (Derossi *et al.* 1994; Vives *et al.* 1997; Pooga *et al.* 1998; Futaki *et al.* 2001), whereas most common CPPs are polycationic by the nature, followed by amphipathic and hydrophobic peptides (Milletti 2012).

Although the cell internalization of CPPs may depend of various characteristics of the compounds and the origin of the cells, at least two main cellular uptake mechanisms can be distinguished: direct membrane translocation and endocytic pathways (macropinocytosis, clathrin-mediated endocytosis, caveolae/lipid-raft-mediated endocytosis, and caveolae-independent endocytosis) (Duchardt *et al.* 2007). For cationic arginine-rich CPPs it has been suggested that endocytosis (macropinocytosis and clathrin/caveolae-mediated endocytosis) has major role in the cellular uptake, while direct cell plasma membrane penetration is dependent on peptide concentration and incubation conditions (Futaki 2006; Duchardt *et al.* 2007; Brock 2014).

Intracellular activity of the CPP transported cargo may be highly dependent on its endosomal escape. Therefore, a number of different strategies have been purposed for achievement of improved delivery of the cargos into cytoplasm or intracellular compartments, including lipidation of the CPPs (El-Sayed *et al.* 2009; Lee and Tung 2010; Erazo-Oliveras *et al.* 2012). For example, it has been demonstrated that oligoarginine peptides modified with fatty acid moieties (N-myristoylation or N-acylation with other fatty acids) possess much better cellular uptake than their non-acylated arginine-rich peptide counterparts (Nelson *et al.* 2007; Lee and Tung 2010; Lee and Tung 2012). Moreover, as the awareness of different transport mechanisms that initiate endocytic processes (*e.g.,* membrane receptor-mediated transport) has increased (Letoha *et al.* 2010; Kawaguchi *et al.* 2016), therefore considering those findings when designing new delivery systems that would be efficient for cellular uptake of arginine-rich CPPs is also important.

Some CPPs (bioportides) possess bioactivity on their own (by this definition arginine-rich ARCs are also bioportides, but although high inhibitory potency of ARCs towards PKs was disclosed much earlier than the term bioportide was introduced, ARCs have not been reported as bioportides in relevant literature) and therefore they are used directly as research tools or therapeutic/diagnostic agents (Vasconcelos *et al.* 2013; Lukanowska *et al.* 2013; Jones *et al.* 2016).

1.2.3. Characterisation of protein kinase inhibitors

In order to assess the potency of PK inhibitors mainly two types of biochemical assay can be distinguished: kinetic inhibition assays and equilibrium binding assays (Jia *et al.* 2008; Smyth and Collins 2009). Both assay technologies have found applicability in screening of PK inhibitors. However, depending on their advantages and drawbacks, some methods are more preferred than others.

Traditionally, kinetic inhibition assays were preferred for screening of PK inhibitors as they enable direct detection of phosphorylation of the substrate peptide/protein by a kinase of interest. PK inhibitors are characterised by determining their inhibitory effect on the rate of the kinase-catalysed phosphorylation reaction of the peptide/protein substrate, which can be monitored in different ways (Smyth and Collins 2009; Uri *et al.* 2010). For performing an

inhibition assay, main requirements include the availability of a suitable substrate for phosphorylation, sufficient catalytic activity of the kinase, and specific reagents for monitoring of the phosphorylation reaction (Lebakken *et al.* 2009). For quantification of the phosphorylated substrate and detection of the inhibition of the phosphorylation activity of kinase, common techniques involve radioactive [γ-^{32}P]-ATP, fluorescently labelled peptide substrates, specific phosphopeptide antibodies, or separation-based procedures for simultaneous monitoring of phosphorylated and non-phosphorylated substrates (Olive 2004; Jia *et al.* 2008; Smyth and Collins 2009). However, these methods can be expensive, time-consuming, and too complicated for development of high throughput screening (HTS) assays. Therefore, screening platforms which determine binding of the inhibitor to the PK not paying attention to the rate of PK-catalysed phosphorylation reaction have become increasingly popular (Lebakken *et al.* 2009; Vaasa *et al.* 2009; Rudolf *et al.* 2014).

Competitive binding assays are progressively used for screening PK inhibitors. Generally, these assays involve fluorescently labelled compounds (fluorescent probes/ligands) that bind to the ATP-binding site of the PK (Lebakken *et al.* 2007). Thereafter, either the direct binding of a labelled compound is measured or the displacement of the labelled probe from its complex with the kinase by an unlabelled inhibitor is examined (Vaasa *et al.* 2009; Ansideri *et al.* 2016). For detection of the binding (or displacement) of the probe, the changes in the photoluminescent characteristics (*e.g.,* intensity, lifetime, polarization) of the system are usually determined (Huang 2003; Lebakken *et al.* 2007; Uri *et al.* 2010; Slatter *et al.* 2013). These assays do not give information about the catalytic properties of the enzyme, but reveal whether binding of the probe would be disturbed in the presence of the inhibitor (Lavogina *et al.* 2010a). Most of fluorescence-based binding assays in active use are homogeneous and allow quick measurements in HTS format (Lea and Simeonov 2011). However, the applicability of these assays may be restricted if the reporter probes do not possess sufficiently high affinity towards the target kinase (Huang 2003; Vaasa *et al.* 2009). On the other hand, special heterogeneous assays based on the application of non-fluorescent detection techniques (*e.g.,* surface plasmon resonance) may have great value for performing precise measurements as well (Viht *et al.* 2007).

For the characterisation of inhibitors, the IC_{50} values are in wider use. In case of an inhibition assay IC_{50} value represents the concentration of the inhibitor that causes 50 % reduction of the enzymatic/catalytic activity of the PK at specific concentration of the substrate. For a displacement assay, IC_{50} value is the concentration of the inhibitor at which 50 % of the probe is displaced from its complex with the PK. In both cases, the IC_{50} value is dependent on the conditions used in the assay (*e.g.*, the origin of the substrate or probe, the concentration of the substrate or probe, and the concentration of the PK) and may therefore vary for a specific inhibitor in different experiments. In case of competitive inhibition, the IC_{50} values can be converted to comparable absolute

inhibition (K_i) or displacement (K_d) constants according to the Cheng-Prusoff equation (if [S] >> K_m or [L] >> K_D, respectively) (Cheng and Prusoff 1973):

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}} \quad (1), \qquad K_d = \frac{IC_{50}}{1 + \frac{[L]}{K_D}} \quad (2),$$

where [S] is the concentration of the substrate and K_m is the Michaelis constant of the substrate. [L] is the concentration of the probe and K_D is the dissociation constant of the complex between the probe and PK.

For binding/displacement assays more suitable equations have been disclosed, which also consider the effect introduced by the protein concentration in the sample (equation 3) (Nikolovska-Coleska *et al.* 2004):

$$K_d = \frac{IC_{50}}{1 + \frac{[L]_{50}}{K_D} + \frac{[P]_0}{K_D}} \quad (3),$$

where $[L]_{50}$ is the concentration of free probe at 50 % displacement, $[P]_0$ is the total concentration of PK, and K_D is the dissociation constant of the complex between the probe and PK.

1.3. Application of photoluminescence techniques in binding/displacement assays

Photoluminescence-based assay methods are widely applied for different biochemical as well biological applications. Photoluminescence is an optical emission of photons from molecule's electronically excited states after absorption of photons. Depending on the nature of the excited state, photoluminescence is formally divided into two phenomena: fluorescence and phosphorescence (Lakowicz 2006).

Following the light (photon) absorption, outer electrons of the luminophore molecules are excited from the ground state (S_0) to some higher vibrational levels of the excited singlet state (S_1 or S_2) (Figure 2). Thereafter, the molecule rapidly relaxes through vibrational relaxation (VR) or internal conversion (IC) to the lowest vibrational level of S_1. From the excited state S_1, the molecule can return to the ground state (S_0) either by emitting a photon in the form of fluorescence or by a radiationless mechanism, like energy dissipation as heat, collisional quenching, or photobleaching. Due to the non-radiative processes between different vibrational levels of excited states and ground states, the emission of the luminophore occurs at lower energies (longer wavelengths) and the difference between positions of the band maxima of the absorption and

emission spectra is called the Stokes shift. Additionally, the spin of an excited electron can be also reversed by intersystem crossing (ISC) to triplet state (T_1), followed by radiationless processes or emission of a photon termed as phosphorescence. As a result of the spin reversion, the electron in the excited orbital has the same spin orientation as the ground-state electron. Therefore, the transition from the triplet excited state to the singlet ground state is formally forbidden and proceeds on much slower time scale than fluorescence (Lakowicz 2006; Demchenko 2015).

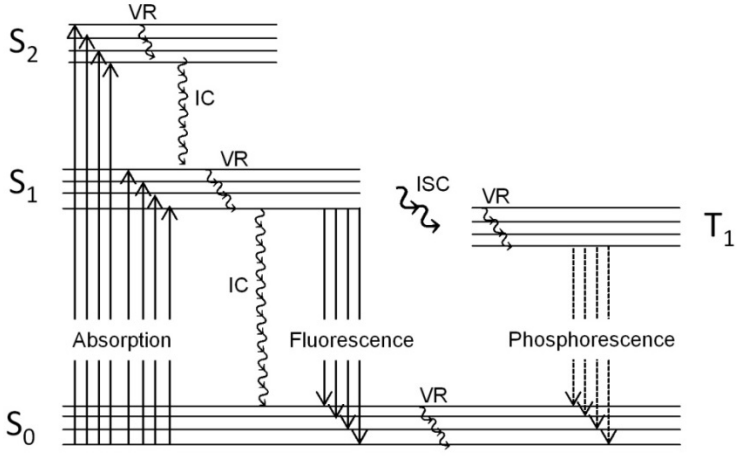


Figure 2. The Jablonski diagram for photoluminescence (Hemmilä 1991). The diagram illustrates electronic states of a molecule (luminophore) and transitions between them. When a luminophore absorbs light (photon), its electrons are excited from the ground state (S_0) to some higher vibrational levels of an excited singlet state (S_1 or S_2). The molecule rapidly relaxes through vibrational relaxation (VR) or internal conversion (IC) to lowest vibrational level of S_1. Thereafter, it can return to the ground state (S_0) either by emitting a photon in the form of fluorescence or without emission. The spin of an excited electron can also be reversed by intersystem crossing (ISC) to triplet state (T_1), which is followed by radiationless processes or emission of a photon termed as phosphorescence.

The lifetime (τ) of the excited state is defined by the average time the molecule spends in the excited state prior to return to the ground state. The value of τ depends on both the origin of the luminophore and its interactions with the local environment. Typical fluorescence lifetime is within the pico- to nanosecond range. As phosphorescence involves a spin forbidden process, the emission rates are slow and phosphorescence lifetimes are typically in the range of milliseconds to seconds (Lakowicz 2006).

The development of a potential drug candidate from a PK inhibitor is time-consuming and costly. Therefore, homogeneous photoluminescence-based binding/displacement assays are in an increasing demand for characterisation of

PKs and their inhibitors, allowing more accurate, cost-effective and HTS measurements. Most commonly applied techniques are based on the measurements of fluorescence intensity, fluorescence polarization/anisotropy (FP/FA), or efficiency (FI) of Förster-type resonant energy transfer (FRET, also used as Förster resonance energy transfer), whereas the measurements can be performed in a steady-state or time-resolved (TR) mode.

1.3.1. Fluorescence polarization/anisotropy

FP/FA-based measurements are widely used in biochemical applications for the analysis of protein-protein, protein-DNA, or protein-ligand binding events (Lakowicz 2006). They are based on the photoselective excitation of fluorophores by polarized light, where polarized light selectively excites those fluorophore molecules whose absorption transition dipole is parallel to the electric vector of the excitation. This selective excitation results in a partially oriented population of fluorophores, and in partially polarized fluorescence emission. The degree of polarization is determined by the measurements of the emission light intensity vertically ($\parallel$) and horizontally ($\perp$) to the excitation light plane, and is expressed as FP (P; equation 4) or FA (r; equation 5) (Lakowicz 2006).

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \qquad (4)$$

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \qquad (5)$$

The polarization and anisotropy are two representations of the same phenomenon and can be used interchangeably. The use of anisotropy is preferred as it is normalized by the total intensity.

FA of the fluorescent probe correlates with its rotation speed and thus it is dependent on the molecular weight of the fluorophore-containing complex. A free small-molecule fluorescent probe is rotating rapidly and displaying a low value of anisotropy. On binding of the probe to the target biopolymer (*e.g.,* PK) the size of the rotating unit increases, leading to a sharp rise in FA (Lakowicz 2006; Demchenko 2015).

Due to these features, FA-based measurements can be used for determination of binding affinity of the probe to the protein and for characterisation of unlabelled inhibitors in displacement assays (Iyer *et al.* 2008; Vaasa *et al.* 2009; Ansideri *et al.* 2016). However, the affinity of a fluorescent probe limits the range of the inhibitor affinities that can be tested in the displacement assay (Huang 2003) and it also settles the lowest concentration of the binding protein that can be used in the FA-based assay. Moreover, the level of autofluorescence and other factors that affect the rate of rotational diffusion or binding of the probe to target molecules in biological samples reduce the applicability of anisotropy-based assays.

1.3.2. Förster-type resonant energy transfer

Some of the abovementioned drawbacks of FA-based measurements can be succeeded with the application of FRET-based assays. FRET is a non-radiative energy transfer from an excited donor (D) molecule to an acceptor (A) molecule in the ground state (Lakowicz 2006). The latter chromophore can be excited by this transferred energy and as a result the emission intensity of the donor is reduced and, instead, emission intensity of the acceptor is increased. The rate of energy transfer depends upon the extent of spectral overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor, the quantum yield of the donor, the relative orientation of the donor and acceptor transition dipoles, and the distance (usually in the range of 20 to 90 Å) between the donor and acceptor molecules (Lakowicz 2006).

The transfer efficiency is typically measured by using either luminescence intensity (equation 6) or luminescence lifetime (equation 7) of the donor in the absence or presence of the acceptor:

$$E = 1 - \frac{FI_{DA}}{FI_D} \quad (6), \qquad E = 1 - \frac{\tau_{DA}}{\tau_D} \quad (7),$$

where the FI_{DA} and FI_D are the fluorescence intensities of the donor in the presence (DA) and absence (D) of the acceptor, and τ_{DA} and τ_D the luminescence lifetimes, respectively.

FRET-based binding/displacement assays describe whether the unlabelled inhibitors are capable of binding to PK by distribution of FRET between the fluorophores (fluorescently labelled PK and probe). It has been demonstrated that these studies can be efficiently performed in both biochemical as well cell-based assay formats with different PKs (Kwan *et al.* 2009; Vaasa *et al.* 2010; Manoharan *et al.* 2016). However, biological samples may still possess some disturbing background autofluorescence which interferes with the signal detection. Therefore, TR measurement techniques in combination with long-lifetime donors luminophores (*e.g.,* lanthanides) are utilized (Soini and Hemmila 1979; Li *et al.* 2008).

1.3.3. Time-resolved Förster-type resonant energy transfer

TR-FRET refers to FRET measurements which are performed in TR format. The difference of TR-FRET and the conventional steady-state FRET stems from the luminescence properties of the donor luminophore that possess long luminescence decay time in case of TR-FRET assays. Introducing a time delay (usually 50-150 microseconds) between the initial donor excitation with flash of radiation and measurement of acceptor emission, the non-specific nanosecond-

scaled background fluorescence is ceased leading to increased sensitivity and precision of the assay (Soini and Hemmila 1979).

Most commonly used donors in TR-FRET measurements are luminescent complexes (*e.g.,* chelates and cryptates) of lanthanide ions. They are widely used in various bioanalytical FRET methods (*e.g.,* homogeneous immunoassays) for monitoring biological binding events (Lebakken *et al.* 2009; Hagan and Zuchner 2011; Rajendran *et al.* 2013; Geissler *et al.* 2014). Furthermore, some of the lanthanide complexes are also successfully applied in several commercially available assays for detection of different biomarkers: TRACE®/ Kryptor® system of Brahms, HTRF® of CisBio, and Lanthascreen® of Invitrogen (Hemmilä and Laitala 2005; Moore *et al.* 2009).

Ions of lanthanides (*e.g.,* europium, terbium, samarium, or dysprosium) are trivalent cations and they display emission, which is the result of the formally forbidden transitions of the *f* electrons. Because of their low extinction coefficients ($\varepsilon \sim 1\ M^{-1}cm^{-1}$) in bioanalytical measurements lanthanides are usually not excited directly but rather through light-absorbing organic heterocycles in proximity to the lanthanide ion. These light-harvesting antennas increase extinction coefficients of the complex above 10,000 $M^{-1}cm^{-1}$ (Demchenko 2015). In addition, these complexes are important for protecting the ion from the interactions with the solvent and other possible quenchers of the luminescence in the buffer. Indirect excitation (or antenna effect) occurs through organic chelating ligands that after light absorption are excited to the singlet state, followed by the non-radiative energy transfer to their triplet state. Thereafter, the energy is transferred to the emitting lanthanide ion (Soini and Hemmila 1979; Bünzli and Piguet 2005). Lanthanide-chelator complexes exhibit luminescence lifetimes in the µs–ms timescale. Moreover, due to the structure of the lanthanide complexes and several ground states of lanthanide ions, they express very specific (narrow band line-type) luminescence emission bands and exhibit large Stokes shifts after excited in the near-UV region (Hemmilä and Laitala 2005; Bünzli and Piguet 2005). Hence, a wide selection of different acceptor fluorophores (organic dyes, fluorescent proteins, quantum dots, *etc.*) can be applied as FRET acceptors (Hötzer *et al.* 2012; Geissler *et al.* 2014).

In addition to the TR readout that eliminates background fluorescence, another advantage of using lanthanide complexes as FRET donors is the possibility of spectral isolation of lanthanide emission signals and performance of dual wavelength detection. This allows the normalization of acceptor emission, taking into account the sample interferences and assay medium variability (Mathis 1995).

1.4. Methods for measurement of protein phosphorylation and kinase activity in cells

Measurement of both protein phosphorylation extent and PK activity in cells is useful for evaluating the cellular uptake of PK inhibitors and describing biological activity of the compounds. If the inhibitor is capable for penetrating cell plasma membrane and its intracellular localisation is favourable for inhibition of activity of target PK, therefore several changes (*e.g.,* phosphorylation of substrate peptide/protein, protein production) in cell signalling pathways could be detected (Delghandi *et al.* 2005; Stebbins *et al.* 2011; van Wandelen *et al.* 2013).

A classical method for identification of phosphorylated proteins in signalling pathways involves the incubation of cells with $^{32}PO_4$ (^{32}P-ortophosphate). During the incubation, the cellular ATP equilibrates with ^{32}P, and the radiolabelled ATP is thereafter used by PKs for the phosphorylation of their substrates (de Graauw *et al.* 2006). Subsequently, the phosphorylated proteins are separated (*e.g.,* by SDS gel-electrophoresis) and the phosphorylation status of the protein is determined using scintillation counting. However, radiolabelled methods have many disadvantages, including the short half-life of ^{32}P, personal radioactivity risks, and environmental pollution, which has promoted the development of numerous non-radiometric assays.

Immunoblotting is a useful technique for identifying proteins and their phosphorylation states, providing an important tool for studying signal transduction pathways and activities of PKs in cells. One important requirement for a successful immunoblotting assay is that an antibody, either poly- or monoclonal, is available for the target protein or in case of phosphorylation, for phosphorylated tyrosine, threonine, or serine residues (Kaufmann *et al.* 2001). In addition, a secondary antibody, conjugated with specific enzyme [alkaline phosphatase (AP), horseradish peroxidase (HRP)] or a fluorescent dye is needed for visualization of the separated proteins.

In order to visualize PK activity and determine cellular inhibitory potency of PK inhibitors in living cells (and in real-time), several genetically encoded FRET-based biosensors have been created (Nagai *et al.* 2000; Zhang *et al.* 2001; Zaccolo 2004; Friedrich *et al.* 2010; Prével *et al.* 2014). Typically a FRET-based biosensor consists of a recognition element for the target PK (a substrate sequence and binding domain) which is fused to fluorescent proteins. Conformational changes in the biosensor report on activity of the target PK through changes in FRET efficiency (Zhou *et al.* 2012; Prével *et al.* 2014).

A-kinase activity reporter (AKAR) and the indicator of CREB activation due to phosphorylation (ICAP) are FRET-based biosensors that can be used for the determination PKA activity in live cells (Zhang *et al.* 2001; Zaccolo 2004; Friedrich *et al.* 2010). AKAR is a four-part chimeric protein consisting of cyan fluorescent protein, phosphoamino acid binding domain (14-3-3), substrate peptide (kemptide) that is phosphorylated by PKAc, and yellow fluorescent

protein. The phosphorylation of kemptide leads to the conformational change of the probe, bringing fluorescent proteins to closer proximity which results in increase of FRET intensity. Similar principle has been applied for imaging CREB activation in live cells, where the ICAP sensor reports phosphorylation of the Ser133 residue in the kinase-inducible domain (KID). To monitor CREB activation, the KID domain of CREB is fused with CREB interaction domain (KIX) of CBP, which specifically recognizes phospho-Ser133. Phosphorylation at Ser133 leads to a conformational change and subsequent FRET change.

PK activity in cells can be also detected with the aid of TR measurements in homogeneous cellular kinase assays (Saville *et al.* 2012; Hermanson *et al.* 2012). In this case a suitable PK substrate can be expressed as a fluorescent fusion protein and increase of the signal is detected when the lanthanide-labelled antiphospho-antibody binds to the phosphorylated product, permitting energy transfer from the lanthanide label to the acceptor fluorophore.

Reporter-gene assays, on the other hand, follow a general principle, where changes in intracellular signalling pathways (*e.g.,* cAMP concentration changes, PKA and CREB activation) are detected via changes in the expression level of a particular gene (the reporter-gene) (Jiang *et al.* 2008). In order to generate a reporter-gene assay for a single signalling cascade, a synthetic promoter containing a single type of transcription factor binding site, such as CRE, can be constructed (Hill *et al.* 2001). The transcription of the reporter-gene occurs when phosphorylated CREB binds to CRE. In order to measure transcription rates, numerous reporter systems have been developed in which production of β-galactosidase, green fluorescent protein, firefly luciferase, growth hormone, AP, or β-lactamase take place (Schenborn and Groskreutz 1999; Hill *et al.* 2001).

2. AIMS OF THE STUDY

The aims of the present study proceeded from the requirements of biomedical research. A great need remains for small-molecule regulators of the activity of PKs in cells and for monitoring systems of PK activity based on synthetic organic photoluminescent probes. Most important objectives for the study were following:

- Establishment of effect of the structure of adenosine-mimicking fragment of bisubstrate ARC-inhibitors on their inhibitory potency towards basophilic PKs that possess similar consensus sequence (PKAc, PKBγ, ROCK-II).
- Testing of new ARC-based photoluminescent probes in combination with europium-labelled antibodies targeted to the tag of the PK to work out a homogeneous binding assay for analysis of a specific PK in biological solutions using TR measurement mode of luminescence intensity.
- Characterisation of the applicability of ARC-based photoluminescent probes in biochemical assay formats as well in the complicated biological systems (live cells or cell lysates).
- Adaption of methods that enable monitoring of the phosphorylation of CREB in the cell nuclei for ARC-based inhibitors of PKs.
- Demonstration of ability of ARC-inhibitors to affect the PK-catalysed phosphorylation of proteins in living cells. Establishment of the structural elements of ARCs that affect their inhibitory potency and cell plasma membrane penetration properties.

3. MATERIALS AND METHODS

3.1. Peptide phosphorylation assay for determination of activity and inhibition of protein kinases

The peptide phosphorylation was carried out in 96-well polystyrene plates (40 µl reaction volume) thermostated at 30 °C. The reaction mixture contained 50 mM Hepes hemisodium salt (pH = 7.5), 10 mM magnesium acetate, 0.2 mg/ml bovine serum albumin (BSA), and 5 mM dithiothreitol (DTT). The kinase (PKAc or PKBγ), ATP and substrate (5-TAMRA-kemptide or 5-TAMRA-Ahx-RPRAATF, respectively) were added in various concentrations.

The phosphorylation reactions were initiated by the addition of ATP. At fixed time points, the reaction was terminated by a 7-fold dilution of aliquots (2 µl) from the incubation medium with 75 mM aqueous phosphoric acid. Obtained mixtures were analysed by thin-layer chromatography (TLC).

Samples (2 µl) were spotted onto a silica gel TLC plate 5–6 mm apart. The plate was dried over a hotplate (100 °C for 10–15 min) and developed with 1-butanol/pyridine/acetic acid/water (15/10/3/12 by volume). After drying, the fluorescence imaging of the plate was performed on a Molecular Imager FX Pro Plus (Bio-Rad; ex 532 nm, em 555 nm long-pass filter). The concentration of the phosphorylated substrate in the sample was calculated from the ratio of the integrated intensity of the substrate's spot to the sum of the intensities of the substrate spot and its phosphorylated product spot.

The inhibitory potency was determined in the presence of a concentration series of inhibitors (three-fold dilutions), and the inhibition curves were fitted to a sigmoidal dose–response model to yield IC_{50} values (corresponding to the concentration of the inhibitor decreasing the activity of the enzyme twofold).

3.2. Binding/displacement assays in time-resolved measurement mode

All biochemical binding/displacement experiments were performed on black low-volume 384-well nonbonding-surface microplates (cat. no. 3676, Corning). Experiments were performed in the assay buffer containing 50 mM HEPES hemisodium salt (pH = 7.5), 150 mM NaCl, 5 mM DTT, 0.5 mg/ml BSA, and 0.005 % Tween-20 with final volume of 20 µl. The binding assay was used for the determination of the concentration of active PK by titrating PK with ARC-Lum probe. 3-fold dilutions of PK were made in the assay buffer and the fixed concentrations of luminescent probe were added to each well. The microplates were incubated for 15 min at 30 °C before each measurement. After incubation, the luminescence intensities were measured and the results were analysed with GraphPad prism 5.0 (GraphPad Software), using non-linear regression analysis (equation 8):

$$TGL = B + M\frac{\left[L_t + K_D + kE_0 - \sqrt{\left(L_t + K_D + kE_0\right)^2 - 4L_t kE_0}\right]}{2} \quad (8),$$

where B is the background signal; M is the luminescence intensity of the PK/ARC-Lum complex; L_t is the total concentration of ARC-Lum probe; E_0 is the nominal concentration of the kinase; K_D is the dissociation constant between ARC-Lum and PK; and k is the fraction of the active kinase.

The displacement assay was performed by adding the fixed concentration of ARC-Lum probe in the complex with PK to the concentration series of the competitive compound (3-fold dilutions) in the assay buffer. After 15 min incubation time at 30 °C, the luminescence intensity was measured and the results were fitted to a sigmoidal dose-response model with GraphPad prism software to obtain IC_{50}-values. Displacement constant K_d values were calculated according to equation 2 or equation 3 (Cheng and Prusoff 1973; Nikolovska-Coleska *et al.* 2004).

For both, the binding and displacement assays, luminescence measurements were performed with a PHERAstar platereader (BMG Labtech) using 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] in the TR fluorescence measurement mode (50 μs delay time and 150 μs acquisition time).

3.3. Transient transfection and luciferase assay

For monitoring the activity of PKA in cell nuclei that leads to the phosphorylation of CREB at Ser133 resulting in increased luciferase expression and activity, the cells were transfected with pGAL4-CREB and p$(GAL4)_5$-E1b-Luc plasmids by using transfection reagents Lipofectamine2000 (Invitrogen) or ExGen500 (Fermentas) according to the instructions from the manufactures. After treating the cell cultures with transfection mixtures for 3-4 h, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and then serum-starved for 20–24 h in 0.2 % serum until the performance of the experiment.

One day after the transfection, the growth medium from each well was displaced with a fresh medium, free of serum and antibiotics. Thereafter, the cells were incubated with different inhibitors at desired concentrations at 37 °C and 5 % CO_2 for 1 h. Thereafter, forskolin at an appropriate concentration was added and the cells were incubated for 3 h. After incubation, the reaction mixture was removed and the cells were washed carefully with DPBS. Thereafter, the lysis buffer containing TROPIX Lysis solution (Applied Biosystems) and 0.5 mM DTT or Cell Lysis Buffer (Invitrogen) with 1x Protease Inhibitor Cocktail, 1 % Triton-X, and 0.5 mM DTT was added to the cells. The cell lysates were scraped, transferred into microtubes, and centrifuged for 2 min at 15 000 g.

For measuring of the activity of luciferase in cell lysates, a Luciferase Reporter 1000 Assay System (Promega) or the solution containing 100 μM D-luciferin monopotassium salt (Thermo Scientific) in 30 mM tricine buffer (pH = 7.8) with 8 mM magnesium acetate and 1.5 mM ATP was used. Luciferase activity was assessed by using Labsystems Luminoscan RT luminometer (em 560 nm) or PHERAstar platereader with optical module [em 515 (30) nm, em 410 (80) nm], respectively.

3.4. Immunoblot analyses

All immunoblot analyses were performed with extracts obtained from the cells grown on 12-well plates. The day before the experiment, the cells were serum-starved in 0.2 % serum for overnight. The cultured cells were treated with different inhibitors for 1 h and with forskolin for 30 min. After that, the cells were washed twice with DPBS, harvested in lysis buffer [40 μL, DTT (0.1 M), NuPAGE LDS Sample Buffer (1x), and ddH_2O], heated to 70 °C for 10 min, and sonicated two times for 3 s. Samples were analysed by gradient or 10 % SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.45 μm polyvinylidene difluoride (PVDF) membrane. The blots were blocked with TTBS buffer [Tris-HCl (100 mM, pH = 7.4), Tween-20 (0.1 %), NaCl (0.9 %)] containing BSA (3 %) for 1 h at room temperature. Thereafter, the blots were incubated with primary antibodies, phospho-CREB Ser133 (Santa Cruz) and α-tubulin (Abcam), overnight at 4 °C in TTBS buffer containing BSA (1 %). After overnight incubation, blots were washed with TTBS buffer and incubated for 1 h at room temperature with AP-conjugated anti-rabbit (AnaSpec) and anti-mouse (Applied Biosystems) antibodies. After washing with TTBS, the blots were equilibrated with AP substrate buffer [Tris-HCl (100 mM, pH = 9.5), NaCl (100 mM), $MgCl_2$ (5mM)] and visualized with a NBT (BioChemica) and BCIP (Thermo Scientific) substrate mixture. The blots were scanned with CanoScan LiDE 35 scanner and analysed with ImageJ software.

4. RESULTS AND DISCUSSION

4.1. Characterisation of high-affinity bisubstrate-analogue inhibitors of AGC kinases

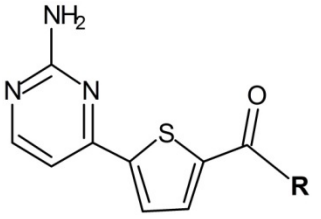
The importance of PKs in cell signalling pathways and their involvement in various pathological processes has made PK inhibitors into important therapeutic agents. In addition, the need for more effective screening assays for discovering PK inhibitors as well for detecting the cellular functioning of PKs has increased. Numerous approaches and strategies describe how to target PKs and design a potential drug (Sharma *et al.* 2016). In the present study a structure-guided design was followed to improve the properties (affinity and selectivity) of ARC-based inhibitors towards AGC kinases. This activity was aimed to obtain potent compounds for the development of biochemical assays that would allow more accurate measurement of the activity of PKs, and screening and characterisation of PK inhibitors.

Previously obtained 3-D structures of ARC/PK co-crystals have served as the basis for the development ARCs of the third generation (Figure 1). These ARCs incorporate a chiral spacer and two flexible organic linkers. They possess nanomolar affinity towards several basophilic PKs (Lavogina *et al.* 2009). In the present study, we aimed to increase the potency of ARC-based inhibitors to the subnanomolar region. Therefore, a previously described compound Adc-Ahx-DLys-Ahx-$(DArg)_2$-NH_2 (ARC-1012) was used as the lead compound and Adc (adenosine 4'-dehydroxymethyl-4'-carboxylic acid) moiety was substituted by different structural fragments (**Paper I** Table 1). The choice of new fragments was made based on the previously reported co-crystal structures of the compounds that bind to the ATP pocket of PKs (Lin *et al.* 2006; Lavogina *et al.* 2009).

To compare the newly synthesized compounds as inhibitors of PKs, their inhibitory potency or affinity towards several PKs (PKAc, PKBγ, and ROCK-II) was determined (**Paper I** Table 1). A TLC-based kinetic method (Viht *et al.* 2005) enabling the separation of the substrate peptide 5-TAMRA-Kemptide (5-TAMRA-Leu-Arg-Arg-Ala-Ser-Leu-Gly) and its phosphorylated counterpart was used for characterisation of inhibitors of PKAc. To assess kinase activity and evaluate the inhibitors for PKBγ, a new substrate peptide Arg-Pro-Arg-Ala-Ala-Thr-Phe was chosen for measurements. The TLC-based method was adapted for measurements with the 5-TAMRA derivative of the latter peptide. A FA-based binding/displacement assay was applied for determination of affinity of the inhibitors towards ROCK-II (Vaasa *et al.* 2009).

ARC-1102, the conjugate of AMTH [5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid] (Table 1) revealed the highest inhibitory potency of the tested compounds towards all PKs. Although other compounds showed some selectivity towards certain kinases, in general they were still 30–500-fold weaker inhibitors than their Adc-comprising counterpart (**Paper I** Table 1).

Table 1. Conjugates of 5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid (AMTH)

Nucleoside mimicking fragment	R	Code
	-Ahx-DLys-Ahx-$(DArg)_2$-NH_2	ARC-1102
	-Ahx-DLys-Ahx-$(DArg)_6$-NH_2	ARC-664
	-Ahx-DAla-Ahx-$(DArg)_6$-DLys-NH_2	ARC-663

Thereafter, ARC-1102, the inhibitor with the highest inhibitory potency in the set of tested compounds was taken for further structural modifications (Table 1). Variation of the chiral spacer as well as the peptide part of the conjugate caused similar effect on potency of the compounds (Table 2) as these modifications had caused to the inhibitory potency of their Adc-comprising counterparts (Lavogina *et al.* 2009). The conjugates with six D-arginine residues (ARC-664 and ARC-663) were 10–25-fold more potent than the compound comprising two D-arginine residues (ARC-1102). Moreover, as PKAc equally well tolerates several D-amino acids (D-alanine, D-lysine, or D-arginine) in the role of the chiral spacer, ARC-664 with D-lysine spacer and ARC-663 with D-alanine spacer revealed similar inhibitory potency towards PKAc. However, PKBγ, which prefers larger basic amino acids (D-lysine and D-arginine) demonstrated 3.5-fold higher potency of ARC-664 compared to ARC-663. Hence, D-alanine as the chiral spacer also improved the selectivity of the conjugate towards PKAc.

Table 2. Inhibitory potency of conjugates of 5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid (AMTH) towards PKAcα and PKBγ

Compound	Inhibition of PKAcα		Inhibition of PKBγ	
	IC_{50} (nM)	pIC_{50} ± SE	IC_{50} (nM)	pIC_{50} ± SE
ARC-1102	82	7.08 ± 0.15	270	6.57 ± 0.14
ARC-664	5.5	8.26 ± 0.27	12	7.92 ± 0.30
ARC-663	4.9	8.31 ± 0.23	42	7.38 ± 0.19

Standard error (SE; 95 % confidence interval)
1000 μM ATP (K_m = 20 μM) was used in kinetic assay of PKAcα and 100 μM ATP (K_m = 100 μM) was used in kinetic assay of PKBγ.

The compounds comprising synthetic non-natural fragments and D-arginine residues are expected to be resistant to enzymatic degradation (Elmquist and Langel 2003; Enkvist *et al.* 2006) and thus have great potential for application in experiments with living cells and tissues. In addition, higher affinity of those

compounds gives the opportunity and advantages to apply them for construction of photoluminescent probes that could be used in binding/displacement assays for the measurement of concentration of PKs and screening of inhibitors of PKs. Because the lowest inhibitor K_d value that can be resolved in an FA-based binding assay is approximately equal to the K_D value of the probe, inhibitors with low-nanomolar affinity can be used for characterisation of non-labelled inhibitors with affinities in whole nanomolar and micromolar range (Huang 2003; Vaasa *et al.* 2009). Moreover, as the selectivity panel of PK inhibition showed that ARC-664 efficiently inhibited most of the tested basophilic kinases, a single ARC-based probe can be used for testing of several PKs.

Although homogeneous, rapid, and inexpensive binding-based bioanalysis methods have great potential as tools for HTS of small-molecule inhibitors, the "classical" kinetic inhibition assays maintain their importance as they give the direct information about the PK-catalysed phosphorylation reactions. However, Vaasa *et al*. have demonstrated good linear dependency (R^2 = 0.95) between the inhibitory potency (IC_{50} as measured by the TLC-based kinetic inhibition assay) and affinity (K_d as determined in FA assay with an ARC-based fluorescent probe) of various inhibitors of PKAc (Vaasa *et al.* 2009). In the course of the present study, the linear dependency (R^2 = 0.97) was also shown between the inhibitory potency and affinity of different ARC-inhibitors and H89 for PKBγ (Figure 3 unpublished data). Therefore, binding/displacement assays can be efficiently used for characterising PK inhibitors and support the further usage of those tested compounds as inhibitors or regulators of protein phosphorylation balances in live cells.

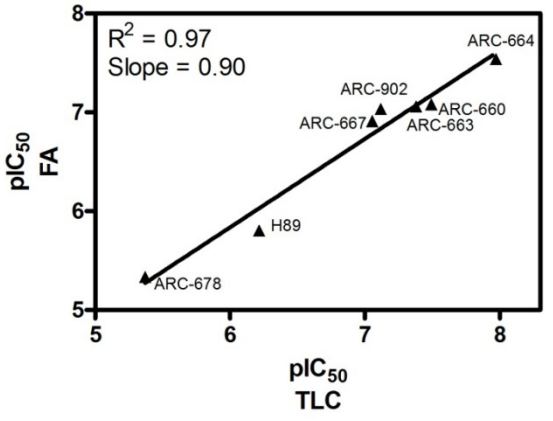


Figure 3. Correlation between the inhibitory potency (pIC_{50} TLC, measured with TLC-based kinetic inhibition assay) and affinity (pIC_{50} FA, measured with the FA-based binding/displacement assay) for various inhibitors towards PKBγ. The data set contains H89 (well-characterised commercially available ATP-competitive inhibitor), ARC-902 (II generation Adc-containing ARC-based inhibitor) (Enkvist *et al.* 2006), ARC-663 and ARC-664 (AMTH-containing ARC-based inhibitors) (**Paper I**). Structures of three tested ARC-inhibitors (ARC-678, ARC-667 and ARC-660) have not been disclosed.

4.2. Development and application of bioassays based on photoluminescent probes

4.2.1. Discovery of ARC-Lum probes

Many photoluminescence-based assays have been disclosed for determination of PKs as specific biomarkers or for screening and characterisation of small-molecule inhibitors (Lebakken *et al.* 2007; Kwan *et al.* 2009; Uri *et al.* 2010; Morris 2013). The development of biochemical assays based on the application of ARC-based photoluminescent probes has moved along together with the improvement of affinity of ARCs towards PKs. ARC-Fluo probes have been successfully used in FA-based assays for determination of concentration of different basophilic PKs or characterisation of PK inhibitors possessing affinity in micromolar and nanomolar range (Vaasa *et al.* 2009; Uri *et al.* 2010; Lavogina *et al.* 2012). ARC-Fluo probes have been also successfully applied for FRET-based assays in pair with PKs that were either chemically labelled with fluorescent dyes or expressed as fusions with a fluorescent protein (Vaasa *et al.* 2010; Manoharan *et al.* 2016). Moreover, labelling of ARCs with lanthanide (*e.g.,* terbium) chelates or cryptates resulted in photoluminescent probes, whose emission decay time is in the range of 100–2000 μs, which is suitable for the TR measurement format (Uri *et al.* 2010).

An europium chelate-based binding assay for PKs using TR-FRET measurement format has been described (Lebakken *et al.* 2009). The assay works with recombinantly tagged kinases and requires the application of antibodies that bind to the tag. A drawback of the assay is the usage of fluorescently labelled ATP-competitive kinase inhibitors as the tracers (fluorescent probes), thus, the assay is suitable for characterisation of the compounds that bind to ATP-binding site (or to an allosteric site that changes the conformation of the ATP site).

An advantage of ARC-probes in binding/displacement assays is their bisubstrate nature that makes possible their application for characterisation of inhibitors that are targeting binding sites of both substrates of the PK (Vaasa *et al.* 2009; Lavogina *et al.* 2010b). In order to analyse PKs in complicated biological samples a TR-FRET-based three-component assay system with the application of ARC-probes and anti-tag antibodies labelled with organic chromophore antenna-sensitized europium cryptates was taken under study (Figure 4).

For the development of a TR luminescence-based bioassay, new high-affinity ARC-inhibitors comprising AMTH moiety were used (Table 1). Substituting the chiral spacer D-alanine in ARC-663 with D-arginine gave the compound ARC-668 [AMTH-Ahx-DArg-Ahx-$(DArg)_6$-DLys-NH_2]. ARC-668 was labelled at D-lysine residue with the fluorescent dye Alexa Fluor 647, leading to the labelled compound ARC-1063 (**Paper II** Figure 1B). The latter compound (FRET acceptor) was used for the development of a homogeneous three-component analysis method, using it together with 6His-tagged recombinant human PK PKBγ (Akt3) and europium-labelled anti-6His antibody

(FRET donor) (Figure 4). In case of simultaneous association of both europium-labelled antibody and Alexa Fluor 647-labelled ARC with the kinase, emission signal with long decay time was awaited at 675 (50) nm upon pulse-excitation of the solution at 337 (50) nm.

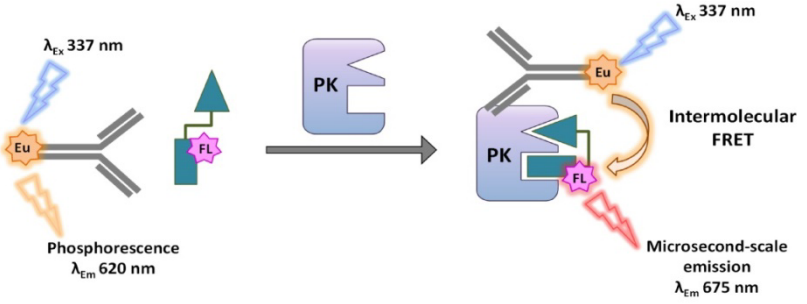


Figure 4. Schematic presentation of a homogeneous TR-FRET-based protein kinase (PK) assay with the application of Alexa Fluor 647-labelled ARC-probe (FL) and europium-labelled antibody (Eu). Simultaneous association of europium-labelled antibody and fluorescently-labelled ARC-probe with the PK leads to the formation of triple complex and intermolecular FRET. After pulse-excitation of the formed complex at 337 (50) nm, acceptor's (FL) emission signal intensity with long decay time increases at 675 (50) nm, while donor's (Eu) emission signal intensity decreases at 620 (20) nm.

However, we discovered a strong luminescence signal [measurements performed with 50 μs delay time after pulse-excitation at 337 (50) nm] if ARC-1063 was titrated with PKBγ, without the presence of the europium-labelled antibody (Figure 5).

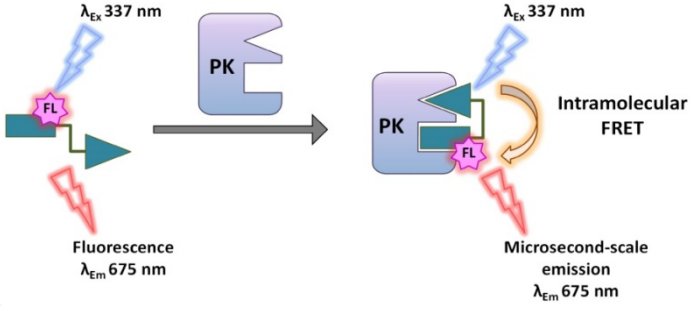


Figure 5. Schematic presentation of a homogeneous ARC-based assay for determination of protein kinases (PKs). Upon pulse-excitation at 337 (50) nm of free Alexa Fluor-647 labelled ARC-Lum probe (FL) a short-lived fluorescence signal of acceptor dye is detected at 675 (50) nm. Binding of probe with PK leads to the intramolecular FRET after pulse-excitation at 337 (50) nm and photoluminescence with microsecond-scale decay time is emitted at 675 (50) nm.

Closer examination of this phenomenon showed that the measured signal was directly proportional to the concentration of the complex of ARC-1063 and PKBγ and the displacement of ARC-1063 from the complex by competitive inhibitors decreased the luminescence signal in the concentration-dependent manner. Novel organic protein binding-responsive long life-time photoluminescent probes (ARC-Lum probes) were discovered.

4.2.2. Photoluminescence signal of ARC-Lum probes

Upon binding to a PK and excitation with a pulse of near-UV radiation ARC-Lum probes produce long lifetime (τ = 19–266 μs) photoluminescence signal, whereas free probe gives negligible long-lifetime emission signal (Figure 5). ARC-Lum probes are divided into two categories: ARC-Lum(-) and ARC-Lum(Fluo) probes, where the latter compounds are fluorescent dye labelled counterparts of the corresponding ARC-Lum(-) probes.

During these studies we observed that ARC-Lum(-) probes possess weak photoluminescence (phosphorescence) signal at 500–650 nm in complex with a PK, which is more than 1000-fold weaker compared to the luminescence of the respective ARC-Lum(Fluo) probes. This effect could be explained with the intramolecular FRET between the thiophene-comprising fragment (donor phosphor, D) and conjugated fluorescent dye (acceptor fluorophore, A) of an ARC-Lum(Fluo) probe (**Paper II** Figure 1D). The thiophene-comprising fragment has an absorption maximum at 340 nm. Flash-excitation of the thiophene-comprising fragment at 337 (50) nm while bound to the active site of the PK leads to the excited singlet state $S(D)_1$*, followed by ISC to the excited triplet state $T(D)_1$* of the fragment. Thereafter, FRET from donor phosphor $T(D)_1$* to the singlet state of the acceptor fluorophore $S(A)_1$ takes place, which leads to the excited singlet state of the acceptor $S(A)_1$*. Due to the forbidden energy transfer from $T(D)_1$* to fluorophore $S(A)_1$, slow emission of light from the excited fluorophore occurs.

Luminescence signal is largely intensified through efficient energy transfer and is dependent on the quantum yield and molar absorption coefficient of the fluorescent dye and spectral overlap of the donor phosphorescence emission spectrum with the absorption spectrum of acceptor fluorescent dye. Here we demonstrated that fluorescent dyes (*e.g.,* HilyteFluor 488, TAMRA, Alexa Fluor 647, PromoFluor-647) with strong absorption in the wavelength range of 500–650 nm could be used as effective luminescence-sensitizing acceptors (**Paper II** Supplementary Figures 3, 4A, and 5A). Additionally, it was determined that the acceptor-mediated amplification of the luminescence signal of the donor also takes place intermolecularly. PromoFluor-647-labelled PKAc was used together with different ARC-Lum(-) probes and energy transfer was detected when donor phosphor moiety of ARC-Lum(-) probe and the PromoFluor-647 fluorescent label of the kinase were in close proximity due to the binding event (**Paper II** Figure 2D).

The formation of the PK-induced luminescence signal was also demonstrated by changing the sulfur atom in the conjugates with heavier selenium atom. Selenium atom in the aromatic system increases the probability of the energy transfer to the triplet state after photon absorption and the rate of triplet to singlet energy transfer (Kuijt *et al.* 2003), resulting in a higher intensity of the emitted light and its shorter lifetime (**Paper II** Table 1). Selenophene-comprising conjugates of ARC-Lum(-) probes gave a 30- to 100-fold stronger phosphorescence signal compared to the thiophene-comprising analogue, pointing to the positive heavy-atom effect. Moreover, the phosphorescence signal was strong enough to be used for the measurement of the concentration of the probe/PK complex. However, it was still 20- to 100-fold weaker than luminescence intensity of the corresponding ARC-Lum(Fluo) probes. No long lifetime luminescence signal was detected for adenosine-comprising ARCs labelled with fluorescent dye.

ARC-Lum probes gave a long-lifetime luminescence signal with all the tested PKs of the AGC group, whereas different PKs complexed with ARC-Lum probes led to considerable differences in the intensities and lifetimes of emitted light (**Paper II** Figure 2A and B, Table 1). These differences may come from the structure of the active centre of PKs, which behave as a binding, fixing, and protecting framework for ARC-Lum probes, and higher the rigidness of the binary complexes, the greater the protection of the probe from quenching effect of dissolved molecular oxygen and the buffer components.

4.2.3. Characterisation of protein kinases and their inhibitors in biochemical assays

The great value that ARC-Lum probes hold due to their binding-responsive photoluminescence properties is that they could be effectively used for the determination of activity of basophilic PKs, characterisation of inhibitors of PKs, and as cAMP sensors in biochemical as well in biological assay systems. The measurements are performed with a microsecond-scale time delay after pulse-excitation of the probe. Therefore, the measured signal is free from the nanosecond-scaled background fluorescence of organic compounds and autofluorescence of cells. Moreover, as ARC-based bisubstrate inhibitors efficiently bind only to the active form(s) of PKs, ARC-Lum probes can be used for measuring the concentration of the active form of the kinase in the sample. Binding/displacement assays based on the use of ARC-Lum probes are simple, quick, and do not need any additional reagents (*e.g.*, substrates or antibodies). In addition to TR measurement of luminescence, ARC-Lum(Fluo) probes can be in parallel applied for FA- or FI-based steady-state measurements.

The affinity of the probe towards PKs (K_D) or the activity (active fraction) of PKs (k) can be determined by performing titration of the ARC-Lum probe with the PK (**Paper II** Figure 2A, B) and fitting the data to the equation 8, similarly to the FA-based assay with ARC-Fluo probes (Vaasa *et al.* 2009). Additionally,

as free ARC-Lum probe does not give long-lifetime photoluminesence signal, it was possible to perform the titration of the fixed concentration of protein with varying concentration of the probe (**Paper II** Supplementary Figure 5A). The usability of both titration variants was confirmed by comparable K_D values.

The ARC-Lum-based displacement assay can be used for characterisation of inhibitors for several PKs, like PKAc, PKBγ, ROCK-II. The intensity of PK-induced long-lifetime photoluminescence signal was reduced by various ATP-competitive, protein substrate-competitive, or bisubstrate inhibitors. The obtained displacement constant (K_d) values determined with the ARC-Lum-based displacement assay were in good agreement with the inhibition constant (K_i) values determined using the TLC-based kinetic inhibition assay (R^2 = 0.92; Figure 6A).

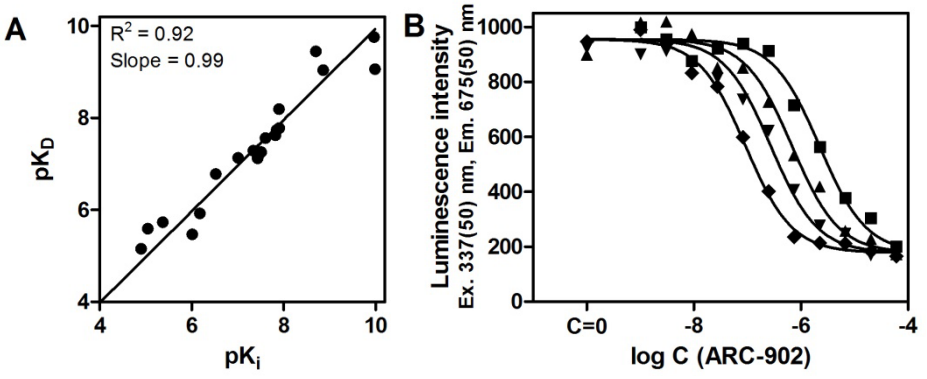


Figure 6. Displacement of ARC-Lum(Fluo) probes from the complex with PKs by various inhibitors. (**A**) Correlation between the values of inhibition constant (K_i) measured in TLC kinetic inhibition assay and the values of displacement constant (K_d) determined in displacement assay with the probe ARC-1063 for various PKAc inhibitors. (**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.

The absence of long-lifetime photoluminescence signal of free ARC-Lum probe gives the opportunity to use the probe at relatively high concentration in the displacement assays. Therefore, if [L] >> K_D, the results of the measurements do not depend on the concentration of active PK and the K_d values for competing inhibitor can be calculated from displacement curves (Figure 6B) according to the Cheng-Prusoff equation (equation 2) (Cheng and Prusoff 1973). Furthermore, high concentration of the probe allows determination of affinities for highly potent compounds by shifting their displacement curves away from the tight-binding region (Copeland 2005). Additionally, it is possible to apply a single ARC-Lum probe for experiments with PKs possessing different binding properties. Even if the probe has low affinity towards the PK, increasing con-

centration of the probe ensures that sufficient fraction of the kinase is still bound to the probe.

4.2.4. Characterisation of protein kinase activity in live cells and cell lysates

Methods enabling the monitoring of activity of PKs in complicated biological solutions, such as blood serum, cell lysates, tissue extracts, and living cells, have great value for biological research. ARC-Lum probes have the potential for becoming useful tools in this field of study, as they do not need fluorescently labelled target proteins for monitoring changes in activity of PKs in native biological systems.

In the present study human embryonic kidney cells (HEK293) possessing native expression level of PKA were incubated with solutions of thiopehene- or selenophene-comprising ARC-Lum(Fluo) probes (c = 10 μM). After removal of excess of the probe, intracellular localisation of the probe was followed with a fluorescence microscope (Figure 7). The localisation of the ARC-Lum(Fluo) probe was similar to that of ARC-Fluo probes (Vaasa *et al.* 2010). Fluorescently labelled ARC-Lum probes efficiently penetrated the cell plasma membrane and located into the cytoplasm and nucleus of the cell. Similarly to ARC-Fluo probes, ARC-Lum(Fluo) probes showed tendency to concentrate in nuclear subdomains, apparently nucleoli.

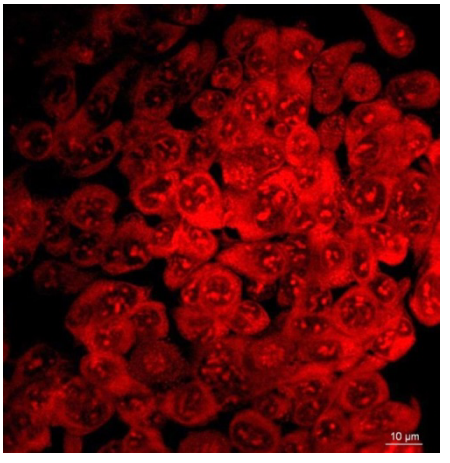


Figure 7. Cellular uptake and localisation of ARC-1139 [selenophene-containing ARC-Lum(Fluo) probe] in HEK293 cells. HEK293 cells were incubated with 10 μM ARC-1139 at 37 °C for 1 h.

Activation of PKA by forskolin (the activator of AC) in HEK293 cells led to an increased level of free catalytic subunit PKAc whose association with ARC-Lum(Fluo) probe resulted in elevated long-lifetime luminescence intensity

signal (Figure 8A, B). Furthermore, even bigger effect was demonstrated in case of Chinese hamster ovary cells, C9H6, that are recombinantly overexpressing both subunits of PKA (Figure 8A). Increased long-lifetime luminescence intensity was also achieved through activation of β-adrenergic receptors natively expressed in HEK293 cells with the agonist isoproterenol (Figure 8B). A cell-permeable ATP-competitive PKAc inhibitor H89 reversed the effect of both compounds (Figure 8A, B). The obtained results point to ARC-Lum probes as promising sensors for real-time monitoring of cAMP concentration in live cells.

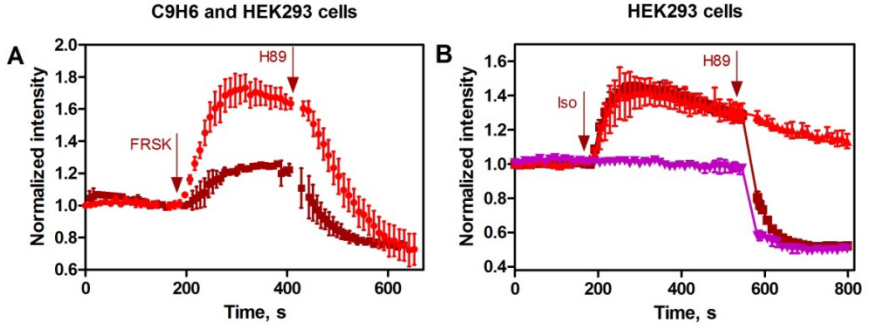


Figure 8. Measurement of long-lifetime luminescence of ARC-Lum(Fluo) probes in biological systems. Real-time monitoring of PKA activity in HEK293 cells (**A**: ■; **B**) and C9H6 (**A**: ●) cells using intracellular ARC-1139. Long-lifetime luminescence of cells on the bottom of the wells of a microtiter plate was detected with a PHERAstar platereader (λ_{ex} = 337 (50) nm, λ_{em} = 675 (50) nm, delay time = 50 μs). Time points of addition of forskolin (FRSK, 25 μM; A: ■, ●), isoproterenol (Iso, 10 μM; B: ■, ▲), and H89 (100 μM; A: ■, ●; B: ■, ▼) are marked with arrows.

Lower concentrations of ARC-Lum probes were needed for monitoring the PK activity in cells after their lysis (**Paper II** Figure 5D). Forskolin increased the long-lifetime luminescence signal in a concentration-dependent manner compared to nonactivated cells, whereas specific PKA inhibitor PKI reduced the signal by displacing ARC-Lum probe from its complex with the PKAc.

ARC-Lum probes can be successfully used for real-time monitoring of activity of PKs in cells possessing the native expression level of PKs with no need for recombinant expression of fusions with fluorescent proteins. ARC-Lum probes have been efficiently adapted for determination of the concentration of a putative biomarker extra-cellular PKA (ECPKA) in patients' blood plasma samples and for mapping activity of PKs in live cells with time-gated luminescence microscopy (Kasari *et al.* 2012; Vaasa *et al.* 2012).

4.3. Biological effect of bisubstrate-analogue inhibitors in living cells

We have shown that ARC-inhibitors possess high affinity towards several PKs (PKAc, PKBγ, ROCK-II, *etc*.) and they are chemically and biologically stable. ARCs labelled with fluorescent dyes [ARC-Fluo and ARC-Lum(Fluo) probes] are capable for penetrating cell plasma membrane and interacting with PKs in cellular milieu. Therefore, ARC-probes are useful tools for biochemical and cell-based assays to determine activity of PKs and characterise inhibitors of PKs (Vaasa *et al.* 2009; Vaasa *et al.* 2010; Kasari *et al.* 2012; Vaasa *et al.* 2012). Although ARC/PK co-crystal studies and biochemical inhibition assays have demonstrated that ARCs inhibit PK-catalysed phosphorylation reactions by the bisubstrate mechanism, the capability of ARCs to regulate phosphorylation of proteins in living cells has not been well characterised. Overall, only a limited number of publications describe the potency of bisubstrate inhibitors to affect the phosphorylation of proteins in living cells (Räägel *et al.* 2008; Stebbins *et al.* 2011; Lamba and Ghosh 2012; van Wandelen *et al.* 2013; Gower *et al.* 2014) and therefore it was essential to take this potential of ARCs under investigation.

Several important cellular signalling cascades are initiated by the activation of PKA (Tasken 2004; Turnham and Scott 2016). In this study, the effect of ARCs on the PKA-catalysed phosphorylation of CREB was studied in mammalian cells. CREB (MW = 43 kDa) is a transcription factor that induces gene transcription after being phosphorylated at Ser133 by PKA or other PKs. Moreover, in case of the forskolin-activated pathway, PKA is the main CREB-phosphorylating PK (Johannessen *et al.* 2004a; Delghandi *et al.* 2005). Therefore, the reaction of CREB phosphorylation is a good target for monitoring the inhibitory efficiency of ARC-inhibitors in living cells.

A series of ARCs with varying PK-binding and plasma membrane-penetrating properties was tested in cells as regulators of protein phosphorylation. In order to test the cellular PK inhibitory potency of structurally diverse ARCs, several new structural modifications were introduced into previously biochemically characterised compounds (Table 3). In addition to the effect of structural modification of the adenosine-mimicking moiety (Adc-, AMTH-, and dPurp-moiety) of ARCs, the effect of hydrophobic groups attached to the lysine residue of the conjugates to improve their cell-penetrating properties, was studied.

Table 3. Structures and codes of the tested compounds; K_d values determined with the displacement assay

Compound	**Code**	K_d **(nM)**
N-[2-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide	H89	11
Adc-Ahx-$(_D\text{Arg})_6$-DLys-NH_2	ARC-904	0.4
Adc-Ahx-$(_D\text{Arg})_6$-DLys(Myr)-NH_2	ARC-1222	3.7
Adc-Ahx-$(_D\text{Arg})_6$-DLys(Hex)-NH_2	ARC-1171	0.2
Adc-Ahx-$(_D\text{Arg})_6$-DLys(C(O)$(CH_2)_2$-$P^+(Ph)_3$)-NH_2	ARC-1172	0.1
dPurp-C(O)$(CH_2)_7$C(O)-$(_D\text{Arg})_6$-DLys(Myr)- NH_2	ARC-1412	0.1
AMTH-Ahx-Ala-$(_D\text{Arg})_6$-DLys(Myr)-Gly	ARC-1143	2.3
AMTH-Ahx-(DArg)-Ahx-$(_D\text{Arg})_6$-DLys(Myr)-NH_2	ARC-681	1.8

HPLC-based analysis of intracellular concentration of labelled and unlabelled ARCs in cells has demonstrated an intense uptake of the conjugates (up to 10-fold augmentation of the concentration of some ARCs in cells compared to their concentration in incubation solution) (Hedi Sinijärv, unpublished data). Meanwhile, myristoylated ARCs were taken up by the cells even more intensively (Hedi Sinijärv, unpublished data). This result, together with the great structural stability of ARCs comprising only D-amino acid residues in cellular milieu (Enkvist *et al.* 2006), pointed to the applicability of ARCs for regulation of intracellular protein phosphorylation balances.

The biochemical affinity (K_d) of each compound towards PKAc was determined with ARC-Lum-based assay using the selenophene-comprising probe ARC-1139 (**Paper II** Figure 1). The affinities of the compounds towards PKAc were in nanomolar or subnanomolar range (Table 3). Although myristoylation decreased the affinity of the conjugates, the acylated ARCs still bound to PKAc three- to 100-fold more tightly than H89. The latter compound is a well-characterised commercially available small-molecule ATP-competitive inhibitor of PKAc and other PKs of the AGC group (MSK1, ROCK-II, *etc*.), which has been used as a reference compound for evaluating cellular inhibition of PKs (Davies *et al.* 2000). It has been shown that H89 at 10 μM concentration abrogates forskolin-induced phosphorylation of CREB (Delghandi *et al.* 2005).

The biochemically characterised ARCs were further tested in mammalian cells to establish their effect on the phosphorylation of CREB catalysed by

PKAc. The effect of inhibitors on CREB-dependent transcription and CREB phosphorylation was monitored with both a luciferase reporter assay and an immunoblot-based protein phosphorylation assay. Luciferase reporter assay is a relatively simple, rapid, and sensitive technique for studying gene expression at the transcriptional level. It has been used for the determination of the inhibition effects in cell signalling pathways (Delghandi *et al.* 2005; Mano *et al.* 2014). However, it has been noticed that some PK inhibitors (*e.g.,* H89) affect luciferase-mediated bioluminescence directly (Herbst *et al.* 2009; Dranchak *et al.* 2013), therefore determination of CREB phosphorylation with phospho-specific antibodies was also performed. The obtained inhibition profiles from both methods were similar and the dependency between pIC_{50} values from the luciferase assay and the immunoblot analysis (R^2 = 0.87; **Paper III** Figure 7) demonstrates that both of the methods can be used for assessing the inhibitory potential of compounds on PKAc activity in living cells.

All tested myristoylated conjugates (ARC-1222, ARC-1143, ARC-1412, and ARC-681) caused dose-dependent decrease in CREB-mediated luciferase activity and CREB phosphorylation (**Paper III**, Figure 4, 6). Some inhibitors (*e.g.,* ARC-681) led to a substantial decrease in the activity of CREB at 5, 10, and 20 μM concentration and to relatively weak inhibition at 1 μM concentration. This observation is in accordance with the results of earlier studies using ARC-Fluo probes in cell cultures, which demonstrated that a "critical" threshold of the ARC-Fluo concentration had to be overcome in order to obtain effective internalization of conjugates into cells (Vaasa *et al.* 2010). The same phenomenon, endocytosis-independent concentration threshold-sensitive transport mechanism via localised regions of the plasma membrane has also been described for arginine-rich transport peptides (Duchardt *et al.* 2007; Brock 2014).

Because of the high intracellular concentration of ATP (1–5 mM) (Beis and Newsholme 1975; Ando *et al.* 2012), ATP-competitive inhibitors H89 and ARCs reveal cellular IC_{50} values in the low micromolar range. The comparison of IC_{50} values (Table 4) with the K_d values from the biochemical measurements (Table 3) shows that myristoylated compounds with lower K_d values (ARC-1412, ARC-681) act more effectively in living cells than compounds possessing higher K_d values (ARC-1222 and ARC-1143). However, another set of compounds (ARC-904, ARC-1711, and ARC-1172) did not have a significant influence on the activity of CREB (**Paper III** Figure 2B), although their affinity towards PKAc was generally higher than that of the myristoylated conjugates. H89, on the other hand, has good inhibitory potency in cells, although its biochemical K_d value is higher than these of ARCs. Therefore, more efficient inhibition of the cAMP/PKA pathway by H89 (compared to ARCs) may be (partially) due to its better internalization into cells and localisation to the cell nucleus where it can act on a target PK, PKAc.

Table 4. IC_{50} values for the effect of inhibitors on the activity of PKAc in Chinese hamster ovary cells (CHO-K1) analysed by luciferase reporter assay and phospho-CREB immunoblotting assay

Compound	Luciferase assay		Immunoblot assay	
	IC_{50} (µM)	pIC_{50} ± SE	IC_{50} (µM)	pIC_{50} ± SE
H89	2.6	5.59 ± 0.09	6.4	5.19 ± 0.06
ARC-1222	4.2	5.38 ± 0.19	5.8	5.23 ± 0.41
ARC-1412	1.9	5.73 ± 0.17	4.7	5.33 ± 0.49
ARC-1143	18	4.75 ± 0.21	13	4.87 ± 0.45
ARC-681	1.4	5.85 ± 0.18	2.8	5.56 ± 0.46

Standard error (SE; 95 % confidence interval)

A cascade of biological and physical processes, including final co-localisation of CREB and PKAc, spatially and temporally regulates PKAc-mediated phosphorylation of CREB. Our previous fluorescence microscopy studies with ARCs labelled with fluorescent dyes have revealed that these compounds have a tendency to concentrate into nuclear subdomains, nucleoli (Figure 7), whereas CREB proteins are almost fully excluded from these nuclear subdomains (Uhlen *et al.* 2010). Thus, different intra-nuclear compartmentalization of CREB proteins (and PKAc) on one hand and ARCs on the other hand may be a cause of lower than expected intracellular inhibitory potency of ARCs. However, the improvement of cellular inhibitory potency of ARCs could be achieved with a simple structural modification of the conjugates, N-myristoylation. These results point to the importance of further research on structural modifications of ARCs that could lead to their better intracellular targeting and improved availability by nuclear PKs for regulating the phosphorylation of functionally important proteins. Moreover, those improved compounds could be useful for the construction of photoluminescent probes for monitoring and mapping the activity of PKs in living cells.

5. CONCLUSIONS

This thesis describes the development and characterisation of high-affinity bisubstrate-analogue inhibitors (ARC-inhibitors) with improved properties for further applications in biochemical and biomedical research for determining the activity of PKs, screening and characterising of PK inhibitors, and for regulating protein phosphorylation balances in signalling pathways of cells. The main results of the present research can be summarized as follows:

- Several heteroaromatic structures were used as adenosine mimics of ARCs to produce bisubstrate inhibitors for basophilic PKs with up to picomolar potency.
- A conjugate with thiophene-containing moiety [AMTH-Ahx-DLys-Ahx-$(DArg)_2$-NH_2] was shown to be a more potent inhibitor than its adenosine counterpart towards all three tested PKs (PKAc, PKBγ, ROCK-II).
- The inhibitory potency of AMTH-Ahx-DLys-Ahx-$(DArg)_2$-NH_2 was increased to subnanomolar region by increasing the number of D-arginine residues in the peptidic fragment.
- Long-lived emission of a thiophene-comprising fluorescently labelled ARC in complex with PKBγ was discovered in the course of development of a TR-FRET assay based on the application of organic chromophore antenna-sensitized lanthanide cryptates.
- Binding of a thiophene- or a selenophene-comprising heteroaromatic moiety (luminescence donor) to the purine-binding pocket of PK induced long lifetime photoluminescence (phosphorescence) signal that was largely intensified through efficient energy transfer to a fluorescent dye present in close proximity to the luminescence donor.
- The developed ARC-Lum probes were successfully used for determination of the concentration of active PKs as well for characterising various PK inhibitors in biochemical assays.
- ARC-Lum probes were used for real-time monitoring of activity of PKs in cells possessing native expression level of PKs with no need for recombinant expression of PK fusions with fluorescent proteins.
- ARC-inhibitors with varying PK-binding and plasma membrane-penetrating properties were tested as regulators of protein phosphorylation in living cells through the effect on cAMP/PKA/CREB signalling pathway.
- Low micromolar extracellular concentration of N-myristoylated ARCs was shown to be capable for reducing the activity of the transcription factor CREB through inhibition of PKAc. The cellular inhibitory potency of the compounds was depended on both their cell-penetration capability and PKAc affinity.

6. SUMMARY IN ESTONIAN

Proteiinkinaaside aktiivsust registreerivate fotoluminestsents-sondide ja rakusiseste inhibiitorite arendamine

Proteiinkinaasid (PK-d) on olulised valkude aktiivsust reguleerivad ensüümid, katalüüsides nende fosforüülimist ning olles seega oluliseks lüliks rakusisestes signaalülekande radades. Hälbeid PK-de normaalses funktsioneerimises, mis on enamasti tingitud nende liigsest aktiivsusest, üleekspressioonist või geenimutatsioonidest, on seotud mitmete haigustega, nagu vähkkasvajad, diabeet, südame-veresoonkonna haigused. Sellest tulenevalt on PK-dest saanud olulised ravimiarenduse sihtmärgid ning üle 30 PK inhibiitori on jõudnud vähiravimina kasutusele (Rask-Andersen *et al.* 2014; Wu *et al.* 2015). Lisaks ravimiarendusele on toimunud erinevate biokeemiliste analüüsimeetodite arendamine nii uute efektiivsete ravimikandidaatide võimalikult kiireks väljatöötamiseks, PK-de toimemehhanismide ja signaalradade uurimiseks kui ka PK-de kui haiguste biomarkerite tuvastamiseks.

Käesoleva töö eesmärgiks oli uudsete kõrge afiinsusega bisubstraatsete ARC-inhibiitorite kasutatavuse selgitamine biokeemilistes ja bioloogilistes (elavad rakud ja nende lüsaadid) proovides PK-de aktiivsuse määramiseks ning erinevate inhibiitorite iseloomustamiseks. Lisaks uuriti nende ühendite rakendatavust PK-de signaalradade uurimiseks ning kinaaside aktiivsuse reguleerimiseks.

Töö käigus iseloomustati mitmeid erineva struktuuriga ARC-inhibiitoreid, et tuvastada olulised ATP sidumistaskusse seostumist mõjutavad struktuurifragmendid, mis lubaksid arendada pikomolaarse afiinsusega selektiivseid inhibiitoreid basofiilisete PK-de jaoks. Kõige suurema inhibeerimisvõimega ühendiks kolme analüüsitud PK-i (PKAc, PKBγ, ROCK-II) suhtes osutus konjugaat, mille ATP sidumistaskusse seonduvaks osaks oli tiofeeni fragmenti sisaldav aromaatne struktuur. Järgnevalt optimeeriti linkerit ning PK substraatvalgu sidumistaskusse seonduvat osa, mille tulemusel saadi ARC-inhibiitor, mida rakendati Försteri energia resonantsülekandel (FRET) põhineva meetodi arendamiseks PK-de aktiivsuse määramiseks ning inhibiitorite iseloomustamiseks nii biokeemilistes kui ka bioloogilistes proovides.

Meetodi väljatöötamiseks võeti aluseks kolmekomponendiline süsteem, mis koosnes sihtmärk kinaasist, orgaanilise kromofoorantenniga sensibiliseeritud lantaniidi kelaadiga märgistatud antikehast (FRET-doonor) ning fluorestsentsmärgisega ARC-inhibiitorist (FRET-aktseptor). Lantaniidi kelaatide kui pika luminestsentsi elueaga ühendite kasutamine võimaldas teostada viivitusega FRET signaali mõõtmist, mis omakorda aitas kaasa uudsete tiofeeni sisaldavate ARC-inhibiitorite optiliste omaduste kirjeldamisele ning nendel põhinevate ARC-Lum-sondide avastamisele. Töö käigus tuvastati, et uute ühendite seostumisel PK-dega ning selle kompleksi ergastamisel kiirgusega lähis-UV alas emiteerivad nad oranžis või punases spektri piirkonnas aeglase sumbumisega

(20–250 μs) valgust. Seega kasutades luminestsentsi viivitusega mõõtmistehnikaid on võimalik efektiivselt hinnata PK-de aktiivust, iseloomustada erinevaid inhibiitoriteid ning uurida huvipakkuvaid protsesse reaalajas nii geneetiliselt muundamata rakkudes kui ka teistes keerulistes bioloogilistes proovides.

ARC-sondide abil on võimalik määrata aktiivse PK osakaalu lahuses või jälgida PK kontsentratsiooni muutusi bioloogilistes süsteemides sõltuvalt erinevate inhibeerivate või aktiveerivate ühendite toimest. ARC-de inhibeerimisvõime PK-de suhtes ning nende mõju rakusisestele signaalradadele oli varem kirjeldamata. Selle uurimiseks kasutati nii varem sünteesitud ARC-inhibiitoreid kui ka antud töö käigus arendatud ühendeid, mis erinesid eelnevatest nii inhibeerimisomaduste kui ka rakkude plasmamembraani läbimise võime poolest. Töös uuriti nende ainete mõju cAMP/PKA/CREB signaalrajale. Selgus, et kuigi oligoarginiini sisaldavad ARC-d on võimelised läbima raku plasmamembraani, siis sihtmärkvalguni (PKAc) jõudmiseks ja inhibeeriva toime avaldumiseks on vajalik ühendite struktuuri edasine suunatud modifitseerimine. Ühendid, mille struktuuri oli lisatud hüdrofoobne müristiinhappe jääk, olid võimelised rakkudes inhibeerima PKAc-d ning vähendama sellega transkriptsioonifaktor CREB-i aktiivsust. Sealjuures oli nende ainete inhibeeriv efekt sõltuv ainete biokeemilisest afiinsusest PKAc suhtes.

Saadud tulemused on aluseks uute inhibiitorite konstrueerimisele, mis võimaldavad PK-de aktiivsuse suunatud reguleerimist rakkudes, ning biosensorite arendamisele, mida saab kasutada PK-de aktiivsuse seireks ja kaardistamiseks rakkudes.

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2. Enkvist, E., Vaasa, A., Kasari, M., **Kriisa, M.**, Ivan, T., Ligi, K., Raidaru, G., Uri, A. (2011) Protein-induced long lifetime luminescence of non-metal probes. ACS Chemical Biology, 6(10), 1052–1062.
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Alates 2010 Eesti biokeemia seltsi liige

Scientific publications

1. Enkvist, E., **Kriisa, M.**, Roben, M., Kadak, G., Raidaru, G., Uri, A. (2009) Effect of the structure of adenosine mimic of bisubstrate-analog inhibitors on their activity towards basophilic protein kinases. Bioorganic and Medicinal Chemistry Letters, 19(21), 6098–6101.
2. Enkvist, E., Vaasa, A., Kasari, M., **Kriisa, M.**, Ivan, T., Ligi, K., Raidaru, G., Uri, A. (2011) Protein-induced long lifetime luminescence of non-metal probes. ACS Chemical Biology, 6(10), 1052–1062.
3. **Kriisa, M.**, Sinijärv, H., Vaasa, A., Enkvist, E., Kostenko, S., Moens, U., Uri, A. (2015) Inhibition of CREB phosphorylation by conjugates of adenosine analogues and arginine-rich peptides, inhibitors of PKA catalytic subunit. ChemBioChem, 16(2), 312–319.

DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS

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