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ANGELA VAASA

Development of fluorescence-based kinetic and binding assays for characterization of protein kinases and their inhibitors

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Author's contribution

Paper I: The author participated in the planning of experiments, worked out and characterized fluorometric TLC protein kinase activity assay, analyzed the data and contributed to the writing of the manuscript.

Paper II: The author contributed to the planning of research, performed most of the experiments, was responsible for data analysis and writing of the manuscript.

Paper III: The author participated in the planning of experiments, performed fluorescence anisotropy based measurements and cellular experiments, was responsible for writing of the respective part of the manuscript.

Paper IV: The author planned the experiments for biological testing of compounds, performed biochemical tests and worked out assays for testing of compounds with live cells, analyzed the data and wrote the manuscript.

ABBREVIATIONS

AC	adenylate cyclase
Adc	adenosine 4’-dehydroxymethyl-4’-carboxylic acid moiety
ADP	adenosine 5'-diphosphate
Ahx	6-aminohexanoic acid
AKAP	A-kinase anchoring protein
AMP	adenosine 5’-monophosphate
AMTH	5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid
ARC	adenosine analogue-oligoarginine conjugate
ARC-306	Adc-Ahx-(L-Arg)$_6$-OH
ARC-341	Adc-Ahx-(L-Arg)$_6$-NH$_2$
ARC-902	Adc-Ahx-(D-Arg)$_6$-NH$_2$
ARC-904	Adc-Ahx-(D-Arg)$_6$-D-Lys-NH$_2$
ARC-Photo	fluorescently labelled ARC
ATP	adenosine 5'-triphosphate
Bodipy FL	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid
Bodipy 564/570	4,4-difluoro-5-styryl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid
cAMP	cyclic adenosine 3',5'-monophosphate
CFP	cyan fluorescent protein
CPP	cell-penetrating peptide
ELISA	enzyme-linked immunosorbent assay
FA	fluorescence anisotropy
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
FP	fluorescence polarization
FRET	Förster resonance energy transfer
FRSK	Forskolin
GFP	green fluorescent protein
H89	*N*-{2-[(p-bromocinnamyl)amino]ethyl}-5-isoquinolinesulfonamide
H9	*N*-(2-aminoethyl)-5-isoquinolinesulfonamide
HA-1077	1-(5-isoquinolinesulfonyl)-homopiperazine
HTS	high-throughput screening
IC_{50}	inhibitor concentration causing 50% reduction of the kinase enzymatic/catalytic activity in the used assay conditions
ITC	isothermal titration calorimetry
K_D	equilibrium dissociation constant obtained from binding assays
K_d	dissociation constant obtained from displacement assays
K_i	inhibition constant
K_m	Michaelis constant
MW	molecular weight
NAD^+	nicotinamide adenine dinucleotide, oxidized form

NADH	nicotinamide adenine dinucleotide, reduced form
NMR	nuclear magnetic resonance
PK	protein kinase
PKA	cAMP-dependent protein kinase
PKAc	PKA catalytic subunit
PKAcα	PKA catalytic subunit, α-isoform
PKAr	PKA regulatory subunit
PKArI	PKA regulatory subunit type I
PKArII	PKA regulatory subunit type II
PKI	heat-stable protein kinase inhibitor
RFP	red fluorescent protein
R_t	retention time
SPR	surface plasmon resonance
TAMRA	carboxytetramethylrhodamine
TLC	thin layer chromatography
TRF	time-resolved fluorescence
V_{max}	maximum velocity of the enzyme-catalyzed reaction
YFP	yellow fluorescent protein

INTRODUCTION

Protein kinases are enzymes which catalyze the phosphorylation of substrate proteins by transferring the γ-phosphoryl group of ATP to the acceptor amino acid of the protein/peptide substrate. Protein phosphorylation serves as a regulatory mechanism for most cellular processes such as cell division, proliferation, apoptosis, and differentiation, hence aberrant functioning of protein kinases is associated with serious diseases including cancer, diabetes, and Alzheimer`s disease. For this reason, protein kinases have become one of the major therapeutic targets [Cohen 2002; Morphy 2010; Knight *et al.* 2010]. Currently, 10 small-molecule kinase-inhibiting compounds and 4 antibodies have been approved by FDA as cancer drugs, and about 150 compounds are at various stages of clinical development as drugs against different diseases [Fedorov *et al.* 2010; Gill *et al.* 2007].

Several types of active site-binding protein kinase inhibitors have been developed, the major classes represented by ATP-site and protein/peptide substrate-site directed compounds. Despite serious selectivity problems arising from the similarities of ATP-binding sites in different kinases, the majority of developed protein kinase inhibitors belong to the first class. Inhibitors directed to the less conserved protein/peptide substrate-site are endowed with higher selectivity potential compared to ATP-competitive inhibitors, but are less advantageous from the aspect of other characteristics, *e.g.,* binding energy *per* heavy atom, stability, cell membrane-penetrative properties, *etc.* Combining the two aforementioned types of inhibitor into one molecule may yield selective and potent bisubstrate (biligand) inhibitors [Parang and Cole 2002; Hines and Cole 2004]. The conjugates of adenosine analogues and arginine-rich peptides (ARCs) [Loog *et al.* 1999; Enkvist *et al.* 2006; Lavogina *et al.* 2009] developed by our research group also take advantage of bisubstrate inhibitor approach.

The importance of the development of protein kinase inhibitors as drug candidates has caused an increasing need for the elaboration and improvement of analytical methods for high-throughput screening (HTS) and characterization of new compounds. Additionally, the transfer of biochemical assays to cell-based format is required in order to assess the degree of cellular internalization, subcellular localization, stability and potency of the drug candidate at the early stages of research.

This thesis describes a progress in assay development leading from a biochemical kinetic method to the binding assays applicable in biochemical format as well as in living cells for the screening and assessment of the inhibitory properties of ARCs and other inhibitors of cAMP-dependent protein kinase. The study demonstrated the cell-penetrative properties and intracellular activity of ARCs, pointing to the potential of ARC-based fluorescent probes for both biochemical and intracellular applications.

LITERATURE OVERVIEW

Protein kinases

Protein kinases (PKs) that catalyze phosphorylation of proteins belong to the enzyme family of transferases. Phosphorylation results in the structural modification of the substrate protein, triggering changes in its enzymatic activity, cellular location, or association with other proteins. Functioning of up to 30% of all proteins is modified by PK-catalyzed phosphorylation [Manning 2005]. PKs are therefore involved in regulation of large variety of cellular processes including metabolism, transcription, cell cycle progression, cytoskeletal rearrangement and cell movement, apoptosis, and differentiation.

The human genome contains 518 PK encoding genes, constituting about 2% of all human genes [Manning *et al.* 2002; Manning 2005]. The most widely used basis for the systematization of PKs is the sequence similarity of their catalytic domain. According to this criterion, PKs are divided into seven main groups [Manning *et al*. 2002]:

- AGC group
  Contains the cyclic nucleotide-regulated protein kinase (PKA and PKG) family, diacylglycerol-activated/phospholipid-dependent protein kinase C (PKC) family and RAC/Akt family PKs.
- CaMK group
  Contains kinases regulated by Ca^{2+}/CaM (*e.g.,* CaMKII), KIN1/SNF1/Nim1 family and other CaMK-related kinase families (*e.g.,* MAP).
- CMGC group
  Contains cyclin-dependent kinase (CDK), extracellular signal-regulated/ mitogen-activated protein kinase ERK (MAP), glycogen synthase kinase 3 (GSK3), casein kinase II (CK2) and Clk families, and other CMGC related kinases (*e.g.,* Yak1).
- CK1 group
  Contains caseine kinase 1 (CK1), tau tubulin kinase (TTBK), and vaccinia-related kinase (VRK) families.
- STE group
  Includes many PKs involved in MAP kinase cascades as Ste7/MAP2K, Ste11/MAP3K, and Ste20/MAP4K.
- TK group
  Includes non-membrane spanning protein tyrosine kinases (*e.g.,* Src, Tec/ Atk, Csk, Fes (Fps), Abl, Syk/ZAP70, Tyk2/Jak1, Ack and focal adhesion kinase (Fak) families), and membrane spanning protein tyrosine kinases (*e.g.,* epidermal growth factor receptor family, fibroblast growth factor receptor family, insulin receptor family, *etc*).
- TKL group
  Tyrosine kinase-like group, received its name because of close sequence similarity to tyrosine kinase group. TKL contains mixed-lineage kinase

(MLK), LISK (LIMK/TESK), IRAK [interleukin-1 (IL-1) receptor-associated kinase], Raf, RIPK [receptor-interacting protein kinase (RIP)], and STRK (activin and TGF-receptors) families.

PKs belonging to the same group tend to show similar substrate specificities and modes of regulation [Hanks and Hunter 1995].

The second criterion for PK systematization is the phosphorylatable amino acid of the PK substrate. In the course of phosphorylation the γ-phosphoryl group of ATP is transferred to the protein/peptide substrate, and according to the origin of the amino acid residue of the substrate to which the phosphoryl is transferred, PKs are classified into serine/threonine, tyrosine and dual-specificity kinases. In other organisms (*e.g.,* bacteria, fungi and plants), there are also PKs that phosphorylate other amino acids, *e.g.,* histidine kinases that phosphorylate a histidine residue of the target protein [Besant and Attwood 2009].

Finally, the substrate consensus sequence flanking the phosphorylatable residues of the substrate can also serve as the basis for the classification of PKs. According to their substrate preferences, Ser/Thr kinases can be classified into three main categories [Pinna and Ruzzene 1996]:

1. Basophilic kinases prefer basic and hydrophobic amino acid residues as determinants. This category includes members of the AGC group (with a couple of exceptions), CaMK group kinases and several kinases not falling into seven main groups.
2. Proline-directed PKs also prefer basic amino acids in the substrate recognition sequence, but additionally require a proline residue at the position P+1. Essentially, members of CMGC group catalyze the phosphorylation of substrates possessing corresponding sequences.
3. Acidophilic/phosphate directed kinases, which prefer carboxylic acid and phosphorylated residues in their consensus sequences. This is a comparatively small group of PKs compared to basophilic kinases, and it comprises members of various superfamilies of PKs.

cAMP-dependent protein kinase (PKA)

AGC group contains 60 of the 518 human PKs [Manning *et al.* 2002], including different isoforms of PKA, PKB, PKC, PKG, MSK, ROCK, *etc*. PKs belonging to this group are mainly basophilic, as they phosphorylate substrates containing basic amino acids Arg and Lys in close proximity to the phosphorylatable Ser/Thr residues (Tabel 1) [Pearce *et al.* 2010].

Table 1. Substrates of AGC kinases [Pearce *et al.* 2010]

Kinase	**Consensus motif for substrate**
Akt (PKB)	Arg-X-Arg-X-X-**Ser/Thr**-φ
DMPK	Arg-X-X-**Ser/Thr**-Leu/Val-Arg
LATS	His-X-Arg/His/Lys-X-X-**Ser/Thr**
MSK	Arg-Arg/Lys-X-**Ser** or Arg-Lys-**Ser**
NDR	His-X-Arg/His/Lys-X-X-**Ser/Thr**
PDK1	Thr-Phe-Cys-Gly-**Thr**
PKA	Arg-X-**Ser/Thr** or Arg-Arg/Lys-X-**Ser/Thr**
PKC	Arg-Lys-X-**Ser/Thr**-X-Arg/Lys
PKG	Arg-$Lys_{2–3}$-X-**Ser/Thr**
PKN	Arg-X-**Ser/Thr**-X-Arg/Lys
ROCK	Arg/Lys-X-**Ser/Thr** or Arg/Lys-X-X-**Ser/Thr**
RSK	Arg/Lys-X-Arg-X-X-**Ser/Thr**
S6K	Arg/Lys-X-Arg-X-X-**Ser/Thr**
SGK	Arg/Lys-X-Arg-X-X-**Ser/Thr**

X denotes any amino acid and φ is a bulky hydrophobic residue.

The most extensively studied representative of kinases of the AGC group, cAMP-dependent protein kinase (PKA) was discovered in 1968 [Walsh *et al.* 1968]. Due to its structural simplicity and easiness of production, PKA has served as a model kinase for the general research of PKs, further supported by the fact that the catalytic subunit of PKA was the first PK whose 3-dimensional crystal structure was reported [Knighton *et al.* 1991].

The activation mechanism of PKA has also been a subject of thorough investigation. The catalytically active form of PKA is its catalytic subunit (PKAc), whereas in the holoenzyme form PKA resides in its inactive state. The holoenzyme of PKA consists of two catalytic subunits and a dimer of two regulatory subunits (PKAr) [Gill and Garren 1969]. In human organism, there are three major isoforms of PKAc (α, β and γ) [Zhang *et al.* 2004; Gamm 1996] and four different isoforms of PKAr (Iα, Iβ, IIα and IIβ) [Cheng *et al.* 2001], which all differ in their distribution, functioning and expression level. The binding of cyclic adenosine 3',5'-monophosphate (cAMP) molecules to the regulatory subunits of holoenzyme causes the dissociation of PKA into a $PKAr_2$ dimer and two monomeric PKAc subunits.

cAMP is a cyclic nucleotide produced as the result of ATP cyclization catalyzed by adenylate cyclases (ACs). cAMP is an ubiquitous second messenger in intracellular signal transduction, being responsible for activation of protein kinase A and other cAMP-dependent signalling cascades. The concentration level and gradients of intracellular cAMP are regulated by the activity of ACs on one hand and cAMP-degrading phosphodiesterases on the other hand. In turn, the activity of ACs is regulated by special G-proteins that are coupled to membrane receptors and can therefore respond to external stimuli, or directly by specific activators (*e.g.,* Forskolin).

Apart from the cAMP-induced activation, PKAc requires the phosphorylation of specific amino acid residues of its activation loop in order to become catalytically fully active; the latter mechanism for regulation of PK catalytic activity is shared by nearly whole kinome [Pearce *et al.* 2010]. In case of PKAc, the phosphorylation of Thr197 residue triggers formation of several key contacts crucial for active conformation of the catalytic subunit, and thereby enhances catalytic activity of the kinase by approximately three orders of magnitude [Johnson *et al.* 2001].

PKAc is a 40 kDa monomeric protein that possesses an ATP-binding site and a protein/peptide substrate-binging site. During PKAc-catalyzed phosphorylation, both substrates simultaneously associate with the catalytic site resulting in formation of a ternary complex [Johnson *et al.* 2001], and the direct transfer of the phosphoryl group from ATP to the protein/peptide substrate follows. The recognition of a substrate sequence by a PK is influenced by the local environment around the phosphorylation site of the substrate (so-called substrate consensus sequence), which in case of PKAc substrates is Arg-Arg-X-Ser-X (RRXSX) [Smith *et al.* 1999]. The important selectivity determinants are therefore arginines at the positions –3 and –2 to the N-terminus of the posphorylatable residue, and a large hydrophobic residue at the +1 position of the posphorylatable residue; the residue X in position –1 can be any neutral amino acid [Adams 2001]. The consensus sequence of a substrate for its target kinase may be determined either by a synthetic peptide library screening, or by detailed analysis of amino acid sequences of natural substrates; for instance, the widely used PKAc synthetic substrate peptide Kemptide (LRRASLG) was designed based on the phosphorylatable domain of pyruvate kinase, a PKAc target protein [Kemp 1977].

In mammalian organisms, PKA is highly abundant in tissues and its activity inside cells is modulated by a variety of mechanisms. Firstly, the intracellular localization of the enzyme is controlled by AKAPs (A-kinase anchoring proteins) that anchor PKA holoenzyme to cell membranes *via* interactions with the regulatory subunits; AKAPs for both PKArI and PKArII [Huang *et al.* 1997; Carnegie *et al.* 2009] have been identified. Secondly, the physiological inhibition of PKA is performed by pseudo-substrates, *i.e.,* PKArI and PKArII and heat-stable protein kinase inhibitors (PKIs) [Johnson *et al.* 2001; Herberg and Taylor 1993; Collins and Uhler 1997]. All of those inhibitors contain an amino acid sequence similar to the consensus sequence of substrates, thus the association of pseudo-substrates with the kinase blocks the access of substrates to kinase binding sites. However, most of pseudo-substrates cannot be phosphorylated, except for the PKArII subunit of PKA that contains a Ser residue which is autophosphorylated in the holoenzyme complex [Johnson *et al.* 2001].

Finally, it is possible to affect the activity of PKAc by non-physiological compounds, including synthetic inhibitors.

Inhibitors of protein kinases

Given the fact that PKs are involved in the majority of cellular processes, it is not surprising that false regulation of PKs is associated with several diseases. In recent years, the unveiling importance of PKs as drug targets has caused a substantial interest in and need for the design of selective and potent inhibitors of PKs. The most successful example on the drug market that urged even more intense research in the field was small-molecule PK inhibitor Imatinib (Gleevec) [Deininger *et al.* 2005]. Imatinib is relatively selective towards mutated Brc-Abl protein kinase, and it has been successfully used for the treatment of chronic myelogenous leukemia [Deininger *et al.* 2005; Deininger 2007].

The reversible inhibitors of PKs targeting the active sites of the catalytic domain may be classified into the following groups: ATP-site directed inhibitors, protein/peptide substrate-site directed inhibitors, and bisubstrate-analogue inhibitors. Despite the fact that the majority of the described inhibitors of PKs are ATP-site directed, the applicability of representatives of this group is potentially subjected to several problematic issues. First, it is a substantial challenge to design sufficiently selective ATP-competitive inhibitors, as all 518 kinases and more than 1500 other proteins bind purine nucleotides [Davies *et al.* 2000; Fischer 2004; Bain *et al.* 2003; Bain *et al.* 2007]; furthermore, inhibitors that are directed to the ATP-binding site must compete with the high (1–10 mM) intracellular concentration of ATP.

The first highly potent ATP-site directed inhibitor discovered was staurosporine, a natural compound isolated from bacterium *Streptomyces staurosporeus* in 1977 [Omura *et al.* 1977]. By virtue of possessing low nanomolar K_i values for a wide range of PKs, staurosporine has remained in use as a potent generic kinase inhibitor. Another widely known class of ATP-site directed inhibitors are known as H-series inhibitors, disclosed by Hidaka *et al.* in 1984 [Hidaka *et al.* 1984]. H-series inhibitors are derivatives of isoquinolinesulfonamides. The first representatives of these compounds showed micromolar activity towards several PKs of the AGC group, but further modification of the structures has yielded more active and selective inhibitors [Ono-Saito *et al.* 1999]. Furthermore, the first clinically applied PK inhibitor HA-1077 (Fasudil) is also an isoquinolinesulfonamide derivative [Tamura *et al.* 2005]. Fasudil is moderately selective towards Rho-kinase and it has been used in Japan since 1995 for the treatment of cerebral vasospasm after subarachnoid hemorrhage [Olson 2008].

Inhibitors directed to the protein/peptide substrate-binding site of the PKs target non-conserved residues responsible for recognition and development of specific interactions with substrates of a given PK; hence, this class of compounds should possess considerably higher potential for selectivity. Still, due to the nature of protein/peptide-binding site (relative openness to solvent and absence of clearly defined cavity) inhibitors directed to this site must generally incorporate longer peptidic structures in order to achieve high inhibitory potency [Bogoyevitch *et al.* 2005; Lawrence 2005]. The resulting increased molecular weight often accompanied by increased polarity of compounds leads

to complications related to cellular uptake and proteolytic stability. Nevertheless, several more or less selective peptide-based inhibitors have been recently developed and successfully used for the regulation of physiological processes in cells and tissues [Dostmann *et al.* 2002; Lawrence 2005; Shiga *et al.* 2009; Bogoyevitch *et al.* 2010; Ekokoski *et al.* 2010; Nickl *et al.* 2010].

Bisubstrate-analogue (bisubstrate) inhibitors are compounds that simultaneously associate with the ATP- and protein/peptide substrate-binding sites of the enzyme. Therefore, the bisubstrate inhibition strategy is aimed at enhancement of both the selectivity and potency of inhibitors [Ricouart *et al.* 1991; Parang *et al.* 2001; Parang and Cole 2002; Hines and Cole 2004; Schneider *et al.* 2005; Lavogina *et al.* 2010]. There are three acknowledged methods to reveal the bisubstrate character of a PK inhibitor [Lavogina *et al*. 2010]: analysis of the structure of the inhibitor-PK co-crystal, displacement of the inhibitor from its complex with PK by either ATP- or protein/peptide substrate-competitive inhibitors, and kinetic analysis of the competitiveness of the inhibitor versus either substrate. The principle advantage of bisubstrate inhibitors is their higher binding energy (up to three orders of magnitude) as compared to the sum of binding energies of the two single motifs [Jencks 1981; Saxty *et al.* 2007]. This increase in affinity results from the interaction of a single molecule rather than multiple ligands with the protein, and may be attributed to additional energetic gain such as entropic win, cooperative binding effects, interaction of linking chain with the enzyme, *etc*. In the most promising cases, the conjugation of two fragments with millimolar affinities has resulted in an inhibitor with nanomolar affinity [Saxty *et al.* 2007].

Adenosine analogue-oligoarginine conjugates (ARCs)

ARC-type inhibitors designed according to the bisubstrate inhibitor strategy comprise analogues of both substrates (inhibitors) of a PK connected *via* a linker: an adenosine mimic that is targeted to the ATP-binding site, and an arginine-rich peptide that is targeted to the protein/peptide substrate-binding site.

The construction of ARCs was started in 1999 [Loog *et al.* 1999]. ARCs of the first generation consisted of adenosine 5′-carboxylic acid and hexa(L-arginine) tethered *via* a linker whose structure was optimized in structure-activity studies (Figure 1). The most potent compound (ARC-341) incorporating 6-aminohexanoic acid moiety showed sub-micromolar potency towards kinases PKA, PKC and calcium-dependent protein kinase [Loog *et al.* 1999].

The next steps in the ARC design included the amidation of the C-terminus of the conjugates and the substitution of D-amino acids for L-amino acids (Figure 1). These modifications increased the potency of ARCs to the low nanomolar range, and also provided ARC(II) compounds with the proteolytic stability [Enkvist *et al.* 2006].

The crystal structure of the complex of a representative of ARC(II) inhibitors, ARC-1034 [Adc-Ahx-(D-Arg)$_2$-NH$_2$] with PKAc provided information for the development of the third generation of ARCs, ARC(III) (Figure 1). The main structural modification was the introduction of a chiral spacer and the 2nd linker, which resulted in the extra increase in potency of these conjugates [K_d up to 30-fold lower as compared to ARC(II); Lavogina *et al.* 2009]. All in all, the inhibitory potency of ARCs could be increased by more than a 1000-fold by spatial repositioning of the main pharmacophoric elements of ARCs (*i.e.,* adenosine analogue and guanidino groups).

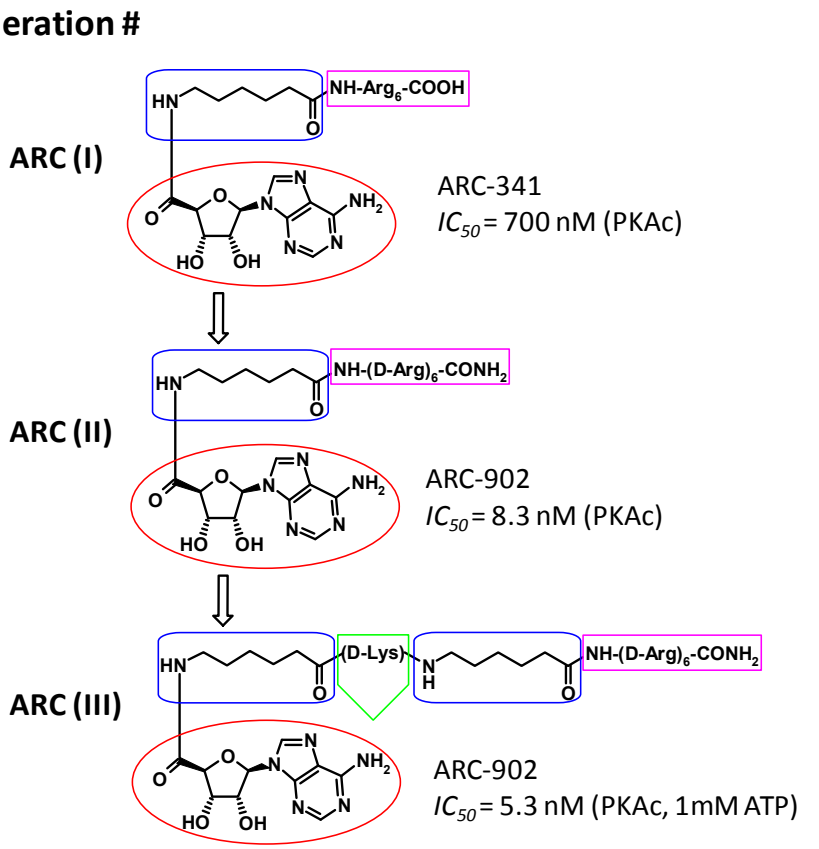

Figure 1. Structures of the ARCs of different generations. The nucleosidic part is surrounded with red ellipse, the linker(s) with blue rounded rectangle, the chiral spacer with green polygon, and the peptidic part with magneta rectangle.

Selectivity studies have shown that ARCs are group-selective inhibitors of basophilic PKs of AGC and CAMK groups [Enkvist *et al.* 2006; Lavogina *et al.* 2009], which might be attributed to the incorporation of the oligo-arginine moiety in the structure of ARCs. This arginine-rich moiety also enables

penetration of ARCs through the cell plasma membrane (cf. Cell-penetrating peptides). The additional advantages of ARCs include possibility of their further derivatization without reducing of the binding affinity. ARCs tolerate the attachment of voluminous tags possessing some beneficial biological (*e.g.,* biotin moiety for binding avidin or streptavidin) or physical (*e.g.,* a fluorescent dye) properties, which allows the application of ARCs in several bioanalytical assays.

Cell-penetrating peptides (CPPs)

An important prerequisite for the development of new inhibitors for intracellular applications is to ensure the penetration of the compounds through biological barriers. A large number of potential drug candidates have failed in the pre-clinical and clinical trials due to the lack of ability to reach their targets caused by poor translocation into the cell [Heitz *et al.* 2009]. One of the possible solutions for this issue is offered by peptides endowed with plasma membrane penetrative properties. Cell-penetrating peptides (CPPs) representing a variety of structures have found wide application in biotechnology, as CPPs can induce the cellular uptake of bioactive molecules (*e.g.,* synthetic small molecules, peptides, proteins, oligonucleotides, *etc*) otherwise not able to cross biological barriers. The best-characterized CPPs include peptides rich in basic amino acids arginine and lysine, such as the HIV-Tat peptide, TP10 and synthetic oligo-arginines (R6–R18) [Vives *et al.* 1997; Zorko and Langel 2005; Nakase *et al.* 2008].

There has been a lot of discussion considering the mechanism of penetration of CPPs in scientific literature, and the two main pathways contributing to CPP internalization have been proposed: the direct cell membrane penetration, and the endosomal pathway [Heitz *et al.* 2009]. However, the exact mechanism of cellular translocation of CPPs is still disputable and is most likely not the same for different types of CPPs [Ter-Avetisyan *et al.* 2008; Watkins *et al.* 2009; Fischer *et al.* 2002]. Moreover, variations in cellular uptake of CPPs may be caused by different characteristics of cell lines or tissues used for the experiment [Räägel *et al.* 2010].

Methods for the characterization of the inhibitors of protein kinases

Since the discovery of importance of PK signalling, vast variety of assays has been reported for the assessment of potency of PK inhibitors [Jia *et al.* 2008]. These assays may generally be divided into two types according to the measured characteristic of the inhibitor.

The first group is represented by the kinetic inhibition assays, where the inhibitors are characterized on the basis of their retarding effect on the rate of

phosphorylation of the protein/peptide substrate catalyzed by the kinase. The degree of incorporation of phosphoryl moiety into protein/peptide substrate is quantified either directly (*e.g.,* by utilization of radioactive [γ-^{32}P]ATP or fluorescently labelled peptide substrate) or indirectly (by implementation of antibodies). Regardless of their popularity, inhibition assays are often time-consuming and comprise several steps, which complicate the automation of the assay for a HTS format.

The second group involves binding/displacement assays where either association of the labelled compound with the target kinase is monitored, or the competitive displacement of the labelled probe from its complex with the kinase by an unlabelled inhibitor is measured. The most common format is represented by equilibrium binding assays where the measurement of the binding potency of compounds to the kinase is performed with the aid of fluorescently labelled small molecular weight-probes. Instead of a fluorescent label, it is also possible to use a radioactive tag or a suitable isotope, or measure direct binding without labelling (ITC, NMR or SPR) [Nordin *et al.* 2005; Smith and Windsor 2007; Viht *et al.* 2007; Masterson *et al.* 2008].

The binding and inhibition efficiency of inhibitors can be characterized by the values of dissociation and inhibition constants, respectively. The equilibrium dissociation constant (K_D) obtained from binding assays characterizes the affinity between the ligand and enzyme, and is expressed as:

$$K_D = \frac{[E][L]}{[EL]} \quad (1)\,,$$

where [E], [L] and [EL] represent the equilibrium concentrations of the enzyme, ligand and complex, respectively. In this case, ligand can act as an inhibitor or a substrate, or bind to an allosteric site.

The mathematical equation for K_i value is dependent on the inhibition model; *e.g.,* in case of competitive inhibition, the K_i is expressed analogically to K_D, whereas inhibitor concentration [I] is used instead of ligand concentration [L]. However, as the K_i value cannot generally be directly estimated from the experimental data (*i.e.,* it needs to be calculated), other inhibition characteristics (*e.g.,* IC_{50} value) are frequently used in order to characterize the ability of an inhibitor to reduce the catalytic activity of a kinase at given conditions. IC_{50} value corresponds to the inhibitor concentration causing 50% reduction of the kinase enzymatic/catalytic activity in the used assay conditions. Thus, IC_{50} is not a direct indicator of inhibitory potency and the value may vary between experiments depending on substrate concentration. IC_{50} can be converted to an absolute inhibition constant (K_i) using the Cheng-Prusoff equation [Cheng and Prusoff 1973]:

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

where [S] is substrate concentration and K_m indicates the affinity of the substrate for the enzyme. Alternatively, in competition binding assays, the IC_{50} can express the concentration of inhibitor which displaces 50% of the ligand from its complex with enzyme. In this case the Cheng-Prusoff equation can be expressed as:

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

where K_d is the dissociation constant of the complex between the inhibitor and the kinase, K_D is the dissociation constant of the complex between the ligand and kinase, and [L] is the concentration of the ligand.

Assays for the measurement of kinase activity

Historically, the majority of kinase inhibitor tests have been performed in the form of kinetic inhibition studies, and the kinetic format has still preserved its popularity. The traditional assay for the determination of PK activity is the phospho-cellulose paper method in which the transfer of radioactive phosphate (^{32}P or ^{33}P) from ATP to a protein or a synthetic peptide substrate is measured [Witt and Roskoski 1975; Glass *et al.* 1978]. However, as radiometric methods have several drawbacks (*e.g.,* short half-life of ^{32}P, personal risks, environmental pollution), a number of non-isotopic methods have been developed [Zaman *et al.* 2003]. The detection techniques often take advantage of fluorescence phenomena, for example several assays utilize the separation of fluorescently labelled product from the non-phosphorylated substrate (by electrophoresis [Promega Inc. 2001] or chromatography [Wu *et al.* 2006]), or measure change in fluorescence intensity accompanying phosphorylation reaction [Wright *et al.* 1981; Kondo *et al.* 1984; Loving *et al.* 2010].

The kinase activity can also be assayed *via* a cascade of reactions triggered by the catalytic functioning of the PK of interest. For instance, in Cook's assay [Cook *et al.* 1982] the PK-catalyzed conversion of ATP to ADP is coupled to the ADP-utilizing oxidation of NADH by pyruvate kinase and lactate dehydrogenase. NADH oxidation to NAD leads to the change of spectral properties of the assay solution, and the resulting decrease of absorbance at 340 nm is measured spectrophotometrically.

The availability of high-quality phospho-antibodies has largely increased the application of immunoassays in kinase research. The most widely known example is probably Enzyme-Linked ImmunoSorbent Assay (ELISA), which utilizes monoclonal antibodies specific for the phosphorylated amino acid [Alberta and Stiles 1997]. These antibodies capture the phosphorylated substrate and the amount of the latter is subsequently detected using the secondary antibodies linked with enzymes that catalyze a reaction producing an

optically detectable signal. Alternatively, the detection antibody may be tagged with fluorophores, enabling direct quantification of the phosphorylated substrate in sample. The growing variety of fluorescently labelled phospho-antibodies and small-molecule fluorescence probes has triggered intensive development of assays based on fluorescence polarizarion (FP), Förster resonance energy transfer (FRET), or time-resolved fluorescence (TRF) read-outs [Zaman *et al.* 2003; Olive 2004].

Fluorescence techniques

During the recent years, the fluorescence measurement has become the main methodology used in biotechnology including cell and tissue imaging. The reason for such popularity lies in the ability of fluorescence measurements to provide information about a wide horizon of molecular processes, starting from rotational diffusion of molecules and reaching to intermolecular conformational changes [Lakowicz 2006].

Fluorescence is a radiative process in which the molecule exited to higher energy levels (S_1 or S_2) by absorbing a photon returns to its ground state (S_0) by emitting a photon (fluorescence emission), whereas the energy of the emitted photon is generally smaller than that of the absorbed photon (Figure 2). The latter phenomenon may be explained by the internal conversion (S_2 to S_1 in case of higher energy level) and/or vibrational relaxation of molecule to the lowest vibrational level of the first excited state (S_1). Therefore, the emission wavelength of the fluorophore is longer than its excitation wavelength and this difference between excitation and emission wavelength is called the Stokes shift.

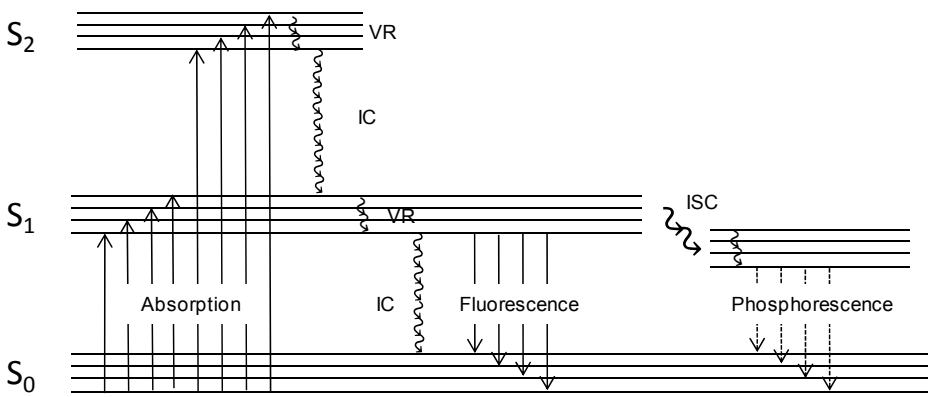

Figure 2. Jablonski diagram for fluorescence and phosphorescence. IC – internal conversion, VR – vibrational relaxation, ISC-intersystem crossing [Hemmilä 1991].

Several other relaxation pathways compete with the fluorescence emission process, for instance, non-radiative dissipation of energy (as heat), or energy transfer to another molecule (*e.g.,* quenching). Additionally, a phenomenon known as intersystem crossing to the lowest excited triplet state may occur which if followed by the radiative decay from an excited triplet state to a singlet ground state that is termed phosphorescence.

The average time the molecule stays in its excited state before emitting a photon is referred to as a fluorescence lifetime and is typically less than 10 ns, whereas the phosphorescence lifetime is longer, from milliseconds to seconds. The lifetime depends on both the origin of the fluorophore and its interaction with the local environment, and thus can be used to trace the molecular events such as molecular movement, rotation, collision and participation in different reactions. In addition to lifetime measurements, other fluorescence emission parameters such as fluorescence intensity and emission anisotropy may be used in fluorescence-based assays.

Fluorescence anisotropy/polarization (FA/FP)

Fluorescence anisotropy (FA) is a homogeneous, non-radioactive, sensitive, and robust detection method based on the assessment of the rotational motions of fluorescent probes in solution [Lakowicz 2006; Jameson and Ross 2010]. Upon irradiation with polarized light, fluorescent molecules possessing absorption transition moments parallel to the electric vector of the incident light are preferentially excited; still, the polarization of the emitted light is randomized due to the fast rotational diffusion, as a small molecular weight fluorophore or a fluorescently labelled probe can freely rotate several times during its excited-state lifetime in solution. The randomization effect may however be decreased as a result of binding of probe to a larger molecule, hence the degree of anisotropy is directly related to the amount of complexed fluorophore in solution, allowing direct quantification of the strength of binding interaction and/or the amount of binding partners in the sample. Anisotropy measurements have been applied for studies of protein-protein [Roehrl *et al.* 2004], protein-DNA, and receptor-ligand association, and for immunoassays of numerous substances [Lakowicz 2006].

Fluorescence anisotropy is frequently used in combination with the term "fluorescence polarization". The fluorescence anisotropy (r) and polarization (P) are defined as:

$$r = \frac{I_{II} - I_{\perp}}{I_{II} + 2I_{\perp}} \quad (4), \qquad P = \frac{I_{II} - I_{\perp}}{I_{II} + I_{\perp}} \quad (5),$$

where I_{II} and $I_{\perp}$ are the fluorescence intensities of vertically and horizontally polarized emission when the sample is excited with vertically polarized light.

Although the polarization and anisotropy express the same phenomenon and the corresponding values are interchangeable quantities:

$$P = \frac{3r}{(2+r)} \quad (6)\,,$$

the anisotropy is generally preferable as it is normalized by the total intensity [Lakowicz 2006].

Detection of fluorescence anisotropy/polarization has been one of the most widely used techniques for *in vitro* HTS in drug discovery [Owicki 2000]. However, application of this technique for characterization of inhibitors of PKs is limited due to low affinity (submicromolar or micromolar) of majority of fluorescent probes toward kinases, which in turn leads to the requirement for high concentration of the kinase in assay. Furthermore, FA-based assays are not well suitable for measurements in biological solutions (*e.g.,* blood plasma, cell lysate) rich in non-target proteins. Non-specific interactions of these proteins with hydrophobic fluorescent dyes may cause a false increase of anisotropy value that disturbs the measurement of specific interaction of the fluorescent probe with the target protein [Roehrl *et al.* 2004]. Still, some of the drawbacks of FA-based methods can be overcome by the application of Förster-resonance energy transfer (FRET) detection [Förster 1948] assays, which are less sensitive to side effects.

Förster resonance energy transfer (FRET)

Förster resonance energy transfer (FRET) [Förster 1948] is a non-radiative energy transfer from an excited donor molecule to an acceptor molecule. As a consequence of FRET, the emission of the donor is decreased and the emission of the acceptor is increased [Lakowicz 2006]. The efficiency of this process is dependent on several factors, including the spectral overlap and the distance between a donor and an acceptor, the quantum yield of the donor, and the relative orientation of the donor and acceptor transition dipole moments.

Both the efficiency (E) and the rate of energy transfer [k_T (r)] are dependent on the distance between the donor and acceptor, and can be calculated according to equations 7 and 8, respectively:

$$k_T(r) = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6 \quad (7)\,, \quad E = \frac{R_0^6}{R_0^6 + r^6} \quad (8)\,,$$

where r is the distance between the donor and the acceptor, τ_D is the decay time of the donor in the absence of the acceptor, and R_0 is the Förster distance at which the energy transfer efficiency is 50%. R_0 can be calculated as followed:

$$R_0 = 0.211[\kappa^2 n^{-1} Q_D J(\lambda)]^{\frac{1}{6}} \quad (9)\,,$$

where n is the refractive index of the medium (typically ~1.4 for aqueous solutions), Q_D is the quantum yield of the donor in the absence of the acceptor, $J(\lambda)$ is the spectral overlap between the donor-acceptor pair, and κ^2 is a factor describing the relative orientation of the transition dipoles of the donor and the acceptor in space (usually assumed to be equal to 2/3). The Förster distance is typically in the range of 20 to 90 Å and thus appropriate for studies of biological macromolecules.

In general, the occurring of FRET can be measured using three basic principles: the measurements of changes in donor fluorophore emission, acceptor fluorophore emission, or both donor and acceptor emissions [Clegg 2009]. FRET technology has been one of the most promising spectroscopic tools for studying biochemical processes such as protein-protein or protein-ligand interactions, protein cleavage, and conformational changes of proteins [Clegg 2009; Du *et al.* 2006; Jares-Erijman and Jovin 2006] (Figure 3).

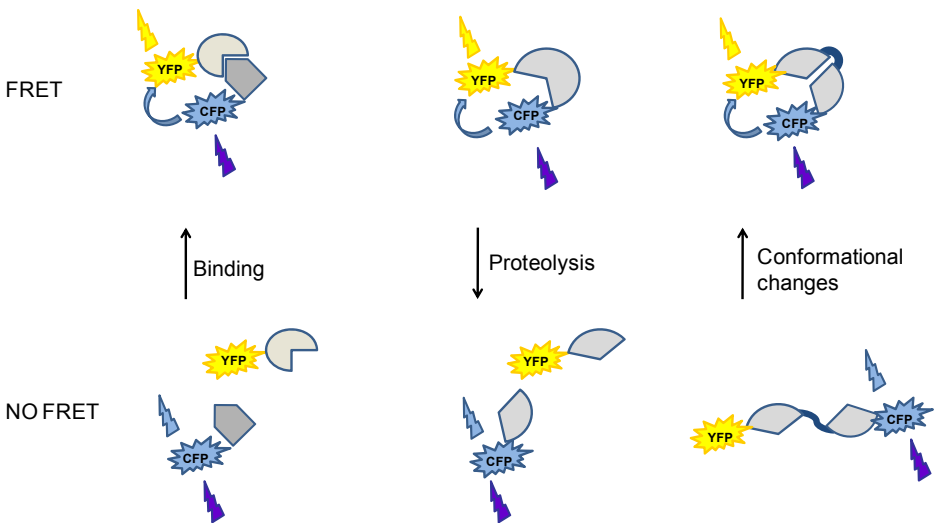

Figure 3. Some formats of the application of FRET in biological systems [Du *et al.* 2006].

The major limitations of traditional FRET measurements related to *in vivo* applications are caused by background fluorescence from biological samples such as cell lysate and blood plasma; in order to minimize these fluorescence interferences, the use of long-lived fluorophores combined with time-resolved detection is a powerful solution [Selvin 2002].

In time-resolved fluorescence (TRF) measurements, the sample is exposed to a pulse of light whereas the pulse width is shorter than the decay time of the donor fluorophore emission [Lakowicz 2006]. The emission of the acceptor is detected after a short delay (up to 400 μs), within which the possible background fluorescence of the sample components and autofluorescence of other interfering materials has decayed. As TRF necessitates the application of donors with an extremely long half-life (from microseconds to milliseconds), lanthanides (mainly europium, terbium, samarium or dysprosium) are commonly used for these measurements [Hemmilä and Laitala 2005]. Additional advantages of lanthanides further widening the field of their application involve large Stokes shifts and unique spectral properties that allow combination with a large variety of acceptors.

Fluorophores in fluorescence measurements

The most common fluorophores applied in fluorescence measurements may be divided into two groups represented by the synthetic organic dyes and fluorescent proteins. The number of fluorophores has increased dramatically during the past decade and nowadays, a variety of dyes with a sufficient wavelength range, Stokes shift and spectral bandwidth are commercially available. In addition to the aforementioned properties, extinction coefficient, fluorescence quantum yield and photostability of the dye are major factors that may affect the performance of fluorophores in various applications.

Structures of some organic fluorophores widely used in assays with both FA- and FRET-detection, are shown in Figure 4. The major advantages of organic dyes as compared to fluorescent proteins lies in the small size, high quantum yield, photostability and in a wide choice of available fluorophore spectral characteristics. However, due to the incorporation of several aromatic rings in their structure, synthetic fluorophores tend to be hydrophobic, which might be problematic for conjugation of these dyes to other molecules and for use in aqueous solutions.

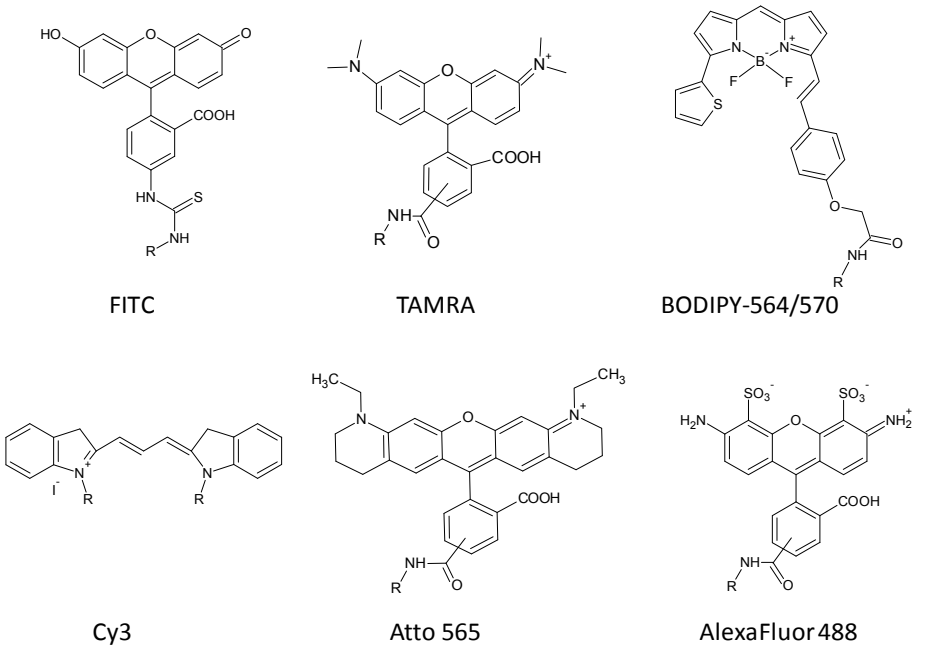

Figure 4. Some representatives widely used fluorescence dyes of different classes, FITC, TAMRA, BODIPY 564/570, Cy3, ATTO 565, Alexa Fluor 488 [Demchenko 2009].

Green fluorescent protein (GFP) isolated from jellyfish *Aequorea Victoria* by Shimomura in 1962 [Shimomura *et al.* 1962] was the first fluorescent protein described; since that time derivatives of GFP (blue, cyan and yellow fluorescent proteins) have been widely used in a variety of *in vitro* and *in vivo* studies [Heim *et al.* 1994; Heim and Tsien 1996]. Fluorescent proteins are increasingly used to monitor cellular processes, including gene expression, protein localization, protein degradation, and many biomolecular interactions, whereas the latter field of application generally utilizes fluorescent proteins as donors or acceptors in the FRET format [Nienhaus 2008]. In addition to the traditional cyan and yellow FRET pair, other donor-acceptor combinations have recently evolved (*e.g.,* GFP with RFP), which can provide both sufficient spectral separation of the donor and acceptor fluorescence as well as higher FRET efficiency. The recent development of orange, red and far-red fluorescent proteins [Merzlyak *et al.* 2007; Tsien 2009] has further enriched the palette of available genetically encoded fluorescent proteins and contributed to the growing popularity of these fluorescent proteins by virtue of low cellular autofluorescence in red region of the spectrum, which is ideal for *in vivo* imaging.

AIMS OF THE STUDY

The main aims of this study were:

1. Elaboration of a biochemical inhibition assay with chromatographic separation and fluorescence detection for the characterization of inhibitors of PKs, including ARC-type inhibitors.
2. Development of a biochemical binding/displacement assay based on the application of ARC-Photo probes for high-throughput screening of inhibitors and determination of kinase activity.
3. Establishment of the structural elements of ARC-Photo probes affecting their cellular uptake efficiency and intracellular localization to transfer the assays applying ARC-Photo probes from biochemical to cellular format.
4. Development of a FRET readout method based on ARC-Photo probes for monitoring of protein kinase activity in living cells.

RESULTS AND DISCUSSION

Fluorometric TLC assay for evaluation of protein kinase inhibitors

The connection of falsely regulated PK signalling with a variety of diseases has stimulated the development of both PK inhibitors and assays for their evaluation. The advantage of kinetic assays is the fact that these enable direct assessment of retarding effect of an inhibitor on the PK-catalyzed reaction, and thus allow estimation of the ability of the inhibitor to protect PK substrates from phosphorylation. Traditionally, the kinetic methods for the assessment of potencies of PK inhibitors as well as for the measurement of PK activity have been performed in the radioactive assay format. Despite the robust character and widespread popularity of these "classical" reference methods, radioactive assays have several drawbacks such as short half-life of ^{32}P, personal and environmental risks. All of the aforementioned disadvantages gave motivation for the development of a non-radioactive assay for the assessment of kinase activity and for characterization of ARC-type inhibitors. The assay developed as a part of the present study was based on the separation of a fluorescently labelled substrate of PKAc 5-TAMRA-Kemptide from its phosphorylated counterpart by thin layer chromatography (TLC) and subsequent ratiometric quantification of the product by fluorescence imaging.

First, it was demonstrated that the attachment of a fluorescent dye to the N-terminus of Kemptide had little influence on its substrate characteristics towards PKAc. 5-TAMRA was chosen as the fluorescent marker due to its relatively high extinction coefficient, high photostability, low sensitivity to environmental effects, and widespread application of the filter sets appropriate for TAMRA detection in fluorescence devices.

Second, it was ensured that the phosphorylation of 5-TAMRA-Kemptide by PKAc did not change the fluorescence characteristics (*i.e.,* absorption and emission maxima and fluorescence intensity) of the labelled peptide, but expectedly changed its elution characteristics. The product could be separated from the substrate on normal-phase silica gel TLC plates by elution with 1-butanol/ pyridine/acetic acid/water (15/10/3/12 by volume) mixture, yielding well-separated symmetric spots with R_f = 0.47 and R_f = 0.33 for 5-TAMRA-Kemptide and 5-TAMRA-phospho-Kemptide, respectively.

The reliability of the assay was demonstrated by comparison of kinetic parameters, K_m and V_{max} obtained from the new TLC assay with parameters from the traditional phosphocellulose radiometric assay. Table 2 demonstrates that both methods gave coinciding results for 5-TAMRA-Kemptide, and reveals that the attachment of a bulky fluorescent dye 5-TAMRA to the N-terminus of Kemptide improved its substrate characteristics resulting in lower K_m value of the TAMRA-labelled substrate.

Table 2. Kinetic parameters of phosphorylation of Kemptide and TAMRA-Kemptide catalyzed by PKAc

Parameter	Kemptide substrate	5-TAMRA-Kemptide substrate	
	By radioactivity (P81)	By radioactivity (P81)	By fluorescence (TLC)
K_m^{app} (μM)	12.8 ± 2.5	2.3 ± 0.9	2.0 ± 0.5
V_{max} (μmol min^{-1} mg^{-1})	6.4 ± 0.4	6.0 ± 0.7	5.2 ± 0.4

In addition, the inhibitory potencies of three PKAc inhibitors (H89, H9 and ARC-306) that had been tested previously with the phosphocellulose paper method were assessed with the novel TLC method (Figure 5). The similarity of inhibition values (**I** Table 2) obtained by both methods proved once again the reliability of the new assay.

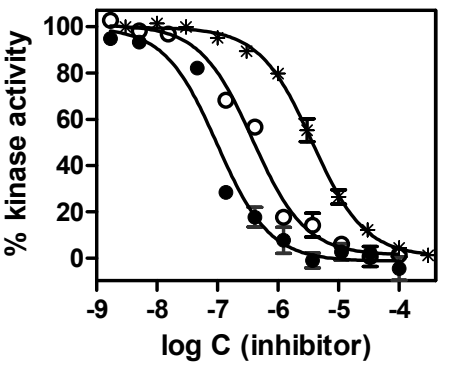

Figure 5. Inhibition of PKAc by H89 (●), Adc-Ahx-Arg$_6$ (○), and H9 (*).

The simultaneous quantification of the substrate and the reaction product make the developed assay ratiometric, allowing the reduction the effect of the fluorescent compound concentration and pippetting errors connected to sample preparation, reaction termination, and spotting of the sample onto a TLC plate. In addition, the assay is time- and reagent economizing, as up to 25 samples can be analyzed simultaneously on a 20 cm wide standard TLC plate. Finally, the throwaway format of the analysis matrix supports the use of the method with biological samples, *e.g.,* cell lysate and blood plasma.

TLC assay has been widely applied for the characterization of ARC-type inhibitors in the later research [Enkvist *et al.* 2006]. The method was initially developed for PKAc, but it has now been applied for other kinases that utilize Kemptide as the substrate, or even for kinases that phosphorylate other substrate peptides (for example, this method was recently adapted for assessment of PKBγ inhibitors [Lavogina *et al.* 2009; Enkvist *et al.* 2009].

Fluorescence anisotropy binding/displacement assays utilizing high-affinity bisubstrate probes

Another group of assays applicable for the assessment of properties of PK inhibitors as well as for the determination of concentration the active form of PK are binding assays that have been gaining wide approval due to their homogeneous character and quickness of measurements. Among the binding assays based on the measurement of fluorescent properties of the sample solution, fluorescence anisotropy/polarization [Kashem *et al.* 2007] and FRET [Lebakken *et al.* 2007; Lebakken *et al.* 2009] readouts have become the most popular for the assessment of PK inhibitors. The main general downside of these assays frequently pointed out is their requirement for high-affinity fluorescent probes (*i.e.,* with K_D values of less than 10 nM) in order to avoid the requirement of high concentration of a target PK for the analysis.

Several ARCs possess subnanomolar inhibitory potency and binding affinity towards PKAc, therefore it was decided to use high affinity and derivatization potential of ARCs for the development of homogeneous binding/displacement assay with FA-detection. First, fluorescent probe ARC-583 was constructed by attaching 5-TAMRA dye to the C terminus of ARC-902 (Figure 6); analogically to the TLC assay, TAMRA was chosen as the fluorescent label due to its good optical properties and wide application of appropriate filter sets in fluorescence plate readers.

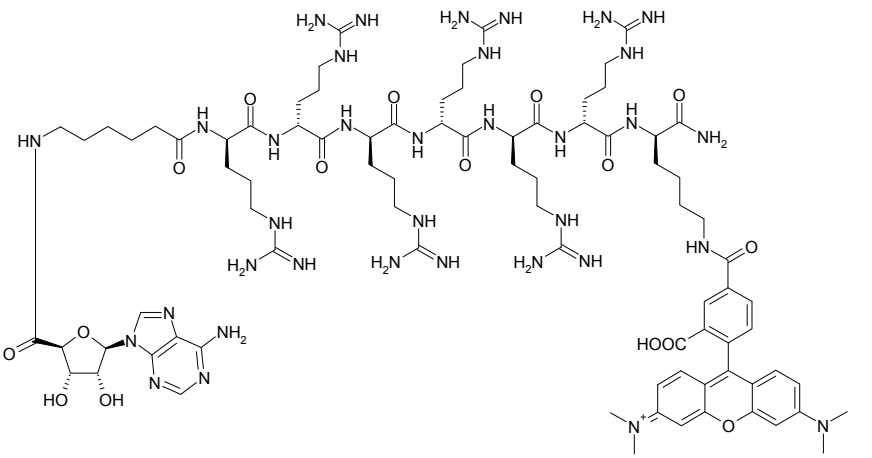

Figure 6. The structure of ARC-583.

Based on ARC-583, an assay was designed that enables the measurement of both direct binding of ARC-583 to the kinase, or competitive displacement of ARC-583 from its kinase complex by compounds targeted to ATP-site of PKAc, protein/peptide substrate-site of PKAc, or both sites (Figure 7).

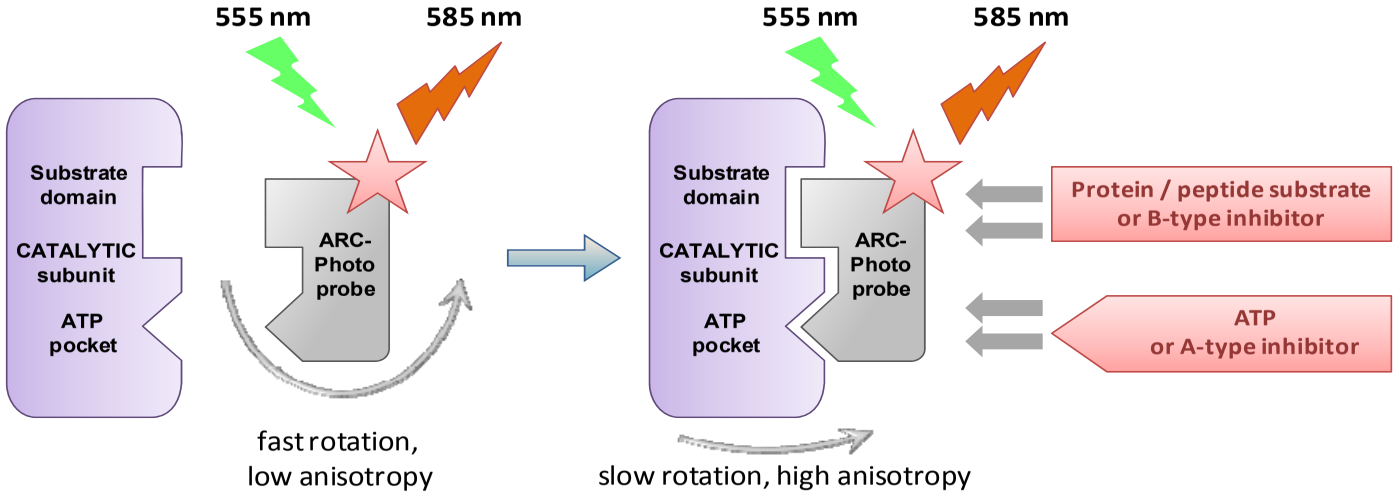

Figure 7. Schematic illustration of the principle of ARC-based FA-assay. Upon formation of high molecular weight complex between the kinase and the low molecular weight fluorescent probe ARC-Photo, the fluorescence anisotropy of ARC-Photo is increased. The displacement of ARC-Photo from this complex by either an ATP-competitive or a substrate-competitive inhibitor leads to the decrease of anisotropy to the initial level.

To establish the affinity of the probe towards PKAc, the titration of ARC-583 with the kinase was performed (Figure 8). The increase in fluorescence anisotropy resulting from the association of small molecular weight (MW < 2000) probe with high molecular weight (MW = 40000) kinase was plotted against the nominal kinase concentration, and the dissociation constant (K_D) of the ARC-583-PKAc complex was calculated by the application of nonlinear regression analysis:

$$A = A_f + \left(A_b - A_f\right)\frac{[L_t + K_D + kE_0 - \sqrt{(L_t + K_D + kE_0)^2 - 4L_t kE_0}]}{2L_t} \quad (10),$$

where A is the measured anisotropy; A_f is the anisotropy of free ARC-583; A_b is the anisotropy of ARC-583 associated with the kinase; L_t is the total concentration of ARC-583; E_0 is the nominal concentration of the kinase; K_D is the dissociation constant between ARC-583 and PKAc; and k is the fraction of the active kinase.

The K_D values of 0.48 and 0.66 nM were obtained in the absence and presence of magnesium ions, respectively, being in good accordance with the inhibitory potency of unlabelled analogue of ARC-583 as determined in the kinetic inhibition assay.

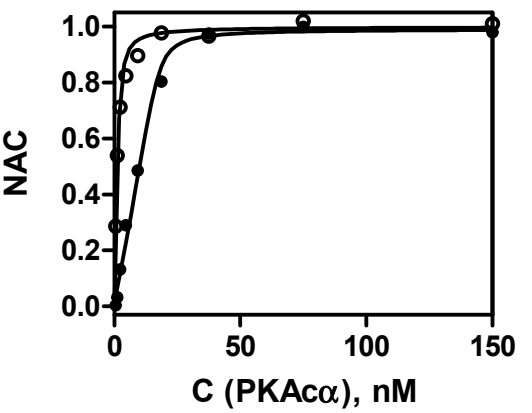

Figure 8. Titration of the fluorescent probe ARC-583 [2 nM (○) and 20 nM (●); magnesium ions not added] with PKAc. The binding constant K_D value of 0.48 nM was calculated from these data according to Eq. 10. Normalized anisotropy change (NAC) was calculated as NA= $(A-A_f)/(A_b-A_f)$.

The same direct binding format of the assay also allows assessment of the concentration of catalytically active form (*i.e.,* the form binding to ARC-583) of the kinase, allowing the determination of 2 fmol (80 pg) of PKAc. The fraction of the active kinase (k) was determined by performing titration of the fluorescent probe with kinase at high concentration of ARC-583 (20 nM;

Figure 8), and the binding data were fit to Eq. 10. Higher ARC-583 probe concentration was needed, as according to Equation (1):

$$E = \frac{K_D[EL]}{[L]} \quad (11)\,,$$

and the total enzyme concentration is expressed as

$$E_0 = [E] + [EL] \quad (12)$$

Hence

$$E_0 = \frac{K_D[EL]}{[L]} + [EL] = [EL]\left(\frac{K_D}{[L]} + 1\right) \quad (13)\,,$$

and in case of L >> K_D and $E_0 < L_t$

$$E_0 = [EL] \quad (14)$$

It was established that there was a good correlation between the concentration of the binding protein (as established with the probe ARC-583) and the phosphorylation activity of the solution of PKAc (as determined with TLC kinetic inhibition assay) (Figure 9).

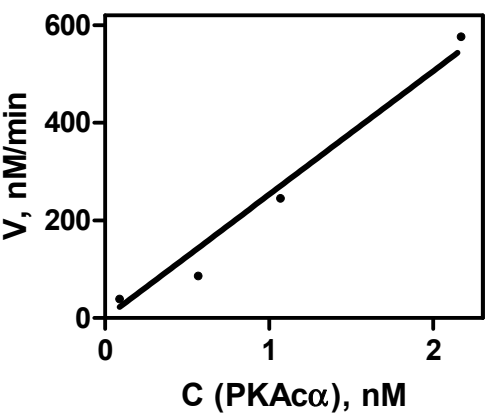

Figure 9. Correlation between the catalytic activity (V, determined with the TLC kinetic assay) and the active concentration (C, as titrated with the fluorescent probe ARC-583) of the solution of PKAc.

The subnanomolar affinity of ARC-583 appointed it of the most potent fluorescent probes ever described for PKs, enabling its utilization for the characterization of non-labelled inhibitors with nanomolar to micromolar potency. Moreover, the unique bisubstrate character of the probe (*i.e.,* simultaneous association with both ATP-binding site and substrate-binding site of a PK) allows its application for the characterization of both ATP- and protein/peptide substrate-competitive inhibitors of kinases. The bisubstrate character and very high affinity make the ARC-Photo probe different from previously described ATP-competitive [Chen and Poenie 1993] and protein substrate competitive [Schneider *et al.* 2005; Saldanha *et al.* 2006] fluorescent probes for PKs. The bisubtrate character of the probe was confirmed by its full displacement from the complex with the PKAc by compounds targeted to the ATP-binding pocket (H89, ATP and ADP; **II** Fig. 6) or to the protein/peptide substrate-binding site (PKArI, PKArII and PKI; in the presence of ATP,**II** Fig. 7) and by bisubstrate-inhibitors (ARC-902 and ARC-341; **II** Fig. 6) (Figure 10). The values of displacement constants determined for tested compounds ranged from subnanomolar to millimolar values, and were in a good agreement with the values obtained with TLC kinetic inhibition assay or other assays (Figure 11; **II** Table 2).

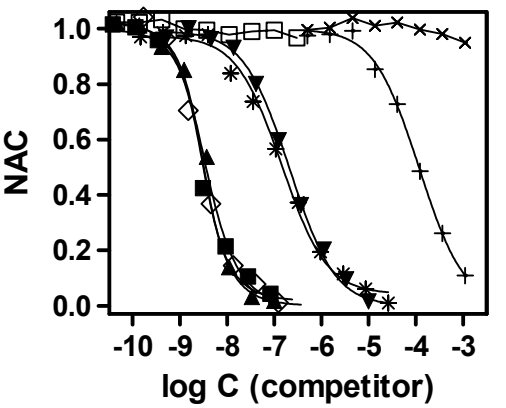

Figure 10. Displacement of fluorescent probe ARC-583 from its complex with PKAc by RIα (■, K_d=0.3 nM) and PKIα (◇, K_d=0.2 nM), in the presence of both ATP and Mg^{2+}; RIIα (▲, K_d=0.3 nM), H89 (*, K_d=23 nM), ARC-341 (▼, K_d=38 nM), RIα (□, no displacement below 300 nM), all in the absence of ATP and Mg^{2+}; ATP in the presence (+, K_d=17 μM) and in the absence (×, no displacement below 1 mM) of Mg^{2+}.

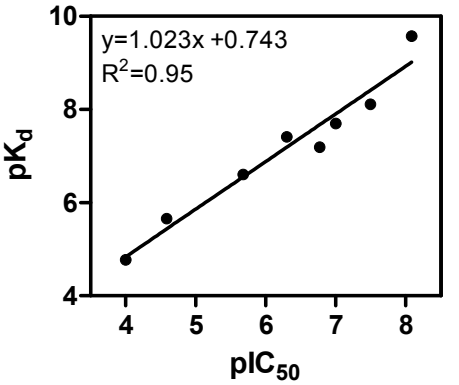

Figure 11. Correlation between the inhibitory potencies (IC_{50}) measured with the TLC kinetic inhibition assay and the displacement constants (K_d) determined in FA assay with fluorescent probe ARC-583 for eight inhibitors of PKAc.

The demonstration of competitive displacement of ARC-583 from its complex with PKAc by both synthetic and natural inhibitors, the latter category including PKI and PKAr subunits, demonstrated the potential of ARC-Photo probes and ARC-type inhibitors for measurements in living cells and tissues. Moreover, the previously demonstrated ability of ARC-Photo probes to serve as indicators of the concentration of free PKAc lead to conclusion that the probes could be used for assessment of cAMP concentration *via* effect of cAMP on PKA holoenzyme dissociation. By titration of PKA holoenzyme with cAMP in the presence of ARC-583 (Figure 12) it was indeed demonstrated that ARC-Photo probes may also be used for the characterization of compounds that realize their activity on PKA as agonists or antagonists of cAMP-regulated activation of PKA holo-enzyme.

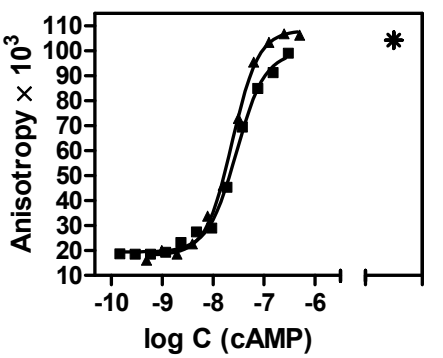

Figure 12. Effect of cAMP on anisotropy of the solution of ARC-583 (2 nM) and PKA holoenzyme, formed from PKAc and either PKArI (▲) or PKArII (■). EC_{50} values of 22 nM and 28 nM were calculated for PKAc/rI and PKAc/rII, respectively.

Finally, it was shown that the ARC-Photo-based binding/displacement assay with FA readout may be utilized not only for PKAc, but also for several other basophilic PKs. Titration of ARC-583 with ROCKII (**II** Fig. 3) yielded the K_D values of 3.6 and 9.2 nM in the absence and presence of magnesium ions, respectively (**II** Table 1), which allowed the utilization of ARC-583 for characterization of a wide range of inhibitors of ROCKII. Recently, a new generation of ARC-type inhibitors has been designed by focussed structural modification of ARCs from previous generations. Fluorescence labelling of those novel ARCs yielded ARC-Photo probes with wider selectivity profiles [*e.g.,* K_D values of 5.3 nM for ARC-1059 towards MSK1 and of 0.8 nM for ARC-669 towards PKCδ were obtained (**III** Fig. 1)].

To sum up, the use of the developed probes in optimized FA-based assay has several properties that support the use of the assay for HTS and precise characterization of inhibitors of PKs, including homogeneity, single step performance, quickness, no need for special substrates and capricious antibodies. Moreover, all of the aforementioned applications of ARC-Photo probes offer extremely interesting possibilities for the study of pathways of PKAc and several other basophilic PKs in cells and tissues; however, the latter goal would require converting the assays from *in vitro* biochemical to intracellular format.

ARC-type inhibitors in cells

Low success rate of the emergence of new drugs to the market has caused pharmaceutical industry to turn substantially more attention to the performance of primary and especially secondary screening of potential inhibitors in the living cells [Starkuviene and Pepperkok 2007]. As ARCs contain cell plasma membrane-penetrating oligoarginine moiety and it has been previously shown that multiple arginine residues-containing ARCs labelled with fluorescent dyes are able to penetrate plasma membrane [Uri *et al.* 2002], the idea to use ARCs and ARC-Photo-based assays in living cells emerged.

First, the effect of structure of an ARC-Photo probe on its cellular internalization efficiency and intracellular localization was studied. For this purpose, compounds differing in the structure of adenosine mimics (Figure 13), the number of arginine residues, the labelling position and the origin of the dye were constructed (Table 3) and tested in living CHO cells.

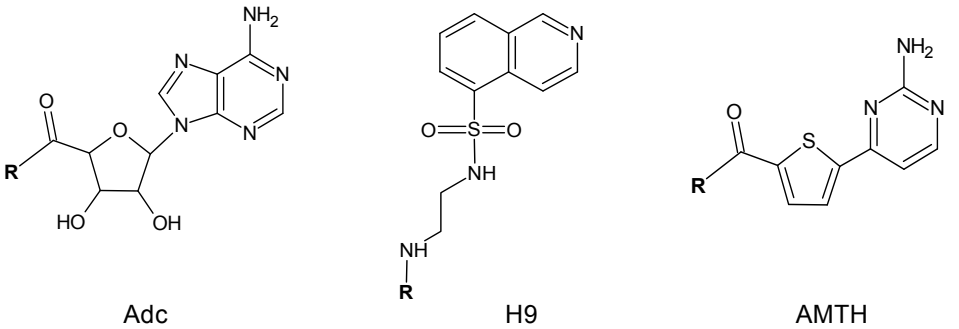

Figure 13. Nucleoside mimics in structures of ARCs, where R is denoting the fragment incorporating the linker and the peptide.

Table 3. Structures and codes of fluorescently labelled ARCs

ARC-Photo	**Fluorophore X**	**Code**
Adc-Ahx-(D-Lys-X)-Ahx-(D-Arg)$_2$-NH_2	5-TAMRA	ARC-1101
Adc-Ahx-(D-Arg)$_6$-(D-Lys-X)-NH_2	5-TAMRA	ARC-583
	Bodipy 564/570	ARC-1221
	ATTO 565	ARC-1046
	FITC	ARC-1045
	Bodipy FL	ARC-1204
Adc-Ahx-(D-Arg)-Ahx-(D-Arg)$_6$-(D-Lys-X)-NH_2	5-TAMRA	ARC-1042
	Bodipy 564/570	ARC-1219
H9-Ahx-(D-Arg)$_6$-(D-Lys-X)-NH_2	5-TAMRA	ARC-1059
AMTH-Ahx-(D-Arg)-Ahx-(D-Arg)$_6$-(D-Lys-X)-NH_2	5-TAMRA	ARC-669

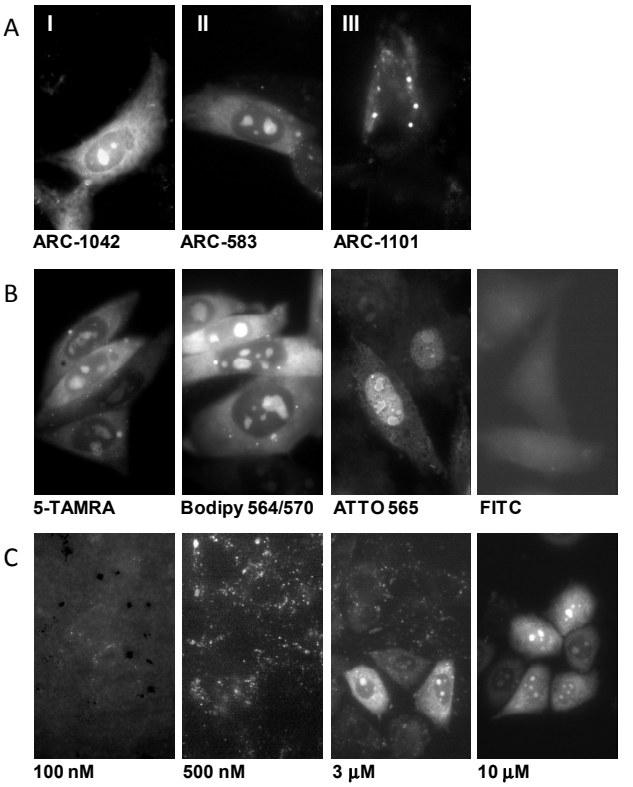

Figure 14. Localization of ARC-Photo probes in CHO cells after incubation at 37°C for 1 h. (A) Cells incubated with 10 µM ARC-1042 (I), ARC-583 (II), or ARC-1101 (III). (B) Cells incubated with ARCs labelled with different fluorescent dyes (ARC-583, ARC-1221, ARC-1046, ARC-1045). (C) Cells incubated with different concentrations (0.1 – 10 µM) of ARC-1042.

Extracellularly applied compounds that contain six or seven D-arginine residues in the peptide moiety (*e.g.,* ARC-583, ARC-1042) efficiently entered the cell and accumulated in its cytoplasm and nucleus. In the nucleus, these compounds concentrated to special regions, apparently nucleoli (Figure 14A I and II). In contrast, an ARC containing only two D-arginine residues (ARC-1101) stuck to the plasma membrane and did not reach the cytoplasm or nucleus of the cell (Figure 14A III). Cytosolic diffusion and nuclear accumulation of probes was less sensitive to the structure of the adenosine mimics (*e.g.,* ARC-1059, ARC-669).

In addition, cellular uptake and localization of the labelled inhibitors was influenced by other factors such as the concentration of extracellularly applied compounds and the nature of the fluorescent dye. The comparison of ARCs labelled with orange dyes demonstrated that 5-TAMRA- and Bodipy 564/570-labelled ARCs (ARC-1042 and ARC-1221, respectively) were both diffusely

distributed in the cytoplasm and localized in the nucleus (with accumulation in nucleoli; Figure 14B), whereas the ATTO 565-labelled ARC-1046 gave strong fluorescence only in the nucleus of the cells. In comparison to the Bodipy 564/570-labelled compound, the TAMRA-labelled compounds exhibited a stronger tendency to accumulate in a vesicular pattern. The examination of the uptake of ARCs carrying green dyes revealed that ARCs labelled with FITC gave a hardly detectable fluorescence signal in cells. The latter observation may be caused by unfavorable optical properties of FITC (*e.g.,* relatively high rate of photobleaching or pH-sensitive fluorescence) as well as by retarding effects of negatively charged FITC to membrane penetration. In contrast, high cellular fluorescence signal was detected in cells that were incubated with Bodipy FL-labelled ARC (ARC-1204), whereas the intracellular localization of ARC-1204 was very similar to that of the Bodipy 564/570-labelled conjugate (ARC-1221).

The influence of the concentration of the conjugates in the cell medium was demonstrated by varying the concentration in 0.1–10 μM range. As shown in Figure 14C, a vesicular pattern of the distribution of fluorescence was seen in cells incubated with ARC-1042 at concentrations lower than 1 μM, but upon the increase of concentration to 3–10 μM, diffuse cytoplasmic and nuclear fluorescence was observed. These results are in agreement with earlier studies, which indicated that increasing the concentration of CPPs above a certain threshold leads to strong increase in the level of diffuse cytosolic colouring of the cells [Kosuge *et al*. 2008].

The next step after confirmation of cell membrane-penetrative properties of ARCs was the application of ARC-Photo probes in intracellular assays. FA-based assays are not always well suited for measurements in biological solutions (*e.g.,* blood plasma, cell lysate) rich in non-target proteins that bind non-specifically to hydrophobic dyes of the fluorescent probes. The latter process can lead to increased anisotropy value of the free ligand, and thus reduce the measurement window (the effect was detected in the case of ARC-Photo probes in the case of the presence of larger concentration of albumin [**II** Fig. 2]). Therefore, it was decided to take advantage of FRET phenomenon to develop an ARC-Photo-based assay for the characterization of PK inhibitors in living cells.

To test the general applicability of ARC-Photo probes for assays with FRET readout, measurements of inter-molecular FRET between ARC-based 5-TAMRA-labelled fluorescent probe ARC-583 and PKAc chemically labelled with fluorescent dye FITC (PKAc-FITC) were performed in a biochemical assay format. FITC and TAMRA have sufficient spectral overlap between the donor emission and acceptor excitation spectra (Figure 15) to allow FRET measurements, and the excitation of the green donor fluorophore FITC at 485 nm led indeed to the detectable emission from the orange acceptor TAMRA at 590 nm as the result energy transfer. This FRET effect was disrupted by competing inhibitors H89, H9 and ARC-902 (Figure 16), whereas displacement of ARC-583 from the complex caused nearly 1.6-fold decrease in the ratio of fluorescence emission intensities at 590/520 nm.

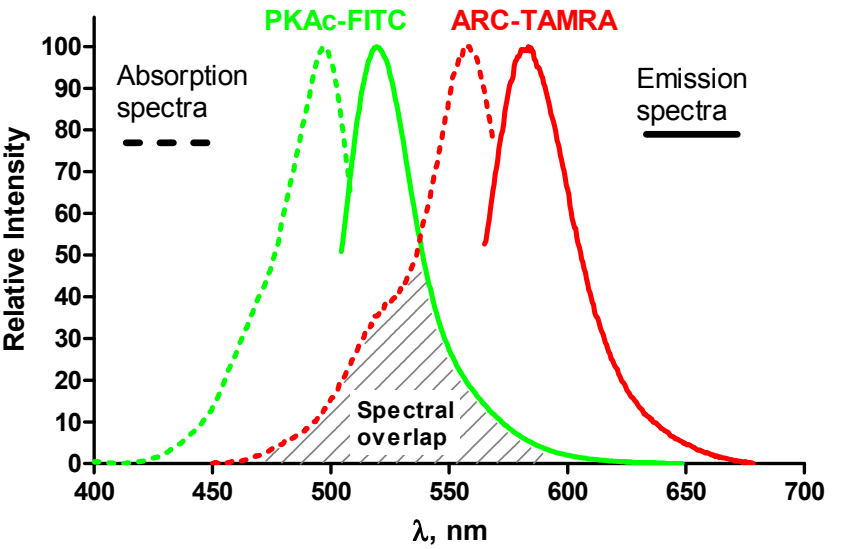

Figure 15. Spectral overlap between the donor (PKAc-FITC) emission and the acceptor (ARC-583) excitation spectra.

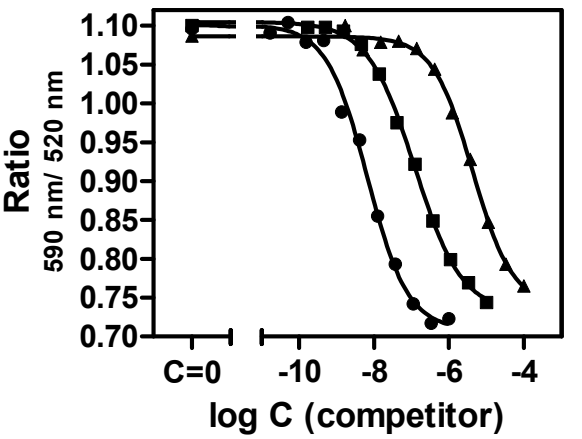

Figure 16. The competitive displacement of the ARC-Photo probe from the complex by a PKAc inhibitor. Graphs describing disruption of FRET between FITC-labelled PKAc and ARC-583 with ARC 902 (●), H89 (■) and H9 (▲) are expressed as the ratio of intensities (590/520).

The results of biochemical FRET experiments as well as the demonstration of cell membrane-penetrative properties of ARC-Photos encouraged testing of the applicability of ARC-Photo probes for FRET measurements in live C9H6 cells expressing the fusion protein PKAc-YFP (Figure 17).

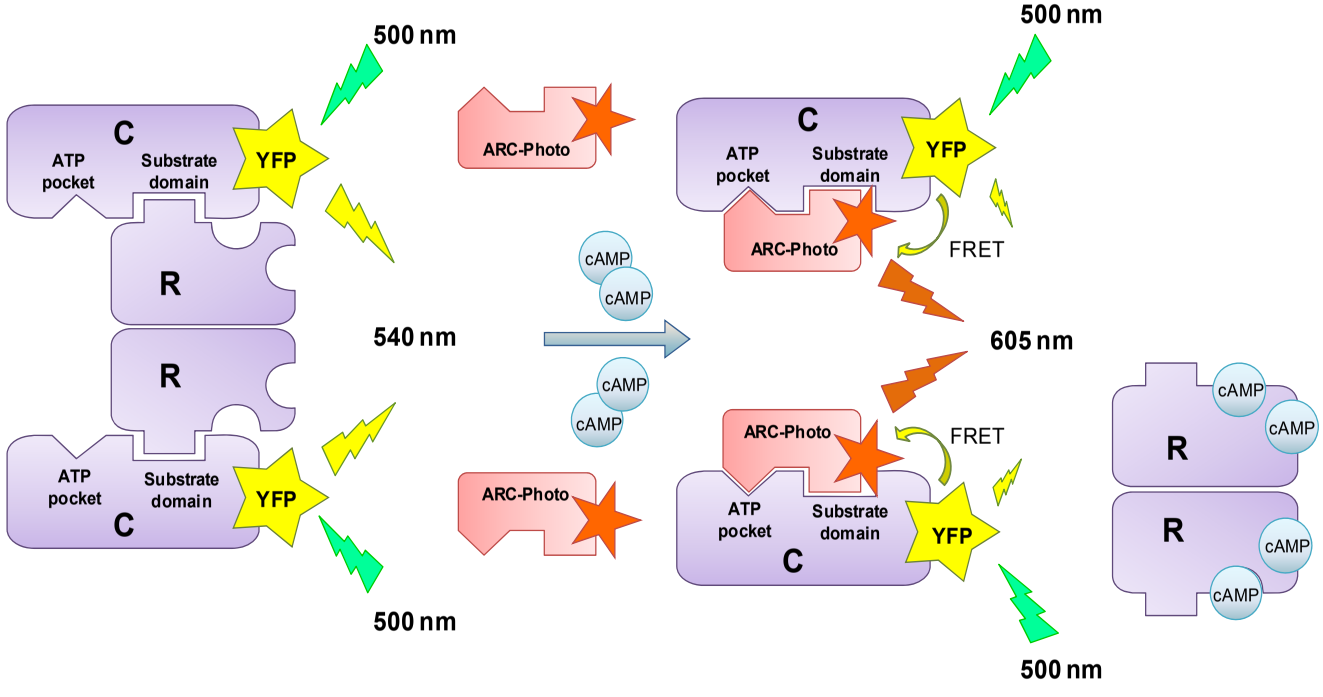

Figure 17. Schematic illustration of PKA activation: free PKAc-YFP binds the ARC-Photo probe, thus bringing the YFP donor fluorophore and the acceptor fluorophore of the ARC-Photo probe to close proximity that results in increased energy transfer between the fluorophores.

The occurrence of energy transfer between fluorescent labels of the two interacting partners (YFP of PKAc and 5-TAMRA of ARC-1042) was detected with a fluorescence microscope. Upon the treatment of cells with the cell-permeable adenylate cyclase activator Forskolin that leads to dissociation of PKAc-YFP from the holoenzyme and interaction of PKAc-YFP with ARC-1042, significant decrease (20–25%) in the donor emission at 540 nm and increase (18–23%) in acceptor emission at 605 nm (ex. at 500 nm) was measured (Figure 18). That effect was reversed by a cell-permeable PKA inhibitor, H89 that at 10 μM concentration competitively displaced ARC-1042 from its complex with PKAc-YFP and thereby prevented energy transfer between the fluorophores.

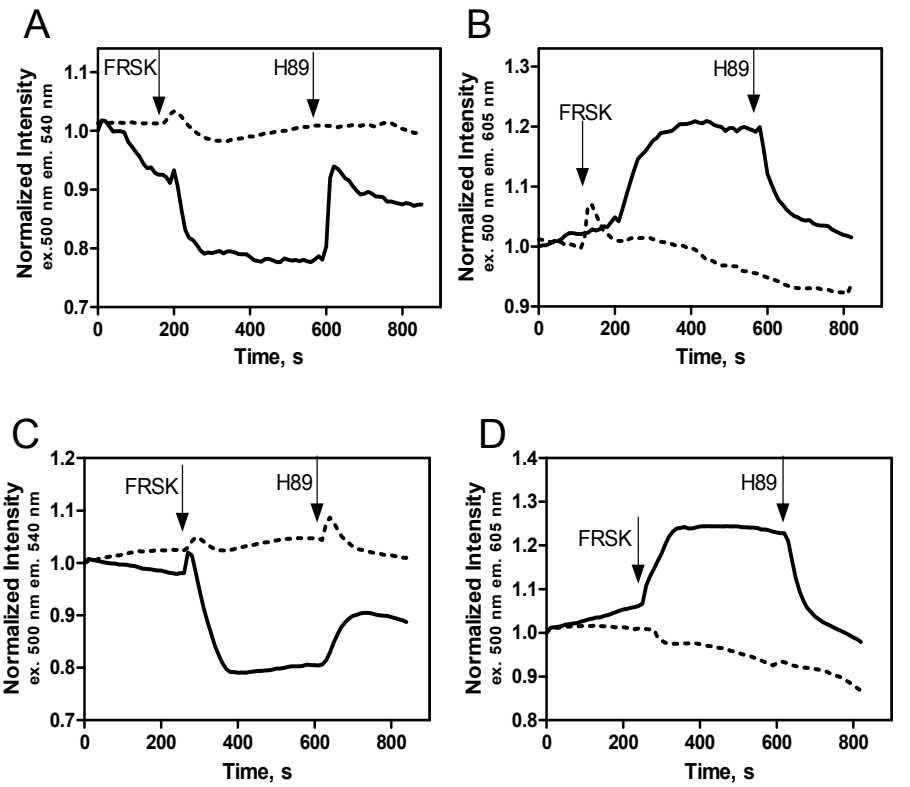

Figure 18. Single-cell detection of FRET with the fluorescence microscope. (A) Graph representing emission intensity of the donor fluorophore (ex.500 nm, em.540 nm) in cells in the presence (solid line) or absence (dotted line) of ARC-1042 (acceptor); n=10. (B) Emission intensity of acceptor ARC-1042 (ex. 500 nm, em. 605 nm) in cells expressing (solid line) or not expressing (dotted line) PKAc-YFP (donor); n=10. (C) Emission intensity of donor (ex. 500 nm, em. 540 nm) in cells in the presence (solid line) or absence (dotted line) of ARC-1219 (acceptor); n=7. (D) Emission intensity of acceptor ARC-1219 (ex. 500 nm, em. 605 nm) in cells expressing (solid line) or not expressing (dotted line) PKAc-YFP (donor); n=7. (A, B, C, and D). Arrows indicate the time points when Forskolin (25 μM) and H89 (10 μM) were added to the cell medium.

Subsequently, the Bodipy 564/570-labelled probe (ARC-1219) was used as a FRET acceptor instead of ARC-1042 with the same cell line, and even larger changes in fluorescence intensity were observed after application of Forskolin and H89 (Figure 18C and D).

Finally, the experiment was carried out in 96-well microtiter plates and the FRET measurements were performed with a fluorescence microplate reader to test the applicability of ARC-Photo-based FRET-readout assay in a high-throughput screening format (Figure 19). As expected, cell-to-cell signal heterogeneity caused lower sensitivity of the recorded signals and reduced signal-to-noise ratio as compared to the single cell-based assay. Still, the results were well reproducible, hence, the assay showed significant potential as a simple cellular assay for monitoring activity of PKA or effectors of PKA-mediated signalling pathways in high-throughput applications.

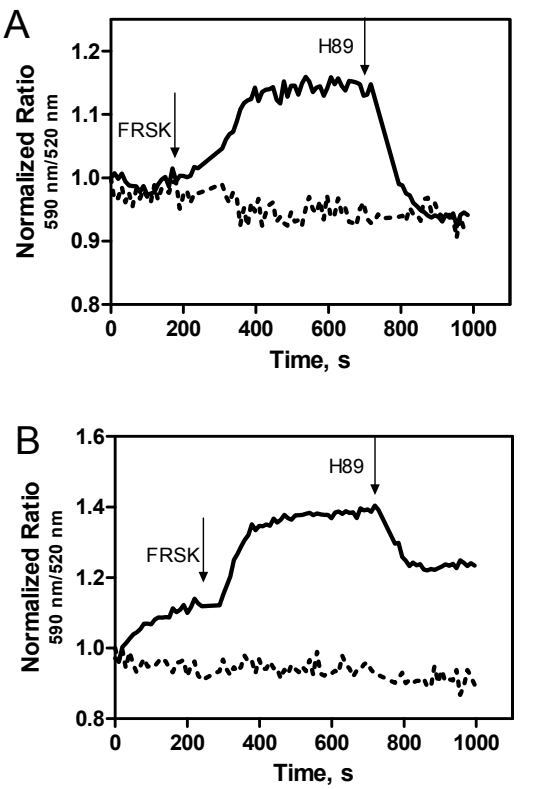

Figure 19. Detection of FRET changes in living cells using a fluorescence microplate reader. FRET efficiency between the donor fluorophore of PKAc-YFP and the acceptor dye of the ARC-Photo probe was measured as the ratio of fluorescence emission intensities (590/520) in C9H6 cells nontreated (dotted line) or treated (solid line) with (A) ARC-1042; n=10 (B) ARC-1219; n=10. (A and B). Arrows indicate the time points when Forskolin (25 μM) and H89 (10 μM) were added to the cell medium.

The novel assay may be used for both evaluation of non-labelled inhibitors of PKAc, and for monitoring cAMP signalling pathway *via* detection of changes in the activity of the cAMP downstream effector PKA. Furthermore, as ARC-s possesses high affinity towards several representatives of PKs of the AGC-group [Lavogina *et al.* 2009; Enkvist *et al.* 2009], the developed method may be extended into a format suitable for other basophilic PKs. In latter case, the specificity of interactions between the non-selective fluorescent probe and the target kinase is achieved *via* tagging of the target kinase with a fluorescent protein.

Further optimization of the system would be required to increase the signal window; for instance, the application of a high-content analysis reader (*e.g.,* an automated fluorescence microscopic imaging system) could greatly improve the quality of this method. The signal-to-noise ratio of the assay can be further improved if time-resolved (TR) version of FRET is applied (**III** Fig. 5), as this would enable overcoming of both the excitation cross-talk and background fluorescence occurring in complicated biological samples such as live cells, cell or tissue extracts, and blood plasma.

CONCLUSIONS

This thesis describes the progress with the development of fluorescence-based assays for protein kinase research, starting with a biochemical kinetic method and getting at a binding assay applicable for monitoring of protein kinase activity in living cells**.**

- A kinetic assay based on the chromatographic separation of the fluorescently labelled substrate peptide TAMRA-Kemptide from its phosphorylated counterpart and quantification of the phosphorylation extent ratiometrically with a fluorescence imager was worked out. The assay can be used as both a reliable control method and a sensitive method for the determination kinase activity in complicated biological samples. The assay has been successfully applied in research to support the development of ARC-type inhibitors (adenosine analogue-oligoarginine conjugates) and ARC-Photo probes (fluorescently labelled ARCs).

- The fluorescent probe ARC-583 was developed and its bisubstrate character (simultaneous association with both binding sites of the kinase) demonstrated. The probe was applied for the characterization of both ATP- and protein/peptide substrate-competitive inhibitors of protein kinases in fluorescence polarization-based assay. High affinity of ARC-583 (K_D = 0.48 nM towards PKA) enabled its application in competition assay for the characterization of inhibitors with nanomolar to micromolar potency. ARC-583 binding/displacement assay with fluorescence polarization readout could also be used for determination of the concentration of the active form of PKA and as a sensor for cAMP concentration.

- The effect of structural elements of ARC-Photo probes on the cellular internalization efficiency and intracellular localization of the probes was studied. It was demonstrated that extracellularly applied compounds that contained six or seven D-arginines in the peptide moiety efficiently entered the cell and accumulated in its cytoplasm and nucleus; inhibitors containing only two arginine residues stuck to the membrane and did not reach the cytoplasm. Cell entry of probes was less sensitive to the structure of the adenosine mimics of the conjugates. The cellular uptake and localization of the labelled inhibitors were also influenced by several other factors such as the concentration of extracellularly applied compounds and the origin of the fluorescent dye of the ARC-Photo probe.

- The ability ARC-Photo probes to bind with high affinity to the free catalytic subunit of PKA was used to construct a cellular sensor for monitoring PKA activity in living cells. CHO cells stably expressing PKAc fused with the yellow fluorescent protein (PKAc-YFP) were loaded with ARC-Photo probes. Increased Förster resonance energy transfer efficiency between the

fluorescent labels of the two interacting partners, the fusion protein and ARC-Photo probe, was detected with a fluorescence microscope or plate reader when PKA was activated. The novel assay can be used for both the evaluation of non-labelled inhibitors of PKAc and for monitoring of cAMP signalling *via* detection of changes in the activity of PKA as a cAMP downstream effector.

SUMMARY IN ESTONIAN

Fluorestsentsmeetodite arendamine proteiinkinaaside ning nende inhibiitorite uurimiseks

Proteiinkinaaside (PK) poolt katalüüsitav valkude fosforüleerimine on üks tähtsamaid valkude aktiivsuse reguleerimise mehhanisme, mis võimaldab rakkudel reageerida väliskeskkonna muutustele ning suunata elutegevuse tagamiseks olulisi rakusiseseid protsesse. PK-de funktsioneerimishäiretega (eelkõige nende üleekspressiooni ning anomaalselt kõrge aktiivsususega) on seotud mitmed haigused, sealhulgas vähkkasvajad, suhkruhaigus, südame-veresoonkonna haigused ja Alzheimeri tõbi. Seetõttu on PK-de uurimine ja PK-de aktiivsust blokeerivate inhibiitorite arendamine muutunud keskseks teemaks nii teadusasutustes kui ka ravimitööstustes. Sellest tulenevalt on viimaste aastatega oluliselt kasvanud ka vajadus usaldusväärsete ja kiirete kinaaside analüüsimeetodite järele.

Käesoleva töö raames töötati välja fluorestentsil põhinevad meetodid kinaasi aktiivsuse määramiseks ning kinaasi-inhibiitorite iseloomustamiseks, mille rakendatavust demonstreeriti nii kinaase sisaldavates lahustes kui ka elusrakkudes. Esimene arendatud meetod põhineb fluorestsentsmärgistatud cAMP-sõltuva proteiinkinaasi substraadi (5-TAMRA-kemptiid) lahutamisel fosforüleeritud produktist planaarkromatograafiliselt ning fosforüleerimisreaktsiooni komponentide kvantifitseerimisel fluorestsentsskänneriga. Fosforüleerimisreaktsiooni sügavus määrati tekkinud produkti ja lähtesubstraadi signaalide intensiivsuse suhete kaudu, seejuures suurendas kasutatud sisemine normeerimine oluliselt analüüsi täpsust. Seda meetodit rakendati edukalt kinaaside inhibiitorite, sealhulgas ARC-tüüpi inhibiitorite, iseloomustamiseks.

Teine arendatud meetod on fluorestentsanisotroopia mõõtmisel põhinev sidumismeetod, mis kasutab fluorestsentsvärviga märgistatud adenosiini analoogi ja arginiini-rikka peptiidi konjugaate, ARC-Photo sonde. Uudset fluorestsentssondi kasutati kinaaside PKAc ja ROCKII inhibiitorite tuvastamiseks ning sidumisomaduste määramiseks. Tõestati ARC-Photo sondide bisubstraatne iseloom ning nende sobivus kinaaside aktiivsuse määramiseks ning rakendatavus cAMP-sensorina. Sondi unikaalsed omadused võimaldasid iseloomustada nii ATP kui ka substraatvalgu sidumistaskusse seonduvaid inhibiitoreid, mille afiinsus varieerus väga laias ulatuses, nanomolaarsest kuni millimolaarseni.

Eespool kirjeldatud meetodite abil saadud inhibeerimistulemuste omavaheline võrdlus ning kontroll laialt kasutatava radioaktiivse referentsmeetodiga andis hea korrelatsiooni, mis näitab mõlema arendatud meetodi usaldusväärsust.

Uurimustöö järgmiseks etapiks oli kinaaside analüüsimeetodite juurutamine mõõtmisteks elusrakkudes, kuna just rakkudes toimuvate protsesside jälgimine võimaldab adekvaatselt hinnata inhibiitorite omadusi ning proteiinkinaaside inhibeerimisest tingitud füsioloogilisi efekte. Esmalt tehti kindlaks ARC-Photo

sondide võimekus läbida rakkude plasmamembraani. Töös näidati, et fluorestsentssondid, mis sisaldavad peptiidses osas kuut või seitset arginiini jääki, on võimelised efektiivselt tungima rakkudesse ning paigutuma tsütosooli ja tuuma. Lisaks arginiinide arvule sõltub ARC-Photo sondide rakumembraani läbimise efektiivsus ja paiknemine rakus sondide kontsentratsioonist ja fluorestsentsvärvi keemilisest loomusest.

ARC-Photo sonde kasutati Försteri resonantsienergia ülekande (FRET) efektiivsuse määramisel põhineva meetodi väljatöötamiseks PKA aktiivsuse jälgimiseks elusrakkudes. PKAc ja kollase fluorestseeruva valgu (YFP) liitvalku (PKAc-YFP) tootvates rakkudes põhjustas PKA aktiveerimine aktiivse PKAc-YFP vabanemise ning selle seondumise ARC-Photo sondiga. Sellest tulenes FRETi efektiivsuse oluline muutumine fluorofooride vahel, mida oli võimalik mõõta fluorestsentsmikroskoobi või fluorestsentsplaadilugeja abil. Uut meetodit on võimalik kasutada kinaaside aktiivsuse jälgimiseks rakkudes, PKA ja teiste kinaaside inhibiitorite kiirkatsetamiseks ning cAMP-regulatsiooniga seotud retseptoritele toimivate ligandide iseloomustamiseks.

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