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Development of bisubstrate inhibitors for protein kinase CK2

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

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II Viht, K., Saaver, S., **Vahter, J.**, Enkvist, E., Lavogina, D., Sinijärv, H., Raidaru, G., Guerra, B., Issinger, O.-G., Uri, A. (2015) Acetoxymethyl ester of tetrabromobenzimidazole-peptoid conjugate for inhibition of protein kinase CK2 in living cells, Bioconjug. Chem., 12, 2324–2335

III **Vahter, J.**, Viht, K., Uri, A., Enkvist, E. (2017) Oligo-aspartic acid conjugates with benzo[c][2,6]naphthyridine-8-carboxylic acid scaffold as picomolar inhibitors of CK2, Bioorg Med Chem, 25(7), 2277–2284

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Autor's contribution

I The author synthesized new precursor and most of the new bisubstrate inhibitors described in the article, also participated in planning the experiments.

II The author synthesized group of bisubstrate inhibitors, containing oligo L-Asp, described in the paper.

III The author synthesized new precursor and most of the new bisubstrate inhibitors described in the article, planned and performed measurements for characterization of novel compounds.

IV The author synthesized new precursor and new bisubstrate inhibitors described in the article, planned and performed most of the measurements for characterization of novel compounds.

ABBREVIATIONS

5-TAMRA	5-carboxytetramethylrhodamine
ARC	formerly adenosine analogue and oligoarginine conjugates, presently biligand inhibitor or probe for protein kinases designed in Asko Uri's research group at the University of Tartu
ARC-Lum(-)	ARC-probe incorporating a phosphorescent heteroaromatic fragment
ARC-Lum(Fluo)	ARC-probe incorporating a phosphorescent heteroaromatic fragment and a fluorescent dye
ASB	4-(2-amino-1,3-selenazol-5-yl)benzoic acid
ATB	4-(2-amino-1,3-thiazol-5-yl)benzoic acid
ATP	adenosine-5'-triphosphate
Boc	tert-butyloxycarbonyl protecting group, used in solid phase peptide synthesis
BSA	bovine serum albumin
CK2	protein kinase formerly known as casein kinase 2
CX-4945	5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid
DMF	N,N-dimethylformamide
FA	fluorescence anisotropy
FDA	US Food and Drug Administration
Fmoc	fluorenylmethyloxycarbonyl protecting group, used in solid phase peptide synthesis
FRET	Förster's resonant energy transfer
GO	Glucose oxidase
HBTU	O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOBt	1-hydroxybenzotriazole
HPLC	high performance/pressure liquid chromatography
IC_{50}	the total concentration of the inhibitor when 50% displacement has occurred
K_D	dissociation constant
NIH-3T3 cells	mouse embryonic fibroblast cells
PDB	Protein Data Bank
PF	PromoFluor
PK	protein kinase
PKA	cAMP-dependent protein kinase A holoenzyme

RET	resonance energy transfer
SPPS	solid phase peptide synthesis
TBBi	4,5,6,7-tetrabromobenzimidazole
TGLI	time-gated luminescence intensity
TFA	trifluoroacetic acid
TIPS	triisopropylsilane

1. INTRODUCTION

Protein kinase (PK) superfamily with more than 530 members plays a crucial role in cell life cycle by transferring terminal phosphoryl group from ATP to substrate proteins. The present thesis is focusing on one of the members of the kinase family called CK2.

CK2 is a highly conserved and acidophilic serine/threonine type of protein kinase. With its high cellular concentration, constitutive activity and having over 300 protein substrates, CK2 plays important role in cell regulation. Research has shown that deregulation of CK2 expression is also related to serious diseases including various types of cancer, Alzheimer's disease, diabetes and inflammation. For that reason, CK2 has become an important target for drug discovery.

Over the years, many ATP-competitive inhibitors of CK2 have been reported. Since many protein kinases have structural similarities and most of them use ATP as the phosphoryl group donor, ATP-competitive inhibitors often have high affinities but poor selectivity toward a target kinase.

A bisubstrate inhibitor approach allows to take into account the structural aspects of two regions of the active sites (ATP and substrate binding site) of protein kinase and designing inhibitor binding to both sites simultaneously. This method could improve the selectivity and affinity of the inhibitor.

Within this thesis, we introduce three scaffolds of new CK2 bisubstrate inhibitors. Using TBBi, CX-4945 and ATB/ASB as ATP-competitive fragments and conjugating them with negatively charged peptides led to inhibitors of CK2 with high selectivity and affinity. The ability to label these inhibitors with fluorescence dyes gives a chance to use them as probes for characterization of new CK2 inhibitors and for measurement of kinase activity by using fluorescence anisotropy method and/or long-lifetime luminescence properties. Bright CK2 dependent luminescence of ATB/ASB fragment allows us to measure low concentrations of CK2 and preform measurements with high signal to noise ratio. These probes could be used to measure the CK2 activity and localization in cells and they simplify characterization of novel inhibitors of CK2 by using displacement assays.

2. LITERATURE OVERVIEW

2.1. Protein kinases

Protein kinases (PKs) are a protein superfamily with more than 530 enzymes. Protein kinases are transferases that catalyze the transfer of the terminal phosphoryl group from nucleoside triphosphates to target substrate proteins/ peptides. Based on the sequence identity, kinases are divided into eight main groups: TK, TKL, STE, CK1, CMGC, AGC, CAMK, RGC, and a group called OTHERS, which all the remaining typical kinases are assigned to.[1] In case of phosphorylation sites, PKs are divided into two types: Ser/Thr and Tyr kinases. [2] In addition to PKs, there is also a group of enzymes that have protein kinase activity, but do not share detectable sequence similarity with typical kinases. These enzymes are called atypical kinases or α-kinases. [3]

By adding the phosphoryl group to target proteins, PKs control many cellular processes, including metabolism; transcription; cell division, movement, survival; and also programmed cell death. PKs also have multiple roles in the immune response and nervous systems. Because of PKs high influence in cell regulation, the misregulation of these enzymes is related to a number of different diseases, including diabetes, Alzheimer's disease, inflammation and various types of cancer. This is the reason why several protein kinases are target molecules for novel drugs and compounds in current drug development.[1,2]

2.2. Protein kinases as drug targets

Many protein kinases are drug targets due to their important roles in cell regulation. In addition to that, misregulation of the signal transduction networks of kinases has been shown to be related with different diseases.[2] Because of the high drug target value of kinases, the effort to determine their structures has led to over 3600 X-ray structures of human kinases that are available in Protein Data Bank (PDB).[4]

Because of the druggability of the highly conserved ATP-binding site of these proteins, Type I inhibitors, which bind to the ATP sites of the active form of kinase, are the most commonly developed.[5] Type II inhibitors are also common drugs that stabilize the inactive conformations of kinases. Type III inhibitors are usually defined as allosteric inhibitors and have been discovered for some kinases. [6,7] Because of the high conservation of the ATP-binding site, many of ATP-competitive drugs discovered possess a low selectivity and have many off-targets in addition to the key target.[8]

After the first FDA approved kinase inhibitor called Trastuzumab [9], which is a monoclonal antibody used in breast cancer treatment [10], the field of kinase inhibitors has grown drastically. Over the years, more than 250 kinase inhibitors are undergoing clinical trials and 42 small molecule kinase inhibitors have

become FDA approved drugs (Table 1).[11] Most of these inhibitors are for treating different types of cancer, but there are also drugs for the treatment of some other diseases, including arthritis and chronic immune thrombocytopenia. Comparing the targets of those inhibitors, most of them are designed for inhibiting protein-tyrosine kinases.[12]

Even though the amount of FDA approved protein kinase makes up almost 3% of all approved drugs [13,14], the fact that 35 of 42 small molecular protein kinase inhibitors have received approval over the last 16 years highlights the importance of PK inhibitors in the field of drug discovery. [15,16]

Table 1. List of small molecule drugs and their FDA approved protein kinase inhibitor targets (based on [11,12],).

Targeted kinase	Type of phosphorylation	Drug
ALK	Tyrosine	Alectinib, brigatinib, crizotinib, ceritinib
BCR-Abl	Tyrosine	Bosutinib, dasatinib, imatinib, nilotinib, ponatinib, regorafenib
EGFR family	Tyrosine	Gefitinib, brigatinib, erlotinib, lapatinib, vandetanib, afatinib, osimertinib
IGFR	Tyrosine	Brigatinib
PDGFRα/β	Tyrosine	Axitinib, gefitinib, imatinib, lenvatinib, nintedanib, pazopanib, regorafenib, sorafenib, sunitinib
VEGFR family	Tyrosine	Axitinib, lenvatinib, nintedanib, regorafenib, pazopanib, sorafenib, sunitinib, ponatinib
c-Met	Tyrosine	Crizotinib, cabozantinib
RET	Tyrosine	Alectinib, vandetanib, ponatinib
BTK	Tyrosine	Acalabrutinib, ibrutinib
JAK family	Tyrosine	Baricitinib, ruxolitinib, tofacitinib
Src family	Tyrosine	Bosutinib, dasatinib, ponatinib, vandetanib
Syk	Tyrosine	Fostamatinib
FLT3	Tyrosine	Midostaurin, ponatinib
ERBB2	Tyrosine	Neratinib
CDK family	Serine/threonine	Abemaciclib, palbociclib, sorafenib, ribociclib
mTOR	Serine/threonine	Everolimus, sirolimus, temsirolimus
B-Raf	Serine/threonine	Vemurafenib, dabrafenib, encorafenib, regorafenib
MEK1/2	Dual specificity	Bininetinib, cobimetinib, trametinib

2.3. Protein kinase CK2

Protein kinase CK2 is a highly conserved serine/threonine type protein kinase usually presented in cells as a tetramer, containing two catalytic subunits (α or α', expressed by different genes) and two regulatory subunits (β) (Figure 1). CK2 is always found to be active and ubiquitously expressed.[17] Differently from some kinases (for example PKA), the regulatory subunit does not inhibit the activity of CK2. β-subunits are modulators that regulate the stability of holoenzyme and affect the affinity towards some CK2 substrates, which are CK2β dependent.[18] There are more than 300 protein substrates reported for CK2, and because of that it is considered to be one of the most pleiotropic protein kinase.[19] Previously, it was estimated that CK2 is responsible for about 20% of protein phosphorylation [20], but recent studies have shown that it might be an overestimation. Proteomics suggests that the amount of phosphorylation affected by CK2 is only about 10%.[21]. Nonetheless, the role of CK2 in cell regulation should not be undervalued.

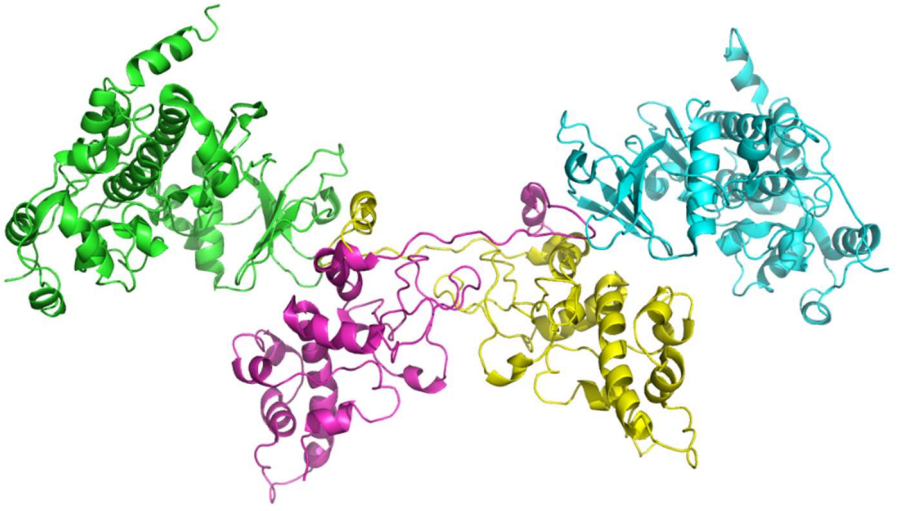


Figure 1. Crystal structure of CK2 holoenzyme. Catalytic subunits are colored green and cyan; regulatory subunits are colored yellow and magenta. (PDB code: 1JWH, [32])

Because of the high amount of substrates, CK2 is involved in many regulative processes in a cell, including gene expression, cell growth, cell viability, cell cycle and anti-apoptosis.[22–25] Moreover, CK2 seems to be involved in the regulation of tRNA and rRNA synthesis process and also plays an important role in cell DNA damage response system.[26]

Because of the overexpression and elevated activity of CK2 and unbalanced expression of its subunits, CK2 is reported to be related to a number of different diseases, but is mostly associated with different types of cancer.[27] CK2 has been shown to be highly overexpressed in cancer cells. It also favors rapid pro-

liferation, survival of cancer cells and supports angiogenesis.[28–30] Research has shown that cancers cells are “addicted” to CK2 [27],downregulating its activity will induce apoptosis and suppress angiogenesis. [23,31] In addition to that, downregulation of CK2 seems to have a more significant effect on cell survival in cancer cells than it does in normal cells.[23] CK2’s important role in cell functioning and its noticeable role in the development of cancer makes it a potential target for the treatment of cancer.

2.4. Protein kinase CK2 inhibitors

Because of the importance of CK2 in cellular processes and the fact that its overexpression is involved in multiple different diseases, many CK2 inhibitors have been developed throughout the years. Most of these inhibitors are designed to compete directly with the binding of ATP to the nucleotide triphosphate binding site of the kinase and are therefore Type I inhibitors (Figure 2).[33] These inhibitors are usually small molecules with one or more aromatic cycles. Type I CK2 inhibitors include naturally occurring compounds such as flavones [34] and coumarins [35], but also different benzimidazole derivatives [36,37] and carboxylic acid derivatives, including CX-4945. Lastly mentioned CX-4945 was the first CK2 inhibitor included in clinical trials as a cancer drug.[38]

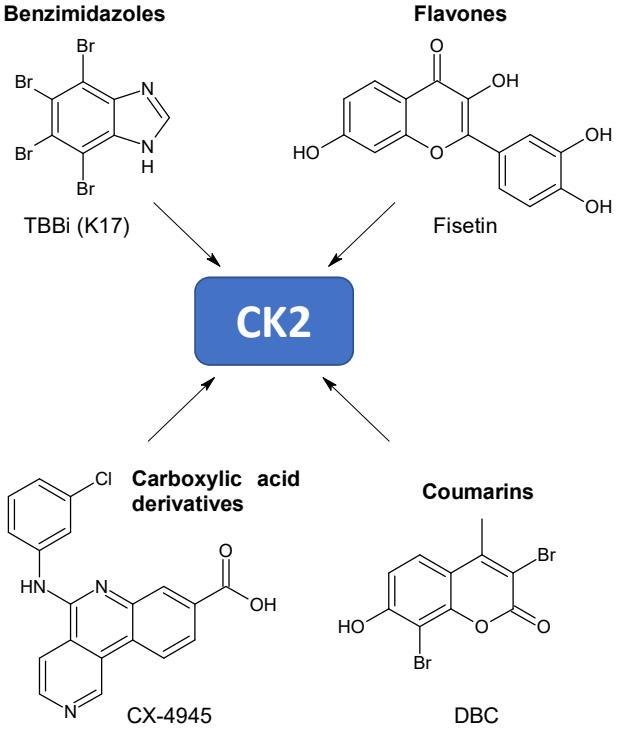


Figure 2. Types of ATP-competitive inhibitors of CK2. (Modified figure from [33])

In addition to ATP-competitive inhibitors, attempts to find substrate-competitive inhibitors have been also reported. CK2 is an acidophilic protein kinase, meaning that it prefers negatively charged amino acids in the consensus sequence of the protein substrates.[39] Negatively charged peptides could be used to inhibit the substrate binding to the kinase. Taking this into account, heparin, a negatively charged oligosaccharide has been reported as a substrate-competitive inhibitor.[40,41]

2.5. Bisubstrate inhibitors

Bisubstrate inhibitors are compounds consisting of two conjugated fragments, which are related to both substrates of a specific protein kinase. While site-directed or monosubstrate inhibitors occupy only one binding site of one substrate, a bisubstrate approach exploits interactions of both binding sites that are simultaneously occupied by the inhibitor. This approach results in the increase of the affinity and selectivity of the inhibitor, but also its weight. Bisubstrate inhibitors usually have a high molecular weight of 800–3000 Da. This does not fit in with the Lipinski's rule of five that states that the molecular mass should be less than 500 daltons.[42,43] On the other hand, bisubstrate inhibitors has well conjugatable (addition of fluorescence dyes [44], etc), which could make them a multipurpose tool for studying kinases.

In recent years, the development of bisubstrate inhibitors and their conjugated probes for protein kinases has been a main focus in the medicinal chemistry workgroup at the University of Tartu. Potent inhibitors for several kinases (including PKAc [45], ROCK [45], PKG [42], PKB [44], etc.) have been reported. High selectivity, low nanomolar affinities and probes possessing kinase dependent room temperature phosphorescence for time-gated luminescence measurements are the outcome of this work.[44,46,47] (Figure 3)

Using ATP-competitive moieties of CK2 inhibitors and conjugating them with kinase substrate mimics (usually negatively charged peptide consisting aspartic or glutamic acid residues) would lead to bisubstrate inhibitors and possible probes for CK2. Before this research only one attempt for developing a multisite inhibitor for CK2 was reported [48], but as a result of this research other papers describing a bisubstrate inhibitor approach for CK2 have followed.[49,50]

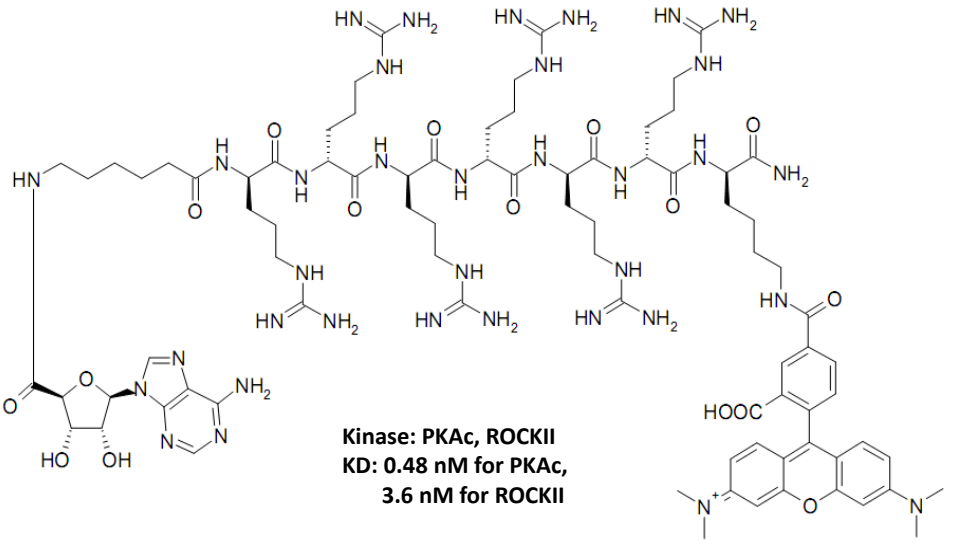


Figure 3. Structure and characteristics of bisubstrate inhibitor ARC-583. [45]

2.6. ARC-Lum probes

In 2011 our work group reported probes containing metal-free organic molecules with an ability to emit long (microsecond-scale) lifetime luminescence when they were bound to a complex with an enzyme and excited with UV-radiation.[46,51] Those probes can be divided in two: ARC-Lum(-) probes, that are conjugates of sulfur or selenium containing aromatic adenosine analogues that possess kinase dependent room-temperature phosphorescence; ARC-Lum(Fluo) probes that have similar structures as ARC-Lum(-), but with additional fluorescence dye conjugated with the molecule. ARC-Lum(Fluo) have significantly higher luminescence intensity resulting from efficient triplet-singlet energy transfer from donor phosphor to fluorescent acceptor dye. [46,49,51] In case of ARC-Lum(-), the measured signal is phosphorescence emission from the complex of a small organic molecule with a kinase. The long lifetime signal of an ARC-Lum(Fluo) probe kinase complex is mainly the result of a FRET (Förster's resonant energy transfer) and corresponds to the spectra of an acceptor dye.[51,52] Excitation spectra of long lifetime emission of ARC-Lum(Fluo) probes coincide always with the absorption spectra of phosphorescent donor molecule (S or Se containing adenosine analogue).[47,49,51,52] The excitation is performed at a low wavelength where a phosphorescent donor absorbs photons and the signal is measured at the wavelength of the emission of the acceptor molecule (fluorescence dye). Efficient triplet-singlet energy transfer requires an overlap of the phosphorescence emission spectrum of the donor molecule and the excitation spectrum of the acceptor molecule. Also, the donor and acceptor moieties need to be close enough to each other.[53–56] Emission from the triplet state is a forbidden process and the corresponding

transition dipole is very weak.[57] Therefore, phosphorescence emission has a long lifetime and needs the triplet state to be protected from quenching. Dipole-dipole type triplet-singlet energy transfer has similar characteristics and accords slowly and needs protection of the triplet state.[58] At short distances T-S FRET might be faster and more efficient than phosphorescence, leading to enhanced emission of tandem probes (D-A systems) compared to emission of pure phosphors. Signal of long-lifetime luminescence is usually measured by using time delayed detection that removes background signals, fluorescence and light scattering.[51–53] Usually the delay time is at least 50 μs to eliminate the afterglow of the xenon flash lamp that is the most common light source of commercial platereaders (Figure 4).[59]

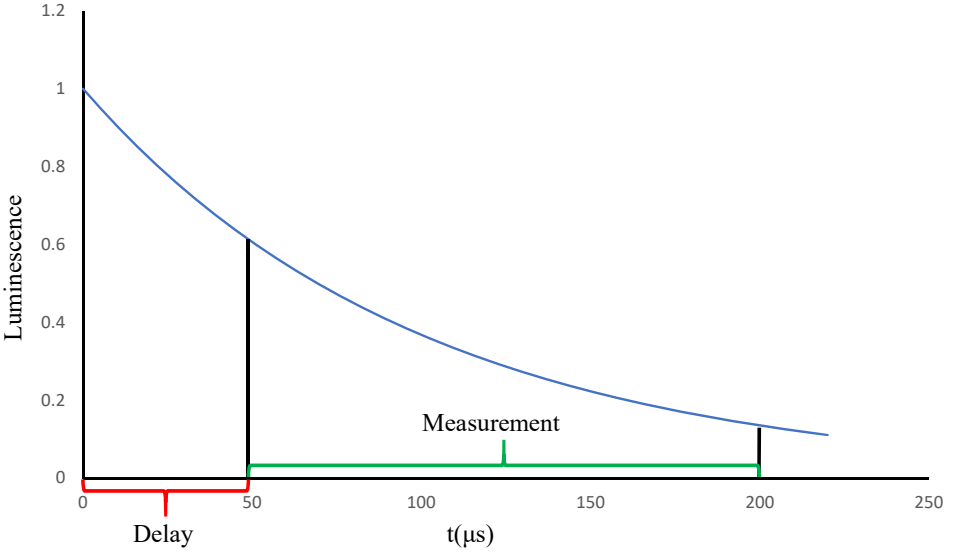


Figure 4. General schematic of the delayed measurement system.

2.7. Synthesis of peptides and peptide conjugates

Peptide synthesis is mostly carried out on a solid supporter, but methods for synthesis in a solution are also available. Both methods follow similar principles but differ by the protecting groups and purification procedures used. Solid phase peptide synthesis (SPPS) (Scheme 1) was first described by R. B. Merrifield in 1963.[60] and is the most common method in use.

In case of SPPS, two main synthesis strategies have been developed, differing by the protecting group used for N-terminus of the conjugate.[61] The first method is based on using Boc as the protecting group. The Boc method uses strong acids (trifluoro acetic acid (TFA)) to remove the protecting group from the N-terminus of the peptide. In case of the Fmoc method, a mild base (for example piperidine) is used to remove the protecting group from terminal

amino group.[62] Because of the milder reagents the Fmoc strategy (compared to the use of hydrofluoric acid in the Boc method) is usually preferred in laboratory scale synthesis.

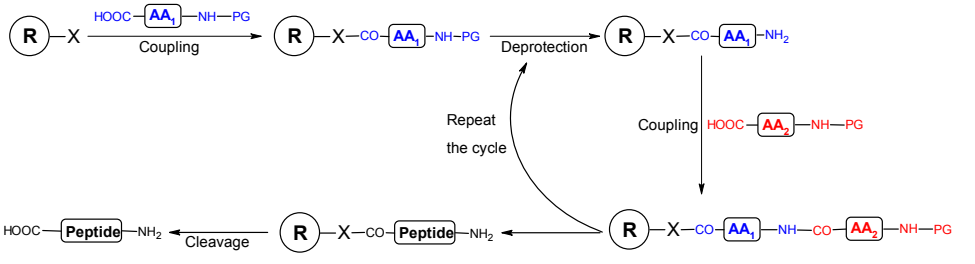
SPPS overall method consists of four steps: coupling of the first amino acid to resin, removal of the protecting group and elongation of peptide, cleavage of the peptide from the resin (Scheme 1).

The first amino acid is attached to the resin using amide or ester bond, depending on the type of the resin used. Merrifield introduced a method using chloromethylpolystyrene, which forms ester bond between carboxylic group of the first N-protected amino acid and solid supporter.[63]

Elongation of the peptide contains amino group deprotection and amino acid coupling reactions. In case of Fmoc method the protecting group is removed by a mild base (piperidine).[64] Coupling of amino acids needs activators to activate the carboxylic group of the next amino acid added to the solid supporter. Over the years many different types of activators have developed, including different carbodiimides and phosphonium or uronium based reagents.[65] The activated carboxylic acid group reacts with the deprotected amino group of the amino acid on the resin and forms an amide bond. This cycle of deprotection and coupling can be repeated until the desired peptide is synthesized.

Depending on the method, removing peptide from the solid supporter differs. In case of the Fmoc-method the peptide is removed from the resin using strong acids (TFA for example).[66] The strong acid environment usually also removes the protecting groups form the side groups of the amino acids. Boc-method usually uses an acidic environment for removing the peptide from the solid supporter. The purification of the peptide is usually executed by using a type of liquid chromatography.

Conjugation of ATP-competitive moiety with the peptide follows a procedure similar to peptide synthesis itself and could be carried out on the solid supporter. In that case, a fragment equivalent compared to amount of amino acids used SPPS is usually smaller to avoid any type of cross-reactions and to save on reagents that are not commercially available. The fragment should have one functional group that can react with the N-terminal of the peptide and protecting groups of the fragment should be similarly removed as the ones on the peptide.



Scheme 1. General synthesis scheme of solid phase peptide synthesis.

3. AIMS OF THE STUDY

The general aim of this study was to develop novel CK2 inhibitors with high affinity and selectivity by using a bisubstrate inhibitor approach. To achieve the goal, several ATP competitive fragments and negatively charged peptides were chosen. The study included several subtasks:

- Selection of a precursor that has good inhibition properties towards CK2 and a functional group for linkage to a peptide moiety.
- Optimization of the length and the structure of the linker connecting the ATP-competitive fragment and the peptide fragment.
- Optimization of the length and the composition of the peptide chain.
- Biochemical characterization of synthesized inhibitors and fluorescence probes.

4. METHODS

4.1. Binding and displacement assays

4.1.1. Measurement of fluorescence anisotropy

The binding affinities of ligands were determined in the binding/displacement assays as described earlier [45,46]. The assay was carried out in a 4-component buffer (pH = 7.5) consisting of 50 mM HEPES hemisodium salt (Sigma), 150 mM NaCl (Riedel-de Haën), 0.005% Tween® 20 (Sigma) and 5 mM dithiothreitol (Sigma). The final volume of measurements was 20 μL on black 384-well polystyrene microplates with a non-binding surface (Corning category number 3676 or 4514). The microplates and sample solutions were preincubated and measured at 30 °C.

The fluorescence anisotropy (FA) was registered on a PHERAstar plate reader (BMG Labtech). For FA measurements, optical modules suitable for detecting PromoFluor-647 [ex 590 (50) nm, em 675 (50) nm] and 5-TAMRA/ PromoFluor-555 [ex 540 (20) nm, em 590 (20) nm] were applied.

The concentration of active protein kinase was determined by titration of a fixed concentration fluorescence probe with the solution of the enzyme (2-fold dilutions). Fluorescence anisotropy was measured after incubation for 15 minutes and results were analyzed in GraphPad Prism software (version 5.04, GraphPad), as described in [45].

4.1.2. Time-resolved measurement of luminescence intensity

Measurements of luminescence intensity followed protocol similar to the one used in fluorescence anisotropy measurements. [45,46]. The assay was carried out in a 4-component buffer (pH = 7.5) consisting of 50 mM HEPES hemi-sodium salt (Sigma), 150 mM NaCl (Riedel-de Haën), 0.005% Tween® 20 (Sigma) and 5 mM dithiothreitol (Sigma) in the final volume of 20 μL on black 384-well polystyrene microplates with a nonbinding surface (Corning category number 3676 or 4514). The microplates and sample solutions were preincubated and measured at 30 °C.

Time gated luminescence intensity (TGLI) was registered on a PHERAstar plate reader (BMG Labtech), using an optical module [ex 337(300…360) nm, em 590(50) nm], a delay time of 50 μs, and a 150 μs integration time.

The luminescence decay curves were fitted to the equation:

$$I = (I_0 - I_{bg}) \cdot e^{-t/\tau} + I_{bg} \quad (1)$$

where I is the intensity of the luminescence signal measured at time t, I_0 is the intensity of the luminescence signal at $t = 0$, I_{bg} is the intensity of the signal of the background and τ is the luminescence lifetime.

4.2. Solid phase peptide synthesis

A traditional Fmoc solid phase peptide synthesis (Fmoc-SPPS) method was used for the synthesis of peptide fragments.[66] Fmoc-L-Asp(tBu)-Wang resin (loading 0.6 mmol/g), Fmoc-D-Asp(tBu)-Wang resin (loading 0.6 mmol/g) or Fmoc-L-Lys-(Boc)-Wang resin (loading 0.6 mmol/g) was used for the synthesis. In the beginning of the SPPS the resin was swollen for 30 minutes in N,N-dimethylformamide (DMF).

The coupling step started with the weighing of an amino acid (3 equiv. compared to resin) HBTU (2.85 equiv.), HOBt (2.85 equiv.) and N-methyl-morpholine (NMM, 10 equiv.). The amino acid and activators were dissolved, transferred to a 5 ml tube and mixed properly. Then the solution with activated amino acid was incubated for 3 minutes and then transferred to the resin. The reaction flask was put on a shaker for 60–90 minutes. After every acylation reaction, the resin was washed 5 times with DMF and the completeness of the reaction was controlled with a Kaiser test.

Solution A (5% ninhydrin in ethanol) and B (80% phenol in ethanol) were used for Kaiser test. On a few beads of resin 50 µl of solution A and B were added. The mixture was heated for 5–10 minutes. In case of an unsuccessful coupling the beads turned blue and the coupling reaction was repeated.

In case of a successful coupling, adding the next amino acid required removal of the Fmoc protecting group from N-terminal of the peptide. The removal was carried out using 20% piperidine solution in DMF for 3+17 minutes. After every step (coupling or cleavage of Fmoc) the resin was washed five times with DMF.

To measure the loading (number of activated groups) of the resin, Fmoc cleaving and washing solutions were collected. With a UV-Vis spectrometer, the spectrum of the solution was measured, and the Fmoc concentration was calculated using the Lambert-Beer law (λ=301 nm, ε=7100 l/mol*cm).

Coupling of the ATP-competitive fragment to the peptide followed procedure similar to adding an amino acid. To save on the reagents that are not commercially available, smaller equivalents were used. 1.2 equivalent of ATP-competitive fragment was mixed with HBTU (1 equiv.), HOBt (1 equiv) and NMM (5 equiv.) in DMF and added to the resin. The reaction mixture was put on the shaker for 90 minutes.

After the last coupling the resin was washed 5 times with DMF, 5 times with isopropyl alcohol and 5 times with 1,2-dichloroethane. Thereafter the resin was dried. The compound was cleaved from the resin by treatment with TFA/TIPS/H_2O (95:2.5:2.5, v:v:v, 3h) and purified by reverse phase HPLC.

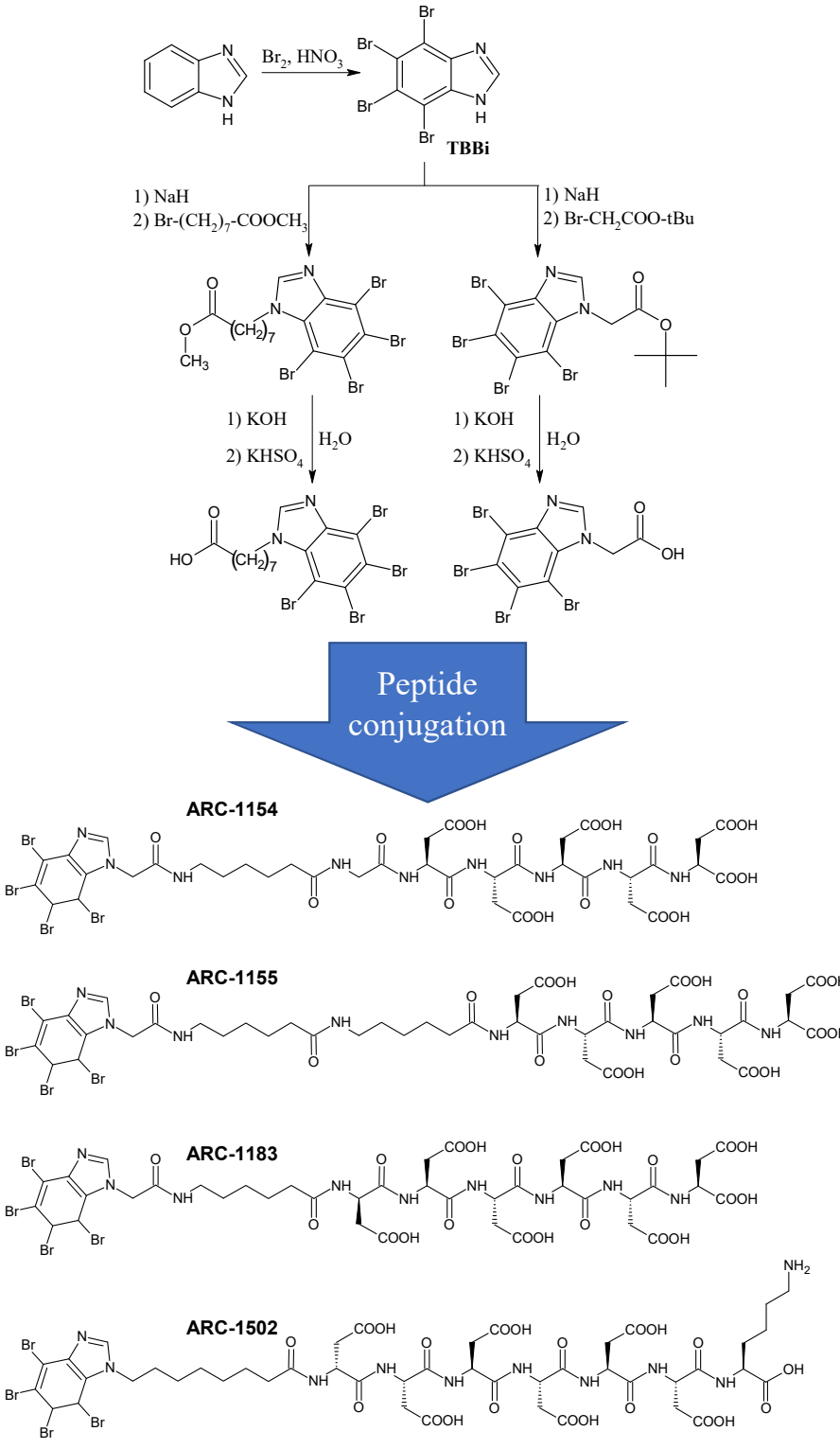
5. SUMMARY OF RESULTS AND DISCUSSIONS

5.1. Development and characterization of TBBi-based bisubstrate inhibitors (Paper I, Paper II)

Over the years, many different ATP-competitive inhibitors have been reported for CK2. In 2003 a paper about a new selective and potent inhibitor of CK2 TBBi (Scheme 2), was published.[36] TBBi is a simple scaffold and it has the NH group that could be easily alkylated to connect the linker structures. Using that knowledge TBBi was chosen to be used as a fragment to develop a bisubstrate inhibitor for CK2.

CK2 is an acidophilic protein kinase that prefers substrates that contain negatively charged amino acids (Asp, Glu) around the phosphorylation site.[39] For this reason, oligo-aspartic acid peptide was selected for a substrate mimetic part in the bisubstrate inhibitor.

In bisubstrate inhibitor approach, two inhibitory moieties are assembled with a linker. The lengths and the structures of the linkers play a crucial role in the design of the inhibitor. Well optimized linker enables simultaneous binding of both fragments with low conformational strain and steric repulsion. Correctly chosen linker may lead to significant increase of inhibition potency.[12,13]



Scheme 2. Synthesis of alkylated TBBi moieties and peptide conjugation of TBBi moieties.

5.1.1. Development of TBBI-based bisubstrate inhibitors (Paper I)

Firstly, benzimidazole was brominated to 4,5,6,7-tetrabromo-1*H*-benzimidasole (TBBi) [36] and the product was alkylated with an ester of bromoacetic acid. Hydrolysis of the ester gave TBBi-acetic acid (K68). Thereafter the fragment was coupled on the solid phase to oligo-aspartic acid peptide as a substrate mimetic (Scheme 2).

In case of TBBi-acetic acid additional amino acid-based linker was used between the TBBi and the oligo aspartic moiety. Three different products were synthesized by combining 6-aminohexanoic acid and glycine.

These first bisubstrate compounds had up to 190-fold higher inhibitory potency compared to the initial K68 (Tabel 2). Co-crystallization studies were carried up in the group of professor Karsten Niefind (Köln) to get a more detailed understanding of the binding of these inhibitors to CK2α. Crystallization results showed potential strain in the linker region and its amide function was considered to be too rigid. This strain does not allow the binding of the peptide fragment of the inhibitor with full potential to the protein substrate binding site. Since ARC-1183 had the lowest IC_{50} value (30 nM) in this series, it was taken as a starting point for further linker optimization. The amide group was removed and an octanoic acid linker was used to lose the rigidity and increase the flexibility of the linker, keeping the linker length similar to ARC-1183. TBBi was alkylated with 8-bromooctanoic acid methyl ester (Scheme 2). The ester group of the product was thereafter hydrolyzed and the corresponding carboxylic acid was coupled to the peptide. A lysin residue was added to the peptide sequence for the possibility of attachment of fluorescence dye. Cleavage of the compound from the resin resulted in the compound ARC-1502. The inhibitory potency of ARC-1502 (IC_{50} = 2.7 nM) was about 10-fold higher than that of ARC-1183 and over 1000-fold higher than the IC_{50} value of K68. This indicates that using a flexible linker in this bisubstrate inhibitor approach improves the simultaneous interaction of TBBi and the oligo-aspartate fragment with CK2α.

Table 2. Affinities of compounds towards CK2α. Modified table form **Paper I, Table 1**.

Compounds	**FA assay, K_D (SE), nM**
K68	1500 (100)
ARC-1154	22 (3)
ARC-1155	56 (7)
ARC-1183	12 (2)
ARC-1502	0.52 (0.06)
ARC-1504	0.39 (0.06)

5.1.2. Co-crystallization of ARC-1154 and CK2α

One of the initial interests to develop bisubstrate inhibitors for CK2 was based on the idea that these compounds could be useful tools for crystallization studies. There was no structural data on how substrate proteins and peptides interact with CK2α because these weak and flexible interactions did not give visible electron density in X-ray studies. Karten Niefind had the hypothesis that high affinity bisubstrate inhibitors may help solve these challenges. Crystal structures are also useful for rationalization of the design of inhibitors. Co-crystallization with ARC-1154 and CK2α resulted in a medium resolution crystal structure (PDB 4FBX). Study showed that as expected, the TBBi fragment of the inhibitor occupied the ATP binding site. Unfortunately, only the electronic density of ATP-competitive moiety and the beginning of the linker was visible and the peptidic fragment of the inhibitor was not defined by electron density.

Compared to the positioning of K68/ CK2α (PDB 3PVG) [68], the moiety of TBBi within ARC-1154 was turned more than 60°. K68 orientation allows the acetic acid carboxylate group to have an interaction with the Lys68, which is also noticed in case of other CK2α ATP-competitive inhibitors possessing a carboxylate group. [69,70] This is not possible in case of ARC-1154, because the carboxyl group is not present due to the formation of an amide bond in the linker. In case of ARC-1154, the TBBi moiety could be oriented such that the linker and the peptide part could be positioned toward the substrate binding site of CK2α. Even though it was not possible to determine the specific positioning of the peptidic fragment of ARC-1154, increase of the affinity shows that the peptide still interacts with the enzyme (Figure 5).

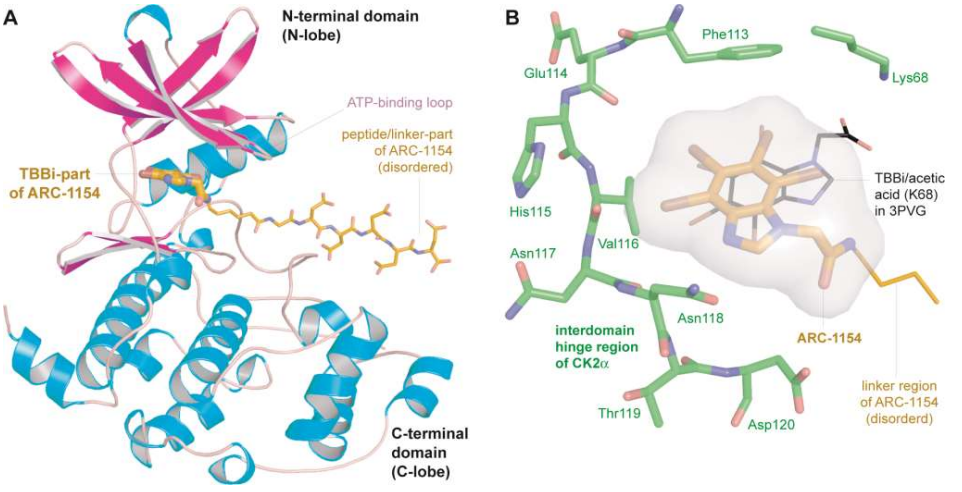


Figure 5. (A) Complex structure of the CK2α/ARC-1154 (PDB code: 4FBX). Only the TBBi-moiety was defined by electron density, the peptide fragment and the linker are disordered. (B) A magnified view of the ATP-site occupied by TBBi-moiety of ARC-1154. The structure of the conformation of the inhibitor K68 (TBBi-acetic acid) is added for the comparison (PDB code: 3PVG). (Full picture available in **Paper I**, **Figure 3**).

5.1.3. Selectivity of ARC-1502 (Paper I)

A goal of the bisubstrate approach was also to increase the inhibitory selectivity toward CK2. In case of ATP-competitive inhibitors binding to many off-targets are common because all PKs use ATP as its phosphoryl group source. It has been shown that using a bisubstrate inhibitor approach helps to increase the selectivity of the inhibitor.[42] The inhibition selectivity of ARC-1502 was tested with 140 different PKs at 1 μM concentration (with the radiometric filter-binding assay on commercial basis by International Centre for Kinase Profiling, University of Dundee) and only ten kinases from that panel showed more than 50% inhibition. (Table 3) Those PK-s mostly belonged to the protein kinase CMGC group. Compared to the selectivity of TBBi, the increase of selectivity toward CK2 was considerable. TBBi is an ATP-competitive inhibitor of CK2 and it had higher inhibition of PKD1,kinases of DYRK and PIM families.[43, 46] Based on the results of the panel, a Gini coefficient of 0.616 was calculated [72] for ARC-1502. This result is comparable to data obtained for inhibitors of CX-series.[73]

Table 3. 10 most inhibited protein kinases from the selectivity panel data of ARC-1502 (1 μM) and TBBi (10 μM, data from published selectivity panel [74]). Modified table form **Paper I, Table 2**. Full table in **Supplementary Data** of **Paper I**.

Protein kinase	Residual activity (%)[a]	
	ARC-1502, 1 μM	TBBi, 10 μM [74]
CK2	1±0	10
DYRK2	17±1	9
PLK1	18±3	29
CLK2	24±2	
ERK8	25±0	11
DYRK3	30±2	6
DYRK1A	36±3	3
HIPK2	36±1	3
GSK3β	46±7	45
CK1δ	49±5	

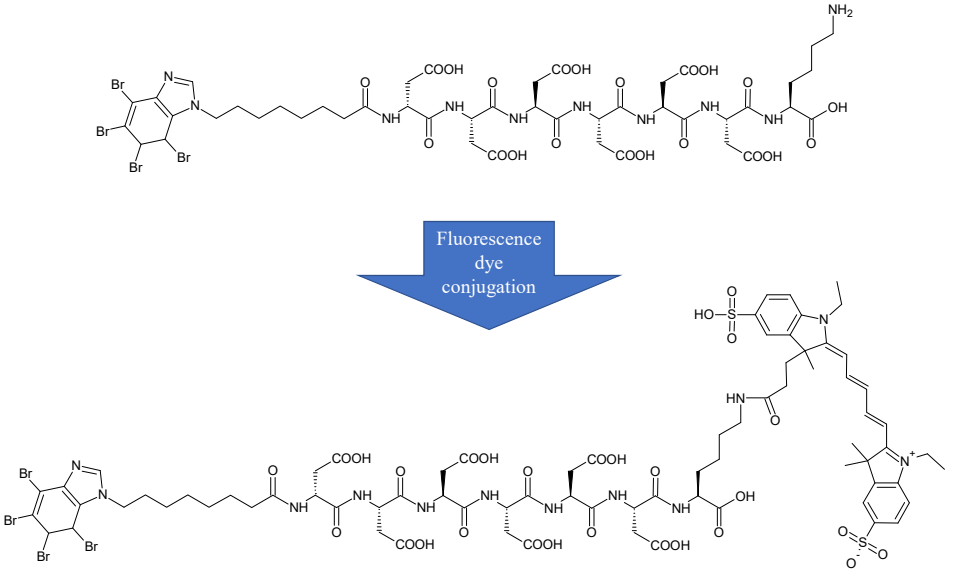
[a] Residual activities are expressed as a percentage of the control without an inhibitor (by means of duplicate determination).

5.1.4. Biochemical characterization CK2 inhibitors by using fluorescence anisotropy based binding assay (Paper I)

Labeling of the inhibitors with fluorescence dyes gives a great possibility to use that kind of compounds in biochemical and medicinal research. These inhibitors could be used for determination of active kinase concentration and screen inhibitors in assays and to study kinase localization in cells, tissues and living organism. These kind of fluorescence probes for PKs have described previously, for example for PKA.[45]

ARC-1502 was labelled with the fluorescence dye PromoFluor-647, yielding fluorescence probe ARC-1504 (Scheme 3). To our knowledge, this was the first fluorescence probe reported for CK2. The probe was characterized using fluorescence anisotropy (FA) readout and the K_D value of 0.4 nM was measured for the ARC-1504.

High affinity and fluorescence label gave the possibility to use ARC-1504 to measure active CK2α concentration in the solution and also characterize unlabeled inhibitors for CK2 by using displacement experiments (Figure 6)



Scheme 3. The conjugation of ARC-1502 with PromoFluor-647 yields with the fluorescence probe ARC-1504.

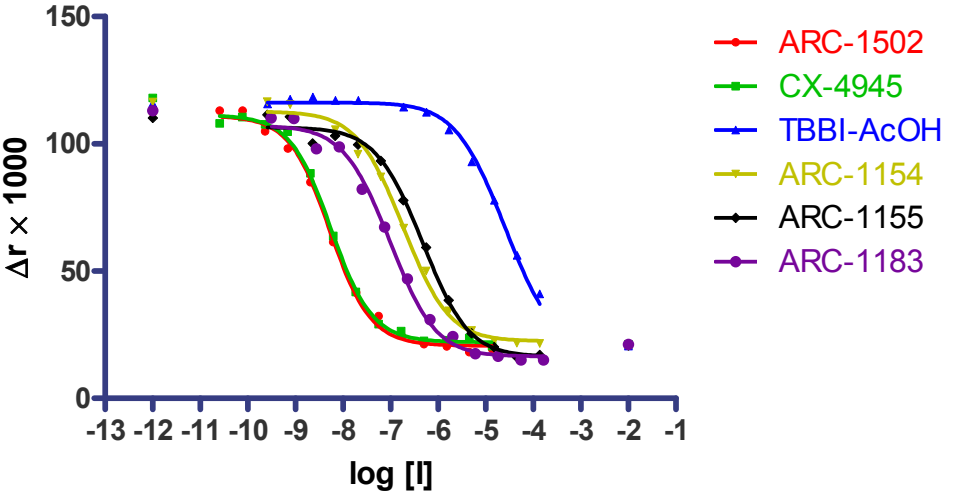


Figure 6. Displacement of ARC-1504 (3 nM) form the complex with CK2α$^{1–335}$ (2 nM). – Anisotropy changes are given relative to free ligand (3 nM).

5.1.5. Modification and optimization of the peptide fragment of the TBBi containing bisubstrate inhibitors (Paper II)

In the case of adenosine oligo-arginine conjugates as the inhibitors of basophilic PK-s it has been shown previously that chirality and the number of amino acids in the peptide fragment of the inhibitor influence the inhibitory potency. Affinity depends on the amount and chirality of arginine residues in the peptide.[44] Control of similar trend for inhibitors of CK2 was performed by varying the number and the chirality of aspartic acid residues.

Series of bisubstrate inhibitors containing TBBi moiety as an adenosine analogue, octanoic acid as a linker and different oligo-aspartic acid peptides were prepared.

Length of the peptide in the compounds varied from one to five aspartic acid residues. Two groups of inhibitors were synthesized differing by the L- or D-amino acids used in the peptide moiety.

The results of the experiments followed somewhat the same trend as previously reported for positively charged ARC-s. The amount of aspartic acids in the peptide fragment of the bisubstrate inhibitor affected the affinities of the compounds as with the increase of the length of the peptide the affinities also increased. (Table 4). Using different isomers of the amino acids (L or D) did not affect the inhibitory potency of the conjugates and CK2α seemed not to have a preference regarding the chirality of aspartic acid residues in inhibitors. Chirality of some positions in peptide has shown to be very important in the case of oligo-arginine containing bisubstrate inhibitors. Systematic variation of the structure gives information that could help to optimize the effective usage of aspartic acids in the peptide part and also gave the reason to use biologically more stable D-aspartic acids in the peptide moiety of the conjugate.

Table 4. Dissociation constants of the compounds varying length of and amino acid isomers used in the peptide moiety

Number of aspartic acids	K_D using D-aspartic acid (nM)	K_D using L-aspartic acid (nM)
1	63±5	45±5
2	21±4	25±7
3	5.6±0.1	7.2±2.5
4	1.8±0.5	1.9±0.6
5	0.7±0.4	1.2±0.1

5.2. Development and characterization of CX-4945 based bisubstrate inhibitors (Paper III and unpublished material)

High hydrophobicity of TBBi fragment in bisubstrate inhibitors complicates the HPLC purification of the compounds and increases the non-specific binding with cellular components.[75] In 2011, new and highly potent CK2 ATP-competitive inhibitor CX-4945 was reported [69], which came to be the first CK2 inhibitor to enter the clinical trials.[73] Because of its lower hydrophobicity and higher inhibitory potency, the key pharmacore of CX-4945, benzo[c][2,6]naphthyridine-8-carboxylic acid (Figure 7), was chosen to be used as an ATP-competitive fragment for new series of bisubstrate inhibitors of CK2.

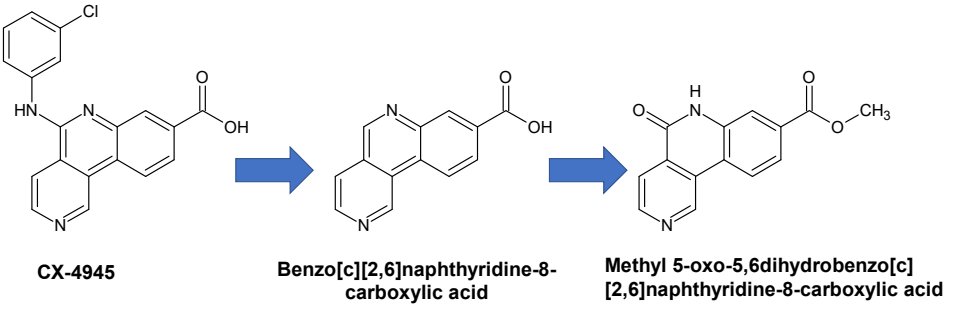


Figure 7. CX-4945, its key pharmacophoric fragment and corresponding precursor used for synthesis bisubstrate inhibitors of CK2

5.2.1. Construction and synthesis of CX-4945-based bisubstrate inhibitors (Paper III)

As mentioned earlier, beside the ATP-competitive moiety and the peptide fragment, usage of the optimized linker has a great importance because it allows both parts of the inhibitor to bind simultaneously to the active sites of the kinase. CX-4945 has a carboxylic group that seems to be the most convenient

way to attach the linker, but reportedly, the negative charge of this functional group gives important ionic interaction with CK2 and modification of the carboxylic acid group leads to great loss of inhibitory potency.[69] The tricyclic benzo[c][2,6]naphthyridine-8-carboxylate moiety is the core fragment of CX-4945, contributing most to the binding energy with CK2α.[73], The middle ring of this structure is directed outside of the ATP-binding pocket and could be used for attachment of structural elements without disturbing the binding of the molecule.[76]

Taking this information into account, the synthesis started from the preparation of methyl 5-oxo-5,6-dihydrobenzo[c][2,6]naphthyridine-8-carboxylate[69] that was further used as a fragment for bisubstrate inhibitor synthesis. The K_D value measured for 5-oxo-5,6-dihydrobenzo[c][2,6]naphthyridine-8-carboxylic acid was 8.9 nM. Even the affinity of the fragment towards CK2 was a magnitude lower than the one measured for CX-4945 (K_D = 0.56 nM, **Paper I**), the compound was still more affine than TBBI. Methyl 5-oxo-5,6-dihydrobenzo[c][2,6]naphthyridine-8-carboxylate was alkylated with esters of 7-bromoheptanoic acid or 9-bromononanoic acid. The alkylation of the fragment yielded in a mixture of 6N- and 5O-alkylated compounds (Figure 8, Scheme 4). The mixture of 6N- and 5O-alkylated compounds was then hydrolyzed and a mixture of compounds containing two carboxylic groups was yielded. Latter measurements showed that compounds with alkylation in position 5O showed higher affinities than the derivatives with alkylation in position 6N (Figure 10B). For that reason, the optimization of the alkylation was carried out using KOtBu, NaH, Li_2CO_3 as the base for the reaction (Table 5). The best reaction yields were achieved with KOtBu. Using KOtBu also gave the best isomeric ratio in favor of 5O-alkylated product that were the inhibitors with higher affinities towards CK2. Since it has been reported that fragments with an NH-group at the 5th position has better inhibitory potency [76], a respective compound was latter synthesized. Fragments were then coupled to the oligo-aspartic acid peptides using solid phase peptide synthesis. For some peptides lysine residue was also added for the attachment of a fluorescent dye (PromoFluor-555 or PromoFluor-647) (Scheme 5).

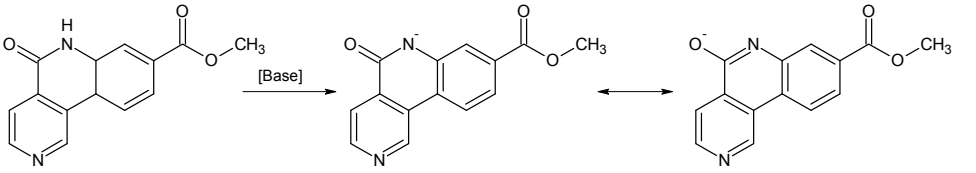
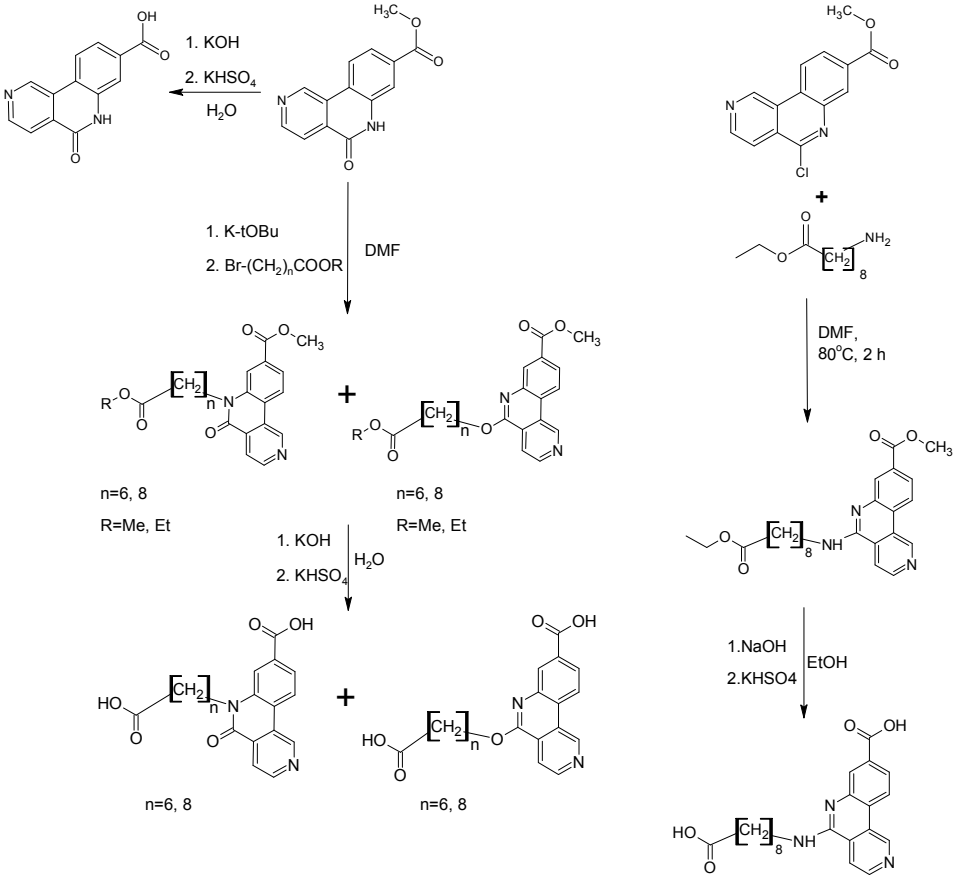


Figure 8. Resonance structures of anion from methyl 5-oxo-5,6-dihydrobenzo[c]-[2,6]naphthyridine-8-carboxylate that was used in alkylation reaction that gave two regioisomers.

After cleaving the products from solid phase, all isomers, including different acylation products, were isolated with HPLC. The different acylated products were identified by their biological activity towards CK2α, because the carboxylic group in the position 8 improves the affinity of the inhibitor and conjugated through this groups yields compounds with low affinity.[73] 5O- and 6N-alkylated isomers were distinguished by the differences in UV spectra (Figure 9).



Scheme 4. Synthesis of ATP-competitive fragments based on the pharmacore of CX-4945

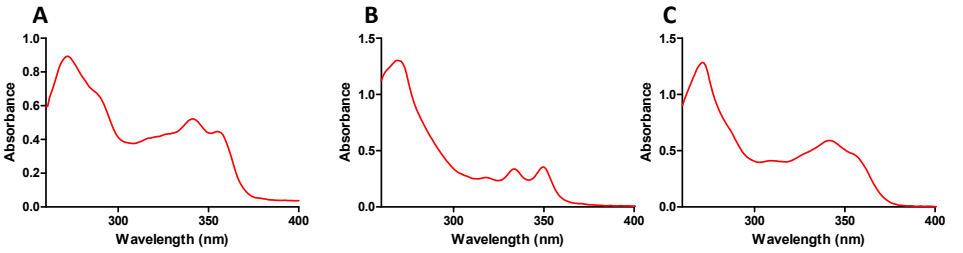
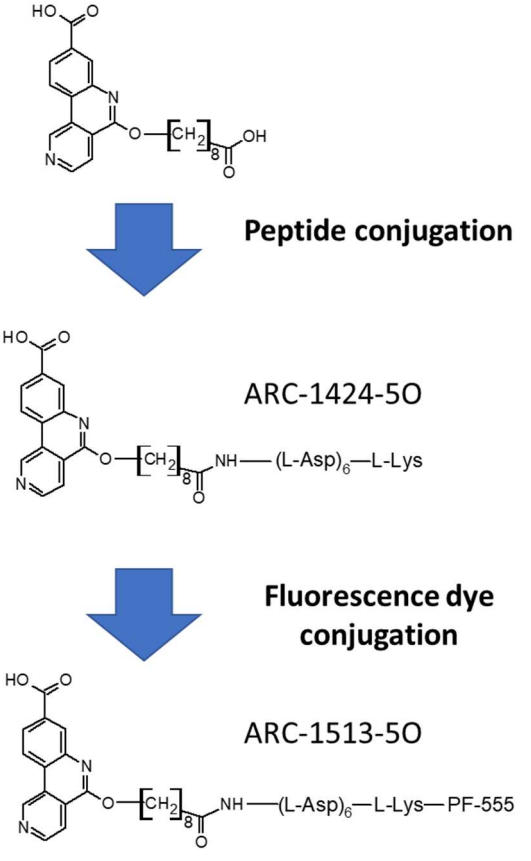


Figure 9. Comparison of the UV-spectra of 5-oxo-5,6-dihydrobenzo[c][2,6]-naphthyridine-8-carboxylate fragment, 5O-alkylated (B) and 6N-alkylated (C) fragments.



Scheme 5. Synthesis of ARC-1424-5O and ARC-1513-5O.

5.2.2. Biochemical characterization of synthesized compounds (Paper III)

The first series of characterized compounds all comprised $(L\text{-}Asp)_3$ moiety and their K_D-values were determined. The conjugates differed by the length of the linker, the position and atom which the linker is connected to the aromatic system. Displacement studies for compounds ARC-1199-5O/6N, ARC-1458-5N and ARC-1417-5O/6N resulted in K_D values in low nanomolar region (Table 6). In comparison of n-heptanoic acid and n-nonanoic acid, isomers with longer linker showed clearly higher affinity (Figure 10A). Compounds with alkylation in position 5 showed higher affinities than the derivatives with alkylation in position 6 (Figure 10B). Substitution of oxygen in the fifth position with nitrogen increased the affinity of the compound more than two-fold (ARC-1458-5N compared to its oxygen counterpart of ARC-1199-O5). Most importantly, all bisubstrate inhibitors had higher affinities, than the starting fragment 5-oxo-5,6-dihydrobenzo[c][2,6]naphthyridine-8-carboxylic acid and analogous TBBi-conjugates comprising $(L\text{-}Asp)_3$ fragment (K_D values ranging 5–9 nM (**Paper II**)). These results support the using of fragment from CX-4945 as the ATP-competitive moiety instead of TBBi.

Table 5. Optimization of conditions for O-alkylation of methyl 5-oxo-5,6-dihydro-benzo[c][2,6]naphthyridine-8-carboxylate

Reaction number	Base	Solvent	Temperature (ºC)	Ratio between N- and O-alkylation	Reaction depth*
1.	NaH	DMF	r.t.	O-alkylated isomer was not detected	19%
2.	NaH	DMF	50	1.7:1	24%
3.	NaH	DMSO	50	4.6:1	69%
4.	KOtBu	DMF	r.t.	0.8:1	75%
5.	KOtBu	DMF	50	1.9:1	51%
6.	KOtBu	DMSO	50	3.2:1	58%
7.	Li_2CO_3	DMF	r.t.	No product detected	–

* – calculated based on the peak areas of HPLC spectrum

For the next set of inhibitors, compounds ARC-1418-5O/6N were synthesized. L-Lys was added to the middle of the peptide fragment to have the chance to label the inhibitor. We have shown that increasing the number of aspartic acid residues in the peptide fragment also increases the affinity of the inhibitor (**Paper II**), although the positive charge of L-Lys may have a small decreasing effect on the affinity of the compound. The affinities measured for this set were about 4–5 times higher than the ones measured for the compounds comprising three aspartic acid residues in the peptide fragment (K_D(ARC-1418-5O) = 0.094 nM, K_D(ARC-1418-6N) = 0.71 nM) (Table 6) This indicates that the

L-lysine is well positioned and its positive charge does not affect the binding of the peptide fragment to the kinase. ARC-1418-5O was labelled with PromoFluor-647 yielding fluorescence probe ARC-1419-5O with the measured K_D value of 0.55 nM. The decrease of the affinity could be explained with the steric strain caused by high molecular weight fluorescence dye positioned close to the oligo-aspartic acid peptide fragment that interacts with the CK2.

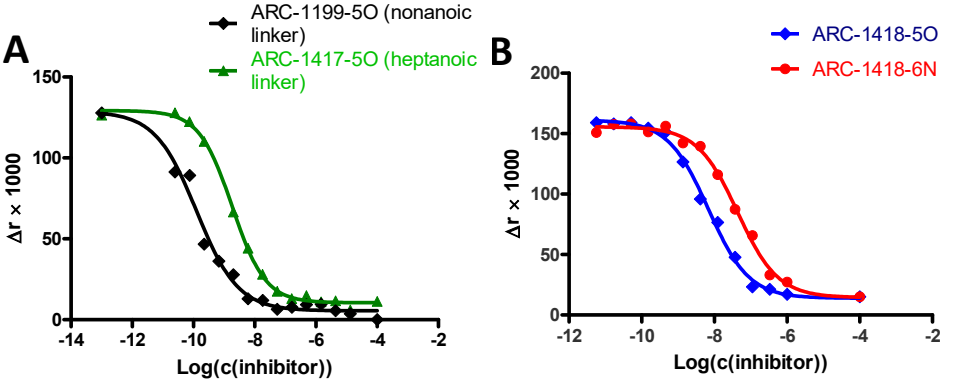


Figure 10. Comparison of inhibitors with different linker length (A) and N- or O-alkylated inhibitors (B).

Table 6. Structures and affinities of benzo[c][2,6]naphthyridine-8-carboxylic acid scaffold inhibitors towards CK2α

Inhibitor code	Inhibitor structure	K_D (nM)	pK_D (SD)
	R_1 = H	8.9	8.05 (0.25)
ARC-1199-5O	R_2 = -$(CH_2)_8$CO-(L-Asp)$_3$	0.42	9.38 (0.30)
ARC-1199-6N	R_1 = -$(CH_2)_8$CO-(L-Asp)$_3$	2.38	8.62 (0.21)
ARC-1417-5O	R_2 = -$(CH_2)_6$CO-(L-Asp)$_3$	1.57	8.80 (0.18)
ARC-1417-6N	R_1 = -$(CH_2)_6$CO-(L-Asp)$_3$	5.89	8.23 (0.17)
ARC-1458-5N	R_3 = -$(CH_2)_8$CO-(L-Asp)$_3$	0.17	9.79 (0.16)
ARC-1418-5O	R_2 = -$(CH_2)_8$CO-L-Lys-(L-Asp)$_4$	0.094	10.03 (0.18)
ARC-1418-6N	R_1 = -$(CH_2)_8$CO-L-Lys-(L-Asp)$_4$	0.71	9.15 (0.17)
ARC-1419-5O	R_2 = -$(CH_2)_8$CO-L-Lys(PF647)-(L-Asp)$_4$	0.55	9.26 (0.17)
ARC-1419-6N	R_1 = -$(CH_2)_8$CO-L-Lys(PF647)-(L-Asp)$_4$	2.403	8.62 (0.21)
ARC-1424-5O	R_2 = -$(CH_2)_8$CO-(L-Asp)$_6$-L-Lys	0.037	10.50 (0.29)
ARC-1513-5O	R_2 = -$(CH_2)_8$CO-(L-Asp)$_6$-L-Lys(PF555)	0.016	10.89 (0.34)

The final compounds of this series were inhibitors comprised of six L-Asp residues and n-nonanoic acid as the linker that were prepared to achieve the highest inhibitory potency towards CK2. L-lysine was added to the C-terminus of the peptide to conjugate the compound with a fluorescence dye. Synthesis resulted in ARC-1424-5O that possessed the K_D value of 37 pM. Conjugation of the inhibitor with fluorescence label yielded in ARC-1513-5O with the measured K_D value of 16 pM. This kind of fluorescence probe with such high affinity could be useful characterizing non-labelled CK2 inhibitors possessing subnanomolar potency [77], which are not measurable with other methods because of the "tight-binding" effect [78]. The affinity range of the inhibitors that could be reliably analyzed with the fluorescence polarization assays is defined by the affinity of the probe.[77]

Ligands with such high affinity might have very slow dissociation kinetics when bound to the protein. It is important to take the effect of the slow kinetics into account when using high affinity inhibitors in assays. Experiment to monitor the dissociation kinetics of the ARC-1513-5O/CK2α was performed by measuring the time dependence of fluorescence anisotropy decay after addition of large excess of a displacer (Figure 11). The complex dissociation half-life of 297 ± 36 s at 30°C was obtained. Therefore, using of ARC-1513-5O in a binding or displacement assays requires at least 25 minutes long (5 dissociation half-lives) incubation time before measurement, in order to establish equilibrium. In 2017, a paper was published by our research group which shows that apparent dissociation rate of bisubstrate inhibitors are highly dependent on concentration and nature of displacer that is used [79]. It means that the dissociation rate presented for ARC-1513-5O could be somewhat different, if other ligand or concentration is used.

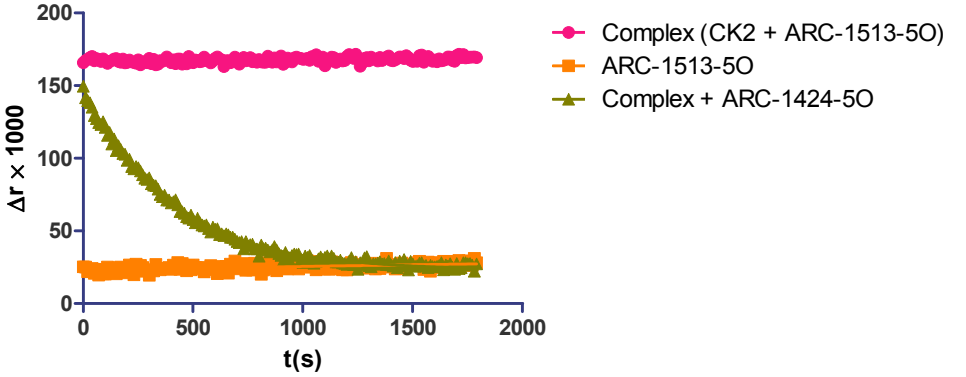


Figure 11. Dissociation kinetics measurement of ARC-1513-5O (3 nM) in complex with CK2 (6 nM), displaced with ARC-1424-5O (3 μM).

An assay using labeled compounds (ARC-1504 and ARC-1419-5O) was carried out to see the extent of non-specific binding of the probes. Titration with BSA was used to estimate the non-specific binding of the probes. The result was that

ARC-1504 had much higher affinity towards the protein compared to ARC-1419-5O (Figure 12). This results shows that, in addition to higher affinity, inhibitors containing benzo[c][2,6]naphthyridine-8-carboxylic acid moiety have advantage over the TBBi-based conjugates for studies in biological systems because of lesser hydrophobicity driven non-specific binding.

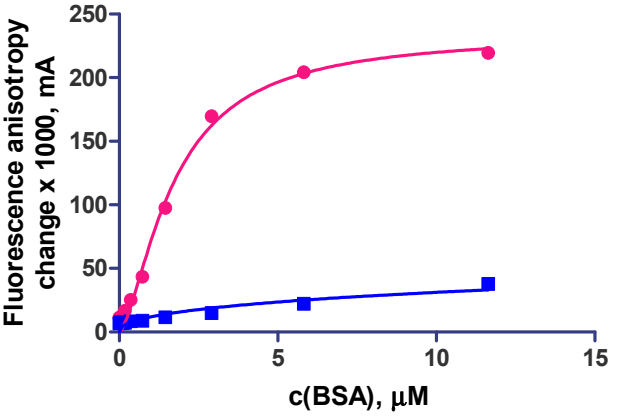


Figure 12. Binding ARC-1504 (purple) and ARC-1419-5O (blue) to BSA.

5.2.3. CK2 selectivity of ARC-1424-5O (Paper III)

The target selectivity of ARC-1424-5O at 1 µM concentration was tested in a panel of 140 protein kinases (Table 7). The panel was conducted to obtain info about the off-targets of the compound and to compare it with CX-4945 and our previous bisubstrate inhibitors. The data shows high CK2 selectivity of ARC-1424-5O, as the enzyme had the highest extent of inhibition compared with other PKs in the panel. Besides of CK2, also the kinases of the DYRK and HIP families showed high inhibition by ARC-1424-5O. From the panel of 140 protein kinases, 23 enzymes were inhibited more than 50% and 6 of those PKs had more than 90% inhibition (including CK2). The selectivity profiles of ARC-1424-5O and CX-4945 were generally similar [73], because of their structural similarities, but CLK2 (a known off-target of CX-4945, possessing even lower IC_{50} value compared to CK2 [80]) was only moderately inhibited by ARC-1424-5O. Taking these results into consideration, we believe that ARC-1424-5O, having more than 10 fold higher affinity towards CK2, is more selective inhibitor for CK2 than CX-4945. It is important to consider that apparent selectivity of the compounds in one concentration point test in panels depends on the used concentration and lower used concentrations leads to higher apparent selectivity. This is because of inhibition of the off targets are lower at lower concentrations while the main target may have already full inhibition.

Table 7. Selectivity panel for ARC-1424-5O. The final total concentration of the inhibitor used in the panel was 1 μM. Modified table form **Paper III, Table 2**

Protein kinase	Residual activity %	SD	Protein kinase	Residual activity %	SD
CK2	**1**	0	PLK1	**23**	0
DYRK3	**4**	0	GSK3b	**26**	3
HIPK2	**5**	1	S6K1	**31**	3
DYRK2	**5**	1	TBK1	**34**	0
HIPK3	**8**	1	ERK5	**39**	3
DYRK1A	**9**	3	Lck	**39**	13
ERK8	**10**	1	RIPK2	**39**	7
AMPK (hum)	**11**	0	TIE2	**41**	0
IKKe	**13**	0	MLK1	**42**	2
GCK	**15**	0	EIF2AK3	**47**	3
CLK2	**19**	1	TTK	**48**	3
MLK3	**22**	3			

5.2.4. Leu-scan of ARC-1424-5O

Determination of the contribution of each amino acid residues in peptide could lead into better understanding of the binding of the peptide. For that reason, leucine scanning was performed by substituting aspartic acid residues one-by-one in sequence with a leucine. Similar approach (for example with alanine) is a widely used method to study peptide interactions.[81–83] ARC-1424-5O was taken to the starting point to determine the importance of every position in $(L\text{-}Asp)_6$ peptide fragment. Leucine was chosen after analyzing abundancy of different amino acids near the phosphorylation site of substrate, using data from previously published list of CK2 substrates.[84] Knowing of which of the aspartic acid residues contributing most to the binding with CK2 could give possibility to reduce the number of negative amino acid residues in the peptide and to make high affinity compounds with reduced molecular weight and smaller overall negative charge. By substituting one-by-one every aspartic acid residue in the peptide fragment with leucine, new group on inhibitors were synthesized and their affinities towards CK2 were determined. (Table 8)

Table 8. Affinities of the inhibitors from leucine scan and ARC-1424-5O as comparison

Inhibitor code	Structure	K_D (SD), nM
ARC-1424-5O	CX-$(CH_2)_8$-CO-(L-Asp)$_6$-L-Lys	0.037 (**Paper III**)
ARC-1520-5O	CX-$(CH_2)_8$-CO-(L-Asp)$_5$-L-Leu-L-Lys	0.083 (0.028)
ARC-1521-5O	CX-$(CH_2)_8$-CO-(L-Asp)$_4$-L-Leu-L-Asp-L-Lys	0.11 (0.02)
ARC-1522-5O	CX-$(CH_2)_8$-CO-(L-Asp)$_3$-L-Leu-(L-Asp)$_2$-L-Lys	0.15 (0.03)
ARC-1523-5O	CX-$(CH_2)_8$-CO-(L-Asp)$_2$-L-Leu-(L-Asp)$_3$-L-Lys	0.18 (0.041)
ARC-1524-5O	CX-$(CH_2)_8$-CO-L-Asp-L-Leu-(L-Asp)$_4$-L-Lys	0.37 (0.11)
ARC-1525-5O	CX-$(CH_2)_8$-CO-L-Leu-(L-Asp)$_5$-L-Lys	0.24 (0.14)

Comparing of the affinities of the compounds from leucine scan shows that first two aspartic acid residues of the peptide contribute most to the binding with CK2α. Substituting those positions with leucine, the K_D values had the highest increase, up to 10-fold. Aspartic acids in the next four positions seemed to have lesser contribution to binding, but still in all cases the affinities of the inhibitors decreased. It might be concluded that in the peptide fragment of ARC-1424-5O, the negatively charged amino acids are more essential in the area closer to the ATP binding site.

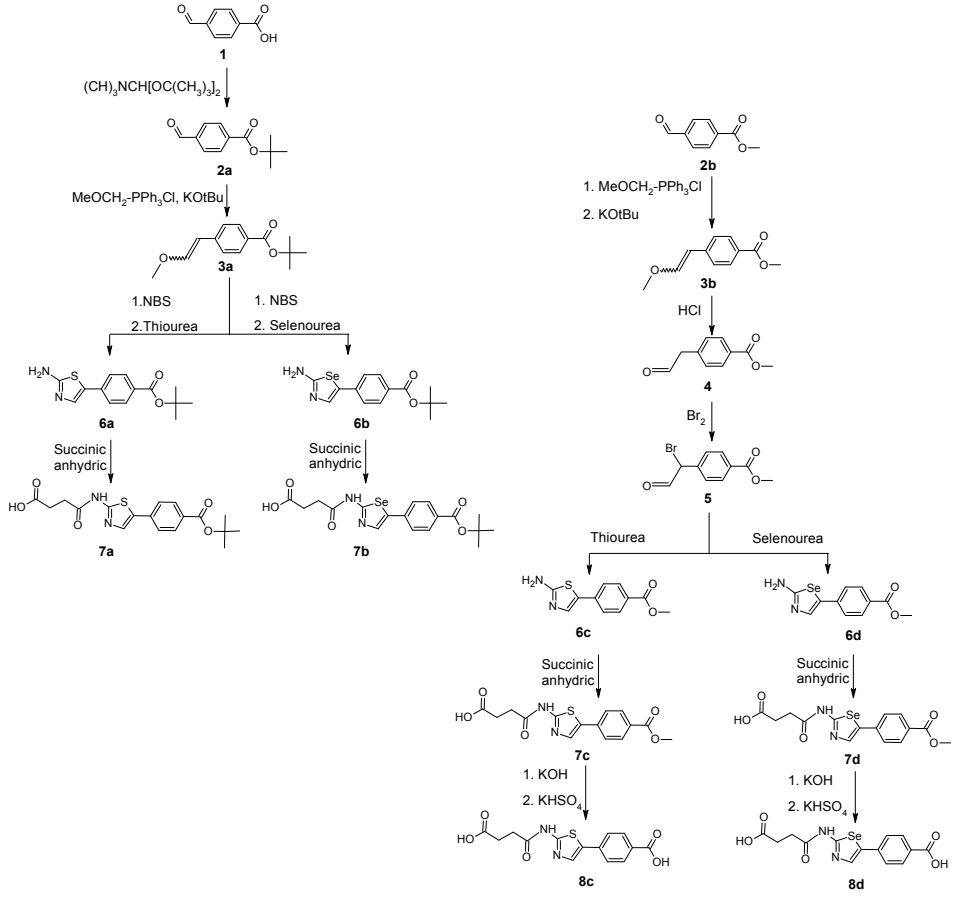
5.3. Development and characterization of bisubstrate inhibitors with long-lifetime luminescence properties (Paper IV)

Our work group has previously shown that protein kinase inhibitors with sulfur or selenium atoms in aromatic structures possess room temperature long-lifetime luminescence properties in complex with an enzyme.[47,49,51] Taking this knowledge into account, previously reported CK2 inhibitor 4-(2-amino-1,3-thiazol-5-yl)benzoic acid (ATB)[85] and its selenium-containing counterpart 4-(2-amino-1,3-selenazol-5-yl)benzoic acid (ASB) were chosen to construct new series of bisubstrate inhibitors for CK2. As presumed, both compounds possessed room temperature phosphorescence in CK2 complex.

5.3.1. Synthesis of bisubstrate inhibitors with long-lifetime luminescence properties

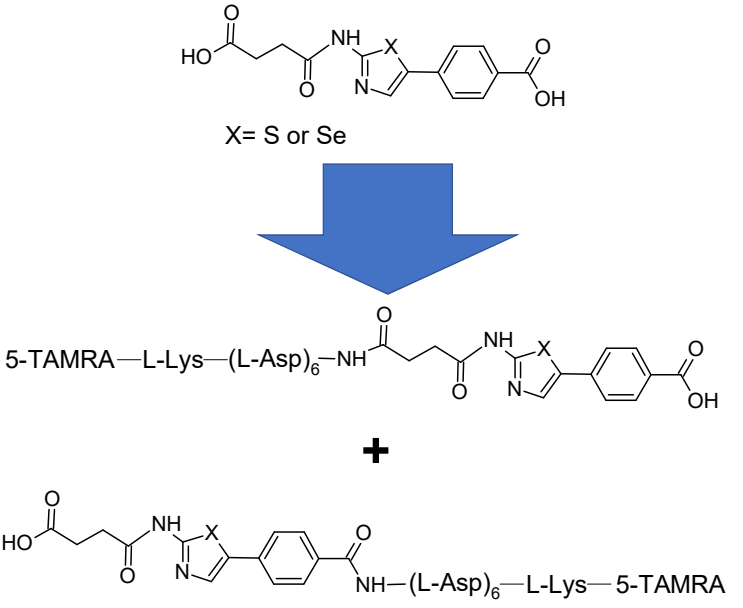
Two different synthesis routes were used for the synthesis of ATB and ASB. In the first case a previously described method was used to prepare the ATB scaffold.[85] Methyl 4-formyl benzoate (compound **2b**, Scheme 6) was added to a mixture of potassium *tert*-butoxide and (methoxymethyl)triphenylphosphonium chloride, yielding in corresponding enol ether (**3b**). Treatment of enol ether with

HCl resulted in aldehyde **4** that was thereafter brominated with bromine to give compound **5**. Reaction of compound **5** with thiourea or selenourea produced methyl 4-(2-amino-1,3-thiazol-5-yl)benzoate or methyl 4-(2-amino-1,3-selenazol-5-yl)benzoate, respectively. Both compounds were thereafter reacted with succinic anhydride to result in compounds **7c** and **7d**, which were then hydrolyzed to ATB-Suc and ASB-Suc. In case of described synthesis route, reaction with thiourea resulted with moderate yields and reaction with selenourea resulted in really poor or sometimes even not detectable yield of the product. In this route the precursors to be connected to peptide have two carboxylic acids functional groups that results in two isomers of peptide conjugates, but only one of them has the desired structure (Scheme 7). Also, there exists the possibility of cross linking reactions between a dicarboxylic acid and two peptide chains on the resin. These side reactions resulted in low yields of the more active compound and complicated the chromatographic separation.



Scheme 6. Synthesis routes for ATB and ASB synthesis.

The second improved synthesis route started with synthesis of *tert*-butoxy ester of 4-formyl benzoic acid by using *N*,*N*-dimethylformamide di-*tert*-butyl acetal(compound 1, Scheme 6).[86] The next step was similar to the first route and resulted in analogical enol ether. The enol ether was brominated by using N-bromosuccinimide and then directly reacted with thiourea or selenourea in one pot, yielding *tert*-butyl 4-(2-amino-1,3-thiazol-5-yl)benzoate or *tert*-butyl 4-(2-amino-1,3-selenazol-5-yl)benzoate in good yields.[87] Similarly to the first route, both compounds were reacted with succinic anhydride resulting in *t*Bu-ATB-Suc and *t*Bu-ASB-Suc. Coupling of a *tert*-butyl group containing pre-cursors to peptides resulted in only single products. *t*-Butyl esters were hydrolyzed together with the removing the peptides from the resin during the TFA treatment. This procedure yielded only the desired isomers of the inhibitors in higher yield and simplified chromatographic separation of the final products.



Scheme 7. Favored (top structure) and unfavored (bottom structure) acylation products of ATB/ASB fragments.

Both compounds were coupled to a peptide that contained amino hexanoic acid linker and $(L\text{-}Asp)_6$ moiety. Lysine residue was also included to the peptide fragment for the latter attachment of the fluorescence dye 5-TAMRA. Synthesis yielded in ARC-1527 and ARC-1529. Both contained Ahx-$(L\text{-}Asp)_6$ -L-Lys peptide and differed from the heavy atom (S or Se) in the ATP-competitive fragment. Labelling both compounds with 5-TAMRA resulted in compounds ARC-1528 and ARC-1530.

5.3.2. Biochemical characterization of ATB and ASB containing bisubstrate inhibitors

Both compounds without fluorescence label (ARC-1527, ARC-1529) showed similar, subnanomolar, K_D values. (Table 9) ARC-1527 possessed significantly higher affinity towards CK2 than its fragments ATB and ATB-Suc. Labelled compounds gave similar dissociation constant values as their non-labelled counterparts.

Long-lifetime luminescence properties of all these four compounds in complex with CK2α were measured and the data is presented in Table 9. Labeled compound showed shorter luminescence lifetimes and higher intensities in complex with CK2 than their unlabeled counterparts. ARC-1527 and ARC-1529 (Figure 13A) possessed luminescence lifetimes of 82 μs and 55 μs, respectively and labeled probes ARC-1528 and ARC-1530 (Figure 13B) showed luminescence lifetimes of 62 μs and 36 μs, respectively. (Table 9) This effect could be explained by the effective RET (resonance energy transfer) between the long-lifetime possessing moiety and the fluorescence label. Even though compounds containing selenium possessed stronger luminescence intensity, the efficiency of inter-chromophore triplet-singlet RET were not much different (24% for ARC-1528 and 29% for ARC-1530). The efficiency of RET was calculated using equation:

$$E = 1 - \tau(DA)/\ \tau(D)$$

where E is the triplet-singlet RET efficiency; τ(D) the phosphorescence lifetime of donor molecule without the acceptor; τ(DA) the luminescence lifetime of the molecule, when both, donor and acceptor, are present.

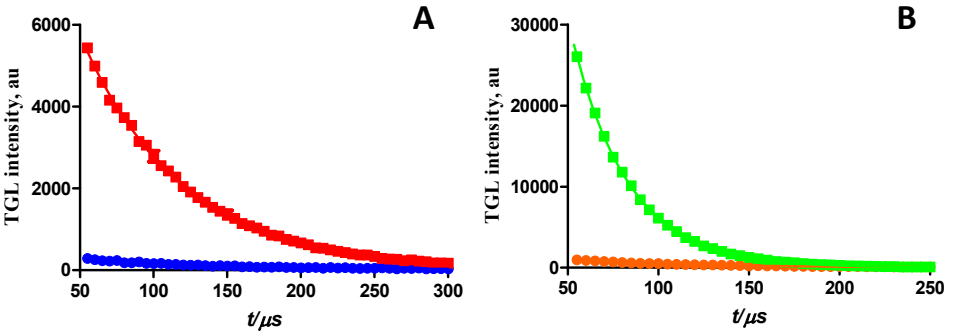


Figure 13. Decay of the intensity of long-lifetime luminescence signal of ARC-Lum(-) probes ARC-1527 (100 nM, blue) and ARC-1528 (100 nM, red) in complex with 100 nM CK2α (A); ARC-Lum(Fluo) probes ARC-1529 (10 nM, orange) and ARC-1530 (10 nM, green) in complex with 100 nM CK2α (B)

Table 9. Affinities and luminescence signal properties of compounds (Modified table from **Paper IV**)

Compound		CK2 affinity K_d/nM	Luminescence lifetime τ/µs of ARC-CK2α complex	I_0
Code	**Strucure**			
ATB	–	48 ± 7	n.d.	n.d.
ATB-Suc	–	11 ± 2	n.d.	n.d.
ARC-1527	ATB-Suc-Ahx-(L-Asp)$_6$	0.58 ± 0.14	82 ± 2	1 ± 0.12
ARC-1528	ATB-Suc-Ahx-(L-Asp)$_6$-L-Lys(5-TAMRA)	0.40 ± 0.12	62 ± 2	76 ± 7.7
ARC-1529	ASB-Suc-Ahx-(L-Asp)$_6$	0.34 ± 0.12	55 ± 2	46 ± 9.2
ARC-1530	ASB-Suc-Ahx-(L-Asp)$_6$-L-Lys(5-TAMRA)	0.85 ± 0.15	36 ± 2	5300 ± 370

Compounds with fluorescence labels also possessed higher I_0 values (Figure 14) than their non-labelled counterparts, which could be explained by the fact that in case of non-labelled compounds the phosphorescence emission is measured that has very slow emission rate and could not efficiently compete with non-radiative pathways. In the case of labelled compounds much faster RET is taking place competing successfully with non-radiative processes. The results are slightly shorter lifetime of the emission, but much higher initial intensities and therefore also increased overall intensities. I_0 and τ values are both important characteristics for probes with long-lifetime luminescence properties. Higher I_0 values often leads greater sensitivity and allows to measure lower concentrations whereas higher τ gives the possibility to use longer delay time, which increases the signal to noise ratio by decreasing the background.

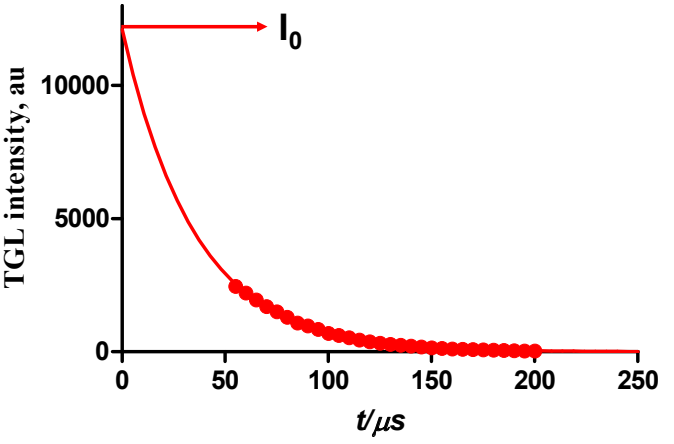


Figure 14. Decay of the complex between ARC-Lum probe and CK2α. I_0 is the maximal intensity from extrapolation of decay curve.

Similar RET efficiencies of thiazole and selenazole could be explained with spectral overlapping of phosphorescence emission of donor and fluorescence dye absorption. The emissions spectrum of thiazole has better overlapping with 5-TAMRA absorption spectrum compared to selenazole spectrum (Figure 15). Therefor the RET efficiency in case of compounds containing thiazole is not much lower than the compounds containing selenazole that has notably higher phosphorescence. Comparing this new set of compounds with long-lifetime luminescence probes described previously selenadiazole based probes for CK2 [49], new compounds possess about 50 times higher affinity towards CK2 and ARC-1530 also has more than magnitude stronger signal intensity of long-lifetime luminescence than compounds described before. This gives the opportunity to use new probes in wide range of application in biomedical studies.

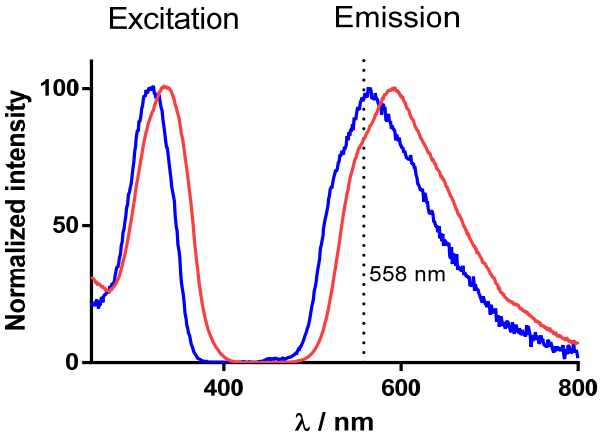


Figure 15. Normalized excitation and emission spectra of room temperature phosphorescence of ARC-1527 (blue) and ARC-1529 (red) in solid PVA matrix. 558 nm marks the absorption maximum of 5-TAMRA.

Long lifetime photoluminescence is very rare in solutions at room temperature and the time delayed detection of it allows sensitive and specific measurements. TGL makes possible to construct number valuable assays by using probes with long lifetime properties. Due to the high long-lifetime luminescence signal intensity of ARC-1530-CK2 complex, it allows the measurement of CK2 in complicated samples by using time delay between excitation and detection. Usage of the time delay eliminates the background signal from fluorescence and light scattering. Probe ARC-1530 was used to measure CK2 in cell lysates. Two sets of lysates were used: native NIH-3T3 cells and NIH-3T3 cells with overexpressed CK2. (Figure 16) Solutions of ARC-1530 were titrated by using of different concentration of lysates and the concentration of the probe was kept fixed. In both cases, increase of the lysate concentration also increased the luminescence signal intensity. In case of cell line with over-expressed CK2, the measured signal was much stronger and saturated at higher lysate concentrations,

but in the native cell line the signal was linearly dependent on lysate content through the entire range of measurement. Initial slope of the graph is estimated to be proportional with the expression level of the CK2 and point where the upper plateau begins contains equal amount of the probe and CK2. To estimate the possible effect of nonspecific binding of the probe, CX-4945 was used for displacement, which brought the signals down to base levels, indicating that the effect of nonspecific binding is insignificant, but since CX-4945 is not the most selective inhibitor, we cannot say that the total signal comes from binding only CK2.

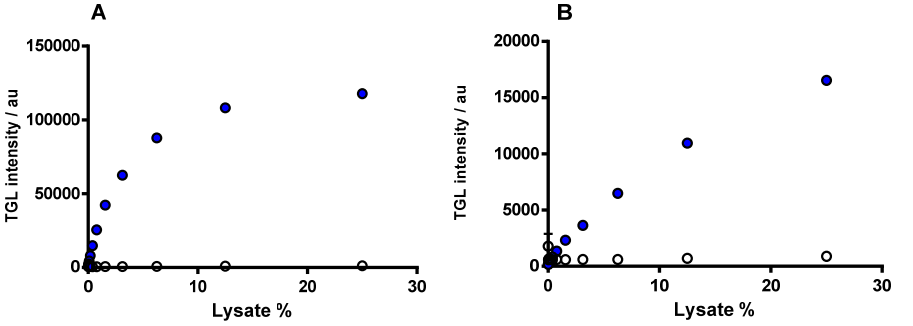
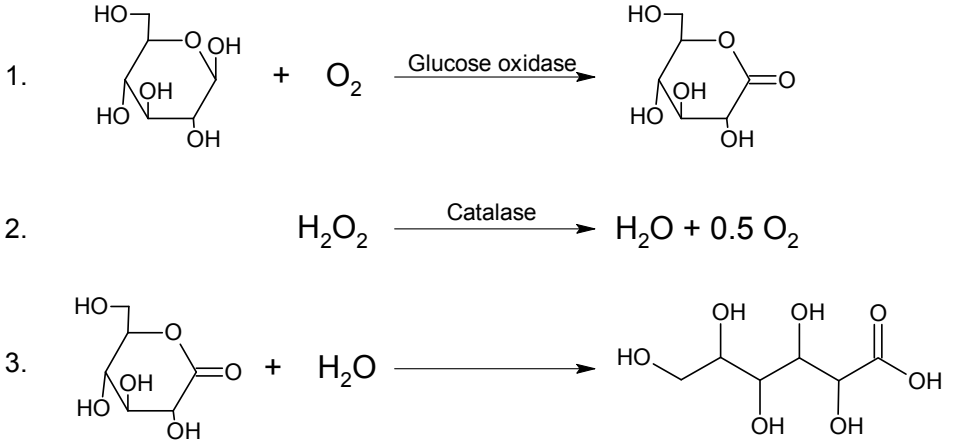


Figure 16. Luminescence intensity of the probe ARC-1530 (10 nM) upon binding to CK2 in diluted cell lysates before (filled circles) and after (open circles) treatment with 10 μM CX-4945. Lysates of NIH-3T3 cells with overexpressed RFP-tagged CK2 (A) and native NIH-3T3 cells (B) were studied.

5.3.3. Deoxygenation effect on properties of long lifetime luminescence

Molecular oxygen is a very efficient quencher of the triplet exited state and therefore phosphorescence signals are strongly dependent on the present concentration of oxygen. In previous reports has been shown, that dissolved oxygen plays a crucial role in quenching long-lifetime luminescence signals of ARC-Lum probes. A method using glucose oxidase (GO), catalase and glucose was used to deoxygenate the samples.[53] This method is based on two reactions: firstly GO catalyzes the oxidation of glucose by dissolved molecular oxygen; secondly catalase removes the hydrogen peroxide from the solution that is produced during the oxidation of glucose (Scheme 8).[53,88] Deoxygenation of the samples lead to the increase of luminescence lifetime of the ARC-1528-CK2 complex form 82 μs to 165 μs and in case of ARC-1530-CK2 complex, the increase of lifetime was from 36 μs to 58 μs. In case of PKA probes, the reported changes of luminescence lifetimes of probes in kinase complex after deoxygenation were even larger. This could indicate that incase of CK2 the other quenching mechanisms beside the effect of oxygen are also important.



Scheme 8. The overall scheme of deoxygenation using GO/catalase method. [88,89]

5.3.4. Selectivity of ARC-1527

A selectivity panel was run with 100 nM ARC-1527 for 50 protein kinases. Data of this panel showed high selectivity of ARC-1527 towards CK2α, which was inhibited by the highest extent with residual activity of 1.9%. (Table 10) In this panel, three other kinases were inhibited more than 50%. These include DYRK1a, GSK3β and HIPK2. These results were somewhat expected, as they are known off-targets of CK2 inhibitors (**Paber III**). These results can indicate that ARC-1530, which has similar structure and affinity compared to ARC-1527, but much higher long-lifetime luminescence intensity, could be used for rather selective detection of CK2 concentration in biological solutions.

Table 10. Residual activities (%) of PKs inhibited more than 50% by ARC-1527 (100 nM) (Modified table form full table of **Paper IV, Table 2**)

PK	Residual activity %	SD (N = 2)
CK2α	1.9	0.5
DYRK1a	11.5	0.6
GSK3ß	19.0	0.7
HIPK2	23	8
TAK1	62	17
SmMLCK	68	29
AMPK (hum)	69	2
SRPK1	69	1
MLK3	69	4

6. CONSTRUCTION OF CELL-PENETRATING BISUBSTRATE INHIBITORS OF CK2

Since highly negatively charged compounds do not penetrate cellular membrane, an approach to make the inhibitors cell plasma membrane permeable was needed. In our research group a prodrug approach was developed to hide negative charges of the peptide part of compounds with conjugating them to acetoxymethyl esters. (**Paper II**) This method allows the compounds to penetrate the cell plasma membrane, after what they are cleaved by the esterases form the carboxylic acid groups.

First scaffold of cell-penetrating bisubstrate inhibitors were developed using the backbone of TBBi-based compounds. Derivatization of oligo-aspartate peptide to acetoxymethyl esters was complicated by the efficient side reaction that lead to formation of cyclic imides. That side reaction is not possible with achiral peptoids that were used as the substrate mimetic in this approach (Figure 17).

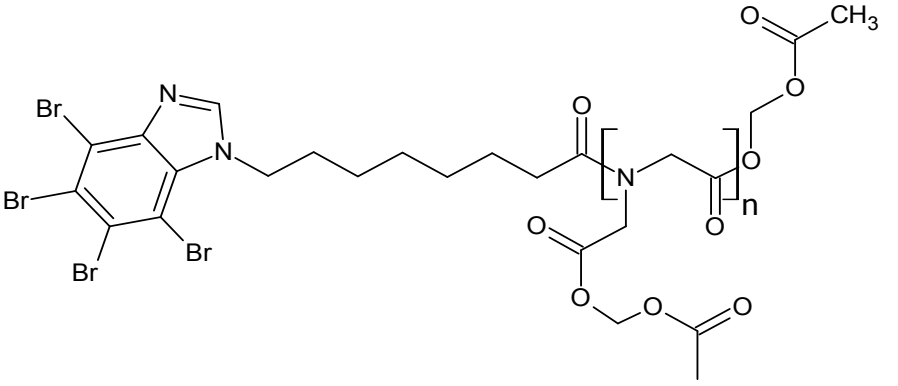


Figure 17. TBBi-peptoid conjugated to acetoxymethyl esters.

The uptake of the conjugated compounds was visualized by monitoring two CK2-related phosphosites in live cells. The reduction of the phosphorylation of Cdc37 pSer13 and NFκB pSer529 was demonstrated by Western blot analysis (Figure 18).

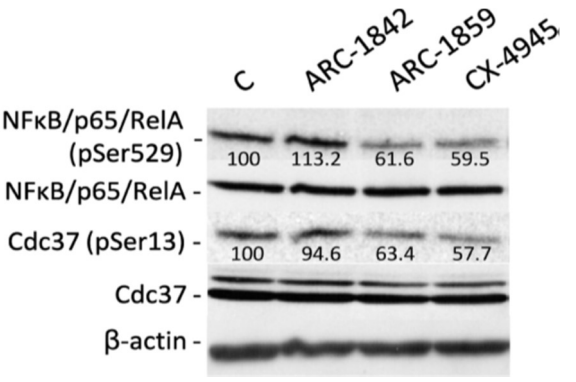


Figure 18. Western blot analysis of whole lysates from MIA PaCa-2 cells. Cells were incubated with ARC-1842 (compound without acetoxymethyl esters), ARC-1859 (prodrug of ARC-1842, conjugated with acetoxymethyl esters) and CX-4945 at the final concentration of μ10 M for 5 h. The phosphorylation level of two known CK2-regulated phosphosites, Cdc37 at Ser13 and NFκB at Ser529, were monitored. (Modified figure from **Paper II**, figure 4.)

Using the prodrug approach, novel ATB-based cell-permeable compounds were synthesized (Figure 19) and their uptake was monitored with different cell lines.[90] The activity of the compounds in cells was demonstrated by the reduction of the phosphorylation of Cdc37 at Ser13 and platelet aggregation.[90]

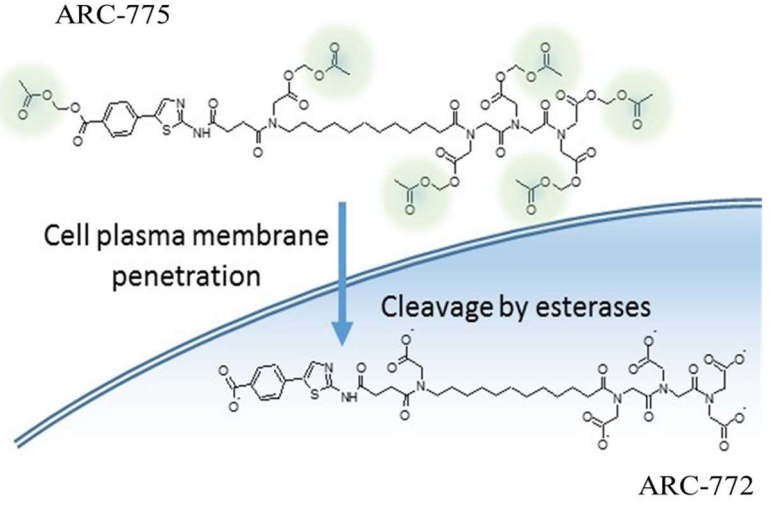


Figure 19. Figure of prodrug ARC-775 and the active compound ARC-772 after cleavage by esterases in cell. (Modified figure from Figure 5 in [90].)

Successful examples of prodrug approach show that bisubstrate inhibitors of CK2 described in this thesis could be converted to intracellulary active derivatives. This enables the construction of new probes and inhibitors with great potential for various applications.

7. CONCLUSIONS

The main aim of the present thesis was to develop novel inhibitors of protein kinase CK2 using a bisubstrate inhibitor approach. Taking the known ATP-competitive inhibitors of CK2 and conjugating them with negatively charged peptides to get inhibitors possessing higher selectivity and affinity toward target kinase. This kind of inhibitors and their conjugated probes could be used to in biochemical studies of CK2 in test tubes. But have also potential to be converted to compounds that monitor or regulate cellular activity of the kinase. The results of the study presented in present thesis can be summarized as follows.

- TBBi was chosen for the first ATP-competitive scaffold of bisubstrate inhibitors for CK2. Conjugation of TBBi to peptides led initially to free bisubstrate inhibitors that possessed sub-micromolar affinities toward CK2 in binding assays. Optimization of the structure of the linker led to bisubstrate inhibitor ARC-1502 with a K_D value of 0.52 nM, which was more than 500 times more potent than TBBi. Also the selectivity of ARC-1502 toward CK2 was considerably higher than the selectivity of TBBi. Labelling ARC-1502 with fluorescence dye PromoFluor-647 led to fluorescence probe ARC-1504 (K_D = 0.4 nM), possessing highest affinity of a probe for CK2 ever described at that time. This showed that using of bisubstrate approach on known ATP-competitive inhibitors of CK2 results in inhibitors possessing higher affinity and selectivity towards target kinase.
- The second used pharmacore originated from the high affinity ATP-competitive inhibitor CX-4945 (the first CK2 inhibitor accepted to clinical trials). This led to the second series of bisubstrate inhibitors of CK2. Conjugation of this pharmacore with oligo aspartic acid peptide gave sets of isomeric bisubstrate inhibitors with various alkylation sites. Effects of different lengths and composition of the peptide part and linkers were studied. As the result of this research bisubstrate inhibitor ARC-1424-5O was synthesized that possessed K_D value as low as 37 pM. Labelling ARC-1424-5O with PromoFluor-555 led to a fluorescence probe ARC-1513-5O with K_D value of 16 pM. According to our knowledge, those are the lowest values ever reported for inhibitors and fluorescence probes of CK2.
- The third scaffold tested was previously reported CK2 inhibitor 4-(2-amino-1,3-thiazol-5-yl)benzoic acid (ATB) and its selenium-containing counterpart 4-(2-amino-1,3-selenazol-5-yl)benzoic acid (ASB). These compounds were used as the precursors. Conjugation ATB and ASB to peptide fragment led to high affinity bisubstrate inhibitors ARC-1527 and ARC-1529 possessing room-temperature phosphorescence properties in CK2 complexes with signal lifetimes in micro-second range. Labelling those inhibitors with 5-TAMRA led to ARC(Lum)-Fluo probes ARC-1528 and ARC-1530. Labelled probes showed increased long lifetime luminescence signal in CK2 complex where ARC-1530 had also the highest luminescence signal intensity,

compared to its sulfur counterpart and compounds without the fluorescence dye. It was shown that probes with microsecond-scale luminescence properties could be used in biological samples, where the time gated measurements eliminate the autofluorescence background of biological components and leads to higher signal sensitivity.

In conclusion, we have synthesized new tools for of protein kinase CK2 related biochemical research. Our created inhibitors and probes have high selectivity and affinity. The third scaffold-based probes possess also room-temperature phosphorescence properties. These compounds are useful tools to characterize content of CK2 in complicated environments, for example in cell lysates.

SUMMARY IN ESTONIAN

Bisubstraatsete inhibiitorite arendus proteiinkinaasile CK2

Proteiinkinaasid (PKd) on ensüümide perekond, millesse kuulub üle 530 erineva kinaasi.[1] Nende ensüümide ülesandeks on katalüüsida fosforüülrühma ülekannet ATP-lt sihtvalgule. Kuna tegemist on eluliselt olulise funktsiooniga, siis juba väiksemad muutused kinaaside aktiivsuse reguleerimises mõjutavad oluliselt raku elutegevust.[1,2] Aastate jooksul on leitud, et taolised PK-de aktiivsuse ala- ja ülereguleerimised on seotud paljude haigustega ning PK-d on muutunud oluliseks ravimiuuringute sihtmärkideks. Tänaseks on välja töötatud ja FDA (Food and Drug Administration) poolt kinnitatud juba 42 kinaasi inhibiitorit erinevate haiguste (erinevat tüüpi vähid, artriit jt.) raviks.[11,15]

Üheks PK-de perekonna liikmeks on CK2, mis on rakus olulise tähtsusega proteiinkinaas kuna vastutab hinnanguliselt vähemalt 10% kõigis rakus toimuvate fosforüleerimiste eest.[17,21] CK2 kõrgenenud aktiivust seostatakse mitmete haigustega, seal hulgas erinevat tüüpi vähktõbedega. Kuna on leitud, et vähirakkude elutegevus on tugevalt seotud CK2 kõrge aktiivsusega [27], siis on see kinaas leidnud suurt huvi vähiravi arendamise vallas. CK2 uuringute käigus on välja töötatud paljusid ATP-konkurentseid inhibiitoreid [33], kuid tihtipeale sellised väikesed molekulid ei oma piisavalt suurt afiinsust või selektiivust sihtmärkensüümi suhtes. Käesolevas töös arendati ja sünteesiti CK2-le bisubstraatseid inhibiitorid, mis seovad kinaasi mõlema sidumistaskuga korraga ning seetõttu omavad enamasti ka kõrgemat afiinsust ning selektiivust.

Uurimustööd alustati proteiinkinaasile CK2 sobiva ATP-konkurentse ja peptiidse fragmendi valimisega. Bisubstraatse inhibiitori ATP-konkurentseks fragmendiks valiti varem kirjeldatud CK2 inhibiitor TBBi. Kuna CK2 on atsidofiilne kinaas ja eelistab peptiidses osas negatiivse laenguga aminohappeid, siis oligopeptiid sünteesiti kasutades asparagiinhappe jääke. Algselt kasutati kahe fragmendi ühendamisena variatsioone glütsiinist ja 6-aminoheksaanhappest. Selle tulemusel sünteesiti bisubstraatsed inhibiitorid, mille inhibeerimisvõime oli märkimisväärselt kõrgem TBBi-le kirjeldatud väärtusest. Esimeste kristallstruktuuri uuringutega leiti, et kasutatav ATP-konkurentset ja substraadikonkurentset fragmenti ühendav osa ei ole piisavalt painduv ning vastavad aminohapped asendati oktaanhappega. Selle tulemusel sünteesiti ARC-1502, mis koosnes TBBi-st, kuut asparagiinhappe ja üht lüsiini jääki sisaldavast peptiidsest fragmendist ning neid ühendavast oktaanhappest. ARC-1502 sidumiskonstandiks (K_D) mõõdeti 0,52 nM. Märgistades ARC-1502 fluorestsentsmärgisega PromoFluor-647 saadi fluorestsentssond ARC-1504 (KD = 0,4 nM). Varasemalt nii kõrge afiinsusega bisubstraatset inhibiitorit ja fluorestsentssondi CK2-le kirjeldatud ei olnud. Lisaks näitas ARC-1502 väga head selektiivust CK2 suhtes.

Töö teises osas kasutati ATP-konkurentse fragmendina kliinilistes katsetustes oleva CK2 inhibiitori CX-4945 farmakofoori benso[c][2,6]naftüridiin-8-karboksüülhapet. Lähtudes varasematest teadmistest, et alküülahel on sobivaks

ühendajaks ning peptiidahela pikkus mõjutab oluliselt bisubstraatse inhibiitori sidumisvõimet, sünteesiti inhibiitoreid, milles varieeriti nii siduja ja peptiidahela pikkust ning fluorestsentsmärgise asukohta peptiidahelas. Uurimustöö tulemusena sünteesiti madal-pikomolaarne bisubstraatne inhibiitor ARC-1424-5O (K_D = 37 pM). Konjugeerides selle fluorestsentsmärgise PromoFluor-555-ga sünteesiti fluorestsentssond ARC-1513-5O (K_D = 16 pM). Meile teadaolevalt nii kõrge afiinsusega ühendeid proteiinkinaasile CK2 varem sünteesitud ei ole.

Töö kolmandas osas tegeleti pika luminestsentsi elueaga sondi arendamisega CK2-le ATP-konkurentse osana kasutati väävlit sisaldavat 4-(2-amino-1,3-tiasool-5-üül)bensoe hapet (ATB) ja sellele vastavat seleeni sisaldavat analoogi (ASB). Antud fragmendid konjugeeriti kuut asparagiinhappe ja üht lüsiini jääki sisaldava peptiidi külge ning sünteesiti bisubstraatsed inhibiitorid ARC-1527 (kasutades ATB-d) ja ARC-1529 (kasutades ASB-d). Neid ühendeid CK2-ga seondunult ultravioletse valgusega kiiritades emiteerivad need pikaajalist fotoluminestsentskiirgust, mille eluiga jääb kümnete mikrosekundite suurusjärku. ARC-1527 ja ARC-1529 fotoluminestsents kiirguse eluigadeks mõõdeti vastavalt 82 μs ja 55 μs. Konjugeerides vastavad inhibiitorid fluorestsentsvärviga 5-TAMRA sünteesiti ARC(Lum)Fluo sondid ARC-1528 ja ARC-1530. Kõik sünteesitud inhibiitorid ning sondid omasid alla nanomolaarset sidumiskonstanti ja mikrosekundilises suurusjärgus fotoluminestsentsi eluiga olles kompleksis CK2-ga. Nii kõrge afiinsuse ja pika fotoluminestsentsi elueaga ühendeid pole CK2-le varem kirjeldatud.

Töö käigus sünteesiti ja uuriti erinevate omadustega CK2 bisubstraatseid inhibiitoreid ja fluorestsentssonde. Töös väljatöötatud kõrge afiinsusega sondid võimaldavad CK2 aktiivsuse määramist ning uute, potentsiaalsete CK2 inhibiitorite karakteriseerimist. Pika elueaga fotoluminestsentsiga ühendite rakendamine bioloogilistes süsteemides võimaldavad kasutada aegviivitusega mõõtmisemeetodeid, mis parandab signaal-müra suhet ning tagab parema mõõtmismeetodite tundlikkuse.

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PUBLICATIONS

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1. Enkvist, E., Viht, K., Bischoff, N., Vahter, J., Saaver, S., Raidaru, G., Issinger, O.-G., Niefind, K., Uri, A. (2012) A subnanomolar fluorescent probe for protein kinase CK2 interaction studies, Org. Biomol. Chem., 10, 8645–8653
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