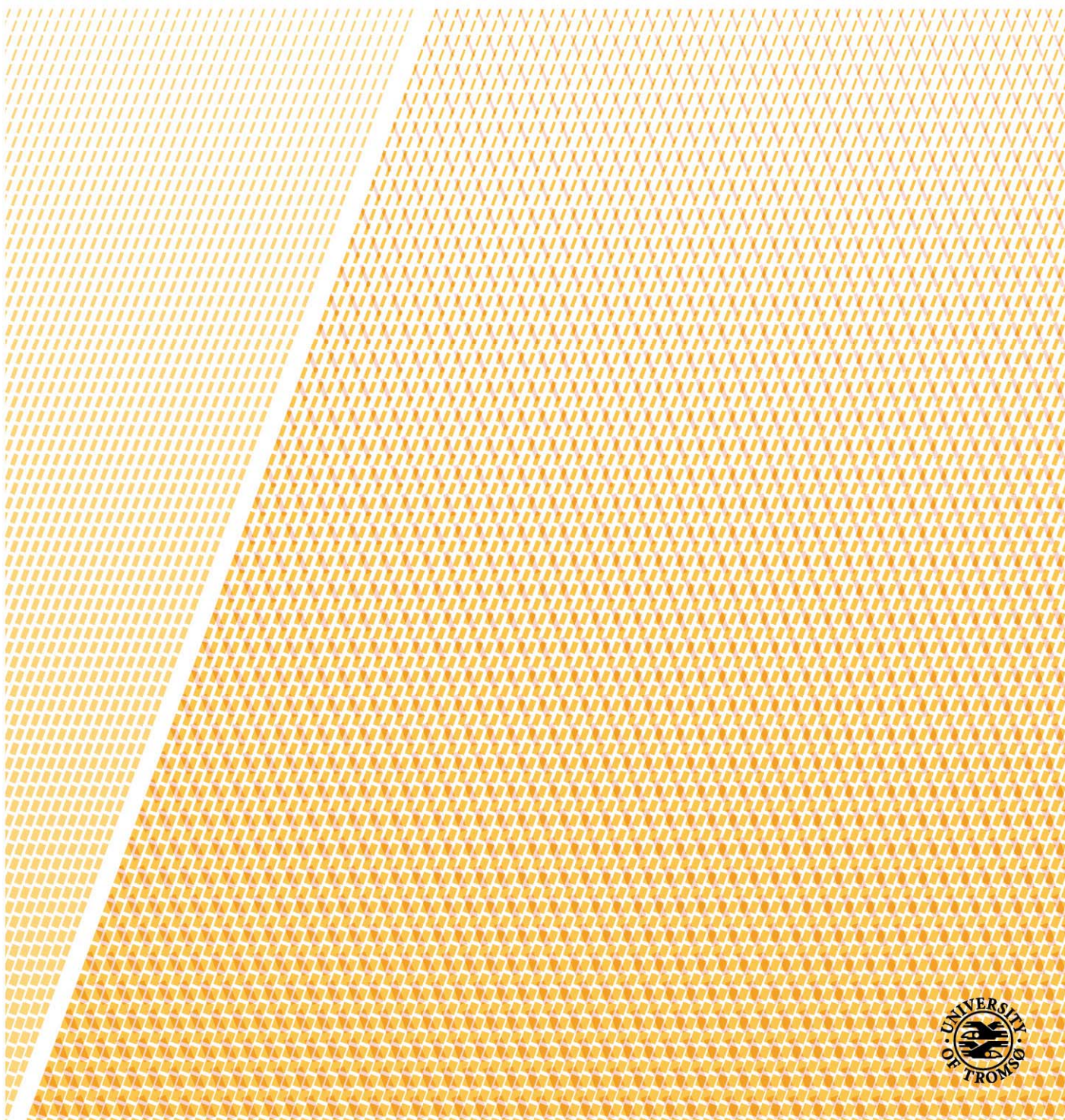Faculty of Science and Technology

Department of Chemistry

Studies on selectivity determinants of protein kinase inhibitor binding
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Kazi Asraful Alam

A dissertation for the degree of Philosophiae Doctor – March 2017

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March, 2017.

To my beloved Family

Acknowledgements

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Summary

Protein kinases are involved in many essential cellular processes, and are regulated in a dynamic manner by the movement of domains or motifs via interaction with various proteins and substrates. Protein kinase deregulation can lead to a variety of diseases, including cancer and diabetes. Protein kinase A (PKA) has been a prototype to study the entire family, including for studies in drug discovery research. However, the high sequence similarities in the kinase domain of protein kinases hinders the development of target-specific inhibitors, and the use of PKA alone is insufficient. Mutants of PKA can act as surrogate targets to aid the design of target specificity. In this work, we use a combination of several biophysical methods to investigate the properties of PKA, PKA based surrogate kinases (PKAB3 & PKA-Au6), the cancer target Aurora kinase, including especially their interactions with inhibitors. We characterized a series of 32 enantiomerically pure inhibitors with respect to interactions with protein kinase A (PKA) and its mutant PKAB3 as a PKB surrogate. The ligands bind to the hinge region, ribose pocket, while their substituted aromatic rings (phenyl, thienyl) bind to the glycine-rich loop at the ATP site. Biological assays show their high potency against both PKA and PKAB3, generally with preference to PKA. The crystal structures reveal a multi-facetted network of ligand–glycine-rich loop interactions, with efficient water replacement contributing to the binding strength. Site-directed mutagenesis and biophysical characterization of the glycine–rich loop mutants (T51G and G55A-PKA) show weakened affinities against tested inhibitors: 1i, 1p, 1c and H-89. We also show structural effects of the aromatic residue phenylalanine that is highly conserved among the AGC kinase group, corresponding to Phe327 in PKA, and its role in inhibitor binding. The inhibitor-Phe327 interactions reveal a complex mix of favorable and unfavorable contacts: Phe327 can block inhibitors from occupying the ATP-binding pocket, but may also be displaced to enable tight binding. The PKA-based Aurora kinase B (PKA-AU6) model, which shares this Phe327, provides examples of crystal structures (PDB ID: 5N23) showing effects on the binding affinities of the pan-Aurora

inhibitor AT9283, and also the novel tricyclic dianilino-pyrimidine inhibitors (1B & 2A). The pan-kinome inhibitor staurosporine is known to be able to displace Phe327. We also observe how bisubstrate inhibitors can be used for studies of strong protein peptide-interactions. In summary, this work advances the understanding of how subtle aspects of flexibility, water structure, and chemical interactions determine inhibitor binding kinetics and energetics. Such detailed understanding is required for truly effective structure based drug design.

List of Papers

Paper I

Addressing the Glycine-Rich Loop of Protein Kinases by a Multi-Facetted Interaction Network: Inhibition of PKA and a PKB Mimic. Chem. Eur. J., 22: 211–221. doi:10.1002/chem.201503552.

Birgit S. Lauber, Leo A. Hardegger, **Alam K. Asraful**, Bjarte A. Lund, Oliver Dumele, Michael Harder, Bernd Kuhn, Richard A. Engh, and François Diederich.

Paper II

Flexibility properties of the protein kinase glycine-rich loop are critical for inhibitor binding.

Kazi A. Alam, Osman A. B. S. M. Gani, Tony Christopeit, and Richard A. Engh. (Manuscript).

Paper III

Inhibitor induced structural effects involving Phe327 in AGC kinases.

Kazi A. Alam, Ulli Rothweiler, Osman A. B. S. M. Gani, and Richard A. Engh. (Manuscript).

Paper IV

Bifunctional Ligands for Inhibition of Tight-Binding Protein–Protein Interactions. **Bioconjugate Chem**., 2016, 27 (8), pp 1900–1910. DOI: 10.1021/acs.bioconjchem.6b00293.

Taavi Ivan, Erki Enkvist, Birgit Viira, Ganesh babu Manoharan, Gerda Raidaru, Alexander Pflug, **Kazi Asraful Alam,** Manuela Zaccolo, Richard Alan Engh, and Asko Uri.

Paper V

Assessing protein kinase target similarity: Comparing sequence, structure, and cheminformatics approaches. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics. Volume 1854, Issue 10, Part B, October 2015, Pages 1605–1616.

Gani, Osman A.B.S.M.; Thakkar, Balmukund; Narayanan, Dilip; **Alam, Kazi Asraful**; Kyomuhendo, Peter; Rothweiler, Ulli; Tello-Franco, Veronica; Engh, Richard Alan.

Paper VI

On methionine as a gatekeeper residue for protein kinase inhibition targets.

Dilip Narayanan, **Kazi A. Alam**, Matthias M. Engh, Richard A. Engh, Osman A.B.S.M Gani. (Manuscript).

Abbreviations

Aurora A	Aurora kinase A
Aurora B	Aurora kinase B
Aurora C	Aurora kinase C
ABL	Abelson murine leukemia viral oncogene homolog 1
AGC	Protein kinase A, G, and C families
ATP	Adenosine -5'- triphosphate
AMP-PNP	Adenylyl-imidodiphosphate
BRCA1	Breast cancer-associated gene 1
CDK	Cyclin-dependent kinase
cAMP	Cyclic-3',5'-adenosine monophosphate
CPC	Chromosomal Passenger Complex
DNA	Deoxyribonucleic acid
DSF	Dynamic scanning fluorimetry
GTP	Guanosine -5'- triphosphate
HM	Hydrophobic motif
INCENP	Inner centromere protein
IC_{50}	The half maximal inhibitory concentration
JAK	Janus kinase
kDa	kilodalton
LB	Luria-Bertani
MAP	Mitogen-activated protein kinase
MPD	2-Methyl-2,4-pentanediol
nM	Nanomolar
PKA	Protein Kinase A
PKAc	Catalytic subunit of Protein Kinase A
PKAr	Regulatory subunit of Protein Kinase A
PKB	Protein Kinase B
PI3K	Phosphatidylinositol 3 Kinase
PtdIns	Phosphatidylinositol
PDK1	Phosphoinositide dependent protein kinase-1
PDK2	Phosphoinositide-dependent protein kinase-2
PKI	Heat stable protein kinase A inhibitor peptide
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PH	Pleckstrin homology
PTEN	Phosphatase and tensin homolog
PEP	Phosphoenolpyruvic acid
RTK	Receptor tyrosine kinase
RNA	Ribonucleic acid
SPR	Surface Plasmon Resonance
SUMO	Small Ubiquitin-like Modifier
TPX2	Targeting Protein for Xenopus kinesin-like protein 2
TACC	Transforming acidic coiled coil
TB	Terrific Broth
uM	Micromolar
wtPKA	Wild type Protein Kinase A

1.Introduction

1.1. Protein phosphorylation

1.1.1. Mechanism

Protein phosphorylation plays a central role in biological processes. Almost every cellular event is regulated in part through protein phosphorylation which is responsible for modulating biological activity and controlling localization in the cells [1]. The fascination of the discovery of protein phosphorylation was in recognizing how information about an external signal could be transferred to the correct intracellular recipient by a clearly defined chemical process. By doing this across a wide range of signals and cellular components, phosphorylation orchestrates the cellular activities for total harmonious function. Phosphorylation of proteins is carried out by protein kinases which transfer terminal phosphate of ATP to the substrates or other proteins and thus modify the target proteins (Figure 1.1).
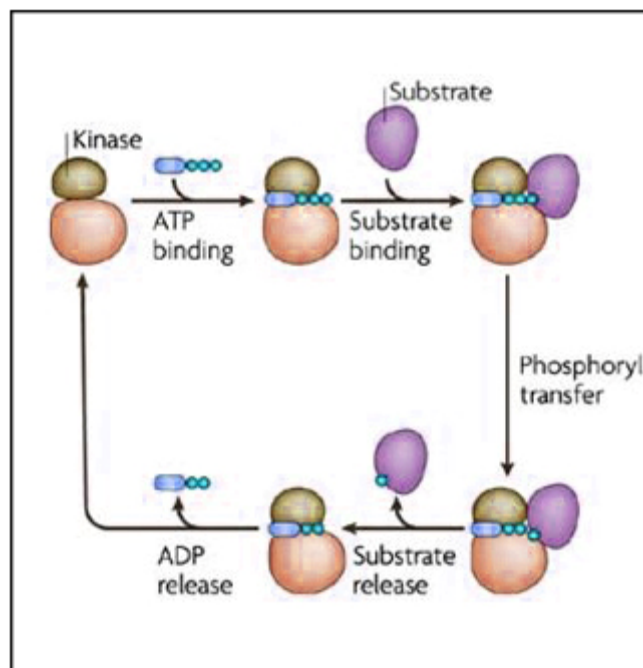
Figure 1.1: Protein kinases carry out phosphorylation using a mechanism whereby ATP first binds to the active site of the enzyme, followed by substrate binding. The kinase phosphorylates the substrate by transferring the terminal phosphate from ATP to the substrate. The figure is adapted with permission (Ubersax and Ferrell) [2].

There are nine amino acids which can be phosphorylated, using either ATP, GTP or PEP as a phosphate donor, under physiological conditions [3]. The phosphorylation of different amino acids is typically carried out by different protein kinases. Thus, serine/threonine kinase responsible for serine/threonine phosphorylation and tyrosine kinase for tyrosine residues. Phospho-threonine and serine residues are more abundant than phospho-tyrosine [4]. Although most commonly studied event of the cell signaling processes is protein phosphorylation, much information regarding these processes remains unknown. About 30% of cellular proteins are estimated to be phosphorylated, and the human genome encodes more than 500 protein kinases [5, 6].

1.1.2. Brief history of protein phosphorylation

The activity of protein kinase was first described by George Burnett and Eugene P. Kennedy in 1954 during their work on rat liver mitochondria. They found that casein protein is phosphorylated by mitochondrial enzyme [7]. In 1956, Krebs and Fischer observed that the phosphorylase enzyme catalyzes glycogen breakdown, is phosphorylated by phosphorylase kinase, and dephosphorylated by another enzyme called phosphorylase phosphatase [8]. Protein phosphorylation studies increased significantly after the discovery of cyclic AMP-dependent protein kinase (PKA) by Walsh, Perkins, and Krebs, in 1968, which provide clues that it can phosphorylate more than one protein [9]. Calcium-dependent protein kinase activity was observed in rat brain, attributed to diacylglycerol activated protein kinase C activity [10, 11] . In 1991, Sir Philip Cohen discovered that glycogen synthase is phosphorylated at nine serine residues by different kinases [12]. The protein v-src is encoded by the transforming gene of Rous sarcoma virus. In the late 1970s, Ray Erikson discovered that v-src is a kinase which phosphorylates tyrosine residues [4, 13] and can act as an oncogene. The discovery of JAK kinases led to expanded interest in signaling pathways. The discovery of MAP kinase in the 1986 by Ray and Sturgill added more information regarding protein kinase cascade. They discovered insulin dependent

activation of a serine threonine kinase that phosphorylated microtubule-associated protein-2 (MAP2) [14]. With the discovery that therapeutic inhibition of protein kinases is a viable approach, research into protein kinases and their inhibitors has increased dramatically over the last few decades.

1.2. Summary of kinases in focus for this thesis

1.2.1. Protein kinase A

PKA has long been a prototype for general studies on protein kinases. The activity of PKA, also known as cAMP-dependent protein kinase, or phosphorylase kinase kinase, is dependent on the level of cAMP [9]. cAMP binding dissociates the regulatory subunit from an inactive tetrameric PKA, releasing the active catalytic subunit. The active enzyme catalytic domain has been used as a research and structural model for studies of a broad range of kinase properties, including regulatory mechanisms, recognition of substrate and binding, phosphoryl transfer mechanism, in part because of well established bacterial expression protocols and reproducible crystallizability [15, 16].

1.2.1.1. Structure of protein kinase A

In the early 1990s, the first ternary complex of the catalytic subunit of PKA with a pseudosubstrate inhibitor peptide, PKI (5-24) (TTYADFIASGRTGRRNAIHD) in the presence of the MgATP analogue MnAMP-PNP was solved, at resolutions around 2.0 Å [16-18]. The full length PKI inhibitor, heat stable up to 90° C, contains at its amino terminus (5-24) the kinase inhibitory domain [19, 20]. In the presence of MgATP, it has a very high affinity towards PKA (with a Ki of approximately 2nM) [21, 22]. PKA is a tetramer holoenzyme which contains a regulatory (R) homodimer subunit and two catalytic (C) subunits in the inactivated physiological state [18]. The enzyme consists of two lobes, the N-terminal lobe is quite flexible, consisting mostly of beta-sheets. The C-terminal lobe is mostly helical and contains a segment for activation that often includes residues whose phosphorylation activates the kinase. The two lobes are connected by a polypeptide segment called the hinge. A cleft between the two lobes

forms the ATP binding pocket and contains the catalytic residues [16, 18, 23] (Figure 1.2).

The N-terminal lobe has five anti-parallel beta stands and an alpha helix which is located between the β3 and β4 strands. The N-lobe is also primarily responsible for nucleotide binding, as most of the interactions with MgATP involve this lobe. There is a consensus sequence of GxGxxGxV in the N-lobe, called the glycine-rich loop (in PKA numbering: Gly50-x-Gly52-x-x-Gly55-x-Val57), which covers the nucleotide. In contrast, the C-lobe contains seven α-helices and, compared to the conserved protein kinase core, has an additional C-terminal extension. This domain contains the substrate recognition elements and catalytic residues. The hinge region (residues 120-127) between two lobes also plays a role in peptide recognition [16, 18].
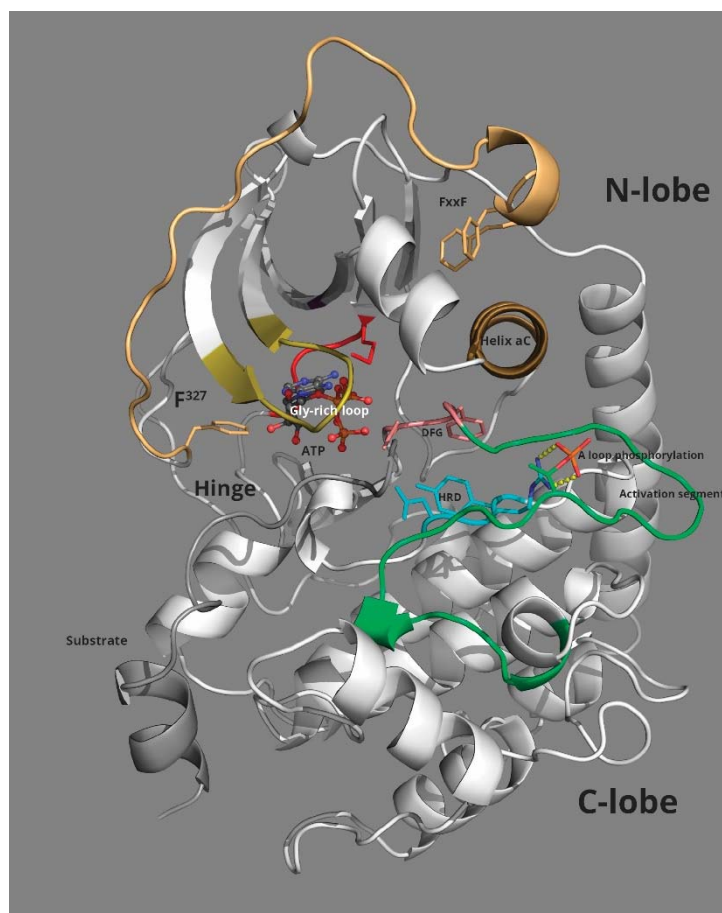
Figure 1.2: The catalytic subunit of PKA as a prototype for AGC kinases. The N-lobe and C-lobe joined by hinge (grey), ATP (red) binds in the pocket formed by N and C-lobe under the glycine-rich loop (dark yellow). The DFG, HRD, activation segment and Helix C show as dark magenta, cyan, green and dark yellow, respectively.

A lysine residue (Lys72) from the β3 strand of the N-lobe coordinates the α- and β-phosphates of the ATP molecule, but is unable to make contacts with the γ-phosphate without the movement of β-strands [16]. Biochemical data also shows the critical role of this lysine residue in recognition of phosphates [24, 25], and it is often mutated to create "kinase dead" mutants as a control for activity studies.

As mentioned above, the C-lobe, large compared to N-lobe, is primarily α-helical and is associated mostly with peptide binding and catalysis. The helices (from αD-αI) are conserved in eukaryotic protein kinases (EPKs) [26]. In PKA, helices E and F are hydrophobic, connected by a β-structure at their surface. The catalytic loop connected by the β- strand 6 and 7. The activation segment of PKA starts with widely known conserved motif called DFG motif (Asp184-Phe185-Gly186) and end of αF helix. The DFG motif is present between β strands 8 and 9 [27] . The role of Asp184 of DFG is to bind to the Mg ion, which coordinates the β and γ-phosphate [16]. The activation segment contains Thr197 which appears to be autophosphorylated and important for kinase activity. This phosphorylation also observed in recombinant mouse enzyme [16, 18, 28].

1.2.1.2. Function and Regulation

Protein kinases are modular proteins whose activities are modulated dynamically by chemical modification (especially phosphorylation, but also e.g. the attachment of membrane binding tethers), intra- and intermolecular complex formation, including attachment to scaffolding proteins. PKA is ubiquitously expressed and regulates key biological processes in differentiation and proliferation of the cell, metabolism and memory formation [29, 30]. Like protein kinases in general, the regulation of PKA also includes associated with their interacting proteins, domains and transient movement of motif or linker [31].

The cAMP-dependent PKA is a tetramer complex composed of two regulatory and two catalytic subunits in mammalian cells. The holoenzyme—the inactive state—exists when there are low levels of cAMP (Figure 1.3). Above a threshold concentration of

cAMP in cells, cAMP binding to the regulatory subunit releases the catalytic subunit which is then active and carries out its function of protein phosphorylation [32, 33]. Thus, the regulatory subunit acts as a docking site for the cAMP. Additionally, the holoenzyme (two regulatory with two catalytic subunits) plays a role in cell localization. Usually, the holoenzyme present in cytoplasm but when regulatory subunits are released, the catalytic subunit is moved to the nucleus [34-36]. There are three isoforms (Cα, Cβ and Cγ) for catalytic subunit and four isoforms of the regulatory subunit (RIα, RIβ, RIIα and RIIβ) present in mammalian cells.
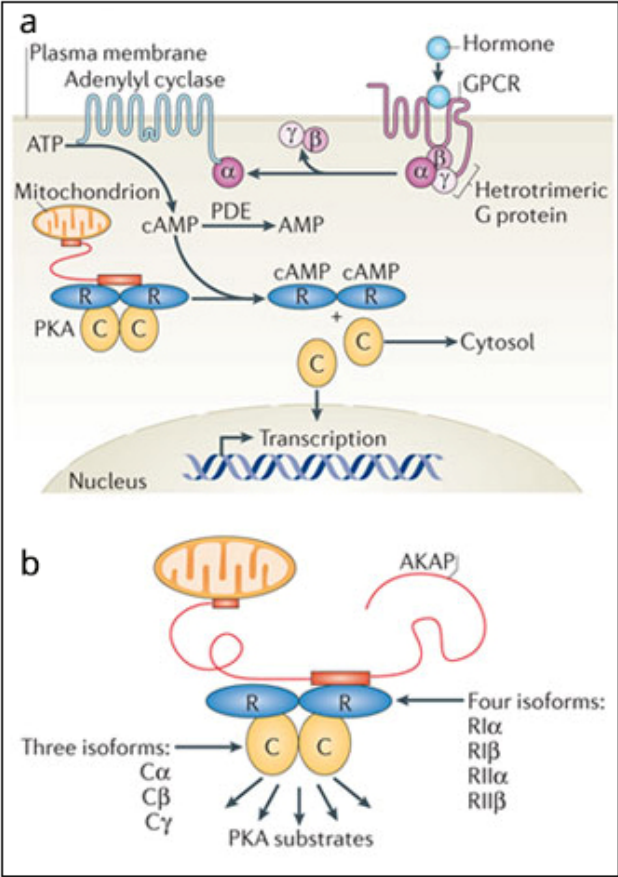
Figure 1.3: Protein Kinase A activated through the external signal via cAMP. a). PKA has a function in cytoplasm and nucleus. The catalytic subunit is released from regulatory part due to the abundance of cAMP in cells and the kinase ultimately acts in the biological event by phosphorylation. b). The catalytic and regulatory subunit has three (Cα, Cβ, and Cγ) and four isoforms (RIα, RIβ, RIIα, and RIIβ). The figure has taken from Susan S. Taylor, et.al. with permission [31].

The Protein Kinase A (PKA) has been widely used as a model for the understanding of cellular signaling mechanisms. As activation of PKA depends on the external signal (cAMP), and its total spectrum of activity depends on other proteins and in turn their phosphorylation or other translational modification states, fine tuning of PKA and substrates is pivotal to maintain signaling process fidelity and robustness. The first substrates of PKA were identified in 1980 and the number of known sites has increased dramatically since. So far, more than 100 PKA substrates have been identified. PKA phosphorylation consensus sequences have been described as Arg-Arg-X-Ser/Thr (RRX [S/T]), Arg/Lys-X-X-Ser/Thr ([R/K] XX[S/T]), and Arg/Lys-X-Ser/Thr([R/K] X [S/T]) [37].

1.2.2. Protein kinase B

Protein kinase B (PKB, also known as AKT) is a serine /threonine kinase which is a central enzyme in several intracellular signaling pathways. PKB has a major role in the PI3K pathway and responds to various type of signals. These responses include phosphorylation of a wide range of proteins involved in cell growth, survival, proliferation, and regulation of metabolism [38, 39]. Three PKB/AKT isoforms have been found identified, known as PKB_α, PKB_β and PKB_γ, respectively (also known as AKT1, AKT2 and AKT3). All PKB isoforms show similar domain structures and almost 80% sequence identity, but differ with respect to gene location (on chromosomes (14, 19, and 1) [40-44]. The function of PKB isoforms appear to be specific for survival, growth and metabolism, depending on the stimulus or localization [45]. PKB_α is necessary for trophoblast differentiation and regulates and maintenance placental development, PKB_β plays roles in animal growth, angiogenesis, β-cell function and glucose metabolism and PKB_γ help in brain development [46-49].

1.2.2.1. Protein kinase B /AKT and PI3K signaling pathway

The phosphatidylinositol 3-kinases (PI3K)/PKB pathway is a master regulator of various cellular response and plays a pivotal role in the growth, development, and metabolism. The PI3-kinase regulates different protein kinases upon phosphorylation

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and induces an array of responses. Receptor tyrosine kinases (RTK) are autophosphorylated by G-protein coupled receptors and binding to e.g. insulin or growth factors or integrin, and then activate PI3 kinase (Figure 1.4). The PI3-kinase generates the second messenger lipid product PtdIns(3,4,5)P3 from PtdIns(4,5)P2, and PKB interacts with the PtdIns(3,4,5)P3 via its PH domain, and is thereby translocated to the plasma membrane from the cytoplasm. It is believed that binding of PtdIns(3,4,5)P3 induces a conformational change in PKB upon which kinases phosphoinositide-dependent kinase1and 2 (PDK1 and PDK2) phosphorylate Thr308 and Ser473 residues of PKB [38, 50-55] . Deregulation of PKB by mutation or overexpression has been found in many cancer types, including melanomas, breast, ovarian, lung and pancreatic [39, 56-58]. More than 60% PKB overexpression is observed in prostate tumor cells, and an approximately 50% increase of PKB activity is found in breast and prostate tumor cells [59-62]. All three isoforms of PKB are found overexpressed in different cancers [63].
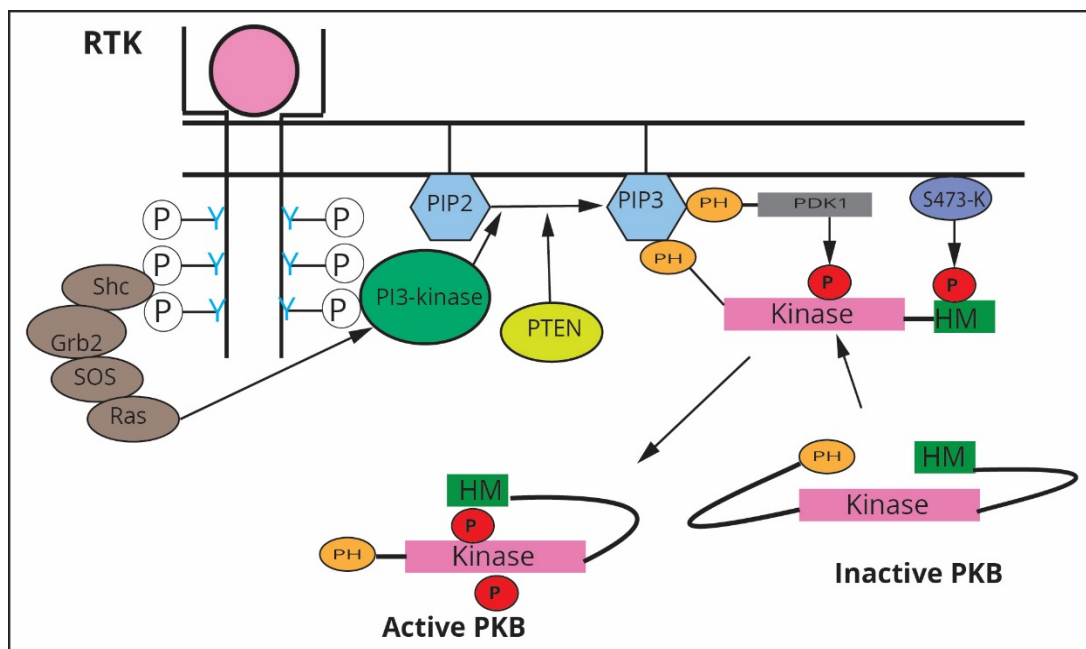
Figure 1.4: PKB/AKT activation mechanism. Receptor tyrosine kinase (RTK) activates by ligand followed by phosphatidylinositol 3-kinase (PI3-kinase) to produce phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PH domain of PKB and PDk1 interact with PIP3. PKB becomes phosphorylated by PDK1 and S473-K and activated PKB moved from cytosol to nucleus.

1.2.2.2. Structural features and regulation of PKB

Protein kinase B, as a member of the AGC kinase group (PKA, PKC, PKC, PDK1, SGK, etc.), phosphorylates serine or threonine amino acid residues and share structural similarities with the rest of the group [64, 65]. More than 80% sequence identity are shared between PKB_α, PKB_β and PKB_γ [66] (Figure 1.5). All PKB isoforms share a conserved domain architecture, including a kinase domain, an N-terminal pleckstrin homology (PH) domain, and a C-terminal regulatory domain which contains the AGC kinase characteristic hydrophobic motif [67] (Figure 1.6).

The N-terminal PH domain is well known as a modular domain, contains approximately 100 amino acids, and is present in over 200 gene encoding proteins [68, 69]. The PH domain was first discovered in platelet protein pleckstrin; later, Bruce J. Mayer and co-workers termed it the pleckstrin homology (PH) domain [69, 70]. The PH domain of PKB and PDK1 coordinates PIP3 in different orientations [67]. It provides the mechanism for PKB activation through interacting with PI3-kinase products and anchoring the PH domain-containing proteins to the membrane [71, 72]. PI3-kinase signaling from platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) via PKB is dependent on PH domain [71, 73].
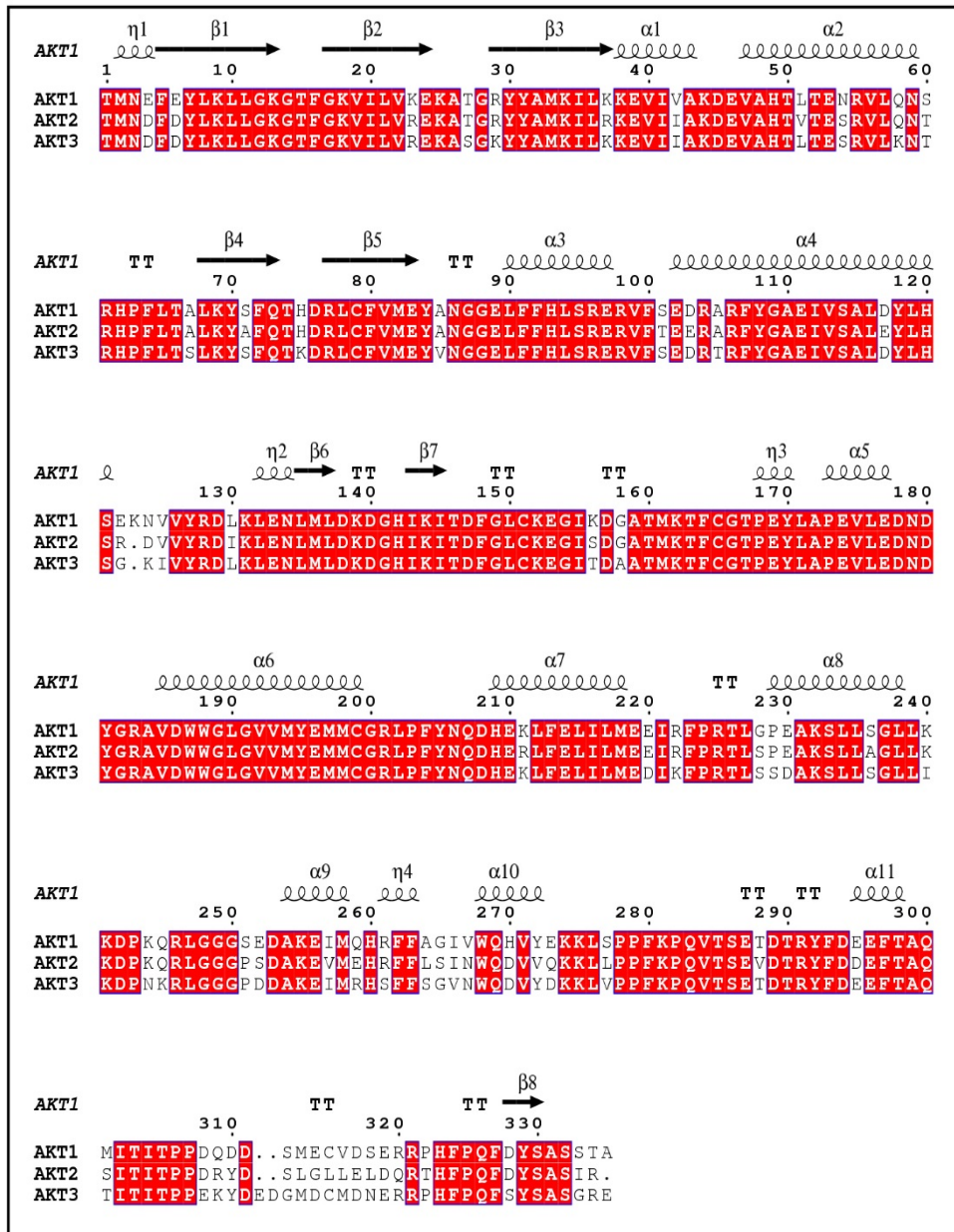
Figure 1.5: Multiple sequence alignment of PKB/AKT isoforms (AKT1, AKT2, and AKT3, respectively) of *Homo sapiens.* The numbering and secondary information refers to AKT1.

The similarities that the catalytic domain of PKB kinase shares with the rest of the AGC kinase family include its dependence on the phosphorylation of conserved thr308 residues in the activation loop. The activation loop is defined between DFG and APE motifs [74, 75]. Along with the Thr308, there is another phosphorylation site present in the C-terminal strand of the kinase domain, at a segment known as a regulatory hydrophobic motif. The consensus sequence is F-X-X-F/Y-S/T-Y/F (where X is any amino acid) and is common not only to all PKB isoforms but also to most other

members of the AGC kinase family. For PKB, phosphorylation of Ser474 in the hydrophobic motif appears to be essential to acquire full kinase activity and the crystal structure of PKB shows that interactions with the phosphorylated segment order the αC helix from a previously disordered state [76-78].
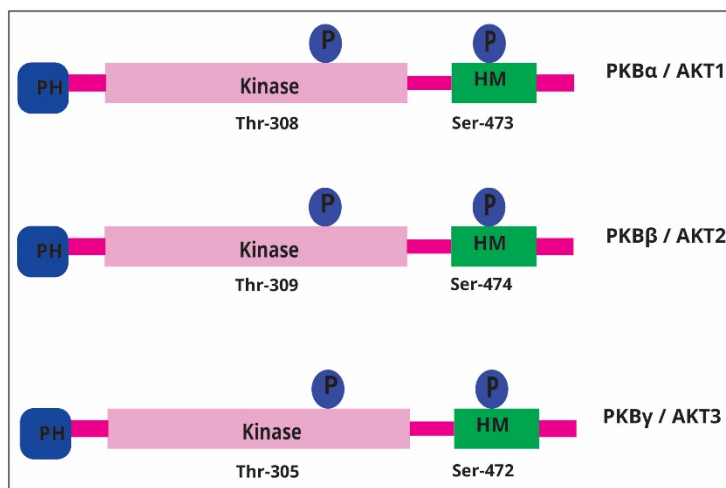
Figure 1.6: The architecture of PKB/AKT isoforms. The kinase domain in the central region. The PH (pleckstrin homology) and hydrophobic motif (HM) is located at the N and C-terminal adjacent to the kinase domain. Phosphorylation sites are indicated as a blue circle.

1.2.2.3. Drug discovery (PKA as surrogate)

PKA is a close homologue of PKB and their kinase domains and the ATP binding sites share approximately 45% and 80% sequence identity, respectively [79]. The α-isoforms of PKA and PKB have only two residues that differ in their adenine-binding site; these are valine123 and veucine173 in PKA, corresponding to alanine and methionine, respectively, in PKB [80]. PKA has been used for the studies of ligand-protein interactions, especially for anti-cancer drug design of PKB because of rapid and easy crystallization process, and difficulties of getting native PKB crystals. It has also provided additional features to study selectivity between PKA and PKB [79]. The crystal structure of PKAB2 (PKAα V123A, L173M) shows the side chain of Q181 residue close to the hinge region of ATP binding site which is distant in the native PKA crystal structure. An additional mutation in PKA (Q181K) corrected the model, as confirmed by the crystal structure of PKAB3 (PKAα V123A, L173M. Q181K) and surface plasmon resonance studies [80].

The challenge of developing PKB inhibitors is to get not only selectivity and specificity among the three isoforms, but also against other protein kinases, as they share high sequence similarity in ATP binding pocket. Most of the tight binding inhibitors target the ATP site. However, there are examples that show inhibitors may be based on additional or allosteric sites, providing specificity derived from PH domain interactions, for example, to thereby specifically block activation [81-83]. More than 10 PKB inhibitors are in clinical testing stage, and approximately 5 inhibitors are in clinical phase 2.

1.2.2.4. ATP Site targeting inhibitors

Compounds developed in recent drug discovery programs include ATP-competitive PKB inhibitors based on chemical moieties azepane (balanol), aminofurazans (GSK690693), pyrazole (AT7867, AT13148) and thiophenecarbxamide derivatives (GSK2110183, GSK2141795) [84]. Afuresertib (GSK2110183) is a pan-PKB inhibitor which is in clinical phase 2. The inhibitor is orally administered, potent with Ki values of 0.08 nM, 2 nM, and 2.6 nM for Akt1, Akt2, and Akt3, respectively [85]. Afuresertib was developed using high throughput screening which identified a 2-pyrimidyl-5-amidothiophene derivative which, based on structure-activity analyses and replacement of the pyrimidine with pyrazole, lead to the potent PKB inhibitor (Figure 1.7). The replacement of thiophene ring with a furan ring in afuresertib resulted in uprosertib which is also a potent pan-PKB inhibitor[85, 86] .
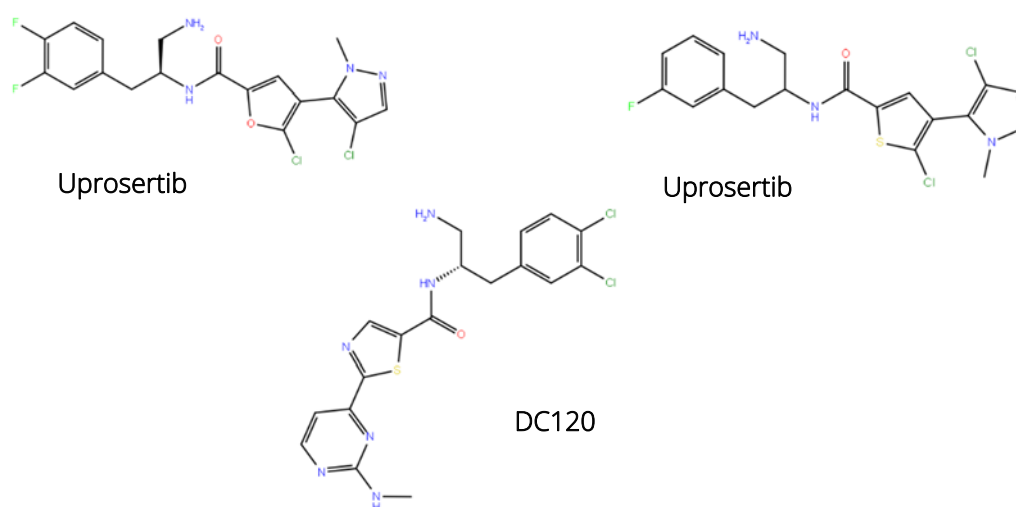
Figure 1.7: PKB inhibitors based on thiophene derivatives.

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1.2.2.5. Allosteric site targeting inhibitors

Due to high similarity of the kinase sequence in the ATP binding site, and the difficulty of making quantitative predictions of binding energy, retrospective analyses of structure-activity relationships (SAR) and rational design are gaining in importance. Although many ATP-competitive PKB inhibitors are selective against other kinases, their selectivity among isoforms is usually not promising, as shown by the binding affinity of afuresertib. Therefore, the researchers have been trying to exploit non-competitive ATP sites to develop more selective inhibitors. Wen-I Wu, et al., showed PH domain and inhibitor interactions by determining the inhibitor complex of the full length PKBα protein, which includes its PH domain. Their findings show a "PH-in" conformation which resulted in close contact between the kinase and PH domains away from membrane [87]. The MK2206 inhibitor also acted as a "PH-in" binder, locking the interface between kinase and PH domain into a close conformation of PKB [88].

1.2.3. Aurora kinases

1.2.3.1. Overview of cell cycle

The cell cycle process is an sequence of events which occur during cell duplication, division and is tightly regulated through series of checkpoints. The cycle is typically described to start from G1 (Gap), and procede through S (Synthesis), G2 (Gap) and M (Mitosis and cytokinesis) phases (Figure 1.8). The G1 phase prepares everything for DNA synthesis and, together with S and G2, is known as interphase. The cell size increases at this phase. Prior to entering M phase, checkpoints of the G2 phase make sure that everything is prepared for continuation. Finally, the M phase is comprised of prophase, metaphase, anaphase, telophase and cytokinesis. In this phase, chromosomes are separated and distribute into two daughter cells. During these cellular processes, protein kinases play roles through phosphorylation.
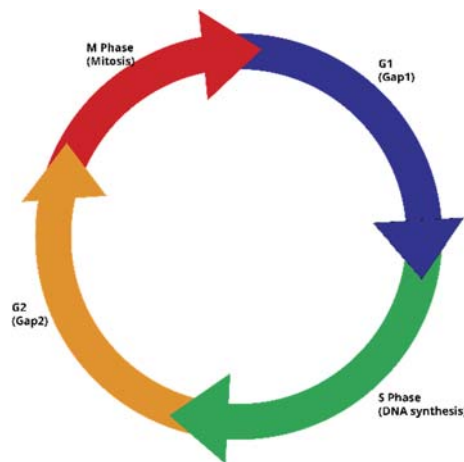
Figure 1.8: Schematic representation of the cell cycle. The M phase stands for mitosis where chromosome separation and cytokinesis occurs. The G1 and G2 phase indicates gap phase 1 and 2, respectively. The S phase (synthesis) where DNA replication occurs.

Interplay between mitotic spindle assembly and disassembly is important for the reproduction of genome during cellular reproduction [89]. The Aurora kinases are serine-threonine kinases that regulate many of the processes during cell division. They are the key regulators of mitosis and are involved in controlling spindle function, chromosome condensation, orientation, chromatid segregation and cytokinesis [90]. Glover and co-workers first identified the Aurora gene during screening from a *Drosophila melanogaster* that was defective in spindle-pole function and named Aurora, an "exotic beauty of north pole region" [91]. The Aurora genes are also found in yeast (*S.pombe, S.cerevisiae*), *Drosophila*, *C.elegans* and *Xenopus* [92].

The human has three Aurora isoforms, namely Aurora A, B, and C, respectively. Human Aurora A and Aurora B share 71 % sequence identity. Aurora kinases contain three domains: a conserved catalytic domain, a variable N-terminal domain (residues, 39-129) which is varied in size and sequence, and a short segment of C-terminal (15-20, residues). Aurora kinases contain characteristic short amino acid motifs in the C-terminal domain of the kinase called the D-box, an A-box in the N-terminal domain, and the activation loop in the kinase domain (Figure 1.9). Phosphorylation (Thr) of the activation loop of the aurora kinases is needed for their kinase activity. Aurora kinases display their dynamic properties by interacting with different proteins, and by changing localization and function [90, 93, 94]. In the following sections, only Aurora kinase A and B will be discussed.
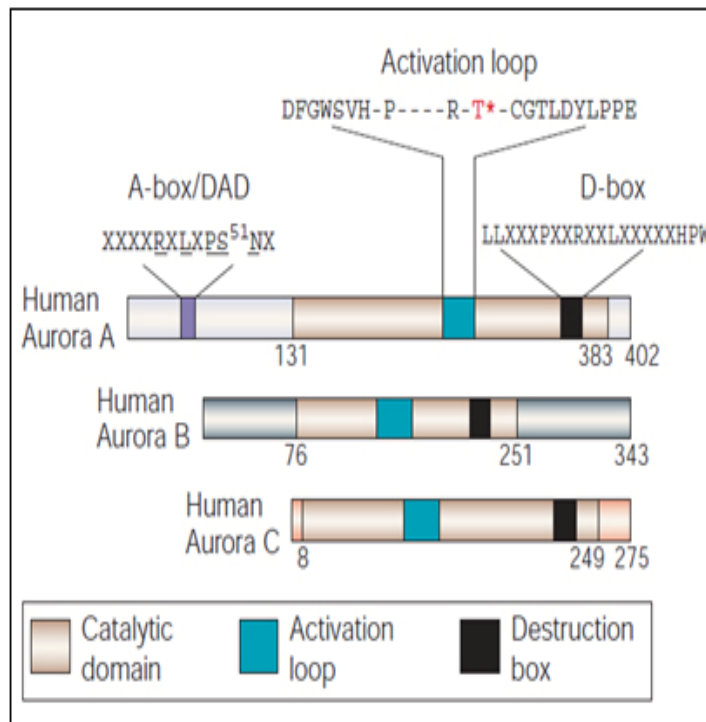
Figure 1.9: Representation of human Aurora kinases. The catalytic, activation loop and D-box are colored with "light wheat", "deep cyan", and black, respectively. The phosphorylatable threonine residue marked as red with asteric. The figure has been adapted with permission (Carmena and Earnshaw) [90].

1.2.3.3. Aurora kinase A

The human Aurora kinases share very similar sequences in their catalytic domain but diverge in their functions and subcellular localizations (Figure 1.10). Aurora kinase A plays multiple roles in the cell division from G2 to M, including centrosome separation, maturation and spindle assembly [95]. Mutations in Aurora can lead to failure of chromosome separation and formed monopolar spindles in Drosophila [91]. The centrosome separation is facilitated by Aurora A which phosphorylates Eg5, a kinesin-like motor protein [93]. At the end of S phase and at the beginning of the G1 phase, the presence of Aurora A in duplicated centrosomes was found by indirect immunofluorescence studies. Its localization at the centrosome depends on its N-terminal domain and the presence of microtubules, while the C-terminal domain acts independently [96, 97].
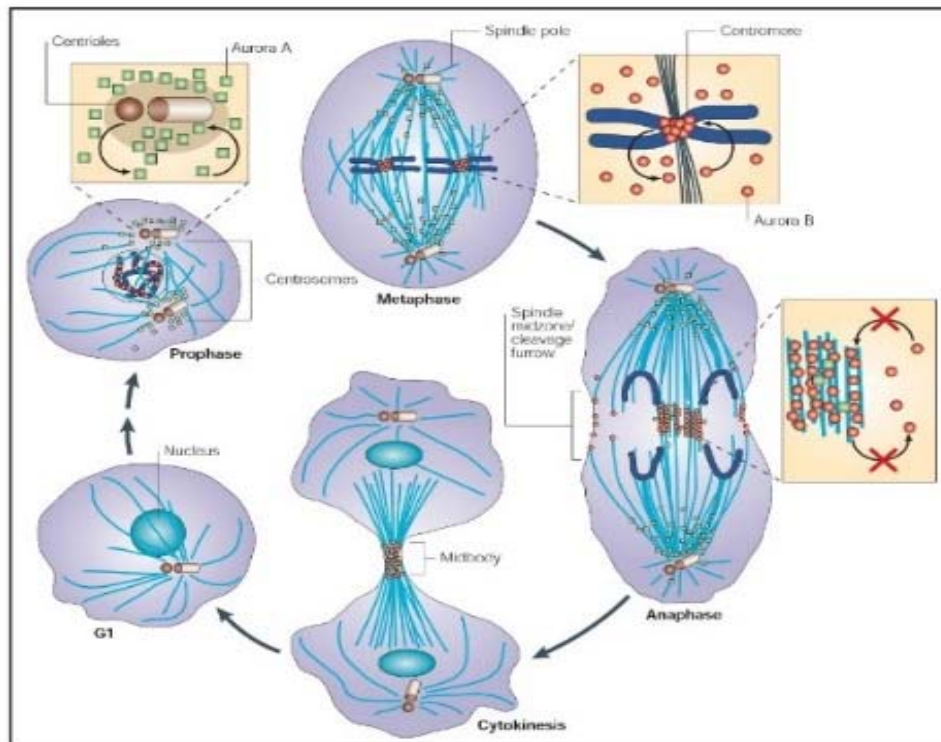
Figure 1.10: Cellular localization of Aurora A and B in mitotic cells. Aurora A and B indicated as green box and red circle during different stages of cell cycle. The figure is adapted with permission (Carmena and Earnshaw) [90].

During maturation, the centrosome recruits various proteins to become completely active. Aurora A plays an important role here by phosphorylating TACC (transforming acidic coiled coil) which locates to the centrosome and interacts with microtubule-associated protein (MAP) Msps/ch-TOG that promotes the growth of MTs [98, 99]. When Aurora is not present in the process, the mass of microtubule spindles is reduced about 60% and centrosome fails to recruit other proteins [95, 100].

Aurora A also plays a role in mitotic spindle assembly. Roghi and co-worker studied *Xenopus* egg extract and discovered the Ran-TPX2 pathway. Ran-GTP is a small GTPase that is needed for nuclear envelope assembly, mitotic spindle assembly and nuclear transport [101-103]. Usually, TPX2 is present in a complex, bound to importin α or β; it is released upon Ran-GTPase gradient formation, and then binds to Aurora A. TPX2 binding activates Aurora A (Figure 1.11). RNAi studies show that depletion of TPX2 cause less compact and multiples spindles, showing the importance of TPX2 for spindle assembly [104, 105].
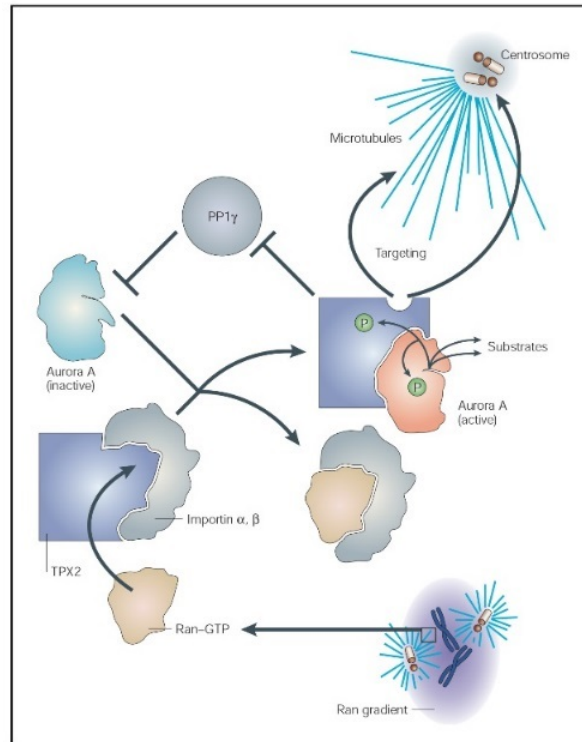
Figure 1.11: Aurora A activity is regulated by TPX2 and Ran-GTP. TPX2 is associated as a complex with importins α or β. The release of TPX2 from complex occur by a gradient of Ran-GTP and subsequently binds to Aurora A. The figure has been adapted (Carmena and Earnshaw) [90].

As described above, TPX2 (Targeting Protein for Xenopus kinesin-like protein 2) is a microtubule-binding protein which plays a significant role in regulating the localization and the activities of Aurora A. TPX2 is a substrate for Aurora A, but it was first found as a substrate for Xklp2 (Xenopus kinesin-like protein 2) [106, 107] . After release of TPX2 from importin α and β by Ran-GTPase, TPX2 binds to Aurora A which is activated and phosphorylates TPX2 [102, 108, 109]. The binding mechanism of TPX2 with Aurora A was revealed by biochemical and structural studies which gave more insight of their mutual cooperativity. TPX2 interacts with Aurora A with the N-terminal domain, similar to the binding of the hydrophobic motif in AGC kinases. Binding similarly activates the kinase, in part due to interactions with helix C, and also protects it from dephosphorylation by PP1 [110].

Toru Hirota and co-workers discovered another Aurora A binding partner called Ajuba. Ajuba is Aurora A specific, and does not activate Aurora kinase B. Ajuba is a lim protein which interacts with Aurora A in the mitotic cells and activates them by inducing

autophosphorylation on Thr288 residue. This was shown by a mutant of Ajuba and the absence of Thr288 phosphorylation *in vitro*, along with a lack of Aurora A kinase activity in the late G2 phase when the Ajuba level is depleted [111].

Bora is an approximately 64 kDa protein which is conserved from *C. elegans* to humans [112, 113]. Bora is a nuclear protein but it is translocated to the cytoplasm in a Cdc2-dependent manner, where they activate Aurora A (Figure 1.12). Andrea Hutterer and co-workers showed that Bora increases Aurora A kinase activity about 8 fold [113]. Overexpression of Bora can rescue a defective Aurora A mutant, which suggests Bora and Aurora A interact [95, 113]. Biochemical and mutational studies also indicate that Bora interacts with Aurora A through their N-terminal domains (65-247, residue); this domain is more conserved than other part of the Bora [113]. The Aurora kinase is no longer active in mitosis with *bora* mutants, further indicating that Aurora A is regulated by Bora [114].
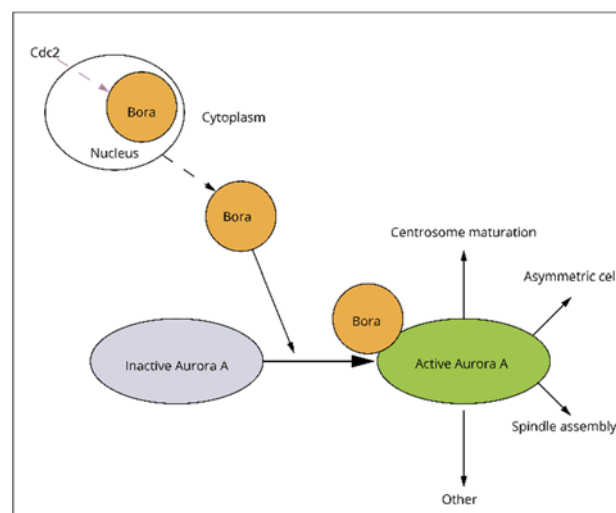
Figure 1.12: Mechanism of Aurora A activation by Bora in Cdc2 dependent manner from nucleus to cytoplasm.

Aurora B is also a serine/threonine kinase with roles in cell cycle regulation. James Bischoff and co-workers discovered Aurora B from a PCR-based screen [115]. Aurora B is the main component of the Chromosomal Passenger Complex (CPC), along with inner centromere protein (INCENP), survivin and borealin. Aurora B plays a role to synchronize chromosome condensation, segregation kinetochore function, spindle-assembly checkpoint and cytokinesis [90, 116]. During mitosis, Aurora B is mostly active and its expression level approaches maximum at the G2-M transition [115]. The localization of Aurora B is maintained in prophase, prometaphase, metaphase and anaphase. Moreover, during metaphase the association of Aurora B in the centromeres is dynamic, as they exchange with proteins in the neighboring cytoplasmic pool [116, 117]. The regulation and localization of Aurora B are modulated by phosphorylation and their interactions with other proteins, such as INCENP, whose binding increases its kinase activity and help to locate to the centromeres in mitosis [118]. Aurora B localization and activity is also modulated by sumoylation. The Small Ubiquitin-like Modifiers (SUMO) are a group of small proteins which act by attaching covalently to the target protein. The importance of sumoylation in the mitotic cellular process is evident in the kinetochore, centromere function and CPC complex [119-127]. The Aurora B kinase domain contains a conserved SUMO motif (ψ- Lys-x-Glu, in which ψ is a hydrophobic residue and x, any residue); the possible interaction occurs through covalent attachment at the lysine residue (K207). Biochemical and mutational studies indicate abnormal chromosome segregation and decreased cell viability when sumoylation is disrupted. Intriguingly, a K207R mutation of Aurora B does not show reduced kinase activity but the probable changes in localization [128]. The function of Aurora B is also modulated by acetylation. Acetylation at lysine215 (K215) of Aurora B by TIPO promotes its activity at kinetochores, chromosome bi-orientation. Acetylation also protects Aurora B activation from the phosphatase PP2A mediated dephosphorylation [129].

As mentioned above, Aurora B is regulated by phosphorylation-dependent manner along with interacting other proteins, including INCENP. This is a microtubule-binding protein that serves as a positive regulator and substrate for Aurora B [130, 131]. The C-terminal of INCENP binds to the Aurora B and is phosphorylated by the kinase at two conserved serine residues; this generates fullly active kinase [131, 132] (Figure 1.13). Survivin is a member of CPC which forms potential Aurora B substrates. Aurora B phosphorylates the C-terminus of human survivin at threonine117. Biochemical data indicates that Aurora B mediates phosphorylation of survivin and regulates its activity. Survivin also binds another passenger of CPC [133, 134]. Borealin is a 31-kD protein which is a part of CPC, first discovered through proteomic screens. It is conserved in all vertebrates. Borealin binds INCENP and Survivin *in vitro* [135]. Borealin localization depends on other CPC member and RNAi studies show delayed mitotic progression and incorrect kinetochore-spindle assembly when it is depleted [135]. Borealin is phosphorylated by Aurora B and acts as a substrate. Co-expression of Borealin with other passenger members did not change the kinase activity [135]. Aurora B also interacts with heat-shock protein 70 (HSP70). HSP70 protein is usually present in the cytoplasm, but methylated HSP70 (Lys561) observed in the nucleus of cancer cells. Hyun-Soo Cho and co-workers show that methylated HSP70 directly interacts with Aurora B and thus promotes activity *in vitro* and *in vivo* [136].
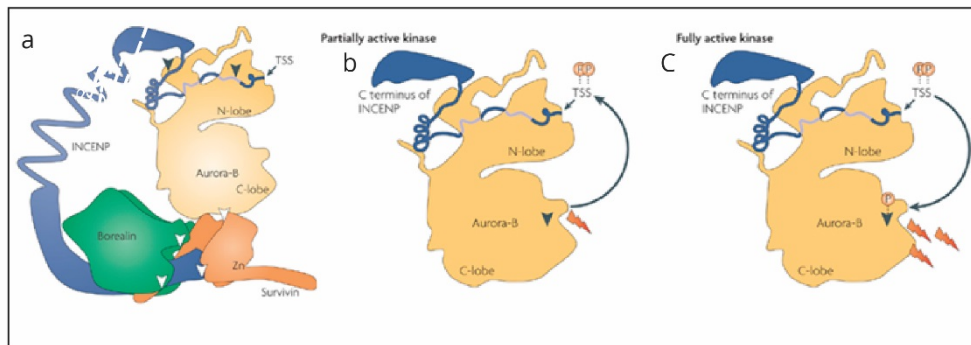
Figure 1.13: Aurora B and chromosomal passenger complex. a) Interactions of Aurora B, INCENP, Borealin, and Survivin. The N-terminus of Aurora B binds to the IN box of INCENP whereas Survivin and borealin interact with the N-terminus of INCENP. b & c) INCENP activates Aurora B by two-step activation. At first, INCENP partially activates Aurora B which autophosphorylated in the activation loop and another phosphorylation at the TSS motif in the C-terminus of INCENP increase kinase activity. The figure is adapted with permission (Ruchaud, Carmena and Earnshaw) [137].

1.2.3.5. Structures of Aurora kinases

Aurora kinases have three domains: an N-terminal domain that is variable in length (39-129 amino acid residues), a catalytic kinase domain and a small C-terminal tail. The Aurora kinase catalytic domain presents the typical bilobal kinase fold, comprised of an N-terminal β-strand lobe and a C-terminal α-helical lobe, linked by a segment that acts as a hinge, about which the two lobes can rotate somewhat with respect to one another. The ATP binding site is in the interface between the two lobes. The ATP interaction is achieved via hydrogen bonds from the adenine moiety to the hinge, contacts from the ribose ring and the initial portion of the C-terminal lobe, coordination of the triphosphate by two metal cations bound by residues on the DGF motif (activation loop or T-loop) and catalytic loop (HRD motif), and triphosphate polar interactions with residues within the glycine-rich loop (G-loop).

The Aurora kinase activity depends on regulatory mechanisms which include a toggle between active and inactive conformations via changes of orientation on the activation loop upon phosphorylation (Thr288 in Aurora A) together with changes in the orientation of the C-helix. A cognate of the so-called "PIF-pocket", which is located on the back of the N-terminal lobe, is also involved in the regulation Auroras, like the closely related AGC kinases. The Aurora consensus phosphorylation site is identified

as [KR]X[TS][ILV] [138], with Aurora B and C being less strict than Aurora A with respect to the hydrophobic residues.

The first crystal structure of Aurora A was solved at 2.9 Å by Graham and co-worker in 2002 [139]. The following year, Richard Bayliss and his group determined a phosphorylated (T287, T288) Aurora A kinase structure with binding partner TPX2 [110] . The complex structure revealed how TPX2 (1-43) binds at two distinct sites (Figure 1.14), the upstream segment TPX2 (residue 7-21) binds at the back of N-lobe and the downstream TPX2 (residue 30-43) binds in the αC helix between N and C-lobe of Aurora A kinase [110]. Both segments of TPX2 engaged main and side chain atoms of Aurora A, most notably, Tyr8,10 and Ala12 of upstream TPX2 established interactions in the hydrophobic groove between the β sheet, helix αB and helix αC. The conserved tyrosine residues 8 and 10 from TPX2 show aromatic interactions with Tyr199 of Aurora A and cation-pi interactions with Lys166 and Arg179. Additionally, hydrophobic interactions between upstream TPX2 and Leu169, 178, Val182, Tyr199 and Val206 of Aurora A are observed. The downstream part of TPX2 also exhibits interaction with Aurora A. The TPX2 bound Aurora A shows a typical active kinase conformation, like other AGC kinase active conformations [110]. Nevertheless, comparison of monomeric and TPX2 bound Aurora A structures show differences. The most prominent difference is in the activation loop where Thr 288 is exposed to solvent in the absence of TPX2 but interacts with Arg255 in the presence of TPX2 bound Aurora A [110]. The phosphorylated [110] and unphosphorylated [139] structures adopt different conformation. The unphosphorylated structure shows a closed conformation [139]. The phosphorylated Aurora A has well-ordered activation segment [110]. The conserved Lys162 (β-3) aligns with phosphate, and Asp274 from the DFG motif coordinates a magnesium ion, while the catalytic base Asp256 is poised to transfer γ phosphate from ATP to substrates. The salt bridge interaction between Lys162 to catalytic residue Asp274 is not present in the unphosphorylated structure.
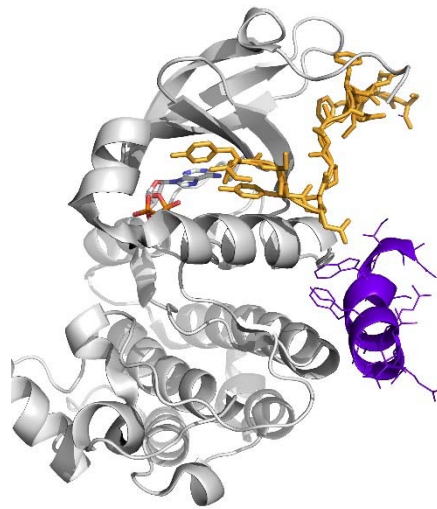
Figure 1.14: The structure of Aurora A-TPX2 complex (PDB code: 1OL5). Aurora A represents as gray, the residues of TPX27-21 and 30-43 showing here as orange and purple-blue, respectively.

The structures of Aurora B kinase domain with INCENP from *Xenopus laevis* and *Homo sapiens* have been solved. Both structures (Aurora B: INCENP) were solved in the presence of inhibitor, hesperidin in the *Xenopus* and VX-680 in the human Aurora B, respectively [131, 140]. The Aurora B: INCENP and Aurora A: TPX2 complex structures are different from TPX2, as INCENP lines the N-lobe of Aurora B kinase on the side opposite to TPX2 [131] (Figure 1.15).
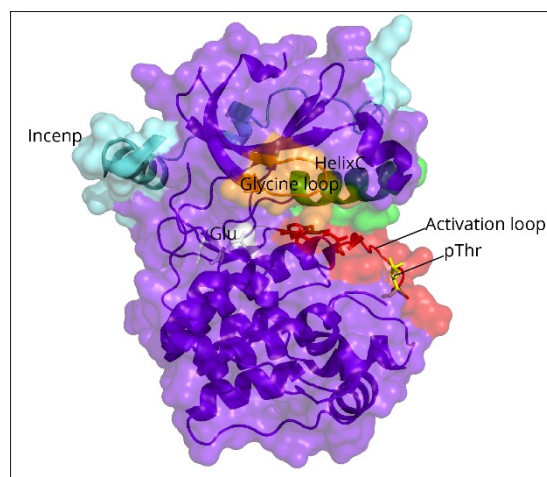
Figure 1.15: Aurora B kinase domain with Incenp 790–847 (PDB code: 2BFX). The Kinase domain of Aurora B represent here as purple -blue, INCENP as aqua-marine, Helix C as green, activation loop as red, phosphorylated Thr as yellow stick, Glu residue in the exit of hinge as white gray and glycine-rich loop as orange.

Human Aurora B: INCENP was dimeric and resulted in the domain swap of the activation loop. Additionally, the DFG motif was found in a different conformation with VX-680, also in comparison to other kinase complexes [140-143]. The binding mechanism of INCENP is based on interactions with Aurora B in two sites called "site 1" and "site 2". The N-terminal part of INCENP (residues 842-863) interacts through site 1 and the pattern of interaction is almost similar to *X. laevis* Aurora B: INCENP structure, except for nonconserved interactions with Ile856 of INCENP and Leu210 of Aurora B (which match Arg812 and Tyr228 in *X. laevis*). The C-terminus of INCENP (residues 868-881) interacting at site 2 and the αC helix of Aurora B are displaced and the INCENP backbone shows a different conformation [140]. The salt bridge interaction between Lys122-Glu141 (the equivalent to Lys162-Glu182 in Aurora A) is suppressed.

1.2.3.6. Aurora kinases and cancer

The overexpression of Aurora kinases is observed in many tumor samples, such as, breast, colon, pancreatic, ovarian and gastric cells [115, 144-149]. The deregulated activity of Aurora kinases is tumorigenic and thus, Aurora kinases are considered as an important drug targets for cancer therapy.

Systematic analyses of mRNA expression levels of Aurora kinases from different stages of primary tumour samples often revealed Aurora A and B overexpression [150]. TPX2, a partner protein of Aurora A, is also overexpressed in many tumor types. Their co-overexpression is frequent, indicating the existence of a novel functional unit with oncogenic properties [151].

Aurora A regulates breast cancer-associated gene1 (BRCA1) by phosphorylation at Ser308 [152]. It is also modulates the inhibition of centrosome function by decreasing E3 ubiquitin ligase activity of BRCA1 [153]. Aurora A and Aurora B genes are upregulated by Myc *in vitro* and *in vivo* and maintain Myc-driven lymphoma [154]. William Clay and his co-workers developed a class of inhibitors that disrupts the native

conformation of Aurora A and consequently destabilizes the Aurora A and Myc interaction [155].

1.2.4. Targeting Aurora kinase

The frequent overexpression of Aurora in many tumour cells put it in focus as a cancer drug target [115]. However, adequate specificity and selectivity has been difficult to achieve with recent methods of small-molecule kinase inhibitors design. The sequence and structure of highly conserved kinase domain, making the task is challenging to gain inhibitor specificity. In addition, the inhibitors should ideally target only cancer cells, as Aurora is important also in healthy cells, particularly for cell division.

As mentioned earlier, Aurora A and Aurora B both play key roles in cell division, but the two Auroras act separately in time and space. Thus, the relative selectivity of inhibitors will be a key determinant of their therapeutic profiles. The kinase domain of Aurora A and B share 76% sequence identity (Figure 1.16) and only three amino acids differ from twenty-six residues in the ATP binding site. These are Leu215, Thr217 and Arg220 in human Aurora A, corresponding to Arg175, Glu177 and Lys164 in human Aurora B.

Biochemical studies suggest that the amino acid residue Thr217 can play a key role in selectivity for Aurora A [160-163]. Vassilios Bavetsias and his co-worker mutated Thr217 into glutamic acid (as in Aurora B) and revealed that the compounds (28c and 40f) were less sensitive to the mutant compared to wild-type Aurora A [156]. Moreover, introducing Ala, Gly, Ser, or Cys, at this position, does not affect inhibitor binding, but replacing it with a negatively charge residue prevents Aurora A inhibition [157]. Most of the Aurora A specific inhibitors were developed to exploit the Thr217 sequence difference[158-161]. There are other features that distinguish Aurora kinases from other kinases, such as small gatekeeper Leu210, compared e.g. to CDK2 with a bulky Phe80, and PKA with an extended Met120.
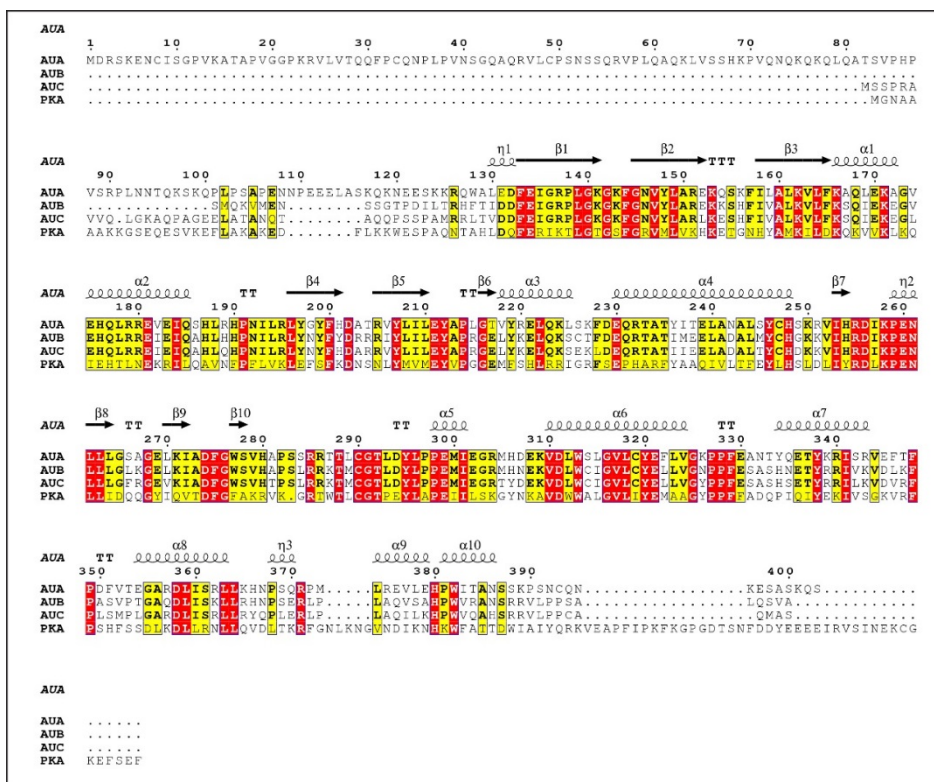
Figure 1.16: Multiple sequence alignment of Aurora and PKA kinases (AUA, AUB, AUC and PKA represents as Aurora A, Aurora B, Aurora C and Protein Kinase A, respectively. The identical residues color as red box.

1.3. Protein kinases and drug design

1.3.1. Protein-ligand interactions

Goood understanding of protein-ligand interactions is crucial in the drug discovery process. The protein-ligand binding affinities is measured and described by the binding constant K_i (Eq. 1.).

$$K_i = [\text{ligand}] \times [\text{protein}] / [\text{ligand--protein}] \qquad (1)$$

K_i has the dimensions of a concentration usually the units of mol/L (M) and the binding constant is often determined by an enzymatic assay.

Protein-ligand complex formation occurs most generally through non-covalent interactions. Hydrogen bonds (H-bonds) are the strongest and are typically the most critical for recognition, i.e. selectivity [162-164]. Typical hydrogen bonds can be grouped by the donor-acceptor distance and defined as; very strong (1.2-1.5 Å),

strong (1.5-2.2 Å), moderate (2.2-3.2 Å), and weak (3.2-4.0Å), respectively [165]. Analysis of the 233 crystal structures of kinases revealed in addition to strong N–H···O, O–H···O, N–H···N hydrogen bonds, weak ones such as C–H···O, C–H···N that may be important [166]. Ionic interactions occur when the charged group of the ligand interacts with the oppositely charged group in the protein, forming so-called salt bridges when there are separated from one another by a distance between 2.7-3.0 Å [167], but the complementary charges also have a long-range energetic effect. Hydrophobic interactions are also observed in protein-ligand binding. The interaction between lipophilic groups of ligand and non-polar amino acids of protein are attractive, directly via dispersion forces, but more importantly via the displacement of poorly bound water molecules at the surfaces of the uncomplexed molecules [167]. Other interactions are energetically important, such as pi-pi interactions, cation-pi interactions and metal complexation. For example, Gallivan and Dougherty showed that arginine was more prone to participate in cation-pi interaction than lysine by analyzing high resolution structures. They conclude the preference of aromatic side chains in cation-pi interactions to be Trp > Tyr > Phe [168].

1.3.2. Strategies of kinase inhibitor design and current status

The design of selective and specific kinase inhibitors is a major challenge due to the high sequence similarities of so many kinase domains. There have been many approaches used to discover selective kinase inhibitors, and three classes have emerged: Type I, Type II, and Type III [169-171].

Type I inhibitors most closely mimic ATP, form hydrogen bonds (typically 1-3 hydrogen bonds) to the hinge region [172], and are ATP competitive inhibitors. Their hydrophobic interactions with the kinase have been systematically mapped [172, 173]. The interaction region of Type I molecules can be divided into hydrophobic regions I and II, the adenine region, the ribose region and the phosphate-binding region [173] (Figure 1.17 & 1.18). Some of the known type I kinase inhibitors are as

follows; Vx-680 [174], MK-5108 [175], dasatinib [169], sunitinib [176] erlotinib [177] and gefitinib [178].

Figure 1.17: ATP binding site grouped into various region of kinase. (Adapted from Liu and Gray, 2006) [169].

Type II inhibitors bind to specific inactive forms of a protein kinase. They are ATP competitive and use the ATP binding cleft. Additionally, type II inhibitors use an adjacent hydrophobic pocket which is created by the conformational change of the activation loop known as "DFG-out". The conformational changes involve the release of the phenylalanine of the "DFG segment, creating an additional hydrophobic pocket which is not available in the activated kinase. The amino acids at this site are less conserved than ATP site, and the DFG-out forms of kinases are more diverse. As a result, it is likely that Type II inhibitors are more selective compared to Type I [179-183] . Imatinib, the first approved type II kinase inhibitor showed promising selectivity over closely related kinase SRC[182]. The inhibitors: imatinib [184], BIRB796 [185], sorafenib [186], AAL993[187], indole amide[188], anilinoquinazoline [179] and 4-aminopyrimidinoquinazoline [180] are well known type II inhibitors.

Figure 1.18: Schematic representation of kinase inhibitors interaction sites and different binding modes. (Adapted from Susanne et.al.) [189].

Type III inhibitors do not target ATP binding site, rather the allosteric sites of the kinase domain, away from ATP pocket. Therefore, they are considered as non-ATP competitive. They are expected to be highly selective as targeting allosteric pockets [190-194]. Several type III inhibitors are developed in the kinase drug discovery process; notably, trametinib; a MEK kinase inhibitor [189], and MK-2206, an allosteric site AKT inhibitor which are in clinical trials [195].

1.3.3. Importance and function of glycine-rich loop

Many nucleotide binding proteins contain a cluster of glycine, as a special feature for this kind of protein family. For example, the motif GxxGxGx was found to bind mononucleotides, while GxGxxG, bound dinucleotides [196, 197]. The catalytic kinase domain of protein kinases encompasses the 'glycine–rich loop' formed between β1 and β2 of the N-lobe. The glycine-rich loop (G-loop), with the conserved motif glycine-x-glycine-x-aromatic-glycine (G50xG52xaG55, residues numbering according to PKA), covers the ATP binding site and plays an important role in the interaction of the adenine ring and phosphates [16]. The G-loop acts as a multifunctional structural element by engaging itself in the substrates recognition, nucleotide binding, and regulation of enzymatic activity [198]. It is generally accepted that the G-loop is the key determinant of the nucleotide positioning. In PKA numbering, the extended consensus sequence of the G-loop is Y49-G50-X-G52-X-[F/Y]54-G55-X-V57. The first and second glycine of the loop (Gly50 and Gly52) are nearly without exception among protein kinases; the two glycine residues provide space for ribose and an oxygen of the β-phosphates, respectively [198]. In PKA, the amino acid residue Thr51 of the G-loop, which is quite variable across the kinome, interacts with arginine residue of inhibitor peptide. This position is varied across serine/threonine and tyrosine kinases [199]. Val57 of the G-loop has hydrophobic interactions with the adenosine moiety [198]. The aromatic residue adjacent to third glycine is conserved across the kinome, either as tyrosine or phenylalanine. In PKA it is phenylalanine (F54), and is often involved in interaction with inhibitors.

An exceptional pattern is observed in ABL kinases which have an extra glycine in the second residue position. It is the target for leukemia drug imatinib, which induces a conformational shift, and was the first type II inhibitor observed. Moreover, ABL kinase has tyrosine as the G-loop aromatic residue, while PKA, Aurora, PKC, and PKB have phenylalanine. Another exceptional pattern is seen in atypical protein kinase C, which lacks the third glycine. The Aurora kinase inhibitor, VX680 induces conformation changes in both Abl and Aurora, but Aurora lacks the extra glycine of ABL. The

sequences of G-loop is GGGQYG, GKGKFG, GTGSFG, in ABL, Aurora, and PKA, respectively (Figure 1.19).
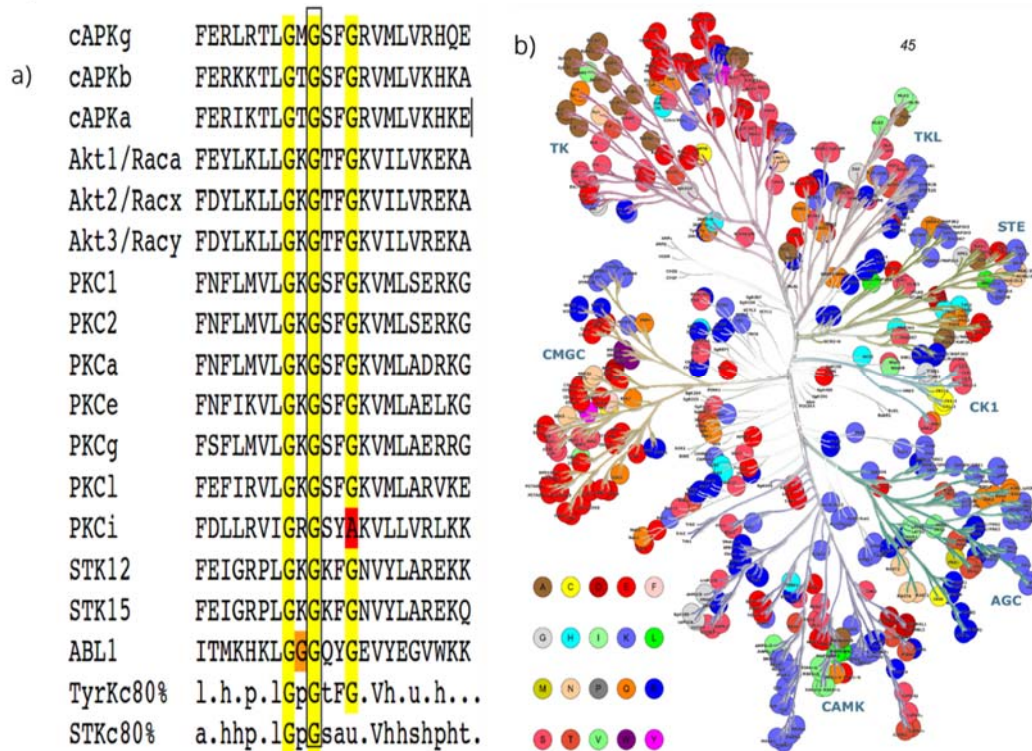
Figure 1.19: a) The glycine rich loop motif GxGxxG for PKA, PKB, PKC, Aurora, Abl, and consensus sequences, with anomalies marked for PKCi and ABL1. b) The distribution of residues at the G**X**GxxG position in the kinome.

The G-loop has long been considered highly flexible, often with unpredictable ligand interactions.

1.3.4. Water molecules and ligand binding

1.3.4.1. General features about water and ligand binding

Biological macromolecules are surrounded by water, a property which fundamentally influences their interactions. In order for ligands to bind in a cavity of the protein, water must either be displaced or mediate the interaction. Thus, water molecules are essential factors of protein-inhibitor binding [200-206], and binding affinities depends also on details of water interactions with the complexed and uncomplexed molecules [207, 208]. Although the ATP binding mechanisms of protein kinases are highly similar, variations in side chains also create variations in water structure. This provides another opportunity for kinase inhibitor selectivity, as has been studied also in other enzyme classes. For example, in thrombin, displacing a well-ordered structural water molecule from a hydrophobic pocket correlates with 40-fold increase in binding affinity [209]. An example of water-mediated hydrogen bonding as a selectivity determinant for protein kinases is described for the approved leukemia drug bosutinib [210]. Another example describes the variation of binding energies due to different sizes of cavities in the binding interface [211].

Water molecules are observed in the hinge region and ATP pocket of protein kinases. Aurora A has conserved water molecules in the different binding region in ATP site (Figure 1.20).
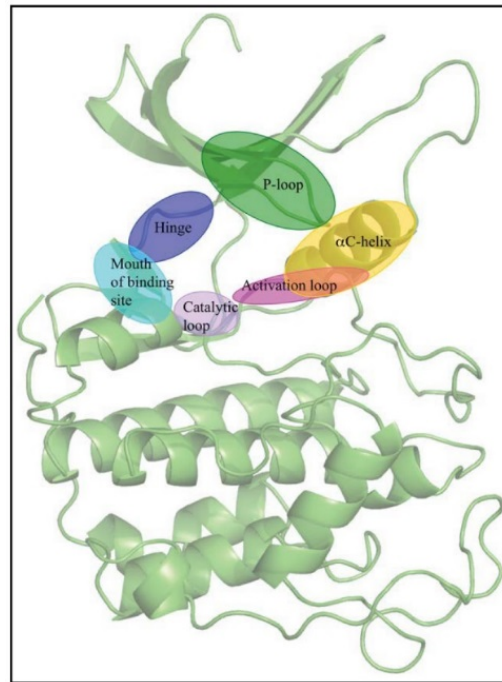
Figure 1.20: The catalytic domain of kinase is showing conserved water molecules in the areas of the binding site. The areas where water molecules are found areindicated as follows: The Gly-rich loop/P-loop (green), hinge(violet), the entrance of ATP binding site (light green), catalytic loop(lilac), activation loop(purple), and aC-helix (yellow). Adapted from Barillari, C., et al. [212].

Caterina Barillari and co-workers analyzed the high-resolution crystal structures of 171 protein kinases and showed that Aurora A has four conserved regions in the binding site where water molecules are present and nine conserved water molecules in Pim-1 kinase, both represent serine/threonine protein kinases. There were four conserved water molecule areas located in Aurora A: between the activation and αC-helix, near the G-loop, at the hinge and at the entrance of the binding site (Figure 1.21). The superposition of Aurora A structures with inhibitors show variations of water pattern due to diverse inhibitor scaffold and kinase conformations. Intriguingly, water patterns different across the kinome despite high sequence similarity of ATP binding site [212, 213]. Structural water molecules have been used to obtain

selectivity and affinity for Auroras B and C over Aurora A, and also for CDK kinase [214]. Moreover, Aurora A-ADP structures show a water mediate interaction with purine ring and threonine residue (Thr 217) [214]. The inhibitor VX-680, a Pan-Aurora inhibitor, interacts with Aurora kinase A (PDB code: 3E5A) through conserved water in the activation loop and beneath the G-loop, constraining the inhibitor to similar interactions otherwise only in ribosomal protein S6 kinase B1(PDB code: 3WE4) and JAK3 kinase PDB code: 4QPS). These examples show how conserved structural water molecules may play a vital role in selectivity and thus in structure based drug design.

a)

b)
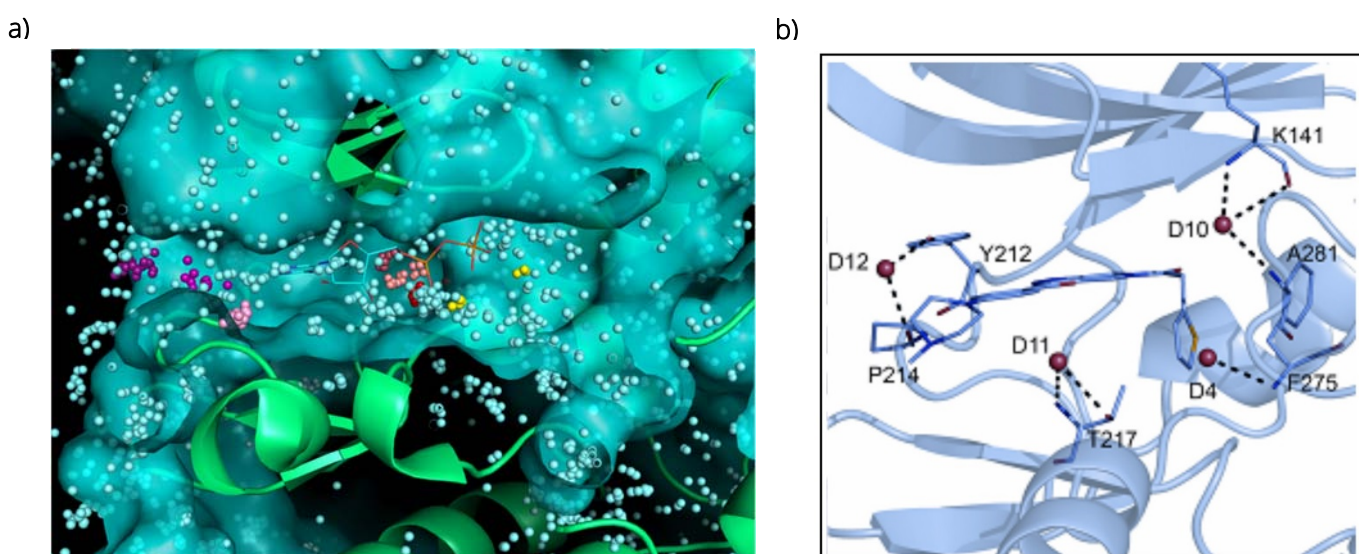
Figure 1.21: a) Superpositions of Aurora A crystal structures has found the presence of conserved water molecules, b) Four conserved water molecules found in Aurora A (pdb code: 2J4z). The figure b adapted from Barillari, C., et al. [212].

1.3.5. Fragment-based drug design in protein kinases

Fragment-based drug discovery (FBDD) has become a common practice in the drug discovery process, and is routinely practiced to facilitate small molecule drug discovery. There are now some 30 drugs in clinical trials which originated from fragment-based approaches [215] (Table 1). A fragment is defined as a small organic molecule which generally follows the Rule of 3: molecular weight < 300Da, the number of hydrogen bond donors and acceptors ≤ 3, and ClogP < 3, as proposed by Astex researchers, and additionally the number of rotatable bonds is ≤ 3 and the polar surface area is ≤ 60 $Å^2$ [216]. Fragments are low-molecular weight, low-affinity molecules which may later be developed into lead compounds by building them up employing medicinal chemistry knowledge and structure-activity relationships (SAR) for their target interactions. One specific approach is to link two fragments that are found to bind close to one another in the target protein binding cavity [217-219]. However, as fragments bind weakly to the target protein, it is often difficult to detect and measure their binding strength in conventional enzymatic assay techniques. These problems may be overcome by biophysical techniques such as X-ray crystallography, nuclear magnetic resonance spectroscopy(NMR), surface plasmon resonance, microscale thermophoresis (MST), differential scanning fluorimetry (DSF) and Isothermal titration calorimetry (ITC). X-ray crystallography, NMR and SPR are widely used methods in fragment screening, and newer methods are coming into use, such as mass-spectrometry, weak affinity chromatography and computational methods [217]. FBDD has been a well-known paradigm in protein kinase drug discovery; in fact, the first fragment-derived BRAF mutant kinase inhibitor is vemurafenib [220, 221] which was approved for the treatment of malignant melanoma. The progression started with an initial biochemical screening of 20,000 low-molecular weight compounds (MW:150-350 Da), and 200 compounds resulted in inhibitory kinase activity against Pim-1, p38, and CSK [221]. Protein crystallography studied these fragments and explored the binding of 7-azaindole to the ATP pocket. Further optimization resulted in vemurafenib with activities against wild-type BRAF

and mutant with IC_{50} values of 160 and 13 nM, respectively [221]. The successful story of vemurafenib progression led other researchers to focus on FBDD approaches. There are many kinase inhibitors from FBDD approaches in clinical trials, including AT9283, pyrazole based inhibitor against Aurora kinases, JAK2 and Abl (T315I) [222] SNS-314, an Aurora kinase inhibitor developed from low uM to potent nM affinity [223], and SGX523, a MET kinase inhibitor [224]. An FBDD approach identified a fragment scaffold (IC_{50} = 80 uM) against PKB which later was developed into high affinity inhibitor using rigorous analysis of SAR [225].

FBDD provides opportunities to obtain new chemotypes for new inhibitors that may be derived from known inhibitors, such as AZD5363, which as a protein kinase B inhibitor, but a fragment of this inhibitor also showed inhibitory activity against RHO-associated protein kinase 2 (ROCK2) [226, 227]. The progression of fragment-based drug discovery is not limited to kinases; it has been used widely in other target related proteins. The development of NVP-AUY922 [201, 228] and Onalespib inhibitor [229] against HSP90 has entered into clinical trials. FBDD has played a significant role in kinase inhibitor development; most of the fragment-based inhibitors now in clinical trials target protein kinases. However, there are still challenges to overcome for fragment-based drug discovery process, including the development of methods which detect fragment binding more efficiently, and better predictivity in structure based optimization of a fragments to lead compounds.

Table 1: Name of the FBDD drug which are in different stages of clinical phase[215].

Drug	Name of the company	Target	Status
Vemurafenib	Plexxikon	B-Raf (V600E)	Approved
Venetoclax	AbbVie/Genentech	Bcl-2	Approved
PLX3397	Plexxikon	FMS, KIT, and FLT-3-ITD	Phase 3
Verubecestat	Merck	BACE1	Phase 3
AZD3293	AstraZeneca/Astex/Lilly	BACE1	Phase 3
AT7519	Astex	CDK1, CDK2,CDK4,CDK5& CDK9	Phase 2
AT9283	Astex	Aurora & JAK2	Phase 2
AZD5363	AstraZeneca/Astex/CR-UK	AKT	Phase 2
Erdafitinib	Johnson & Johnson, Astex	FGFR1-FGFR4	Phase 2
Indeglitazar	Plexxikon	PPAR agonist	Phase 2
LY2886721	Lilly	BACE1	Phase 2
LY517717	Lilly/Protherics	FXa	Phase 2
Navitoclax	Abbott	Bcl-2/Bcl-xL	Phase 2
NVP-AUY922	Vernalis/Novartis	HSP90	Phase 2
Onalespib	Astex	HSP90	Phase 2
ABL001	Novartis	BCR-ABL	Phase 1
ABT-518	Abbott	MMP2 & MMP9	Phase 1
ABT-737	Abbott	Bcl-2 & Bcl-xL	Phase 1
ASTX600	Astex	XIAP & cIAP1	Phase 1
AT13148	Astex	AKT, p70S6K & ROCK	Phase 1
AZD3839	AstraZeneca	BACE1	Phase 1
AZD5099	AstraZeneca	Bacterial topoisomerase II	Phase 1
BCL201	Vernalis/Servier/Roche	BCL-2	Phase 1
DG-051	deCode Genetics	LTA4H	Phase 1
IC-776	Lilly/ICOS	LFA-1	Phase 1
LP-261	Locus	Tubulin	Phase 1
LY2811376	Lilly	BACE1	Phase 1
PF06650833	Pfizer	IRAK4	Phase 1

PLX5568	Plexxikon	RAF	Phase 1
SGX-393	SGX	BCR-ABL	Phase 1
SGX-523	SGX	Met	Phase 1
SNS-314	Sunesis	Aurora	Phase 1

2. Aims of the study

Objective

The overall objective of this study was to use crystallography, experimental and theoretical biophysical tools, and surrogate kinases to investigate key structural and flexibility determinants of protein kinase ligand binding properties.

Specific Objectives

1) Investigate structural and dynamic features of the glycine-rich loop and inhibitors interactions.

2) Studies presence of structural water molecules and their role in the kinase binding region.

3) Studies kinase ligand interactions by PKA as a surrogate kinase.

3. Experimental methods

3.1. Aurora A expression and purification

Aurora A (residue 123-389) with N-terminal hexahistidine purification tag and TEV protease cleavage site was cloned into the vector, pNIC28-Bsa4 and expressed in *E.coli* (BL21(DE3)- pRARER) cells. There were few colonies used to inoculate 50 ml LB (Luria-Bertani) medium containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol and the cultures were grown overnight in a 250 mL flask at 37 °C with shaking 200 rpm. The following day, pre-cultures were transferred to large scale TB (Terrific Broth) medium (10 ml pre-cultures were added to 1L TB) containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol into TB medium and grown at 37 °C with shaking 200 rpm. When the OD_{600} of cells reached 0.5, the temperature reduced at 16 °C and Aurora A expression was induced at 0.8 (OD_{600}) by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside and left overnight. The Aurora A expressed cells were subsequently harvested by centrifugation at 6500 rpm for 25 minutes and resuspended in binding buffer containing 50 mM Tris-HCl, pH 7.8, 400 mM NaCl, 20mM imidazole,10% glycerol, 5mM β-mercaptoethanol and protease inhibitor cocktail (Roche). The resuspended cells were lysed using sonication and the lysate was centrifuged at 14500 rpm for 1 hour at 4°C. The supernatant was passed through nickel affinity HisTrap column and eluted with buffer containing 50 mM Tris-HCl, pH 7.8, 400 mM NaCl, 500mM imidazole, 10% glycerol and 5mM mercaptoethanol. The hexahistidine tag was removed from the eluted protein using TEV protease overnight at 4 °C and subsequently, protein was pre-equilibrated in buffer 50mM Tris-HCl pH 7.5, 200mM NaCl, 10% glycerol and 5mM mercaptoethanol, and purified using size exclusion chromatography.

3.2. PKA and PKA variants expression and purification

The PKA variants, T51G, G55A, E127T, and T51G were made following a site-directed mutagenesis protocol [230]. The catalytic domain of PKA (wtPKA) and variants containing a N-terminal hexahistidine purification tag with a TEV protease cleavage site in the pET28-b vector was expressed in *E.coli* (BL21(DE3)- pRARER) cells using TB medium. The expression was carried out overnight at 17 °C by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside when OD_{600} reached 0.8. The expressed cells were collected by centrifugation at 6500 rpm for 25 minutes and solubilized in binding buffer (binding buffer: 50 mM Tris-HCl 7.5, 300 mM NaCl, 20 mM imidazole and protease cocktail) and stored at -80 °C or -20 °C. The following day, the resuspended cells were lysed using sonication and the lysate was centrifuged at 14500 rpm for 1 hour at 4 °C. The supernatant was filtered and protein was purified using a HisTrap column in affinity chromatography. After tag removal by TEV protease, the protein was purified using cation exchange chromatography using prepacked Resource S column. The column was equilibrated with 20mM K-phosphate, 20 mM KCl pH 6.5 and protein was eluted using a gradient from 20 mM to 500 mM KCl pH 6.5. The second cation exchange was done to get more homogeneity by changing pH 7.15 from 6.5 [231]. The phosphorylation states were confirmed by MS-MS analysis.

3.3. PKAB3 expression and purification

The catalytic subunit of PKAB3 was expressed in the vector pT7-7 in *E. coli* BL21(DE3) RIL cells. The cultures were grown at 37 °C in 2YT media containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol and induced by at 0.7 (OD_{600}) by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside. The expressed cells were subsequently harvested by centrifugation at 6500 rpm for 25 minutes and resuspended in binding buffer containing 50 mM Tris-HCl pH 7.5 and 50mM NaCl. The resuspended cells were lysed using sonication and the cells debris removed by centrifugation at 14500 rpm for 1 hour at 4°C. The supernatant was adjusted by adding 5mM $MgCl_2$ and 400 uM ATP as the final concentration which assured binding of PKA to the PKI mix Affi -gel

resin and kept for 4 hours at 4°C. The PKAB3 supernatant purified by PKI mediated through gravity flow columns (PD-10 Columns, GE Healthcare) using 50 mM Tris-HCl pH 7.5, 2mM $MgCl_2$, 400 uM ATP, 50 and 250 mM NaCl as washing buffer. Finally, the protein eluted with 200 mM L-arginine contained lysis buffer. Further, the phosphorylation states were purified cation exchange chromatography as described in wtPKA purification section.

3.4. Kinase activity assay

The activity of kinase variants was measured using NADH-coupled assay (Cook assay) and binding strengths of small molecule measured in an ATP-competitive manner [232]. Since protein kinase needed to transfer phosphate from ATP to respective substrates, Kemptide (LRRASLG) was used as a known substrate. The Cook assay was performed by adding kinase to the assay mixture (100 mM MOPS, pH 6.8, 100 mM KCl, 10 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.1 mM Kemtide, 1 mM β-mercaptoethanol, 15 U/ml lactate dehydrogenase, 10 U/ml pyruvate kinase, and 0.21 mM NADH). More than 10 dilution series of the small molecule in triplicates were used to the measured remaining activity of the kinase (decrease absorption of NADH) at 340 nM using Molecular Devices "SpectraMax M2e" over a period of 10 minutes. Consequently, the inhibitory concentration at which the substrate conversion reduced 50 %(IC_{50}) were calculated with GraphPad Prism 5 following dose-response-function. Finally, the binding constant (Ki) was calculated using by Cheng and Prusoff equation [233]:

$$K_i = IC_{50}/[1+([S]/K_{M}, ATP)] \qquad (3)$$

where [S] = Substrate concentration, and K_M = Michaelis-Menten constant.

3.5. Thermal shift assay

The thermal shift assay was carried out by using a 4uM PKA-Au6 as a final concentration in 25 mM Bis-Tris HCl, pH 7.0, 150 mM KCl buffer, 10× Sypro Orange (Invitrogen). The final concentration of Maybridge fragments and inhibitors (AT9283 & NVP-TAE684) were 10 mM and 1mM, respectively. The experiments performed in quadruplicate, where 4% vol/vol DMSO used in the positive control and the negative control without DMSO, using Real-Time PCR detection system (BioRad) on Multiplate Low-Profile 48-Well Plates (BioRad). The samples were heated at 1°C per min, from 10° C to 95° C. The data were analyzed with Opticon Monitor software (Bio-Rad) and the thermal shifts were calculated from subtracted control, $\Delta T_m = T_{sample} - T_{control.}$

3.6. Surface plasmon resonance (SPR)

SPR binding studies were performed at 25 °C using a Biacore T200 (GE Healthcare). Aurora A, PKA-Au6, T51G PKA, and G55A PKA were diluted to 25 to 30 ug/mL in 10 mM Bis-Tris pH 6.5 separately. The immobilization was carried out at 25 °C at a flow rate 30 uL/min using 10mM HEPES pH 7.4 with 150 mM NaCl. The proteins were covalently coupled to the S-series CM5 sensor chips (GE Healthcare) through standard NHS/EDC amine coupling. The amount of immobilized proteins was determined to a level between 5000 to 8000 RU. Interaction studies were started by injections of dilution series of inhibitors concentrations at a flow rate of 30 μL/min for the 60 seconds (association) and 600 seconds (dissociation) at 25 °C. The running buffer contained 20 mM HEPES pH 6.8, 150 mM NaCl, 2mM $MgCl_2$, 0.05 % Tween and 2% DMSO, respectively. The inhibitors were dissolved in 100 % DMSO and diluted such a way with running buffer (without DMSO) to keep DMSO level at 2% in the inhibitors dilution series. In the blank injections, running buffer was used and the bulk effects were adjusted using solvent correction. The unspecific binding was removed by subtracting SPR signals from a blank flow cell. After each cycle, the sensor chip was regenerated by injecting 100mM NaHCO3 pH 8.6. The kinetics data were analyzed using Biacore T200 Evaluation Software 2.0 (GE Healthcare).

3.7. Crystallization

PKA was crystallized at 4 °C in hanging drops. The droplets contained 10 to 16 mg/ml protein with 25 mM Bis-Tris-HCl pH 7.0, 150 mM KCl, 1.5 mM octanoyl-N-methylglucamide and 0.8 mM PKI peptide. The droplets were equilibrated against 12-26 % (v/v) methanol and crystals appeared within a week. The crystals were soaked with ligand (1c, 1b,1e, 1i and 1p) as final concertation of 4mM overnight at 4 °C. PKA-Au6 was crystalized in the same manner as wtPKA and crystals were soaked with inhibitor AT9283 as 4mM final concentration. The wtPKA:ARC-1416 complex was successfully crystallized in the conditions of 18% PEG3350, 0.2 M Li2SO4 at 4 °C. PKAB3 was co-crystalized with ligands (1c and 1p) at 4 °C in hanging drops. The drops contained 10 to 16 mg/mL protein, 1mM ligand with 25 mM Bis-Tris-HCl, pH 7.0, 150 mM KCl, 1.5 mM octanoyl-N-methylglucamide and 0.8 mM PKI peptide and equilibrated against 12-26 % (v/v) methanol. All the crystals were frozen in 30-40 % MPD for data collection.

3.8. X-ray diffraction and structure determination

The diffraction data of crystals were collected at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) and at the 14.1 beamline at Berlin Electron Storage Ring Society for Synchrotron Radiation (BESSY, Berlin). The diffracted images were processed, integrated and scaled by using either XDS and XSCALE [234] or with MOSFILM [235]and SCALA [236]. Molecular replacement and structure refinement were done by MOLREP [237] , PHASER [238], REFMAC5 and Phenix [239]. The coordinate and molecular topology files for ligands were created with PRODRG [240] and elbow [239].

4. Results and discussion

4.1. Side chain determinants of ligand binding

The structure-based design of kinase inhibitors have focused mostly on interactions with rigid regions of the kinase, in the hinge region with its surrounding ATP binding pockets. Flexible elements such as the glycine-rich loop have been recognized as important, but have not been the focus for optimization. Here, we studied a series of PKA and PKAB3 inhibitors using enzyme activity assay and determined their binding modes by protein crystallography. The inhibitors were designed and synthesized to study the ligand binding properties of the glycine-rich loop by incorporating different halogenated benzoyl or carbothienyl substituents along with the hinge region and ribose pocket. Kinase activity assays showed that most of the inhibitors have nanomolar binding affinity against PKA and PKAB3 (**Paper I**). The crystal structure of wtPKA with inhibitor (+)-1b is found interact to the Glu121 and Val123 in the hinge. The benzoyl C=O group of (+)-1b has contact with Gly50 from glycine-rich loop at a distance of 3.0 Å. Additional interactions are observed from residues Glu127, Glu170 and Asn171, respectively. The side chain of Asp184 from the DFG loop also interacts with the inhibitor (Figure 4.1).

Figure 4.1: Binding mode of (+)-1b with wtPKA (PDB code: 4UJB). Color code: C green, N blue, O red, and F cyan, respectively.

The Glu127, Asp184 interactions with the ribose pockets are predominant among AGC and CMAK families of kinases. Only two tyrosine kinases, PTK2 and PTK2B, have similar interactions [241]. Glu127 is the key selectivity determinant that distinguishes Aurora kinase A from B; Aurora A has Thr217 and Aurora B has a glutamic acid residue in the similar position [156, 157].

The crystal structure of PKAB3 with inhibitors (+)-1p (PDB code: 4Z83) revealed the conformational flip of the Met173 side chain. The Leu173 residue (Met173 in PKAB3) in PKA showed favorable interaction with quinazolinone ring (Figure 3, **Paper I**). Due to side chain flip of Met173 in PKAB3, the inhibitors interactions are less favorable which might have explained why all inhibitors were more potent against PKA than against PKAB3. Biochemical studies showed inhibitors with *para*-substituted phenyl and thienyl derivatives have higher affinities than *meta*-substituted derivatives. The crystal structure of PKA with the para-substituted inhibitor (with a F_3C-phenyl ring) (+)-1I (PDB code: 4UJ9) showed extensive interactions between the inhibitor and the glycine-rich loop (Figure 4.2). The binding affinities in para-substituted inhibitors (except (+)-1h) are stronger than meta-substituted inhibitors. (The inhibitor (+)-1h has fluorine as substituents which are small compared to rest.) The thiophene derivatives are tight binding (e.g. (+)-1n with Ki=170 nm) inhibitors, and the addition of halide in ortho-position to the sulfur atom of the thiophene substituent increases affinity ((+)-1p, Ki = 0.9 nm).
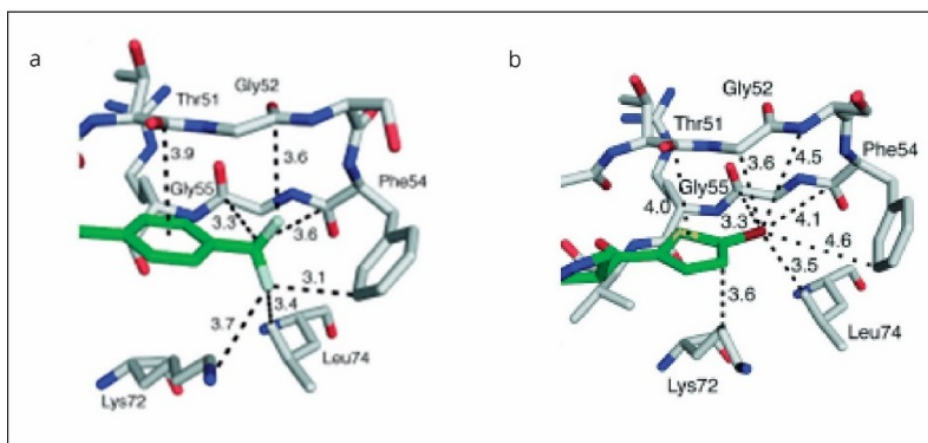
Figure 4.2: Crystal structure of compound (+)-1l (a, PDB code: 4UJ9) and (+)-1p (b, PDB code: 4UJA) bound to PKA. a). The interaction of F_3C-phenyl ring ((+)-1l) in to the glycine-rich loop. b) Interactions between the Br atom and the glycine-rich loop. Color code: ligand green; O red, N blue, F light blue, S yellow, Br dark red. Figures adapted (Lauber, B, et. al.) [242].

The adenosine analogue-oligoarginine conjugate ARC1416 is a bifunctional inhibitor that also interacts with wtPKA through hinge interactions combined with peptide binding site interactions, as shown by crystal structures **(Paper IV)**. The inhibitor developed using 7-deazapurine as adenine moiety with α, ω-nonanedioicacid linker and five number of D-arginine residues were added to the target peptide binding site. Dynamic scanning fluorimetry showed ΔT_m of 16.7 °C for wtPKA which is higher than previously reported PKA inhibitors [243], but 5 fold less sensitive for ATK3/PKB_γ. The co-crystal structures revealed deazapurine to interact with Glu121, Val123 at 2.9 and 3.8 Å, respectively. The D-Arg2 interacts with Glu127 at 3.2 Å (Figure 4.3). The residue Glu127 interactions with inhibitor were also mentioned earlier. Moreover, The D-Arg4 interacts with both the Glu residues at positions 170 and 230, at 2.8 and 3.0 Å respectively.
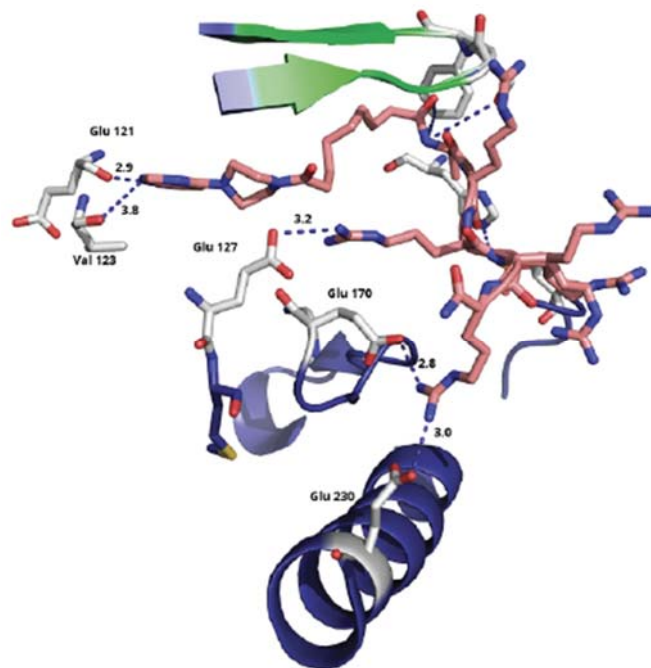
Figure 4.3: The co-crystal structure of wtPKAc: ARC1416 (PDB code: 5J5X). ARC1416 interacts with wtPKA through hinge region and peptide binding site.

4.2. Dynamic properties of ligand binding

Protein kinases are very dynamic in nature and the glycine-rich loop represents one of the most flexible regions of protein kinase A. The amino acid residue Thr51 (PKA) of the glycine-rich loop interacts with arginine residues of inhibitor peptide. This position is typically either threonine or serine in serine/threonine or tyrosine kinases [199]. However, an exceptional pattern is observed in ABL kinase which has an extra glycine in the second residue position and is the target for leukemia drug imatinib, which induces a conformational shift in the shape of the glycine-rich loop. Another exception is seen in atypical protein kinase C, which lacks the third glycine. The Aurora kinase inhibitor VX680 is interesting as a cross-reactive inhibitor of ABL as well, and it induces conformation changes in the glycine-rich loops of both proteins. However, Aurora does not have the extra glycine of ABL. In order to study the dynamic properties of this loop, we have mutated residues of T51G and G55A in PKA **(Paper II)**. The enzymatic assays showed significant changes in their binding affinities (Ki) with respect to wtPKA for ATP-competitive inhibitors (Table 2).

Table 2. Binding affinities (K_i) measured based on ATP competitive enzymatic assay and presented in uM.

Kinase	1l	1p	1c	H89
G55A	0.5	0.6	3.87	0.44
T51G	0.09	1	n.d.	n.d.
WtPKA	0.002*	0.0009*	0.3*	0.063‡

* from Lauber et al.[242].
‡ from Plug et.al.[244].
n.d.-not determined.

The inhibitors have decreased affinities (Ki) against PKA mutants compared to wtPKA (**Paper II**). Investigating wtPKA-1l, 1p, crystal structure (PDB code: 4UJ9 & 4UJA, respectively) revealed an extended interaction network in the glycine-rich loop and peptide bond Thr51-Gly52 where the para-F_3C –phenyl ring made multiple favorable contacts [242, 245, 246]. The loss of binding affinities is due to conformation changes. Replacement of threonine with glycine seems likely to facilitate conformational changes, and increase flexibility. The inhibitor 1c shows a 12 fold decrease in affinities with the G55A mutation, similar to H89. Susan Taylor and co-workers [247] studied the three glycine sites via mutation to serine, showing an approximate 10 fold reduction of affinity for ATP for the first two sites. However, the third glycine mutation G55S had no apparent affect on nucleotide binding [247]. To summarize results, we mutated T51G and G55A to the corresponding residue of ABL and PKC kinases, respectively. The affinity for ATP almost same (4-6 uM) for wtPKA and both mutants but there are dramatic shifts with respect to inhibitor binding energies.

4.3. Water determinants of ligand binding

Water molecules play an essential role in protein-ligand binding mechanisms [200-207]. In order to bind the ligand to the cavity of the protein, water molecules need either be displaced or mediate the interaction. As a consequence, the binding affinity depends on details of the water interactions [207, 208]. There is a water molecule

(**Paper I**) present in the meta-aryl-substituted inhibitors 1c and1e complex with PKA close to Phe54 from the glycine-rich loop. Intriguingly, this water molecular is also present as a cluster in the eight of the thirteen PKA structures with similar loop conformation (Figure 4.4) [242]. For the para-substituted inhibitor 1l (Ki = 2 nM), the CF_3 substituent displaced the water molecule and gained approximately 40 and 150-fold binding affinities compared to 1c (Ki= 300 nM) and 1e (Ki= 80 nM) [242]. This is analogous to effects characterized for e.g. thrombin, where displacing a well-ordered structural water molecule from a hydrophobic pocket correlates increase binding affinity 40-fold [209].
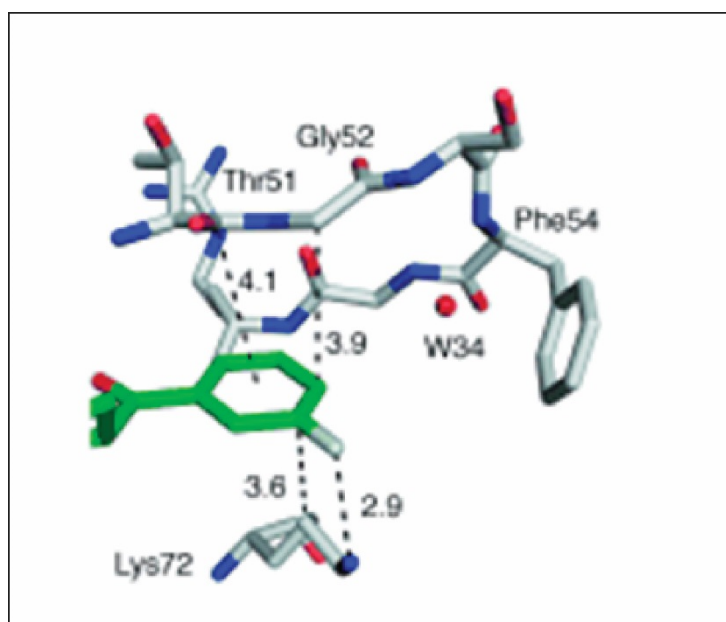
Figure 4.4: Presence of water molecule in front of Phe54 in the presence of iodo-substituted inhibitor (PDB code: 4UJ2).

The ATP binding mechanisms of the Aurora kinases are highly similar to the other protein kinases. As most approved protein kinase inhibitor drugs target ATP binding sites, adequate selectivity for target kinases has been demonstrated, but it remains a major challenge to achieve target specificity. The specificity of protein kinase inhibitors is of course determined by the amino acids surrounding the ATP sites (e.g. Thr217 in Aurora A vs Glu in Aurora B), but structural water molecules also play a key role. An example of water mediated hydrogen bonding as a selectivity determinant for protein

kinases is shown by the approved leukemia drug bosutinib [210] and it can play role in getting pharmacological selectivity [206]. Both effects, the role of conserved water molecules and the changes of single amino acid, contribute to affinity and specificity [211]. The conserved clusters of water molecules in Aurora A were identified by superimposing crystal structures **(Paper V)**. There were 42 Aurora kinase A crystal structure used in this study (Figure 4.5). Aurora A structures with resolution better than 2.5 Å were selected.
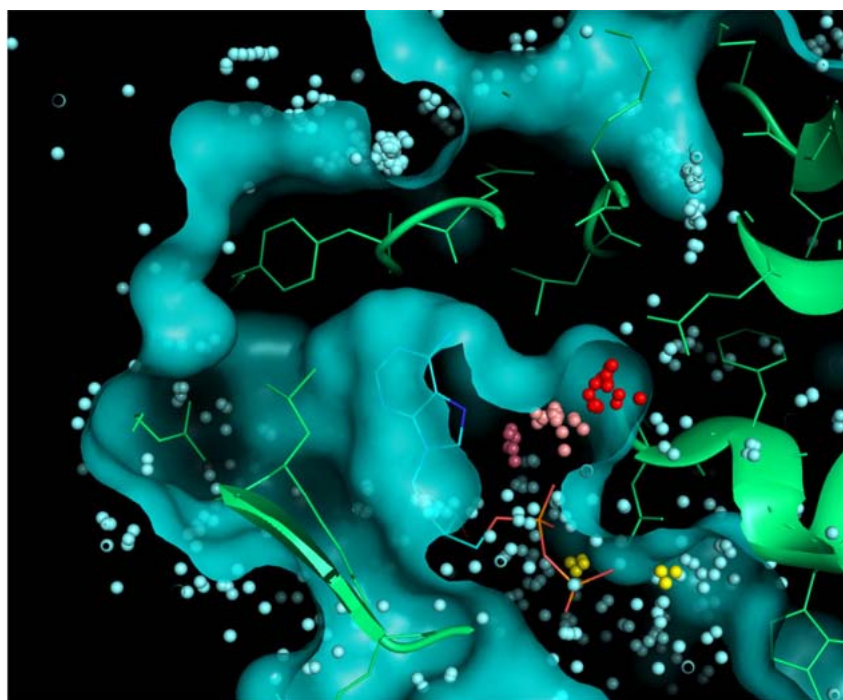
Figure 4.5: Clusters and scattered distribution of water molecules at the ATP binding pocket. Spheres with shades of red mark different clusters at or near a pocket found between ATP, the gate-keeper, and the aspartic acid of DFG. The figure has been taken with permission (O.A. Gani, et.al.) [213].

Rational drug design based on the targeted structural water molecules increase the ligand binding affinity [248]. Structural water molecules can be used for increasing ligand efficiency in Aurora kinase A.

4.4. Kinase–ligand interactions studies using thermal shift assay

Studies of protein kinases-ligand interactions are critical to improve drug discovery and design processes. The establishment of screening assays often require time and

are expensive as well. Sometimes the methods are not compatible to assess all kinds of inhibitors due to technique limitations. However, there are several techniques that are simple and inexpensive. Differential scanning fluorometry (thermal shift) is a popular, rapid, inexpensive and powerful technique to study protein ligand stability interactions, especially for low molecular weight compound like fragments [249, 250]. Thermal shift assays monitor the stabilization introduced by ligand binding to the protein which is measured in the presence and absence of inhibitors. The measured ΔT_m values correlate with K_D and IC_{50} values [249, 251]. In this study, we used a thermal shift assay to investigate Maybridge fragments, and known kinase inhibitors AT9283, and NVP-TAE684 on the PKA-Au6 kinase (Figures 4.6 and 4.7).
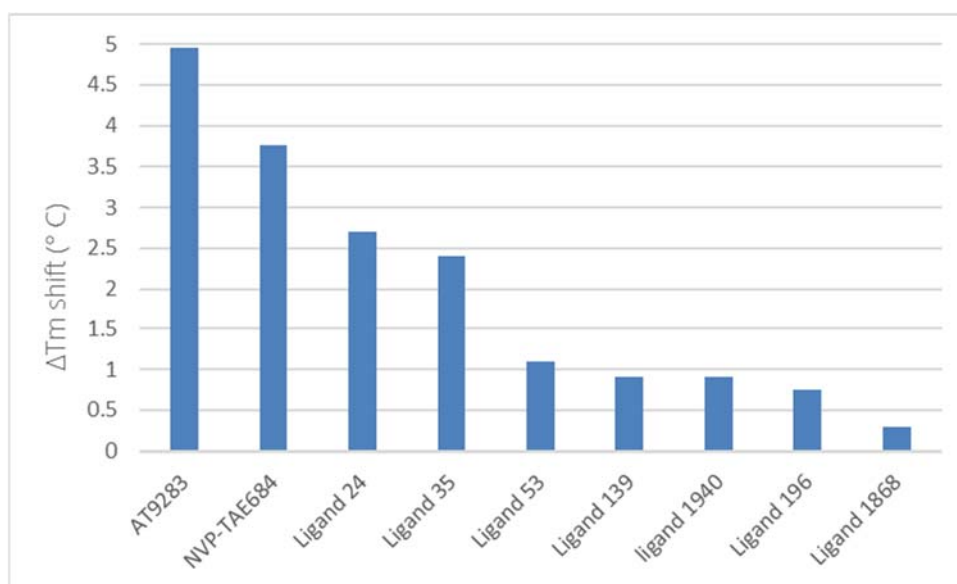
Figure 4.6: Thermal shift assays with kinase inhibitors and fragments.
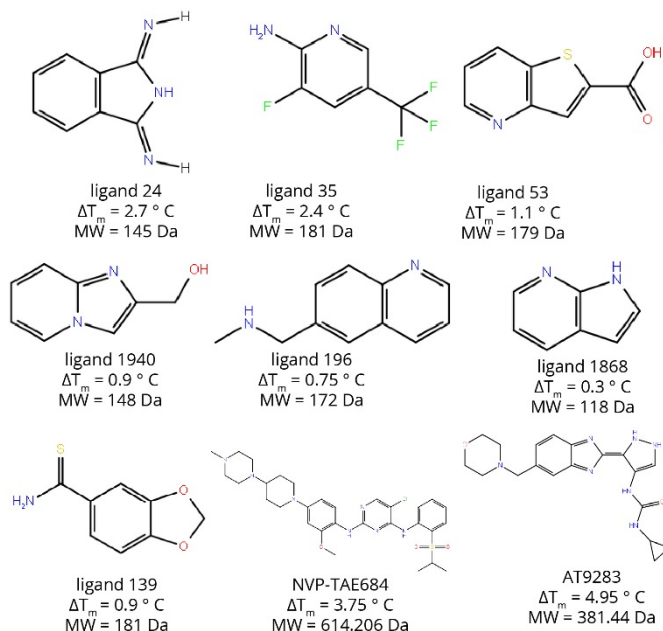
Figure 4.7: The thermal shift screen exhibited a varied range in ΔT_m (from 0.3 to 5.0 °C) to Aurora mimic PKA.

Multitargeted kinase inhibitors AT9283 and NVP-TAE684 showed ΔT_m 4.95 °C and 3.75 °C, respectively. The crystal structure of PKA-Au6 with AT9283 complex revealed classical hinge hydrogen bond interactions with the pyrazole scaffold of the inhibitor. The glutamic acid (E121) and alanine (A123) formed three hydrogen bonds with AT9283 (Figure 4.9). Similar hinge-pyrazole interactions are observed in Aurora kinase A [222]. Thus, these interactions might be the cause of the higher ΔT_m. Moreover, the known kinase inhibitors such as VX-680, staurosporine, sunitinib and JNJ-7706621 have ΔT_m values higher than 5 °C [249]. The binding affinities (K_i) of AT9283 and NVP-TAE684 are measured based on an ATP competitive enzymatic assay against PKA-Au6. Both compounds show tighter binding and K_i values are 33 and 36 nM, respectively. The correlation of thermal shifts with K_i values indicate that T_m shifts > 4 °C likely represent binding affinities in nanomolar range (<1 uM) [243, 252].

The Maybridge fragments which were selected based on molecular docking were tested by the thermal shift assay. The fragments ligand_24 (ΔT_m=2.7 °C) and ligand_35 (ΔT_m= 2.4 °C) resulted ΔT_m > 2 °C. Only ligand_1868 (ΔT_m=0.3 °C) showed ΔT_m < 0.5 °C. Fragments which exhibited ΔT_m > 0.5 °C can be used as a hit for further development [253].
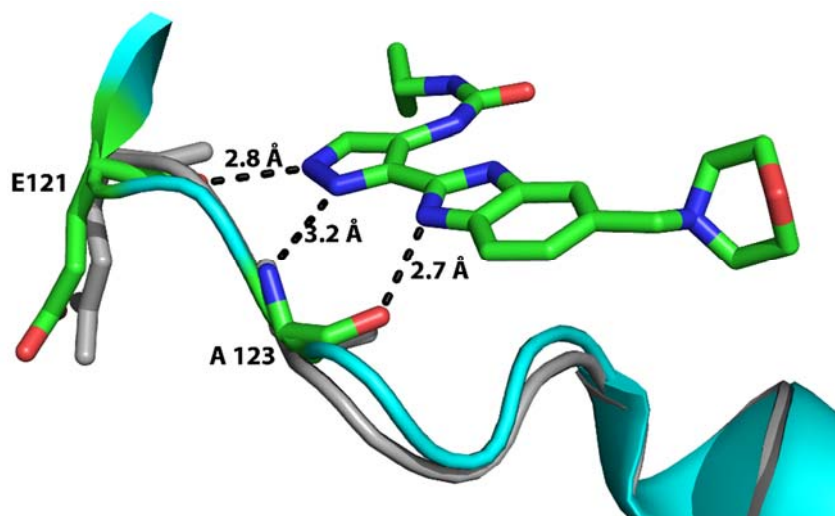
Figure 4.8: X-ray structure of PKA-Au6 and Aurora A with AT9283. Analogously, AT9283 interacted in the in the hinge region of both kinases. PKA-Au6 is colored as cyan and Aurora A as gray. Only hinge region is shown here for clarity. Color code for AT9283: C green, O red, N blue.

5. Summaries of papers

5.1. Paper I

Addressing the Glycine-Rich Loop of Protein Kinases by a Multi-Facetted Interaction Network: Inhibition of PKA and a PKB Mimic.

Birgit S. Lauber, Leo A. Hardegger, **Alam K. Asraful**, Bjarte A. Lund, Oliver Dumele, Michael Harder, Bernd Kuhn, Richard A. Engh, and François Diederich.

Protein kinase A is a prototype for protein kinase studies, including its use a kinase for other protein kinase targets. We described here the design, synthesis, and tests of a series of 32 enantiomerically pure inhibitors of protein kinase A (PKA) and its mutant PKAB3 as the mimic of PKB. The ligands bind to the hinge region, ribose pocket, and their substituted aromatic rings (phenyl, thienyl) target interactions with the glycine-rich loop at the ATP site of the proteins. Biological assays showed high potency against both PKA and PKAB3, with a preference for PKA. The crystal structures revealed a multi-facetted network of ligand–glycine-rich loop interactions, whereby efficient water replacement contributes to the binding strength. The results suggest that aromatic rings of the inhibitors with para-CF3-phenyl and 5 bromothienyl substituents are involved in arene interactions with optimzed interactions with the glycine-rich loop. The para-substituted or thiophene-substituted inhibitor weres 10-100-fold more active that the meta-substituted compounds, because the meta-substituted inhibitors lacked interaction with the glycine-rich loop.

Flexibility properties of the protein kinase glycine-rich loop are critical for inhibitor binding

Kazi A. Alam, Osman A. B. S. M. Gani, Tony Christopeit, and Richard A. Engh. (Manuscript).

The glycine-rich loop is the dynamic and flexible part of the kinase domain of protein kinases. The glycine-rich loop with the conserved motif glycine-x-glycine-x-aromatic-glycine (G50xG52xaG55, residues numbering according to PKA), involved in interactions with the ATP binding site. The amino acid sequence of this motif is not strictly conserved across the kinome. An exceptional pattern is observed in ABL kinases which have an extra glycine in the second residue positions (GGGxxG), and in atypical PKC, which lacks the third glycine (GxGxxA). In this paper, we studied the effects of the inhibitor binding with corresponding mutations in the glycine-rich loop (T51G and G55A in PKA). We tested inhibitors against T51G and G55A. The binding affinities of 1i, 1p, 1c and H-89 is weakened by several orders of magnitude. The results revealed the higher sensitivity of the inhibitors which were developed to target the loop. However, the binding of staurosporine, a pan-kinome inhibitor, is strengthened by both mutations. The increased affinities of staurosporine may be explained by the structural changes of the loop which resulted in faster association rates and better accommodation for the inhibitor.

Inhibitor induced structural effects involving Phe327 in AGC kinases.

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Protein kinases are regulated in dynamic manner by interacting with various proteins, substrates and movement of domains or motifs. As such, the C-terminal stand (C-tail) of AGC kinases also regulates its kinase activity. In this work, we show the highly conserved aromatic residue phenylalanine (Phe327 in PKA) in AGC kinases and its role in kinase-inhibitor binding. The inhibitor-Phe327 interactions showed complex behavior. Phe327 can block inhibitors from occupying the ATP-binding pocket, or may be moved to enable good binding interactions. The PKA-based Aurora kinase (PKA-Au6) model which has Phe327 show the displacement of this residue in crystal structures (PDB ID: 5N23) and its affects on binding affinities of pan-aurora inhibitor AT9283. Binding studies of tricyclic dianilino-pyrimidine scaffold based 1B and 2A show the former inhibitor exhibit more potent against PKA-Au6 while the latter inhibitor is more effective against to Aurora A. The weaker affinity of 2A is might be effects from Phe327. Similarly, Barasertib is a potent Aurora B inhibitor with weaker binding affinity to the PKA based model. Inhibitors induced displacement was previously observed as staurosporine was able to displace Phe327.

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Bifunctional or bisubstrate ligands can be used for studies of strong protein peptide interactions. In this work, we report adenosine analogue-oligoarginine derivatives ARC 14108, 1411 and 1416 inhibitors interaction against the catalytic subunit of wtPKA. Biophysical studies revealed 7-deazapurine as a tight binding nucleotide moiety with the addition of linker. The number of d arginine residue increase in ARC1416 based on 1409 (1000-fold higher affinity toward PKAcα). The co-crystal structures show interactions with hinge and peptide binding region including different glycine-rich loop refolding configurations. The bifunctional ligand Arc1411 disrupted tight binding tetrameric holoenzyme of cAMP-dependent protein kinase (PKA) whereas the ATP site inhibitor H89 was not able to dissociate them. Thus, these studies show a new route to develop inhibitors for protein protein interactions which may be used as an important tool in drug discovery.

Assessing protein kinase target similarity: Comparing sequence, structure, and cheminformatics approaches.

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Although protein crystallography provides us atomic resolution data which shows protein ligand interactions in great detail; the energies of ligand binding cannot be predicted precisely from individual structures. However, the availability of kinase inhibitors structures along with biophysical results enable statistical methods to assess similarity and identify key factors. In this paper, we have analyzed kinase target similarity by sequence, structure, and binding data. Examples of the gatekeeper and "gatekeeper +2" residues distribution provide examples of specific selectivity determinants. Statistical measures based on residue identities and also inhibition profiles identify similarities between targets. These measures are only statistical, however. For example, Aurora and ABL kinases are quite distinct, but share tight binding with VX680. Statistical measures also identify clusters of conserved water positions. The superpositions provide information regarding the importance of the water molecule positions.

5.6 Paper VI

On methionine as a gatekeeper residue for protein kinase inhibition targets.

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The gatekeeper residue in kinase domain of protein kinases acts as an important selectivity determinant for kinase inhibitor interactions. Methionine is the most frequent gatekeeper residue in protein kinases; approximately 40% of the gatekeeper residues are methionine in the human kinome. EGFR has threonine, however, and the mutation of this gatekeeper residue to methionine creates drug resistance. Methionine side chain interactions, especially the sulfur interactions are interesting for their contributions to the binding affinity. In this paper, we show the side chain and rotamer distribution of methionine and their inhibitor binding pattern using available structures and binding data from different databases. We choose PKA as a model for study as it has methionine as a gatekeeper residue. We screen fragments from an in-house library by using molecular modeling and solve the crystal structures of two fragment (Frag195_Cl and Frag 414_S) complexes in PKA. The structures reveal different methionine rotamer conformations in Frag195_Cl and for Frag414_S, the structure is flipped by 180 about an axis perpendicular to the ring. These studies should aid the design of new protein kinase inhibitors against gatekeeper Met protein kinases, including drug resistant EGFR.

6. Conclusions and future perspectives

Protein kinase A, surrogate kinase, and Aurora kinase A were used to study kinase inhibitor binding mechanisms. Several biophysical techniques were applied to investigate kinase-ligand interactions in dynamic and atomic level. Ligands were designed and synthesized to targeting the ATP binding site and in particular the glycine-rich loop of PKA and PKB. Although known to be important, this loop has not generally been addressed specifically before in most protein kinase inhibitor design programs. Crystal structures with inhibitors revealed details of their binding modes and showed that the ligand interactions with the glycine-rich loop enhanced binding affinities. Biophysical characterization of the glycine–rich loop mutants (T51G and G55A) showed weaker affinities against the inhibitors which have interactions with the loop. Structural and biochemical studies of glycine-rich loop addressed inhibitor considered the loop is a hot spot for developing kinase inhibitors. However, further work is needed to test these inhibitors against native PKB if that is the best target, which may lead to better understanding of the energetics of glycine-rich loop interactions. The co-crystal structures of wtPKA with bisubstrate inhibitors showed differences in glycine-rich loop conformations. The bisubstrate approach targets dual sites of both ATP and substrate binding, enabling high specificity and the disruption of tight-binding protein complexes.

PKA-Au6 (Aurora B model) was used to study selectivity determinants. Different inhibitors tested showed the residue Phe327 of PKA-Au6 to play a significant role in inhibitor selectivity, for example either by accommodating or blocking inhibitors in the pocket. Tricyclic dianilinopyrimidine compounds (1B & 2A) showed intriguing results where 1B is selective against PKA-Au6 but 2A was weakened binding affinities, like Barasertib. Structural analysis of these inhibitors with PKA-Au6 model suggests that there might be a clash between the side chain of Phe327 and inhibitors (2A & Barasertib). However, additional studies required to make such a statement.

Analysis of Aurora A crystal structures demonstrated the presence of structural water molecules in the different region of ATP binding pocket. Water replacement by the ligand in the loop region contributes to the binding strength and careful examination facilitates the design of better selectivity among kinases. Fragment-based approach aids to find new scaffold and novel kinase inhibitors. PKA-Au6 was also used for the studies of the fragments. The fragments named ligand_24 (ΔT_m=2.7 °C) and ligand_35 (ΔT_m= 2.4 °C) show ΔT_m > 2 °C which indicates the possibility of the fragments to develop as inhibitors. The crystal structures of two fragment (Frag195_Cl and Frag 414_S) complexes in wtPKA revealed different methionine rotamer conformations. PKA has methionine as a gatekeeper residue. The abundances of methionine in the gatekeeper position are high in the protein kinases; and methionine side chain interactions, especially the sulfur interactions are interesting for their contributions to the binding affinity. In sum, these studies provide a range of information that can aid drug design, particularly against AGC kinase targets.

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8. Papers I-VI