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# Chemical Proteomics for Characterization of Small Molecule Kinase Inhibitors in Cancer and Inflammation

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# Abstract

Protein kinases have been established as promising drug targets for treatment of various types of cancers. Small molecule kinase inhibitors have become increasingly important in cancer therapeutics. Currently, more than 33FDA-approved kinase inhibitors have been used as promising targeted therapeutics, and numerous (>250) inhibitors are in various stages of clinical evaluation. Kinobeadstechnology in combination with high-resolution MS has emerged a powerful tool to understand the mode of action, to identify new targets, and to characterize potential off-target effects of small molecule kinase inhibitors. In this thesis, a dose-resolved quantitative chemical proteomics approach has been used to understand the mechanism of small molecule kinase inhibitors and to highlight their off-targets.

CDKs are a family of protein kinases that play the most important role in the regulation of cell cycle transitions, and four CDKs (CDK1, CDK2, CDK4, and CDK6) are essential components of the cell cycle machinery with key functions in both human normal cells and in human cancer cells. Over-expression of CDKs is highly related with human cancers, and 14 drugs that target CDKs activity have emerged and have been tested in various stages of clinic. The other CDK inhibitors are still in clinical trials beside FDA approved CDK4/6 inhibitor Palbociclib. This study characterized 14 CDK inhibitors, which include both first- and second-generation CDK inhibitors. These inhibitors were tested using Kinobeadstechnology in a competitive approach against a large number of kinases, including CDKs and their complex partners. The results of this work show the successful development of a new generation of CDK inhibitors, but they also offer good explanations why CDK inhibitors have not been able to withstand FDA testing in the past.

Salt-inducible kinase 2 (SIK2) has been found to be a potential target in inflammation and autoimmune diseases. In this study, SIK2 has been detected as an off-target by screening 242 small molecule kinase inhibitors through Kinobeadspulldown assay. Notably, treatment of various immune responding models (THP-1, BMDCs, PBMCs, and human DCs) with SIK2 inhibitors particularly induced the production of anti-inflammatory cytokine IL-10 and reduced the production of pro-inflammatory cytokine TNF-α. Anti-TNF-α therapy and IL-10 therapy have been demonstrated to be exploited in various clinical trials for the development of therapies of several inflammatory diseases. Nevertheless, no small molecule kinase inhibitor has shown therapeutic benefits in these therapies. This work provides strong reasons to drive low molecular weight SIK2 inhibitors in the investigation of inflammatory and autoimmune diseases.

# Zusammenfassung

Proteinkinasen wurden als vielversprechende therapeutische Ziele zur Behandlung verschiedener Krebsarten etabliert. Niedermolekulare Kinaseinhibitoren nehmen eine zunehmend wichtige Bedeutung als Krebsmedikamente ein. Mehr als 33 Kinaseinhibitoren wurden bereits von der FDA als vielversprechende zielgerichtete Therapeutika zugelassen und eine Vielzahl von Inhibitoren (>250) werden derzeit in verschiedenen klinischen Phasen getestet. Die KinobeadsTechnologie, kombiniert mit hochauflösender Massenspektrometrie, ist zu einer effektiven Methode geworden, um die Wirkungsweise niedermolekularer Kinaseinhibitoren zu verstehen, neue Zielproteine zu identifizieren und potentielle Nebenwirkungen zu charakterisieren. In dieser Arbeit wurde dieser chemisch-proteomische Ansatz dosis-aufgelöst angewandt, um die Wirkweise niedermolekularer Kinaseinhibitoren zu erfassen und deren weitere Zielproteine aufzuzeigen.

CDKs umfassen eine Familie von Proteinkinasen, die die wichtigste Rolle in der Regulation des Zellzyklus spielen; vier dieser CDKs sind essentielle Komponenten der Zellzyklus Maschinerie mit Schlüsselfunktionen sowohl in gesunden als auch in Krebszellen: CDK1, CDK2, CDK4 und CD6. Die Überexpression von CDKs ist eng verknüpft mit der Entstehung von Krebs und es wurden bereits 14 Medikamente, die die Aktivität von CDKs beeinflussen, entwickelt und in verschiedenen klinischen Phasen getestet. Ausser dem durch die FDA zugelassenen CDK4/6 Inhibitor Palbociclib, sind alle CDK Inhibitoren noch in klinischen Studien. Diese Arbeit charakterisiert 14 CDK Inhibitoren, welche sowohl die erste als auch die zweite Generation von CDK Inhibitoren umfassen. Diese Inhibitoren wurden mit Hilfe der KinobeadsTechnologie in einem kompetitiven Ansatz gegen eine grosse Anzahl von Kinasen, darunter auch CDKs und deren Komplexpartner, getestet. Die Ergebnisse dieser Arbeit zeigen zum einen die erfolgreiche Entwicklung einer neuen Generation von CDK Inhibitoren auf, bieten allerdings auch triftige Erklärungen weshalb CDK Inhibitoren der Prüfung durch die FDA in der Vergangenheit nicht standhalten konnten.

*Salt-inducible kinase 2* (SIK2) wurde als potentielles therapeutisches Ziel in Entzündungs- und Autoimmunerkrankungen beschrieben. In der vorliegenden Studie wurden 242 niedermolekulare Kinaseinhibitoren mit Hilfe der KinobeadsTechnologie getestet und SIK2 wurde als unbeabsichtigt getroffenes Zielprotein einiger Inhibitoren identifiziert. Die Behandlung verschiedener präklinischer Modelle für Immunantwort (THP-1, BMDCs, PBMCs und humane DCs) mit SIK2 Inhibitoren induzierte die Produktion des anti-inflammatorischen Zytokins IL-10 und reduzierte die Produktion

des pro-inflammatorischen Zytokins TNF-α. Anti-TNF-α und IL-10 basierte Therapien wurden bereits in verschiedenen klinischen Studien zur Behandlung einiger Entzündungskrankheiten getestet. Allerdings konnte bisher kein niedermolekularer Inhibitor einen therapeutischen Effekt vorweisen. Diese Arbeit liefert starke Gründe dafür niedermolekulare SIK2 Inhibitoren in der Erforschung von Entzündungs- und Autoimmunerkrankungen voranzutreiben.

# CHAPTERI
# General Introduction

## 1.1 Targeted cancer therapies

Cancer is a group of diseases characterized by somatic cells becoming abnormal, having out-of-control cell growth, and becoming malignant[1, 2]. Over 100 different types of cancers have been detected, and each of them has been classified by the initially affected cell type so far[3]. High morbidity and mortality rates worldwide are caused by cancer, with a total of 14.1 million new cancer cases emerging and 8.2 million cancer deaths occurring in the world in 2012[4]; approximately 1.7 million new cases emerged and 0.6 million cancer-related deaths occurred in the United States in 2014[5].Population growth and aging and risk factors such as obesity and tobacco and alcohol use have been established as factors that increase the occurrence of cancer, and smoking is the most important risk factor for around 20% of cancer-related deaths[6-8]. Elucidation of the molecular mechanism of cancer development has consequently devoted considerable efforts. There are various types of cancer therapies: surgery, radiation therapy, chemotherapy, immunotherapy, targeted therapy, hormone therapy, and stem cell transplant[9, 10]. The understanding of cancer biology contributes toward identifying the pathways of different tumor entities. These findings are being used to generate targeted cancer therapy by selectively targeting certain proteins that have different expression levels in cancer cells and in normal cells or tissues[11].

More recently, targeted cancer therapy has increasingly attracted greater attention[6, 12, 13]. Specific molecules, also called molecular targets, have been detected to play an essential role in tumor growth, progression, and spreading[14]. Thereby, targetedcancer

therapy enables utilization of drugs or other substances to block the tumor growth and spreading by interfering with specific molecular targets[15]. Targeted cancer therapy exhibits some differences when compared with the conventional chemotherapeutic treatment[16]. While targeted cancer therapy works on specific tumor-associated molecular targets, most standard chemotherapies are non-specific when interfering with the growth of all rapidly dividing cells, including both normal and cancer cells. The design of targeted therapy is aimed at blocking the interaction with specific tumor-associated molecular targets, whereas most standard chemotherapies are identified because of their cytotoxicity on cells[17]. Targeted therapy often blocks the proliferation of tumor cells, while standard chemotherapy drugs enable tumor cell killing[18].Owing to these characteristics, targeted therapy shows significant benefits over standard chemotherapy for the treatment of cancer. Currently, targeted therapies have become the focus of anti-cancer drug development. A number of different types of targeted therapies, including immunotherapies, ligand-targeted therapies, antisense therapies, angiogenesis inhibitors, tyrosine kinase inhibitors, metalloproteinase inhibitors, apoptosis agonists, have been approved for the treatment of cancer[15, 19-22].

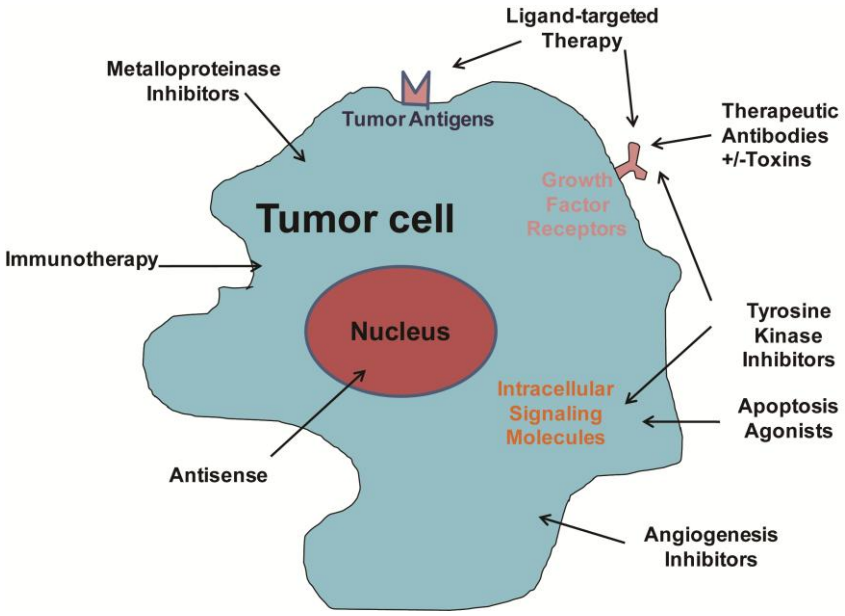


**Figure 1.1 An overview of targeted cancer therapy.**

The identification of specific targets that play a critical role in cancer cell growth and survival is required in the development of targeted therapies (Figure 1.1)[23].Some approaches have been identified to carry out target identification. The first approach is to compare the expression level of individual proteins in cancer and normal cells[24]. The specific proteins that are expressed only in cancer cells or more abundantly in cancer cells, especially if they have been demonstrated to be involved in cell growth or survival, would be potential targets. For example, human epidermal growth factor receptor 2 protein (HER-2) is present in breast and stomach cancer cells with high expression level. Trastuzumab is designed to directly work against HER-2 and is approved to treat certain breast and stomach cancers with over-expression of HER-2[25]. The second approach of potential target identification is determining the mutant proteins that drive tumor progression[26]. An example of such cell growth signaling protein BRAF mutant V600E is present in many melanomas. Vemurafenib has been developed to work against this mutant, which has been approved to treat metastatic melanoma with BRAF V600E[27]. The third approach is to identify certain chromosome abnormalities that are present only in cancer cells, which result in a fusion protein and can drive tumor development[28]. For example, Imatinibhas been developed to target Breakpoint cluster region protein-Abelson murine leukemia viral oncogene homolog (BCR-Abl) fusion protein and has been approved to treat BCR-Abl-driven leukemia[29].

After the identification of the candidate target, there is a key problem in the development of targeted therapy to interfere with the ability of target that induces cancer cell growth or survival. Small molecules that focus on the intracellular targets and monoclonal antibodies that focus on the extracellular targets are two common approaches of targeted therapies[30]. Small molecule agents are designed to easily enter the cells to work against the intracellular targets. “High-throughput screen” is used to identify the candidate small molecules by determining the effects of test compounds on a specific target protein in compounds libraries[31]. Numerous versions of related effective compounds have been produced by chemical modification and

testing to determine the most effective and specific molecules for targeted therapy[31, 32]. Monoclonal antibodies, which are relatively large and difficult to enter cells, are used to work against the extracellular targets[33]. Purified target proteins are used to inject animals such as mice, enabling the animals to produce many different types of antibodies against the target. The most effective antibody that binds best to the target protein is detected from these antibodies libraries[34].

Although targeted cancer therapies exhibit many advantages in cancer treatment, they still have some limitations. The most serious issue is the acquired resistance after long-term drug treatment[35, 36]. The mechanism of acquired resistance includes two ways: mutation of the targets that mutes the interaction between targeted therapy drugs and the identified targets, and introduction of a new pathway that is independent of the target and occurs to promote tumor growth. Therefore, a combination of targeted therapies that target different parts of cell signaling pathway works better in cancer treatment. Recent research has showed that a combination drug therapy slowly induces drug resistance and disease progression in melanoma with B-Raf V600E mutation[37]. Combination of targeted therapy with traditional chemotherapy also works well in cancer treatment. The combination of the targeted therapy drug trastuzumab with the traditional chemotherapy drug docetaxel was approved for the treatment of metastatic breast cancer with over-expression of HER-2[38].

## 1.2 Kinases and their inhibitors

### 1.2.1 Protein kinases

Protein kinases, which play aessential role in the regulation of cell processes, have been found to be closely involved in tumor cell survival and proliferation, and represent a magnetic target in oncology drug discovery[39]. The human genome consists of 518 protein kinases genes, which constitute about 2% of all human genes[40]. Among these 518 kinases, over 150 are demonstrated to be misregulated or

mutated in various diseases, especially in cancer[25]. Based on the sequence similarities of catalytic domain, protein kinases can be grouped into seven families: AGCK (PKA, PKC, and PKG kinases), CaMK (calcium/calmodulin-dependent protein kinases), CK1 (casein kinase 1), CMGC (CDK, GSK3, and CLK kinases), mitogen-activated protein kinase (MAPK), STE (homologs of yeast Sterile 7, Sterile 11, and Sterile 20 kinases), TK (tyrosine kinases), and TKL (tyrosine kinase-like group of kinases) (Figure 1.2A)[40]. By adding phosphate groups to substrate proteins, the function of substrate proteins is changed by changing the enzyme activity and cellular location (Figure 1.2B)[41]. Protein kinases have been reported to be the most active in allthe cellular processes because of the kinase phosphorylation[42].

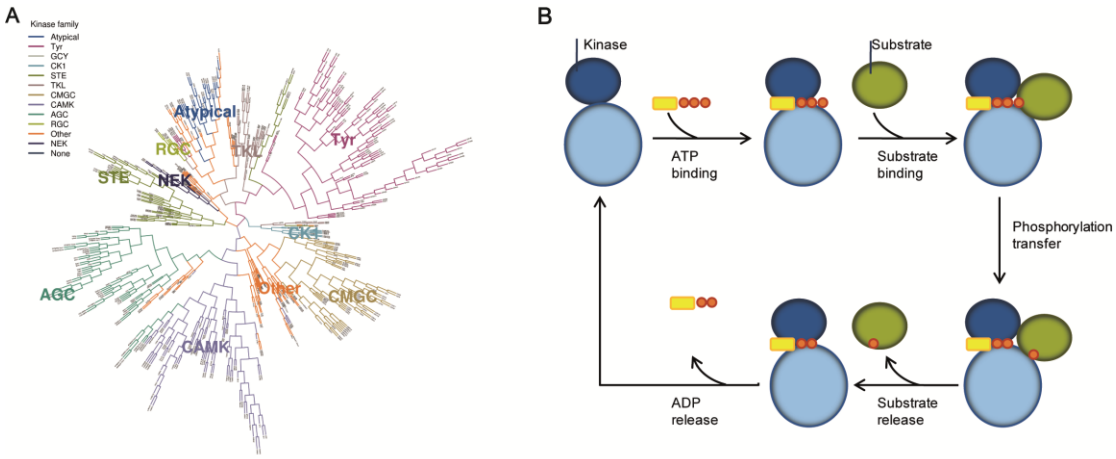


**Figure 1.2 Classification of protein kinases and catalytic cycle of protein kinases.** (A) Dendrogram of the human kinome, modified from (Manning, G. et al., 2002). (B)The catalytic cycle of kinase phosphorylation, adapted from (Ubersax, J.A. & Ferrell, J.E., 2007). At first, the active site of kinase occurs the ATP binding, followed by the substrate protein binding. After ATP and substrate binding, the substrate residue including Ser, Tyr or Thr accepted a γ-phosphate of ATP (red). Then the substrate is released from the kinase after phosphorylation. Then the substrate and ADP is released in turns from the active site of the kinase.

On comparison of the 3D structures, it can be seen that most of the human kinases are very similar, especially the ATP-binding pocket, which is located in the active kinase domain[43].ATP binding pocket comprises three components: an α helix-dominated C-terminal lobe (C-lobe), a β sheet containing N-terminal lobe

(N-lobe), and a connecting hinge region[44]. The binding region of ATP, which is located between N- and C- terminal lobes, is perturbed by most kinase inhibitors. A conserved amino-acid sequence Asp-Phe-Gly (DFG) is the start of an activation loop, which controls the access to the active site[45].Based on the binding mode, there are two groups of kinase inhibitors: reversible and irreversible. Irreversible inhibitors normally react with the kinase and change it chemically via covalent bond conformation[46, 47].Reversible kinase inhibitors can be classified into four types according to the DFG motif and binding pocket conformation. As ATP-competitive inhibitors, Type I inhibitors enable to bind to the active conformation of kinases to control the residue of DFG motif fact into the kinase active site (DFG-Asp in). TypeII inhibitors enable to bind to the inactive conformation of kinases to protrude the residue of DFG motif outward from the kinase ATP-binding site (DFG-Asp out). In the binding mode of type III, inhibitors bind to an allosteric pocket adjacent to ATP, and there is no interaction between the allosteric pocket and the ATP-binding pocket. In the type IV binding mode, inhibitors enable to bind to an allosteric site remote from the ATP-binding pocket. Type Vinhibitors including bivalent andbisubstrate inhibitors, bind to more than one protein kinases conformations (Figure 1.3)[44, 48].

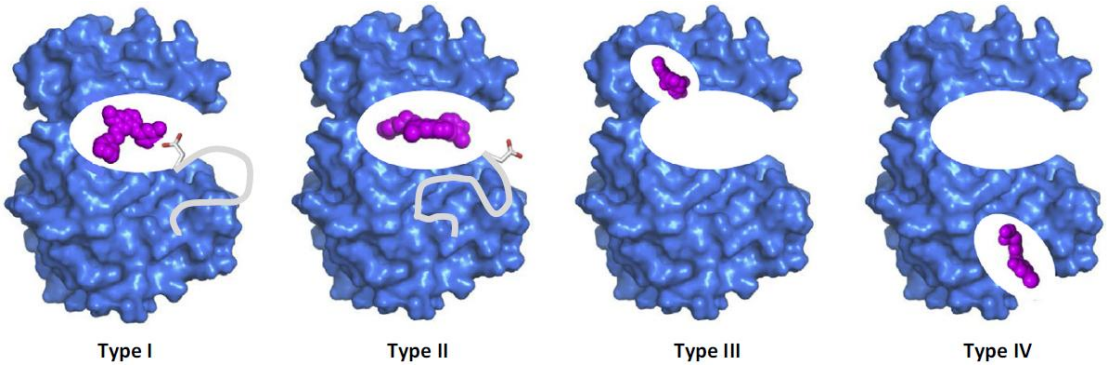


**Figure 1.3 Different binding modes of reversible small molecule kinase inhibitors.**Four binding modes of reversible small molecule kinase inhibitors (Copied from Wu, P. et al., 2015). In type I binding mode,inhibitors bind to the kianse active forms with the DFG motif aspartate residue (white backbone). In type II binding mode, inhibitors bind to the kianse inactive forms with the DFG motif flipped aspartate residue (white backbone). In type III binding mode, inhibitors bind to an allosteric pocket adjacent of ATP. In the type IV binding mode, inhibitors bind an allosteric site remote from the ATP-binding pocket.

### 1.2.2 Small molecule kinase inhibitors

Since protein kinases play an important role in the regulation of cellular activities and signal transductions, they have been established as promising drug targets for treatment of various cancers[9, 19, 25]. The first Food and Drug Administration (FDA)-approved kinase inhibitor Imatinib, which was designed for the treatment of chronic myeloid leukemia (CML), drives the development of kinase inhibitors in the field[49]. Currently, more than 33 kinase inhibitors have been approved by FDA as promising targeted therapeutics, and numerous inhibitors are in various stages of clinical evaluation[44]. Among these 33 oncology drugs, most of them are tyrosine kinase inhibitors, the othersare serine/threosine kinase inhibitors except lipid kinase inhibitorIdelalisib[50]. Classified by binding mode, covalent inhibitors Afatinib[51] and Ibrutinib[52] are irreversible inhibitors, and the others are reversible inhibitors (Figure 1.4).In the current research, we have assessed the therapeutic indications and the mechanism of function of these FDA-approved kinase inhibitors.

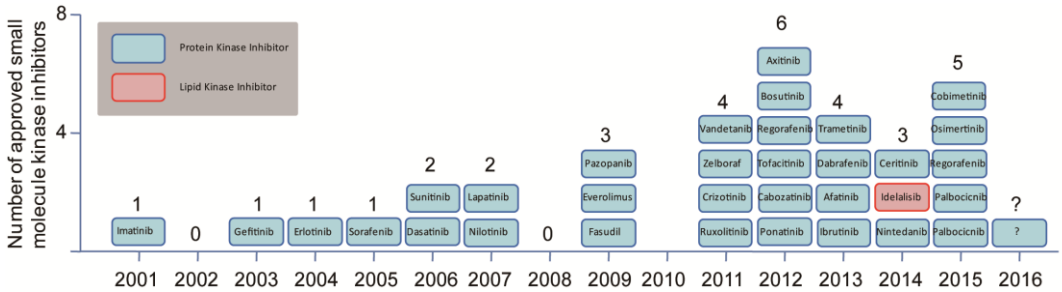


**Figure 1.4 Small molecule kinase inhibitors which approved by FDA were present here (from 2001 to 2015).**

#### 1.2.2.1 Approved tyrosine kinase inhibitors

##### 1.2.2.1.1 Reversible non-receptor tyrosine kinase inhibitors

Imatinib(Gleevec, Novartis, 2001) was the first approved small molecule inhibitor that targeted BCR-Abl in 2001[49, 53]. BCR-Abl exists in cancer cells only, and Imatinib blocks BCR-Abl's activity by stopping the addition of phosphate groups, which leads to apoptosis.Imatinib is the first drug that has been approved for the treatment of CML, and the structure and binding mode of Imatinib provide a basic understanding of the

design of the next-generation BCR-Abl inhibitors[54]. Imatinib's resistancedrives the development of BCR-Abl inhibitors, and the most important BCR-Abl mutations that cause Imatinib resistance are T315I mutation and P-loop mutations[55, 56]. Beside the treatment of CML, Imatinib has been assessed in the treatment of patients with gastrointestinal stromal tumors (GIST)[57], Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL)[58], dermatofibrosarcoma protuberans (DFSP)[59], and hypereosinophilic syndromes/chronic eosinophilic leukemia[60]. Imatinib also works against the activity of PDGFRA and c-KIT[61].

So far, there are five BCR-Abl inhibitors which were approved by FDA: Imatinib (Gleevec, Novartis, 2001), Dasatinib (Sprycel, Bristol-Myers Squibb, 2006), Nilotinib (Tasigna, Novartis, 2007), Ponatinib (Iclusig, Ariad Pharmaceuticals, 2012), and Bosutinib (Bosulif, Wyeth, 2012)[62]. Imatinib, Ponatinib, and Nilotinib have been found to bind BCR-Ablinactive conformation with DFG motif; Dasatinib and Bosutinib have been reported to bind BCR-Abl active conformation with the activation loop[63].

Janus kinases (JAKs) family, which is one of the intracellular, non-receptor tyrosine kinases, mediates cytokine transduction via the JAK-signal transducer and activator of transcription (STAT) pathway[64]. JAKs family has four isomers (JAK1, 2, 3, and tyrosine kinase 2 [TYK2]). JAK1 plays an essential role in cytokine secretion; it induces the production of IL-2, 4, 3, and 9[65]. JAK2 is also important for the secretion of hormone-like cytokines, such as growth hormone (GH), prolactin (PRL), erythropoietin (EPO), and thrombopoietin (TPO)[66-68]. JAK3 plays an important role in the immune system, and it is exclusively expressed in lymphoid lineage. TYK2 is essential for IL-12 signaling[69]. The high sequence similarity of these JAK isomers limits the development of selective JAK inhibitors[70], and JAK inhibitors are mainly focused on the treatment of autoimmune diseases and malignant neoplastic diseases so far[71].

The first FDA-approved JAK inhibitor wasRuxolitinib (Jakafi, Incyte Corp. 2011), which

inhibits JAK1, 2 and was approved for the treatment of patients with myeloproliferative disorders e.g. myelofibrosis[72]. Since JAK3 is essential for the immune system and malignant neoplastic diseases, the first FDA-approved JAK3-selective inhibitor Tofacitinib(Xeljanz, Pfizer, 2012) was developed and used for the treatment of rheumatoid arthritis[73]. However, European regulatory agencies didn't approve Tofacitinib as a JAK3-selective inhibitor because of its side effects including neutropenia and anemia, probably because of the pan-JAK inhibition[74, 75]. Both of these JAK inhibitors have been reported to bind with the JAKsATP pocket. The development of next-generation JAK inhibitors is mainly focused on improving JAK3 inhibition to reduce its side effects[76].

#### 1.2.2.1.2 Reversible receptor tyrosine kinase inhibitors

ErbB is a family of receptor tyrosine kinases, which includes four members: ErbB1/epidermal growth factor receptor (EGFR), ErbB2/HER-2, ErbB3/HER3, and ErbB4/HER4[77]. Over-expression of ErbBs is associated with various types of solid tumor development[78]. The activation of receptor tyrosine kinases phosphorylate EGFR leads to the activation of Ras/MAPK pathway, PI3K/Akt pathway, and signal transducers and activators of transcription signaling pathways[77]. EGFR mutations have been detected frequently in non-small cell lung cancer(NSCLC), which leads to the development of the cancer. Over 90% of activating EGFR mutations are L858R point mutation and exon 19 deletions, which enhance the sensitivity of Gefitinib and Erlotinib[79, 80].Drug resistance that is conferred by a secondary mutation, which includes T790M mutation and MET amplification, has been found as the most important factor to limit the efficacy of EGFR tyrosine kinase inhibitors (TKIs)[81]. The study of ErbB signaling transduction pathway becomes more and more obvious, and the family of ErbB inhibitors is the largest group of approved kinase inhibitors[77, 78].

So far, five ErbB inhibitors have been approved: Gefitinib (Iressa, AstraZeneca, 2003), Erlotinib (Tarceva, OSI Pharmaceuticals, 2004), Lapatinib (Tykerb, GlaxoSmithKline, 2007), Vandetanib (Caprelsa, AstraZeneca, 2011), and Afatinib (Gilotrif,

BoehringerIngelheim, 2013)[79, 82]. Gefitinib and Erlotinib are EGFR inhibitors and are used for certain breast, lung, and other cancers[81].The binding mode of Gefitinib is the same as Erlotinib in type I binding because of the high structural similarity[83]. Lapatinib a dual ErbB2 and EGFR inhibitor, used for the treatment of ErbB2-positive breast cancer. Lapatinib act to bind the inactive conformation of EGFR and ErbB4 was bind by Lapatinib[84].Vandetanib is determined as a multiple kinase inhibitor that works against EGFR, VEGFR, and RET[85]. Unlike the other reversible inhibitors, Afatinib acts as an irreversible covalent inhibitor of EGFR and ErbB2[51, 86].

Vascular endothelial growth factor receptors (VEGFRs) are the receptors of VEGF and play an important role in vasculogenesis and angiogenesis[82]. There are three main subtypes of VEGFR: VEGFR1, VEGFR2, and VEGFR3. VEGFR1 is essential in tumor progression and dissemination, and the expression of VEGFR1 increases tumor invasiveness. VEGFR2 also expresses in tumor cells, such as melanoma and hematological malignancies. VEGFR3 is essential in tumor microenvironment. Anti-VEGF therapy is an important option for the management of several human malignancies[87].

So far, there are seven FDA-approved VEGFR inhibitors: Sorafenib (Nexavar, Bayer, 2005), Sunitinib (Sutent, Pfizer, 2006), Pazopanib (Votrient, GlaxoSmithKline, 2009), Axitinib (Inlyta, Pfizer, 2012), RegoRafenib (Stivarga, Bayer, 2012), Nintedanib (Ofev, BoehringerIngelheim, 2014), and Lenvatinib (Lenvima, Eisai Inc., 2015)[87, 88]. Many SAR studies demonstrate the binding mode of Sorafenib, Sunitinib, Axitinib, and Nintedanib with VEFGR2 in Cys919 and Glu917. Sorafenib has been shown to inhibit p38α and B-Raf[82, 89].

Anaplastic lymphoma kinase (ALK), which belongs to insulin receptor kinase family, shares a similar sequence with leukocyte tyrosine kinase (LTK) and plays an essential role in development of brain and nervous system[90]. ALK was detected to constitutively activate in large-cell lymphomas, and the diffusion of ALK fusions were also identified

in inflammatory myofibroblastic tumors, NSCLC, systemic histiocytosis, esophageal squamous cell carcinomas, and large B-cell lymphomas. Over-expression of ALK has been detected in neuroblastomas[91, 92]. It has been established that ALK is an anticancer target due to the expression level of ALK in various types of cancers[93].

Crizotinib (Xalkori, Pfizer, 2011) is the first FDA-approved ALK inhibitor used for the treatment of anaplastic large cell lymphoma, neuroblastoma, and other advanced solid tumors[94]. In addition, Crizotinib was the first specific drug for the treatment of some NSCLC[95]. Besides the main target being ALK, Crizotinib also inhibits ROS1, MET, and hepatocyte growth factor receptor (HGFR)[96]. Crizotinibhas been found to bind the ATP-binding pocket of ALK in type I mode, but does not fully utilize the ALK ATP-binding pocket, which explains the poor selectivity[97]. The second-generation ALK inhibitor, Ceritinib (Zykadia, Novartis, 2014) was approved for the treatment of NSCLC patients with acquired resistance to Crizotinibaccording to rearrangements of the ALK gene[98]. Ceritinib was shown to be potently effective in overcoming ALK with L1196M, G1269 A, I1171T, and S120Y mutations, but not effective in the case of ALK with G1202R and F1174 C mutations. The binding mode of Ceritinib is similar to that of Crizotinib, but the isopropyl-sulfonylphenyl binds deeper inside the hydrophobic pocket[99].

Tyrosine-protein kinase Met (c-MET) or HGFR is the only know receptor of Hepatocyte growth factor (HGF). MET activates various cellular signaling pathways and is involved in a wide range of cellular processes, such as proliferation, motility, migration, and invasion[100]. MET signaling pathway plays an important role in cancer development through RAS, PI3K, STAT3, and angiogenesis. The aberrant activation of MET (mutation, amplification, and protein over-expression) was identified in human cancers[101]. Thereby, MET has been confirmed as a potential candidate and biomarker for cancer treatment.

There are two approved MET inhibitors: Crizotinib (Xalkori, Pfizer, 2011) and

Cabozantinib (Cometriq, Exelixis, 2012)[102].Crizotinibhas been detected to bind MET with the ATP-binding pocket in type I mode, and Cabozantinib binds MET in a potent type II binding mode[103]. These MET inhibitors have multiple targets; Crizotinib works against both ALK and MET, and Cabozantinib is a dual MET and VEGFR2 inhibitor[97, 103]. More recently, Cabozantinib has been shown to overcome Crizotinib resistance by blocking proto-oncogene tyrosine-protein kinase 1 (ROS1)-acquired mutations[104].

#### 1.2.2.2 Approved irreversible protein kinase inhibitors

Targeted covalent inhibitors (TCIs) react with their target proteins to form a covalent complex in which the protein has lost its function. The advantages of covalent inhibitors are high potency, high selectivity against the related proteins, and low side effects[105]. The high selectivity of covalent inhibitors is because of a specific cysteine targeting. Moreover, the covalent inhibitors were determined to overcome and prevent the acquired resistance that is conferred by mutations[106]. These covalent compounds provide new and broad application for the cancer therapies.

In 2013, Afatinib was firstly approved by FDAas an irreversible pan ErbB inhibitor, followed by AZD-9291[51]. These two molecules covalently bind with a cysteine residue in the active site of kinases. Even though irreversible inhibitor has potential toxicities, these two inhibitors are expected to achieve better potency and selectivity. Compared with the first-generation TKIs, Afatinib is active against both EGFR-sensitive mutation (L858R) and EGFR-resistant mutation (T790M)[107]. AZD-9291 is only active against resistance mutation (T790M); therefore, AZD-9291 is used for the treatment of EGFR-TKI resistance advanced NSCLC[108].

Ibrutinib (Imbruvica, Pharmacyclics Inc., 2013) is an FDA-approved Bruton’s tyrosine kinase (BTK) inhibitor that is used for the treatment of mantle cell lymphoma and chronic lymphocytic leukemia (CLL)[109]. BTK is an important component of B-cell receptor signaling, which plays an essential role in regulating the survival and proliferation of CLL cells. Ibrutinib binds with BTK ATP-binding pocket with the

activation loop displaying a “DFG-in” conformation[110].

### 1.2.2.3 Approved serine/threonine kinase inhibitors

Raf serine/threonine kinases are essential signaling integrators that drive cancer cell proliferation[111]. There are three members in RAF family: A-Raf, B-Raf, and C-Raf. B-Raf was validated as an anticancer target in melanoma and other cancer cell lines[112]. About 90% of B-Raf mutations include replacement of Val600 with Glu600 in the kinase domain, and result in elevating the activation of the MAPK pathway, and then promote the cell survival and proliferation[113]. The B-Raf mutations are common present in cancer patients, for instance, melanoma (60%), thyroid cancer (15%), colorectal cancer (10%) and lung cancer (3%)[114]. The specific role of B-Raf drives B-Raf as a therapeutic target in melanoma, thyroid cancers and colorectal. The first B-Raf approved inhibitor, Vemurafenib (ZelboRaf, Roche, 2011), was designed to inhibit B-Raf V600E mutation, and is used for the treatment of patients with metastatic melanoma and thyroid tumors[115]. Vemurafenib bind with B-Raf in a type I binding mode by occupying ATP-binding site with a FDG-in conformation[116].Dabrafenib (Tafinlar, GlaxoSmithKline, 2013) was approved by FDA in 2013 to against both B-Raf V600E mutation and C-Raf with a type I binding mode[117, 118].

Mitogen-activated protein kinase kinase (MEK) is a member of the MAPK signaling pathway, and it is active in melanoma. The related dual-specificity kinases MEK1 and MEK2 phosphorylate extracellular signal-regulated kinase 1 (ERK1) and ERK2[119]. As ERK is the only known substrate of MEK and as ERK is implicated in tumors, MEK inhibitors are developed to block cell proliferation and to induce apoptosis by blocking ERK activation[120]. Only one FDA-approved MEK inhibitor, Trametinib (Mekinist, GlaxoSmithKline, 2013), is used for the treatment of metastatic melanoma with the B-Raf V600E mutation[121]. In addition, Trametinib has also been approved to overcome the resistance of B-Raf inhibitors[122].

The serine/threonine kinase protein kinase B (PKB), also named as Akt, plays an

important role in glucose metabolism, apoptosis, cell proliferation, transcription, and cell migration[123]. Akt and mammalian target of rapamycin (mTOR) are also promising targets in the PI3K/Akt/mTOR pathway[50]. Even though no Akt or mTOR inhibitors have been approved, a lot of their candidates are currently in various clinical trials. Three mTOR inhibitors (sirolimustem, sirolimus, and everolimus) were approved in 1999, 2007, and 2009, respectively[124].

Cyclin-dependent kinases (CDKs) play a key role in regulating cell cycle, transcription, and mRNA processing[125]. Four CDKs(CDK1, CDK2, CDK4, and CDK6) are important components of the cell cycle machinery with key functions both in human normal cells and in human cancer cells. Over-expression of CDK1 is highly related with some human cancers, such as ovarian cancer, colorectal cancer, and breast cancer. CDK2 has been reported as a target for cancer therapy as the over-expression of CDK2 regulates cell cycle abnormality in cancer cells. CDK4/6 activation leads to uncontrolled cell cycle in many types of cancers, especially in breast cancer[126]. Owing to these features, numerous drugs that target CDKs activity have emerged and have been tested in various stages of clinic over the past 2 decades[125, 127, 128].Palbociclib (Ibrane, Pfizer, 2015) was approved for the treatment of breast cancer[129]. As a dual CDK4 and CDK6 inhibitor, Palbociclib potently inhibits CDK6 compared with other pan-CDK inhibitors[130].

#### 1.2.2.4 Approved lipid kinase inhibitors

Lipid kinases, such as phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3Ks), and other components of PI3K signaling pathway play important roles in tumor development[131].Idelalisib (Zydelig, Gilead Sciences, 2014) was approved as the first PI3K inhibitor for the treatment of CLL in combination with rituximab[132]. As a PI3K inhibitor, Idelalisib binds with PI3Kδ in a type II mode. There are more than 20 PIK3 inhibitors in clinical trials, which include single isoform inhibitors, dual PI3K mTOR inhibitors, pan-PI3K inhibitors, and selective-isoform inhibitors[133].

#### 1.2.2.5 Limitations and challenges of kinase-based drug discovery

Over the last decade, the development and discovery of kinase-based cancer drugs has received tremendous progress. Most notably, small molecule kinase inhibitors that focus on particular kinase dependencies and mutation of cancer cells have established individual drug strategy. The discovery and development of small molecule kinase inhibitors reflect several significant challenges, such as lack of inhibitor selectivity and efficacy, drug resistance, and difficulty of drug target validation[15, 134].

## 1.3 Profiling of small molecules by chemical proteomics

### 1.3.1 Chemical proteomics

Chemical proteomics, which is a mass spectrometry (MS)-based affinity chromatography approach, provides a powerful tool for the identification and validation of novel drug targets and/or natural products[135-137]. The major applications of chemical proteomics include activity-based probe profiling (ABPP)[138], which uses active site-directed chemical probes to assess the enzymatic activity of a particular protein family and compound-centric chemical proteomics (CCCP)[139], which focuses on target discovery and drug modes of action (MoA)(Figure 1.5).

### 1.3.2 Activity-based probe profiling

ABPP is used both in the detection of active enzymes in disease to identify the new targets and in the determination of selectivity profiling of drugs targeting an enzyme family. As a functional proteomics technology, the basic part of ABPP is the chemical probe. The probe is used to react with the enzymes and can be divided into three key components[138]: a reactive functional group (“warhead”), a flexible linker, and a reporter tag (Figure 1.5). The reactive functional group is the most important element in ABPP, and it becomes the biggest challenge in the design of affinity-based probe (ABP). In the reactive group, a covalent attachment of the probe is provided to attach the catalytic residue of the protease active site. Therefore, specific and high activity

interactions with target proteins are necessary.

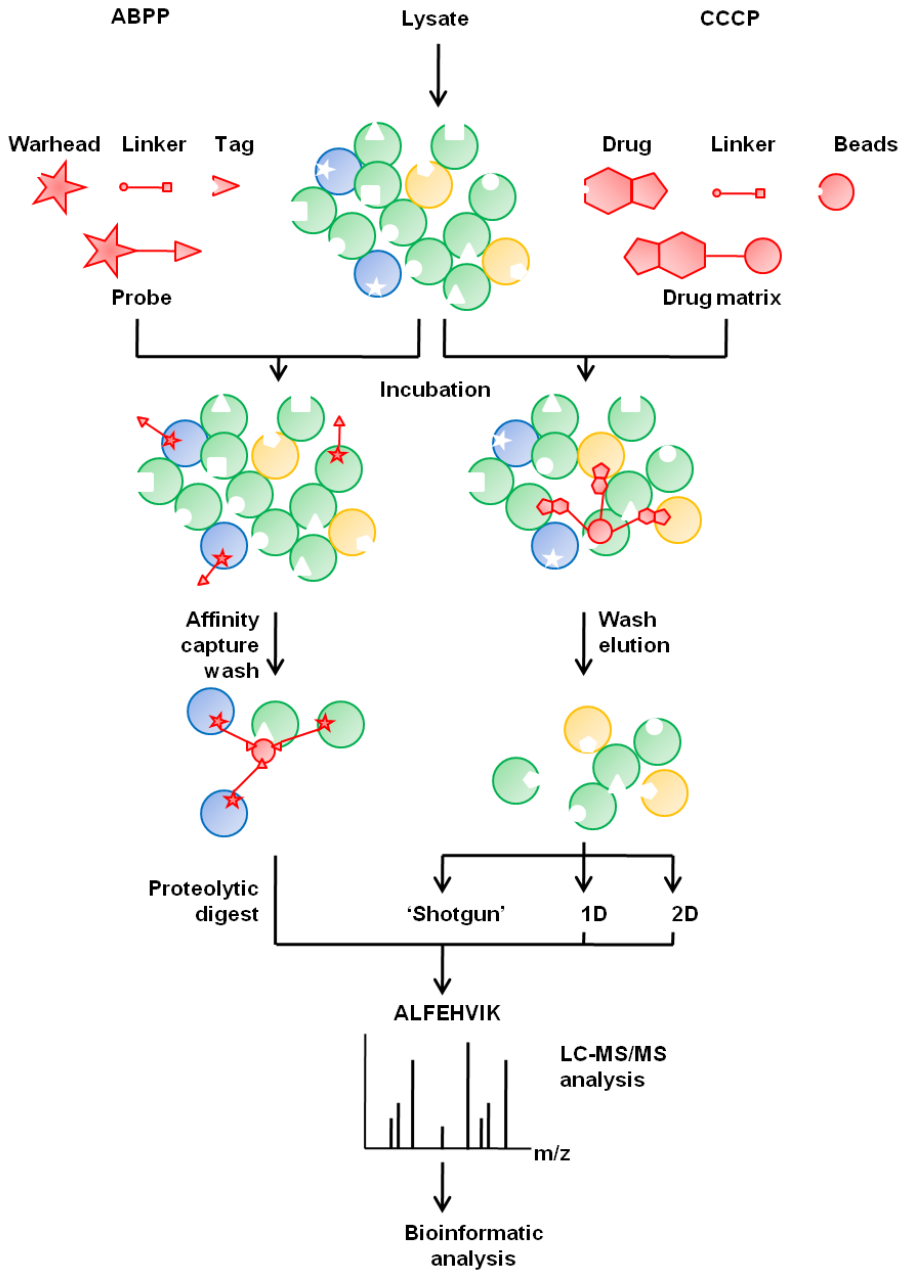


**Figure 1.5 Ageneral workfolw of chemical proteomics approaches by using activity-based probe profiling (ABPP) and compound-centric chemical proteomics (CCCP).**(adapted fromLiu Y. & Guo M., 2013)

There are two elements in ABP: a photo-reactive group and a non-specific covalent bond forming event. The photo-reactive group is designed from a non-covalent bond to a specific site on a protein. Both “directed ABPP” and “non-directed ABPP” are used for the design of reactive groups. A flexible linker is also necessary in the design

of ABP, which provides a space between the reactive group and the reporter tag. The function of the reporter tag is easy detection. Biotin, fluorophores, isotope, and hemagglutinin (HA) are commonly used in the design of reporter tag[140, 141].

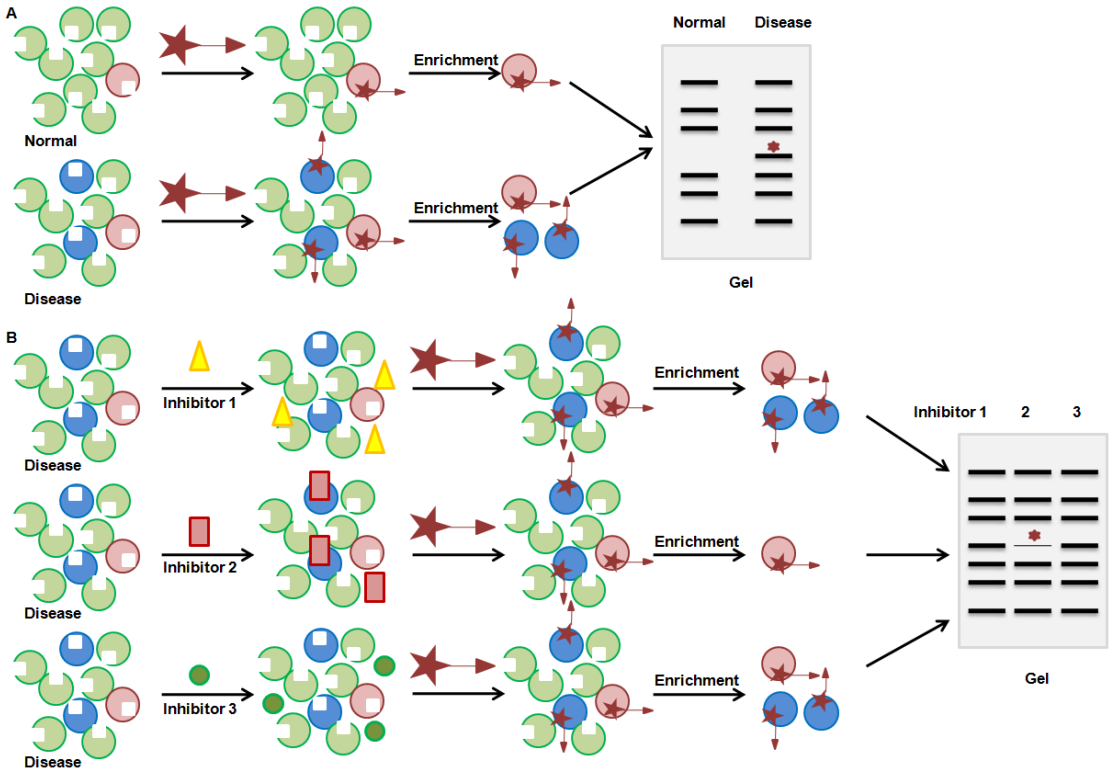


**Figure 1.6 Experimental workflows of (A) comparative profiling ABPP and (B) competitive profiling ABPP.**(A) Comparative ABPP: Same ABP is used to label the normal and disease samples. SDS-PAGE is used to resolve the target proteins to identify the specific disease-related drug target candidates (*). (B) Competitive proteomics: all the disease samples are incubated with individual small-molecule inhibitors and labeled with ABP. The small molecule selective inhibitor specifically binds with the target protein, then ABP can't label the target protein. Therefore, the target protein is missing on SDS-PAGE (adapted from Liu Y. & Guo M., 2014).

Comparative profiling and competitive profiling are two common usages of ABPP[142]. Comparative ABPP is used to compare the healthy and diseased state in biological systems to identify different activity levels of enzyme(s); therefore, the major application of comparative ABPP is target discovery[143]. Competitive ABPP is used to discover the potent and selective inhibitors for discrete enzymes in native proteomes (reversible and irreversible inhibitors) from large compound libraries; therefore, the

major application of competitive ABPP is inhibitor screening[144]. In comparative ABPP, two or more proteomes are analyzed by ABPP to detect the specific enzyme activity (Figure 1.6A). In competitive ABPP, proteomes are incubated with small molecule inhibitors, and a broad probe is used to react with most enzymes (Figure 1.6B).

### 1.3.3 Compound-centric chemical proteomics

CCCP is mainly used for target discovery and drug MoA. It can identify both the enzymatic function binder and the non-enzymatic function binder[139, 145]. Affinity chromatography and advanced MS techniques contribute to the global profiling of a bioactive compound in CCCP identification[146] (Figure 1.5).

In the typical CCCP, firstly, a hydrophilic linker and a commercially available solid matrix (agarose, sepharoseor magnetic beads) are used to immobilize a bioactive compound. A specific chemical group (sulfhydryl, amino, hydroxyl, or carboxyl group) of a bioactive compound is necessary for the attachment of the linker. Secondly, target proteins are captured by the target compound after the incubation of protein extracts and drug-immobilized matrix. Thirdly, salts, pH changes, and free compounds are used to achieve the ligand-bound proteins. Finally, the ligand-bound proteins are resolved by SDS-PAGE, digested by trypsin, and identified by LC-MS/MS[136].

The biggest problem of CCCP is that low abundance and low binding affinity of the target candidates result in non-specific binding to the drug-immobilized matrix[147, 148]. Several essential control experiments are necessary to solve this problem (Figure 1.7). An activated resins without drug molecule control is necessary for the identification of non-specific binding proteins. The optimization of low-abundance targets (nuclear proteins and membrane-bound proteins) is also important. If the target proteins are membrane-associated, the specific cell lysis conditions are important, and affinity-based techniques are used in the application of membrane proteins because of the advantages such as specificity and enrichment[149, 150].

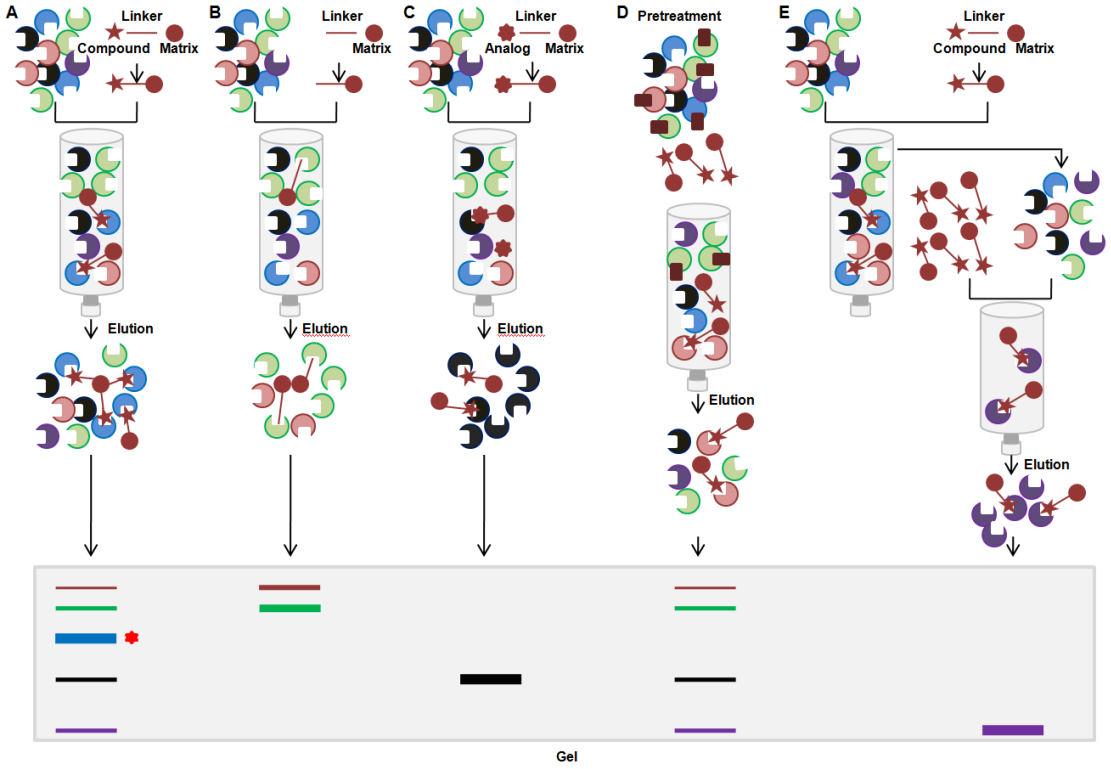


**Figure 1.7 CCCP in drug target deconvolution.** (A) Matrix immobilizing compound is incubated with cell lysates to bind protein targets. (B) Matrix alone is incubated with cell lysated. (C) Matrix immobilizing an inactive analog is incubated with cell lysates. The target proteins of nonspecific inhibitor are identified because of the inactive analog binding. (D) Excess free compound is pre-incubated with cell lysates, following by labeling with immobilized compound. Proteins are considered to be real targets only competed by free compound. (E) Matrix immobilizing compound is incubated with cell lysates, the binding protein is removed. Fresh matrix immobilizing compound is incubated with the remaining extract and the nonspecific binding proteins are similar in both matrices. The interest protein target is represented with asterisk (Adapted from K. Wang et al. 2012).

Chemical proteomics has been used to solve many problems, which include the drug action mechanism and novel medical usages. Candidate compounds, such as small molecule kinase inhibitors, natural products, and established drugs, have been profiled by affinity chromatography-based proteomics in the past few years[15, 136, 151, 152]. Affinity chromatography-based proteomics is commonly used for target identification of small molecule kinase inhibitors of drug development[153]. Protein kinases play an important role in a wide range of diseases, such as cancer and autoimmune diseases[45, 46].

"Kinobeads$^{TM}$", which immobilize non-specific kinase inhibitor to capture a broad range of expressed kinome, have been used to "profile the targets of kinase inhibitors[154, 155]. In native proteins, Kinobeads, which are immobilized chemical probes to affinity resins, can reversibly bind the ATP pockets. Our group first published versions of Kinobeads(Figure 1.8) that can bind about 350 kinases as well as over 2000 additional ATP and nucleotide binding proteins from different cell lines[156].As a powerful tool in kinome research, Kinobeadscan determine chaperones, ATPases, transporters, and metabolic enzymes[157-159].

In the typical Kinobeadspulldown experiment[160, 161], at first, a concentration range of inhibitor is incubated with four different cancer cell line lysate mixture. The aim of cell lysate mixture is to improve the kinome coverage. The ATP-competitive inhibitor competes with the immobilized inhibitors on the affinity matrix to bind the kinasesin with the lysate. Kinases, which are the targets of the inhibitor, compete in a dose-dependent manner because they lose their binding ability to the matrix; the non-targeted kinases are not affected. Kinobeadsselectivity screenings can be performed in high throughput and engage a public–private partnership to screen thousands of advanced drug molecules from the pharmaceutical industry with the purpose to unlock the current state of the drug-able kinome. This will allow the detection of new chemical probes or give a starting point for medicinal chemistry in the future.

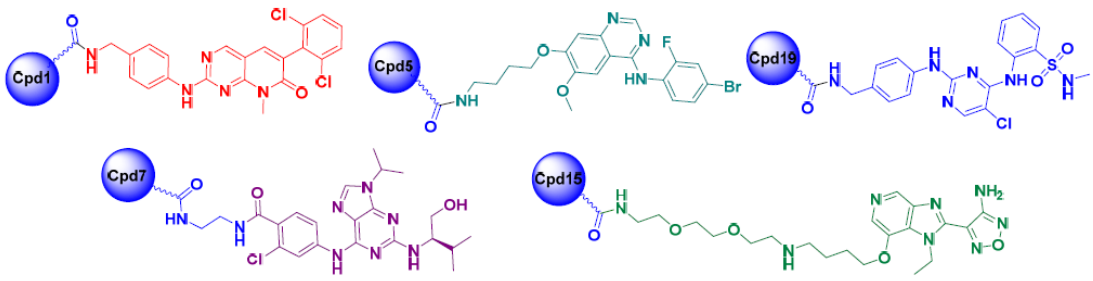


**Figure 1.8 Immobilized Kinobeads.**Each compound was covalently bond with Sepharose beads and equal amount Kinobeadswere added in the kinome profiling.

Another application of affinity chromatography-based proteomics is the profiling of natural product candidates[162, 163]. However, the immobilization of the natural products to the matrix is difficult because of their structural complexity and diversity[162]. In addition to maximizing novel therapeutics for other diseases and/or to minimize the side effects and toxicity, emerging technologies were used to re-examine the established drugs in order to detect the drug action mechanism in primary disease therapy. For example, functional proteomics approach was used to detect the mechanism of quinolines in malaria treatment[164]. In addition to identifying novel quinoline-binding proteins, this method exploited the similarities of the structure between ATP purine ring and quinoline compounds. ALDH1 and QR2 were found as the novel targets of quinolines[165], and these findings might provide new applications of these established compounds.

## 1.4 Mass spectrometry

The application of MS, which measures mass-to-charge ratios (m/z) of ions for the identification and quantification of molecules in simple and complex mixtures, has significantly improved in the study of chemical proteomics and has become an essential tool in proteomics analysis over last 15 years[166-168]. Chemical proteomics exhibit a large-scale study of the expression, structures, and functions of proteins and the high-throughput large-scale protein studies called “shotgun proteomics”[169, 170]. The “shotgun proteomics” consists of high pressure liquid chromatography (HPLC) coupled with high-performance tandem MS[170].

### 1.4.1 Instrumentation

In general, allmass spectrometers consist of three basic components[171, 172]: an ion source to convert the sample into ions; a mass analyzer to take ionized masses and separate them according to m/z; and a detector to register the ions at m/z value. Ionization refers to the production of gas phase ions suitable for resolution in the mass analyzer or mass filter, and it occurs in the ion source. Hard ionization techniques (i.e.

electron ionization [EI]) are processes that impart high quantity of residual energy in the subject molecule, invoking great degree of fragmentation[173]. Soft ionization (i.e. electrospray ionization [ESI]) and matrix-assisted laser desorption/ionization [MALDI]) refer to the processes that impart little residual energy onto the subject molecule and as such result in little fragmentation[174-176].

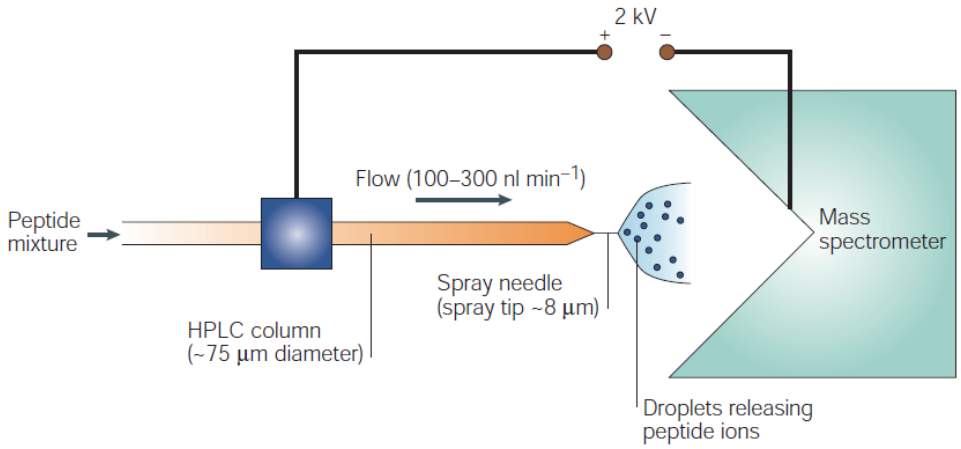


**Figure1.9 Electrospray ionization (ESI) techniques.**(copied from Steen and Mann, 2004).

In the typical experiment (Figure 1.9), proteins are digested into peptides by specific protease before the subjection of LC-MS/MS analysis. Trypsin is commonly utilized to cleave proteins on the carboxyl-terminal side of lysine and arginine residues. In the current study, additionally, alternative proteases including Glu-C, LysN, Lys-C, Asp-N, or chymotrypsin were used to increase the individual protein sequence coverage and/or the generation of unique peptide sequences[177]. Peptide mixtures were eluted by reverse phase liquid chromatography with volatile solvents, such as acetonitrile, water, and organic acids. Soft ionization (ESI) was used to facilitate the measurement of large bio-molecules. After the elution of peptide mixtures, they were sprayed into an electric field from a fine capillary on which a high voltage was applied. Electrically charged spray is the start of the ionization process that leads to the formation of small charged droplets. Next, the continuous evaporation of solvent was broken down into smaller highly charged droplets when the Coulomb repulsion overcame the surface tension of a droplet. There are two major types of MS: the scanning MS that includes

quadrupole (Q) and time-of-flight (TOF) analyzer and the trapping MS that includes ion traps (linear and 3D) and orbitrap analyzers[178]. Orbitrap analyzer has dominated proteomics since it was developed in 2000[179] and has been used in this project as well.

### 1.4.2 Mass spectrometry-based proteomics

In general, MS-based proteomics is important in the disease-associated biomarker discovery and new target identification, and it provides a powerful tool in protein and peptide identification and quantification[168, 180]. Top-down proteomics[181] ,which focuses on the identification and characterization of intact proteins, and bottom-up proteomics[182] ,which focuses on the identification of proteins and characterization of protein amino acid sequences and post-translational modifications (PTMs), are two main MS-based proteomics strategies. Top-down approach has many advantages, such as the detection of degradation products, sequence variants, and combinations of PTM[183]. Bottom-up proteomics achieves higher resolution separation compared with top-down approach[184].

### 1.4.3 Protein identification and protein quantification

The most versatile and widely used application of tandem MS/MS is high resolution MS-based peptide identification[184-186]. The full-scan mass spectrum and second mass spectrum are required for this purpose. The m/z values of the intact peptides are determined by the full-scan mass spectrum. The second mass spectrum enables the isolation and fragmentation of the interested precursors, and records various fragment ions. In the full MS results, b-(N-terminal) and y-ions (C-terminal) are generated based on the fragmentation of the precursor ion, and the amino acid sequences are determined according to the differences between these ions series (Figure 1.10)[187, 188].

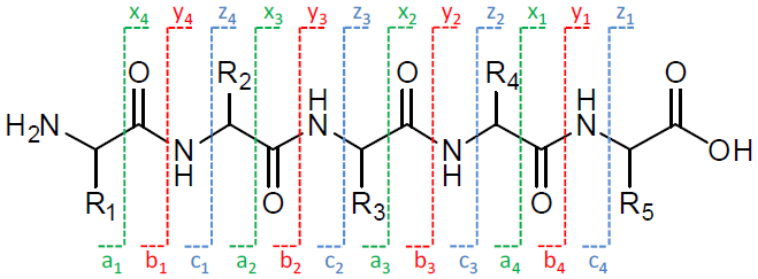


**Figure 1.10 The nomenclature of peptide fragmentation.** N-terminal are named a, b, c ions, and C-terminal fragments are named x, y, z ions. (adapted from Roepstorff and Fohlman, 1984).

The most common application of peptide and protein identification is database searching in large-scale proteomics studies[189, 190]. The protein sequence database searching approach containing the theoretical fragmentation of each peptide is performed to compare with the experimental peptides. Then, the peptide is assigned to the best match and is defined based on the database scores and specific parameters. Lastly, the defined peptide is assigned to the proteins. The target-decoy searches, which search all tandem spectra against the target, are used to assess the quality of protein identification[191]. An assumption that any matches in the decoy search are false assignments is used to compute the false discovery rate (FDR)[192].

Quantitative proteomics has provided powerful tools to determine large-scale protein expression in complex biological systems in recent years[168, 180, 193, 194]. Isotope labeling and label-free approaches are two major methods in the application of protein quantification (Figure 1.11). The most popular methods for isotope labeling, which include stable isotope labeling by amino acids in cell culture (SILAC)[195], trypsin-catalyzed $^{18}O$ labeling[196], isotope-coded affinity tagging (ICAT)[197], and isobaric tags for relative and absolute quantitation (iTRAQ)[198] enable the achievement of internal standard. Protein quantification enables us to compare the mass spectrometric signal intensities of the same peptide with different labelings to offer an accurate quantification. More recently, label-free quantification that determines the relative amount of proteins in different biological samples has attracted more and

more attention[199]. The label-free quantification methods consist of precursor ion intensity or spectral counting of the same protein. Spectral counting enables us to count the number of peptides of a protein that are collected by tandem mass spectra, and then integrates the quantified results for all the measured peptides of the protein. In contrast, precursor ion intensity method provides a significant advantage in the integration and comparison of the ion intensity for same peptide in different biological samples[194, 200-202].Owing to these advantages, label-free approach is the most accurate technique in the mass spectrometric quantification method. In addition, label-free approach offers few additonal advantages, such as allowing the comparison of unlimited biological samples, being inexpensive, and consuming less time[169, 182].

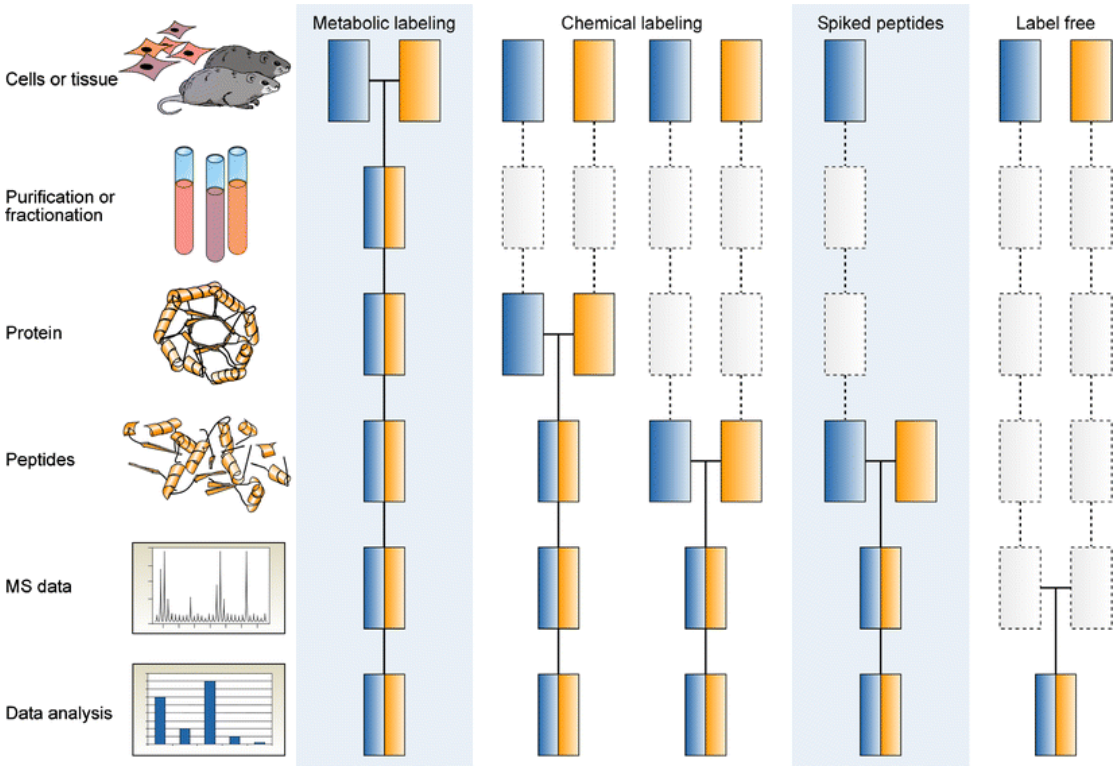


**Figure 1.11 An overview of general common quantitative MS workflows.** (copied from M. Bantscheff et al., 2007). Two different experimental conditions are represented with blue and yellow boxes. Horizontal lines indicate that the samples are pulled together. The experimental variation is indicated with dotted lines.

# Aim of thesis

Protein kinases have been established as promising drug targets for treatment of various types of cancers. Small molecule kinase inhibitors have become increasingly important in cancer therapeutics. Currently, more than 33 kinase inhibitors have been approved by FDA as promising targeted therapeutics, and numerous (>250) inhibitors are in various stages of clinical evaluation. Kinobeadstechnology in combination with high-resolution MS has emerged a powerful tool to understand the mode of action, to identify new targets, and to identify potential off-target of small molecule kinase inhibitors.

In this thesis, a dose-resolved quantitative chemical proteomics approach has been used to understand the mechanism of small molecule kinase inhibitors and to identify the off-targets. In chapter II, 14 CDK inhibitors, which include both first- and second-generation CDK inhibitors were characterized. These inhibitors were tested using Kinobeadstechnology in a competitive approach against a large number of kinases, including CDKs and their complex partners.Since only one CDK inhibitor Palbociclib has been approved by FDA, our results try to explain the success of Palbociclib and the failure of the other CDK inhibitors in clinical stages at the protein level. In addition, CDK inhibitors can be classified in effecting the regulation of cell cycle and/or transcription in our study. The identification of off-targets of CDK inhibitors may drive them into new anti-cancer therapies. In chapter III, salt-inducible kinase 2 (SIK2) has been detected as an off-target by screening 242 small molecule kinase inhibitors through Kinobeadspulldown assay. Furthermore, immunity cell models have been used to characterize the role of SIK2 inhibition in immune response.No small molecule kinase inhibitor has shown therapeutic benefits in inflammatory therapies. This work enables to provide strong reasons to drive low molecular weight SIK2 inhibitors in the investigation of inflammatory and autoimmune diseases.

# CHAPTER II
# Selectivity Profiling of Cyclin-dependent Kinase Inhibitors

## 2.1 Introduction

CDKs are a family of protein kinases that play the most important role in the regulation of cell cycle transitions[125]. They also participate in regulating mRNA processing, transcription, and differentiation of nerve cells. CDK sub-family comprises 13 members (CDK1-13) based on function and similarities in sequence[203]. Four CDKs (CDK1, CDK2, CDK4, and CDK6) are essential components of the cell cycle machinery with key functions in both human normal cells and in human cancer cells[204].In activation of human cell cycle[125], at first, CDK-4/6 facilitates the progression of the cell cycle from the growth phase (G1) to the S-phase, which is associated with DNA synthesis. Retinoblastoma protein (Rb) was phosphorylated by CDK4/6-cyclin D and CDK2/cyclin E complexes, and the inactivation Rb protein is necessary in G1/S phase. The formed CDK2/cyclin A complex facilitates to execute the S phase events e.g. DNA replication and centrosome cycle. The CDK1/cyclin A and CDK1/cyclin B are required for DNA damage checkpoint control and G2/M phase transition and initiation of mitosis, respectively (Figure 2.1).

Normal controls of the cell cycle are deranged in all cancers based on the role of CDKs in cell division control[205]. Over-expression of CDK1 is highly related with some human cancers, such as ovarian cancer, colorectal cancer, and breast

cancer[206].CDK2 has been reported as a target for cancer therapy as the over-expression of CDK2 regulates cell cycle abnormality in cancer cells[207]. CDK4/6 activation leads to uncontrolled cell cycle in many types of cancers, especially in breast cancer[208]. Owing to these features, numerous drugs that target CDKs activity have emerged and have been tested in various stages of clinic over the past 2 decades[125, 209, 210].

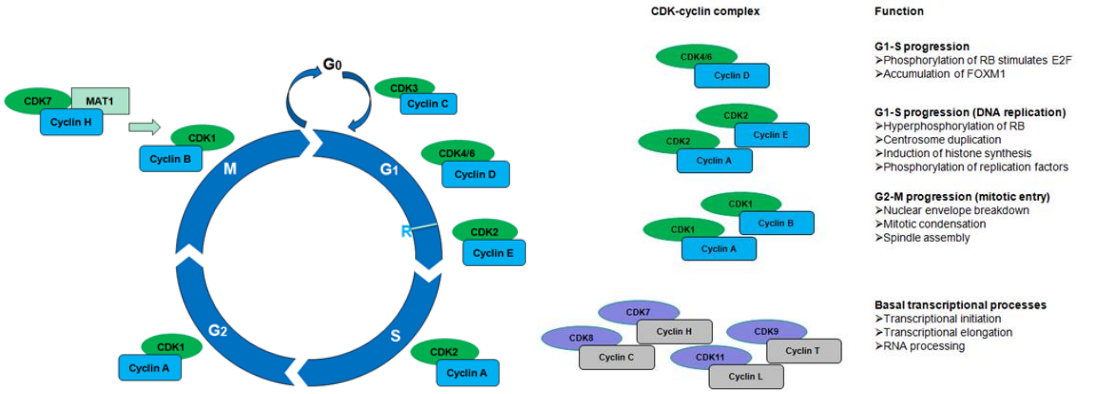


**Figure 2.1 An overview of human cell cycle activation and transcriptional regulation, and an overview of the CDK/cyclin complexes function in both cell cycle regulation and transcription.**

The first generation of CDK inhibitors that includeFlavopiridol (Alvocidib; developed by Sanofi-Aventis) and Roscovitine (Seliciclib; developed by Cyclacel) were developed to work against most of the CDK sub-units[128]. Alvocidib has been shown to inhibit CDK1, 2, 4, 6, 7, and 9 in cell free assay[211]. It can do both, induce cell cycle arrest in G1/2 phase by targeting CDK1 and CDK2, and lead to suppression of transcription by targeting CDK7 and CDK9. Alvocidib has also been shown to have substantially high level of clinical activity in vitro and low level of clinical activity in vivo[212]. Clinical studies have indicated that Alvocidib has a weakly effect in solid tumors and a strong effect in mantle cell lymphoma and CLL[213, 214]. Roscovitine was evaluated to target CDK1, 2, 5, 7, and 9 that lead to cell cycle arrest and the inhibition of transcription[215]. It has been tested for the treatment of leukemia and NSCLC[216, 217]. Due to the broadspectrum of CDK sub-unit inhibition, the non-specific-based first-generation CDK inhibitors were referred to “pan-CDK” inhibitors[218].

The development of second-generation CDK inhibitors aims to either increase the selectivity of CDK1 and CDK2 or increase the overall potency[205]. Numerous second-generation CDK inhibitors have been developed following Alvocidib and Roscovitine (Table 2.1).Palbociclib (PD-0332991, developed by Pfizer) was approved under FDA as a treatment (in combination with letrozole) for patients with estrogen receptor positive advanced breast cancer in 2015[129]. Besides Palbociclib, other CDK inhibitors are still in clinicaltrilas, and few of them have been reported to have remarkable effects in clinical research studies[219, 220]. Dinaciclib (MK7965 and SCH-727965; developed by Merck) was designed to specific potent inhibit the activity of CDK1, CDK2, CDK5, and CDK9[221]. Compared with Alvocidib and Roscovitine,Dinaciclib suppresses cell cycle progression in various tumor cell lines and induces the regression of solid tumors[221, 222]. It has been evaluated in clinical trials for various cancer indications, such as NSCLC, advanced acute myeloid leukaemia, and CLL[222-225]. AT-7519 (developed by Astex) was developed to inhibit CDK1, CDK2, CDK4, CDK6, and CDK9, and it has been evaluated in combination with bortezomib to treat the patients with multiple myeloma (MM)[226]. BMS-387032 (SNS-032; developed by Bristol-Myers Squibb) was developed as a high selectivity CDK2 inhibitor[227], and it was also reported to weekly inhibit CDK1, CDK4, CDK7, and CDK9[228].

Numerous CDK inhibitors have been tested in various stages of clinic over the past 2 decades, but a lot of them were failed in clinical trials. There are at least three key principles that explain the failure of non-selective CDK inhibitors[205].Firstly, there is a lack of clear understanding of the action mechanism for many low-specificity CDK inhibitors. The lack of clear recognition regarding which CDK is actually inhibited in vivo may become the corresponding mechanism of the therapeutic effect; secondly, appropriate patient selection is another restriction. There are no key biomarkers for the sensitive sub-populations that respond to this class of inhibitors; thirdly, there is no suitable therapeutic window. Many CDK inhibitors inhibit the critical proliferation of

protein CDK1 and the survival of associated protein CDK9 in both cancer cells and normal cells. Therefore, these non-selective CDK inhibitors cannot discriminate between cancerous and healthy tissues and result in side effects. The limitation of non-selective CDK inhibitors suggests that increase in certain CDK selectivity is important for the development of CDK inhibitors. More recently, selective CDK2 inhibitors became the new research direction in cyclin E-derived tumors[229]. In certain contexts, targeting CDK1 is synthetically lethal with KRAS mutation and MYC[230, 231]. As cancer cells may harbor unique vulnerabilities to selective suppression, targeting basal transcription-associated protein CDK7, CDK8, and CDK9 is another alternative strategy[205, 232].

| Inhibitors | Name | Company | Reported targets | STATU |
|---|---|---|---|---|
| HMR-1275 | Alvocidib | Sanofi-aventis | CDK | Phase III |
| AZD-5438 | | AstraZeneca | CDK1/2/9 | Phase I |
| PHA-793887 | | Nerviano Medical Sciences | CDK1/2/4 | Phase I |
| SCH-727965 | Dinaciclib | Schering-Plough, Pharmacopeia | CDK1/2/5/9 | Phase II |
| R-547 | | Hoffmann-La Roche Inc | CDK1/2/4/9 | Phase II |
| P-276-00 | Riviciclib | Piramal Life Sciences | CDK1/4/9 | Phase II |
| BMS-387032 | | Bristol-Mvers Squibb | CDK2 | Phase I |
| PHA-848125 | Milciclib | Nerviano Medical Sciences | CDK1/2/4 | Phase II |
| AT-7519 | | Astex Therapeutics | CDK2/4/5/9 | Phase II |
| CYC-202 | Roscovitine, Seliciclib | Cyclacel Pharmaceuticals | CDK2/7/9 | Phase II |
| PD-332991 | Palbociclib | Pfizer | CDK4/6 | Approved |
| LY-2835219 | Abemaciclib | Lilly | CDK4/6 | Phase I |
| LEE-011 | Ribociclib | Nocartis | CDK4/6 | Phase II |
| RGB-286638 | | Agennix AG | CDK1/2/3/4/9 | Phase I |

**Table 2.1 CDK inhibitors and their targets present in the profiling system panel.**

More recently, CDK4/6 has been a growing concern, and the CDK4/6 dual inhibitor Palbociclib was reviewed and approved by FDA in 2015[129]. CDK4/6 activation leads to uncontrolled cell cycle in many types of cancers, especially in breast cancer[233]. The development of CDK4/6 inhibitors includes three important features[208]. Firstly, CDK4/6 inhibitor may arrest tumor cells in G0/G1 phase; secondly, CDK4/6 inhibitor may directly result in the RB protein suppressing gene expression and proliferation; thirdly, CDK4/6 inhibitor may result in CDK4/6 deregulation tumors.LY-2835219 (Abemaciclib;

developed by Eli Lilly) and LEE-011 (developed by Novartis) were developed as CDK4/6 dual inhibitors and have been evaluated in clinical trials besidesPalbociclib. These CDK4/6 inhibitors demonstrate a similar structure and distinct from pan-CDK inhibitors.

In this study, 14 CDK inhibitors, which include both first- and second-generation CDK inhibitors,have been evaluated to against the maximum number of kinases, including both CDKs and their complex partners in Kinobeadscompetition assay. CDKs have little kinase activity without cyclins, and cyclins bound different CDKs. Kinobeadscompetition assay provides a powerful tool in indicating the interaction between CDKs inhibitors and their targets in vitro. The characterization of CDK inhibitors enabled us to understand the effectiveness of CDK inhibitors in multiple biological processes including cell cycle, transcription, and some other processes. Only one CDK inhibitor Palbociclib has been approved by FDA, our results try to find evidence to the success of Palbociclib. Meanwhile, the failure of the other CDK inhibitors in clinical stages should also detected in Kinobeads assay.Additionally, the identification of off-targets might provide a positive proof to drive CDK inhibitors into new cancer therapies.

## 2.2 Methods and materials

### 2.2.1 Four cell lines mix

MV4-11, K562, SK-N-BE2 and Colo205 were cultured in cell culture incubator with 37°C and 5% $CO_2$. The cell culture medium was supplemented with 10% FBS (Sigma-12003C) and 1% antibiotic solution (Sigma-A5955).MV4-11 and K562 cells were cultured in roller bottle culture in RPMI-1640 medium. Cells were harvested upon density (approx. 5 x $10^6$/ml), centrifuged with 1000 rpm in 5 minutes and washed with 2 times cold PBS (without $Ca^{2+}$ and $Mg^{2+}$).The cells were lysed in lysis buffer andthe cell lysate was centrifuged for 20 minutes at 4 ºC in 148,000g and afterwards stored at -80 °C until usage.SK-N-BE2 cells and Colo205 cells were cultured in stationary culture (15 cm dish) in DMEM/HAMS F-12 medium and RPMI-1640 medium, respectively. Cells were harvested upon 90% dish's surface area. Lysis buffer were added to dish and confluence by mechanical detachment. The lysate was centrifuged for 20 minutes at 4 °C in 148,000g and afterwards stored at -80 °C until usage.

### 2.2.2 Kinobeadscomprtition assay

Kinobead competition assay (96-well plate) has been performed as described previously[156].. In total 1 ml cell lysates mixture containing 5 mg/ml proteins were used in each well. Different compound which diluted in DMSO (0 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 µM, 3 µM, and 30 µM) were incubated with cell lysates for 45 min at 4 °C in a head-over shaker. 35 µl settled beads containing KBγ or KBɖ, F2, Compound 18, VI compound and Compound 2 were incubated with cell lysates for 30 min at 4 °C. DMSO control was recovered to calculate the depletion factor. Beads were washed by CP buffer and LDS buffer containing DTT were used to elute the bound proteins. The coupling of the inhibitors to the sepharose beads was kindly performed by Stefanie Heinzlmeier.

### 2.2.3 LC/MS-MS measurements

LC/MS-MS mensurement has been performed as described previously[156].. Briefly, a nanoLC-Ultra 1D+ (Eksigent, Dublin, CA) coupled to an Orbitrap Elitemass spectrometerwere used to analyze the peptides which generated by in-gel trypsin digestion. Then peptides were delivered to a trap column (100 µm × 2 cm, packed in-house with Reprosil-Pur C18-AQ 5 µm resin, Dr.Maisch) at a flow rate of 5 µl/min in 100% solvent A. An analytical column (75 µm × 40 cm, packed in-house with Reprosil-Gold C18, 3 µm resin, Dr. Maisch) and a flow rate of 300 nl/minin 100 min gradient ranging from 4-32% solvent Bin A were used to separate the peptides.The mass spectrometer was operated in data dependent mode, automatically switching between MS and MS2 spectra. Up to 15 peptide precursors were subjected to fragmentation by higher energy collision-induced dissociation (HCD) and analyzed in the Orbitrap. Dynamic exclusion was set to 20s. A Kinase peptide inclusion list (including 3 most intense peptides per kinase) was enabled.Measurements were kindly performed by Susan Kläger.

### 2.2.4 Proteins and peptides identification and quantification

MaxQuant(version1.4.0.5) and Uniprot database (v22.07.143) were used to process the raw data.Label free quantification and match-between-runs were used, and 1% peptide and protein FDRs were usedto filter the results. Selected dose-inhibition curves where analysed in GraphPad Prism (v. 5.04)[156].

## 2.3 Results and discussions

### 2.3.1 Characterization of “pan-CDK” inhibitor Alvocidib

As a broad spectrum “pan-CDK” inhibitor, Alvocidib competes with ATP to inhibit CDKs, including CDK1, CDK2, CDK4, and CDK6,and prevents the cell cycle progression in either G1 or G2[205]. As a first-generation CDK inhibitor, Alvocidib was reported to be a “pan-CDK” inhibitor, and Alvocidibwas demonstrateda broad CDK inhibition including CDK1, CDK2, CDK5, CDK7, CDK9, and CDK12 with $K_D$< 1 μM (CDK1: 955 nM, CDK2: 861 nM, CDK5: 69 nM, CDK7: 576 nM, CDK9: 7 nM, CDK12: 96 nM, respectively), and a weakly inhibition of CDK6 with the $K_D$ of 1193 nM (Figure 2.2A). These findings also validated that the Kinobeadsassay is quite effective in the research of kinase inhibitors as they are consistent with the previous studies[154]. CDKs have little kinase activity without cyclins, and only CDK-cyclin is an active kinase[126].Compared with recombinant protein technology in small molecule drug discovery, the Kinobeads competition assay provided an advantage in capturing CDK-cyclin complex partners, including CCNA2, CCNB1, CCNB2, CCNH, CCNI, CCNK, CCNT1, and CCNT2.In our study, most CDK/cyclin complexes were detected in Kinobeads assay, such as CDK1/CCNB and CDK2/CCNB complexes (CCNB1: $K_D$= 239 nM, CCNB2: $K_D$=258nM), CDK7/CCNH complex (CCNH: $K_D$=1027 nM, ERCC2: $K_D$=508 nM, MNAT1: $K_D$=692 nM), and CDK9/CCNT complex (CCNT1: $K_D$=3 nM, CCNT2: $K_D$=2nM) (Figure 2.2C). Alvocidib had an effect on in both cell cycle regulation and transcription regulation (Figure 2.2B).Besides the reported targets CDKs, Alvocidib was detected to weekly inhibit TBK1, GSK3A, GSK3B, PRKD2, PRKD3, DYRK1A, CAMKK2, and PRKCD with the $K_D$ of 1949nM, 971nM, 740nM, 1316nM, 856nM, 909nM, 1378nM, and 1592nMin our study (Figure 2.2C).

Alvocidib wasdesigned as a CDK1, CDK2, CDK4, and CDK6 inhibitor from the beginning[234]. Surprisingly, in our study,Alvocidib strongly inhibited transcription

positive regulator CDK9 and its complex (CDK9: $K_D$=9 nM, CCNT1: $K_D$=3nM, CCNT2: $K_D$=2 nM). CDK9 is a sub-unit of the positive transcription elongation factor b (P-TEFb), which binds to T-type cyclin (T1 and T2) that controls RNA polymerse II[235]. CDK9 inhibition can drive cancer cells into apoptosis, and recent publication highlights CDK9 as an anticancer target[236].Cell-based studies also have shown that Alvocidib-induced cell death is independent of the cell cycle[212], demonstrating that CDK9 is the primary target of Alvocidib. According to our data, the primary target of Alvocidib was CDK9 by comparing the $K_D$ of the main target CDK1, CDK2 and CDK9 (955 nM, 861 nMvs.7nM). This finding may provide a new opportunity for research on Alvocidib and drive Alvocidib as a CDK9 inhibitor in the treatment of CDK9-related cancer therapies.

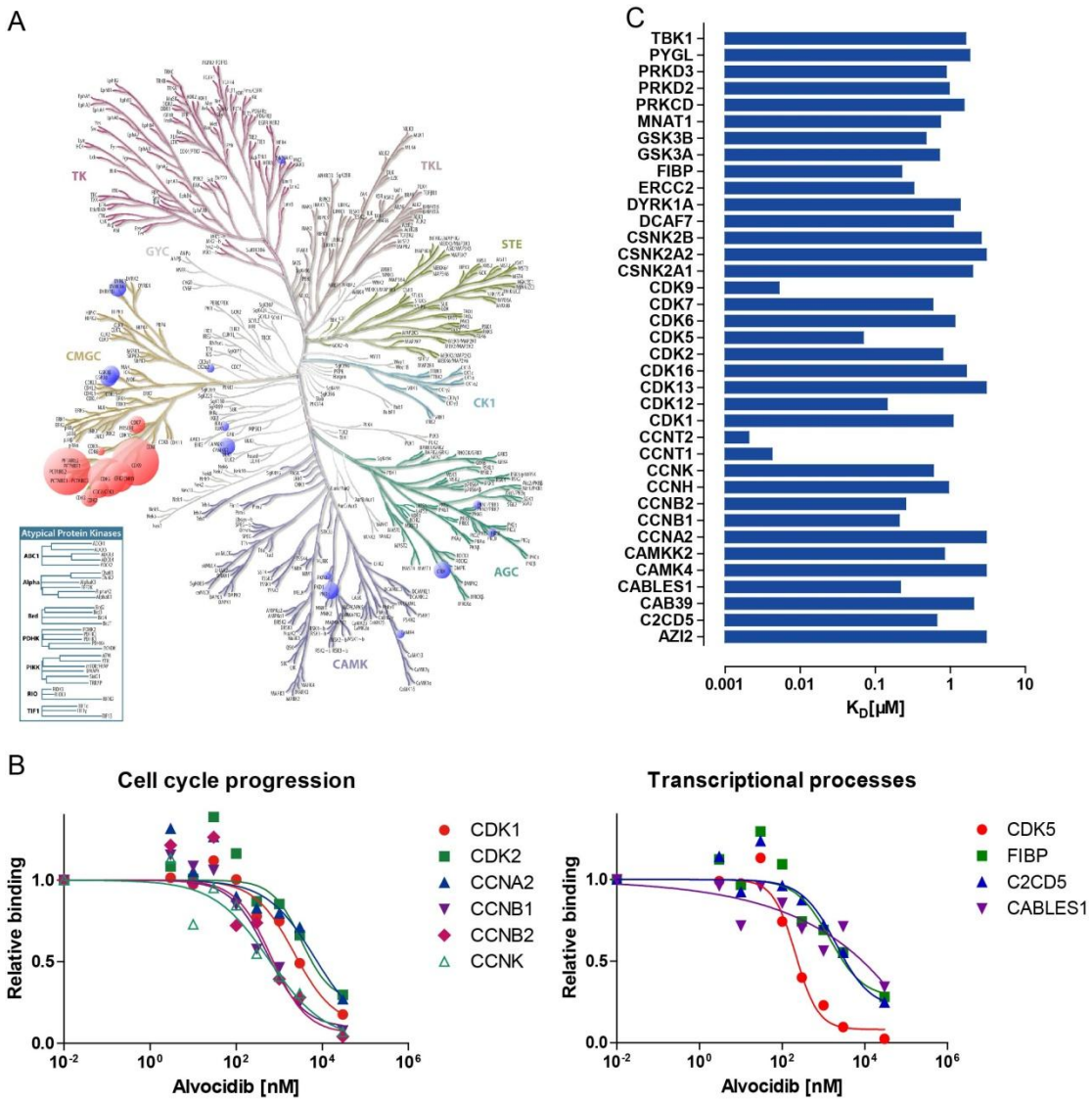


**Figure 2.2 Characterization of "pan-CDK" inhibitor Alvocidib.**(A) Kinometree of Alvocidib. The size of the dots correspond to $K_D$ of targets. (B) Dose response curves for Alvocidib of thecell cycle progression associateddrug targets CDK1, CDK2and their complex partners CCNA2, CCNB1 and CCNB2; the transcriptional processes associated drug targets CDK5 and their complex partners FIBP, C2CD5 and CANLES1. (C) Histogram dissociation constants ($K_D$) obtained for Alvocidib of target proteins.

### 2.3.2 New-generation CDK inhibitors increase CDK2 potency, but not CDK1

One aim of the development of new-generation CDK inhibitors is to increase the selectivity of CDK1 and CDK2 following Alvocidib and Roscovitine[205]. In our study, 12 second-generation CDK inhibitors (AZD-5438, PHA-793887, SCH-727965, R-547, P-276, BMS-387032, AT-7519, PHA-848125, RGB-286638, LEE-011, LY-2835219, PD-0332991, Rociletinib, and Rigosertib) were characterized in

Kinobeadscompetition assay (Figure 2.4).

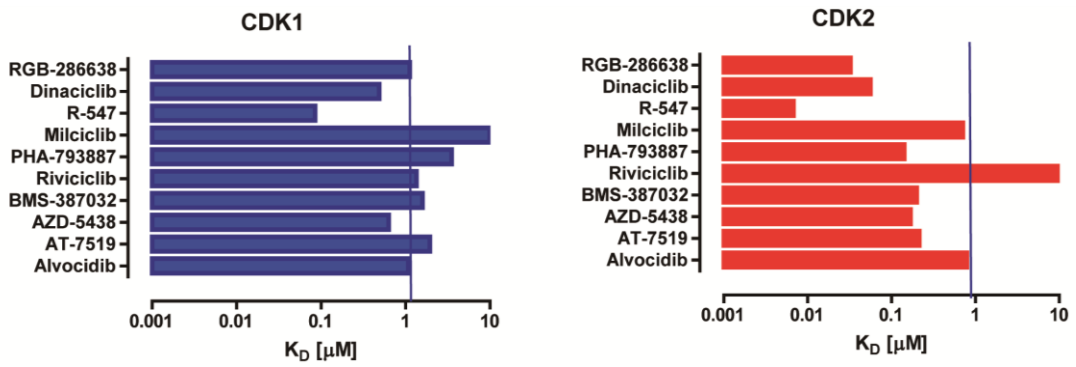


**Figure 2.3 Overview of CDK1 and CDK2 inhibition for CDK inhibitors identified in Kinobeadscompetition assay.** Histogram of dissociation constants ($K_D$) obtained by chemical proteomics for CDK inhibitors.

Our data demonstrated that many second-generation CDK inhibitors enhanced the selectivity of CDK2 (Figure 2.3) and few second-generation CDK inhibitors increased the overall potency(Table 2.2). RGB-286638, Dinaciclib, R-547, PHA-793887, BMS-387032, AZD-5438, and AT-7519 were more potent to work against CDK2 compared with Alvocidib (CDK2: $K_D$ = 36 nM, 44 nM, 5 nM, 157 nM, 198 nM, 252 nM, 161 nM vs. 861 nM). Most of the second-generation CDK inhibitors can't increase CDK1 inhibition except R-547 and Dinaciclib compared with Alvocidib ($K_D$= 90 nM, 465 nM vs. 955 nM). This finding may indicate the success of the second-generation CDK inhibitors is increase the potency of CDK2. CDK2 has been reported as a key regulator in cell cycle, and CDK2 over-expression leads to disorder of the cell cycle control[207].In addition, CDK2 has been reported to be a potentially therapeutic target for cancer therapy, and selective CDK2 inhibitors may become the new research direction in cyclin E-derived tumors[206, 215]. Therefore, the identification of potent CDK2 inhibitors in our study may do the effect in the treatment of CDK2-related cancer.

| targets / Inhibitors | CDK1 | CDK1 2 | CDK1 3 | CDK1 6 | CDK1 7 | CDK2 | CDK4 | CDK5 | CDK6 | CDK7 | CDK9 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Alvocidib | 1079 | 143 | 3196 | 1637 | NA | 791.4 | NA | 68.9 | 1149 | 581.2 | 5.2 |
| R-547 | 85.1 | NA | NA | NA | NA | 6.8 | NA | 7.9 | 3000 | 408.4 | 28.1 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SCH-727965 | 489.6 | 7.6 | 13.6 | 41.1 | 103.3 | 56.1 | 289.5 | 45.4 | 7.4 | 169.3 | 2.7 |

**Table 2.2 CDK inhibitors Alvocidib, R-547, and SCH-727965 and their main target CDK family sub-units present in the profiling system panel.**

Second-generation CDK inhibitors R-547 and SCH-727965 were demonstrated a promising effect in increasing the overall potency of CDK sub-units (Table 2.2). R-547 highly increased the overall potency in CDK1, CDK2, CDK5, and CDK7 inhibition when it was compared with Alvocidib (CDK1: 85 nM vs. 1079 nM, CDK2: 7 nM vs. 791 nM, CDK5: 8 nM vs. 69 nM, CDK7: 408 nM vs. 541 nM). A phase I clinical trial of R-547 in solid tumoralso showed that R547 is a potent and selective CDK1 and CDK2 inhibitor[237]. As an unspecific CDK inhibitor, SCH-727965 increased the overall potency in CDK2, CDK6, CDK12, CDK13, CDK16, and CDK17 inhibition when it was compared with Alvocidib (CDK2: 56.1 nM, CDK6: 7.4 nM, CDK12: 7.6 nM, CDK13: 13.6 nM, CDK16: 41.1 nM, CDK17: 103.3 nM). CDK12 and CDK13 play a key role of regulators of transcription elongation and regulate the expression of genes involved in DNA repair; they are also required for the maintenance of genomic stability[238, 239]. They have been reported as promising new drug targets for cancer treatment[240, 241]. Few CDK12 and CDK13 covalent inhibitors were developed to target the remote cysteine and were evaluated for the treatment of breast cancer, acute leukemia, and ovarian cancer[242]. In our study, SCH-727965 was detected to strongly target CDK12 and CDK13, and this finding may also drive SCH-727965 into the application of cancer therapies.

The goals of development of second-generation CDK inhibitors include either increasing the selectivity of CDK1 and CDK2 or increasing the overall potency[205].Many of second-generation CDK inhibitors such as BMS-387032, R-547, PHA-793887, AT-7519, Dinaciclib and P-276-00 were detected to achieve this aim (Figure 2.4B). However, few second-generation CDK inhibitors including RGB-286638, AZD-5438 and Milciclibwere characterized to fail to achievethese goals in our study (Figure 2.4A). In our results, the most non-selective CDK inhibitor was RGB-286638.

Although RGB-286638 increased the overall potency against CDK sub-units, it also found various off-targets. Meanwhile, AZD-5438 and Milciclib was demonstrated to neither increase the selectivity of CDK1 and CDK2 nor increase the overall potency of CDKs, and it still achieved greaternumber of targets. These findings, on one hand, provided strong evidence to prove the success of second-generation CDK inhibitors and promoted these promising CDK inhibitors in further clinical research studies; on the other hand, may explain the failure of certain CDK inhibitors, such as non-selectivity and low potency on CDK sub-units. In summary, Kinobeadscompetition assay provides a powerful tool in the characterization of CDK inhibitors. Some second-generation CDK inhibitors were indicated to potently work against CDK sub-units, especially the cell cycle regulator CDK2. Some second-generation CDK inhibitors, such as R-547 and SCH-727965, were indicated to increase the overall potency to work against all of the CDK sub-units. Moreover, these findings may help us screen the appropriate compounds in clinical research.

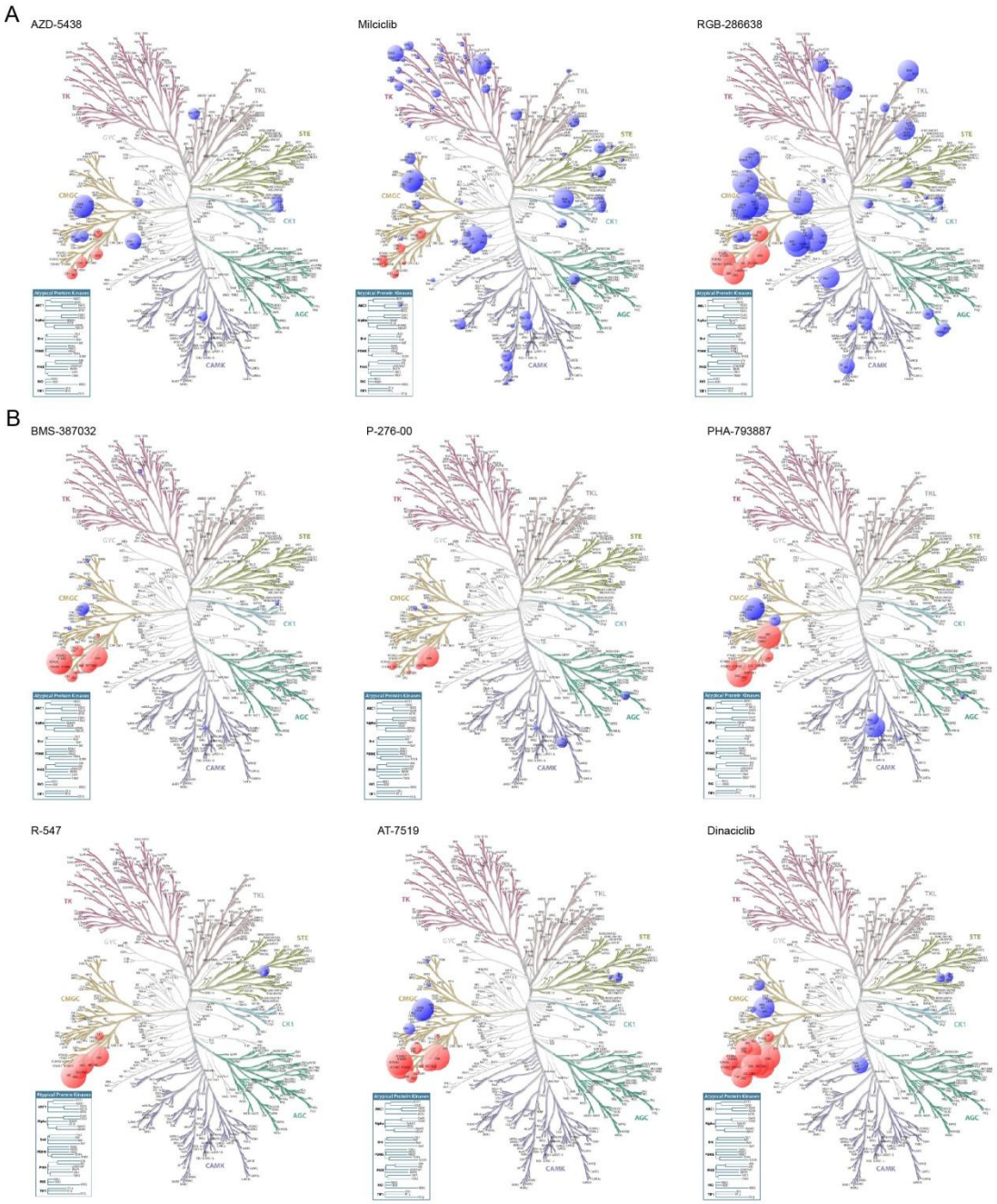


**Figure 2.4 Characterization of second-generation CDK inhibitors.**(A)Kinometrees of AZD-54738, Milciclib, RGB-286638 and (B) BMS-387032, P-276-00, PHA-793887, R-547, AT-7519 and Dinaciclib. The size of the dots corresponds to the $K_D$ of target. Red dots denote CDK sub-units, blue dots denote non-CDKs.

### 2.3.3 CDK4/6 inhibitors are more selective and potent than “pan-CDK” inhibitor Alvocidib

CDK4/6 complexes are a part of the cell cycle regulatory pathway that drive proliferation in the G1 phase in many tumorigenic events, and targeting CDK4/6 has

been reported to be an anticancer strategy[233, 243, 244].Palbociclib (PD-0332991, IBRANCE, Pfizer, 2015) was successfully reviewed and approved under FDA as a treatment (in combination with letrozole) for patients with estrogen receptor positive advanced breast cancer in 2015[129]. In our study, three CDK4/6 dual inhibitors (Abemaciclib, Ribociclib, Palbociclib) were characterized in Kinobeadscompetition assay. For the CDK4/6-specific inhibition, our result showed that all of the CDK4/6 dual inhibitors potently inhibit the activity of CDK6 and/or CDK4 (Abemaciclib: CDK6 with $K_D$ of 214 nM, Ribociclib: CDK6 with $K_D$ of 271 nM, Palbociclib: CDK4 with $K_D$ of 420 nM, CDK6 with $K_D$ of 40 nM)(Figure2.5). As an FDA-approved CDK4/6 inhibitor, Palbociclib was demonstrated to be a very potent and selective CDK4 and CDK6 inhibitor compared with first-generation CDK inhibitors. This may provide an overwhelming evidence of thesuccess of FDA-approved drug Palbociclib. Besides the main target CDKs, Palbociclib weakly inhibited translation initiation factor 3 J (EIF3J), casein kinase 2 sub-units CSNK2A1, CSNK2A2, CSNK2B with the $K_D$ of 700 nM, 760 nM, 507 nM, 447 nM, respectively. EIF3J plays an important role in the recruitment and assembly of the translation initiation machinery[245]. EIF3J has been reported to interact with CSNK2 and be phosphorylated by CSNK2[246]. Casein kinase 2 is involved in cellular processes, such as cell cycle control, DNA repair, and circadian rhythm[247]. Moreover, casein kinase 2 has been reported as an an attractive target for anti-cancer drug design[248].

Previous studies have shown that these CDK4/6 dual inhibitors havea similar structure that is distinct from pan-CDK inhibitors; thereby, these inhibitors should show a similar feature to work against the kinome. Nevertheless, Abemaciclib was detected as a broad spectrum inhibitor in our study. Moreover, the main targets were not CDKs in the selectivity profiling of Abemaciclib. CSNK2A1, CSNK2A2, CSNK2B, GSK3A, GSK3B, PIM1, CAMK2G, CAMK2D, and CLK1 were potently inhibited byPalbociclib with $K_D$ of 4 nM, 20 nM, 34nM, 18 nM, 25 nM, 7 nM, 17nM, 22 nM, and 3 nM, respectively. As mentioned before, casein kinase 2 is involved in cellular processes such as cell cycle control, DNA repair, and circadian rhythm. It has been

reported to phosphorylate the aminoterminal domain CDK11, which leads to the regulation of RNA transcriptionand processing events. GSK3 families are active in cellular proliferation, migration, glucose regulation, and apoptosis, and they have been implicated in inflammation and cancer[249]. GSK3 became a potential target in cancer therapy based on the tumor suppressor functions[250].CDC-like kinase 1 (CLK1) is involved in pre-mRNA processing in the cell nucleus, and it was reported as a novel target for Alzheimer's disease[251].In our result, the characterization of Abemaciclib may drive Abemaciclib in the treatment of Alzheimer's disease based on the most potent target CLK1. In the profiling of Ribociclib, no CDKs were detected as potential targets ($K_D$< 100 nM). CSNK2A2 and CAMKK2 were inhibited with the $K_D$ of 85 nM and 93 nM, respectively. The characterization of Ribociclib provides strong evidence to halt the clinical trials of Ribociclib.

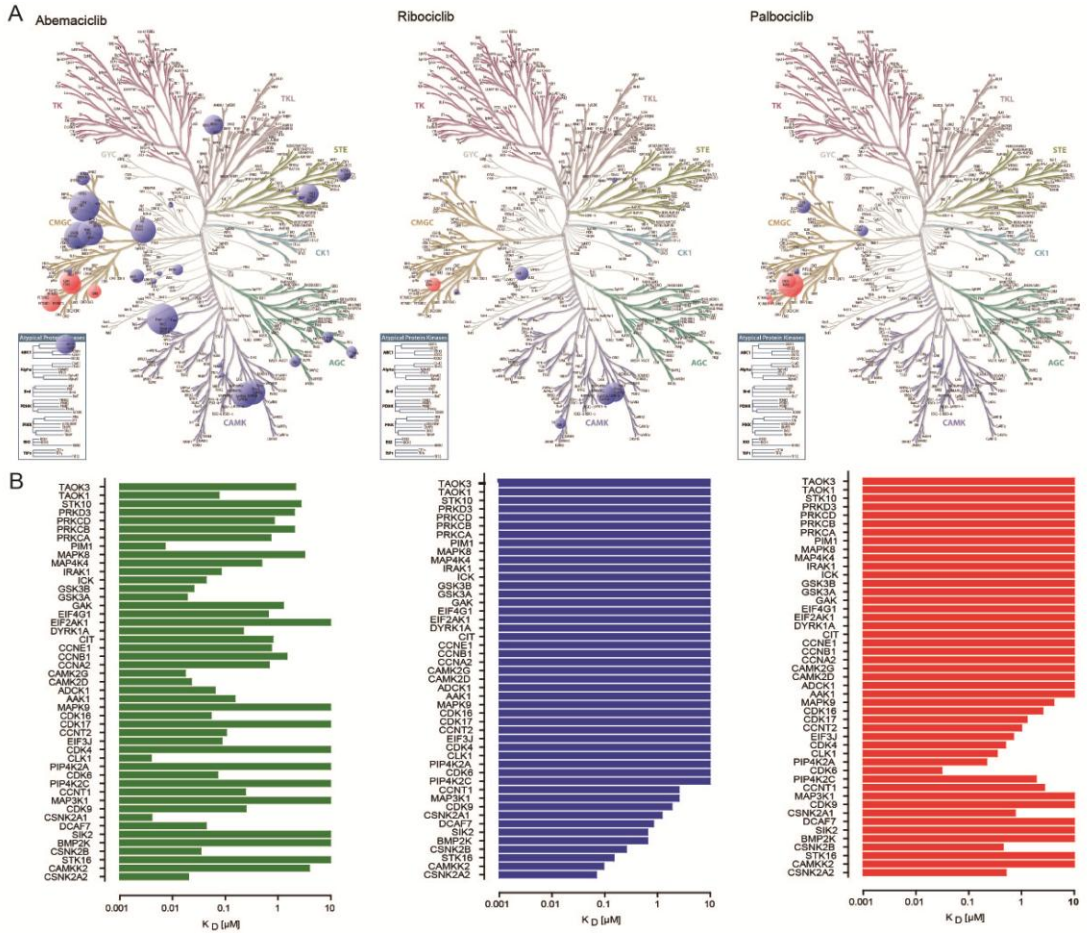


**Figure 2.5 Characterization of CDK4/6 dual inhibitors.**(A) Kinometrees of CDK4/6 inhibitors Abemaciclib, Ribociclib and Palbociclib. The size of the dots corresponds to the $K_D$ of targets.

Red dots denote CDKs, blue dots denote non-CDKs. (B) Histogram dissociation constants ($K_D$) obtained for CDK4/6 inhibitors of target proteins.

### 2.3.4 Off-targets of second-generation CDK inhibitors

Our results enable to detect all of the potential targets, which include both new CDK sub-units and non-CDKs, by screening 14 CDK inhibitors. New CDK sub-units CDK12, CDK13, CDK16, and CDK17 were performed as new targets of some CDK inhibitors (Figure 2.6A). AT-7519, BMS-387032, Dinaciclib, and Alvocidib inhibited CDK12 with $K_D$ of 148 nM, 98 nM, 14 nM, and 98 nM, respectibely.AT-7519, P-276-00, and BMS-387032 were found as CDK13 inhibitors in our results ($K_D$= 203 nM, 4 nM, and 356 nM, respectively). CDK12 and CDK13 play as key regulators of transcription elongation and regulate the expression of genes involved in DNA repair; they are also required for the maintenance of genomic stability[238, 242]. CDK12 and CDK13 bind cyclin K in human cells, and depletion of CycK/Cdk12 induces spontaneous DNA damage, resulting in DNA damage agents becoming sensitive[239]. Our finding detected few new CDK12 and CDK13 inhibitors, and may drive these CDK12/CDK13 inhibitors in the combination with DNA damage agents for cancer treatment.

Abemciclib, AT-7519, and BMS-387032 were demonstrated to inhibit CDK16 with the $K_D$ of 53 nM, 4 nM, and 71 nM. CDK16, which belongs to the cdc2/cdkx complex, is essential in signal transduction cascades. It has been reported to be up-regulated in several transformed cancer lines[252], which may suggest that CDK16 inhibitor can be used in the treatment of some transformed cancers. AT-7519, BMS-387032, and Alvocidib inhibited CDK17 with the $K_D$ of 8 nM, 4 nM, and 7 nM. CDK17 belongs to the cdc2/cdkx complex and has been reported to be involved in terminally differentiated neurons[125]. Meanwhile, silence of CDK17 led to cell death in breast cancer cell line, which may promote CDK17 inhibitors in the treatment of breast cancers to evaluate the effects[232].

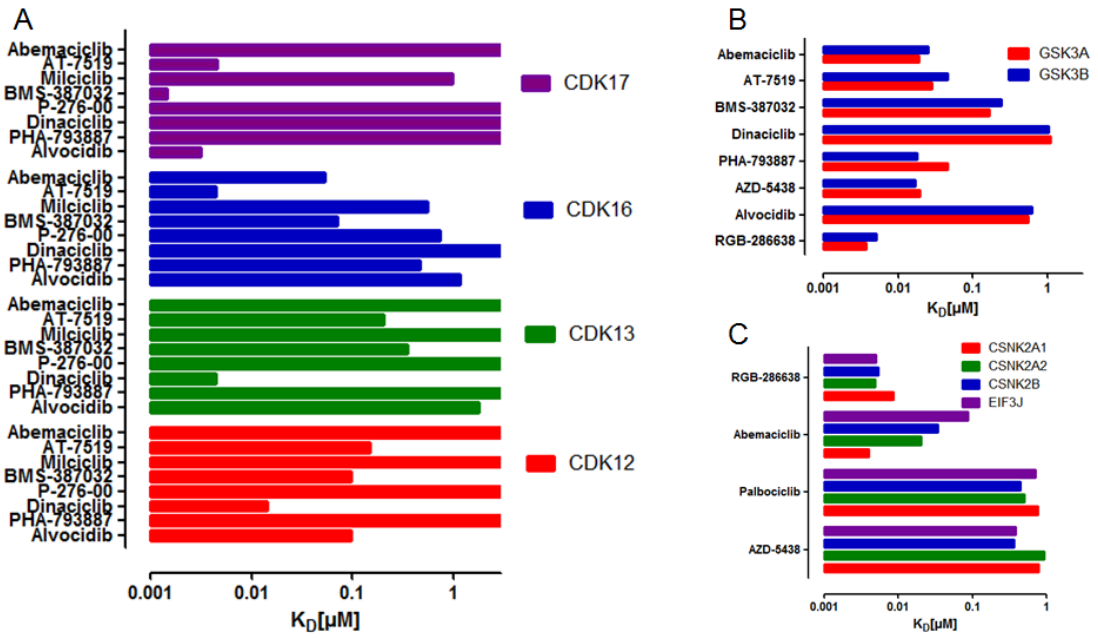


**Figure 2.6 Off-targets of CDK inhibitors.** Histogram dissociation constants ($K_D$) obtained for (A)Alvocidib, PHA-793887, Dinaciclib, P-276-00, BMS-387032, Milciclib, AT-7519 and Abemaciclib of CDK sub-units CDK12, CDK13, CDK16, CDK17; (B) Abemaciclib, AT-7519, BMS-387032, Dinaciclib, PHA-793887, AZD-5438, Alvocidib and RGB-286638 of GSK3A and GSK3B; (C) RGB-286638, Abemaciclib, Palbociclib and AZD-5438 of CSNK2A1, CSNK2A2, CSNK2B and EIF3J.

Besides the CDKs, our results also detected some off-targets, which were non-CDKs in the characterization of CDK inhibitors through Kinobeadscompetition assay. Some CDK inhibitors including Abemaciclib, AT-7519, PHA-793887, AZD-5438, and RGB-286638 were demonstrated to inhibit the activity of glycogen synthase kinase 3 Alpha (GSK3A) and GSK3B in our data (Figure 2.6B). RGB-286638 resulted in the most potential inhibition of GSK3A and GSK3B with $K_D$ of 4 nM and 6nM. As mentioned, GSK3 familiesare active in cellular proliferation, migration, glucose regulation, and apoptosis, and they have been implicated in inflammation and cancer.GSK3 became a potential target in cancer therapy based on the tumor suppressor functions[249, 250, 253], and our data detected some new GSK3A/B inhibitors. Casein kinase 2 is involved in cellular processes such as cell cycle control, DNA repair, and circadian rhythm. Besides CDK4/6 inhibitor Abemaciclib, second-generation CDK inhibitor RGB-286638 was found to inhibit CK2 sub-units very potently(CSNK2A1,

CSNK2A2, CSNK2B, and EIF3J with $K_D$ of 7 nM, 9 nM, 6 nM, and 11 nM, respectively). Casein kinase 2 is a novel player in MM, acute leukemia, and breast cancer phogenesis. Casein kinase 2 specific inhibitor CX-4945 (Silmitasertib) has entered into phase II clinical trials as a potential anticancer drug[254]. Our findings suggest that Abemaciclib and RGB-286638 might be CK2 inhibitors in cancer therapies.

### 2.3.5 Differences and similarities of CDK inhibitors in CDK/cyclin complexes inhibition

By analyzing the highly significant elastic net features associated with response to all the CDK inhibitors for the potential targets, our data demonstrated that (Figure 2.7): (1) Most CDK inhibitors were non-specific to work against CDK sub-units besidesPalbociclib and AZD-5438, which indicated that the development of selective CDK inhibitors was still aformidable challenge; (2) CDK/cyclin complexes including CDK2/CCNA2 complex, CDK1/CCNB1/CCNB2 complex, CDK7/CCNH complex, CDK12/CDK13/CCNK complex, and CDK9/CCNT1/CCNT2 complex were detected in Kinobeadscompetition assay. All of the CDK inhibitors played their biologic role primarily via inhibit the activity of CDK/cyclin complex; (3) CDK9/CCNT1/CCNT2 was inhibited by almost all the CDK inhibitors. There were two CDK inhibitors that potently inhibited CDK9/CCNT with the $K_D$< 10 nM (SCH-727965: 3 nM, Alvocidib: 5 nM). Since CDK9 has been reported to be a promising anti-cancer target because CDK9 inhibition can drive cancer cells into apoptosis[18], this finding may promote SCH-727965 and Alvocidib into CDK9-related cancer therapies; (4) For the CDK6 inhibition, Dinaciclib was detected to be more potent than CDK4/6-specific inhibitorsPalbociclib, Abemaciclib, and Ribociclib ($K_D$= 5 nM vs. 30 nM, 71 nM, 254 nM),which suggests that Dinaciclib can be used in the treatment of estrogen receptor positive advanced breast cancer; (5) First-generation CDK inhibitor Seliciclib, second-generation CDK inhibitors P-276-00, Rociletinib, and Rigosertib did not potentially inhibit any CDKs. This finding may explain the failure of them in the clinical research studies and provides strong evidence to halt their clinical trials. In summary,

these results demonstrated the failure of CDK inhibitors because of the non-specific CDK inhibition. CDK inhibitors function in the CDK/cyclin complex inhibition, and CDK9 complex inhibition might drive some CDK9-specific inhibitors to get new insights into the clinical research.

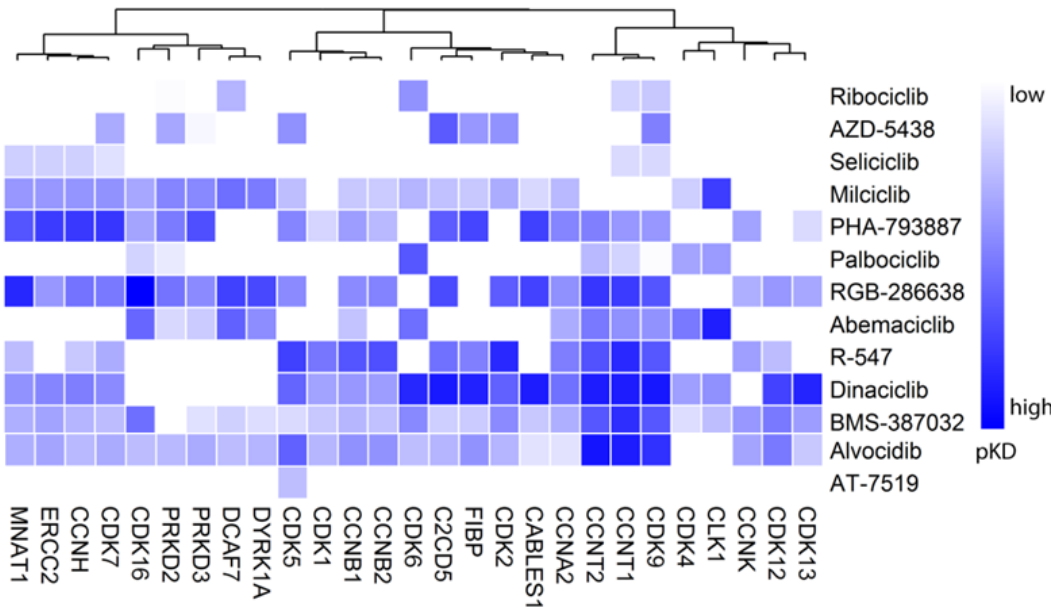


**Figure 2.7 Heatmap of highly significant elastic net features associated with response to all the CDK inhibitors.**The shades of color of the nodes correspond to the $K_D$ of targets. The $K_D$ the lower is, the darker the color is.

### 2.3.6Overview of CDK inhibitors in cell cycle regulation and transcription

Our data demonstrated the different roles of CDK inhibitors in the regulation of cell cycle, transcription, and other processes based on the potency of each CDK inhibitor in different CDK/cyclin complexes(Figure 2.8).Almost all of CDK inhibitors (Abemaciclib, Palbociclib, RGB-286638, Dinaciclib, AT-7519, BMS-387032, PHA-793887, Alvocidib and Milciclib) functioned in cell cycle regulation, transcription and other processes through different CDK/cyclin inhibition. For examples, R-547 regulated cell cycle by mainly targeting CDK2/CCNA/CCNE complex, and regulated transcription by targeting CDK9/CCNT complex; Palbociclibregulated cell cycle by mainly targeting CDK6 complex, and regulated transcription by targeting CDK9/CCNT complex. BMS-387032 regulated cell cycle by mainly targeting CDK16 and CDK17,

and regulated transcription by targeting CDK6. Only few CDK inhibitors functioned in either cell cycle regulation or transcription. For examples, P-276-00regulated the transcription level by mainly targeting CDK9/CCNT complex; Seliciclib also involved intranscriptional regulation by targeting CDK7/CCNH and CDK9/CCNT complexes. This result showed that almost all the CDKs inhibitors were non-specific CDK inhibitors, which is the major problem of the failure of CDK inhibitors in the clinical trials.

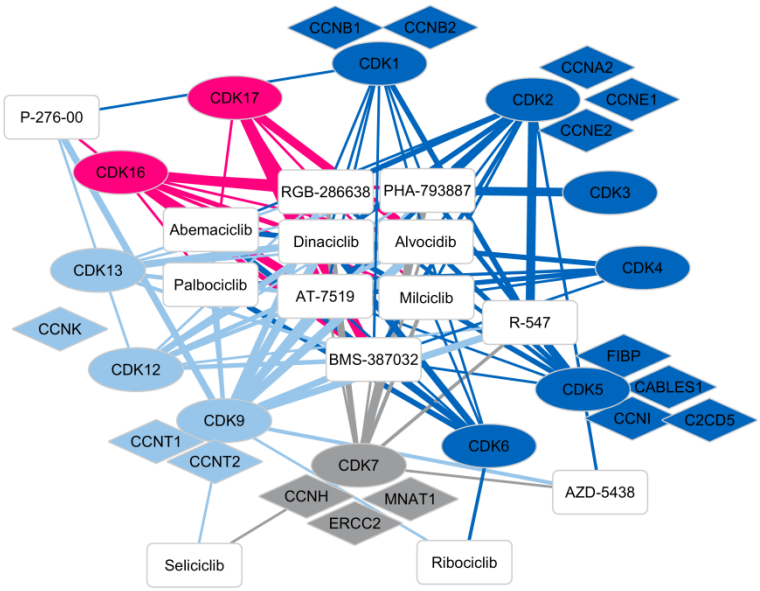


**Figure 2.8 Relationships betweenCDK inhibitors and CDK/cyclin complexes.** Dark blue color denotes the cell cycle-associated complexes. Gray, light blue, and pink color denote transcription-associated complexes. White color denotes CDK inhibitors. The boldness of the lines corresponds to the $K_D$ of targets.

## 2.4 Conclusions

The current study screened 14 CDK inhibitors, which include both first- and second-generation CDK inhibitors, against the maximum number of kinases, including both CDKs and their complex partners in Kinobeadscompetition assay. Kinobeads™ provided an advantage in capturing CDK-cyclin complex partners, including CCNA2, CCNB1, CCNB2, CCNH, CCNI, CCNK, CCNT1, and CCNT2.Our results indicated that most CDK inhibitors were non-specific for targeting CDKs and cyclins beside FDA-approved CDK4/6 inhibitor Palbociclib. Second-generation CDK inhibitors were demonstrated to more potent to work against CDK2, but not against CDK1 compared with first-generation CDK inhibitor Alvocidib. New CDK sub-units (CDK12/13/16/17/18)were detected in the profiling of most CDK inhibitors. Our results also demonstrated that CDK inhibitors were involved in multiplebiological processes including cell cycle, transcription, and some other biological processes, and explained the failure of CDK inhibitors at the protein level. Off targets (Casein kinase 2, GSK3A/B) were detected besides the main target CDKs and cyclins of CDK inhibitors.

To summarize, our results characterized all the CDK inhibitors at the protein level. On one hand, the results of this work offer good explanations why CDK inhibitors have not been able to withstand FDA testing in the past; on the other hand, our data detected new potential off-targets of CDK inhibitors.

# CHAPTER III

# Salt-inducible Kinase 2 Is a Potential Target in Immune Response

## 3.1 Introduction

Salt-inducible kinase (SIK) family is a serine/threonine protein that belongs to adenosine-monophosphate (AMP)-activated protein kinase (AMPK)-related kinase superfamily[255].It modulates the efficiency of insulin signal transduction by phosphorylating insulin receptor substrate 1 (IRS-1) in insulin-stimulated adipocytes[256]. It also phosphorylates the cAMP response element-binding protein (CREB)-specific coactivator TORC, which leads to the inhibition of CREB activity[257, 258].

There are three isoforms in SIK family: SIK1 (SNF1LK), SIK2 (QIK or SNF1LK2), and SIK3 (QSK or KIAA0999)[259]. SIK1 is a key regulator in cell cycle, gluconeogenesis and lipogenesis, and tumor suppression[260]. SIK3 is a diagnostic marker because it is highly expressed in ovarian cancer[261]. SIK3 depletion increases mitotic arrest and cell death, and over-expression of SIK3 promotes G1/S cell cycle progression[262]. SIK2 is a key regulator in insulin secretion by phosphorylating CDK5 activator p35[263], and it is involved in neuron survival[264] and hepatic metabolism[265]. Recent studies have shown that SIK2 is a potential target in ovarian cancer, and SIK2 depletion both delays G1/S transition and sensitizes ovarian cancers to paclitaxel[266]. Moreover, SIK2 has been found to be a potential target in inflammatory and autoimmune diseases[267, 268]. In

classically activated macrophages (M1), regulatory (M2 B) macrophages, and dendritic cells (DCs), SIK2 plays a pro-inflammatory role to suppress the interleukin (IL)-10 secretion by phosphorylating CRTC3[268, 269].

SIK2 plays an essential role in LKB1-SIK-CRTC3 signaling pathway (Figure 3.1)[268]. In the activation of LKB1-SIK-CRTC3 signaling pathway, at first, Toll-like receptor (TLR) agonists activate MAPKs, which leads to the activation of MSK1/2. The activation of MSK1/2 phosphorylates CREB. SIK2 is highly activated by liver kinase B1 (LKB1). CREB co-activator CRTC3 is phosphorylated by SIK2, which increases the interaction with 14-3-3 protein and leads to CRTC3 arrest in the cytosol. SIK2 inhibition leads to dephosphorylation of CRTC3, which results in the increase in CREB-dependent gene transcription, thereby inducing anti-inflammatory cytokine IL-10 and reducing the secretion of pro-inflammatory cytokine TNF-α. TNF-α is involved in acute and chronic inflammatory diseases[270], and IL-10 has been reported to switch from tumor-promoting inflammation to antitumor immunity[271]. These findings suggest that SIK2 inhibition may be important for the immune system and provide an opportunity to utilize SIK2 inhibitors in the treatment of inflammatory diseases.

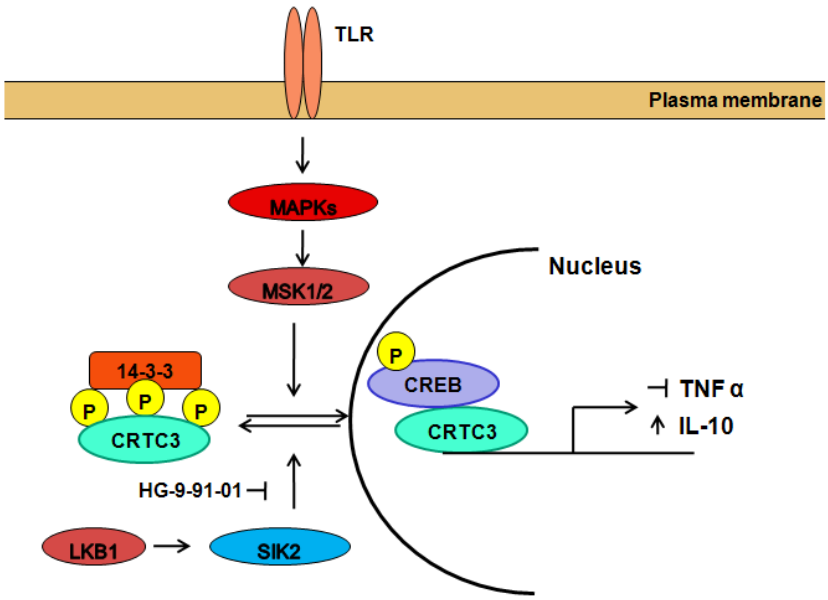


**Figure 3.1 The model for regulation of TNF-α and IL-10 production by LKB1-SIK-CRTC3 signaling pathway.**SIK2 inhibition dephosphorylates CRTC3, and induce anti-inflammatory

cytokine IL-10 and reduce the pro-inflammatory cytokine TNF-α secretion.

Pharmacological inhibition of SIK2 plays an important role in innate immune system, and few FDA-approved drugs such as Dasatinib and Bosutinib were found to induce anti-inflammatory macrophages by inhibiting SIK2[267]. However, there is no global screening of SIK2 inhibitors in kinase inhibitors' library. In this study, SIK2-specific inhibitor HG-9-91-01 was evaluated in Kinobeadsassay to detect the on/off targets.242 kinase inhibitors containing both FDA-approved drugs and small molecule kinase inhibitors in clinic were screened by Kinobeadsassay. The detected SIK2 inhibitors were characterized according to the cytotoxicity in some cancer cell lines, which were highly associated with inflammation. These SIK2 inhibitors were evaluated in immune responding model containing murine and human cell lines (BMDCs, HoxB8, THP-1, PBMCs, and Human DCs) to observe the role of SIK2 inhibition in immune system, such as cytokine (TNF-α and IL-10) secretion. Anti-TNF-α therapy and IL-10 therapy have been demonstrated to be exploited in various clinical trials for the development of therapies of several inflammatory diseases.Nevertheless, no small molecule kinase inhibitor has shown therapeutic benefits in these therapies. This work try to find some evidence to drive low molecular weight SIK2 inhibitors in the investigation of inflammatory and autoimmune diseases.

## 3.2 Methods and materials

### 3.2.1 Reagents

Small molecule kinase inhibitors were dissolved in DMSO as 10mM solutions and stored at −20 °C. HG-9-91-01 was obtained from MedChem Express. Recombinant human GM-CSF, IL-4, mouse GM-CSF and IL-4 were obtained from Peprotech. SIK2 antibody (D28G3) was obtained from Cell Signaling Technology. Human TNF-α (88-7346-88), Human IL-10 (88-7106-88), Mouse TNF-α (88-7324-88), and Mouse IL-10 (88-7105-88) ELISA Ready-SET-Go kits were obtained from eBioscience. The TLR agonists Zymosan (Zym) and lipopolysaccharide (LPS) were kind gifts from Prof. Dr. Jürgen Ruland.

### 3.2.2 Cell lines

THP-1 cell line was grown in RPMI 1640 medium (Biochrom GmbH) supplemented with 10% FBS (Biochrom GmbH), 1% antibiotics solution (Sigma), 10 mM HEPES (Biochrom) and 0.1 mM 2-Mercaptoethanol (Sigma). MV4-11, HL60, CCRF, THP-1, Molt4 K562, SR and RPMI cell lines were grown in RPMI 1640 medium (Biochrom GmbH) supplemented with 10% FBS (Biochrom GmbH), 1% antibiotics solution (Sigma).

### 3.2.3 Bone-marrow–derived dendritic cell (BMDCs) culture

Bone marrow was harvested from femurs and tibias of C57BL/6 mice. Bone-marrow–derived dendritic cells (BMDCs) were differentiated in DMEM medium (Biochrom GmbH) supplemented with 2 mMGlutaMAX (Thermo Fisher Scientific), 10% FBS, 1% antibiotics solution, and 2% mouse GM-CSF. Cells were differentiated for 7 d and analyzed for >90% CD11c (BD Biosciences) by flow cytometry before use in experiments.

### 3.2.4 Peripheral blood mononuclear cells (PBMCs) and human DCs culture

Peripheral blood mononuclear cells (PBMCs) were isolated in using density gradient

centrifugation by Ficoll-Paque (Pharmacia Biotech). Followed by purification of PBMCs by plastic adherence for 1 h and washed 5× with PBS. In PBMCs pool, monocytes were differentiated toward DCs supplemented with IL-4 (50 ng/mL, Peprotech) and GM-CSF (50 ng/mL, Peprotech) for 7 d. Half of cell culture medium supplemented with 2 mMGlutaMAX (Thermo Fisher Scientific), 10% FBS, 1% antibiotics solution, IL-4 (50 ng/mL) and GM-CSF (50 ng/mL) were replaced after 3 d.

### 3.2.5 Cytokine secretion measurement

THP-1 were seeded into 96-well plates at 50000 cells per well in 100 µL RPMI 1640 medium supplemented with 10% FBS, 1% antibiotics, 10 mM HEPES and 0.1 mM 2-Mercaptoethanol followed by incubation at 37°C for 4h. Compounds (50 µL per well) were transferred into 96-well plates for 48 hours followed by stimulation with LPS (100 ng/mL final concentration) dispersed in culture medium (10 µL per well) for 24 hours. After LPS stimulation, the cell culture medium was removed, clarified by centrifugation for 5 min at 14000g. The concentration of TNF-α and IL-10 were determined using human/mouse TNF-α ELISA ready-set-go (eBioscience) and human/mouse IL-10 ELISA ready-set-go (eBioscience).

### 3.2.6 Dose dependent cell viability assay

Alamar blue cell viability assay (Thermo Fisher Scientific) was used to determine cell viability. For THP-1, cells were seeded into 96-well plates at 50000 cells per well in 100 µL RPMI 1640 medium supplemented with 10% FBS, 1% antibiotics, 10 mM HEPES and 0.1 mM 2-Mercaptoethanol followed by incubation at 37°C for 4h. Different concentrations (from 3 nM to 3µM) of each inhibitor were added to each well then the cells were incubated for 72 h at 37 °C. 10% alamar blue reagent was added to each well to perform cell viability assay. The reduction from resazurin to resorufin was measured after 4 h using a fluorescence spectrophotometer (BMG Labtech) at 544 nm (excitation) and 584 nm (emission).

### 3.2.7 Small interfering RNA (siRNA) to knockdown SIK2 expression

THP-1 cells were seeded into 12 well plates (3 × 105 cells per well) and incubated for 24 h at 37 °C. siRNA (Qiagen: SI02665439, SI00604667, SI04439099, SI04439092) were diluted in Opti-MEM medium. INTERFERinTM (PolyPlus) was added to siRNA and mix for 10 s, followed by 10 min incubation at room temperature. Then added the mixture into each well and incubated 48 h. THP-1 cells were harvest for the western blot assay and the supernatant was collected for the ELISA assay.

### 3.2.8 Western blot

Cells were extracted in lysis buffer (150 mMNaCl, 50 mM Tris/HCl, pH 7.5, 1 mM Na3VO4, 5% glycerol, 0.8% NP40, 1.5 mM $MgCl_2$, 0.375 mMNaF, 1 mM DTT). The protein concentration was measured by Bradford assay. To detect SIK2 in cell lysates, 20 μg of protein was separated by SDS/PAGE and transferred to PVDF membranes. SIK2 was detected by SIK2 antibody in immune-blotting.

## 3.3 Results and discussions

### 3.3.1 Characterization of SIK2-specific inhibitor HG-9-91-01 in Kinobeadsassay

SIK-specific inhibitor HG-9-91-01 was characterized in Kinobeadsassay in two different set-ups. For the original Kinobeadspulldown assay, different concentrations of HG-9-91-01 were first incubated with cell lysate mix (SK-N-BE 2, MV4-11, Colo 205, and K562), followed by affinity enrichment of kinome. Quantitative MS was used for the identification of targets. In order to further evaluate HG-9-91-01 in living cells, HG-9-91-01 was pre-incubated with MV4-11 cell line, followed by cell lysis and Kinobeadsassay. In this study, SIK2 was potently inhibited by HG-9-91-01 in both set-ups (Figure 3.2), and it was inhibited stronger when HG-9-91-01 was pre-incubated with MV4-11 ($K_D$ = 0.06 nM vs. 27.4 nM). This result indicated that HG-9-91-01 plays a greater role in the internal environment that is closer to the organism. Surprisingly, SIK2 wasn't the most potent target in the profiling of HG-9-91-01 in the original Kinobeadsassay (Figure 3.2A). The off-targets PTK6, RIPK2, EPHA2, GAK, FRK, and LIMK2 were inhibited more potently than SIK2 inhibition ($K_D$ = 1.2 nM, 2.6 nM, 7.0 nM, 13.4 nM, and 15.6 nM, respectively). When HG-9-91-01 was pre-incubated with MV4-11 cell line, SIK2 was found to be the most potent target with $K_D$ of 0.06 nM (Figure 3.2B). Especially SIKs sub-unit family member SIK3 was determined in the profiling of HG-9-1-01 with $K_D$ of 0.33 nM. Meanwhile, RIPK2, EPHB4, GAK, and LIMK1 were found as off-targets of HG-9-91-01 ($K_D$ = 0.16 nM, 2.6 nM, 2.9 nM, 7.9 nM, and 9.7 nM, respectively). Briefly, the pre-incubation of HG-9-91-01 with MV4-11 identified more off-targets with $K_D$< 1μM (27 vs. 21) compared with original Kinobeadsassay. HG-9-91-01 has been reported as a specific SIK inhibitor in the previous studies[258, 260, 263, 266]; however, our result confirmed that SIK-specific inhibitor HG-9-91-01 was not selective to work against SIKs. The general usage of HG-9-91-01 is as a tool compound in SIK2 research, such as insulin secretionand the regulation of lipid homeostasis and adipogenesis[258, 265].The non-selectivity of HG-9-91-01 in this study indicated that

off-target effect of HG-9-91-01 might have a negative influence on SIK2 research, since few potential off-targets were determined as off-targets of HG-9-91-01in both set-ups, such as RIPK2 and LIMKs. Recently, research studies have shown that RIPK2 plays an important role in the modulation of immune responses[272], and that LIMK inhibition leads to the suppression of tumor growth in vivo[273]. The inhibition of RIPK2 might contribute in SIK2-mediated immune-regulation. The off-targets of HG-9-91-01 provide new research directions concerned and drive HG-9-91-01 as an RIPK2 and LIMK inhibitor in anti-inflammatory and anti-cancer therapies.

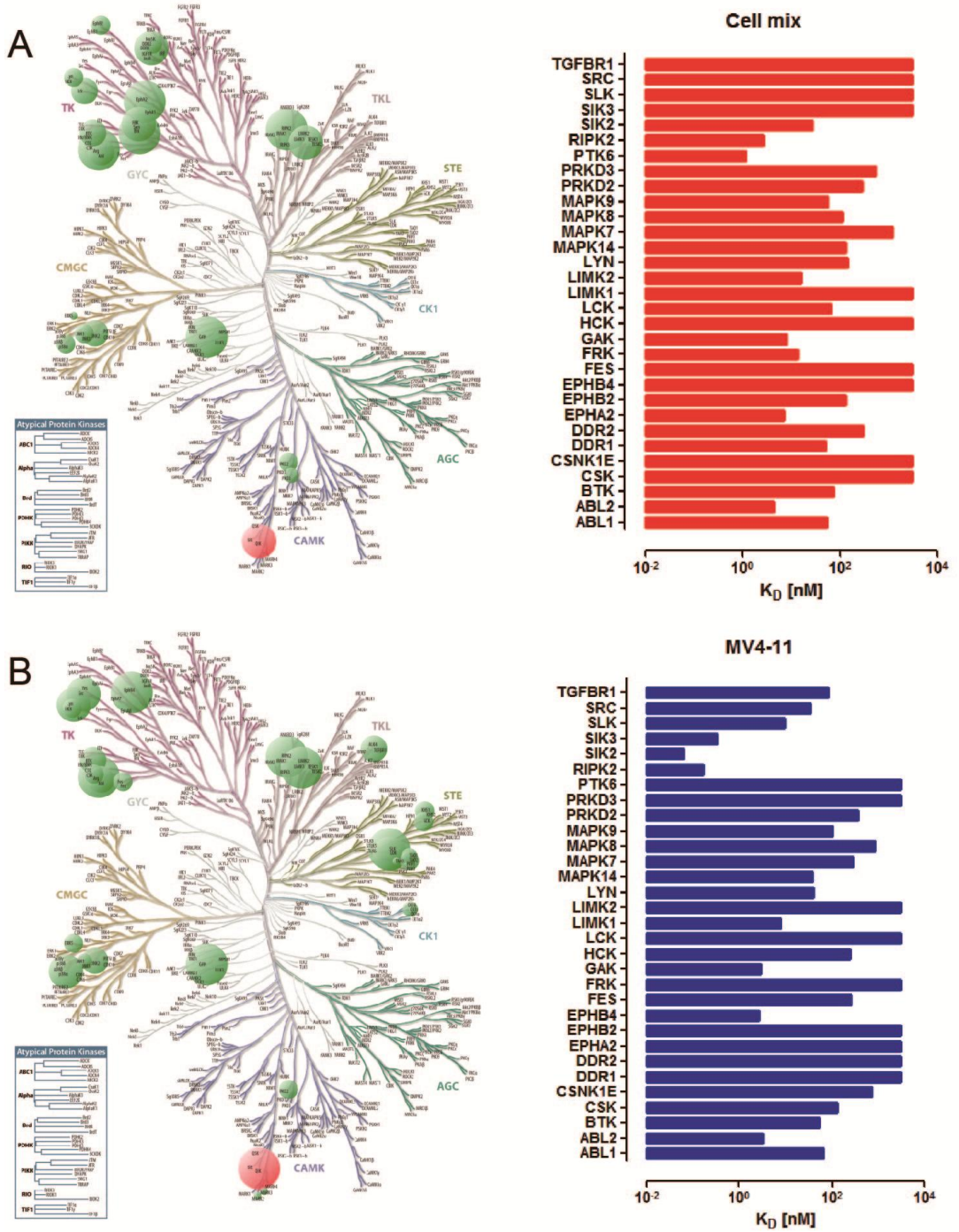


**Figure 3.2 Characterization of SIK2-specific inhibitor HG-9-91-01 in Kinobeadspulldown assay with different set-ups.** (A) HG-9-91-01 was incubated with cell lysis mix (SK-N-BE 2, MV4-11, Colo 205 and K562), followed by affinity enrichment of kinome. Quantitative mass spectrometry was used for the identification of targets. (B) HG-9-91-01 was pre-incubated with MV4-11 cell line, followed by Kinobeadspulldown assay. Kinometrees of HG-9-91-01 in two different set-ups. The sizes of the dots correspond to $K_D$ of target proteins. Red color denotes SIK2 and green color denotes the off-targets of HG-9-91-01. Histogram dissociation constants ($K_D$) obtained for HG-9-91-01 of target proteins.

### 3.3.2 Drug-screen profiling to determine SIK2 inhibitors in inhibitors' library

In this study, 26 small molecule kinase inhibitors were determined to inhibit SIK2 with $K_D$< 1 μM in Kinobeadscompetition assay (Table 3.1) by screening 242 small molecule kinase inhibitors. These 26 SIK2 inhibitorsincluded six FDA-approved anti-cancer and anti-inflammation drugs (Bosutinib, Dasatinib, K252A, Vargatef, CEP-701, and Dabrafenib) and 19 small molecule kinase inhibitors that are currently under clinical trials. Dasatinib and Bosutinib were designed as BCR/ABL inhibitors for the treatment of chronic myeloid leukaemia[274]. K252A and CEP-701 were approved for the treatment of psoriasis by FDA[275]. As a small molecule kinase inhibitor, Vargatef targets VEGFR, FGFR, and PDGFR for NSCLCpatients[276].Dabrafenib was developed for metastatic melanoma patients with BRAF (V600) mutation[277]. The other SIK2 inhibitors in clinic had various targets, such as CDK inhibitors (RGB-286638, PHA-848125), Aurora inhibitors (PF-38914735, AT-9283, AT-9283, GSK1070916), and JAKs inhibitors (PRT062070, XL-019, TG101348). Even though 14 inhibitors have been reported to inhibit SIK2 in various literatures[278, 279], only Dastinib and Boutinib were evaluated in SIK2-associated immune research[267].

In our result, SIK2 was determined as a new potential off-target of AT-9283, PF-477736, PRT062070, RGB-286638, SCH-900776, and ASP-3026 with $K_D$< 100 nM (20 nM, 30 nM, 40 nM, 80 nM, 89 nM, and 94 nM, respectively). AT-9283 and PRT062070, which were designed as JAKs inhibitors, were developed for the patients with advanced solid tumor, CLL or non-hodgkin's lymphoma (NHL), or multiple myloma[280]. PF-477736 and SCH-900776 were developed to inhibit Chk1, and Chk1 inhibition became a novel therapeutic strategy in breast and ovarian cancers[281].RGB-286638 was designed as a CDK inhibitor in the treatment of patients with solid tumurs[209]. Inflammation is crucial for cancer development[282], and SIK2 inhibition plays an important role in the immune system. Therefore, the potential inhibition of SIK2 by these inhibitors may contribute toward suppressing cancer development.

| Inhibitors | Reported targets | SIK2$K_D$ (nM) | STATU | known/unknown target SIK2 |
|---|---|---|---|---|
| UCN-01 | PKC | 5 | Phase I | Known |
| AZD-7762 | Chk1,2 | 5 | Phase I | Known |
| Dasatinib | Src, Abl | 6 | Approved | Known |
| Crenolanib | PDGFRB, FLT3 | 10 | Phase III | Known |
| PF-03814735 | Aurora | 17 | Phase I | Known |
| AT-9283 | Aurora, JAK1, 2 | 20 | Phase II | Unknown |
| PF-477736 | Chk1 | 30 | Phase I | Unknown |
| K252A | TrkA, CaMK, PKA | 34 | Approved | Known |
| PRT062070 | JAKs, Syk | 40 | Phase II | Unknown |
| BMS-690514 | Pan-ErbB, VEGF2 | 58 | Phase II | Known |
| Bosutinib | Src, Abl | 72 | Approved | Known |
| Vargatef | VEGFR, FGFR | 72 | Approved | Known |
| RGB-286638 | CDKs | 80 | Phase II | Unknown |
| SCH-900776 | Chk1 | 89 | Phase I | Unknown |
| ASP-3026 | ALK | 94 | Phase I | Unknown |
| CEP-701 | Flt3, JAK2, TrkA | 100 | Approved | Known |
| XL-019 | JAK2 | 113 | Phase II | Unknown |
| PHA-848125 | CDK1, 2, 4, TrkA | 120 | Phase II | Unknown |
| TAK901 | Aurora | 153 | Phase I | Unknown |
| Dabrafenib | BRAF | 297 | Approved | Known |
| GSK1070916 | Aurora | 365 | Phase I | Known |
| R-406 | SYK | 547 | Phase II | Known |
| Saracatinib | Src, Abl | 630 | Phase III | Unknown |
| TG101348 | JAK2, Flt3, Ret | 670 | Phase II | Known |
| XL-228 | IGF1R, Src, Bcr-Abl | 753 | Phase II | Unknown |
| Danusertib | Abl, FGF1, Ret | 858 | Phase II | Unknown |

**Table 3.1 SIK2 inhibitors and their targets present here.** Kinobeadspulldown assay was used to screen small molecule kinase inhibitors.

### 3.3.3 SIK2 inhibition had no effect in cell growth

After detecting SIK2 inhibitors and characterizing SIK2-specific inhibitor HG-9-91-01 in Kinobeadscompetition assay, five leukemia cell lines (MV4-11, HL60, CCRF, THP-1, Molt4), two lymphoma cell lines (K562, SR), and one melanoma cell line (RPMI), which are highly associated with inflammation, were incubated with SIK2-specific

HG-9-91-01 to observe the cytotoxicity of SIK2 inhibition. In this study, HG-9-91-01 didn't show any cytotoxicity in most of the cell lines besides MV4-11 and K562 (IC50 = 39.6 nM and 291.4 nM) (Figure 3.3 A). An FLT3 inhibitor Crenolanib, which also showed potent SIK2 inhibition with $K_D$ of 10 nM in Kinobeadsassay, was observed for cytotoxicity in these cell lines by cell viability assay (Figure 3.3B). Only MV4-11, which highly expressed FLT3-ITD mutation, was strongly inhibited by FLT3-specific inhibitor Crenolanib. Combining the cell viability results of HG-9-91-01 and Crenolanib, it was confirmed that SIK2 inhibition had no effect on cell growth.Next step, all of the SIK2 inhibitors were used in these cancer models to evaluate the role of SIK2 inhibition in immune response, especially in cytokine secretion.

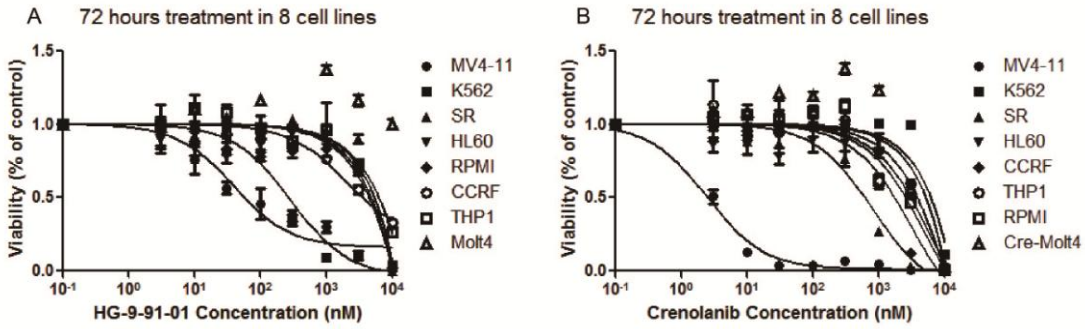


**Figure 3.3 Response curves of (A) HG-9-91-01 and (B) Crenolanib.** Cells were seeded into 96 well plates and different concentration of HG-9-91-01 and Crenolanib were treated with cells for 72 hours. 10% alamar blue reagent was added to each well to perform cell viability assay. The reduction from resazurin to resorufin was measured after 4 h using a fluorescence spectrophotometer (BMG Labtech) at 544 nm (excitation) and 584 nm (emission).

### 3.3.4 SIK2 inhibition reduced TNF- α production in THP-1 cell line

THP-1 cell line, which is an in vitro cell model for immune modulation approach[283], was used to evaluate the cytotoxicity of SIK2 inhibition. In cell viability assay, most of SIK2 inhibitors had no effect in cell growth (Figure 3.4A). Only few SIK2 inhibitors were demonstrated to have very potent cytotoxicity of THP-1. For examples, CDK-specific inhibitor RGB-286638, PKC inhibitor UCN-01, BCR-Abl inhibitor Bosutinib, and MAPK inhibitor K252A suppressed THP-1 cell growth with the IC50s of 6.8 nM, 21.1 nM, 69.7 nM, and 399.2 nM, respectively. The reported targets of these

inhibitors were determined in Kinobeadspulldown assay, and these targets also demonstrated significant potent inhibition in this study (Figure 3.4B). CDK inhibitor RGB-286638 inhibited CDK16, GAK, GSK3A, GSK3B, and CDK9 with $K_D$ of 6.5 nM, 7.6 nM, 7.5 nM, 8.1 nM, and 8.5 nM. PRKCA, PRKCB, PRKCD, MARK2, and MARK3 were determined as potent targets of PKC inhibitor UCN-01 with $K_D$ of 7.4 nM, 7.4 nM, 3 nM, 7.6 nM, and 7.8 nM. BCR-Abl inhibitor Bosutinib inhibited BCR, ABL1, and ABL2 with $K_D$ of 7.7 nM, 3 nM, and 8.5 nM. MAPK family members MAP2K6, MAP3K5, MAP4K2, MAP4K4, and MAP4K5 were potently inhibited by MAPK inhibitor K252A with $K_D$ of 3 nM, 7.1 nM, 5.5 nM, 6.0 nM, and 6.9 nM, respectively. The results indicated that Kinobeadspulldown assay determines the reported targets of these inhibitors in potent inhibition, and the potent inhibition of these targets leads to cell death.

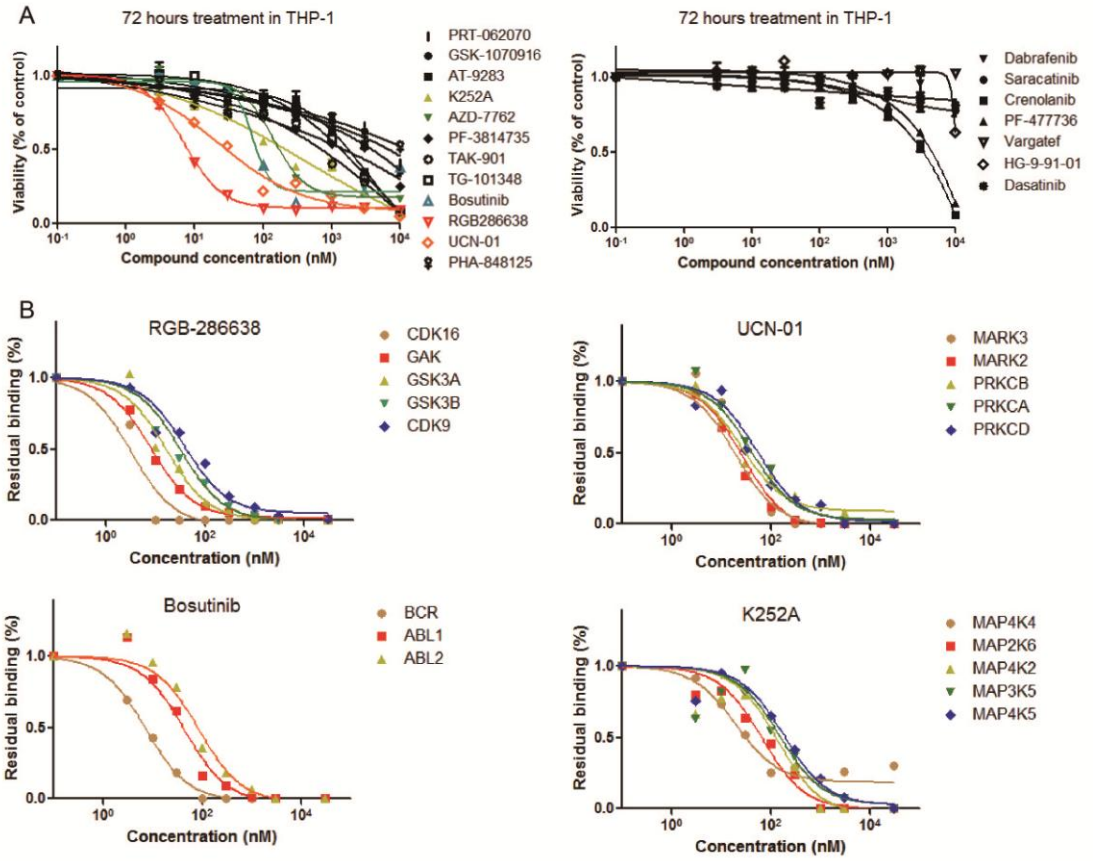


**Figure 3.4 Dependent curves of SIK2 inhibitors in responding to THP-1 and reported targets determined in Kinobeadsassay.** (A) Does dependent curves of SIK2 inhibitors in responding THP-1. THP-1 cells were seeded in 96 well plates and different concentration of

SIK2 inhibitors were treated with cells for 72 hours. 10% alamar blue reagent was added to each well to perform cell viability assay. The reduction from resazurin to resorufin was measured after 4 h using a fluorescence spectrophotometer (BMG Labtech) at 544 nm (excitation) and 584 nm (emission). (B) Reported targets of SIK2 inhibitors were determined in Kinobeadscompetition assay.

Since most of SIK2 inhibitors had no significant influence on the growth of THP-1 cell line (Figure 3.5B), 500 nM of SIK2 inhibitors were treated with THP-1 to evaluate the role of SIK2 inhibition in immune response, especially in cytokine secretion. Two experiments with different treatment times (2 hours and 72 hours) were set up in this study (Figure 3.5A). In 2-hour SIK2 inhibitors treatment condition, many SIK2 inhibitors reduced tumor necrosis factor alpha (TNF-α) secretion. K252A, AZD-7762, PF-3814735, Crenolanib, and HG-9-91-01 had a significant effect on TNF-α secretion. TAK-901, PHA-848125, and Dasatinib reduced around 50% of TNF-α secretion. In 48-hour SIK2 inhibitors treatment condition, TNF-α secretion was inhibited in a certain extent by almost all of the SIK2 inhibitors. GSK-1070916, K252A, AZD-7762, TAK-901, TG-101348, PF-3814735, Crenolanib, HG-9-91-01, Dasatinib, Bosutinib, and PF-477736 completely suppressed TNF-α production. PHA-848125, Saracatinib, and Vargatef reduced around 50% of TNF-α secretion. K252A, AZD-7762, and Bosutinib reduced TNF-α secretion by inhibiting the cell growth with IC50 of 399.7 nM, 139.6 nM, and 69.7 nM, respectively (Figure 3.5B). BRAF inhibitor Dabrafenib increased the TNF-α production at the very beginning, but there was no effect in TNF-α secretion over time. Surprisingly, JAKs inhibitor AT-9283, which was developed for the patients with advanced solid tumor, CLL or NHL, or multiple myloma[280], induced TNF-α secretion in a time-dependent trend. JAK1 and TYK2 were determined as the potential targets of AT-9283 with $K_D$ of 247.5 nM and 369.3 nM (Figure 3.5C). JAKs inhibition in macrophages leads to an increase in lipopolysaccharide-induced cytokine production[284]. This might explain AT-9283 inducing the secretion of TNF-α in this study.

In summary, most SIK2 inhibitors showed time-dependent inhibition in TNF-α

secretion in this study. SIK2 inhibition leads to the decrease of TNF-α secretion. TNF-α, which is chiefly produced by activated macrophages, plays an important role in the regulation of immune cells. As a pro-inflammatory cytokine, TNF-α induces inflammation and is involved in a number of human diseases, such as Alzheimer's disease, cancer, psoriasis, and inflammatory bowel disease (IBD)[270]. The detection of new potential SIK2 inhibitors in this study may drive these inhibitors toward a new research direction in the treatment of immune diseases.

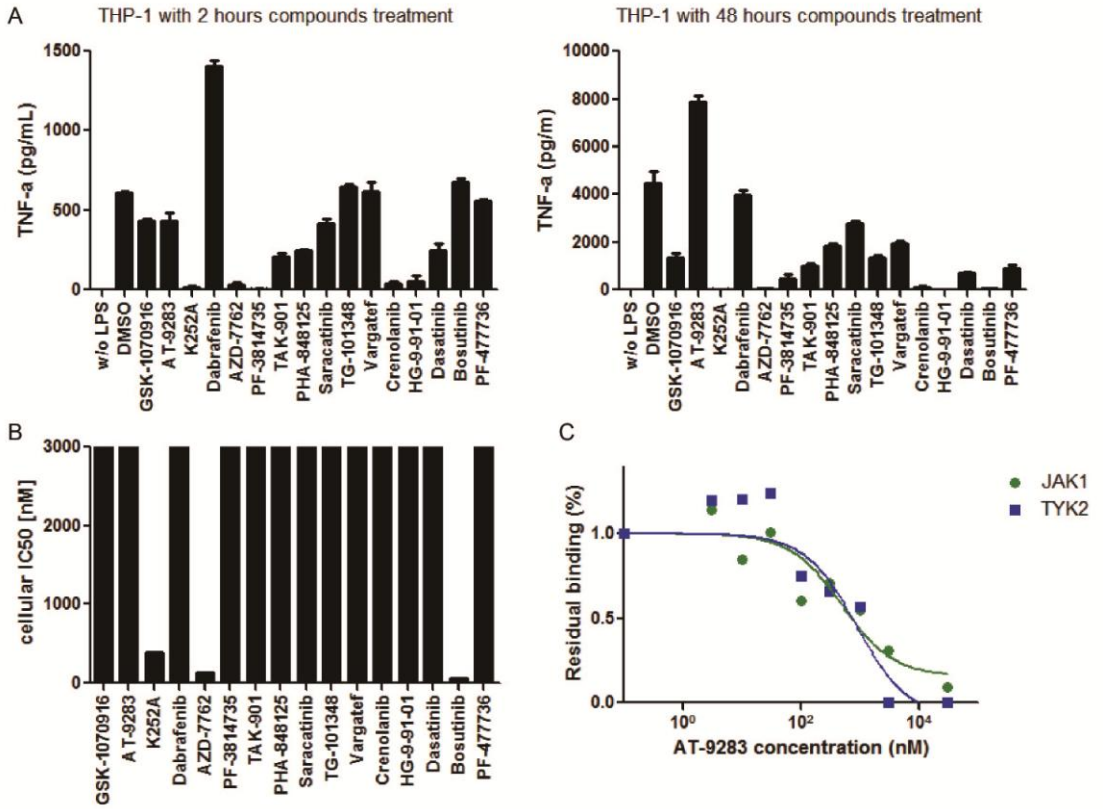


**Figure 3.5Eeffect of SIK2 inhibition on TNF-α secretion inTHP-1 cell line.** (A) TNF-α secretion measurement in ELISA. THP-1 cells were treated with 500 nM of each inhibitor 48 hours followed by 100ng/ml LPS stimulated 24 hours. Supernatants were harvested and an ELISA assay was used to measure TNF-α concentrations by ELISA kits. (B) Cellular IC50s of potential SIK2 inhibitors in THP1 cells were determined by cell viability. (C) Reported targets of AT-9283 were detected by Kinobeadspulldown assay.

### 3.3.5 SIK2 inhibition reduced TNF-α production and induced IL-10 production in HoxB8 cell line

HoxB8 cell line, which indicates important similarity with DCs,represents a unique

inflammatory characteristic in vitro model[285].In this study, SIK2-specific inhibitor HG-9-91-01 was treated with HoxB8 cell line to evaluate the role of SIK2 inhibition in immune response at first. We demonstrated that HG-9-91-01 indicates a dose-dependent inhibition in TNF-α secretion and a dose-dependent trend in increasing IL-10 secretion in the range of 5 nM to 100 nM HG-9-91-01 treatment (Figure 3.6A). In 500 nM HG-9-91-01 treatment condition, the reason of decrease in IL-10 production may be the cytotoxicity of the cell growth (cellular IC50 = 411.5 nM) (Figure 3.6B). Most SIK2 inhibitors had no effect on cell growth; only few SIK2 inhibitors demonstrated cytotoxicity in cell viability assay of HoxB8 cell line (Figure 3.6B). AT-9283, K252A, AZD-7762, and PF-3814735 inhibited cell growth with cellular IC50s of 177.5 nM, 399.7 nM, 128.8 nM, and 54.5 nM, respectively.

According to the cell viability result in HoxB8 (Figure 3.6B), 500 nM of each compound was confirmed in the evaluation of SIK2 inhibition in immune response. In the cytokine secretion assay, many SIK2 inhibitors reduced the TNF-α secretion, and few SIK2 inhibitors induced IL-10 secretion (Figure 3.6C). For TNF-α production measurement, FDA-approved BCR-Abl inhibitor Dasatinib had a significant inhibition on TNF-α secretion, and Dabrafenib, Crenolanib, and Bosutinib reduced around 50% of TNF-α secretion. Meanwhile, HG-9-91-01, AT-9283, K252A, AZD-7762, and PF-3814735 also inhibited TNF-α production significantly because of the very potent cytotoxicity on cell growth (Figure 3.6B). For IL-10 production measurement, FDA-approved drugs Bosutinib and Dabrafeinib significantly induced IL-10 secretion. Interestingly, FLT3 inhibitor Crenolanib and JAK2 inhibitor TG-101348 potently increased IL-10 production. K252A, AZD-7762, and PF-3814735 reduced IL-10 production because of the cytotoxicity on cell growth (Figure 3.6B). As an anti-inflammatory cytokine, IL-10 has been reported to switch from tumor-promoting inflammation to antitumor immunity[271]. The inhibition of SIK2 leads to the increasing of IL-10 secretion in this study, which may drive these potential SIK2 inhibitors in antitumor immunity research.

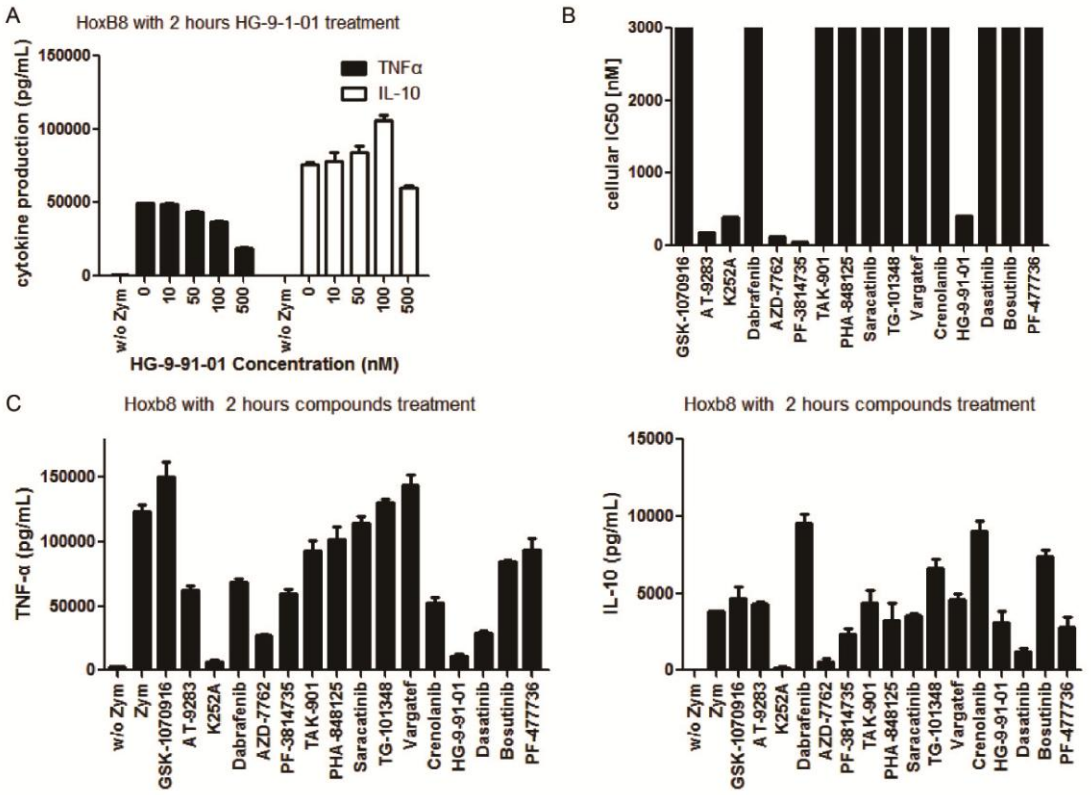


**Figure 3.6 The effect of SIK2 inhibition on TNF-α secretion and IL-10 secretion in HoxB8 cell line.** (A) TNF-α and IL-10 secretion measurement in ELISA. HoxB8 cells were treated with 10 nM to 500 nM of HG-9-91-01 48 hours followed by 4μg/ml zymosan stimulated 24 hours. Supernatants were harvested and an ELISA assay was used to measure TNF-α and IL-10 concentrations. (B) Cellular IC50s of potential SIK2 inhibitors in HoxB8 cells were determined by cell viability. (C) HoxB8 cells were treated with 500 nM of each SIK2 inhibitor 48 hours followed by 4μg/ml zymosan stimulated 24 hours. Supernatants were harvested and an ELISA assay was used to measure TNF-α and IL-10 concentrations.

### 3.3.6 SIK2 inhibition led to the decreasing of TNF-α and increasing of IL-10 in both murine and human immune system

Bone marrow dendritic cells (BMDCs), which are included in the most commonly used model in the immune system, play an important role in immune response and are essential for innate immunity to intracellular infection[286]. In this study, BMDCs were used to characterize the role of SIK2 inhibition in the murine immune system. Most SIK2 inhibitors had no effect on cell growth; only few SIK2 inhibitors demonstrated cytotoxicity in cell viability assay of BMDCs (Figure 3.7A). K252A, Dabrafenib, AZD-7762, and Dasatinib inhibited cell grow with cellular IC50s of 178.4 nM, 363.5

nM, 51.3 nM, and 102.2 nM, respectively. According to the result of cell viability assay, 500 nM of each compound was determined. In the cytokines secretion assay, many SIK2 inhibitors demonstrated the inhibition of TNF-α production, and few SIK2 inhibitors induced IL-10 secretion (Figure 3.7B). For TNF-α production measurement, AT-9283, PF-3814735, Crenolanib, and HG-9-91-01 had a significant effect on TNF-α secretion. Meanwhile, K252A, AZD-7762, and Dasatinib inhibited TNF-α production because of the cytotoxicity on BMDCs. For IL-10 production measurement, almost all of the SIK2 inhibitors slightly induced IL-10 secretion, and only Vargatef significantly induced the IL-10 secretion. Vargatef, which was designed as a PDGFR, VEGFR, and FGFR inhibitor, was approved in the treatment of NSCLC[287]. Vargatef has also shown consistent anti-inflammatory activity in animal models, and our result indicated that Vargatef significantly induced the IL-10 production in the murine system.

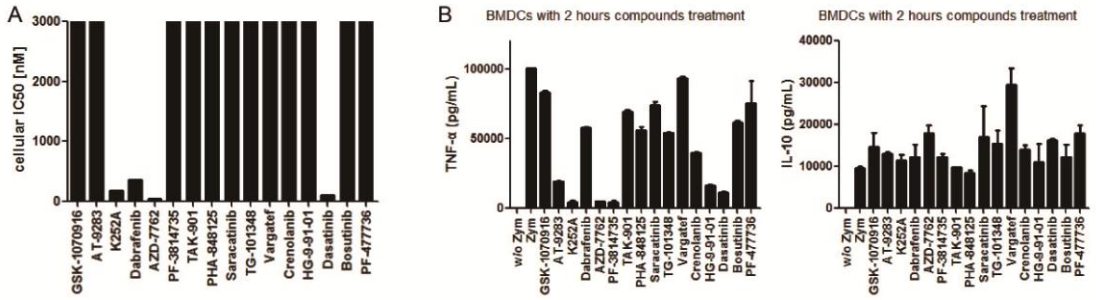


**Figure 3.7 The effect of SIK2 inhibition on TNF-α secretion and IL-10 secretion in BMDCs.** (A) Cellular IC50s of potential SIK2 inhibitors in BMDCs cells were determined by cell viability. (B) TNF-α and IL-10 secretion measurement in ELISA. BMDCs were treated with 500 nM of each SIK2 inhibitor 48 hours followed by 4μg/ml zymosan stimulated 24 hours. Supernatants were harvested and an ELISA assay was used to measure TNF-α and IL-10 concentrations.

Peripheral blood mononuclear cells (PBMCs) and human DC subsets, which are critical for the immune system functioning[288], were also used for the characterization of SIK2 inhibition. SIK2 inhibitors showed similarity in both PBMCs and human DCs in this study (Figure 3.8). For TNF-α production measurement, SIK2-specific inhibitor HG-9-91-01 was indicated to show a significant inhibition in the secretion of TNF-α. FDA-approved drug Dasatinib also obviously reduced TNF-α production. Crenolanib,

AT-9283, Dabrafenib, and PF-3814735 slightly inhibited TNF-α secretion. The inhibition of TNF-α production by K252A might be because of the cytotoxicity in PBMCs and human DCs. For IL-10 production measurement, AT-9283 significantly induced IL-10 production in PBMCs and human DCs. GSK-1070916, TAK-901, and PHA-848125 slightly induced the IL-10 production in human DCs. In general, SIK2 inhibition led to the decrease of TNF-α production, and few SIK2 inhibitors induced the IL-10 production in human immune responding model.

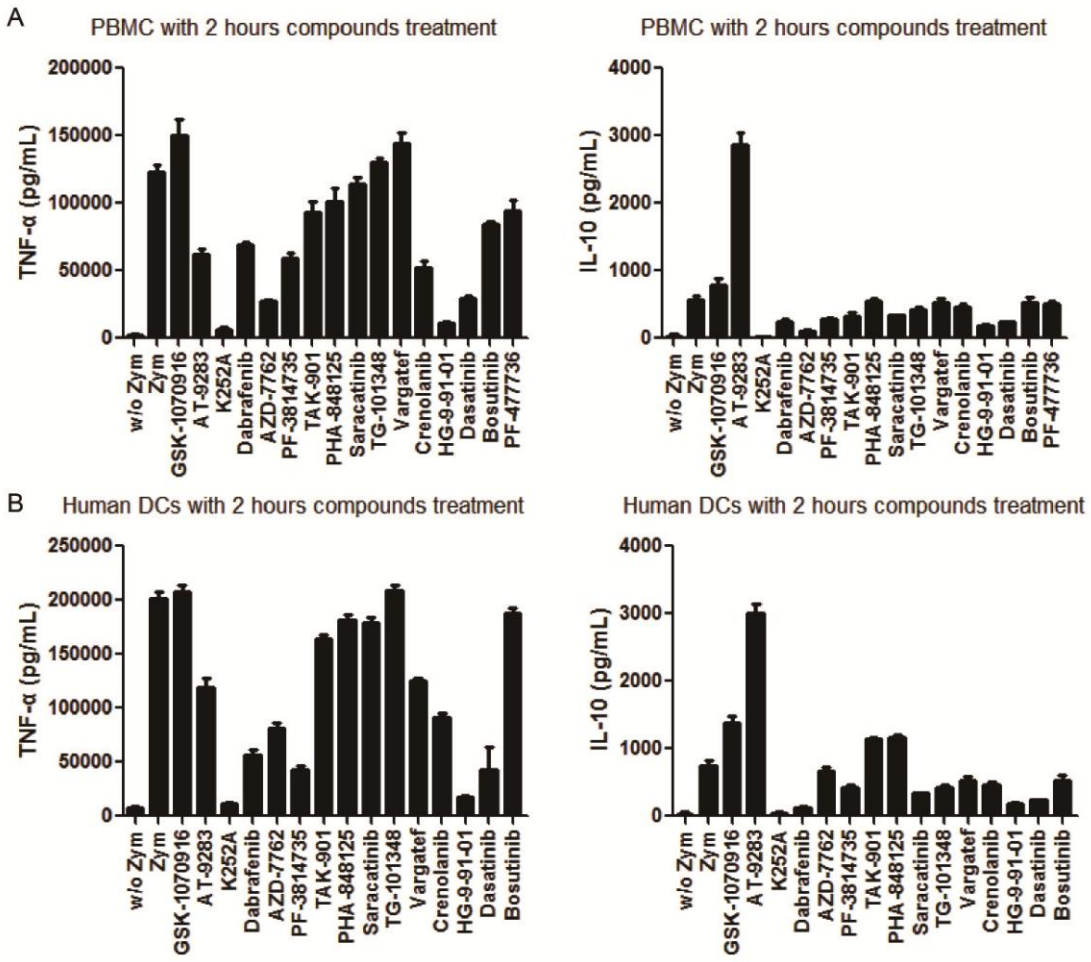


**Figure 3.8 The effect of SIK2 inhibition on TNF-α secretion and IL-10 secretion in PBMCs and human DCs.** (A) PBMCs were treated with 500 nM of each SIK2 inhibitor 48 hours followed by 4μg/ml zymosan stimulated 24 hours. Supernatants were harvested and an ELISA assay was used to measure TNF-α and IL-10 concentrations. (B) Human DCs were treated with 500 nM of each SIK2 inhibitor 48 hours followed by 4μg/ml zymosan stimulated 24 hours. Supernatants were harvested and an ELISA assay was used to measure TNF-α and IL-10 concentrations.

### 3.3.7 Knockdown of SIK2 expression by siRNA induced IL-10 production and reduced TNF-α secretion in THP-1 cell line

Owing to the fact that all of the SIK2 inhibitors were non-selective in SIK2 inhibition, even SIK2 “specific” inhibitor HG-9-91-01 had more than 20 potential targets (Figure 3.2). This can substantially complicate matters for the role of SIK2. Small interfering RNA (siRNA) was used to knockdown SIK2 expression in THP-1 cell line to characterize the role of SIK2 in immune response. In this study, SIK2 was knocked down to 50% and 60% by 5 nM and 20 nM siRNA, respectively (Figure 3.9A). SIK2 knockdown led to the decrease of TNF-α production and the increase of IL-10 production in THP-1 cell line (Figure 3.9B). For TNF-α production measurement, 50% SIK2 knockdown slightly reduced the TNF-α secretion, and 60% SIK2 knockdown observably decreased the TNF-α production. Interestingly, SIK2 knockdown had a significant effect on IL-10 production; even 60% of SIK2 knockdown led to tenfold IL-10 production. Specific SIK2 inhibition was evaluated to play an important role in the immune response by increasing the anti-inflammatory cytokine IL-10 secretion and decreasing the production of pro-inflammatory cytokine TNF-α. Thereby, development of specific SIK2 inhibitor is required in the future and the SIK2 specific inhibitor may play an important role in immune-related disorders by reducing pro-inflammatory cytokine TNF-α secretion and inducing anti-inflammatory cytokine IL-10 production.

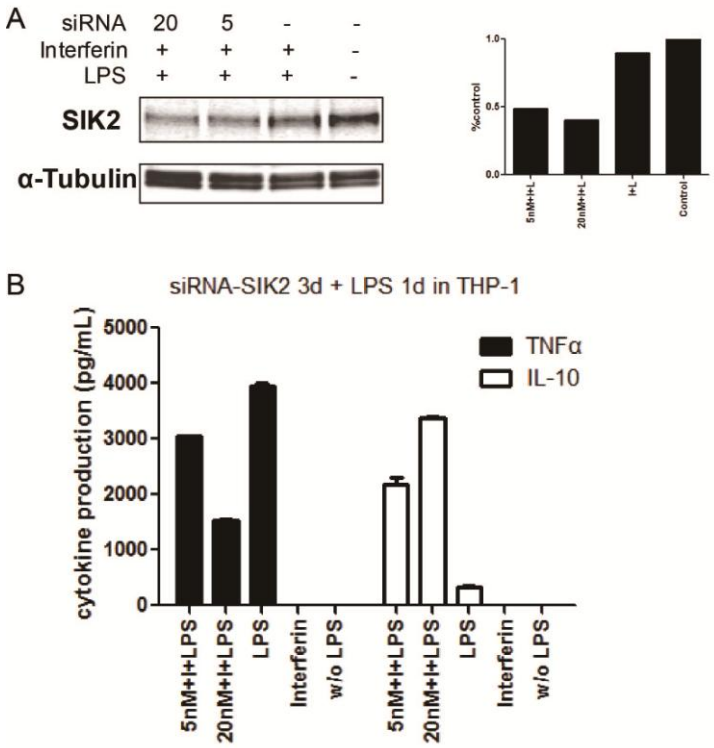


**Figure 3.9 Silencing of SIK2 expression in THP-1 cells.** (A) Knockdown of SIK2 by siRNA in THP-1 cells. Western blot was used to detect the SIK2 expression level in THP-1. (B) SiRNA reagent incubated with THP-1 for 72 hours, followed by harvest the supernatants and ELISA assay.

In summary, the SIK2 inhibition lead to the increase of IL-10 production and the decrease of TNF-α production in human and murine immune response models. Even though these cytokine secretion were analyzed just one time, these results revealed a uniform trend toward similarly reduced pro-inflammatory cytokine TNF-α and induced anti-inflammatory cytokine IL-10. These results were confidence and SIK2 was proved as a promising target in immune response. SIK2 inhibition may provide an advantage for the treatment of inflammatory and autoimmune diseases.

## 3.4 Conclusions

In this study, we evaluated the selective SIKs inhibitor HG-9-91-01, and the data demonstrated HG-9-91-01 as a “non-specific” SIKs inhibitor with more than 20 off-targets. We screened 26 small molecule kinase inhibitors containing approved drugs and inhibitors in the clinical trials that inhibit SIK2. Notably, treatment of various immune responding models (THP-1, BMDCs, PBMCs, and human DCs) induced the production of anti-inflammatory cytokine IL-10 and reduced the production of pro-inflammatory cytokine TNF-α. Six FDA-approved drugs (Bosutinib, Dasatinib, K252A, Vargatef, CEP-701, and Dabrafenib) were determined to inhibit SIK2 in the Kinobeadsassay. Dasatinib and Bosutinib were developed as BCR/ABL inhibitors for the treatment of chronic myeloid leukemia; K252A and CEP-701 were approved for the treatment of psoriasis; Vargatef was developed to work against VEGFR and was used for NSCLC patients; and Dabrafenib was designed as an anti-cancer drug for metastatic melanoma patients with BRAF (V600) mutation. Few new potential SIK2 inhibitors were also determined in this study, such as AT-9283, PF-477736, PRT062070, RGB-286638, SCH-900776, and ASP-3026.

The non-selective nature of inflammatory disease therapies, which rely heavily on glucocorticoids and/or immunosuppressants, has multiple side effects. Neutralizing antibodies were developed to inhibit the actions of particular pro-inflammatory cytokines, such as anti-TNF-α humira. TNF-α is produced primarily by activated macrophages. Pro-inflammatory cytokineTNF-α occurs a variety of chronic effects and acute pathologies.Consequently, TNF-α is involved in acute and chronic inflammatory diseases (AIDS, Crohn’s disease, rheumatoid arthritis). IL-10 is produced by many different types of immune cells (TH2, TH17, TReg, B cells, DCs, natural killer cells, and mast cells). As an anti-inflammatory cytokine, IL-10 plays an important role in the prevention of host damage by limiting the immune response, and is being exploited in clinical trials for the development of several inflammatory diseases therapies. The orally available drugs that improve the treating effect of

chronic inflammatory and autoimmune diseases have become a new hot point. SIK2 inhibition that induces anti-inflammatory cytokine production and reduces pro-inflammatory cytokine production may provide an advantage for the treatment of these diseases.

# CHAPTER IV
# General discussion

Label-free LC-MS approaches, which provide higher dynamic range of quantification, play an essential role in quantitative studies[193, 201, 289]. Compared with other protein quantification methods that use isotope labeling to make the compound chemically bind to the protein, label-free quantification has been used to directly determine the relative or absolute proteins through correlating the intact proteolytic peptides signal or counting the peptide sequencing events number with MS in two or more biological samples[199, 200]. Moreover, the significant changes of a complex mixture in a single experiment can be measured by label-free LC-MS approaches[290]. So far, the label-free approach is the most accurate mass spectrometric quantification method as it reflects all the systematic and non-systematic variations in different experimental data in consideration of the overall experiment. Kinobeads™ represents the covalent attachment of kinase inhibitors with sepharose beads to enrich the affinity of kinomes in cell lines or tissue lysates[154]. In this study, Kinobeadsin combinationwith quantitative MS was briefly used to describe the interaction of kinase inhibitors with cancer cell lines' kinome (around260 kinases). Protein kinases have become an attractive target in the development and discovery of oncology drugs because they play a crucial role in promoting cancer cell survival and proliferation[45, 291]. Currently, 33 kinase inhibitors have been approved, and numerous (>250) inhibitors are in various stages of clinical evaluation[44, 46, 134]. In this thesis, 242 small molecule kinase inhibitors including both FDA-approved anti-cancer drugs and small molecule kinase inhibitors that are in the stage of clinical trials were characterized by kinodeads competition assay, a dose-resolved quantitative chemical proteomics approach, to indicate the on and off-targets, understand the mechanism of action, and develop the novel targeted

cancer therapies.

CDKs are a family of protein kinases that play the most important role in the regulation of cell cycle transitions. CDKs have been reported to misregulate in many different human tumors[125, 128]. Over-expressionof CDK1 has been found to highly relate withovarian cancer, colorectal cancer, and breast cancer. CDK2 has been reported as a magnetic target for cancer therapies because the over-expression of CDK2 regulates cell cycle abnormality in cancer cells. CDK4/6 activation leads to the uncontrolled cell cycle in many types of cancers, especially in breast cancer[206, 207, 233].Owing to the central role of CDKs in the control of cell division, numerous drugs that target CDKs activity have emerged and have been tested in the stages of clinical trials. So far, fourteen CDK inhibitors have been developed and reported in various clinical trials,but only CDK4/6 inhibitor Palbociclib (PD-0332991, IBRANCE, Pfizer, 2015) was successfully reviewed and approved under FDA as a treatment (in combination with letrozole) for patients with estrogen receptor positive advanced breast cancer[129]. There are three key principles of the failure of non-selective CDK inhibitors. Firstly, the lack of clear understanding of the action mechanism of many low specificity CDK inhibitors, and the lack of clear recognition regarding which CDK is actually inhibited in vivo may become the corresponding mechanism of the therapeutic effect. Secondly, appropriate patient selection is another restriction,and there are no key biomarkers for the sensitive subpopulations that respond to this class of inhibitors. Thirdly, there is no suitable therapeutic window. The limitation of non-selective CDK inhibitors suggests that increase in certain CDK selectivity is important for the development of CDK inhibitors,which provides a directionfor new research.

In chapterII, 14 CDK inhibitors, which include both first- and second-generation CDK inhibitors against the maximum number of kinases, involving both CDKs and their complex partners were characterized in Kinobeadscompetition assay. As a first-generation CDK inhibitor, Alvocidib was reported to be a “pan-CDK” inhibitor, and

our result demonstrated broad CDK inhibition, including CDK1, CDK2, CDK5, CDK7, CDK9, and CDK12 with $K_D$< 1 μM (Figure 2.2A). These findings, on the other hand, validate that the Kinobeadsassay is quite effective in the research of kinase inhibitors because they are consistent with the previous studies. Moreover, Kinobeads$^{TM}$ provided an advantage in capturing CDK-cyclin complex partners, including CCNA2, CCNB1, CCNB2, CCNH, CCNI, CCNK, CCNT1, and CCNT2 compared with recombinant protein technology in small molecule drug discovery (Figure 2.2, Figure 2.7). Further screening the second-generation CDK inhibitors in Kinobeadscompetition assay, our results indicated that most CDK inhibitors were non-specific for targeting CDKs and cyclins (Figure 2.4, Figure 2.5, Figure 2.7). Since one aim of the development of new-generation CDK inhibitors is to increase the selectivity of CDK1 and CDK2, our results demonstrated that second-generation CDK inhibitors were more potent to work against CDK2, but not against CDK1 compared with Alvocidib (Figure 2.3). This may indicate the success of the second-generation CDK inhibitors. Interestingly, only FDA-approved CDK inhibitor Palbociclib was characterized to possess high selectivity in targeting CDKs in kinodeads pulldown assay (Figure 2.5). Furthermore, new CDK sub-units (CDK12/13/16/17/18), which are involved in transcription and other processes were found in the profiling of most CDK inhibitors. When classifying all CDK inhibitors that show CDK binding according to their $K_D$, almost all of CDK inhibitors functioned in cell cycle regulation(CDK1/2/4/5/6/7), transcription (CDK12/13/16/17), and other processes (CDK16/17/18) (Figure 2.8). These findings demonstrated that CDK inhibitors were involved in multiplebiological processes including cell cycle, transcription, and some other processes, and explained the failure of CDK inhibitors at the protein level. Besides the main target CDKs and cyclins of CDK inhibitors, some kinases such as GSK3A, GSK3B, casein kinase 2 complex partners CSNK2A1, CSNK2A2, CSNK2B, and EIF3J were detected as off-targets of CDK inhibitors (Figure 2.6). GSK3 families are active in cellular proliferation, migration, glucose regulation, and apoptosis, and they have been implicated in inflammation and cancer. GSK3 became a potential target in cancer therapy based on the tumor suppressor functions[249].Casein kinase is

involved in cellular processes, such as cell cycle control, DNA repair, and circadian rhythm, and casein kinase 2 is a novel player in MM, acute leukemia, and breast cancer phogenesis[248, 254]. The detection of off-targets provides strong evidence to drive some CDK inhibitors in GSK3 and casein kinase 2-associated tumor research studies. To summarize, our results characterized all the CDK inhibitors at the protein level. These findings, on one hand, indicate the success of the development of next-generation CDK inhibitors, while on the other hand, provide strong evidence to explain the failure of CDK inhibitors in the review of FDA.

Inflammation is a biological process of self-protection that occurs when body tissues suffer from harmful foreign stimuli (damaged cells,pathogens and irritants) and begin the healing process[282]. Inflammatory diseases, such as Alzheimer's disease, Crohn's disease, arthritis, and asthma, demonstrate high levels of inflammation in the connective tissues[292]. Various disadvantages of the existing drugs have been noticed in the treatment of inflammatory diseases, such as multiple side effects that result from non-selective drugs glucocorticoids and/or immunosuppressants[293] and expensive purchasing because of neutralizing antibodies[294]. Therefore, the orally available drugs that improve the treating effect of chronic inflammatory and autoimmune diseases have become a new hot point.Cancer is a group of diseases characterized by somatic cells becoming abnormal, going through out-of-control cell growth, and becoming malignant[1]. Numerous research studies have shown that inflammation is crucial in the development of cancer, and that infection, chronic irritation, and inflammation lead to many types of cancers. It is clear that inflammatory cells orchestrate the tumor microenvironment and many pro-inflammatory molecules are up-regulated to foster tumor growth in chronic inflammation[282]. Many anti-cancer drugs have been used for the treatment of inflammatory diseases because of the strong correlation between inflammation and cancer. On one hand, low dose of chemotherapy drugs is used for rheumatic or autoimmune diseases because the chemotherapy drugs reduce the production of certain products that cause the inflammatory response through inhibiting the cell reproduction[295]. On the other hand,

few small molecule kinase inhibitors have been identified to have anti-inflammatory properties. For example, Src inhibitor Dasatinib was found to inhibit immune cell activation and reduce the inflammatory responses in vitro experiment[296]. Furthermore, Dasatinib was demonstrated to show therapeutic value in arthritis and allergic asthma[297].

In chapter III, we indicated that SIK2 plays an important role in inflammatory response, especially in cytokine secretion, such as TNF-α and IL-10. In Kinobeadspulldown assay, SIK2 was detected as an off-target by screeningsmall molecule kinase inhibitors' library including both FDA-approved anti-cancer drugs and small molecule kinase inhibitors that are in the stage of clinical trials. Among these 242 inhibitors, 26 small molecule kinase inhibitors, including six FDA-approved anti-cancer drugs (Bosutinib, Dasatinib, K252A, Vargatef, CEP-701, and Dabrafenib) were detected to potently inhibit SIK2 (Table 3.1). Among these 26 SIK2 inhibitors, few new potential SIK2 inhibitors were determined in this study, such as AT-9283, PF-477736, PRT062070, RGB-286638, SCH-900776, and ASP-3026.Dasatinib and Bosutinib were developed as BCR/ABL inhibitors for the treatment of chronic myeloid leukemia; K252A and CEP-701 were approved for the treatment of psoriasis; Vargatef was developed to work against VEGFR and was used for NSCLC patients; and Dabrafenib was designed as an anti-cancer drug for metastatic melanoma patients with BRAF (V600) mutation[118, 267, 287]. In order to characterize the role of SIK2 inhibition in immune response, at first, a tool compound that showed selective SIKs inhibition in the previous study, HG-9-91-01, was characterized in Kinobeadsassay, and our result demonstrated that HG-9-91-01 was a "non-specific" SIK inhibitor with >26 targets (Figure 3.2).Notably, SIK2 inhibition had no effects on the cell survival and proliferation (Figure 3.3, Figure 3.4).These findings strongly promote SIK2 inhibitors in the immune diseases based on low cytotoxicity. Moreover, these SIK2 inhibitorswere treated in various immune responding models (THP-1, BMDCs, PBMCs, and human DCs), indicating that SIK2 inhibition induces the production of anti-inflammatory cytokine IL-10 and reduces the productionof pro-inflammatory cytokine TNF-α (Figure

3.5, Figure 3.6, Figure 3.7, Figure 3.8). Since almost all of SIK2 inhibitors indicated the non-selectivity in targeting SIK2, siRNA was used to knockdown the expression level of SIK2 in THP-1 cell line. This cytokine production result of SIK2 knockdown provided a strong evidence to prove that SIK2 inhibition leads to an increase of IL-10 and a decrease of TNF-α (Figure 3.9).

TNF-α, which is produced primary by activated macrophages, plays a crucial role in mediating the systemic inflammatory response to infection. As a pro-inflammatory cytokine, TNF-α induces inflammation and is involved in a number of human diseases, such as Alzheimer's disease, cancer, psoriasis, rheumatoid arthritis, and IBD. TNF-α inhibitors including both monoclonal antibody (infliximab, adalimumab, and golimumab) and simple molecules (xanthine derivatives, bupropion) were designed and successfully approved in the treatment of autoimmune and immune-mediated diseases[270]. Nevertheless, no small molecule kinase inhibitors have been found in anti-TNF-α therapies. Our results indicated that a number of SIK2 inhibitors suppress TNF-α secretion in many immune response models. This finding provides a potential opportunity to drive these small molecule kinase inhibitors in the treatment of TNF-α-associated immune disease.Anti-inflammatory cytokine IL-10 is produced by many different types of immune cells (TH2, TH17, TReg, B cells, DCs, natural killer cells, and mast cells). IL-10 plays an important role inimmune-regulation and inflammation[271]. Additionally, IL-10 is also involved in increasing B cell survival, blocking NF-κB activity, and regulating JAK-STAT signaling pathway[284]. Moreover, IL-10 suppresses the secretion of pro-inflammatory cytokines, including IL-2, TNF-α, IL-3, IFN-γ, and GM-CSF[298]. Recent studies have shown that recombinant human IL-10 was reviewed in patients with autoimmune diseases in various stages of clinical trials. IL-10 therapy exhibited promising effect in psoriasis, but it had no significant effect in rheumatoid arthritis or Crohn's disease[299]. A mice study showed that IL-10 counteracted the inflammatory effect in the allergic reaction[300]. In summary, IL-10 has been exploited in clinical trials for the development of therapies of several inflammatory diseases based on these features of IL-10. In our study, few small

molecule kinase inhibitors (Dabrafenib, Crenolanib, AT-9283, Bosutinib, vargataf, AT-9283) that showed SIK2 inhibition were detected to particularly induce the IL-10 secretion. These findings may drive these SIK2 inhibitors in the research of some autoimmune diseases, such as psoriasis.

In summary, this study indicated that SIK2 inhibition is a key regulator in the cytokine secretion,which particularly enhances the production of IL-10 and reduces the secretion of TNF-α. Anti-TNF-α therapy and IL-10 therapy have been demonstrated to be exploited in various clinical trials for the development of therapies of several inflammatory diseases. Nevertheless, no small molecule kinase inhibitor has shown therapeutic benefits in these therapies. This study provides strong evidence to drive these SIK2 small molecule kinase inhibitors into research of anti-immune diseases.

# Abbreviations

| | |
|---|---|
| Abl | Abelson murine leukemia viral oncogene homolog |
| ABP | Affinity-based probe |
| ABPP | Activity-based probe profiling |
| AGCK | PKA, PKC, and PKG kinases |
| Akt | Protein kinase B |
| ALK | Anaplastic lymphoma kinase |
| AMP | Adenosine-monophosphate |
| AMPK | AMP-activated protein kinase |
| BCR | Breakpoint cluster region protein |
| BMDC | Bone-marrow–derived dendritic cell |
| CaMK | Calcium/calmodulin-dependent protein kinases |
| CCCP | Compound-centric chemical proteomics |
| CDK | Cyclin-dependent kinase |
| CK1 | Casein kinase 1 |
| CLK1 | CDC-like kinase 1 |
| c-Met | Tyrosine-protein kinase Met |
| CML | Chronic myeloid leukemia |
| CREB | CAMP response element-binding protein |
| DC | Dendritic cell |
| DFG | Asp-Phe-Gly |
| DFSP | Dermatofibrosarcoma protuberans |
| EGFR | Epidermal growth factor receptor |
| EI | Electron ionization |
| EPO | Erythropoietin |
| ERK | Extracellular signal-regulated kinases |
| ESI | Electrospray ionization |
| FDA | Food and Drug Administration |
| FDR | False discovery rate |

| | |
|---|---|
| GH | Growth hormone |
| GIST | Gastrointestinal stromal tumors |
| HA | Hemagglutinin |
| HCD | Higher energy collisional dissociation |
| HER-2 | Human epidermal growth factor receptor 2 |
| HGF | Hepatocyte growth factor |
| HPLC | High pressure liquid chromatography |
| IC50 | Half maximal inhibitory concentration |
| ICAT | Isotope-coded affinity tagging |
| IL-10 | Interleukin 10 |
| iTRAQ | Isobaric tags for relative and absolute quantitation |
| JAK | Janus kinases |
| LFQ | Label free quantification |
| LTK | Leukocyte tyrosine kinase |
| MALDI | Matrix-assisted laser desorption/ionization |
| MAPK | Mitogen-activated protein kinase |
| MEK | Mitogen-activated protein kinase kinase |
| MoA | Modes of action |
| MS | Mass spectrometry |
| mTOR | Mammalian target of rapamycin |
| NSCLC | Non-small cell lung cancer |
| PBMC | Peripheral blood mononuclear cell |
| PI3K | Phosphatidylinositol-4,5-bisphosphate 3-kinase |
| PRL | Prolactin |
| P-TEFb | Positive transcription elongation factor b |
| PTM | Post-translational modification |
| Rb | Retinoblastoma protein |
| SIK2 | Salt-inducible kinase |
| SILAC | Amino acids in cell culture |

| | |
|---|---|
| siRNA | Small interfering RNA |
| STAT | Signal transducer and activator of transcription |
| STE | Homologs of yeast Sterile 7, Sterile 11, and Sterile 20 kinases |
| TCI | Targeted covalent inhibitor |
| TK | Tyrosine kinases |
| TKI | Tyrosine kinase inhibitor |
| TKL | Tyrosine kinase-like group of kinases |
| TNF-α | Tumor necrosis factor alpha |
| TORC | CREB-specific coactivator |
| TPO | Thrombopoietin |
| TYK2 | Tyrosine kinase 2 |
| VEGFR | Vascular endothelial growth factor receptor |

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# List of Publications

Medard, G., Pachl, F., Ruprecht, B., Klaeger, S., Heinzlmeir, S., Helm, D., **Qiao, H.**, Ku, X., Wilhelm, M., Kuehne, T., Wu, Z., Dittmann, A., Hopf, C., Kramer, K. &Kuster, B. Optimized chemical proteomics assay for kinase inhibitor profiling. Journal of proteome research 14, 1574-1586 (2015).

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