DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
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IMMOBILIZABLE BISUBSTRATE-ANALOGUE INHIBITORS OF BASOPHILIC PROTEIN KINASES: DEVELOPMENT AND APPLICATION IN BIOSENSORS

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Dissertation in bioorganic chemistry

Dissertation is accepted for the commencement of the degree of Doctor of Philosophy in Chemistry on April 27th, 2007 by the Doctoral Committee of the Department of Chemistry, University of Tartu.

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Commencement: June 14th, 2007, University of Tartu, 18 Ülikooli St., room 204, 14:00.

ISSN 1406–0299
ISBN 978–9949–11–613–3 (trükis)
ISBN 978–09949–11–614–0 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr 174

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which are referred in the text by Roman numerals:

I. Viht, K., Padari, K., Raidaru, G., Subbi, J., Tammiste, I., Pooga, M., Uri, A. Liquid-Phase Synthesis of a Pegylated Adenosine-Oligoarginine Conjugate, Cell-Permeable Inhibitor of cAMP-Dependent Kinase. – *Bioorg. Med. Chem. Lett.,* **2003**, 13, 3035–3039.

II. Viht, K., Vaasa, A., Raidaru, G., Enkvist, E., Uri, A. Fluorometric TLC assay for evaluation of protein kinase inhibitors. – *Anal. Biochem.,* **2005**, 340, 165–170.

III. Viht, K., Schweinsberg, S., Lust, M., Vaasa, A., Raidaru, G., Lavogina, D., Uri, A., Herberg, F.W. Surface-plasmon-resonance-based biosensor with immobilized bisubstrate analog inhibitor for the determination of affinities of ATP- and protein-competitive ligands of cAMP-dependent protein kinase. – *Anal. Biochem.,* **2007**, 362, 268–277.

Author's contribution

Paper I: Contributed to the planning of the experiments and carried out the experimental work except mass-spectrometric measurements, HPLC and cellular uptake experiments. Responsible for writing the manuscript.

Paper II: Contributed to the planning of the experiments and carried out kinase activity measurements by radiometric assay. Responsible for writing the manuscript.

Paper III: Contributed to the planning of the experiments and carried out the preparation and structure verification of the affinity ligand. Performed most of the SPR-measurements, responsible for data analysis and writing the manuscript.

ABBREVIATIONS

ACN	acetonitrile
Adc	adenosine-5'-carboxylic acid, 1-(6-amino-9*H*-purin-9-yl)-1-deoxy-β-D-ribofuranuronic acid
ADP	adenosine 5'-diphosphate
Ahx	6-aminohexanoic acid
AKAP	A-kinase anchoring protein
Alpha	amplified luminescent proximity homogeneous assay
AMP	adenosine 5'-monophosphate
ARC	adenosine-oligoarginine conjugate
ARC-306	Adc-Ahx-(L-Arg)$_6$-OH
ARC-341	Adc-Ahx-(L-Arg)$_6$-NH$_2$
ARC-701	Adc-Ahx-(L-Arg)$_4$-L-Lys-NH-PEG5000-OMe
ARC-702	Adc-Ahx-(L-Arg)$_4$-L-Lys(BODIPY FL)-NH-PEG5000-OMe
ARC-703	Adc-Ahx-(L-Arg)$_4$-L-Lys(biotin)-NH-PEG5000-OMe
ARC-704	Adc-Ahx-(D-Arg)$_6$-D-Lys(PEO-biotin)-NH$_2$
ARC-902	Adc-Ahx-(D-Arg)$_6$-NH$_2$
ARC-904	Adc-Ahx-(D-Arg)$_6$-D-Lys-NH$_2$
ATP	adenosine 5'-triphosphate
Boc	*tert*-butoxycarbonyl
BODIPY FL	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid
CAMK	calmodulin-dependent protein kinase
cAMP	cyclic adenosine 3',5'-monophosphate
cAPK	cAMP-dependent protein kinase
cAPK C	cAPK catalytic subunit
cAPK Cα	cAPK catalytic subunit, α-isoform
Delfia	delayed enhanced lanthanide fluorescence immunoassay
DHB	2,5-dihydroxybenzoic acid
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
Fmoc	9-fluorenylmethoxycarbonyl
FP	fluorescence polarization
FRET	fluorescence resonance energy transfer
GST	glutathione *S*-transferase
H1152P	(*S*)-(+)-4-methyl-5-(2-methyl-[1,4]diazepane-1-sulfonyl)-isoquinoline
H89	*N*-{2-[(*p*-bromocinnamyl)amino]ethyl}-5-isoquinolinesulfonamide
H9	*N*-(2-aminoethyl)-5-isoquinolinesulfonamide
HPLC	high performance liquid chromatography
IC_{50}	half maximal inhibitory concentration

IMAP	immobilized metal ion affinity-based fluorescence polarization
IR	infrared
Ip	isopropylidene
K_d	dissociation constant
K_i	inhibition constant
K_m	Michaelis constant
Lance	lanthanide chelation excitation assay
LPOS	liquid-phase organic synthesis
MALDI	matrix-assisted laser desorption/ionization
mPEG	methoxypoly(ethylene glycol)
mPEG9500	methoxypoly(ethylene glycol) with average molecular weight of 9500 Da
MS	mass spectrometry
MW	molecular weight
NAD^+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
NMR	nuclear magnetic resonance
P-site	phosphorylation site
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PEG	poly(ethylene glycol)
PEG5000	poly(ethylene glycol) with average molecular weight of 5000 Da
PEO	polyethylene oxide
PEP	phosphoenol pyruvate
PKA	protein kinase A
pK_a	negative logarithm of acid dissociation constant
PKC	protein kinase C
PKI	heat stable protein kinase inhibitor
PP_i	pyrophosphate
PRKX	human X chromosome encoded protein kinase
PRKY	Y homologue of the protein kinase PRKX
RI	regulatory subunit type I
RII	regulatory subunit type II
RP-HPLC	reversed-phase high performance liquid chromatography
R_t	retention time
SPA	scintillation proximity assay
SPOS	solid-phase organic synthesis
SPR	surface plasmon resonance
SPSOS	soluble-polymer-supported organic synthesis
TAMRA	carboxytetramethylrhodamine
TBME	methyl *tert*-butyl ether
TFA	trifluoroacetic acid
TLC	thin layer chromatography

TOF	time-of-flight
UV	ultraviolet
Vis	visible
V_{max}	maximum velocity of the enzyme-catalyzed reaction

3

INTRODUCTION

cAMP-dependent protein kinase

The phosphorylation of proteins, catalyzed by protein kinases, is a common posttranslational modification in eukaryotes (Figure 1). Most cellular processes from proliferation to apoptosis are controlled by the regulation of the phosphorylation equilibrium whereas aberrant functioning of protein kinases is associated with pathological states such as several forms of leukaemia and solid cancers, inflammatory and autoimmune disorders and cardiac diseases. For this reason protein kinases have become one of the major therapeutic targets [1]. The protein kinase superfamily is one of the largest as about 2% of genes in eukaryotic cells encode the kinase catalytic domain. The human genome encodes more than 500 protein kinases that are divided into nine groups and several families on the basis of the sequence similarity of the catalytic domains [2]. Based on the phosphorylatable amino acid residue, protein kinases are classified into serine/threonine, tyrosine and dual-specificity protein kinases.

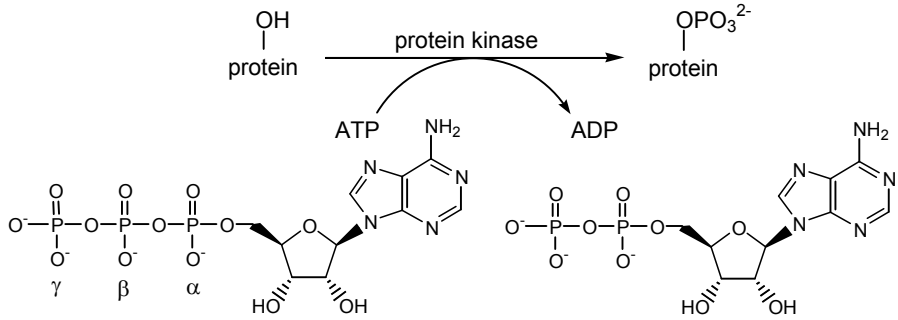

Figure 1. Protein kinases catalyze the transfer of the terminal phosphate group of nucleoside triphosphates to specific amino acid residues of protein substrates.

cAMP-dependent protein kinase (cAPK) is an ubiquitous serine/threonine kinase that belongs to the AGC group of protein kinases. It is the most studied and the best characterized serine/threonine kinase that has served as a prototype for protein kinases, mostly due to its relative structural simplicity and possibility to be expressed and purified in large quantities [3, 4]. cAPK has also been suggested to be a therapeutic target for several forms of cancer and diseases of the immune system [5]. In its inactive state, cAPK exists as a hetero-tetramer composed of a dimer of regulatory subunits (R_2) and two catalytic subunits (C). The enzyme becomes active after the release of the C-subunits from the holoenzyme by cooperative binding of cAMP to two binding sites in the regulatory subunit [6]. The catalytic subunit of cAPK is encoded as several

isoforms (PKAα, PKAβ, PKAγ, PRKX and PRKY) that differ in their expression level, tissue distribution and functionality [7–11]. The structure of C-subunit is comprised of smaller N-terminal domain and larger C-terminal domain, which are connected by a small linker region. The active site of the enzyme is located in the cleft between these domains. The catalytic mechanism involves the formation of ternary complex where both substrates are simultaneously present in the catalytic site. The phosphoryl group is directly transferred from ATP to the peptide substrate without the formation of covalent intermediate with the enzyme [12, 13]. The binding of the nucleotide requires the presence of magnesium ions. The magnesium in the higher-affinity site (K_d=10 μM) bridges the β- and γ-phosphates and coordinates the terminal phosphate with the peptide substrate. The second magnesium ion bridges α- and γ-phosphates with much lower affinity (1.4 mM) and it is considered to be inhibitory in nature. Although it stabilizes the complex between the nucleotide and the enzyme, it also reduces the dissociation of ADP from the catalytic site, which is usually the rate-limiting step of the catalysis [14, 15]. The recognition of the protein substrate by the kinase is based on the amino acid sequence surrounding the phosphorylatable residue (P-site). cAPK is a basophilic protein kinase as it has a preference to phosphorylate proteins rich in basic amino acid residues near the phosphorylation site. The optimal substrate consensus sequence has the following structure: -Arg-Arg-X-Ser/Thr-Y-, where X designates variable and Y hydrophobic amino acid residues [6]. The standard substrate of cAPK is kemptide (Table 1) with K_m value of 16 μM [16] whereas the binding affinity of the substrate is more than an order of magnitude lower (K_d=210 μM) [17]. This discrepancy is explained by the mechanism of the catalysis with rapid phosphoryl transfer step followed by rate-limiting ADP release [14, 18].

Table 1. Sequence comparison of kemptide and the physiological inhibitors (human) of cAPK. Residues corresponding to P-site are highlighted.

Consensus sequence	-RRX$\frac{\mathbf{S}}{\mathbf{T}}$Y-
kemptide	LRRA**S**LG
PKIα	-TTYADFIASGRTGRRN**A**IHDILVSSASGNSNELAL-
RIα	-SPPPPNPVVKGRRRRG**A**ISAEVYTEEDAASYVRKV-
RIIα	-DEDLEVPVPSRFNRRV**S**VCAETYNPDEEEEDTDPR-

The activity of cAPK can be inhibited by compounds that block the active site of the enzyme, binding to ATP-binding site, protein-binding site or associating simultaneously with both of these sites (bisubstrate inhibitors). Additionally the activity of cAPK can be regulated allosterically by substances that bind to cAMP-binding sites on the holoenzyme leading to activation (cAMP agonists) or deactivation (cAMP antagonists) of the kinase.

Physiologically, cAPK is inhibited by two families of inhibitors: regulatory subunits (R) and heat stable protein kinase inhibitors (PKI), which both are competitive towards protein-binding [12]. The regulatory subunits are classified into two types, RI and RII that bind to catalytic subunits with subnanomolar affinity forming type I (RI_2C_2) and type II (RII_2C_2) holoenzymes, respectively. Additionally, the regulatory subunits bind to A-kinase anchoring proteins (AKAP-s) that are responsible for the intracellular localization of the enzyme. The R-subunits are subclassified into four distinct isoforms (RIα, RIβ, RIIα and RIIβ) that differ in the level of expression, tissue distribution and specificity for AKAP-s. PKI-s are also encoded as several isoforms that bind to catalytic subunits with high affinity [19, 20]. In the complex with C-subunit, PKI-s mediate the nuclear export of cAPK [12]. Both RI and PKI possess a pseudosubstrate inhibitory site (Table 1). The high-affinity binding of RI and PKI to C-subunit is synergistically dependent on the presence of MgATP: the affinity of MgATP to the C-PKI complex and the holoenzyme type I is about three orders of magnitude higher than to the free catalytic subunit [21]. In the absence of MgATP, the dissociation constants of the complexes of the catalytic subunit with RI and PKI are in submicromolar range (Figure 2) [12, 22].

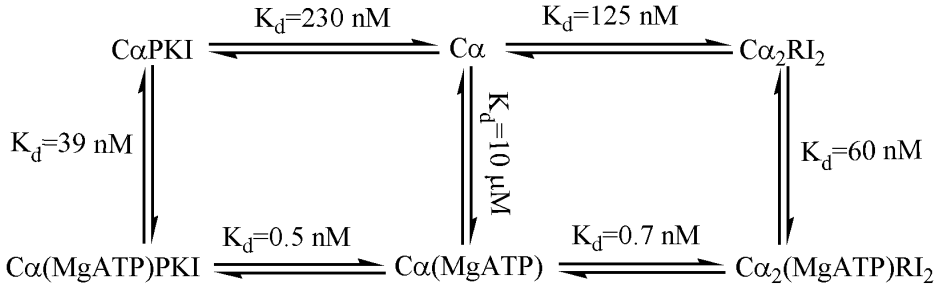

Figure 2. Synergism of the binding of ATP, RI and PKI to cAPK Cα [10, 12 and 22–24].

The type II regulatory subunits do not show such synergism and bind to the C-subunit with high affinity irrespectively of the presence of the nucleotide. The RII subunits incorporate the substrate consensus sequence and are phosphorylated in the holoenzyme complex (Table 1) [12].

Assay principles for the characterization of the inhibitors of protein kinases

The assays used for the evaluation of enzyme inhibitors can be classified into two general categories:

1. functional assays for the determination of the ability of test compounds to inhibit enzyme-catalyzed reaction,
2. binding assays for the determination of the binding affinity of test compounds to enzyme.

The ability of the inhibitor to slow down the catalytic reaction is characterized by IC_{50}-value that corresponds to its concentration causing 50% reduction of the enzymatic activity in the used assay conditions. If the inhibition mechanism is known, the inhibition constant (K_i) can be calculated [25]. Binding assays allow the determination of the dissociation constants (K_d) of the complex between the inhibitor and enzyme and do not directly reveal whether the ligand acts as an inhibitor or substrate or binds to allosteric sites. Since the binding affinity of a test compound is not a direct measure of its inhibitory potency, the inhibitor screening is generally carried out by enzyme activity assays [26, 27].

The best measure of the enzymatic activity is the initial velocity of the enzyme-catalyzed reaction [28]. The reaction rate is usually determined by single time-point measurements of the amount of the formed product or unreacted substrate and, in the initial velocity phase, it is proportional to the enzymatic activity and most sensitive to inhibition. Typical assays are performed at concentrations of the substrates near their K_m values, which are optimal for the detection of inhibitors with different inhibition mechanisms. In these conditions, the initial velocity can be measured with sufficient precision below 10–20% of the substrate consumption. To work with such small changes of the concentrations, the detection methods of the assay must be sufficiently sensitive. At higher substrate conversion, the reaction rates decrease significantly due to substrate depletion and the inhibitory potencies become underestimated. In order to increase the signal quality, it may be acceptable to work at higher substrate conversion where the inhibitory potencies are not drastically deviated from the true values. Wu *et al*. derived a relationship between the IC_{50} values determined by single time point measurements in the initial velocity phase and at higher substrate turnover [29]. They showed that if the reaction proceeds with first-order kinetics, *i.e*., at substrate concentrations much lower than K_m, the IC_{50}-values determined below 75% of substrate conversion were deviated from the true values less than two times. At substrate concentrations much higher than K_m, the reaction becomes of zero-order and the linear velocity phase lasts longer. For example, at substrate concentration of $100K_m$, the reaction rate deviates from the initial velocity at above 90% of the

substrate conversion [29]. At the same time, the assay becomes less sensitive to the detection of competitive inhibitors [28].

The kinase activity is usually determined by the formation of the phosphorylated substrate. The phosphorylation itself does not lead to detectable change in optical characteristics of the molecule; therefore the introduction of labels (fluorescent, luminescent, radioactive) that produce detectable optical signal is required. The labels can be linked with the phosphorylated substrate covalently or non-covalently by affinity-recognition and the labelling may be carried out before, after or in the course of the phosphorylation. The assays can be classified into several categories:

- according to the detection method assays may be radiometric and non-radiometric, mainly based on the measurement of fluorescence or luminescence signals.
- utilizing natural substrates or synthetic substrates.
- homogeneous (do not involve separation steps, "mix-and-measure") and heterogeneous (separation-based) [26]. Homogeneous assays are typically based on fluorescence polarization (FP) or involve distance-dependent energy transfer between two labels that are brought into proximity by specific recognition of phosphorylated substrate. Homogeneous assays are preferred for high-throughput applications because of high speed and possible automatization [30]. Separation-based assays are more time-consuming but afford the possibility to remove substances that interfere with the detection system. Both types of assay may involve recognition of the phosphorylated substrate by antibodies or other types of phosphate-capture particles.
- homogeneous assays can afford continuous monitoring of the reaction or require stopping of the reaction before quantification of the formed product (end-point assays).

The combination of different separation and recognition technologies with different detection methods provides numerous possibilities for the determination of the phosphorylation extent.

Traditional kinase assays are based on radioactive labelling of the substrate by incorporation of (^{32}P or ^{33}P)-labelled phosphoryl group into the protein or peptide substrate in the course of phosphorylation. The labelled product is precipitated or adsorbed onto cation-exchange phosphocellulose paper, the excess ATP is removed by several washing steps and the adsorbed product is quantified by Cerenkov or scintillation counting or by phosphoimaging [31–33]. One of the limitations of this method is incomplete adsorption of the product onto the phosphocellulose matrix [34]. The capture of biotinylated substrates after phosphorylation to streptavidin-coated surfaces affords more efficient separation [35]. Scintillation proximity assay (SPA) [36, 37] quantifies the radioactively labelled phosphopeptide without separation steps. The bio-

tinylated peptides are captured after phosphorylation with [γ–^{33}P]ATP to streptavidin-coated SPA-beads that contain the scintillant. Due to the limited path-length of the beta radiation of ^{33}P, only the radioactivity emitted by the captured product is transformed to optical signal. Radiometric methods involve several shortcomings like hazards associated with ionizing radiation (health risks and production of radioactive waste) and short half-lives of ^{32}P and ^{33}P (14.3 and 25.3 days, respectively) that limit long-term storage.

Several fluorometric assays for the characterization of inhibitors of protein kinases have been developed. The detection of fluorescence signals is prone to interference from other assay components, fluorescent substances or fluorescence quenchers. The background fluorescence can be reduced by the application of fluorescent markers with emission maxima at longer wavelengths or with longer exited state life-time. The chelates of lanthanides (europium, terbium) offer such opportunity with excited state life-times around 1 ms if compared to the life-times of common fluorescent markers in nanosecond range (4 ns of that of fluorescein) [26]. Time-resolved detection of such long-term fluorescence emission cuts down the background signal.

If the phosphorylation changes the emission spectrum of the fluorescently labelled substrate, the reaction can be monitored by direct measurement of the fluorescence intensity. Wright *et al*. developed an assay for cAPK that is based on the phosphorylation of fluorescent analogue of kemptide, LRRWSLG [38]. Analogous assay was developed by Kondo *et al*. who attached dansyl group to the C-terminus of kemptide [39]. Both substrates were comparable to unmodified kemptide by kinetic properties and showed 10–20% fluorescence increase in the course of phosphorylation, affording continuous monitoring of the reaction.

If the fluorescence characteristics are not dependent on phosphorylation, assays can be developed that are based on the separation of the fluorescently labelled product from substrate, *e.g*., by chromatography [40] or electrophoresis [41, 42]. Simultaneous quantification of the phosphorylation product and unreacted substrate affords ratiometric detection.

Assays utilizing phospho-amino acid-specific antibodies may be homogeneous or heterogeneous. In the enzyme-linked immunosorbent assay (ELISA) format [26] the phosphorylated substrate is separated from the reaction mixture or cell lysate by capturing to immobilized antibodies or streptavidin if the substrate is biotinylated. The phosphorylated substrate is detected with enzyme-linked antibodies (detection antibodies) that catalyze a reaction producing optical signal (chemiluminescence or change of absorption). Different configurations of ELISA are possible depending on whether the anti-phospho antibody is captured to immobilized product or *vice versa*. The detection antibody may also be fluorescently labelled. For example, delayed enhanced lanthanide fluorescence immunoassay (Delfia®) utilizes biotinylated substrate that after phosphorylation is captured by immobilized streptavidin [27]. The

product is incubated by lanthanide-labelled anti-phospho antibody and the unbound antibody is removed by washing steps. The extent of phosphorylation is quantified by the fluorescence of the lanthanide label in time-resolved manner.

Homogeneous detection of the antibody-phosphopeptide complex can be carried out by FP or proximity-based assays such as fluorescence resonance energy transfer (FRET) or amplified luminescent proximity homogeneous assay (AlphaScreen®).

In FP format, fluorescently labelled phosphopeptide binds to anti-phospho antibody. The non-phosphorylated peptide as small molecule rotates faster in solution and when illuminated with polarized light, it becomes randomly orientated during the excited state resulting in loss of polarization. In complex with antibody, polarized emission is detected. This method is limited in the size of the substrate for the FP to be efficient and needs labelled substrates. Another possibility is to carry the assay out in competition format with unlabelled substrates that compete after phosphorylation with fluorescently labelled phosphopeptide tracer [43, 44].

FRET uses two fluorescent labels, a donor and an acceptor. The emission spectrum of the donor overlaps with the excitation spectrum of the acceptor. The excitation of the donor leads to energy transfer to the acceptor in distance-dependent manner and the emission of the acceptor is detected. For example, lanthanide chelation excitation assay (Lance®) uses an europium-labelled antibody and streptavidin-coated acceptor bead that are brought to proximity by linking with phosphorylated biotinylated peptide [27]. The phosphorylation extent is determined by time-resolved FRET. A limitation of FRET is the small distance (9 nm) between the donor and acceptor needed for efficient energy transfer.

In the AlphaScreen® format, donor and acceptor beads are brought to proximity in a similar manner [27]. The donor beads contain photosensitizer that under illumination (680 nm) convert the dissolved molecular oxygen to excited singlet form. Within its half-life in water solution (4 μs) singlet oxygen can diffuse about 200 nm, reaching the acceptor bead where the energy is transformed to chemiluminescence with emission maximum in UV range (370 nm) that may be subsequently converted to longer wavelength radiation (500–600 nm) by chromophores immobilized to the acceptor bead. Advantages of this detection system are the requirement for a longer distance between the two labels if compared to the FRET format and an amplification of the signal – one donor bead may create about 60 000 singlet oxygen molecules per second [45].

The assays utilizing immunorecognition rely on the availability of specific anti-phospho antibodies with high affinity and specificity. More universal technology is immobilized metal ion affinity-based fluorescence polarization (IMAP®) that uses nanoparticles bearing immobilized chelate of trivalent metal ion. These nanoparticles bind specifically to phosphate groups in phospho-

serine, phosphothreonine and phosphotyrosine residues. The complexation of fluorescently labelled phosphopeptides with IMAP-particles can be detected by loss of FP or by decrease of fluorescence intensity if nanoparticles are conjugated with fluorescence quenchers [46, 47].

The phosphorylated peptides may also be detected by biosensors. Takeda *et al*. captured phosphorylated peptides to surface plasmon resonance (SPR) biosensor surface by immunorecognition and used anti-phospho antibodies for detection [48]. Another possibility is to carry the phosphorylation out directly with substrates immobilized to the biosensor surface. SPR-based biosensors are sensitive to the molecular mass of the substance binding to the sensor surface. Since the phosphorylation does not increase remarkably the molecular mass of the surface-bound peptides, the signal is amplified by the binding of phosphate-sensitive high-molecular-weight particles to the phosphopeptides like streptavidin-conjugated Zn(II) phosphate-capture complexes or by anti-phospho antibodies [49, 50]. An advantage of this method is that special labels are not needed. A disadvantage is the limited throughput of SPR-based methods.

The kinase activity can also be determined by the extent of the conversion of ATP to ADP. This approach is universal as ATP is a common substrate for all kinases but assays based on this principle detect also ATPase activity that is thus an interfering process. The spectrophotometric assay [51] couples the formation of ADP *via* lactate dehydrogenase- and pyruvate kinase-catalyzed reactions to the oxidation of NADH, monitored as the decrease of absorbance at 340 nm:

$$\text{ATP + protein} \xrightarrow{\text{protein kinase}} \text{phospho-protein + ADP}$$

$$\text{ADP + PEP} \xrightarrow{\text{pyruvate kinase}} \text{pyruvate+ ATP}$$

$$\text{pyruvate + NADH} \xrightarrow{\text{lactate dehydrogenase}} \text{lactate+ } NAD^{+}.$$

Such assay allows continuous monitoring of the phosphorylation reaction. The recycling of the nucleotide holds the concentration of ATP constant that allows the reaction to be performed at zero-order conditions in respect to the nucleotide. The consumption of ATP can be quantified by chemiluminescence produced in oxidative decarboxylation of luciferin (Figure 3) [52]. A disadvantage of this assay is the signal-decrease format, *i.e*., the measured signal is inversely correlated to the enzyme activity that is a potential source of error with low substrate turnover. A general drawback of enzyme-coupled homogeneous assays is that the potential inhibitor of protein kinase may also interfere with the secondary enzymes [28].

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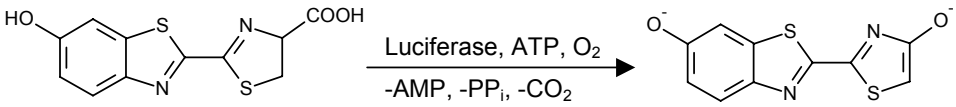

Figure 3. Oxidative decarboxylation of luciferin.

Binding assays present an alternative to functional assays. They can be performed in direct binding or in competition formats. The direct binding assays need labelling or immobilization of one interaction partner and the complex formation can be detected by several detection systems, such as FP, SPR, *etc*. These labelled or immobilized compounds can in turn be used as probes in competition assays where they are displaced from the complex with the enzyme by unlabelled ligands. Both ATP- and peptide-site directed probes for protein kinases have been developed.

Vainshtein *et al*. developed a competition assay for protein kinase inhibitors based on β-galactosidase enzyme fragment complementation [53]. An ATP-competitive inhibitor of protein kinases, staurosporine was conjugated with β-galactosidase donor fragment. The conjugate was able to combine with the β-galactosidase acceptor fragment, forming an active enzyme that catalyzes the hydrolysis of D-galactose, emitting chemiluminescence. The binding of glycogen synthase kinase 3α to staurosporine blocked the binding of the conjugate to the acceptor fragment thus inhibiting the enzymatic reaction. This enzyme-linked binding assay was applied for the determination of the affinities of substances binding to the ATP-binding pocket of the kinase.

Several binding assays rely on PKI-derived probes [54–56]. Saldanha *et al*. developed FP-based ligand-regulated competition assay for characterization of non-ATP-competitive regulators of cAPK holoenzyme type I activity [54]. The fluorescently labelled high-affinity fragment of the heat stable protein kinase inhibitor PKI(5-24) was displaced from the complex with cAPK Cα by regulatory subunit type I, leading to the loss of polarization of the emission signal. This system was applied for the characterization of antagonists and agonists of cAMP. Due to the synergistic binding of the inhibitor peptide and ATP to cAPK C, the high-affinity binding of PKI-based sensors requires the presence of ATP. PKI-based probes can thus be used also for the characterization of substances binding to ATP-binding pocket [55].

Biosensors allow the real time monitoring of the formation of the complexes between the inhibitors and protein kinases. The most powerful technology, SPR-spectroscopy [57] allows the determination of binding processes in heterogeneous system where one of the interaction partners (referred to as ligand) is immobilized to the sensor surface and the binding of the other interactant (referred to as analyte) to this surface is monitored by making use of the SPR phenomenon. The analyte is eluted over the surface in continuous flow that, although increasing the consumption of reagents, holds the concentration

of the analyte constant that facilitates data analysis. A typical interaction analysis consists of three phases. In the association phase, the analyte at fixed concentration is eluted over the surface and its association with the immobilized ligand is monitored. The dissociation of the analyte is monitored by elution of the surface with analyte-free buffer in the dissociation phase. The final phase of the experiment is the regeneration of the surface. The separate monitoring of the dissociation and association kinetics allows simultaneous detection of the kinetic (association and dissociation rate constants) and thermodynamic (equilibrium constant) characteristics of the interaction. The characterization of the inhibitors of protein kinases can be carried out in two configurations. In the first configuration, the binding of kinases from the solution phase to the immobilized inhibitors is detected. This is impractical in inhibitor screening since the immobilization of inhibitors potentially influences the binding characteristics and each inhibitor requires the preparation of a new sensor surface. The binding experiments are thus preferentially carried out in the second configuration with monitoring of the binding of inhibitors to the immobilized kinases [58, 59]. Due to the molecular mass dependence of the SPR-signal, detection of small-molecule inhibitors with this method is problematic. Another problem is the loss of activity of the kinases during immobilization and the low stability of the obtained protein surfaces. SPR-based methods also have low throughput that has limited their application mainly to mechanistic studies.

Adenosine-oligoarginine conjugates as bisubstrate-analogue inhibitors of protein kinases

The majority of the inhibitors of protein kinases reported to date are targeted to the ATP-binding site. The design of selective ATP-competitive inhibitors presents a challenge because the nucleotide-binding pocket is highly conserved within the kinase superfamily and there are more than 1500 other proteins encoded by the human genome, which utilise ATP [60]. In addition, ATP-competitive inhibitors must have sufficient potency to compete with saturating intracellular concentration of ATP (millimolar range if compared to the typical K_m values of ATP towards protein kinases below 100 μM [61]). The peptide/protein-binding domain proposes a more selective target but inhibitors directed to this site must interact with larger surface area to obtain sufficient potency and this strategy is thus considered beyond the scope of small-molecule drug design [62]. Most inhibitors of this kind are peptides that have been derived from the substrate consensus sequence to provide high selectivity but high affinity has been obtained with longer peptide sequences that are limited in therapeutic utility [63].

The ternary complex mechanism of protein kinases makes it possible to combine the aforementioned approaches in the development of bisubstrate-analogue inhibitors that simultaneously interact with both the ATP- and protein-binding site of the enzyme [64]. Although maintaining the competitiveness towards ATP, it is a promising strategy to enhance the selectivity and potency of the inhibition. Due to the additivity of the binding energies, the incorporation of two active fragments into one molecule could be of enthalpic and entropic benefit [28]. Bisubstrate-analogue inhibitors have been constructed by conjugation of the corresponding substrate mimics *via* a linker that allows a suitable spatial arrangement of these fragments to associate with both of the binding sites (Figure 4) [64].

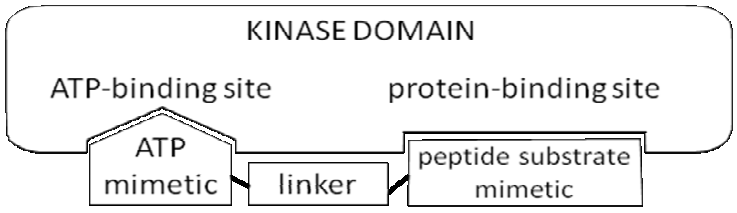

Figure 4. Schematic representation of a bisubstrate-analogue inhibitor in complex with the catalytic domain of protein kinase.

Adenosine-oligoarginine conjugates (ARC), first introduced in 1999, are bisubstrate-analogue inhibitors of protein kinases [65]. The structures of the first generation ARC-s were composed of two moieties, adenosine-5'-carboxylic acid (Adc) and oligo-(L-arginine), which mimic the nucleotide and peptide substrates of basophilic protein kinases, respectively (Figure 5). These two moieties were connected by a linker with optimized structure. The inhibitors had selectivity towards basophilic protein kinases with the most potent compound Adc-Ahx-(L-Arg)$_6$-OH (ARC-306, MW=1332) inhibiting cAPK and PKC at submicromolar concentration. Amidation of the C-terminus of the peptide part of ARC further increased the affinity (Figure 5) [66, 67].

Optimization of the structures yielded ARC-s of the second generation with oligo-(D-arginine) substituted for the oligo-(L-arginine) moiety [66]. Such substantial modification increased the inhibitory potency to low nanomolar range and simultaneously rendered the structures resistant to trypsinolysis. The most active ARC-type inhibitor of this series, Adc-Ahx-(D-Arg)$_6$-NH_2 (ARC-902, Figure 5), was tested in a selectivity panel of 52 protein kinases and it showed a preference to inhibit basophilic protein kinases of AGC and CAMK groups. Kinetic studies revealed that ARC-902 was competitive to ATP and non-competitive to the peptide substrate. The absence of double-competitive behaviour of bisubstrate-analogue inhibitors has been explained by the specific mechanism of the phosphoryl transfer reaction and it does not rule out the two-

site binding model [62, 68 and 69]. Although obligatory ordered binding of the substrates to the catalytic subunit of cAPK has not been suggested as ATP and kemptide can bind to the active site independently [6], ATP has a strong preference for binding first for thermodynamic reasons (K_d=10 μM for ATP [24, 51] *vs* around 210 μM for kemptide [17]). By theory, competitive inhibition is observed when the inhibitor competes with the substrate in a mutually exclusive fashion for the binding to the same enzyme form [62]. If kemptide preferentially associates with enzyme-ATP complex, the competitiveness with the protein substrate will not be registered in kinetic experiments. The weak inhibitory potency of the substrate mimics oligo-(D-arginine) and Adc alone or in a mixture as well as the selectivity of the conjugate for basophilic protein kinases still points to the participation of both of these fragments in the achievement of high affinity.

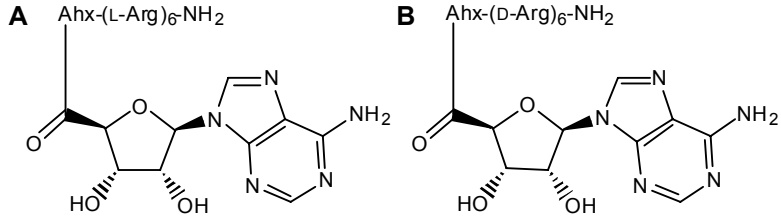

Figure 5. Structures of the first and the second generation ARC-s. **A:** Adc-Ahx-(L-Arg)$_6$-NH_2 (ARC-341, MW=1331), IC_{50}=0.17 μM and **B:** Adc-Ahx-(D-Arg)$_6$-NH_2 (ARC-902, MW=1331), IC_{50}=8.3 nM. Inhibitory potencies are measured at 100 μM ATP, 30 μM TAMRA-kemptide and about 1 nM cAPK Cα [66].

ARC coupled to sepharose produced an affinity chromatography matrix that was successfully used in the separation of cAPK C from cell lysates demonstrating that the immobilized inhibitors retained the ability to bind cAPK C [67]. It was also shown that ARC-s as arginine-rich substances were able to cross plasma membrane [70]. The fluorescently labelled ARC rapidly accumulated into cells and concentrated mainly to cytoplasm. The uptake was not completely abolished at low temperature suggesting partially energy-independent internalization routes. In addition, ARC-s were able to deliver a conjugated protein into cell. The uptake of the complex of biotin-tagged ARC with fluorescently labelled avidin was less efficient and it was strongly impaired at low temperature pointing to the endocytosis as the dominating mechanism of cell entry.

The advantages of bisubstrate ARC-type inhibitors highlighted by these reports are:

- the possibility to be synthesized by standard solid-phase peptide synthesis protocols,

- well-defined optical characteristics that allow their quantification by UV spectroscopy (extinction coefficient of 15 000 $M^{-1} \cdot cm^{-1}$ at 260 nm),
- membrane-permeability that points to the potential of the inhibitors to be used *in vivo* experiments,
- hydrophilicity that affords the exclusion of DMSO from the biological assays,
- possibility to adjust the affinity with structural modifications down to low-nanomolar range and to increase the resistance to proteolytic degradation, and
- the potential to add voluminous labels and tags to the C-terminus of the peptide part without abolishment of the binding ability.

The applications of poly(ethylene glycol) for the conjugation with bioactive substances

The therapeutic value of many bioactive substances is limited by low aqueous solubility, random biodistribution and short plasma half-life due to rapid renal clearance and instability. This requires bigger doses to be administered for maintaining an effective concentration of the drug at the target site and causes side effects. Shortcomings associated with the application of bio-pharmaceuticals such as proteins and nucleic acid-based substances include enzymatic degradation and immunogenicity. Macromolecular drug carrier systems, termed as polymer therapeutics, have become a widely used strategy for the improvement of pharmacokinetic and pharmacodynamic properties of potential drug candidates [71–73].

Figure 6. Structures of poly(ethylene glycol) and its monomethyl ether.

Due to its favourable pharmacokinetic and -dynamic properties, poly(ethylene glycol) (PEG) is the most extensively used polymer in macromolecular drug delivery systems [74]. It is a linear polyether consisting of ethylene oxide units that is produced by anionic polymerization of epoxyethane. The initiation of the polymerization with water or methanol produces PEG with two or one terminal hydroxyl groups, respectively, that can be further modified with various functionalities (Figure 6). The polyether backbone of PEG is chemically relatively inert. PEG with molecular weight of less than 400 Da is degraded by alcohol dehydrogenases to toxic metabolites [75]. At MW>1 kDa, PEG is not toxic and it is excreted from body without degradation. PEG below 20 kDa is

cleared by kidneys; the homologues with higher molar weight are eliminated by liver. PEG lacks immunogenicity and antigenicity. The presence of both hydrophobic and hydrophilic moieties per a monomer unit makes it well soluble in water and in a wide variety of organic solvents [76]. In aqueous environment, the polyether backbone is well hydrated with two or three water molecules associated with each ethylene oxide unit. The resulting large hydrodynamic volume makes the apparent molecular weight of PEG 5–10 times higher than that of a globular protein with similar molecular weight as revealed by size exclusion chromatography and gel electrophoresis [77, 78]. The conjugation of biologically active substances with PEG, referred to as pegylation, transfers the properties of the polymer partly to the conjugates while the biological functions like enzymatic activity or receptor recognition defined by the parent substance may be maintained [74]. The pegylation of proteins has been shown to mask them from the recognition by immune system and hydrolytic enzymes, which reduces their immunogenicity, toxicity and elimination by proteolysis. Pegylation increases the effective size of the substance, which reduces the excretion by kidneys and limits the cellular uptake mainly to endocytotic routes [71]. The combination of these effects results in prolonged circulating half-life and enhanced biodistribution of the therapeutic agent. The drug-polymer conjugates thus need less frequent dosing that is of clinical benefit especially when the drug is administered by parenteral routes.

The effect of pegylation is strongly dependent on the molecular weight of the polymer and the number of PEG chains per biomolecule, the selectivity of pegylation chemistry and used linkers between the polymer and the bioactive substance. The *in vitro* activity of the conjugates is usually inversely correlated to the extent of pegylation because the same mechanisms protecting the pharmacophore from hydrolytic enzymes may also hinder its binding to the target receptor. This effect is often compensated by enhanced *in vivo* profile [74]. Another possibility to overcome this problem is to include a cleavable linker between the therapeutic agent and the polymer [79]. The linker is cleaved under specific conditions, *e.g.*, by lysosomal enzymes after cellular uptake, releasing the drug in its fully active form. Such polymeric prodrug approach possesses a potential to targeted drug delivery to specific organs or cells [71].

Initially pegylation was applied to the modification of proteins. The history of protein pegylation is divided into two generations that differ in the level of heterogeneity of the obtained product [74, 80 and 81].

The first generation pegylation utilized linear low-molecular-weight (5–12 kDa) monofunctional PEG-s with many polymer chains attached to the protein molecule. The pegylation was directed mainly to the modification of the amino groups in the N-terminus of the protein and to the side-chains of lysines by electrophilic alkylating (PEG trichlorotriazine) or acylating (PEG *p*-nitrophenyl carbonate, PEG succinimidyl carbonate) derivatives of PEG. Since there are several amino groups and other nucleophilic groups in the

protein molecule accessible for pegylation, like the side chains of histidine, tyrosine or serine residues, such chemistry was not very selective and produced mixtures of positional isomers with different pegylation stoichiometries and different bioactivities [82]. Strategies for the protection of the bioactivity of the protein were developed, like blocking of the active sites of enzymes with inhibitors or substrates during the pegylation. The selectivity of pegylation could also be increased by changing the reaction conditions. For example, when the coupling is performed at lower pH (between 5.5–6.5) the ε-amino groups of lysines (pK_a=9.3–9.5) are protected by protonation and the conjugation preferentially occurs at less basic α-amino groups in N-terminus (pK_a=7.6–8.0) [74]. Another source of heterogeneity was the high content of dihydroxy-PEG in the starting monofunctional polymer (up to 15%), which originated from the presence of trace amounts of water in the polymerization process. This difunctional impurity increased the diversity in pegylation stoichiometry and caused cross-linking. The heterogeneity of pegylation caused difficulties in purification of the obtained mixtures of conjugates and determination of the sites of pegylation.

The second generation pegylation is characterized by more selective conjugation chemistry and improved polymer quality. The diol content of the monofunctional polymer was reduced by separation of the corresponding carboxyl derivatives of the polymers by ion exchange chromatography [81]. The pegylation was carried out with higher molecular weight polymers or multifunctional branched and dendrimeric PEG-s that were developed to allow higher loading of the therapeutic agent or to increase the steric hindrance of the macromolecule. It was experienced that pegylation at one site with higher molecular weight branched PEG gives better pharmacological profile than the pegylation with lower molecular weight polymers at multiple sites. The methods for the enhancement of the conjugation selectivity involve the reductive alkylation of the terminal α-amino groups by PEG aldehyde, the modification of less-abundant amino acids like the side chains of cystein with PEG maleimide or PEG orthopyridyl disulfide, and enzyme-catalyzed pegylation [83].

The success of pegylation is reflected by an increasing number of pegylated products approved for clinical use [74, 80]. The first product of this kind, pegylated adenosine deaminase (Pegademase, Adagen®), was approved in 1990 for the treatment of severe combined immunodeficiency disease. It was developed with the application of the first generation pegylation chemistry leading to the conjugation of multiple PEG5000 chains per one biomolecule [84]. The conjugate revealed about six-fold longer half-life if compared to the native unmodified form, requiring once-weekly dosing. Another example of the first generation construct is pegylated asparaginase (Pegaspargase, Oncaspar®), which was approved for the treatment of Acute lymphoplastic leukaemia in 1994. The pegylated product shows an extended half-life (from 8–30 h to 15

days) that allows once in 2 weeks administration instead of three times a week dosing of the native form [85].

Pegylated cytokine interferon α2b (PEG-Interferon α2b, PEG-Intron®, MW=31 kDa), approved in 2000 for the treatment of hepatitis C, is an example of the second generation pegylation chemistry [84]. The conjugation was performed with linear 12 kDa PEG succinimidyl carbonate at pH=5 that resulted in the mixture of monopegylated isomers. Although with reduced *in vitro* potency, the conjugate was superior to the free form in *in vivo* conditions showing about eight-fold longer resistance time. Another pegylated cytokine interferon α2a (Pegasys®, MW=60 kDa) is a mixture of six positional isomers monopegylated with branched 40 kDa PEG. The conjugate showed even higher *in vitro* activity and longer plasma half-life than PEG-Intron® and was approved for the treatment of hepatitis C in 2002 [86].

Pegylation is also used for the enzyme-catalyzed organic synthesis [87]. In addition to the improvement of aqueous solubility, enzymes can be solubilized in organic solvents upon modification with PEG while their catalytic activity is preserved. The solvent effects on the enzyme-catalyzed reaction can be studied. In non-polar solvents the reactions can be forced to go in unnatural directions, *e.g*., pegylated lipases and proteases have been shown to catalyze the synthesis of esters and amides in non-hydroxylic solvents.

Polymer-supported organic synthesis

The efficiency of organic synthesis is not only dependent on the analytical yields of the reactions but also on the effectiveness of the isolation and purification procedures. High preparative yields of intermediate products become especially crucial in long synthetic schemes, like the iterative synthesis of oligomers – peptides, oligonucleotides, oligosaccharides, *etc*. Strategies for facilitating the isolation of intermediate products like phase-tagging and polymer-supported organic synthesis are based on the assistance of the accumulation of the products into the phase that is separated from the reagents and catalysts [88, 89].

The solid-phase organic synthesis (SPOS) methodology, first introduced into peptide synthesis by R. B. Merrifield [90], laid a foundation for the high-throughput synthesis of biopolymers [91]. Afterwards it has been expanded into many areas of organic synthesis [92]. In SPOS the substrates are covalently bound to insoluble polymer that is subsequently treated with the solution of reagents and catalysts. The reactions take place inside the swollen polymer matrix [93] and the polymer-bound products are separated after each synthetic step by filtration. The target product is obtained by its cleavage from the polymer during the final step of the synthesis. Such methodology offers many advantages. The intermediate products can be separated without loss. Excess

amounts of reagents can be used that increase the yields and rates of the reactions. The synthesis can be carried out in one reaction vessel and it is easy to automate. There are also many complications associated with the performance of the synthesis on the solid phase. The polymer-bound side-products originating from incomplete or side reactions cannot be separated in the course of the synthesis and their accumulation into the final step causes problems with the purification of the target substance. For these reasons, well optimized synthetic methods with quantitative yields are required. The heterogeneity of the reaction mixture causes diffusion problems and inequivalence of the functional groups bound to the resin matrix. The swelling properties of the polymer determine the choice of the applicable solvents. For example, the classical cross-linked polystyrene-based resins are well swelled in aprotic solvents like in tetrahydrofuran, dimethyl formamide and chloroalkanes while PEG-based resins can also be used in the hydroxylic environment including solutions in water [94, 95]. The choice of solvents in turn sets limits to the synthetic methods because only soluble reagents and catalysts can be used. The synthetic methods developed for homogeneous solution phase are not always directly transferable to the solid-phase conditions. Lelievre *et al*. have performed aminolytic cleavage of peptides with much better yields and purity on PEG-based resins than on polystyrene-based resins that was explained by specific solvatation effects inside the polymer matrix [96]. Aggregation due to insufficient solvatation is a well-known problem in peptide synthesis [97]. One of the major disadvantages of SPOS is the limited arsenal of methods for monitoring of the reaction progress and analyzing the polymer-bound intermediates. The completeness of the reaction is often decided on the basis of colour reactions that are sensitive towards specific functional groups like amino, mercapto, hydroxyl, carboxyl, halogeno or carbonyl groups [98]. UV spectroscopy can be used for the analysis of the solution phase of the reaction mixture. The classical example is the Fmoc-test in peptide synthesis [97]. The removal of N-terminal protecting Fmoc group produces the release of benzofulvene to the solution phase with specific absorbance (301 nm), which can be used for quantification of the coupling yields. The structure of the polymer-bound products can be determined by two approaches. The first possibility is the cleavage of the products from the resin and the use of conventional solution-phase methods for the analysis. Such cleave-and-analyze procedure is destructive and consumes large amounts of the product. The cleavage reaction is an additional time-consuming step and may alter the structure of the intermediate product. The second possibility is to determine the structure directly on solid support by IR [99] and solid-state or gel-phase NMR spectroscopy [100]. As an advantage, this approach is potentially non-destructive but the obtained spectra are more complicated due to the presence of the polymer signals.

Synthesis on soluble polymeric carriers, termed liquid-phase organic synthesis (LPOS) or soluble-polymer-supported organic synthesis (SPSOS [101]), has been proposed as an alternative to SPOS combining the advantages of the latter methodology with these of the classical solution phase organic synthesis [102]. The basic principle of SPSOS is the same with that of SPOS with the difference that instead of the cross-linked insoluble polymer beads the substrate is bound to polymer that is soluble in the reaction mixture. The reactions take place in homogeneous solution phase and the products are isolated after each step by taking advantage of the specific properties of the polymer. The polymer serves three functions: it acts as a protecting group, improves the solubility characteristics of the substrates in the reaction medium and facilitates the separation of low molecular weight reagents and catalysts from the polymer-associated products. Gel filtration, ultracentrifugation, ultrafiltration and dialysis can be used for the isolation of the macromolecular products [89], but all these techniques need special equipment and are time-consuming. Precipitation of the polymer-bound product by the addition of a large amount of solvent in which the polymer is insoluble followed by filtration or centrifugation has turned out to be the most practical technology in means of product purity, high separation speed and yield [102]. Such selective precipitation can be used only in conjugation with reagents which are soluble in the precipitation medium. To avoid the inclusion of low-molecular-weight impurities trapped in the polymer crystals, recrystallization may be needed. For these reasons, this technique consumes quite large volumes of the precipitant. The side-products associated with polymer coprecipitate with the target substance but, differently from SPOS, it is possible to separate polymer-bound side-products chromatographically [103]. Additional advantage of SPSOS over SPOS is the possibility of the analysis of polymer-bound substances by solution phase methods, like TLC, electrophoresis [104], UV-Vis, MS [105, 106], IR and NMR spectroscopy [107]. This allows the monitoring of the reactions without detachment of the intermediate products from polymeric carrier. The soluble supports as synthetic polymers are polydispersed and instead of a single well-defined molecular mass one has to operate with an average molecular mass and mass distribution. For example, instead of the detection of single isotopic distribution of the molecular ion, a mass distribution corresponding to polymer chains with different lengths is registered by high-resolution MS [106].

The synthesis on a soluble support is possible as long as the physicochemical properties like solubility and crystallization tendency are dominated by the properties of the carrier polymer [108]. Therefore the structural characteristics of the molecule that can be synthesized have limits that depend on the synthesis course and loading, *i.e.*, the number of attachment sites per mass unit of the polymer. If the loading is too high, the solubilizing power of the polymer chain is not sufficient and the physicochemical properties of the product-polymer conjugate are too much dependent on the synthesized structure. If the loading is

too small, large amounts of polymer are needed for obtaining the products in reasonable quantity. Therefore the choice of the polymer is a compromise between its loading capacity and solubilizing power [102]. Other important properties are mechanical and chemical stabilities, solubility in a wide variety of solvents and low polydispersity [109].

Linear chloromethylated polystyrene was the first polymer that was used as the soluble support [110]. Although synthetic problems occurred, like the cross-linking of the polymer during the synthesis, the optimization of the conditions produced tetra- and hexapeptides in good yields (above 60%) and purity. Due to recovery problems, polystyrene did not gain popularity but it is still preferred for some applications due to good solubility in many solvents (tetrahydrofuran, chloroalkanes and ethyl acetate) even at low temperatures (–78 °C) and resistance to strong bases that makes it compatible with metalloorganics. Differently from hydrophilic polymers, it allows the application of water/organic solvent extraction for the separation of inorganic catalysts and reagents [102].

Bayer and Mutter used PEG as C-terminal protecting groups in soluble-polymer-supported peptide synthesis [111] and due to the wide solubility profile, good crystallization and spectroscopic properties and chemical resistance, PEG has become the most extensively used soluble support in SPSOS [102]. PEG is well soluble in water, dimethyl formamide, chloro-alkanes, ACN, acetone, methanol, DMSO and aromatic solvents and hardly soluble in hexane, diethyl ether, TBME, isopropanol and cold ethanol [76]. The latter solvents can be used for the precipitation and recrystallization of polymer-bound material. PEG does not disturb remarkably the spectroscopic measurements because of its transparency in UV-Vis range down to 190 nm and simplicity of ^{1}H and ^{13}C NMR spectra. The NMR signal of the terminal methoxy group of the monofunctional PEG can be used as an internal standard for the integration of the signals. The good solubilizing properties of PEG allow the spectroscopic measurements to be performed in solvents where the parent product itself is not soluble [108]. The mass spectrum of PEG reveals a mass distribution with distance of 44 Da between two adjacent peaks, corresponding to the oxyethylene unit. Without the introduction of proton-affine groups, PEG is not protonated but is readily cationized by alkali metal ions [112]. This phenomenon may facilitate the mass-spectral analysis of substances that are difficult to ionize. PEG with average molar mass up to 2 kDa can be separated into oligomers by HPLC, higher oligomers are not chromatographically distinguishable [113]. Monofunctional HO-PEG-OMe (mPEG) between the molecular mass range of 2–20 kDa is usually used for synthetic applications presenting the optimum between the loading capacity and solvatation power. The loading of such monofunctional PEG is relatively small, *e.g.,* the content of hydroxyl groups in HO-PEG-OMe with average molar mass of 5 kDa is 0.2 mmol/g. This means that a small mass portion of low-molecular-weight

impurities makes large molar excess. For example, one equivalent of water in PEG5000 corresponds to only 0.36% by mass.

The PEG-based SPSOS has been successfully applied to the synthesis of oligonucleotides [103] and peptides [76, 108 and 114] up to 20 monomer units. The synthesis of longer sequences depends on the structures of the used monomers, their side chain protecting groups and the conformation of the products [108]. SPSOS can also be automated [111] and applied to combinatorial synthesis [115].

Theoretically, the functional groups attached to soluble polymers should have the same reactivity as their low-molecular-weight analogues [76]. Kinetic studies of the peptide synthesis with PEG-bound and low-molecular-weight amino acids revealed that the rates of the coupling reactions were of the same order of magnitude [116]. In reaction with sterically hindered reagents, however, the conjugation with macromolecule may still have an effect on the reactivity. Bergbreiter *et al*. studied the catalytic hydrogenation of PEG-bound nitroarenes with Pd- and Pt-based catalysts and showed that the reaction rate was dependent on the length of the polymer chain [117]. On sterically more hindered catalysts, like Pd/polystyrene, only low-molecular-weight substances were hydrogenated. Such protecting effect of the macromolecular tail has found applications in pegylation of bioactive substances (see previous Chapter). If optimal conditions are found, insoluble reagents and catalysts can still be used in SPSOS as illustrated by examples of catalytic reductions on heterogeneous catalysts [118, 119].

Another feature of PEG-based SPSOS is that the products are initially pegylated and thus can be used in biochemical assays without cleavage. In this case, PEG is both the synthetic handle and biological carrier. Fischer *et al*. synthesized a dodecapeptide on monofunctional PEG5000 support using Fmoc peptide synthesis strategy [114]. The synthesis was done in parallel on different polymer supports bearing acid-sensitive linkers or allowing permanent connection *via* an amide bond to the C-terminus of the peptide. After cleavage and purification, the target peptide was isolated in 40–50% yield. Both the free peptide and its pegylated counterpart were shown to associate with different types of cyclin and to inhibit the activity of cyclin dependent protein kinase. The pegylated form showed lower affinity. Bonora *et al*. prepared pegylated dodecanucleotides on mPEG9500 support in 70% yield [103]. The pegylated product showed increased resistance to proteolytic degradation.

AIMS OF THE PRESENT STUDY

The present investigation is a part of an ongoing project on the development of ARC-s as bisubstrate-analogue inhibitors of protein kinases.
Specific aims of the present study were:

- The application of soluble-polymer-supported synthetic method for the synthesis of ARC-s,
- Characterization of the effect of pegylation to the inhibitory potency and cellular uptake of ARC,
- Development of fluorescence-based assay for the characterization of ARC-s as inhibitors of protein kinases,
- Characterization of the interactions between cAPK and ARC-s by SPR spectroscopy,
- Development of ARC-based SPR biosensor for the characterization of ligands of cAPK.

MAIN RESULTS AND DISCUSSION

The synthesis and characterization of pegylated ARC [I]

It had been previously established that ARC-type compounds can be attached *via* C-terminus to polymer supports without impairing the binding ability to cAPK C [67]. In the present work, ARC was conjugated C-terminally with linear hydrophilic soluble polymer chain and the effect of the conjugation on the inhibitory potency was characterized quantitatively. For the possibility to tag the conjugate with fluorescent label or biotin, lysine was introduced between ARC and polymer (**I** Figure 1). The pegylated ARC Adc-Ahx-(L-Arg)$_4$-L-Lys-NH-PEG5000-OMe (ARC-701) was synthesized in a step-wise manner by soluble-polymer-supported synthetic method on monofunctional H_2N-PEG5000-OMe (**I** Figure 2). This approach allowed using the advantages of SPSOS and simultaneously attaching the polymer to ARC at defined position. The synthesis was monitored and the structures of the products were verified in combination with chromatographic and spectroscopic methods. The solubility of the polymer-bound material allowed the structure determination both before and after removal of the acid-labile protecting groups (Figures 7 and 8), which is complicated with SPOS strategy.

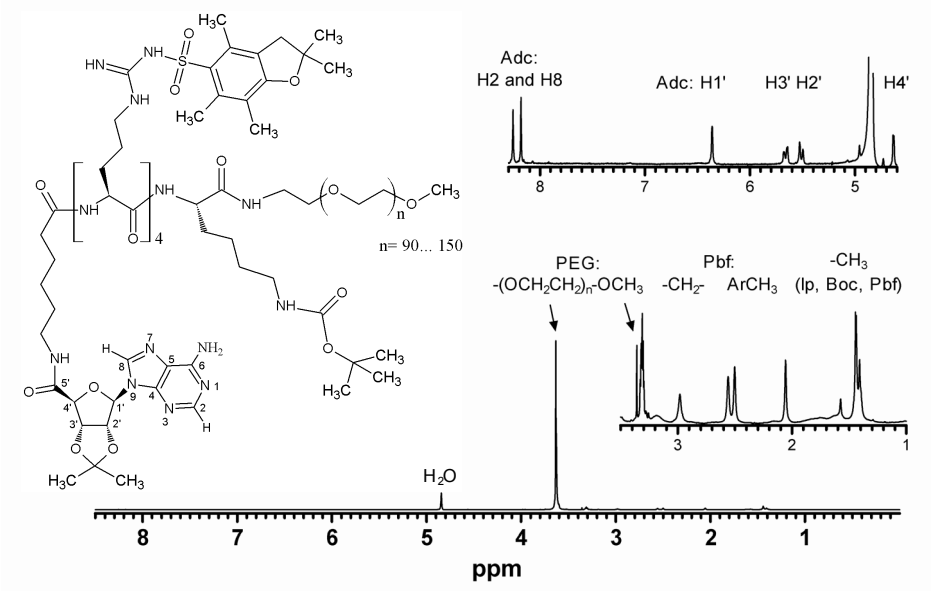

Figure 7. The structure and ^{1}H NMR spectrum (200 MHz) of ARC-701 bearing protecting groups, Adc(Ip)-Ahx-[L-Arg(Pbf)]$_4$-L-Lys(Boc)-NH-PEG5000-OMe (75 mg/ml in CD_3OD). Magnifications are given as insets.

Pegylation rendered the hydrophilic ARC soluble in both polar (water, methanol) and non-polar media (chloroform). This enabled the measurement of spectra in different solvents. UV-Vis spectra were efficiently measured in dichloroethane and aqueous solutions while the best quality NMR spectrum was obtained in methanol (Figure 7). MALDI-TOF spectrum of PEG showed mass distribution between M/z=4000–6500 corresponding to sodium- and potassium-cationized polymer chains (**I** Figure 4). In the course of the synthesis the mass distribution was shifted by attached building blocks towards the formation of the fully protected compound with average molecular mass of 7400 Da (Figure 8B). The removal of the protecting groups exposed the basic guanidino and amino groups in the side chains of arginine and lysine, which resulted in protonation as the dominating ionization mechanism in MS (**I** Figure 4).

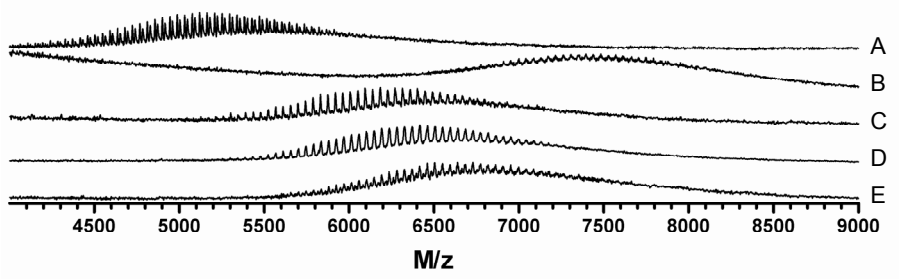

Figure 8. The MALDI-TOF MS (matrix DHB) of **A:** NH_2-PEG5000-OMe, **B:** Adc(Ip)-Ahx-[L-Arg(Pbf)]$_4$-L-Lys(Boc)-NH-PEG5000-OMe, **C:** Adc-Ahx-(L-Arg)$_4$-L-Lys-NH-PEG5000-OMe (ARC-701), **D:** Adc-Ahx-(L-Arg)$_4$-L-Lys(biotin)-NH-PEG5000-OMe (ARC-703) and **E:** Adc-Ahx-(L-Arg)$_4$-L-Lys(BODIPY FL)-NH-PEG5000-OMe (ARC-702).

HPLC showed a surprisingly good performance in separation of different polymer-bound compounds from each other (**I** Figure 5). Even a relatively small change in the structure, the cleavage of one Pbf group, led to considerable change in retention time in reversed phase chromatography (Figure 9). This illustrates the flexibility of SPSOS as it facilitates the separation of polymer-bound material after each synthetic step as well as the removal of polymer-bound side-products. The target product ARC-701 was collected in overall 35% yield, corresponding to an average preparative yield of 93% per one synthetic step. The obtained yield is comparable to the yields of ARC-s produced by SPOS [66].

For cellular uptake studies, ARC-701 was tagged by fluorescent marker BODIPY FL and biotin, yielding ARC-702 and ARC-703, respectively. BODIPY FL was chosen because it has similar excitation and emission maximums (503 and 512, respectively) to that of fluorescein (495 and 520, respectively), a widely used fluorescent marker. At the same time, it has high fluorescence quantum yield and greater photostability and its optical

characteristics are relatively insensitive to solvent polarity and pH. Additionally, the conjugation with BODIPY FL does not add extra charges to the molecule. The ability of pegylated ARC to transport a protein through the plasma membrane was evaluated in the form of complex of the biotin-tagged ARC with fluorescently labelled avidin, which increases the molecular weight by 68 kDa (more than ten times). Both derivatives of ARC entered cells and were able to carry a protein through the plasma membrane (**I** Figure 7). Pegylation decreased the cell-penetration efficiency of ARC and changed the cellular localization pattern.

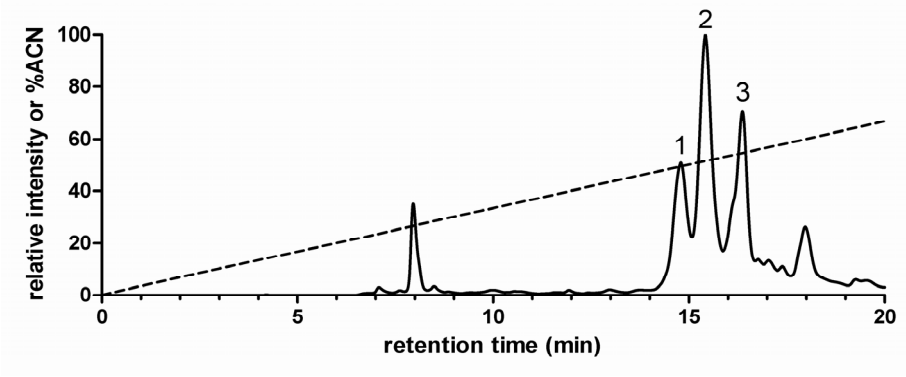

Figure 9. The monitoring of the removal of protecting groups by RP-HPLC. The final product Adc-Ahx-(L-Arg)$_4$-L-Lys-NH-PEG5000-OMe (ARC-701; **1**, R_t=14.8 min) and intermediate products bearing one (**2**, R_t=15.4 min) or two (**3**, R_t=16.4 min) Pbf groups. The dashed line represents linear water-ACN gradient (0–100% ACN/30 min; 0.1% TFA; 1 ml/min).

The potency of pegylated ARC-s to inhibit cAPK was evaluated by kinetic studies of the phosphorylation reaction of kemptide by radiometric phosphocellulose paper assay (**I** Figure 6). It was established that the attachment of a long hydrophilic polymer and biotin tag to the C-terminus of ARC had negligible effect on its inhibitory potency.

Fluorometric assay for the evaluation of inhibitors of protein kinases [II]

The requirement for a non-radioactive assay for the characterization of ARC-type inhibitors initiated us to develop fluorometric assay that is based on the separation of the fluorescently labelled substrate of cAPK, 5-TAMRA-kemptide (Figure 10) from its phosphorylated counterpart by TLC and quantification of the phosphorylation extent ratiometrically by fluorescence imaging. Analogous

assays based on TLC-separation of fluorescence-labelled substrates have been developed for the measurement of tryptic and sialyltransferase activity [120, 121].

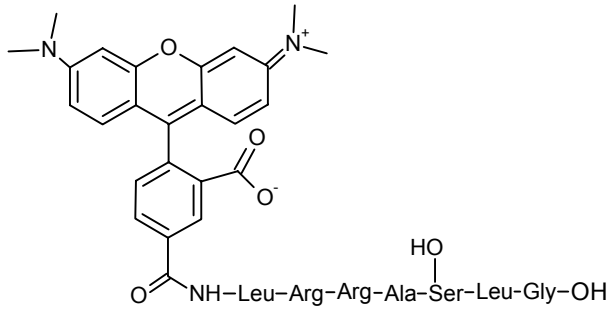

Figure 10. The structure of 5-TAMRA-kemptide.

The attachment of bulky hydrophobic 5-TAMRA to the N-terminus of kemptide slightly improved its substrate characteristics as characterized by similar V_{max} and smaller K_m values if compared to the corresponding parameters of kemptide (**II** Table I). Phosphorylation of the substrate did not influence the fluorescence intensity but changed the polarity of the substance allowing the separation of the product from substrate by normal phase TLC. The reliability of the assay was demonstrated by the monitoring of the time course of the phosphorylation reaction (**II** Figure 2) and characterization of three inhibitors of cAPK, H89, H9 and ARC-306 (**II** Figure 4) in comparison with the traditional radiometric assay.

The developed assay is less hazardous than the radiometric assay. Although it consumes smelling and toxic solvent system (pyridine: acetic acid: butanol: water) for the elution of TLC plates, the chromatographic step can be performed in ordinary fume hood. The assay is separation-based and therefore relatively time-consuming. On the other hand, substances interfering with the fluorescence reading (fluorescence quenchers or fluorescent inhibitors) may be separated. The simultaneous quantification of the substrate and the product allows ratiometric detection. The main disadvantage of the assay is small linear detection range that requires relatively high substrate turnover (>10%). Therefore the assay is not suitable for accurate kinetic measurements and it can be applied for the characterization of inhibitors where the substrate turnover may be higher. An analogous assay was recently developed for cAPK activity that was based on the separation of TAMRA-labelled kemptide from its phosphorylated counterpart by reversed phase microparallel chromatography [40]. This detection method was more sensitive and allowed measuring the inhibitory potencies in the initial velocity phase below 1% of the substrate conversion. On the other hand, the separation by TLC is more robust since the throwaway format of the analysis matrix excludes the requirement for

cumbersome work-up procedures. The developed fluorometric assay has been subsequently applied for the characterization of ARC-type inhibitors [**III,** 66].

Surface-plasmon-resonance-based biosensor with immobilized ARC [III]

Based on the knowledge that the attachment of polymer matrices to the C-terminus of the peptide moiety of ARC does not result in the loss of inhibitory potency [**I**, 67], high-affinity second generation ARC was immobilized to the surface of SPR-based biosensor. Two immobilization strategies were used. The covalent attachment of Adc-Ahx-(D-Arg)$_6$-D-Lys-NH$_2$ (ARC-904) *via* the ε-amino group of C-terminal lysine to the carboxymethylated dextrane surface of the biosensor chip was not successful. Surfaces with too high loading were obtained that showed unstable baseline, high non-specific binding and were not regeneratable. No attempts to optimize the conditions for covalent immobilization were made. The immobilization of the corresponding biotin-tagged ARC, Adc-Ahx-(D-Arg)$_6$-D-Lys(PEO-biotin)-NH$_2$ (ARC-704, **III** Figure 1) *via* the formation of streptavidin-biotin interaction (**III** Figure 2) enabled more efficient adjustment of the loading of the surface and yielded regeneratable surface with excellent stability.

Direct binding experiments (**III** Figure 3) revealed that the obtained surface binds cAPK Cα with high affinity (K_d=16 nM) that was about 1.5 orders of magnitude lower than the affinity of free ARC-704 towards cAPK Cα (K_d=0.5 nM, **III** Table 1). The high-affinity binding does not need the presence of magnesium.

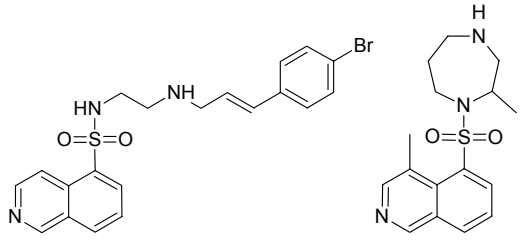

Figure 11. Structures of H89 and H1152P.

The evaluation of the specificity and competitiveness of the interaction was performed in competition assay format (**III** Figure 4). cAPK Cα was displaced from complex with immobilized ARC-704 by substances associating with ATP-binding pocket (ATP, H89 and H1152P, Figure 11), with protein/peptide substrate-binding domain (RIIα and GST-tagged PKIα) and by bisubstrate-analogue inhibitors ARC-341, ARC-704, ARC-904 (**III** Figures 5 and 6). These

results show that the high-affinity binding requires the occupation of both ATP- and peptide-binding sites of cAPK Cα by the bisubstrate-analogue ARC-type inhibitor. Together with the preference to inhibit basophilic protein kinases and low binding affinity of the active fragments of ARC alone (Adc and oligoarginine-containing peptide) [66] these experiments prove the bisubstrate character of ARC and illustrate that binding assays may be useful in verification of the bisubstrate character of an inhibitor when this cannot be unambiguously demonstrated by functional assays.

The competition assays allow the determination of the affinity constants of the competitors. The lower affinity limit that can be reliably determined by this type of assay is defined by the concentration of the enzyme in the assay mixture [28]. The sensitivity of the biosensor towards the enzyme is dependent on the loading of the biosensor surface. The biosensors used in this work were applicable in an affinity range exceeding six orders of magnitude (sub-nanomolar to millimolar, **III** Figure 5). Higher sensitivity could be obtained by increasing the loading, but this would produce higher non-specific binding of the assay components to the surface (**III** Figure 7). The biosensor does not need the presence of magnesium ions that allows investigating the effect of this metal on the binding affinity of competitors as illustrated by the magnesium-dependence of the binding of ATP to cAPK Cα (**III** Table 2). Also the dependence of the interactions on the presence MgATP could be studied below the saturating concentration of the nucleotide (K_d=10 μM) that was shown in the experiment with GST-PKI (**III** Table 2). The affinities of the competitors determined in the present work were consistent with the binding affinities and inhibitory potencies of these compounds measured by other methods (**III** Table 2) that supports the application of the biosensor with immobilized ARC-704 for the determination of the affinities of ATP-, peptide- and bisubstrate-competitive ligands (inhibitors and substrates) of cAPK.

CONCLUSIONS

The conclusions of the present investigation can be summarized as follows:

- Fluorometric assay for the evaluation of inhibitors of cAPK was developed and applied for the determination of inhibitory activities of ARC-s.
- Pegylated ARC was synthesized by soluble-polymer-supported synthetic method with good yield and purity.
- Pegylation had negligible effect to the inhibitory activity of ARC.
- Pegylated ARC was taken up by cells but at smaller extent than the parent compound.
- High-affinity SPR-based biosensor with immobilized ARC was prepared and used for the determination of the affinities of ATP- and substrate protein-competitive ligands of cAPK Cα.
- The competition assays by the prepared SPR-biosensor revealed the bisubstrate character of ARC.

SUMMARY IN ESTONIAN

Immobiliseeritavad basofiilsete proteiinkinaaside bisubstraat-analooginhibiitorid: arendus ja rakendamine biosensorites

Proteiinkinaasid on ensüümid, mis katalüüsivad valkude fosforüleerimist, kandes ATP terminaalse fosfaatrühma üle seriini, treoniini või türosiini hüdroksüülrühmadele. Fosforüleerimisega reguleeritakse suuremat osa raku elutegevusega seotud protsessidest. Häireid fosforüleerimistasakaalus seostatakse paljude patoloogiliste nähtustega, mille tõttu kinaasse aktiivsuse inhibeerimine omab suurt terapeutilist tähtsust.

Üks võimalus inhibeerida proteiinkinaaside katalüütilist toimet on blokeerida ensüümi katalüütiline tasku ühenditega, mis seonduvad ATP või valgu sidumiskohta. Selliseid inhibiitoreid, mis samaaegselt seonduvad mõlema sidumiskohaga, nimetatakse bisubstraatseteks inhibiitoriteks. Adenosiin-oligoarginiinkonjugaadid (ARC) on basofiilsete proteiinkinaaside kõrgefektiivsed bisubstraatsed inhibiitorid. Käesolev töö on osa ARC-de arendamisega seotud projektist.

Vajadusest mitteradioaktiivsete kinaasse aktiivsuse mõõtmise meetodite järele töötati välja fluorestsentsil põhinev meetod inhibiitorite iseloomustamiseks. Meetodi aluseks on fluorestsentsmärgistatud cAMP-sõltuva proteiinkinaasi substraadi fosforüleerimine. Tekkiv produkt lahutatakse fosforüleerimata substraadist planaarkromatograafiliselt. Fosforüleerimisreaktsiooni sügavus määratakse tekkinud produkti ja reageerimata lähteaine signaalide suhte kaudu, mis suurendab analüüsi täpsust. Antud meetod rakendati cAMP-sõltuva proteiinkinaasi inhibiitorite sh ARC-de aktiivsuste iseloomustamiseks.

Eelnevalt oli näidatud [67], et ARC-tüüpi ühendeid on võimalik peptiidse osa C-terminali kaudu ühendada polümeerse kandjaga ilma, et kaoks nende võime kinaasiga seonduda. Et polümeeriga sidumise mõju täpsemalt iseloomustada, sünteesiti käesolevas töös polüetüleenglükooliga seotud ARC. Süntees teostati polümeeri külge järkjärgulise aminohapete kondenseerimisega. Selline strateegia võimaldas eraldada polümeeriga seotud vahesaadusi madalmolekulaarsetest kõrvalproduktidest, kuid erinevalt klassikalisest tahkefaassünteesist kasutada lahusefaasi analüüsimeetodeid: tuuma magnetresonantsspektroskoopiat, mass-spektromeetriat, ultraviolettspektroskoopiat, planaar- ja kõrgefektiivset vedelikkromatograafiat. 14-etapiline süntees kulges summaarse 35-protsendilise saagisega. Eelnevalt oli näidatud, et ARC-d on võimelised rakku sisenema ning transportima sinna ka enda külge haagitud proteiine. Antud töös tehti kindlaks, et polüetüleenglükooliga sidumine vähendab ARC rakku sisenemise võimet ja muudab rakusisest jaotumist. Samuti iseloomustati polümeeriga seotud ARC võimet inhibeerida cAMP-sõltuvat proteiinkinaasi. Tehti

kindlaks, et ARC sidumine polüetüleenglükooliga C-terminali kaudu praktiliselt ei mõjuta tema inhibeerivaid omadusi. Seda teadmist rakendati pinna-plasmonresonantsil põhineva biosensori valmistamisel. ARC immobiliseeriti biosensori külge üle biotiin-streptavidiinühenduse, mis andis tulemuseks väga stabiilse immobiliseeritud inhibiitoriga pinna. Saadud pind sidus kõrge afiinsusega (K_d=17 nM) cAMP-sõltuva proteiinkinaasi katalüütilist alaühikut. Konkurentsuskatsed mitmesuguste ligandidega näitasid, et interaktsioon kinaasi ja ARC vahel on konkurentne nii kinaasi ATP kui peptiidi sidumiskohta seonduvate ligandide suhtes. See tulemus tõestab ARC bisubstraatset iseloomu, mida pole eelnevalt kineetiliste katsetega võimalik olnud üheselt tuvastada. Saadud biosensoriga oli võimalik edukalt mõõta nii ATP-konkurentsete, peptiidikonkurentsete kui bisubstraatsete cAMP-sõltuva kinaasi ligandide sidumiskonstante afiinsuste vahemikus üle kuue suurusjärgu.

ACKNOWLEDGEMENTS

The main body of the investigation presented in this thesis was carried out at the Institute of Organic and Bioorganic Chemistry of the University of Tartu and at Department of Biochemistry of the University of Kassel. The research was supported by grants from the Estonian Science Foundation (grants No 4632, 4007, 5135, 5588 and 6710), the Estonian Ministry of Education and Sciences (grants No 2592 and 0182611s03) and German Ministry for Education and Research (01 GR 0441, NGFN). Financial support from Archimedes Foundation (Kristjan Jaak scholarship), Tartu University Foundation (Heinz Martin Ederma scholarship), Estonian-Revelia Academic Fund Inc. (Harald Raudsepp scholarship) and Rotalia Foundation is greatly acknowledged.

I would like to express my sincere gratitude to all persons who have supported me during my studies, especially:

my supervisor Dr. Asko Uri for accepting me in his group and giving me more opportunities I could use, professional guidance and encouragement during the last nine years, continuous patience and always finding time for scientific and technical questions,

Dr. Friedrich W. Herberg and Dr. Bastian Zimmermann for the opportunity to work in the laboratories of Biaffin GmbH & Co. KG and Department of Biochemistry at the University of Kassel,

colleagues at the Institute of Organic and Bioorganic Chemistry, particularly Erki Enkvist, Darja Lavõgina, Marje Lust, Gerda Raidaru and Angela Vaasa for their contribution to this work and for creating friendly atmosphere,

members of Dr. F. W. Herberg's group and Biaffin team, especially Oliver Bertinetti, Dr. Oliver Diekmann, Mandy Diskar, Dr. Stephan Drewianka, Dr. Frank Gesellchen, Dr. Silke Hutschenreiter, Daniela Moll and Sonja Schweinsberg for their kind acceptance, contribution and support,

co-authors Kärt Padari and Dr. Margus Pooga for their contribution with cellular uptake studies and Indrek Tammiste and Dr. Juhan Subbi for their help with mass-spectrometry and

Dr. Lauri Toom for sending necessary literature.

I would like to thank my friends and family for their love, support and patience.

REFERENCES

1. P. Cohen, Protein kinases: the major drug targets of the twenty-first century? Nat. Rev. Drug Discov. 1 (2002) 309–315.
2. G. Manning, D. B. Whyte, R. Martinez, T. Hunter, S. Sudarsanam, The protein kinase complement of the human genome, Science 298 (2002) 1912–1934.
3. S. S. Taylor, J. Yang, J. Wu, N. M. Haste, E. Radzio-Andzelm, G. Anand, PKA: a portrait of protein kinase dynamics, Biochim. Biophys. Acta. 1697 (2004) 259–269.
4. S. S. Taylor, C. Kim, D. Vigil, N. M. Haste, J. Yang, J. Wu, G. S. Anand, Dynamics of signaling by PKA, Biochim. Biophys. Acta. 1754 (2005) 25–37.
5. B. S. Skålhegg, A. Funderud, H. H. Henanger, T. T. Hafte, A. C. Larsen, A. K. Kvissel, S. Eikvar, S. Orstavik, Protein kinase A (PKA) – a potential target for therapeutic intervention of dysfunctional immune cells, Curr. Drug. Targets 6 (2005) 655–664.
6. J. A. Adams, Kinetic and catalytic mechanisms of protein kinases, Chem. Rev. 101 (2001) 2271–2290.
7. D. M. Gamm, E. J. Baude, M. D. Uhler, The major catalytic subunit isoforms of cAMP-dependent protein kinase have distinct biochemical properties in vitro and in vivo, J. Biol. Chem. 271 (1996) 15736–15742.
8. S. J. Beebe, P. Salomonsky, T. Jahnsen, Y. Li, The Cγ subunit is a unique isozyme of the cAMP-dependent protein kinase, J. Biol. Chem. 267 (1992) 25505–25512.
9. W. Zhang, G. Z. Morris, S. J. Beebe, Characterization of the cAMP-dependent protein kinase catalytic subunit Cγ expressed and purified from sf9 cells, Protein Expr. Purif. 35 (2004) 156–169.
10. B. Zimmermann, J. A. Chiorini, Y. Ma, R. M. Kotin, F. W. Herberg, PrKX is a novel catalytic subunit of the cAMP-dependent protein kinase regulated by the regulatory subunit type I, J. Biol. Chem. 274 (1999) 5370–5378.
11. K. Schiebel, M. Winkelmann, A. Mertz, X. Xu, D. C. Page, D. Weil, C. Petit, G. A. Rappold, Abnormal XY interchange between a novel isolated protein kinase gene, PRKY, and its homologue, PRKX, accounts for one third of all (Y+)XX males and (Y-)XY females, Hum. Mol. Genet. 6 (1997) 1985–1989.
12. D. A. Johnson, P. Akamine, E. Radzio-Andzelm, Madhusudan, S. S. Taylor, Dynamics of cAMP-Dependent Protein Kinase, Chem. Rev. 101 (2001) 2243–2270.
13. M. Ho, H. N. Bramson, D.E. Hansen, Stereochemical Course of the Phospho Group Transfer Catalyzed by cAPM-Dependent Protein Kinase, J. Am. Chem. Soc. 110 (1988) 2680–2681.
14. J. A. Adams, S. S. Taylor, Energetic limits of phosphotransfer in the catalytic subunit of cAMP-dependent protein kinase as measured by viscosity experiments, Biochemistry 31 (1992) 8516–8522.
15. F. W. Herberg, M. L. Doyle, S. Cox, S. S. Taylor, Dissection of the nucleotide and metal-phosphate binding sites in cAMP-dependent protein kinase, Biochemistry 38 (1999) 6352–6360.
16. B. E. Kemp, D. J. Graves, E. Benjamini, E. G. Krebs, Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase, J. Biol. Chem. 252 (1977) 4888–4894.

17. S. Whitehouse, J. R. Feramisco, J. E. Casnellie, E. G. Krebs, D. A. Walsh, Studies on the kinetic mechanism of the catalytic subunit of the cAMP-dependent protein kinase, J. Biol. Chem. 258 (1983) 3693–3701.
18. D. S. Werner, T. R. Lee, D. S. Lawrence, Is protein kinase substrate efficacy a reliable barometer for successful inhibitor design? J. Biol. Chem. 271 (1996) 180–185.
19. M. A. Scarpetta, M. D. Uhler, Evidence for two additional isoforms of the endogenous protein kinase inhibitor of cAMP-dependent protein kinase in mouse, J. Biol. Chem. 268 (1993) 10927–10931.
20. S. P. Collins, M. D. Uhler, Characterization of PKIγ, a novel isoform of the protein kinase inhibitor of cAMP-dependent protein kinase, J. Biol. Chem. 272 (1997) 18169–18178.
21. J. Lew, N. Coruh, I. Tsigelny, S. Garrod, S. S. Taylor, Synergistic binding of nucleotides and inhibitors to cAMP-dependent protein kinase examined by acrylodan fluorescence spectroscopy, J. Biol. Chem. 272 (1997) 1507–1513.
22. F. W. Herberg, S. S. Taylor, Physiological inhibitors of the catalytic subunit of cAMP-dependent protein kinase: effect of MgATP on protein-protein interactions, Biochemistry 32 (1993) 14015–14022.
23. S. Whitehouse, D. A. Walsh, $MgATP^{2-}$-dependent interaction of the inhibitor protein of the cAMP-dependent protein kinase with the catalytic subunit, J. Biol. Chem. 258 (1983) 3682–3692.
24. D. Bhatnagar, R. Roskoski Jr, M. S. Rosendahl, N. J. Leonard, Adenosine cyclic 3',5'-monophosphate dependent protein kinase: a new fluorescence displacement titration technique for characterizing the nucleotide binding site on the catalytic subunit, Biochemistry 22 (1983) 6310–6317.
25. Y-C. Cheng, W. H. Prusoff, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction, Biochem. Pharmacol. 24 (1973) 3099–3108.
26. G. J. Zaman, A. Garritsen, T. de Boer, C. A. van Boeckel CA, Fluorescence assays for high-throughput screening of protein kinases, Comb. Chem. High. Throughput Screen. 6 (2003) 313–320.
27. D. M. Olive, Quantitative methods for the analysis of protein phosphorylation in drug development, Expert Rev. Proteomics 1 (2004) 327–341.
28. R. A. Copeland, Evaluation of Enzyme Inhibitors in Drug Discovery: A Guide for medicinal chemists and pharmacologists, John Wiley & Sons, Inc., New Jersey (2005) pp. 178–213.
29. G. Wu, Y. Yuan, C. N. Hodge, Determining Appropriate Substrate Conversion for Enzymatic Assays in High-Throughput Screening, J. Biomol. Screen. 8 (2003) 694–700.
30. O. von Ahsen, U. Bomer, High-throughput screening for kinase inhibitors, Chembiochem. 6 (2005) 481–490.
31. D. G. Hardie, T. A. J. Haystead, I. P. Salt, S. P. Davies, Assay and Purification of Protein-Serine/Threonine Kinases, in: D. G. Hardie (Ed.), Protein Phosphorylation: A Practical Approach, 2nd Ed., Oxford University Press, New York (1999) pp. 202–207.
32. C. J. Underwood, D. K. Blumenthal, Adaptation of the Protein Kinase Filter Paper Assay to a 96-Well Microtiter Format, Anal. Biochem. 267 (1999) 235–238.

33. C. J. A. Asensio, R. C. Garcia, Determination of a large number of kinase activities using peptide substrates, P81 phosphocellulose paper arrays and phosphor imaging. Anal. Biochem. 319 (2003) 21–33.
34. R. Toomik, P. Ekman, L. Engström, A potential pitfall in protein kinase assay: phosphocellulose paper as an unreliable adsorbent of produced phosphopeptides, Anal. Biochem. 204 (1992) 311–314.
35. C. Lehel, S. Daniel-Issakani, M. Brasseur, B. Strulovici, A chemiluminescent microtiter plate assay for sensitive detection of protein kinase activity, Anal. Biochem. 244 (1997) 340–346.
36. R. Mallari, E. Swearingen, W. Liu, A. Ow, S. W. Young, S. G. Huang, A generic high-throughput screening assay for kinases: protein kinase A as an example, J. Biomol. Screen. 8 (2003) 198–204.
37. G. Sorg, H. D. Schubert, F. H. Buttner, R. Heilker, Automated high throughput screening for serine kinase inhibitors using a LEADseeker™ scintillation proximity assay in the 1536-well format, J. Biomol. Screen. 7 (2002) 11–19.
38. D. E. Wright, E. S. Noiman, P. B. Chock, V. Chau, Fluorometric assay for adenosine 3',5'-cyclic monophosphate-dependent protein kinase and phosphoprotein phosphatase activities, Proc. Natl. Acad. Sci. USA 78 (1981) 6048–6050.
39. H. Kondo, K. Takaki, R. Kuroki, A. Tada, K. Fukumoto, J. Sunamoto, Synthesis of fluorescent peptides and their phosphorylation by the catalytic subunit of cyclic AMP-dependent protein kinase, Bull. Chem. Soc. Jpn. 57 (1984) 2957–2961.
40. J. Wu, R. Barbero, S. Vajjhala, S. D. O'Connor, Real-time analysis of enzyme kinetics via micro parallel liquid chromatography, Assay Drug Dev. Technol. 4 (2006) 653–660.
41. Promega Inc., PepTag® Assay for Non-Radioactive Detection of Protein Kinase C or cAMP-Dependent Protein Kinase, Promega Tech. Bull. 132 (2001) 1–16.
42. S. M. Miick, S. Jalali, B. P. Dwyer, J. Havens, D. Thomas, M. A. Jimenez, M. T. Simpson, B. Zile, K. L. Huss, R. M. Campbell, Development of a microplate-based, electrophoretic fluorescent protein kinase A assay: comparison with filter-binding and fluorescence polarization assay formats, J. Biomol. Screen. 10 (2005) 329–338.
43. R. Seethala, R. Menzel, A fluorescence polarization competition immunoassay for tyrosine kinases, Anal. Biochem. 255 (1998) 257–262.
44. T. C. Turek, E. C. Small, R. W. Bryant, W. A. Hill, Development and validation of a competitive AKT serine/threonine kinase fluorescence polarization assay using a product-specific anti-phospho-serine antibody, Anal. Biochem. 299 (2001) 45–53.
45. PerkinElmer Inc., A practical guide to working with AlphaScreen (2004) 1–52.
46. E. A. Gaudet, K. S. Huang, Y. Zhang, W. Huang, D. Mark, J. R. Sportsman, A homogeneous fluorescence polarization assay adaptable for a range of protein serine/threonine and tyrosine kinases, J. Biomol. Screen. 8 (2003) 164–175.
47. Pierce Biotechnology, Inc., IQ® Assay Platform Technical Handbook (2003) 1–21.
48. H. Takeda, A. Fukumoto, A. Miura, N. Goshima, N. Nomura, High-throughput kinase assay based on surface plasmon resonance suitable for native protein substrates, Anal. Biochem. 357 (2006) 262–271.
49. B. T. Houseman, J. H. Huh, S. J. Kron, M. Mrksich, Peptide chips for the quantitative evaluation of protein kinase activity, Nat. Biotechnol. 20 (2002) 270–274.

50. K. Inamori, M. Kyo, Y. Nishiya, Y. Inoue, T. Sonoda, E. Kinoshita, T. Koike, Y. Katayama, Detection and quantification of on-chip phosphorylated peptides by surface plasmon resonance imaging techniques using a phosphate capture molecule, Anal. Chem. 77 (2005) 3979–3985.
51. P. F. Cook, M. E. Neville Jr, K. E. Vrana, F. T. Hartl, R. Roskoski Jr, Adenosine cyclic 3',5'-monophosphate dependent protein kinase: kinetic mechanism for the bovine skeletal muscle catalytic subunit, Biochemistry 21 (1982) 5794–5799.
52. Promega Inc., Kinase-Glo® Luminescent Kinase Assay, Promega Tech. Bull (2005) 1–16.
53. I. Vainshtein, S. Silveria, P. Kaul, R. Rouhani, R. M. Eglen, J. Wang, A high-throughput, nonisotopic, competitive binding assay for kinases using nonselective inhibitor probes (ED-NSIP™), J. Biomol. Screen. 7 (2002) 507–514.
54. S. A. Saldanha, G. Kaler, H. B. Cottam, R. Abagyan, S. S. Taylor, Assay principle for modulators of protein-protein interactions and its application to non-ATP-competitive ligands targeting protein kinase A, Anal. Chem. 78 (2006) 8265–8272.
55. A. A. Paulucci-Holthauzen, K. L. O'Connor, Use of pseudosubstrate affinity to measure active protein kinase A, Anal. Biochem. 355 (2006) 175–182.
56. H. Xie, O. Braha, L. Q. Gu, S. Cheley, H. Bayley, Single-molecule observation of the catalytic subunit of cAMP-dependent protein kinase binding to an inhibitor peptide, Chem. Biol. 12 (2005) 109–120.
57. W. Huber, F. Mueller, Biomolecular interaction analysis in drug discovery using surface plasmon resonance technology, Curr. Pharm. Des. 12 (2006) 3999–4021.
58. D. Casper, M. Bukhtiyarova, E. B. Springman, A Biacore biosensor method for detailed kinetic binding analysis of small molecule inhibitors of p38alpha mitogen-activated protein kinase, Anal. Biochem. 325 (2004) 126–136.
59. H. Nordin, M. Jungnelius, R. Karlsson, O. P. Karlsson, Kinetic studies of small molecule interactions with protein kinases using biosensor technology, Anal. Biochem. 15 (2005) 359–368.
60. P. M. Fischer, The design of drug candidate molecules as selective inhibitors of therapeutically relevant protein kinases, Curr. Med. Chem. 11 (2004) 1563–1583.
61. Z. A. Knight, K. M. Shokat, Features of selective kinase inhibitors, Chem. Biol. 12 (2005) 621–637.
62. D. S. Lawrence, New design strategies for ligands that target protein kinase-mediated protein-protein interactions, in: L. A. Pinna, P. T. W. Cohen (Eds.), Inhibitors of Protein Kinases and Protein Phosphatases, Handbook of Experimental Pharmacology, vol. 167, Springer-Verlag, Germany (2005) pp. 11–44.
63. M. A. Bogoyevitch, R. K. Barr, A. J. Ketterman, Peptide inhibitors of protein kinases-discovery, characterisation and use, Biochim. Biophys. Acta 1754 (2005) 79–99.
64. K. Parang, P. A. Cole, Designing bisubstrate analog inhibitors for protein kinases, Pharmacol. Ther. 93 (2002) 145–157.
65. M. Loog, A. Uri, G. Raidaru, J. Järv, P. Ek, Adenosine-5′-carboxylic acid peptidyl derivatives as inhibitors of protein kinases, Bioorg. Med. Chem. Lett. 9 (1999) 1447–1452.
66. E. Enkvist, D. Lavogina, G. Raidaru, A. Vaasa, I. Viil, M. Lust, K. Viht, A. Uri, Conjugation of Adenosine and Hexa-(D-Arginine) Leads to a Nanomolar

Bisubstrate-Analog Inhibitor of Basophilic Protein Kinases, J. Med. Chem. 49 (2006) 7150–7159.
67. M. Loog, A. Uri, J. Järv, P. Ek, Bi-substrate analogue ligands for affinity chromatography of protein kinases, FEBS Lett. 480 (2000) 244–248.
68. K. Shen, A. C. Hines, D. Schwarzer, K. A. Pickin, P. A. Cole, Protein kinase structure and function analysis with chemical tools, Biochim. Biophys. Acta. 1754 (2005) 65–78.
69. M. Yu, M. L. Magalhaes, P. F. Cook, J. S. Blanchard, Bisubstrate inhibition: Theory and application to *N*-acetyltransferases, Biochemistry 45 (2006) 14788–14794.
70. A. Uri, G. Raidaru, J. Subbi, K. Padari, M. Pooga, Identification of the ability of highly charged nanomolar inhibitors of protein kinases to cross plasma membranes and carry a protein into cells, Bioorg. Med. Chem. Lett. 12 (2002) 2117–2120.
71. R. Haag, F. Kratz, Polymer therapeutics: concepts and applications, Angew. Chem. Int. Ed. Engl. 45 (2006) 1198–1215.
72. M. Werle, A. Bernkop-Schnürch, Strategies to improve plasma half life time of peptide and protein drugs, Amino Acids 30 (2006) 351–367.
73. T. R. Kumar, K. Soppimath, S. K. Nachaegari, Novel delivery technologies for protein and peptide therapeutics, Curr. Pharm. Biotechnol. 7 (2006) 261–276.
74. G. Pasut, M. F. Veronese, PEGylation of Proteins as Tailored Chemistry for Optimized Bioconjugates, Adv. Polym. Sci. 192 (2006) 95–134.
75. P. K. Working, M. S. Newman, J. Johnson, J. B. Cornacoff, Safety of Poly(ethylene glycol) and Poly(ethylene glycol) Derivatives, in: J. M. Harris, S. Zalipsky (Eds.), Poly(ethylene glycol). Chemistry and Biological Applications, American Chemical Society, Washington, DC (1997) pp. 45–57.
76. M. Mutter, E. Bayer, The Liquid-Phase Method for Peptide Synthesis, in: E. Gross, J. Meienhofer (Eds.), The Peptides. Analysis, Synthesis, Biology, vol 2: Special Methods in Peptide Synthesis, Part A, Academic Press, New York (1980) pp. 285–332.
77. T. Yu, J. A. Traina, E. Pungor 2nd, M. McCaman, Precise and comparative pegylation analysis by microfluidics and mass spectrometry, Anal. Biochem. 359 (2006) 54–62.
78. B. N. Manjula, A. Tsai, R. Upadhya, K. Perumalsamy, P. K. Smith, A. Malavalli, K. Vandegriff, R. M. Winslow, M. Intaglietta, M. Prabhakaran, J. M. Friedman, A. S. Acharya, Site-specific PEGylation of hemoglobin at Cys-93(β): correlation between the colligative properties of the PEGylated protein and the length of the conjugated PEG chain, Bioconjug. Chem. 14 (2003) 464–472.
79. T. Peleg-Shulman, H. Tsubery, M. Mironchik, M. Fridkin, G. Schreiber, Y. Shechter, Reversible PEGylation: a novel technology to release native interferon α2 over a prolonged time period, J. Med. Chem. 47 (2004) 4897–4904.
80. F. M. Veronese, Peptide and protein PEGylation: a review of problems and solutions, Biomaterials 22 (2001) 405–417.
81. M. J. Roberts, M. D. Bentley, J. M. Harris, Chemistry for peptide and protein PEGylation, Adv. Drug. Deliv. Rev. 54 (2002) 459–476.
82. S. P. Monkarsh, C. Spence, J. E. Porter, A. Palleroni, C. Nalin, P. Rosen, P. Bailon, Isolation of Positional Isomers of Mono-poly(ethylene glycol)ylated Interferon/α2a and the determination of their Biochemical and Biological

Characteristics, in: J. M. Harris, S. Zalipsky (Eds.), Poly(ethylene glycol). Chemistry and Biological Applications, American Chemical Society, Washington, DC (1997) pp. 207–216.
83. H. Sato, Enzymatic procedure for site-specific pegylation of proteins, Adv. Drug. Deliv. Rev. 54 (2002) 487–504.
84. R. B. Greenwald, Y. H. Choe, J. McGuire, C. D. Conover, Effective drug delivery by PEGylated drug conjugates, Adv. Drug. Deliv. Rev. 55 (2003) 217–250.
85. M. L. Graham, Pegaspargase: a review of clinical studies, Adv. Drug. Deliv. Rev. 55 (2003) 1293–1302.
86. K. R. Reddy, M. W. Modi, S. Pedder, Use of peginterferon alfa-2a (40 KD) (Pegasys®) for the treatment of hepatitis C, Adv. Drug. Deliv. Rev. 54 (2002) 571–586.
87. P. A. Mabrouk, The Use of Poly(ethylene glycol)-Enzymes in Nonaqueous Enzymology, in: J. M. Harris, S. Zalipsky (Eds.), Poly(ethylene glycol). Chemistry and Biological Applications, American Chemical Society, Washington, DC (1997) pp. 118–133.
88. J. Yoshida, K. Itami, Tag strategy for separation and recovery, Chem. Rev. 102 (2002) 3693–3716.
89. C. C. Tzschucke, C. Markert, W. Bannwarth, S. Roller, A. Hebel, R. Haag, Modern separation techniques for the efficient workup in organic synthesis, Angew. Chem. Int. Ed. Engl. 41(2002) 3964–4000.
90. R. B. Merrifield, Solid phase peptide synthesis. I. The synthesis of a tetrapeptide, J. Am. Chem. Soc. 85 (1963) 2149–2154.
91. P. Virta, J. Katajisto, T. Niittymäki, H. Lönnberg, Solid-supported synthesis of oligomeric bioconjugates, Tetrahedron 59 (2003) 5137–5174.
92. R. Brown, Recent developments in solid-phase organic synthesis, Contemp. Org. Synth. 3 (1997) 216–237.
93. A. R. Vaino, K. D. Janda, Solid-phase organic synthesis: a critical understanding of the resin, J. Comb. Chem. 2 (2000) 579–596.
94. R. Santini, M. C. Griffith, M. Qi, A Measure of Solvent Effects on Swelling of Resins for Solid Phase Organic Synthesis, Tetrahedron Letters 39 (1998) 8951–8954.
95. B.-D. Park, Y.-S. Lee, The effect of PEG groups on swelling properties of PEG-grafted-polystyrene resins in various solvents, React. Funct. Polym. 44 (2000) 41–46.
96. D. Lelievre, O. Turpin, S. E. Kazzouli, A. Delmas, Influence of polar support for the synthesis of large C-terminal peptide aldehyde application of chemoselective ligation, Tetrahedron 58 (2002) 5525–5533.
97. W.C. Chan, P. D. White, Fmoc Solid Phase Peptide Synthesis. Oxford University Press, Oxford, 2000.
98. F. Gaggini, A. Porcheddu, G. Reginato, M. Rodriquez, M. Taddei, Colorimetric tools for solid-phase organic synthesis, J. Comb. Chem. 6 (2004) 805–810.
99. Y. R. de Miguel, A. S. Shearer, Infrared spectroscopy in solid-phase synthesis, Biotechnol. Bioeng. 71 (2001) 119–129.
100. P.A. Keifer, L. Baltusis, D. M. Rice, A. A. Tymiak, J. N. Shoolery, A Comparison of NMR Spectra Obtained for Solid-Phase-Synthesis Resins Using Conventional

High-Resolution, Magic-Angle-Spinning, and High-Resolution Magic-Angle-Spinning Probes, J. Mag. Res. A 119 (1996) 65–75.
101. A. Link, Comments on the Terminology for Applications on Temporarily Attached Solubility-Modifying Moieties in Combinatorial Chemistry, Angew. Chem. Int. Ed. 39 (2000) 4039–4040.
102. D. G. Gravert, K. D. Janda, Organic Synthesis on Soluble Polymer Supports: Liquid-Phase Methodologies, Chem. Rev. 97 (1997) 489–509.
103. G. M. Bonora, E. Ivanova, V. Zarytova, B. Burcovich, F. M. Veronese, Synthesis and Characterization of High-Molecular Mass Polyethylene Glycol-Conjugated Oligonucleotides, Bioconjugate Chem. 8 (1997) 793–797.
104. J. Šebestik, P. Niederhafner, M. Šafarik, J. Hlavaček, Electrophoresis of Derivatized Polyethylene Glycols: A Useful Method for Monitoring of Reactions on Soluble Polymeric Carrier, Int. J. Pept. Res. Ther. 11 (2005) 291–296.
105. C. Enjalbal, F. Lamaty, P. Sanchez, E. Suberchicot, P. Ribiere, S. Varray, R. Lazaro, N. Yadav-Bhatnagar, J. Martinez, J. L. Aubagna, Characterization of soluble polymer supported organic compounds by LC/electrospray ionization MS toward a complete automation of the liquid-phase process in combinatorial chemistry, Anal. Chem. 75 (2003) 175–184.
106. R. Thürmer, M. Meisenbach, H. Echner, A. Weiler, R. A. Al-Qawasmeh, W. Voelter, U. Korff, W. Schmitt-Sody, Monitoring of Liquid-Phase Peptide Synthesis on Soluble Supports via Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry, Rapid Comm. Mass Spectrom 12 (1998) 398–402.
107. J.-Y. Shey, C.-M. Sun, Liquid-phase combinatorial reaction monitoring by conventional ^{1}H NMR spectroscopy, Tetrahedron Lett. 43 (2002) 1725–1729.
108. S. Abt El Rahman, H. Anzinger, M. Mutter, Relationship Between Conformation and Physicochemical Properties of Polypeptides. I. Synthesis of Homo- and Co-Oligopeptides by the Liquid-Phase Method, Biopolymers 19 (1980) 173–187.
109. T. J. Dickerson, N. N. Reed, K. D. Janda, Soluble polymers as scaffolds for recoverable catalysts and reagents, Chem. Rev. 102 (2002) 3325–3344.
110. M. M. Shemyakin, Y. A. Ovchinnikov, A. A. Kinyushkin, I. V. Kozhevnikova, Synthesis of Peptides in Solution on a Polymeric Support, Tetrahedron Lett. 27 (1965) 2323–2327.
111. E. Bayer, M. Mutter, Liquid Phase Synthesis of Peptides, Nature 237 (1972) 512–513.
112. Y. Wang, H. Rashidzadeh, B. Guo, Structural effects on polyether cationization by alkali metal ions in matrix-assisted laser desorption/ionization, J. Am. Soc. Mass Spectrom. 11 (2000) 639–643.
113. C. Sun, M. Baird, J. Simpson, Determination of Poly(ethylene glycol)s by Both Normal-Phase and Reversed-Phase of High-Performance Liquid Chromatography, J. Chrom. A 800 (1998) 231–238.
114. P. M. Fischer, D. I. Zheleva, Liquid-phase peptide synthesis on polyethylene glycol (PEG) supports using strategies based on the 9-fluorenylmethoxycarbonyl amino protecting group: application of PEGylated peptides in biochemical assays, J. Pept. Sci. 8 (2002) 529–542.
115. H. Han, M. M. Wolfe, S. Brenner, K. D. Janda, Liquid-phase combinatorial synthesis, Proc. Natl. Acad. Sci. USA 92 (1995) 6419–6423.

116. E. Bayer, M. Mutter, R. Uhmann, J. Polster, H. Mauser, Kinetic studies of the liquid phase peptide synthesis, J. Am. Chem. Soc. 96 (1974) 7333–7336.
117. D. E. Bergbreiter, T. Kimmel. J. W. Caraway, Modification of Substrate Reactivity Using Soluble Polymeric Supports, Tetrahedron Lett. 36 (1995) 4757–4760.
118. X. Y. Zhao, K. D. Janda, Soluble polymer traceless linker investigations: solvent effects on the desulfonylation of polyethylene glycol (PEG) substituted aryl alkyl sulfones with sodium amalgam, Bioorg. Med. Chem. Lett. 8 (1998) 2439–2442.
119. F.-S. Tjoeng, R. S. Hodges, Cleavage of protected amino acids and peptides from the new o-nitrobenzoyl polyethylene glycol support by catalytic hydrogenolysis, Tetrahedron Lett. 15 (1979) 1273–1276.
120. T. Uchikoba, S. Fukumoto, T. Itakura, M. Okubo, K. Tomokiyo, K. Arima, H. Yonezawa, Detection of Hydrolytic Activity of Trypsin with a Fluorescence-chymotryptic Peptide on a TLC Plate, Biosci. Biotechnol. Biochem. 68 (2004) 222–225.
121. U. Hubl, G. C. Slim, O. V. Zubkova, Thin-layer chromatography and poly-acrylamide gel electrophoresis-based assays for sialyltransferases using tetra-methylrhodamine-labeled acceptors, Anal. Biochem. 285 (2000) 92–99.

PUBLICATIONS

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Scientific publications

1) Viht, K., Schweinsberg, S., Lust, M., Vaasa, A., Raidaru, G., Lavogina, D., Uri, A., Herberg, F.W. Surface-plasmon-resonance-based biosensor with immobilized bisubstrate analog inhibitor for the determination of affinities of ATP- and protein-competitive ligands of cAMP-dependent protein kinase. – *Anal. Biochem.,* **2007**, 362, 268–277.
2) Enkvist, E., Lavogina, D., Raidaru, G., Vaasa, A., Viil, I., Lust, M., Viht, K., Uri, A. Conjugation of adenosine and hexa-(D-arginine) leads to a nanomolar bisubstrate-analog inhibitor of basophilic protein kinases. – *J. Med. Chem*., **2006,** 24, 7150–7159.
3) Viht, K., Vaasa, A., Raidaru, G., Enkvist, E., Uri, A. Fluorometric TLC assay for evaluation of protein kinase inhibitors. – *Anal. Biochem*., **2005**, 340, 165–170.
4) Säälik, P., Elmquist, A., Hansen, M., Padari, K., Saar, K., Viht, K., Langel, U., Pooga, M. Protein cargo delivery properties of cell-penetrating peptides. A comparative study. – *Bioconjug. Chem*., **2004**, 15, 1246–1253.
5) Mahlapuu, R., Viht, K., Balaspiri, L., Bogdanovic, N., Saar, K., Soomets, U., Land, T., Zilmer, M., Karelson, E., Langel, Ü. Amyloid Precursor Protein Carboxy-terminal Fragments Modulate G-proteins and Adenylate Cyclase Activity in Alzheimer's Disease Brain. – *Molecular Brain Research*, **2003**, 117, 73–82.
6) Viht, K., Padari, K., Raidaru, G., Subbi, J., Tammiste, I., Pooga, M., Uri, A. Liquid-Phase Synthesis of a Pegylated Adenosine-Oligoarginine Conjugate, Cell-Permeable Inhibitor of cAMP-Dependent Kinase. – *Bioorg. Med. Chem. Lett*., **2003**, 13, 3035–3039.

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Teaduspublikatsioonid

1) Viht, K., Schweinsberg, S., Lust, M., Vaasa, A., Raidaru, G., Lavogina, D., Uri, A., Herberg, F.W. Surface-plasmon-resonance-based biosensor with immobilized bisubstrate analog inhibitor for the determination of affinities of ATP- and protein-competitive ligands of cAMP-dependent protein kinase.- *Anal. Biochem*., **2007**, 362, 268–277.
2) Enkvist, E., Lavogina, D., Raidaru, G., Vaasa, A., Viil, I., Lust, M., Viht, K., Uri, A. Conjugation of adenosine and hexa-(D-arginine) leads to a nanomolar bisubstrate-analog inhibitor of basophilic protein kinases. – *J. Med. Chem*., **2006,** 24, 7150–7159.
3) Viht, K., Vaasa, A., Raidaru, G., Enkvist, E., Uri, A. Fluorometric TLC assay for evaluation of protein kinase inhibitors. – *Anal. Biochem*., **2005**, 340, 165–170.
4) Säälik, P., Elmquist, A., Hansen, M., Padari, K., Saar, K., Viht, K., Langel, U., Pooga, M. Protein cargo delivery properties of cell-penetrating peptides. A comparative study. – *Bioconjug. Chem*., **2004**, 15, 1246–1253.
5) Mahlapuu, R., Viht, K., Balaspiri, L., Bogdanovic, N., Saar, K., Soomets, U., Land, T., Zilmer, M., Karelson, E., Langel, Ü. Amyloid Precursor Protein Carboxy-terminal Fragments Modulate G-proteins and Adenylate Cyclase Activity in Alzheimer's Disease Brain. – *Molecular Brain Research*, **2003**, 117, 73–82.
6) Viht, K., Padari, K., Raidaru, G., Subbi, J., Tammiste, I., Pooga, M., Uri, A. Liquid-Phase Synthesis of a Pegylated Adenosine-Oligoarginine Conjugate, Cell-Permeable Inhibitor of cAMP-Dependent Kinase. – *Bioorg. Med. Chem. Lett*., **2003**, 13, 3035–3039

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