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Protein kinase selectivity mechanisms and structure assisted drug discovery

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1 Acknowledgement

The list of all persons and institutions that made my PhD possible or more enjoyable would probably exceed the volume of this thesis. I will therefore narrow it down to the biggest contributors (in random order):

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2 Abbreviations

ARCs	adenosine analogue-oligoarginine conjugates
Aurora A	Aurora kinase A
Aurora B	Aurora kinase B
Aurora C	Aurora kinase C
ATP	adenosine-5'-triphosphate
cAMP	Cyclic-3',5'- adenosine monophosphate
CPC	chromosomal passenger complex
PKA	catalytic subunit of Protein kinase A
PKAc	catalytic subunit of Protein kinase A
PKAr	regulatory subunit of Protein kinase A
PKI	heat stable protein kinase A inhibitor peptide

3 Introduction

Dear reader, the title of my thesis 'Protein kinase selectivity mechanisms and structure assisted drug discovery' is not very concise but depicts a whole field of research. This is reflected in the work I conducted as PhD student. The main topics are connected by methodology rather than one common scientific question. However, in all three projects protein kinase A is employed as model to generate crystal structures of the kinase domain in complex with different ligands. The motivation of the work in general was to broaden the knowledge on the design of kinase targeting inhibitors, or in other words: molecules potentially useful in cancer therapy. In order to guide you through my work I will start with a brief introduction on the kinase enzyme family - explain why they are important drug targets and how the targeting works.

3.1 Protein kinases

Protein kinases are a family of enzymes defined by their catalytic activity, the phosphorylation of other proteins. Protein kinases alter the atomic structure of their targets by transferring phosphate groups from Adenosine-5'-triphosphate (ATP) to the hydroxy group of serine, threonine or tyrosine residues; a modification that in most cases goes along with changes of the biological properties of the targeted protein. Through subsequent conformational rearrangements, phosphorylation can trigger the activation or deactivation of enzymes, but also influence the stability of protein molecules. According to which kind of amino acid residues they phosphorylate, eukaryotic protein kinases are divided into serine/threonine kinases and tyrosine kinases. The serine/threonine kinases are further divided into the following groups: AGC, CAMK, CK1, CMGC, STE and TKL (Figure 3.1) [1]; some proteins classified as tyrosine kinases may have serine/threonine kinase activity and vice versa.

The discovery of protein kinases dates back to the middle of the 20th century when Krebs and coworkers investigated the metabolism of glycogen in the liver. Throughout their work they discovered an enzyme that phosphorylated and thereby activated glyco-

gen phosphorylase [2]. This enzyme, phosphorylase kinase, was the first protein kinase to be purified and characterized. At the time, the general importance of protein phosphorylation in the regulation of cellular processes was unknown. Thus it took another 10 years until the second protein kinase was discovered, the mediator of the important second messenger cAMP, protein kinase A [3]. In the course of time more and more kinases were described and their activity linked to vital cellular processes. Eventually in the late 1970s, when the oncogene Src was found to encode for an enzyme with tyrosine kinase activity [4], the first link between dysfunctional kinase activity and the development of cancer was found. Finally, by the 1980s it was clear that protein phosphorylation is the most common mechanism for signal transduction in eukaryotic cells [5]. And with the first crystal structure of a kinase domain published in 1991 [6] protein kinases emerged as one of the major targets for structure based development of cancer treating drugs. Huge investments and efforts finally resulted in the approval of the first protein kinase targeting therapeutics: Fasudil received approval by the Japanese authorities in 1995, and Gleevec/Imatinib by the U.S. Food and Drug Administration in 2001 [7].

Today we know that the genes encoding for protein kinases alone represent 1.7% of the human genome. The human protein kinase family comprises more than 500 members; referred to as the 'kinome' (Figure 3.1) [1]. As most of the kinases are targets of phosphorylation and at the same time phosphorylate other kinases themselves, the kinome forms a vast network of signaling pathways allowing the cell to process information and react to stimuli. Kinase signaling is involved in the regulation of virtually all functional pathways of the eukaryotic cell.

3.1.1 The anatomy of a kinase

While protein kinases feature high variation in length, sequence and structure of their amino- and carboxyl-termini, the catalytic core, the 'kinase domain' is highly conserved. The domain is formed by about 250 amino acids, divided into an amino-terminal (N-terminal) and carboxyl-terminal (C-terminal) lobe (Figure 3.2). The N-terminal lobe represents with about 100 amino acids the smaller portion of the kinase domain. Its shape is dominated by a barrel of five β-sheets and one conserved α-helix (Figure 3.2). The C-terminal lobe is primarily α-helical and accommodates the peptide substrate site. In contrast to the peptide substrate, the binding of ATP requires both lobes, which house the nucleotide at their interface in a deep cleft; called the ATP binding pocket, ATP pocket or ATP cleft. The N- and C-terminal lobe are connected by the 'hinge region', a flexible peptide chain which allows the kinase domain to occupy a variety of

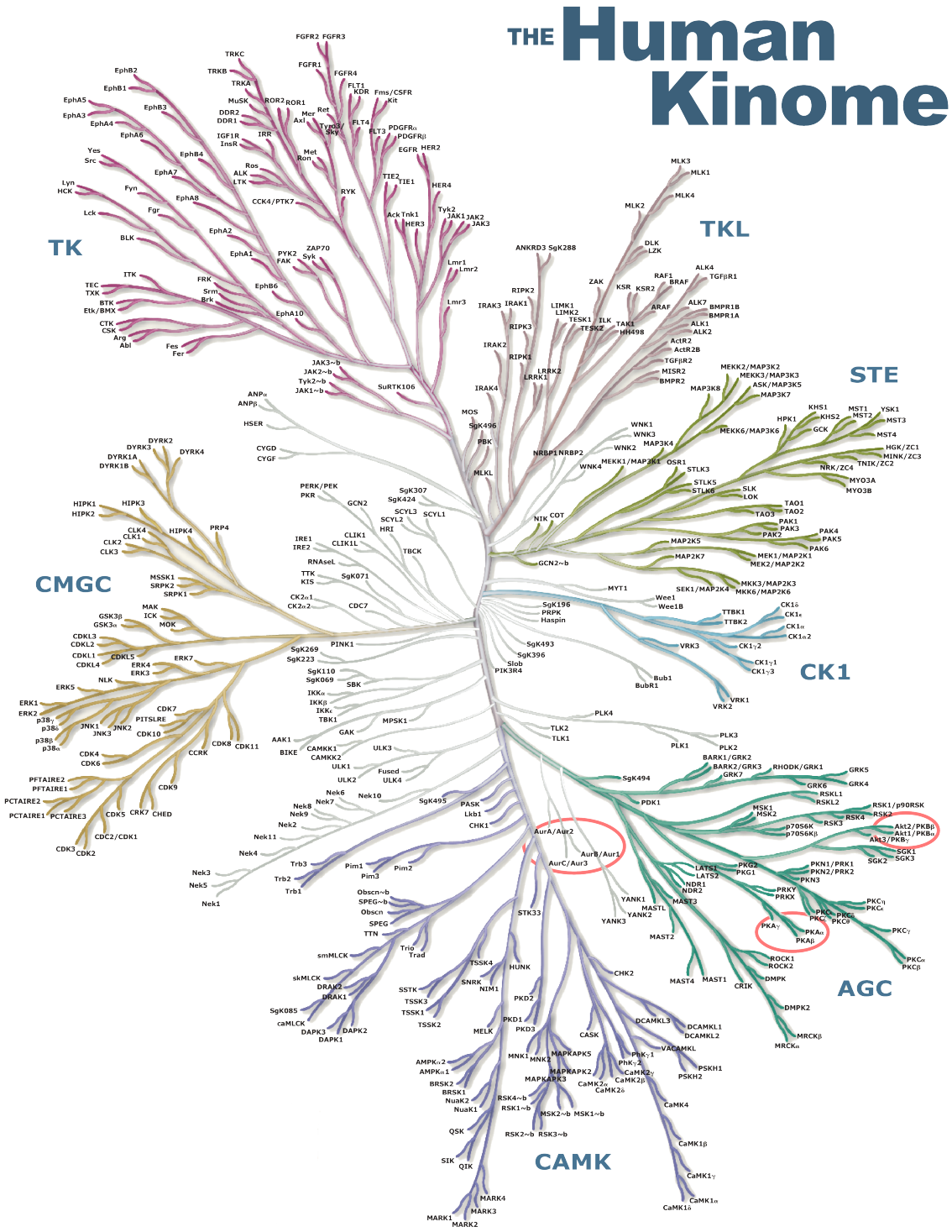

Figure 3.1: Phylogenetic tree of all 518 human kinases, ordered by kinase families. The figure was taken from Manning et al. [1]. TK refers to tyrosine kinases. Serine/threonine kinases are subdivided into: AGC, containing PKA, PKG, PKC families; CAMK, Calcium/calmodulin-dependent protein kinase; CK1, Casein kinase 1; CMGC, containing CDK, MAPK, GSK3 and CLK families; STE, homologs of yeast Sterile 7, Sterile 11 and Sterile 20 kinases; TKL, Tyrosine kinase–like. The Aurora kinases, and protein kinases A and B are highlighted by red ellipses.

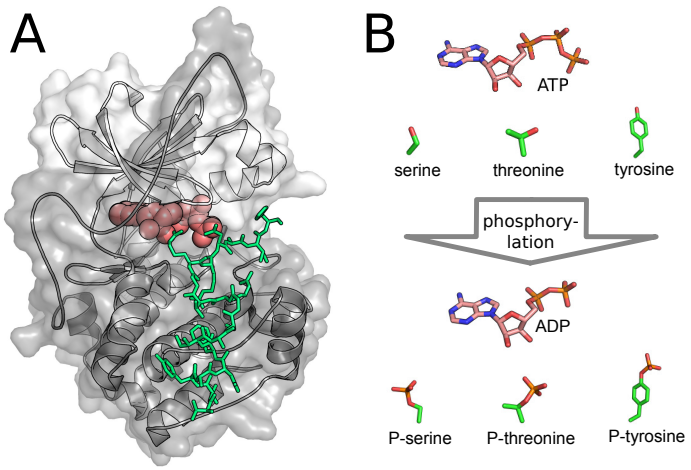

Figure 3.2: (**A**) **Ternary complex of the catalytic subunit α of protein kinase A (PKA)**, bound by ATP (red) and the substrate mimicking peptide 'protein kinase inhibitor' (PKI, green). The N-terminal lobe (white) and the C-terminal lobe (grey) form the ATP binding pocket at their interface. The figure was created utilizing the coordinates of PDB entry 3FJQ [8]. (**B**) Depiction of a phosphorylation reaction. The γ-phospho group of ATP is transferred on a serine-, threonine- or tyrosine residue.

ATP cleft opening states. A unique feature of PKA and some other AGC kinases is the extended C-terminus that coils around the N-terminal lobe (Figure 3.2), anchoring with two phenyl alanine residues in the so called hydrophobic motif.

3.1.2 The phosphotransfer reaction

The universal intracellular energy carrier molecule adenosine-5'-triphosphate (ATP) plays a dual role in the phosphorylation reaction catalyzed by protein kinases. ATP provides the phosphate group to be transferred to the target and contributes energy to the phosphorlylation reaction originating from the high-energy phosphate bond between its β-phosphate and γ-phosphate group. The latter phosphate group is the one to be transferred to the target protein (Figure 3.2).

3.1.3 Regulation by phosphorylation requires regulation of phosphorylation

Transferring the large and electrostatically charged phospho-group to an amino acid side chain most often causes conformational rearrangements in the target protein (Figure 3.2).

This can modify the protein function in multiple ways; act as a molecular 'on-off' switch by strongly increasing or decreasing the enzymatic activity, influence the stability of its tertiary structure, or even mark it for destruction. It is estimated that about 30% of the proteins encoded by the human genome contain covalently bound phosphate, rendering phosphorylation the most abundant and probably most important post-translational modification [9]. Naturally, a process that important needs strict spatial and temporal regulation, which is achieved on various levels.

The activity of kinases can be regulated in time and space on the level of expression and protein stability, through co-factors, scaffolding proteins, or the phosphorylation by kinases of different or the same species. Furthermore, the covalent phosphorylation of proteins is reversible through dephosphorylation, catalyzed by an enzyme family called phosphatases. The orchestrated activity of protein kinases, accessory proteins, and phosphatases allows for a defined temporal activation and deactivation of a target protein.

Another level for the regulation of phosphorylation is the target specificity of the kinase domain itself. Figure 3.2 depicts the interaction of the catalytic domain of protein kinase A and the pseudo-substrate PKI. Both molecules interact extensively with a buried surface area of several hundred Å^2 and a network of multiple polar and non-polar contacts, resulting in nanomolar binding affinity. This affinity strongly depends on the composition of the amino acid side chains flanking the phosphoacceptor site of the PKI peptide. It is due to variations in these amino acids, neighboring the phosphorylation site, that kinases do not possess only selectivity towards serine/threonine or tyrosine residues, but also towards specific sequences. The amino acid composition around the phosphorylatable residue to which a kinase preferably binds is the consensus sequence or motif. However, this selectivity is usually not limited to a single substrate, but usually targets a whole substrate family bearing a common consensus sequence. As in the presented case of protein kinase A and PKI, the regulation of kinase activity through substrate mimicking peptides that possess the consensus sequence but lack a phosphoacceptor site is a common strategy of nature to regulate phosphorylation activity.

3.1.4 Protein kinases as drug targets

Aberrant protein kinase activity is linked to a range of diseases, most notably cancer [10]. Recent large scale sequencing projects revealed that kinases are indeed the most frequently mutated proteins in tumors [11, 12]. Why is that? Cancer is the malignant

growth of cells and tissues, a development that requires independence from organism control in a spectrum of processes; as for example the proliferation, survival and migration of cells. All these functions are orchestrated by protein kinase signaling networks, making kinases a likely starting point for dysregulation. This can be achieved by alteration of expression levels, but also by introducing activity modulating mutations in the kinase domain. Hence it appears logical to fight cancer with kinase activity modulating drugs. With the approval of the first kinase targeting anti-cancer pharmaceutical (Gleevec/Imatinib) in 2001, this approach was surely validated [7]. Imatinib's pharmacological and economical success has made kinases one of the most intensively pursued classes of drug targets in the 21st century [13]. To date, 11 small-molecule kinase inhibitors have received approval for the treatment of cancer from the U.S. Food and Drug Administration and European authorities [14].

3.2 Inhibition of enzymatic activity

The activity of a kinase can be modulated by exposing it to ligands that non-covalently bind to its ATP- or peptide binding site, and by doing so displace the real substrate. This interaction can be quantified as a dissociation constant and described in the form of a thermodynamic state function:

$$K_i = \frac{[\text{ligand}] * [\text{protein}]}{[\text{ligand} * \text{protein}]} \tag{3.1}$$

$$\frac{K_i}{[\text{ligand}]} = \frac{[\text{protein}]}{[\text{ligand} * \text{protein}]} \tag{3.2}$$

K_i, [ligand], [protein] and [ligand*protein] have the dimension of concentration, conventianally with the units mol/l (M), or mmol/l (mM), etc.

In representation 3.2 the function highlights how, when K_i equals the concentration of free ligand [ligand], free protein molecules [protein] and ligand-bound protein molecules [ligand*protein] must be present in ratio 1:1. For both drug application and also *in vitro* protein activity assays, it is standard practice that the drug/inhibitor is dosed in excess over its protein target, rendering the portion of ligand bound to the protein [ligand*protein] to be negligibly small. K_i approximates the concentration of ligand that is needed to saturate 50% of the protein molecules present, resulting in a 50% reduction of protein activity (50% inhibition). The lower the K_i value of a compound, the higher is the affinity to its target.

The K_i can also be described with an equation published by Cheng and Prusoff [15]:

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}} \tag{3.3}$$

$$IC_{50} = K_i * (1 + \frac{[S]}{K_m}) \tag{3.4}$$

IC_{50} is similar to the dissociation- or inhibition constant K_i but describes the ligand concentration that is needed to reach 50% inhibition at a given concentration [S] of the competing natural substrate. The Michaelis constant K_m is the concentration of substrate at which enzyme activity is half maximal. Whereas the IC_{50} value for a compound may vary between experiments depending on experimental conditions (varying substrate concentration [S]), K_i is not affected by competitive substrate concentrations and is generally more suitable to quantify inhibitor affinity.

The Cheng-Prusoff equation (3.4) highlights what factors determine the inhibition through a ligand when competing with the natural substrate. These are the binding strength of the inhibitor (K_i) and the substrate (K_m), and of course the concentration of both competing compounds.

3.3 Protein-ligand interactions

The binding strength between inhibitor and protein (K_i; equations 3.1, 3.2, 3.3 and 3.4) depends on the number of favorable interactions between them. These predominantly non-covalent interactions can be of different forms:

One of the most common and important protein-ligand interaction is the **hydrogen bond** or H-bond. The so called hydrogen-bond donor (usually a $N-H$ or $O-H$ group) carries a proton that is attracted by the hydrogen-bond acceptor, an electronegative atom with a partial negative charge (usually an oxygen or nitrogen atom). Upon binding, electron density is shifted from the donor to the acceptor, and the acceptor group approaches the proton in the H-bond more closely than the van der Waals radii would apparently permit. An H-bond is stronger than a van der Waals interaction, but weaker than covalent or ionic bonds.

An **ionic interaction** or 'salt-bridge' is an electrostatic interaction between charged groups of ligands and oppositely charged groups on the protein. Salt bridges represent the strongest non-covalent interaction. They often occur in combination with hydrogen bonding.

In aqueous solution lipophilic hydrocarbon groups tend to form intermolecular aggregates. This phenomenon results from a complex combination of effects and is termed the **hydrophobic interaction**. Some involves the direct attractive forces (van der Waals) between hydrophobic groups. The main energetic contribution to this type of interaction results from the displacement of water molecules from the hydrophobic environment of the binding pocket and from the hydrophobic moieties of the ligand upon binding. Increased polarizability of aromatic moieties may enhance the effect. Their ability for polarization is also important in cation-π [16] as well as π-π interactions [17].

3.4 Kinase inhibition strategies

Most kinase activity modulating inhibitors are ATP competitive small molecules that block the binding of ATP to the kinase domain and thereby reduce its enzymatic activity. The ATP pocket is a deep cleft with a large surface area and potential for many polar interactions (Figure 3.2 and 3.3), allowing for the design of nanomolar affinity compounds. However, this inhibition strategy is challenged by the high conservation of the pocket among the members of the kinase family and competition from the high intracellular ATP concentrations. This impedes the development of sufficiently specific and affine ATP competitive kinase inhibitors. However, the ATP cleft remains the most druggable site for at least the majority of the kinase targets.

Kinase activity can also be suppressed through ligands that target the peptide substrate site. In fact, this approach is employed by nature itself to regulate the activity of some members of the kinase family (see 3.5.2). Unfortunately, the peptide substrate site of kinases is not a pocket like the ATP binding site, but is surface exposed (Figure 3.2). Consequently, potential ligands might need to exceed drug-like sizes to be able to form the numerous interactions that are necessary for sufficient target specificity and affinity [18]. Additionally, peptide-based compounds, which would be the most logical approach for the design of ligands of a peptide substrate site, suffer generally from low stability *in vivo*.

3.5 Protein kinase A

PKA holds the status as the prototypical kinase, in equal measure due to historical and practical reasons. It was one of the first kinases to be discovered [3], the first to be sequenced [20] and cloned [21], and the first to be crystallized [6] leading to the

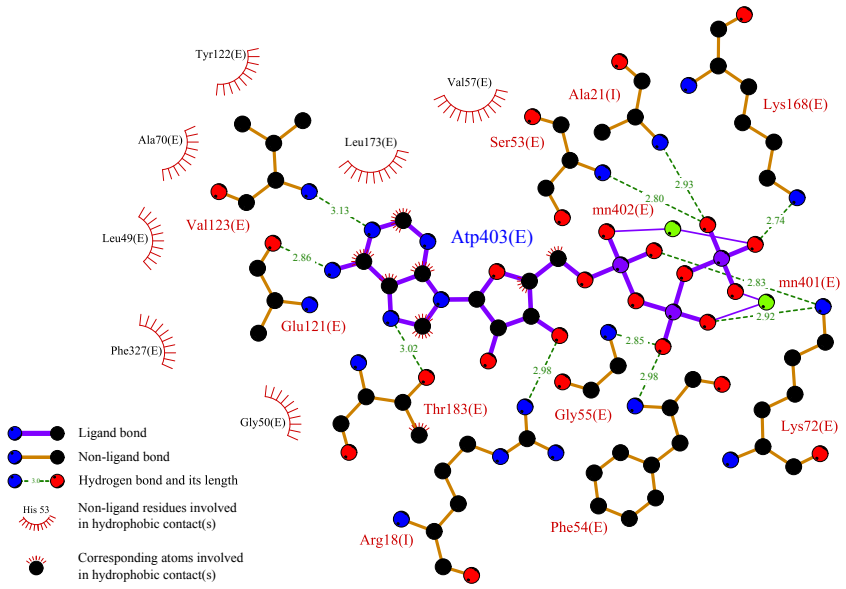

Figure 3.3: Interactions between ATP and the ATP cleft of protein kinase A. The figure was created utilizing the coordinates of PDB entry 3FJQ [8] and the application LigPlot [19]. '(E)' and '(I)' refer to the chains in the PDB file; chain E holds the coordinates of the PKA molecule, chain I the coordinates of the PKI peptide. Carbon atoms are represented in black, oxygen in red, nitrogen in blue and manganese in green.

elucidation of the structure of the kinase domain. PKA is a very important regulator of countless vital cellular functions and beyond that a valuable research tool due to its reproducible recombinant expression, stability, and crystallizability.

In this thesis, functional aspects of PKA were not the main focus of work. Instead, PKA was employed as a model as probably the best crystallizable kinase domain, delivering the highest resolution crystal structures. The work sheds new light on interactions between PKA and its peptide substrate. Understanding and discussing these results requires some background on function and regulation of this kinase, which I will therefore present in the following sections.

3.5.1 The regulatory role of PKA

PKA is a member of the AGC kinases (Figure 3.1). It mediates the response of the second messenger cyclic AMP in the eukaryotic cell and is therefore also called 'cAMP-dependent protein kinase'. PKA is ubiquitously expressed and controls vital cellular mechanisms such as the lipid and glucose metabolism (via phosphorylation of phosphorylase kinase), the formation of memories [22], ion transport (via phosphorylation L-type

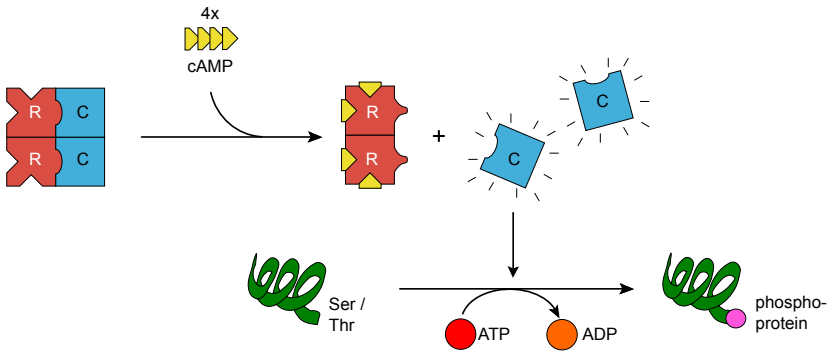

Figure 3.4: Activation of protein kinase A of higher eukaryotes via cAMP. The catalytic (C) subunits freed from the regulatory (R) subunit dimer are phosphorylating various substrate proteins at specific serine or threonine sites, respectively. Hereby ATP is converted to ADP. The figure was taken from Wikipedia (http://en.wikipedia.org/wiki/File:PKARII.svg) and modified.

Ca^{2+} channels [23]), expression of hundreds of genes (via phosphorylation of the transcription factor CREB [24]), as well as the proliferation and differentiation of the cell [25]. Even though PKA activity has been linked to malignancies including several forms of cancer [26], the ubiquitous and essential nature of cAMP-regulated cellular processes renders PKA an 'anti- target' for protein kinase inhibition strategies.

The use of the abbreviation 'PKA' is inconsistent in literature. Mostly it refers to the catalytic subunit of protein kinase A. But 'PKA' is also used to describe the PKA holoenzyme, a quaternary complex composed of a dimer of PKA regulatory subunits and two PKA catalytic subunits (Figure 3.4). In this text 'PKA' and 'PKAc' refer to the catalytic subunit, 'PKAr' to the PKA regulatory subunit, and the holoenzyme is designated 'PKA holoenzyme'.

3.5.2 Activation and regulation of PKA

In the absence of cAMP, PKAc is trapped in the inactive heterotetrameric PKA holoenzyme, formed by a PKAr dimer that maintains two PKAc subunits in a dormant state. Dissociation of the holoenzyme ensues upon binding of cAMP to tandem sites in each PKAr subunit (Figure 3.4). The now released and active PKAc subunits are free to phosphorylate substrates on serine or threonine residues, which are presented in a sequence context of **Arg**-**Arg**-X-Ser/Thr or **Lys**-**Arg**-X-X-Ser/Thr (reviewed by Dell'Acqua et al. [27]).

Mammals carry three PKAc subunit isoforms (Cα, Cβ and Cγ) featuring just minor

differences in their kinetic characteristics but discrete expression patterns. In contrast, the four PKAr subunits exhibit both distinct cAMP binding affinities and differential localization within the cell. They are divided into two families (I and II) accordingly. The cellular localization of the PKA holoenzyme is a consequence of different affinities of the PKAr subunits to the A-kinase anchor proteins (AKAPs), a group of proteins responsible for the association of the PKA holoenzyme with the cytoskeleton and the membrane. While type I PKA holoenzyme (containing either RIα or RIβ) is predominantly cytoplasmic, almost all of the type II PKA holoenzyme (containing either RIIα or RIIβ) is bound to those anchoring proteins (reviewed by Dell'Acqua et al. [27] and JH Schwartz [28]).

The PKAr subunits of PKA are expressed in excess over the PKAc subunits favoring rapid reformation of the holoenzyme in the cytoplasm when cAMP levels return to the basal state. The re-inactivation of catalytic subunits in the nucleus is mediated by peptidic pseudo-substrate PKI, which features a substrate motif of PKAc and a nuclear export signal. When PKI binds to PKAc, the resulting complex is shuttled back into the cytoplasm, where PKAc recombines with PKAr to form the inactive holoenzyme (reviewed by Wiley et al. [29]).

3.6 Aurora kinases

Aurora kinases are a family of serine/threonine kinases that were identified as important regulators of mitosis and cytokinesis. Their dysregulation in several human cancers has drawn attention to them as potential targets for anti-cancer therapeutics [30, 31].

However, the three members of the human Aurora kinase family are difficult proteins to study. Their expression in amounts sufficient for characterization and drug discovery is tedious, due to low expression levels, insolubility and instability. A part of my project was therefore to create a PKA-based model for the Aurora kinases (described in paper II). PKA and Aurora kinases are fairly close relatives (see Figure 3.1). Consequently there are just few differences in the amino acid composition of their ATP clefts determining selectivity to ATP competitive inhibitors (see Figure 1 in paper II). The aim was by stepwise mutating these residues in PKA, to generate a chimaeric kinase which features selectivity towards Aurora kinase inhibitors and at the same time is expressable, stable, and crystallizable as PKA.
Hence, even though I did not directly work with Aurora kinases, I will go on with a brief description of their regulatory function in the cell cycle.

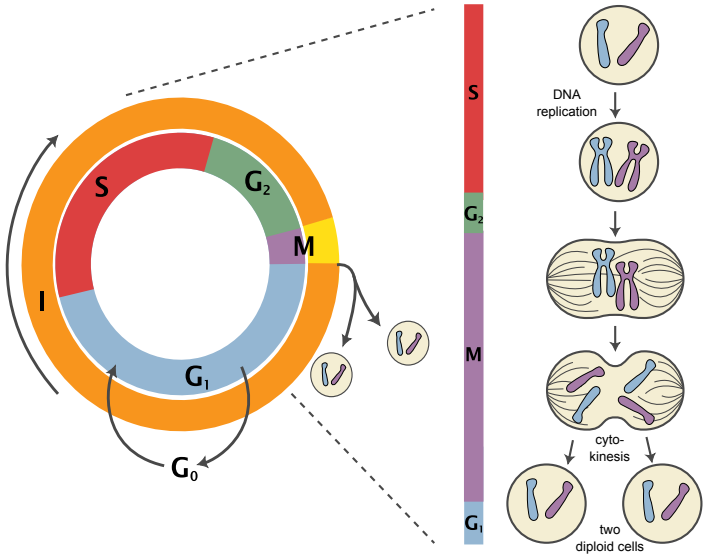

Figure 3.5: Schematic of the cell division cycle in eukaryotics. I=interphase, M=M-phase; G_1=gap 1, G_2=gap 2, S=synthesis, G_0=gap 0 (resting phase). The figure was produced employing the files 'http://en.wikipedia.org/wiki/File:Cell_Cycle_2-2.svg' and 'http://en.wikipedia.org/wiki/File:Major_events_in_mitosis.svg' from Wikipedia.

3.6.1 The cell cycle - a brief recapitulation

The cell cycle is a sequence of events that take place in eukaryotic cells in between two cell divisions. These events belong to two distinct phases: the interphase, during which the cell grows and duplicates its DNA; and the M-phase, during which the mother cell is divided into two daughter cells (Figure 3.5). The interphase is further divided into G_1, synthesis phase and G_2 [32]. The M-phase is defined by two processes, mitosis and cytokinesis (Figure 3.5). The length of the colored bars in Figure 3.5 represent the relative duration of the cell cycle phases. It renders the interphase the dominating period of the cycle and the M-phase as rather short.

G_1 or 'gap 1' is the phase bridging from cytokinesis at the end of the M-phase to the beginning of DNA synthesis. It is also referred to as the growth phase because biosynthetic activities of the cell resume at a high rate in G_1, after being considerably slowed down during M-phase. This includes synthesis of various enzymes needed for DNA replication, which will be required in the subsequent S-phase. The duration of G_1 is highly variable; cells that do not undergo cell division may remain indefinite time in G_1 which then eventually is called G_0 (Figure 3.5) [32].

The following S- or synthesis phase starts when synthesis of DNA begins and ends

when it is complete. Then all of the chromosomes have been replicated so that each chromosome features two sister chromatids (Figure 3.5). Even though the amount of DNA in the cell has doubled, the ploidy of the cell remained the same [33].

When DNA synthesis is complete, the cell enters the G_2 phase. As G_1 also the second gap is characterized by significant biosynthesis activity. In phase G_2 this mainly involves the production of microtubules, which are required for the process of mitosis.

Mitosis is the process by which an eukaryotic cell separates its sister chromatids, resulting in two identical sets of all chromosomes, and distributes them over two nuclei (Figure 3.5 and 3.6). Mitosis is generally followed immediately by cytokinesis, which divides the nuclei, cytoplasm, organelles and cell membrane into two cells containing roughly equal shares of these cellular components (Figure 3.6). With successful cytokinesis the M-phase is completed and the cell cycle may start over again (Figure 3.5).

3.6.2 The regulatory role of Aurora A, B and C

The first Aurora kinase was discovered in *Drosophila melanogaster* in the 1990ies [34]. The knockout mutation of the respective gene resulted in the failure of centrosome separation during mitosis, leading to the formation of monopolar mitotic spindles, reminiscent of Aurora borealis at the North Pole. Accordingly the newly discovered kinase was named 'Aurora'. Homologues of Aurora have been identified in different species including *Homo sapiens*. Humans feature a family of Aurora kinases with three members: isoforms A, B and C. All of them are active in the M-phase of the cell cycle and involved in the regulation of mitosis and cytokinesis.

Aurora isoform A is involved in regulating many of the early events in mitosis. It controls Cdc25b (Figure 3.6), a direct regulator of the cyclin B1-Cdk1 complex, whose activation is an essential requirement for mitotic entry (G_2- to M-phase transition). After inducing mitosis Aurora A moderates the formation of the bipolar mitotic spindle as well as the maturation and separation of the centrosomes (reviewed by Pollard & Mortimore [31]).

Aurora B is the catalytic member of the chromosomal passenger complex (CPC), which consists of Aurora B, the inner centromere protein (INCENP), borealin, and survivin (Figure 3.6). The CPC is initially formed along the chromosome arms before concentrating at the centromeres (Figure 3.6), the region on a chromosome where its two sister chromatids join. After separation of the chromatids the CPC finally localizes to the spindle midzone during cytokinesis (Figure 3.6). The localization of the CPC

is consistent with the multiple roles Aurora B plays in the M-phase. This includes condensation of the chromosomes, formation of the bipolar spindle, attachment of the chromosome to the mitotic spindle, regulation of the spindle checkpoint, and completion of cytokinesis (reviewed by Ruchaud et al. [35]).

Aurora A and B are frequently overexpressed in human cancers and at least isoform A was proven to promote tumorigenic transformation in mice. Upon overexpression both isoforms lead to chromosomal instability with different phenotypes. Increased Aurora A activity causes aberrant progression through mitosis leading to centrosome amplification, aneuploidy and extended telomeres, whereas the overexpression of isoform B results in chromosome segregation errors (reviewed by Katayama & Sen, and Pollard & Mortimore [36, 31]).

Aurora kinase C is present solely in mammals and is the least well studied member of the family. Besides its very low expression in somatic cells it exists in considerably high concentrations in testes. However, it has been reported to rescue human cells depleted of Aurora B in *in vitro* experiments, which indicates a possible functional redundancy between the two isoforms. Aurora C overexpression has been observed in cancer cell lines, though a distinct role in tumorigenesis has not been described (reviewed by Pollard & Mortimore [31]).

Their vital roles in mitotic process during the cell cycle and expression in malignancy renders at least Aurora kinases A and B as promising targets in cancer treatment. Early *in vitro* experiments with the Aurora kinase inhibitor ZM447439 have confirmed the feasibility of the approach and shown a chemotherapeutic-like mode of action [37]. The latter is a result of the temporal expression pattern of Aurora kinases, which is confined to progressively proliferating cells (reviewed by Katayama & Sen, and Pollard & Mortimore [36, 31]).

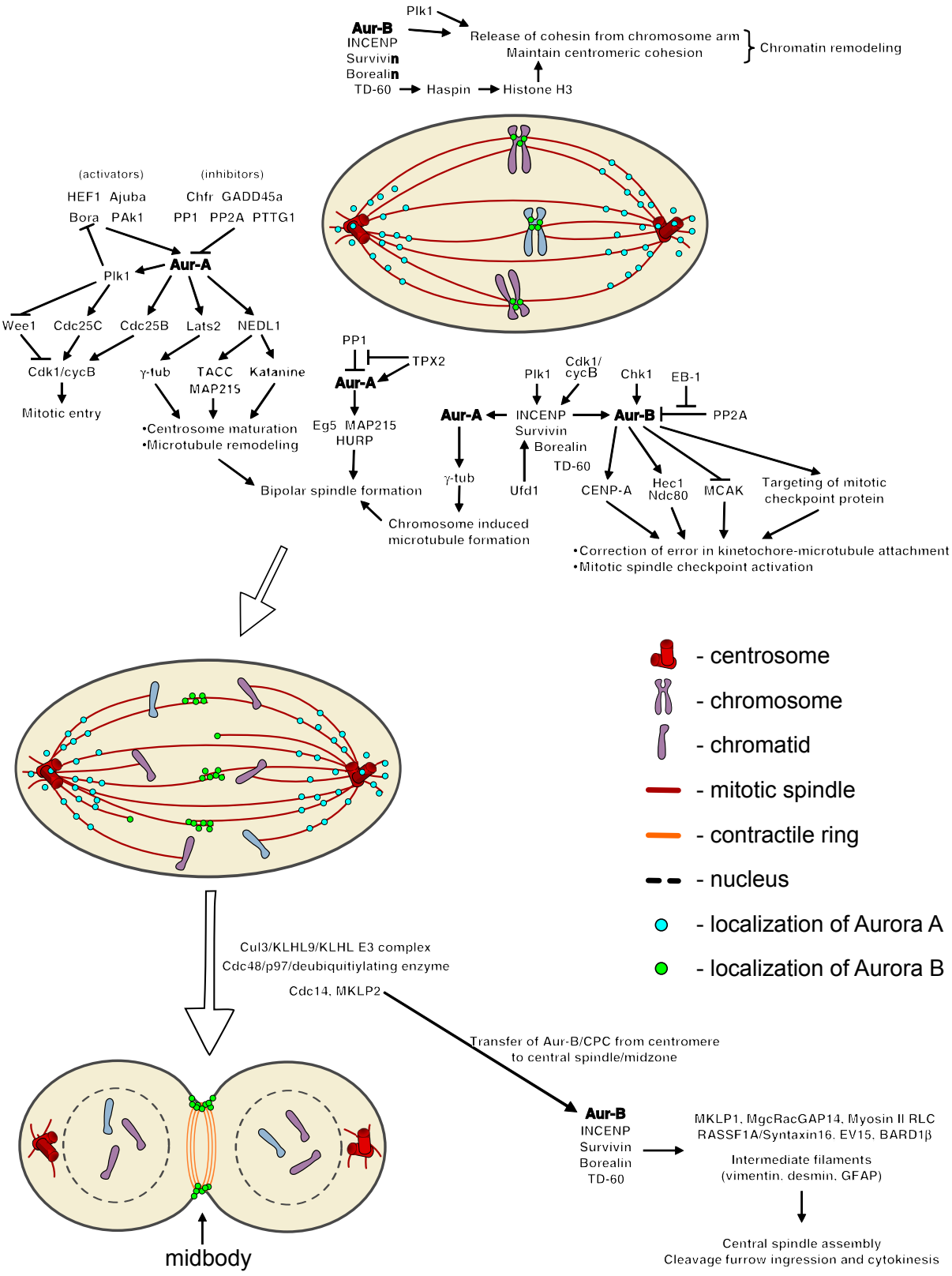

Figure 3.6: Major mitotic signaling pathways controlled by Aurora kinases. The figure was adapted from Katayama and Sen [36]. The three depicted cells represent from top to bottom: early stage of mitosis (metaphase), medium stage (anaphase), and late stage of mitosis (telophase) with beginning cytokinesis.

4 Paper I

4.1 Motivation and summary

This project was conducted in collaboration with the group of Professor Asko Uri at the University of Tartu, Estonia. Professor Uri develops ligands for protein kinases that compete both, with ATP and the peptide substrate for binding. This approach of so-called 'bisubstrate' kinase inhibitors was proposed earlier [38, 39, 40, 41]. However, Professor Uri's group was the first to implement it successfully resulting in compounds featuring extremely high affinity towards PKA and other basophilic kinases (reviewed by Lavogina et al. [42]). Besides the high potency already achieved, combining the advantages of both substrate sites may also enable higher target selectivity than is possible with solely ATP-competitive compounds. This could be a way to overcome the lack of target specificity associated with the 'classical way' of inhibiting kinases through ATP competitors, caused by the high degree of conservation in the kinase domain (as discussed earlier in section 3.4). The first generation of bisubstrate inhibitors designed by Professor Uri's group were adenosine analogue-oligoarginine conjugates, abbreviated 'ARCs'. This description is no longer as directly applicable for the latest compounds where the adenosine as head group or hinge binder was replaced by purine–piperazine pairs or other moieties less analogous to adenosine. However, due to historical reasons the group retained the name 'ARC' for their inhibitors.

The aim of this study was to co-crystallize PKA in complex with ARC compounds in order to study the atomic details of their interaction. One attempt in this direction was conducted earlier in the lab of Dirk Bossemeyer at the 'German Cancer Research Center' in Heidelberg. Even though the structure of one ARC in complex with PKA could be solved [43], the results did not resolve entirely all questions. The complexed compound ARC-1034 is a rather short representative of its kind, with low potency (see Figure 1 and Table 1 in paper I). Consequently the complex structure of ARC-1034 and PKA (PDB ID: 3BWJ) did not verify the bisubstrate character of ARCs nor did it depict the mechanism of action that leads to sub-nanomolar binding affinities of the

best ARC compounds. Additionally, even though the co-crystallization of ARCs and PKA was proven feasible, it actually succeeded in only one case of over ten attempts. This is because the PKI peptide was, and had to be, absent from the crystallization conditions. Usually PKA is crystallized in complex with the 5-24 fragment of the PKI inhibitor peptide bound at the peptide substrate site (Figure 3.2). (5-24)-PKI stabilizes the kinase domain and thereby facilitates the crystallization of PKA. The aim of this project was therefore to produce the crystal structures of more PKA:ARC complexes to prove their bisubstrate binding mode, but also to integrate biophysical binding data with protein-ligand interaction in the complex structures, and to create a knowledge base for further ARC development.

5 Paper II

6 Paper III

6.1 Motivation and summary

Protein molecules and hence also protein crystals consist prevalently of the light atoms carbon, nitrogen, oxygen and sulfur. This allows for tracing of heavy atoms in the crystal lattice through their anomalous scattering signal. Anomalous dispersion methods are mainly used to solve the phase problem in structure determination [44], but also to identify the binding site and orientation of heavy atom-labeled inhibitor fragments in crystal structures [45, 46]. The former company 'Structural Genomix' (re-branded to 'SGX Pharmaceuticals' and eventually merged with the pharmaceutical corporation 'Eli Lilly') employed the latter approach in large-scale in their so-called '$FAST^{TM}$ Library', a collection of molecule fragments also used to develop kinase inhibitors. The efforts to develop enzyme inhibiting drugs of 'Structural Genomix' proved successful. However, many aspects of incorporating heavy atoms (primarily bromine) in the fragments, as the exact method of data processing or the use of the anomalous signal of the fragments in complex crystal structures, remained proprietary and unpublished intellectual property.

The work leading to the following manuscript was a proof of concept to test the use of bromine-labeled inhibitors for structure assisted drug design. Employed as model was protein kinase A and the PKA-targeting inhibitor H-89, which features a bromobenzene moiety [47]. Of additional interest was if the anomalous signal of this moiety would shed light on its binding flexibility which was indicated by the PKA:H-89 co-crystal structure 1YDT [48].

The manuscript 'Anomalous dispersion analysis of inhibitor flexibility - a case study on H-89' is in the state of 'in preparation prior to submission'.

7 Discussion

This chapter represents the general discussion of my PhD work and complementary discussion of some aspects that did not find their way in the publications. However, aspects sufficiently discussed there will not be reviewed.

7.1 Perspectives for the bisubstrate kinase inhibitor design

Several small molecule protein kinase inhibitors with an ATP competitive mode of action are on the market to date [14]. This by itself does not proveATP site inhibition to be the best way of kinase activity modulation in all cases. So it is conceivable that such inhibitors may fail for kinases that feature an ATP binding site not sufficiently unique for drug targeting. But what could be the right targeting strategy in such a case?

Non-ATP competitive inhibitors that bind outside the ATP cleft have been described for a range of protein kinases (reviewed by Kirkland & McInnes [49]). This is the most obvious approach to avoid off-target inhibition and the unfavorable competition with high intracellular ATP concentrations faced by ATP competitive kinase inhibitors. However, off-ATP-cleft inhibition requires the target kinase to feature a 'druggable' binding pocket that is essentially involved in its activity response, as for example a site of substrate- or co-factor binding. Unfortunately, only a few protein kinases are known to be amenable to this strategy of drug targeting.

A second approach for the design of non-ATP competitive protein kinase inhibitors are compounds that bind to a precursor ATP cleft in an inactive conformation [50]. This strategy is of dual benefit. The conformation of such inactive states is less conserved than those of the active kinase domain, as they can be specific to certain families. Targeting inactive states may therefore lead to the identification of more selective inhibitors. Secondly, inhibitors targeting and stabilizing the inactive state will be less likely to compete with ATP for binding and hence may be less impacted by high intracellular concentrations of ATP. However, many kinases, for example PKA, do not present an inactive state

of its kinase domain and require different inhibition strategies.

Due to their extreme potency ARC bisubstrate type inhibitors have proven useful for a wide range of applications for *in vivo* and *in vitro* characterization [51, 42]. However, as pharmaceutical inhibitors they are still in early phases of pre-clinical discovery. The ARCs bisubstrate inhibitor property so far has not been shown to enable sufficient target specifity [52]. In any case, the knowledge gained with ARCs so far could be valuable for optimizing existing ATP competitive compounds by improving affinity and adjusting target selectivity. With increasing finesse in the process of bisubstrate inhibitor design one can hope that soon it will be possible to 'mix and match' existing ATP competitive moieties and peptide pseudo-substrates to tailor compounds to the desired target selectivity.

The bisubstrate approach offers the opportunity, by combining the selectivity potential of the peptide substrate site with the combined affinity and selectivity potential, of the ATP cleft, to make peptidic kinase inhibitors more drug-like in terms of size, proteolytic stability and cell plasma membrane permeability [42]. This enables a new approach of kinase inhibitor design. Instead of focusing on one specific kinase, one could focus on the phosphorylation of one specific consensus site. The respective peptide sequence could be incorporated into an ARC type compound in order to create a selective multi-kinase inhibitor, and thereby overcome the common redundancy of protein kinase activity. Multi-target selectivity was observed for several protein kinase inhibitors already approved for the treatment of patients [53] and might therefore be rather a necessity than a disadvantage; for example by lowering the likelihood of drug resistance mutations.

Above I presented three targeting strategies for small molecule protein kinase inhibitors that have been successfully employed to date: ATP competitive inhibition, inhibition of non-ATP pocket sites, and inhibition or preservation of inactive conformation ATP clefts. All three strategies have proven successful but at the same time are associated with a spectrum of advantages and disadvantages, resulting in their application in specific niches. The niche for ARC type bisubstrate inhibitors in pharmaceutical application remains to be defined. However, it appears promising that in the future bisubstrate kinase inhibitors will complement gaps in the 'druggable kinome'.

7.2 Protein kinase A peptide interactions

The binding modes of ARC-670, ARC-1012 and ARC1039 reflect the conservation of PKAs consensus site in an interesting way. PKA is known to recognize phosphorylation sites presented in a context of basic amino acids; especially the motif **Arg-Arg/Lys**-X-*Ser/Thr*, but also **Arg**-X-*Ser/Thr* and **Lys-Arg**-X-X-*Ser/Thr* [54]. Recent compilations of data expanded this to include the recognition of basic amino acids (preferably arginine) in all positions from position −2 to position −7 N-terminal from the phosphorylizable residue [55]. However, the interaction between PKA and basic residues further than four positions away from the phosphorylation site seems to be less conserved (see publication I) and seems therefore less important.

Just one interaction of PKA and the pseudosubstrate PKI is reproduced by all three ARC compounds in this study, namely the one between PKIs arginine at position −2 from the phosphorylation site and PKAs glutamate 170 and 230 (see Figure 7 in publication I). ARC-1012 and ARC-1039 achieve this with their D-arginine 2, ARC-670 with its D-arginine 6 (see Figure 7 in publication I). In all three cases this is the residue of the inhibitor's peptide moiety to feature the strongest electron density, highlighting the strength and importance of this interaction. In contrast, the interaction of PKI's arginine at position −3 from the phosphorylation site to PKA via glutamate 127 is only imitated by ARC-670 and ARC-1039, even though it is achieved through different conformations (see Figure 7 in publication I). ARC-1012 however, although it seems to have the freedom to take the same conformation as ARC-1039, does not interact with glutamate 127 in the respective structure. Surprisingly, ARC-1012 features a higher affinity to PKA than ARC-1039 (see Table 1 in publication I). This indicates that the basic residue at position −2 in the consensus site is of more importance for peptide substrate binding to PKA than the one at position −3.

7.3 The benefit of PKA-based chimaera

The potential use of PKA-based chimaera is described extensively in publication II. However I would like to use this opportunity to illustrate the benefit of this rather unorthodox approach by summing up the 'career' of the PKA-based chimaera for protein kinase B/Akt published by Gassel et al. [56]. Among others the company Astex Pharmaceuticals employed the chimaera for the structure based screening, characterization and design of ATP competetive inhibitors of protein kinase B, leading to selective

compounds and eight publications [57, 58, 59, 60, 61, 62, 63, 64]. Even though Astex eventually succeeded to crystallize protein kinase B [64], they still continued utilizing the PKA-based chimaera, probably due to better crystallization properties and the high stress tolerance of PKA crystals. Altogether at least 30 crystal structures of the PKA-based chimaera for protein kinase B complexed with different ligands were deposited in in Protein database (www.pdb.org). The chimaera approach was evaluated by Thomas G. Davies from Astex. He came to the following conclusion: "The determination of the structure of activated PKBβ complexed with AMP-PNP was an important step towards using a structure-based approach to PKB inhibitor development, but design studies presented in the literature have until now utilized the experimentally more amenable kinase, PKA, as a surrogate. This has proved to be a useful approach, and in particular, PKA-PKB chimaeric structures have provided important insights into molecular recognition with these kinases." [59].

7.4 The limitations of structure based drug design

One of the motivations for the project leading to publication II 'Mutants of protein kinase A that mimic the ATP-binding site of Aurora kinase' was to identify selectivity determinants for inhibitor binding. Due to the uniform function of protein kinases the ATP cleft is highly conserved throughout the kinome, in both the backbone folding as well as the peptide sequence. Even the least related human protein kinases differ only in a minority of amino acid residues in the respective parts of their sequence. Consequently, these residues must be the determinants of the varying selectivity of small molecule ATP competitors towards different kinases. This point is clearly shown in the paper. The mutations in the ATP cleft obviously modulate inhibitor selectivity and allow to swap the selectivity profile of one kinase to that of another one, consistent with results of earlier PKA-chimaera work [65, 56]. However publication II also demonstrates many limitations of our understanding of protein-ligand interactions. Integrating the physical binding data of the various mutants and the compounds with the protein-ligand interaction seen in the crystal structures is not possible in all cases. Why, for example, does the mutant Q183K_T183A_V123A possess the highest affinity for the compound VX-680, rather than the final Aurora-chimaera, or why do all six mutations alone improve binding of the compound H-89, but in combination in the final chimaera exhibit decreased H-89 affinity? While the data allows for speculation, methods for quantitative prediction are lacking. We not only struggle to predict protein-ligand interactions [66, 67], we also

struggle to understand them after they are measured and observed.

This limited ability to evaluate the interactions of experimental or theoretical protein-ligand complexes shapes the drug development process. Protein kinases might be an especially difficult target class for drug development due to their high degree of structural conservation and ubiquity with more than 500 protein kinases in the human genome [1]. However, *in silico* prediction is inadequately quantitative also for other enzyme inhibitors, as current methods do not adequately address the flexibility and other sources of complexity of protein molecules. Drug development therefore remains a process of trial and error, a repetition of screening, analysis and optimization, while computational prediction methods are supportive in this process.

8 Conclusions

- The crystal structures of ARC type inhibitors in complex with protein kinase A confirm the general model of bisubstrate binding and provide insight into the details of the binding geometries and interactions, thus revealing features of ARCs where modifications are tolerated or desired. This enables further rational development of ARCs, whereby optimization of the compounds may include efforts to reduce their molecular mass, to ease their synthesis, or to modulate target selectivity or cell permeability. Moreover, the bisubstrate nature of ARCs revealed here expands the range of possibilities to 'mix and match' chemical moieties in strategies to target other kinases, by identifying binding sites and linker properties outside of the ATP binding pocket. They also advance our understanding of the substrate- binding patterns of PKA.

- The data on the chimaeric protein kinase A give confidence that its use in co-crystallization with Aurora-specific inhibitors, or in activity-based screenings, will prove valuable in discovering inhibitors and evaluating their binding modes. The transfer of the Aurora kinase ATP binding site on to the crystallographically more accessible PKA opens the door to high throughput inhibitor structure determination, and raises the possibility of dissecting binding and folding processes and analyzing the effect of protein scaffold upon affinity.

- The use of bromine-labeled enzyme inhibitors in co-crystal structures was tested and shown useful for the tight binding PKA inhibitor H-89. The method identified a dual binding mode not apparent with standard methods. Progress toward quantitative understanding of ligand binding energies will require an ability to distinguish such features of protein-ligand interactions.

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