5-2021

Biochemical Characterization of Small Molecule Inhibitor Binding on a Ras Related GTPase and its Effector Interactions

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Biochemical Characterization of Small Molecule Inhibitor Binding on a Ras Related GTPase and its Effector Interactions

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

by

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Hendrix College
Bachelor of Arts in Biochemistry and Molecular Biology, 2012

May 2021
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

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Abstract

The Ras superfamily of GTPases has 167 proteins that are involved in various cellular processes such as proliferation, transformation, migration, and inhibition of cell death. Mutations, abnormal expression, and function of these proteins are observed in many diseases, including several forms of cancer. Even though these GTPases were among the first discovered oncogenes, no successful Ras drug candidate has successfully passed clinical trials. Drugs targeting these proteins have failed mainly because of the complexity of their regulation, their high affinity to GTP, and their structure's dynamic nature. Recently, novel promising targeting approaches have renewed interest in the Ras drug discovery field. One such approach uses small molecules to bind to small pockets on the protein rendering it unable to operate and bind to effectors. In this study, inhibition of Cdc42, a Ras-related GTPase in the Rho subfamily, was investigated. Using various computational, biophysical, and biochemical assays, we characterized the binding of a novel inhibitor to Cdc42. The effects of this inhibitor to Cdc42 protein-protein interactions (PPIs) were also investigated. Results show that the small molecule binds and stabilizes Cdc42 making it more resistant to denaturation. It also affects the protein's hydrolysis activity and binding to PAK1, a crucial Cdc42 effector. A method to efficiently isolate and purify a PAK1 derived peptide was also developed. The results from these experiments show that ZCL278 is an effective Cdc42 inhibitor. However, the low effects of this inhibitor and low solubility in water make it a less attractive drug candidate. Using the results from this study, more soluble, ZCL278-like small molecules with better drug parameters are presented here as potential Cdc42 inhibitors. These small molecules will serve as candidates for investigating and developing better inhibitors of Cdc42-induced abnormal cell signaling.

Acknowledgements

Without the guidance and support of Dr. Adams, this work would not be possible. I want to express my gratitude to him for being my mentor and for guiding me throughout this project. I also want to thank Dr. Kumar for his advice and support. The help of the members of Dr. Adams and Dr. Kumar's labs was also crucial for the success of this project.

The technician staff, KZ and Zay have fixed almost all machines and instruments used in these studies. Without their hard work and willingness to help, experiments performed in this study would have taken longer or not been possible at all. I also want to thank Dr. Mazzanti for helping me to be a better science teacher and communicator.

Throughout this work, the help and encouragement my friends and family are what pushed me to persevere and see it through. I am very thankful for everybody's support and I would not be where I am without you.

Finally, I want to thank my committee members for taking time to discuss my research and teaching me a lot about biochemistry, cell, and molecular biochemistry through classes and in person discussions. I have learned a lot from you and will use your lessons as guidance for my career.

This work is dedicated all scientists, engineers, artists, and innovators. Those with a curious mind, a hunger to learn more, and a desire to solve problems.

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List of Abbreviations

Ras: Rat Sarcoma

Cdc42: Cell division cycle 42

PPI: Protein-Protein Interactions

GEF: Guanine nucleotide Exchange Factors

GTP: Guanosine triphosphate

GDP: Guanosine diphosphate

Kd: Dissociation Constant

GAP: GTPase-Activating Protein

GDI: Guanine Dissociation Inhibitor

DBS: Diazepam binding inhibitor

AZA197: N2-[4-Diethylamino-1-methyl-butyl]-N4-[2-[1H-indol-3-yl]-ethyl]-6-methyl-pyrimidine-2,4-diamine

ZCL278: 4-[3-[2-[4-Bromo-2-chloro-phenoxy]-acetyl]-thioureido]-N-[4,6-dimethyl-pyrimidin-2-yl] benzenesulfonamide

Rho: Ras Homolog

CRIB: Cdc42/Rac interactive binding motif

PAK: p21-activated kinase

ACK: activated Cdc42-associated kinase

WASP: Wiskott–Aldrich syndrome proteins

MD: Molecular Dynamics

MDS: Molecular Dynamics Simulations

FL: Fluorescence

CD: Circular Dichroism

ITC: Isothermal Titration Calorimetry

DSC: Differential Scanning Calorimetry

MOE: Molecular operating environment

PDB: Protein data bank

PBD46: Protein binding domain 46

HSQC: Heteronuclear single quantum coherence spectroscopy

ADMET: absorption, distribution, metabolism, and excretion

sNBD: (succidinimidyl 6-[(7-nitrobenz-2- oxa-1,3-diazol-4-yl)amino]hexanoate).

Chapter I. Introduction

I.1 The Ras Family of GTPases

I.1.A. Introduction and History

It has been 40 years since scientists identified Ras GTPases as the first human oncogenes. The discovery of these proteins stemmed from extensive studies of transforming viruses isolated from sarcomas of rats, chickens, turkeys, and other animals. [1][2] The researchers found that viruses isolated from these animals could cause sarcomas and transform cells in culture. [3] Among the viruses studied, the Harvey and Kirsten murine sarcoma viruses showed high oncogenic potential. The study of their genetic material provided insight into how these genes contribute to various sarcomas in animals. The retroviral oncogenes from these viruses were originally named *src* (short for sarcoma). Due to their ability to cause Rat Sarcomas, they would later be named Ras (HRas for the Harvey murine sarcoma genes and KRas for the Kirsten murine sarcoma). [2][3][4]

The discovery of the Ras GTPase as the first oncogene came from a series of findings from various labs around the world. In 1976, Varmus and colleagues discovered that the viral *src* oncogene was a normal chicken gene transformed by the virus into an oncogenic agent. This was the first discovery that showed oncogenic potential of the normal mammalian genes. [5] In the late 1970s and early 1980s, several studies explored and confirmed the cellular origin of the HRas and KRas viral genes. [6][7] In 1982, Der and colleagues discovered that the Ras genes of human bladder and lung carcinoma cell lines were similar to the Ras genes in Harvey and Kirsten sarcoma viruses. [2][8] Further research showed that various retroviral oncogenes were present in the genomic DNA of mammals. [9][10][11][12] This discovery of the first human oncogene advanced the understanding of cancer in general. Since then, hundreds of crucial oncogenes have been discovered, studied, and successfully targeted in patients with various tumors.

Further studies of Ras GTPases showed that the viral and mammal oncogenes encoded a 21-kilodalton (kDa) protein that was membrane associated and bound to GDP/GTP. [13][14][15][16] They also found that the overexpression of these proteins could transform cells and that GTP hydrolysis was involved in the transformation. [17] [18] Further studies discovered the first GTPase-activating protein (GAP) for Ras GTPases in 1978. The next year, de Vos and colleagues unveiled the first Ras crystal structure. In 1989, Casey and colleagues discovered that Ras is isoprenylated. In 1990, Goldstein, Brown and colleagues identified the first farnesyl transferase and Wolfman, Macara, and colleagues discovered the first guanosine exchange factor (GEF) for Ras GTPases. [1][2] After the identification of Ras proteins in various types of cancer, interest in understanding their GTPase mechanism and involvement increased. Further research showed that the Ras GTPases are among the most important genes in mammals and are found in more than a quarter of human cancers. They play an important role in the growth and metastasis of these diseases. [1][19][20][21][22] Ras proteins are also involved in developmental diseases and neurodegenerative disorders such as Alzheimer's. [23][24]

Research on Ras GTPases has grown quickly in recent years. Ras's function, structure, and mode of action in different pathways have been extensively studied. [22][25] Many more GTPases with similar qualities were discovered and grouped in a superfamily of Ras proteins, comprised of multiple proteins with important features and roles in cells and disease such as proliferation, growth, survival, transformation, and migration. [26][27][28][29][30] Because of the involvement of Ras GTPases in cancer, proteins in the Ras superfamily have garnered therapeutic potential and interest. However, most of the approaches that target Ras proteins for therapeutic purposes have been unsuccessful. [31][32]

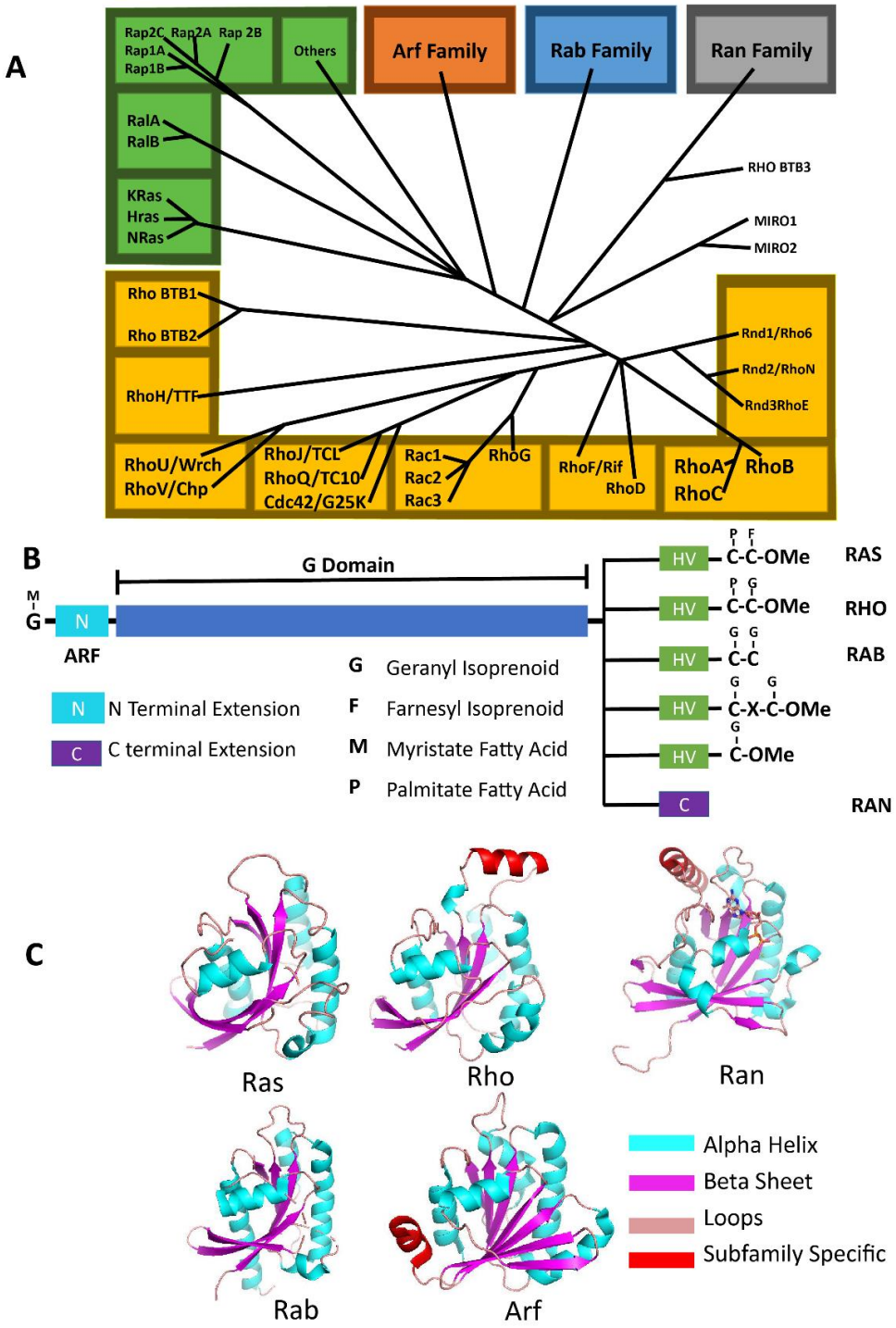

Figure 1:An overview of the Ras Superfamily. A. The entomology of the 5 families in the Ras superfamily. Ras family in green and Rho in Yellow. B. Post translational modifications of proteins in the Ras superfamily. C. Structural Differences between the families in the Ras superfamily. Figure adapted from [33]

I.1.B. The Ras Superfamily

Ras GTPases are grouped into a superfamily of over 167 proteins in humans. [34] These proteins have a similar core G domain but differ in structure, cellular localization, and function in the cell. They are divided into five families—Ras, Rho, Rab, Arf, and Ran. Comparison studies of the superfamily have shown that these proteins have a 30 to 55% homology. [35][36] The defining structural and functional features for each family are discussed below and are illustrated in Figure 1.

As the founding members of the superfamily, the Ras family is the most studied and best understood. [16] These proteins play a role in a multitude of cellular functions, including cell proliferation and survival. They were the first discovered human oncogenes and are involved in many types of cancer. The Ras family contains about 36 members in humans. [12][34][37]

The Rho (Ras homologous) GTPases are also well studied. They are the second-largest family and are comprised of 26 different members grouped in 8 subfamilies. These small GTPases share 30% homology with the Ras family. [38][39] In this group, there is an insert between α4 and β4 known as the "Rho insert". This 12-residue domain is responsible for effector binding and Rho-induced cell transformation. [40] The Rho GTPases are involved in various cell processes such as cytoskeletal organization, cell polarity, proliferation, and apoptosis. [41]

The Rab (Ras related in brain) and Arf (ADP-ribosylation co-factor) families are mainly responsible for cell trafficking. They are involved in all the events of vesicle formation and trafficking (budding, transport, tethering, and fusion). [42][43]

Ran (Ras-related nuclear protein) GTPases are different from the other GTPases. They do not undergo the usual post-translational modifications and are not anchored in the membrane.

These proteins are mostly found in the nucleus and play a role in nuclear transport and mitosis. [40][44][45][46] Structurally, members of the Ran family have a C-terminal helix extension. [47] The Ras superfamily proteins are directly responsible for many processes in the cell—and through the multitude of proteins and pathways they regulate, play a role in most other cellular processes. Researchers have, therefore, been considering the therapeutic potential of the Ras superfamily proteins ever since their discovery. Moving forward, it is important to investigate these proteins' function, regulation, and involvement in various diseases. An interdisciplinary, worldwide effort is currently underway to find the first direct Ras GTPase inhibitors that can successfully treat cancer patients in the clinic. [27]

I.1.C. Structure of Ras GTPases

The first X-Ray crystallography structures of a Ras GTPase were characterized by de Vos et al. [48] The structure of active HRas bound to a nonhydrolyzable GTP analog was also published. [49] Both structures showed that Ras GTPases had six stranded beta sheets and five alpha helices. Analysis of Ras structure revealed a common functional GDP/GTP-binding (G) domain. [50] The G domain (residues 5 to 166 in HRas) contains five G motifs (G1-G5) and is present in all Ras GTPases. This domain is also characteristic of a wider group of G proteins that include transition factors, the dynamin superfamily, SRP, septins, and heteromeric G proteins. [51][52] It is responsible for the proteins' cycling between GTP and GDP-bound forms. The five G motifs work together to bind and stabilize the β-phosphate, the magnesium ion, and the guanine base (Figure 2). [53][54][55][56]

Structural studies show that binding of the GTP nucleotide induces a conformational change (called the "loaded spring" mechanism) in the Switch 1 and Switch 2 regions. The γ-phosphate of the GTP causes Switch 1 and 2 to move close to the nucleotide, this γ-phosphate of

GTP, Thr35 in Switch I, and Gly60 and Gln61 in Switch II all work to stabilize the conformation. After GTP hydrolysis, the conformation is changed to an open position and those interactions are no longer present (Figure 2). [50][51][57]

Other important structural features of Ras GTPases are added later in post-translational modifications. The Ras GTPases are modified at their carboxyl terminal after translation before they are anchored into the membrane. This modification takes place at the cysteine residue of the CAAX motif at the C terminus and involves the addition of a lipophilic group such as palmitoyl, farnesyl, geranylgeranyl, or myristoyl. Subsequently, the AAX part of the motif is removed and replaced by a carboxymethyl group. It is only after this modification that the protein can be anchored in the membrane (Figure 4). The C-terminal CAAX motif serves as the site for post-translational modifications such as phosphorylation, acetylation, ubiquitination, farnesylation, geranylgeranylation, palmitoylation, and carboxyl methylation that facilitate their membrane anchorage and thus, their cellular localization. [58] This hypervariable region also functions as a protein binding site, determining specific binding to regulatory or effector proteins. [59]

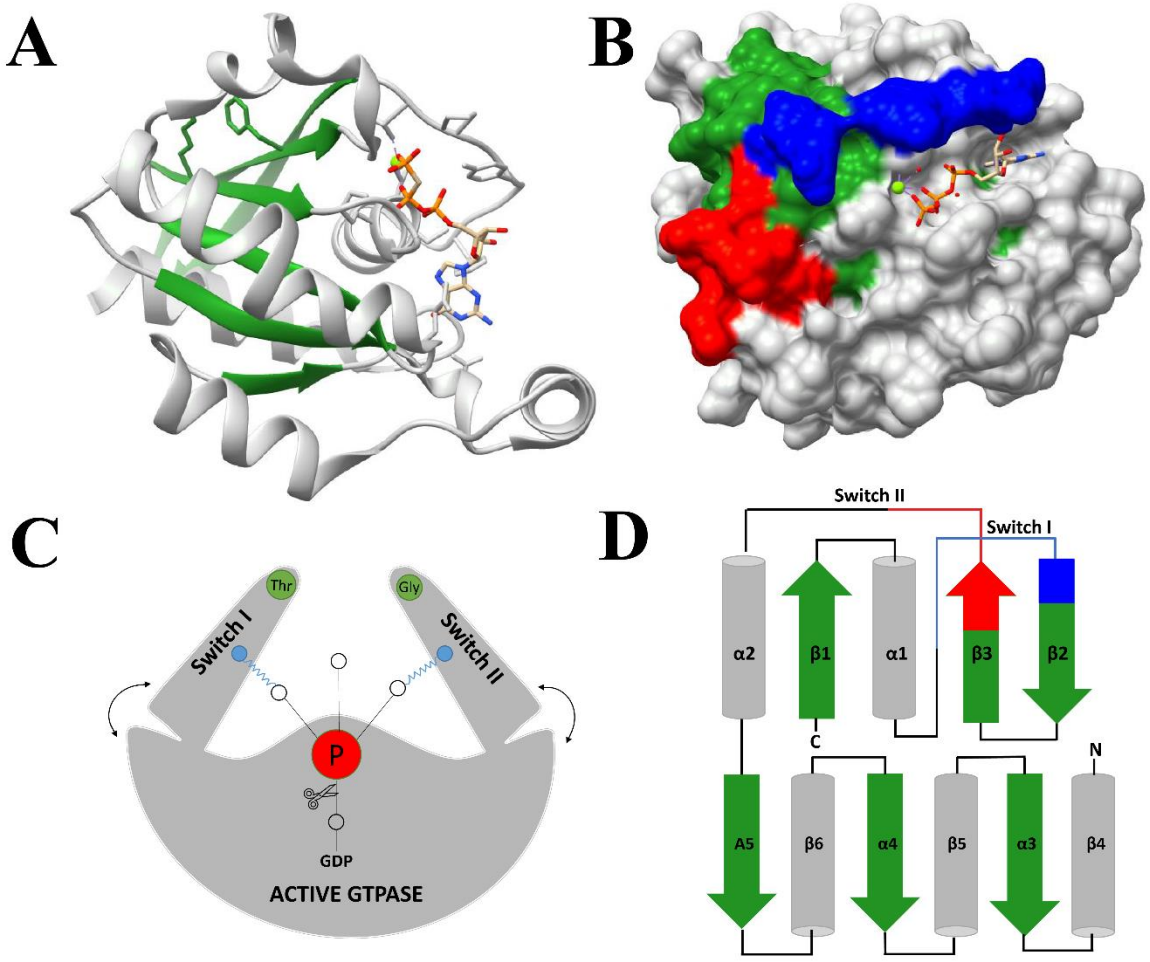

Figure 2: Features of Ras Structure. A. Overall Ras structure. PDB: 121P. Green represents beta sheets while grey represents alpha helices B. Surface Structure of Ras GTPases. Blue Represents the Switch I region and Red represents the Switch 2 region. C. The schematic diagram of the canonical switch mechanism. This is a Ras GTPase abound to GTP showing the relationship between the GTPase switch regions and the nucleotide. [33] D. Structural features of Ras GTPases. Green represents the green represents beta sheets while grey represents alpha helices. Blue Represents the Switch I region and Red represents the Switch 2 region.

I.1.D. Ras Regulation

Ras GTPases are complex, well-regulated proteins. Some GTPases can have up to 20 regulators, and some individual regulators affect several types of GTPases. [60] These regulators are responsible for the Ras GTPase Switch functions. They switch Ras on or off by regulating the protein's hydrolysis and exchange of GTP. Since Ras's intrinsic GTP hydrolysis is poor and the protein's affinity for GTP and GDP is high, the hydrolysis and exchange of these nucleotides needs to be assisted by external factors. In addition to diseased cell states resulting from GTPase variants, mutations in the regulators can contribute to constitutively active GTPases. [61] The various GTPase regulators are discussed below:

Guanine Exchange Factors (GEFs)

Ras GTPases have a high affinity for GTP and GDP nucleotides (low nanomolar to picomolar range, K_d = 10 pM for Ras). In addition, the cellular concentrations of GTP and GDP are high (0.01 mM for GTP and 0.1 mM for GDP). [62] Consequently, this saturation greatly slows down Ras's nucleotide exchange. [60][61] In order to speed up the GTP exchange, the guanine nucleotide exchange factors (GEFs) are needed. These regulators increase the GTP/GDP exchange rate by lowering Ras's affinity for the nucleotides. The GEFs increase GTP/GDP dissociation by up to several orders of magnitude. [60][61]

To increase the rate of GDP dissociation, the GEFs induce changes to the phosphate- and magnesium-binding regions (G1/P-loop, G2/Switch 1, and G3/Switch 2). In these regions, the GEFs induce steric occlusion on phosphate and magnesium binding regions which repulses the magnesium and phosphates. They also stabilize the empty GTPase structure after the nucleotide is removed (Figure 4). [60][61]

GTPase Activating Proteins (GAPs)

Even though Ras proteins can hydrolyze GTP, the intrinsic GTP hydrolysis is very slow in most of the Ras GTPases (10^{-4} to 10^{-5} s^{-1}). [63] Since GTP hydrolysis is needed to return the protein from the active GTP-bound state to the inactive GDP-bound state, a regulator is needed to facilitate the reaction. For the hydrolysis rate to reach functional levels, the activity is stimulated by GTPase activating proteins (GAPs). These proteins can increase the GTP hydrolysis rate by up to 1000-fold (1 to 10 s^{-1}). Most of the GAPs in the Ras family vary in sequence and structure but Ras and Rho GAPs share structural similarities. GAPs increase GTP hydrolysis in three steps. First, they polarize and orient the water molecule for the nucleotide attack on the gamma phosphate. Then they occlude the water from the catalytic site. Lastly, they stabilize the transition state (Figure 4). [61]

Guanosine Dissociation Inhibitors

Another class of small GTPase regulators is guanine nucleotide dissociation inhibitors (GDIs). The GDIs are only present in Rad and Rho family GTPases. These regulators bind to the prenylated GDP-bound forms of the proteins. When bound, the GDIs sequester the inactive GTPase in the cytosol. This prevents the protein's activation and interaction with effectors. GDIs also protect the lipid group of the GTPase from the aqueous environment (Figure 4). [61][64]

Post-Translational Modification and Membrane Localization

A major regulation factor in the Ras GTPase cycle involves post-translational modification and membrane localization. After the discovery of Ras in the late 1970s, researchers soon found out that they require post-translational modifications in order to localize to the plasma membrane. [65][66][67] Further studies showed that members of all five families undergo post-translational modification and membrane localization except Ran. This modification involves the addition of

an isoprenoid group (prenylation), palmitoyl group (palmitoylation), or a myristoyl group (myristoylation). [68][69][70] This modification facilitates the protein-membrane anchoring and is crucial for the function and regulation of small GTPases. [71][72] In the Rho and Rab GTPases, membrane anchoring also affects the interaction with GDIs (Figure 4). [61][64]

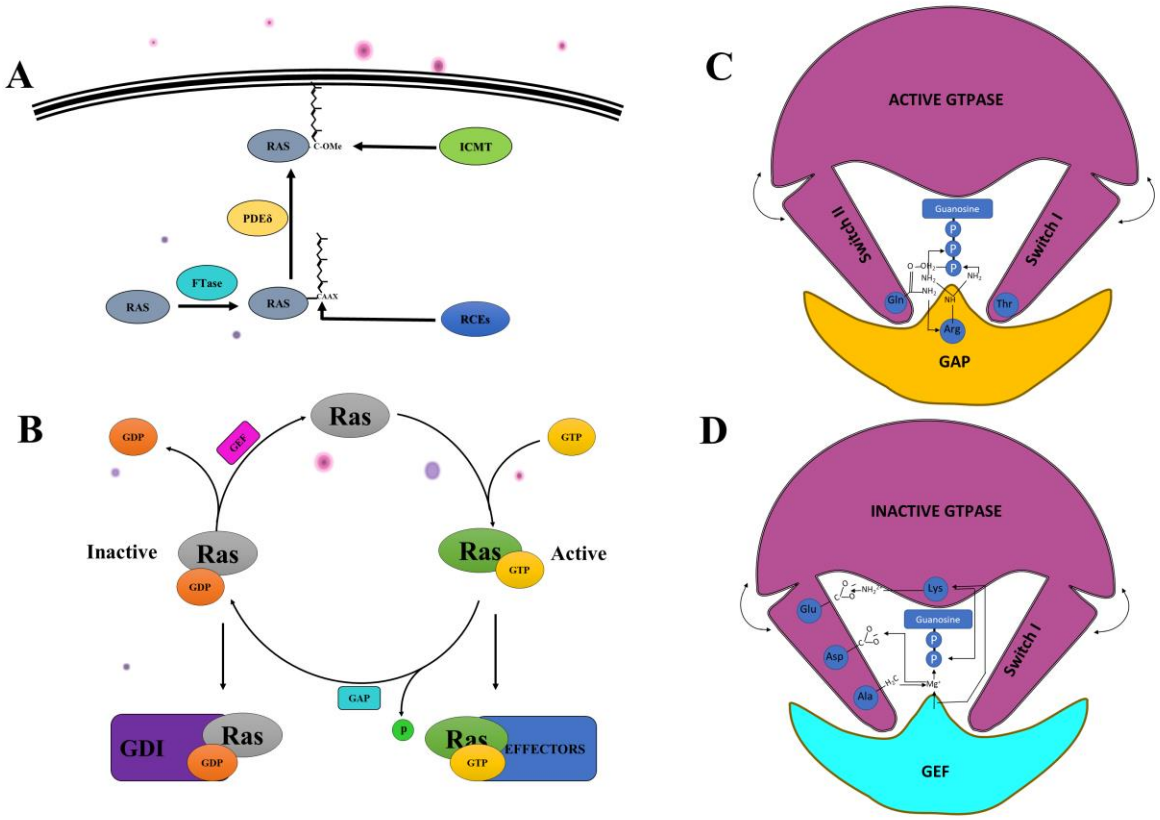

Figure 3. Regulation of Ras GTPases. A. A general overview of the GTPase cycle. B. Post translational steps involved in anchoring the GTPase to the membrane. C. Mechanism of the binding of the GAP to the GTPase. D. Mechanism for GEF binding to the GTPase. Figure adapted from [33][73] and [74]

I.1.E. Ras GTPases and Disease

Due to their role in major cell processes, abnormalities in the Ras superfamily GTPases are present in more than 30% of human cancers. [75][76] In addition, they are also involved in various other diseases such as developmental and neurodegenerative diseases. [23][77] The Ras superfamily–related disease states are mainly the result of mutations. In Ras oncogenes, the most

frequently mutated residues are at 12, 13, and 61. [75] The oncogenic mutations render the GTPase domain unresponsive to GTP hydrolysis and the protein stays locked in the active position. Some mutations in the Ras superfamily are found at higher incidences in certain cancer types. For example, high levels of mutated Ras are present in the cancers of the pancreas (90%), the colon (50%), the lung (30%), and thyroid (50%). Mutations in other Ras superfamily members are also observed in many types of cancer. [75][76]

Ras GTPase–related diseases can also be caused by improper activation and regulation. GAPs and GEFs are responsible for GTP hydrolysis and exchange, and the actions of these regulators keep the protein in the active and inactive states when needed. A malfunction in the GAP or GEF will affect the GTPase activation cycle. For example, the tumor suppressor gene NF1 encodes a GAP for the Ras GTPase. A mutation in NF1 causes neurofibromatosis. When NF1 is mutated, Ras is permanently activated and keeps on activating its effectors. Furthermore, Ras-related malignancies can also be caused by proteins upstream of Ras in different pathways. One example is the growth receptor ErbB; it is located upstream of the Ras GTPase and when it is constitutively activated, Ras will be as well. [78]

In addition to mutations and regulators, Ras-related cancers are also caused by abnormal expression. In some Ras superfamily–related cancers, there are no detectable Ras mutations—but expression studies show that some members of the Ras superfamily are overexpressed in cancer tissues. This overexpression induces an overactivation of downstream effectors of these proteins and that leads to diseased cell states. One example is the overexpression of Rho GTPases observed in colon and breast cancer tissues. [79][80]

Proteins in the Ras superfamily are crucial for cell development, growth, division, and apoptosis. Hence, their mutations, overexpression, and dysregulation; are found in many other

diseases in addition to cancer. For example, Ras superfamily members play a role in neural growth and development; their abnormal expressions and mutations are found in various neurodegenerative and developmental disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis. [23][77]

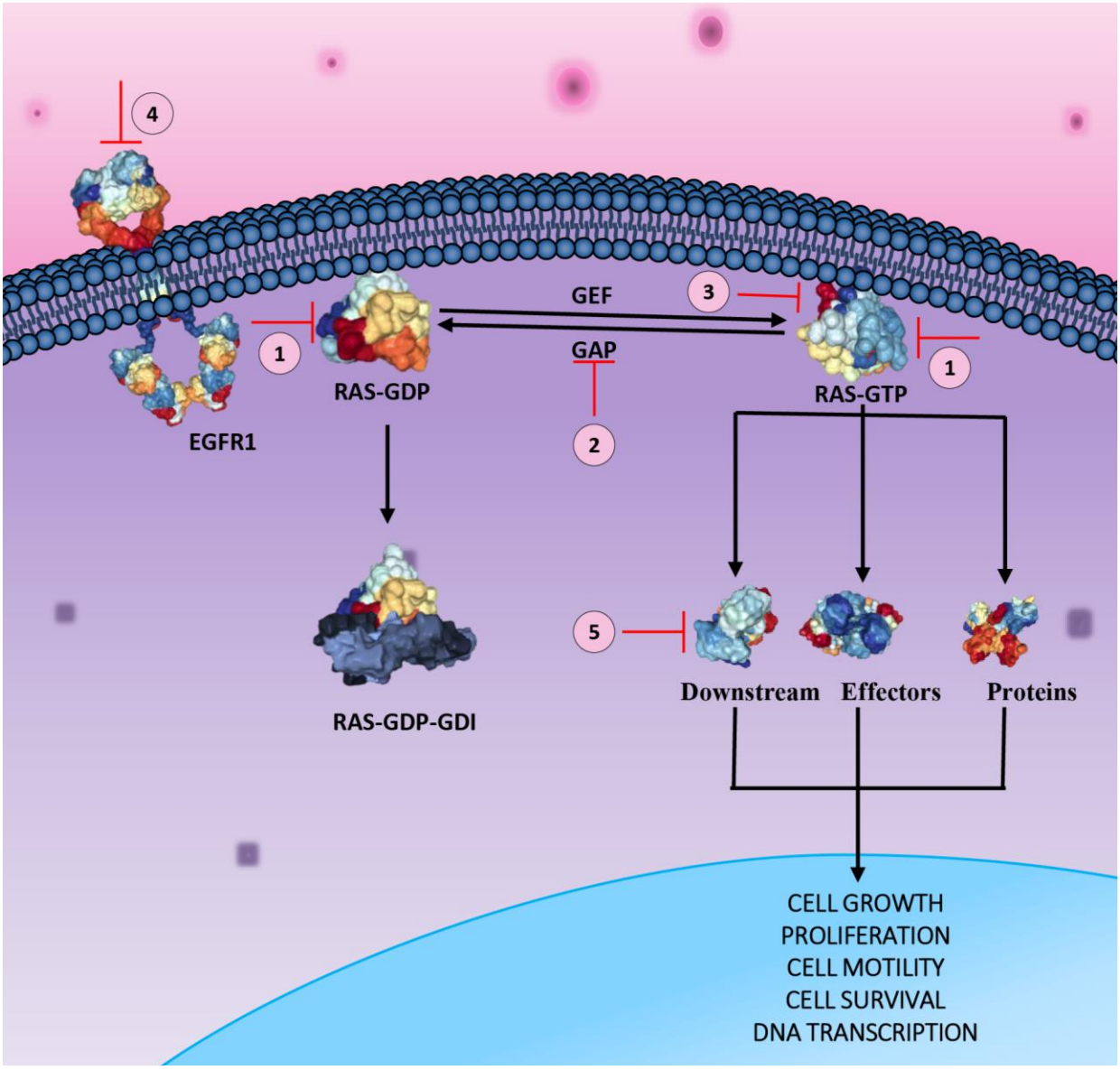

Figure 4. Ras GTPase drug discovery Targets. 1. Direct binding of the drug to the protein. 2. Binding of the drug to regulators like GEFs. 3. Drugs that disrupt the protein's anchoring to the membrane. 4. Drugs that target the proteins upstream of Ras signaling. 5. Drugs that target proteins downstream of Ras signaling. Figure adapted from [81]

I.1.F. Targeting Ras GTPases in Cancer

Since the discovery of Ras GTPases as the first oncogenes in the early 1980s, scientists have been trying to target them with small molecules and macromolecules. [32][82] Drug discovery approaches have targeted all the types of interactions in the Ras signal transduction pathway. Figure 6 illustrates six different mechanisms used to target Ras proteins. First, scientists directly target the GTP binding site, then the GEF and GAP interactions. Next, they target Ras post-translational modification and membrane-anchoring processes. Finally, direct interactions with other effectors can be targeted as well. (Figure 4). [31] In recent years, computer-aided drug discovery, high-throughput screening, shared small-molecule libraries, and abundant tertiary structures have greatly advanced Ras GTPase drug discovery efforts. Over the years, scientists have used many compounds to target Ras—small molecules, cyclic peptides, miRNAs, antibodies, T-cell receptors, and siRNAs. Small molecules have been the most successful and most explored candidates for targeting Ras mutations. [31][32]

Compared to other human oncogenes, Ras GTPases are harder to target. Kinases, for example, are important human oncogenes responsible for signal transduction in multiple pathways that leads to various types of tumors. There are now approximately 30 approved drugs on the market targeting kinases, while Ras proteins have none. [83] There are a few differences between kinases and Ras GTPases that make Ras GTPases harder to target. Kinases bind to ATP while GTPases bind to GTP. The affinity of GTPases for GTP is much higher than kinases' affinity for ATP. [62][83][84] The high affinity for GTP makes finding a competitive Ras GTPase inhibitor much more difficult. Structurally, few or no pockets for binding small molecules are present on Ras GTPases, compared with kinases. Another reason for difficulty in Ras GTPase targeting is the regulation of their cycle. Kinases are autoregulated by their own domains. Ras GTPases on the

other hand, are regulated by various upstream and downstream effectors (GEFs, GAPs, GDIs, etc.) This regulation increases the complexity of Ras signaling and decreases effective inhibition. In summary, a high affinity for GTP, high dynamic structure, lack of binding pockets and a complex regulation cycle all make Ras GTPases harder to target. [31][85]

The first approach used to target aberrant Ras GTPases was to disrupt its post-translational modifications. The first two drugs, lonafarnib and tipifarnib, to enter clinical trials targeting these GTPases inhibited their post-translational modification by targeting farnesyl transferases. Unfortunately, the drugs failed in clinical trials since they didn't provide effective treatment and had severe side effects. [86][87][88][89][90] Another approach explored direct inhibition of Ras-GTP binding. But since the binding affinity of GTPases towards GTP and GDP is very high (In the picomolar range), the design of a GTPase competitive small molecule inhibitor is difficult. [84][62] Several small molecules targeting GTP binding have been discovered but none of them has been effective in treating Ras GTPase related diseases.

Another approach to block Ras GTPase signaling targeted the GEF interaction, because some Ras GTPases' induced tumors can be caused by abnormal expression or mutated Ras regulators. [91][92][93] One example of a Ras-GEF inhibitor is NSC23766243. This compound targets the Rac1 GTPase and interfere with the binding to its GEF. [33]

Another target for Ras GTPase drug discovery is effector interactions. Targeting these effectors is challenging because of the difficulty of finding small molecule binding pockets that interfere with the interaction. However, new methods targeting protein-protein interaction pockets using computer aided discovery, with the help of the availability of crystal structures of the protein complexes and libraries of thousands of small molecules, have provided several drug candidates targeting Ras PPIs in recent years. [94] [73][95]

Recently, approaches targeting Ras mutations have been successful in finding potential drug candidates. Some small molecules are undergoing phase I and II clinical trials. These approaches work mainly by exploiting the reactivity of cysteine 12 in KRas, using a small molecule that covalently binds to this residue. [95] Companies such as Amgen, Jenssen, Mirati, and Eli Lilly are conducting clinical trials involving their prospective drugs using this approach. Table 1 indicates some of the most recent promising small molecules and macromolecules targeting Ras GTPases; these compounds are in the clinical stages.

As computer and research technologies advance, interest and discovery in Ras-related drug design continues to increase. Currently, researchers are using artificial intelligence and advanced computing machines to accelerate the discovery of new binding pockets for different proteins. [96][97] In the future, more promising drugs candidates will be devised and more proteins in the Ras protein superfamily will also be explored to fill the gaps in understanding and targeting these GTPases. Other groups are also investigating the involvement of Ras in other diseases such as developmental and neurological disorders. [23]

Table 1.Molecules and macromolecules undergoing clinical trials for various cancers by targeting Ras proteins. Adapted from [81]

Compound	**Company**	**Target**	**Clinical Phase**	**Trial Number**
AMG 510	Amgen	KRAS G12C	Phase I/II	NCT03600883
MRTX849	Mirati Therapeutics	KRAS G12C	Phase I/II	NCT03785249
JNJ74699157	Johnson & Johnson	KRAS G12C	Phase I	NCT04006301
LY3499446	Eli Lilly	KRAS G12C	Phase I/II	NCT04165031
BI 1701963	Boehringer Ingelheim	SOS1 inhibitor	Phase I	NCT04111458
TNO155	Novartis	SHP2 inhibitor	Phase I	NCT03114319
mRNA-5671/V941	Moderna and Merck	G12D, G12V, G13D, G12C	Phase I	NCT03948763
KRAS TCR	Gilead	KRAS G12D	Phase I/II	NCT03190941
siG12D-LODER™	Silenseed	KRAS G12D	Phase II	NCT01676259

I.2 Rho GTPases

The Ras superfamily of GTPases includes a distinct family of proteins known as Rho proteins, which are small (~21 kDa) monomeric G proteins that share 30% homology with other proteins of the Ras family. [38][39] They act as bimolecular switches and play a role in numerous cellular processes, modulating cytoskeletal organization, cell polarity, transcription, cell cycle progression, and hematopoiesis. [98][99][100] Rho proteins are best known as major regulators of the cytoskeleton and other actin-dependent processes such as cell migration, polarity, and morphology. These proteins alter the contractility of actin fibers, thereby reorganizing the actin cytoskeleton and cellular morphology.

Just like the other Ras GTPases, the activity of the Rho GTPases is regulated by the GEFs, GAPs and GDIs [101] In humans, 22 Rho family proteins have been identified with conserved orthologs across several eukaryotic species. [102] Based on their biological functions and sequence homology, the 22 Rho proteins are divided into 8 distinct subfamilies: Rac (Rac1, Rac2, Rac3, and RhoG), Cdc42 (Cdc42, RhoQ, and RhoJ), the RhoUV (RhoU and RhoV), RhoA (RhoA, RhoB, and RhoC), Rnd (Rnd1, Rnd2, and Rnd3), RhoD (RhoD and RhoF), RhoBTB (RhoBTB1, RhoBTB2, and RhoBTB3) and RhoH. [102] The families and subfamilies of Rho GTPases along with their roles are illustrated in Figure 7.

Of all the members of the Rho GTPase family, RhoA, Rac1, and Cdc42 are the most studied. [80][103] The sequences of Rho, Rac, and Cdc42 are the most highly conserved Rho GTPases in eukaryotic species. [102] Rac1, RhoA, and Cdc42 are key players in the modulation of the actin cytoskeleton, cell polarity, cell cycle progression, and signal transduction–induced changes in gene expression. [104]

Ras homologous A (RhoA) activation by extracellular ligands results in the assembly of actin-myosin filaments, focal adhesion, and integrin adhesion complexes that not only provide rigidity and structure to cells but also facilitate migration by furnishing the necessary contractile forces. [105]

RhoA is involved in the regulation of cell proliferation and cell invasion. [106] RhoA cell signaling is involved in various malignant processes. Therefore, chitosan/siRhoA RNA nanoparticles may be used therapeutically to inhibit cancer aggressivity as well as to promote spinal cord and nerve repair since RhoA inhibits regeneration after injury. [104]

Rac1, with its involvement in reorganization of the actin cytoskeleton, affects the cell cycle, endocytosis, trafficking, adhesion, and migration. [107] Rac1 activation leads to the formation of lamellipodia and the establishment of a network of actin filaments involved in cell motility. [108] Rac1 is also involved in the formation of nascent focal complexes and membrane protrusions. It is also involved in transcriptional modulation of gene expression. [109]

Cell division control protein 42 homolog (Cdc42) upon activation undergoes conformational changes that result in the activation of the PAKs (p21-activated kinases), which in turn lead to filopodia formation [110] and actin reorganization. Cdc42 signaling is involved in cell proliferation, polarity , migration, survival, and invasion. [111][112]

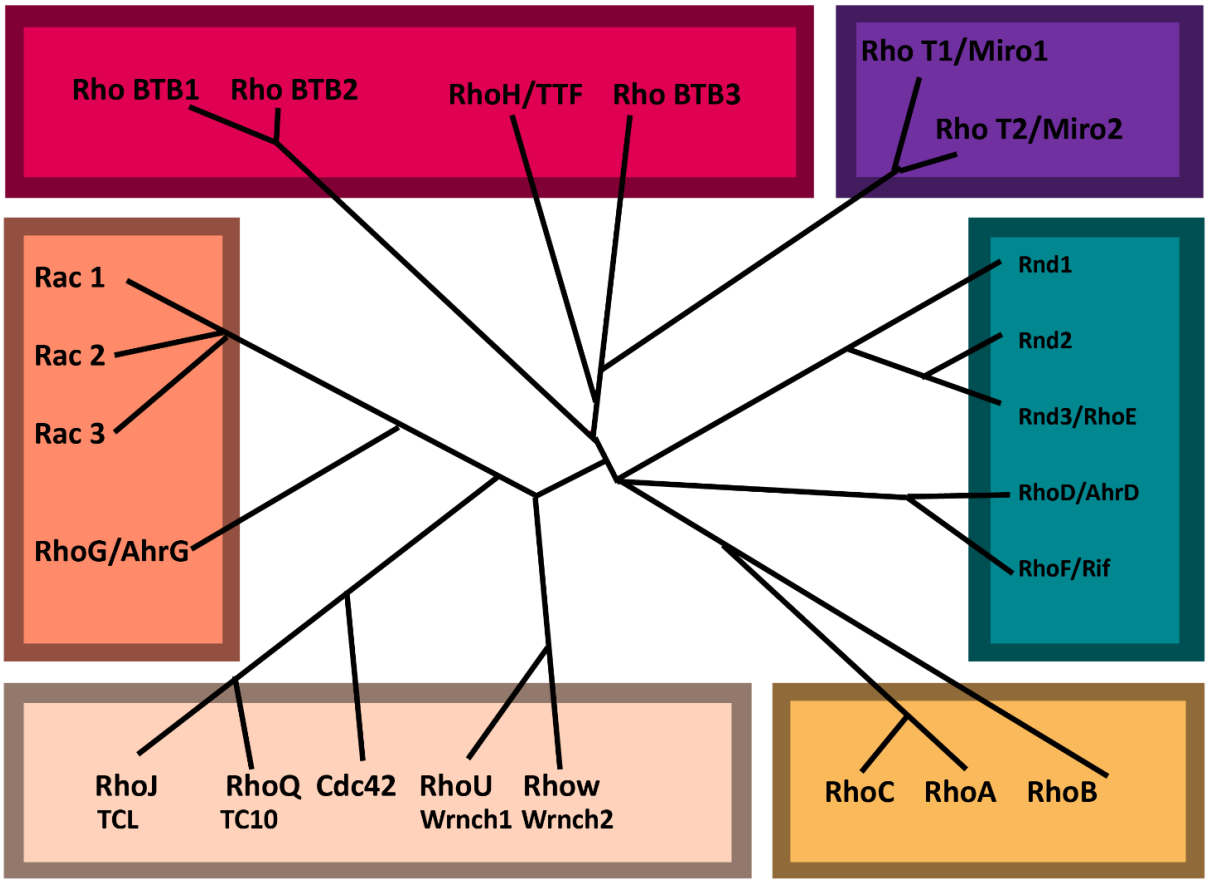

Figure 5. The Rho family of GTPases. Adapted from [113] and [114]

Table 2.The Rho family of GTPases. Adapted from [113] and [114]

Subclass	**Cytoskeletal effect**	**Rho family members**			
Cdc42 subclass	filopodia	Cdc42	RhoQ (TC10)	RhoJ (TCL)	
RhoUV subclass	filopodia and lamellipodia	RhoU (Wrch)		RhoV (Chp)	
Rac	lamellipodia	Rac1	Rac2	Rac3	RhoG
RhoBTB	protein stability	RhoBTB1	RhoBTB2	RhoBTB3	
RhoH	↓Cell growth and survival	RhoH			
Rho (subclass)	↑stress fibres and ↑focal adhesions	RhoA	RhoB		RhoC
Rnd	↓stress fibres and ↓focal adhesions	Rnd1	Rnd2	Rnd3 (RhoE)	
RhoF	Vesicle transport, filopodia	RhoD		RhoF (Rif)	

I.3 Cell Division Control Protein 42

Cell division control 42 (Cdc42) is a small GTPase of the Rho family that was first discovered and characterized in *Saccharomyces cerevisiae* in 1990 [115][116]. [117] The human homolog later found and showed a high degree of sequence similarity to the yeast Cdc42 (80%) and to other members of the Ras and Rho GTPases. More studies on molecular characterization of Cdc42 established its fundamentally important role for this protein in various cellular processes. Cdc42 mainly regulates the membrane trafficking and cytoskeletal reorganization for physiological processes such as cell proliferation, motility, polarity, cytokinesis, and cell growth. [112] Much like other GTPases, Cdc42 functions as a molecular switch in the cell by cycling between the GTP and GDP bound forms. Just like the other Rho GTPases, the activation and inactivation of Cdc42 is controlled by GEFs, GAPs, and GDIs. These regulators help Cdc42 act as a complex molecular switch involved in various cell processes. [61][118][119]

Active Cdc42 binds to and/or activates more than 20 downstream effector molecules that are that are involved in multiple cell functions such as cell polarity, migration, adhesion, actin cytoskeletal remodeling, intracellular trafficking, epidermal growth factor receptor degradation, and cell cycle progression. [106] Since the original discovery of its role in regulation of budding and mating projections in yeast, Cdc42 has also been implicated in the tight regulation of various essential physiological processes including early development, cell cycle progression, cell morphology, and endocytosis, in various species from nematodes to mammals. [120] The deregulation of these cellular processes is associated with numerous pathological conditions, especially proliferation, oncogenic transformation, and metastasis. [41][91] Cdc42's malfunction and overexpression is observed in multiple types of cancer as well as other developmental and neurodegenerative diseases.

I.3.A. Cdc42 Structure

Cdc42 has a conserved domain that contains the guanine nucleotide binding site and comprises of a six-stranded β-sheet surrounded by five α-helices. The G domain of Cdc42 recognizes of the guanine base and its β-phosphate, as well as the magnesium. Within this domain, Switch I and Switch II regions participate not only in nucleotide binding but also engage with regulators, effectors, and Mg^{2+}. [121] The Switch I region is between the α1 and β2 loops and Switch II is between the α2 and β3 loops. (Figure 6) The human Cdc42 gene is on chromosome 1p36.1 and it has a total length of 191 amino acids and a molecular weight of 21.33 kDa.

The Cdc42 subfamily has 11 members, with sizes from 190 to 192 amino acids, and with a very high degree of sequence conservation. For instance, there is 100% similarity between the mouse and human brain versions of the protein. Cdc42 has around 40% sequence homology when compared to other Ras GTPases. The areas of homology in the four domains involved in GTP binding and hydrolysis. [120] However, Ras proteins differ from Cdc42 in the presence of Asn-Lys-X-Asp (NKXD, where X is any amino acid) at amino acids 115 to 118, while all Cdc42 proteins contain the sequence Thr-Gln-X-Asp (TQXD, X is mostly Ile). These sequence differences between Cdc42 and other Ras GTPases may be responsible for the differences in their rates of GTP hydrolysis. [122] Cdc42 proteins are characterized by the presence of the C-terminal sequence Cys-X-X-Leu. The only two exceptions are the brain Cdc42 isoforms that end in a Phenyl residue (Cdc42Mmb and G25K). This conserved domain serves as the site for prenylation and subcellular localization and is therefore necessary for effective anchorage of Cdc42 in the plasma membrane. [123] Abdul-Mannan et. al observed that a GTPase binding domain (GBD) in the CRIB motif of PAK1 binds to the Switch I and Switch II regions of Cdc42. [124] Subsequently, structural comparisons of Cdc42 bound to three of its prominent effectors (WASP, PAK, and

ACK) revealed that an N-termini segment of the three Cdc42 effectors bind to the β2 strand, the α1 and α5 helices of Cdc42. The other residues of the CRIB segment bind to the Switch I and Switch II regions. [125] High resolution X-ray and other structural studies of Cdc42 showed minor differences between the inactive GDP-bound and the active GTP-bound forms. However, effectors are able to distinguish the GTP-bound from the GDP-bound and induce conformational changes in the Switch I and Switch II loops that are crucial for signal transduction cascades. [126]

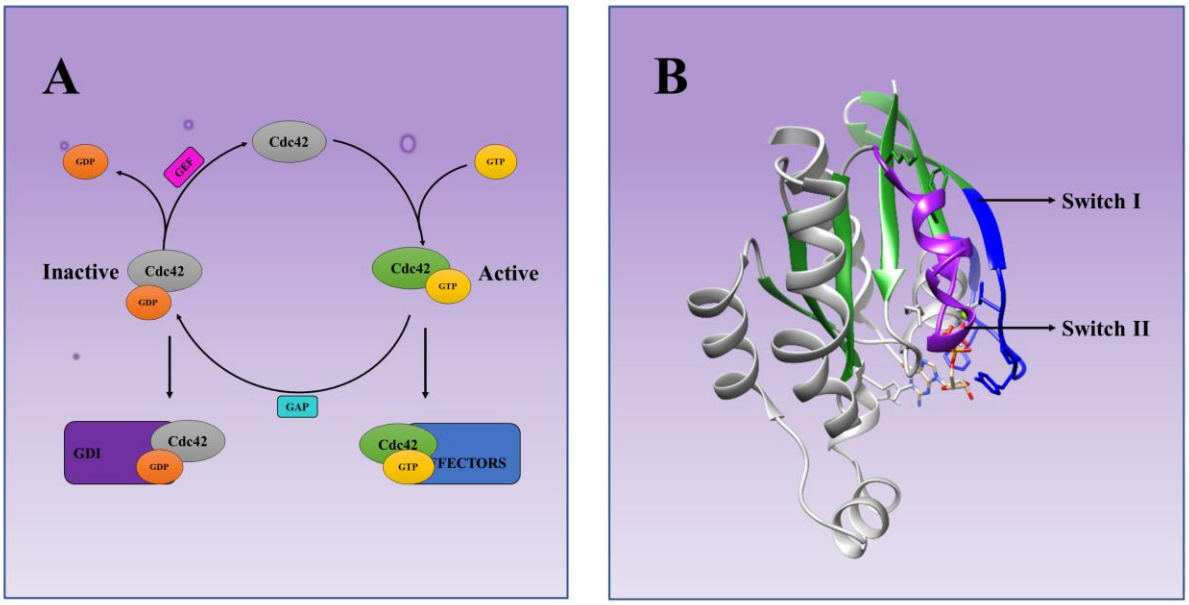

Figure 6. Regulation and Structure of Cdc42. A. The Cdc42 GTPase cycle. Adapted from [73] B. The structural features of Cdc42. Grey represents alpha helices; green represents the beta sheets. Blue represents the Switch 1 region and purple presents the Switch 2 region. PDB: 1AN0

I.3.B. Cdc42 Regulation and Function

As a GTPase, Cdc42 is regulated by GEFs, GAPs, and RhoGDIs. There are multiple regulators of each kind and provide cdc42 with enough control over signaling events. [61][73] Most of these regulators do not distinguish between members of the Rho family, especially Cdc42 and Rac. However, some are Cdc42 specific. For example, some well-known Cdc42 GEFs include intersectin-1, β-Pix1, Zizimin-1, and collybistin. Examples of Cdc42 specific GAPs include dRich, and MEGAP [127] [128] GDIs Inhibit Cdc42 activity by interfering with membrane localization. They also protect these proteins from degradation. RhoGDIα, RhoGDIγ bind to Cdc42 specifically. [129][130] In addition to these regulators, Cdc42 function largely depends on its interaction with various effector proteins and its anchoring process in the cell membrane. Impairment of Cdc42 regulation is observed in various diseases. [131]

Chen and colleagues reported that Cdc42 knockout mice die during implantation, emphasizing the importance of Cdc42 function in vivo. [132] Cdc42 has integral functions in a wide range of cellular activities and biological outcomes. In addition to its essential role as a cytoskeletal regulator along with other Rho proteins, Cdc42 has emerged as an essential protein with broad cellular functionality. One of the roles is in intracellular trafficking and regulation of mammalian cell growth. This is achieved via cell signaling through numerous pathways. Signaling pathways and functions mediated by Cdc42 are mostly tissue- and cell type-specific. Some of the more well-known Cdc42 roles are discussed below.

Cellular polarity is fundamentally important for morphogenesis, differentiation, and proliferation. With the identification of Cdc42 in yeast, it was observed that this protein is necessary and sufficient to promote cell polarization and, hence, budding in yeast [133]. Subsequently role for Cdc42 has been established in eukaryotic cell polarity, which it mediates

through its effects on the actin cytoskeleton and microtubule reorganization, and regulation of the Par-complex (Par6, Par3, and aPKC). [111][120]

Filopodia are protrusive, spike-like actin structures that form at the leading edge of migrating cells. Microinjection studies on the activities of Rho GTPases in mammalian cells demonstrated a role for activated Cdc42 filopodia formation [134] [135][136].

Nobes and Hall described how Cdc42 triggers the formation of the actin-based filopodia at the cell surface. They also described how Cdc42, along with Rho and Rac, is involved not only in the formation of multimolecular focal complexes at the plasma membrane but also the coordinated control of cell motility. [136] The activation of the Par complex by Cdc42 leads to the subsequent stabilization of the microtubule ends at the edge of migrating cells. The stabilized microtubules at the leading edge facilitate the reorientation and migration of the cell. [111][137]

Several studies have shown Cdc42's role in intracellular trafficking. Loss of Cdc42 function induced defects in basolateral transport of the LDL receptor [138] and retrograde transport from Golgi to ER. [139] Cdc42 is also interacts with the exocyst complex to induce phagocytosis. [140]

In the cell cycle, Cdc42 induces S phase entry, promotes proliferation and G1 progression. It also induces anchorage-dependent growth. [141] [142]

Elevated activity of Cdc42 has been associated with aging of hematopoietic stem cells (HSC) and a loss of polarity in aged HSCs. Aged HSCs were functionally rejuvenated and the amount of polarized cells in an aged HSC population increased with pharmacological inhibition of Cdc42 activity.[143]

Cdc42 is also involved in the regulation of the nervous system, cardiovascular system, and the immune system. It is also vital in the development of various organs such as pancreas, blood,

eyes, and skin. [112] This small GTPase is also involved in oligodendrocyte differentiation, neuronal polarity/axon outgrowth, and neuronal migration, and in cell fate determination. [144] [145] Studies carried out in cardiomyocyte-specific Cdc42 knockout mice by Li et. al suggested that Cdc42 is essential for cardiomyocyte proliferation, sarcomere organization, and cell-cell adhesion during heart development. [146]

I.3.C. Cdc42-Protein-Protein Interactions (PPIs)

Upon activation by various stimuli, Cdc42 can interact with multiple downstream effectors to transmit signals that impact cell functions in diverse mechanisms. When active, Cdc42 binds to more 20 effector/adaptor proteins. [147] Cdc42 effectors fall into three major protein families, namely, WASP (Wiskott–Aldrich syndrome proteins), PAK (p21-activated kinases) and ACK (activated Cdc42-associated kinases).

Wiskott-Aldrich Syndrome Protein Interactions

In its activated GTP-bound form, Cdc42 interacts with members of the Wiskott-Aldrich Syndrome proteins (WASP), including the neural isoform N-WASP, to regulate actin polymerization and filopodia formation. Active N-WASP interactions with the Arp2/3 complex (Actin related protein 2 and 3), contribute to a scaffold at the site of actin polymerization and control filopodia outgrowth. [148][149] Furthermore, binding of the Cdc42-GEF Intersectin, through its SH3 domain to the poly-proline region of N-WASP, stimulates the GEF activity and promotes Arp2/3 activation as well as filopodia formation. [150]

The neural WASP (N-WASP) is expressed in all tissues and shares common structural features with WASP family proteins, including the Cdc42-binding domain. It remains in an autoinhibited, folded conformation that represents its inactive form. Upon interaction with Cdc42, N-WASPis activated and subsequently activates the Arp2/3 complex. [151] N-WASP has been

implicated in tumorigenesis and metastasis, as evidenced by several studies over the past decade. N-WASP expression is upregulated in certain cancers and cell lines—cervical cancer, esophageal carcinomas, and certain gliomas—where its overexpression promotes migration and invasion. [152][153][154][155]

Activated Cdc42-associated kinase Interactions

The ACKs (activated Cdc42-associated kinases), also known as TNK2, are nonreceptor tyrosine kinases that are specific targets for activated Cdc42. ACK1 is a ~120 kDa protein with a Cdc42/Rac-interactive domain (CRIB). Cdc42 specifically binds to and directly activates ACK1. ACK1 is related to the subsequently discovered tyrosine kinase, ACK2 (~83 kDa). ACK2 overexpression activates c-Jun NH2-terminal kinase. This suggests that ACK2 is involved in the cellular stress response [128] where it is activated by cell adhesion in a Cdc42-dependent manner and associated with integrin β1.

ACK1, is involved in various cancers, and its overexpression promotes the tumor progression. ACK1 also affects oncogenic processes through both kinase-dependent and kinase-independent (protein-protein interactions) actions. [156] Due to its oncogenic properties and its link to cellular transformation, ACK1 has emerged as a potential target for cancer therapy.

p21-activated Kinase Interactions

PAKs (p21-activated kinase), are one of the best studied Cdc42 effectors. These are serine/threonine kinases that are categorized into two groups based on their structure and function. Group I PAKs are comprised of PAK1, PAK2, and PAK3 and are characterized by two regions: an autoinhibition region and a kinase region. Both these structural features are not present in Group II (PAKs 4-6) and hence, Group II PAKs cannot be activated by Cdc42. PAK1 phosphorylates both Raf1 and MEK that are crucial in the ERK pathway signaling. [157]

Much like some of the other Cdc42 effectors, PAK1 also acts as an oncogene in various cancer cells. PAK1 overexpression was observed hepatocellular carcinoma tissues and induced an increase in invasiveness and tumor growth. [158] [159] [160] Members of the PAK family are involved in several human cancers such as lung, liver, colon, and breast cancers. PAK2 and PAK3 overexpression is observed gastric cancer and cervical cancers, respectively. [156]

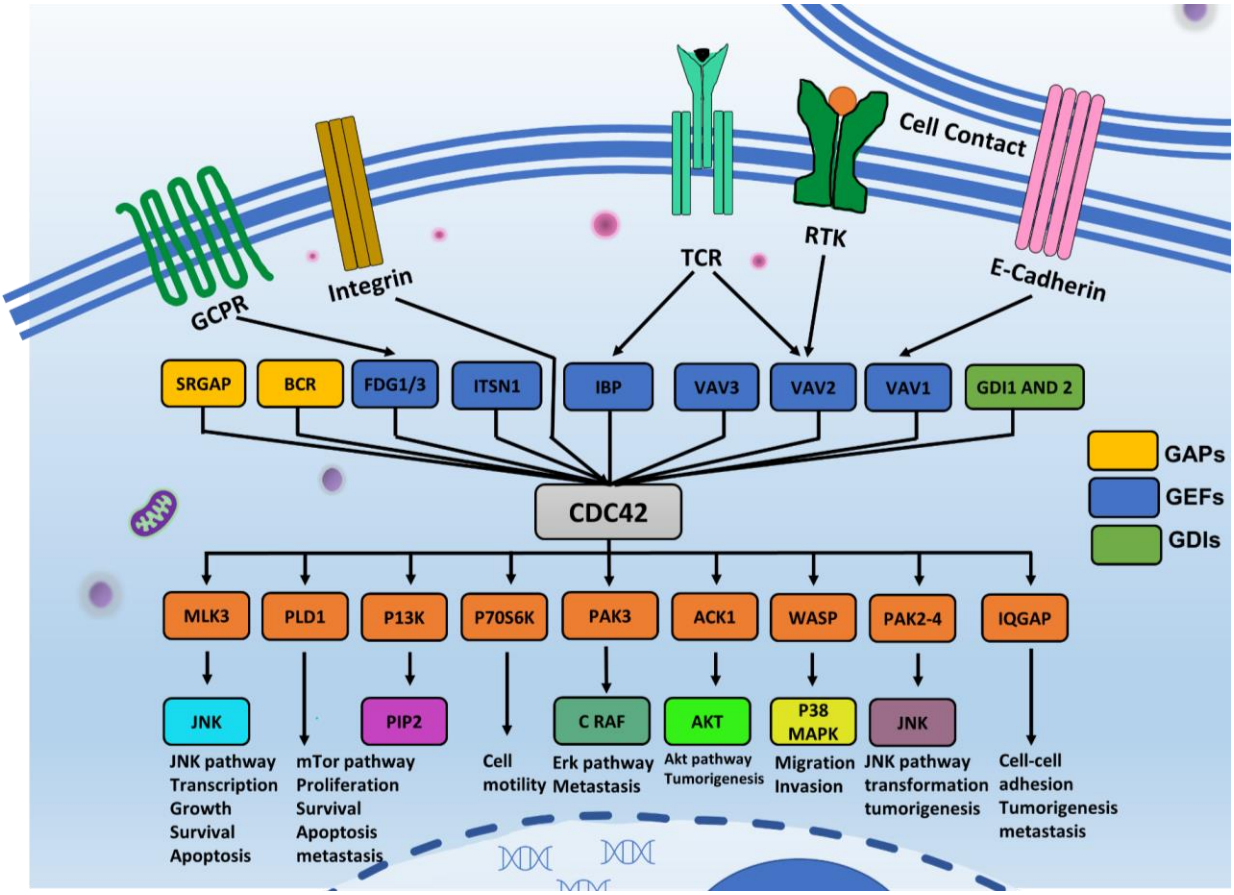

Figure 7. Cdc42 Pathway. Proteins involved in the activation, regulation, and binding of Cdc42. Adapted from [111][156] and [73]

I.3.D. Cdc42 and Cancer

As discussed above, Cdc42 is involved in complex signaling pathways that are cell type- or stimuli-specific under physiological conditions. Analysis of the functional roles of Cdc42 and the characterization of its effectors and regulators indicated that Cdc42 is involved in various human diseases through regulation of various cell processes. Overexpression or knockdown of Cdc42 in experimental animal models resulted in pathological phenotypes that mirrored disease states. Furthermore, deregulation of Cdc42 by its regulators and/or effectors was also associated with several human disease states as well as cell transformation, tumorigenesis, and metastasis. [161][162] Studies on Cdc42 mutants have made opposing revelations, with fast-cycling mutants readily able to transform 3T3 cell lines, and constitutively activated mutants hindering cell growth. [163]

Although Cdc42 mutations have not been found in cancer, Cdc42 expression levels have been found to be both increased and decreased in cancer tissue relative to normal tissue, on the basis of gene expression data from the SAGE database.[164] [163] Cdc42 overexpression is observed testicular and breast cancer, [79][165][166] non-small cell lung cancer, [167] colorectal adenocarcinoma and cutaneous melanoma. [168][169]

There has been major interest in understanding the mechanisms by which Cdc42 overactivation and expression contribute to oncogenesis. Wang et. al postulated that its ability to enhance metabolism contributes to the elevated proliferation of cancer cells. [153][154] Another mechanism of Rho GTPase signaling regulation is the control of GTPase regulators such as GEFs, GAPs, and GDIs. [170][171] These signals involve overexpression of the positive regulators (GEFs) and reduced expression of the negative regulators (GAPs and GDIs) [172] [173] Cdc42 activity is also essential for Ras-induced transformation, as has cellular transformation resulting

from aberrant EGFR signaling. [163] Abnormal Cdc42 expression and regulation is also involved in neurological deficits such as neurodegenerative diseases and developmental disorders. [77]

I.3.E. Cdc42 Inhibition

Because of its role in transformation, oncogenesis and metastasis, Cdc42 is an attractive therapeutic target in cancer and other human diseases. Rho GTPases and Ras in general are difficult to target directly because of their dynamic structure, scarcity of small molecule–binding pockets, their high GTP/GDP binding affinity, and the abundant levels of GTP available in cells. [81] Novel inhibitors of Cdc42 regulators and effectors that are strong candidates for pharmaceutical intervention to block oncogenic effects have been developed. [174] These small molecule inhibitors that have been developed target Cdc42 and its signaling proteins and change the activity of effector proteins of Cdc42. [175] There are several approaches used to target Cdc42. The most explored ones are discussed below.

Nucleotide binding inhibitors are molecules that inhibit Cdc42 activation by targeting GTP binding. One such molecule is MLS000573151, binds to Cdc42 and inhibits the cytoskeletal organization of mast and leukemia cells. However, this small molecule was only effective at higher doses, limiting its pharmacological application. Other nucleotide binding inhibitors that specifically inhibit Cdc42, such as CID2950007 (ML141) and CID44216842 inhibit ovarian cancer cell migration. [176]

ML141, along with its analog, CID44216842, specifically prevents GTP binding to inhibit Cdc42 activity. [177] CID2950007also hampered the migration of ovarian cancer cells and decreased Cdc42 activity in various breast cancer cells. [176]

As discussed, Rho GTPases like Cdc42 require to post translation modifications to be anchored in the membrane which can be affected by membrane anchoring inhibitors. The process

requires multiple steps and enzymes that can be targeted to slow down or inhibit the activation of Cdc42. Common small molecules of this kind are Farnesyltransferase inhibitors such as PAIA6, and GGTI-2418 that target Cdc42 and other Rho GTPases. [178][179][81]

Because of the ability of Rho GDIs modulators to keep the small GTPases in an inactive state, they emerge as attractive targets for Cdc42 inhibition. Thus, as part of a potential inhibitory approach, Secramine was developed as an interfacial adaptor. It is a fast-acting compound isolated from a natural product library and its name comes from its similarity to the yeast *sec* mutant. Secramine inhibits protein trafficking and the actin polymerization in various cell types. [180][181] This small molecule inhibitor also reduces Cdc42 activity in a RhoGDI1-dependent mechanism. [180] Secramine A also inhibits the binding of active Cdc42 to the Golgi membranes. [156]

Potential strategies for Cdc42 inactivation include targeting the binding with other known oncoproteins such as PAKs and ACKs, thereby preventing cancerous effects from downstream signaling from Cdc42 protein-protein interactions (PPIs). There are a few promising PAK inhibitors, such as OSU-03012, FL172, FRAX597 and PF-3758309. Although none of these has emerged as a drug ready for human consumption. Interaction with Ack and Wasp are also targeted extensively. Recently, a cyclic peptide and wiskostatin were developed as anticancer compounds to target N-WASP. [81] Inhibiting the interaction of Cdc42 and Ack1 can also be beneficial, AIM-100, an ACK1 small-molecule inhibitor suppresses ACK1 activation and prostate cancer cells proliferation.[182] Targeting Protein-Protein interactions for Cdc42 is crucial since it focuses on the protein's effect rather than activation. Targeting a regulator interaction, for example, is more inefficient since Cdc42 is regulated by multiple proteins than can substitute for the blocked signal.

As discussed, GEF activity increases the Cdc42 activity by several orders of magnitude. GEF inhibitors can greatly decrease the negative effects of overactive Cdc42 in the cell. Therefore, the most promising Cdc42 inhibitors target the interaction with GEFs.

Pirl, another inhibitor, targets Cdc42 by inhibiting PIP2-mediated GEF activity. [183] A Pirl1 structural derivative named Cdc42 activity–specific inhibitor (CASIN), is a small molecule that disrupts Intersectin-mediated Cdc42 activation. Using CASIN, colon cancer initiation in intestinal tumor cells was inhibited. Furthermore, CASIN inhibited cancer progression in cell, mouse and human tumor models, and it killed HeLa cells within 24 hours. [184][185] CASIN inhibition has also been shown to reverse the aging of LT-HSCs (long-term hematopoietic stem cells). [143]

The small molecule NSC23766 also fits into the surface groove of Cdc42 and reduces growth and invasion in several cancer types. [186] Its derivatives were screened in vitro, leading to the identification of AZA197 and AZA1. [186] AZA197 disrupts the interaction between Cdc42 and GEFs, and thus specifically inhibits Cdc42 activity but does not inhibit Rac1 activity. AZA197 has also shown inhibition of proliferation, migration, and invasion in colon cancer cells. [187] [188] Furthermore, this small molecule reduces the tumor size and increases survival in a model of colon cancer. Meanwhile, AZA1 inhibits Rac1 and Cdc42 activity in prostate cancer cell lines. It was also capable of inhibiting the proliferation and migration of prostate cancer cells while decreasing lamellipodia and filopodia formation. Additionally, both AZA197 and AZA1 inhibit the growth of tumors and prolong survival in mice with colon cancer. [187]

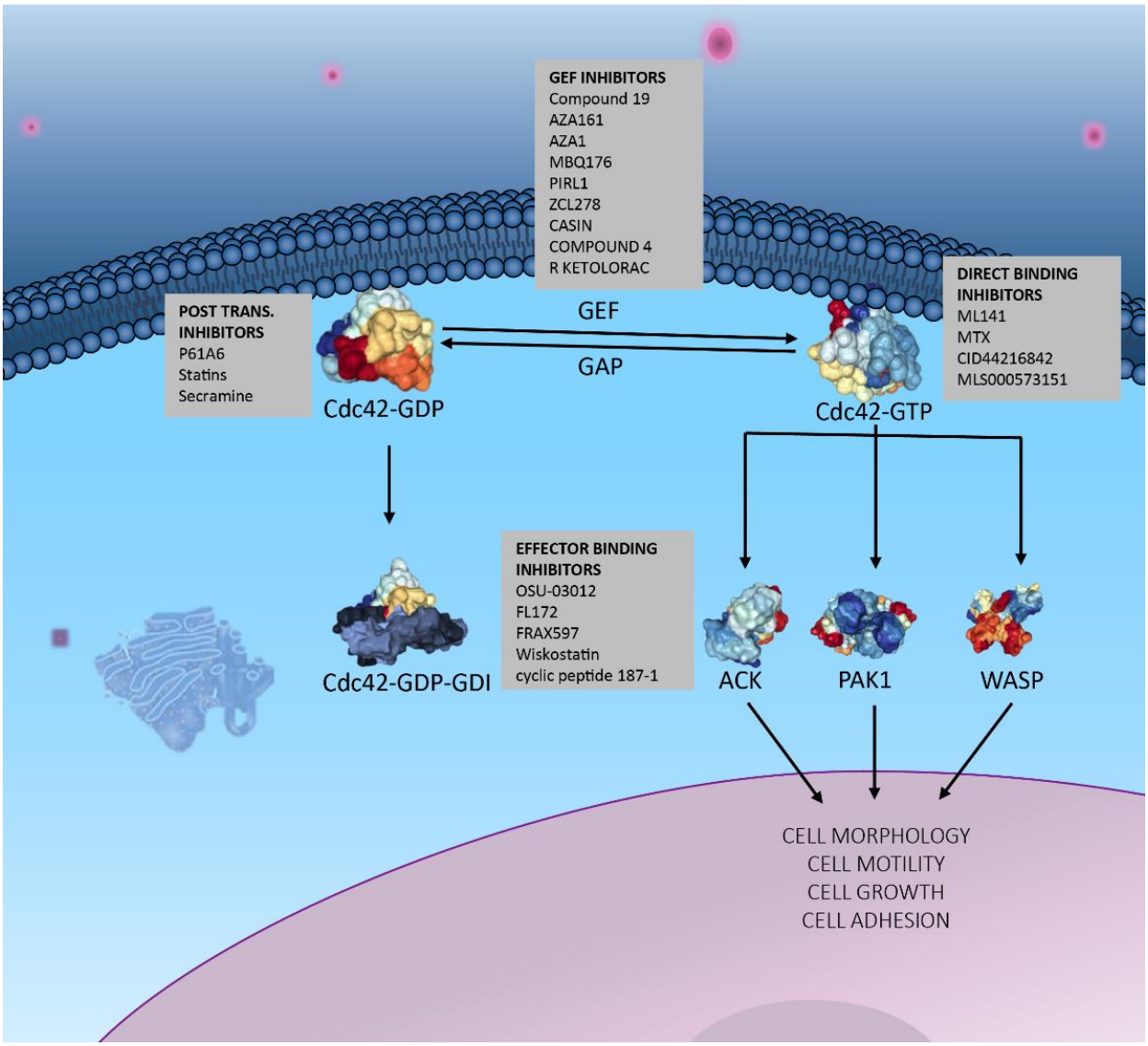

Figure 8. Small molecule targeting of Cdc42. Adapted from [81]

Table 3. Small molecule targeting of Cdc42. Adapted from [170]

Small Molecule	**Target Region**
Compound 19	GEF
AZA161	GEF
aza1	GEF
MBQ-167	GEF
Pirl1	GEF
ZCL278	GEF
CASIN	GEF

Table 4. Small molecule targeting of Cdc42. (Cont.) Adapted from [170]

Small Molecule	**Target Region**
Compound 4	GEF
R ketorolac	GEF
ML141 (CID2950007)	GTP/GDP Binding
CID44216842	GTP/GDP Binding
MLS000573151	GTP/GDP Binding
MTX	GTP/GDP Binding
OSU-03012	PAK1
FL172	PAK1
FRAX597	PAK1
wiskostatin	WASP
cyclic peptide 187-1	WASP
P61A6	Post Translation
statins	Post Translation
Secramine	Post Translation

I.3.F. Cdc42 and ZCL278

One of the most promising inhibitors of Cdc42 was published recently from Dr. Qun Lu's research team at the University of North Carolina and is a Cdc42-GEF inhibitor that lowers the protein's activation and function. To discover this inhibitor, the team investigated the binding between Cdc42 and Intersectin. This interaction showed that there is a surface groove in the Cdc42 structure close to the Switch 1 region that could be exploited for inhibitor binding. Using a large library of small molecules, the team used computational binding models to find molecules that could mimic Cdc42-Intersectin binding. The researchers then used precision docking and high-throughput/in-silico screening to select candidates that were further tested for specific protein binding. From these studies, the best candidate was 4-[3-[2-[4-Bromo-2-chloro-phenoxy]-acetyl]-thioureido]-N-[4,6-dimethyl pyrimidin-2-yl] benzenesulfonamide, later named ZCL278.

Fluorescence spectroscopy titration and surface plasmon resonance showed that ZCL278 bound to Cdc42 with an affinity of 6.4 μM and 11.4 uM respectively. Initial molecular dynamics simulations showed the small molecule binding to Cdc42 in the Switch 1 region and affecting residues Thr35, Val36, Asn39, Phe56, and Asp57. ZCL278 also inhibited Cdc42 activation by disrupting GTP and GDP binding. ZCL278 binding suppressed several Cdc42-stimulated cell functions including abolishment of microspike formation, neuronal branching, and motility. The group also demonstrated that ZCL278 is specific to Cdc42 and not Rac1.

The work by Friesland and colleagues highlights ZCL278 as a potential prime target for the Switch 1 region of Cdc42. This molecule is a good start toward a successful Cdc42 inhibitor. Furthermore, subsequent studies on ZCL278 have shown that it is an effective Cdc42 inhibitor. Figiel et. al showed that the molecule rescues dendritogenesis in dystoglycan-deficient hippocampal neurons by blocking Cdc42 activity. [31] It also inhibits actin polymerization in porcine oocytes through the inhibition of intersectin-Cdc42 interaction. [32] However, details about the ZCL278 interaction with Cdc42 are still lacking. For instance, it is not known whether ZCL278 interferes with Intersectin binding directly, or if it allosterically changes the protein dynamics to affect binding. This information is crucial since it determines whether ZCLl278 affects other Cdc42 protein-protein interactions or not. It is not yet known how binding of this small molecule affects Cdc42 function, stability, conformation, and interaction with other proteins, nor what specific protein residues are affected by the small molecule binding. [13]

In this study, we investigated the ZCL278 and Cdc42 interaction in detail to characterize the mechanisms of its binding and the effects of the binding to effector interactions. We used computational, biophysical, and biochemical methods to study how this small molecule impacts the protein’s structure, function, and stability. We also investigated Pak1 and its interaction with

Cdc42. However, prior to investigating this protein-to-protein interaction, we devised a method to quickly and efficiently express and purify a biologically active Pak1 peptide. Then, our interaction studies investigated how the binding of PAK1 to Cdc42 is affected by ZCL278. In this study, we also highlight the amino acid residues involved in the binding of the small molecule. The results of this study are crucial for the next phase of designing a better Cdc42 inhibitor. Using our results and early drug discovery methods, we here propose novel potential small molecule inhibitors that will be further tested and developed for Cdc42 binding and effects in cells.

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Chapter II. Characterizing the Influence of ZCL278 on Cdc42 Structure, Function and Stability

II.1 Abstract

Ras GTPases play an important role in cell proliferation, transformation, and migration. Abnormal expression and function of these proteins are observed in many diseases including several forms of cancer. Cdc42 is a Ras related protein involved in cell adhesion, migration, and cycle regulation. Ras related protein abnormalities have proven difficult to target and inhibit but recent studies have highlighted potential inhibitors for Cdc42. Studies have found small molecules and miRNAs that can reverse the effects of Cdc42 in cells. However, much is still not understood about the effects of these inhibitors on the protein structure, stability, and function. In this study, a novel Cdc42 inhibitor, ZCL278, was investigated. The binding affinity of ZCL278 to Cdc42 was calculated using various experimental methods. Effects of the ZCL278 on protein conformation and stability of the protein were also studied using computational, biochemical, and biophysical methods. Results from this study show a moderate binding affinity of the drug to the protein. When bound to Cdc42, ZCL278 makes the protein more stable and resistant to denaturation. Regions and amino acids that might be involved in the binding were also identified. This study shows that ZCL278 is a potential Cdc42 inhibitor that stabilizes Cdc42 and blocks its abnormal activity and its effects in the cell. The results will be beneficial in the future design of better Rho GTPase inhibitors.

II.2 Introduction

Proteins in the Ras superfamily have a crucial role in major cell processes and pathways. However, a lot is still unknown about the structure and function of these proteins. Ras proteins were first discovered 50 years ago, and they were among the first identified human oncogenes. Studies have placed Ras proteins into a large family of 167 proteins. Those proteins are involved in more than 30% of human cancers. [1][2][3] Research has now shown that Ras proteins are highly involved in the three of the top four cancer killers in the United States (lung, colon, pancreatic). [4] In several types of cancers, Ras proteins are involved in different stages from growth to metastasis.[5][6][7] Ras proteins are also involved in developmental diseases [7][8] and neurodegenerative disorders such as Alzheimer's [7]. However, despite their crucial role in the body, these proteins have been nicknamed "undruggable" due the inability for scientists to find an effective drug despite decades of approaches and research. [4][4][9]Among the Ras superfamily, Rho GTPases are important to study because they are responsible for many cell functions and can serve as models to study other Ras proteins. [10][11][12] In this study, we investigated a Rho GTPase known as Cell division cycle 42 (Cdc42).

First identified in early 1990s, Cdc42 was discovered as a cell polarity modulator in Saccharomyces cerevisiae cells. [13][14] In subsequent studies, it became clear that Cdc42 was present and essential for different processes in the animals. [15] Further studies in mammals showed that Cdc42 is involved in different systems including the immune system, the nervous system, and the cardiovascular system. It is also involved in developmental process of organs such as the blood cells, eyes, and skin. [15][13] Further studies found that in the cell, Cdc42 is responsible for processes such as proliferation, polarity, adhesion, and migration. It is also involved in regulation of the cytoskeleton and other functions. [16] Mutations, overexpression and

abnormal protein interactions involving Cdc42 play significant roles in events leading to abnormal cellular proliferation and transformation. Cdc42 also plays a big role in cell migration and adhesion and it is therefore involved in cancer metastasis. [17][18][19] [20] In fact, mutational studies of Cdc42 result in early embryonic lethality in knockout Cdc42 mice. [21] These studies show how Cdc42 is crucial to cell development and survival. Studying how this protein works and interacts with others as well as potential inhibitors will not only teach us how target and suppress the overactivity of Cdc42 but also other similar Rho GTPases as well.

Cdc42 binds to GTP and GDP in its active and inactive forms, respectively. The exchange between the GTP and GDP bound forms is controlled by regulatory proteins. Guanine nucleotide exchange factors or GEFs influence GTP dissociation and GDP binding, an action that turns on the protein. GTPase-activating proteins or GAPs are involved in stimulating the GTPase activity of Cdc42 that leads to an inactive GDP bound form of the protein. Guanine dissociation inhibitors or GDIs sequester Cdc42 and prevent nucleotide exchange. [22][23]

Recent advances have focused on interactions with effectors and regulators for inhibitor design to target Cdc42 induced abnormal cell signaling. Recent structure-based drug design approaches as well as fragment-based lead drug discovery methods have discovered potential regions on Cdc42 that could be exploited for small molecule binding to inhibit its overactive. [24] Recently, several small molecules targeting Cdc42 have been developed. Although most of them have shown low affinity, high toxicity, and low effect on Cdc42 related cell functions, some of the inhibitors are promising. Among those small molecule inhibitors, the most notable are ML141, AZA161, CASIN, MBQ-167 and ZCL278. [25][10][26][27][28] One of the most promising Cdc42 inhibitors came from a group of researchers led by Friesland at the University of East Carolina. The team designed, generated, and tested a small molecule that binds to and suppresses the

function of Cdc42 in the cells. The researchers designed the molecule based on the binding of Cdc42 and Intersectin, a known Cdc42 GEF. Since the interaction between Intersectin and Cdc42 is known, molecules that could mimic that interaction were selected in a large library of small molecules. High-throughput screening and precision docking studies were used to select candidates that were later tested for specific residue binding. The group then synthetized ZCL278 and went on to test its effects on Cdc42. Direct binding of ZCL278 to Cdc42 demonstrated a binding affinity value of 6.4μM by fluorescence titration. Different cellular assays showed that ZCL278 binds and inhibits cell functions mediated by Cdc42. The small molecule also inhibited Cdc42 activation. The group also demonstrated that ZCL278 is specific to Cdc42 and not Rac1, a protein in the Rho family and similar to Cdc42 in structure. [27]

Subsequent studies on ZCL278 have highlighted its use as an effective Cdc42 inhibitor. Figiel et. al showed that it rescues dendritogenesis in dystoglycan-deficient hippocampal neurons by blocking Cdc42 activity. [29] It also targets actin polymerization in porcine oocytes by inhibiting the intersectin-Cdc42 interaction. [30] However, there are still unanswered questions regarding ZCL278 interaction with Cdc42. For instance, it is not known whether ZCL278 interferes with Intersectin binding directly or if it changes the protein dynamics to affect binding. It is also not known how binding of this small molecule affects Cdc42 function, stability, conformation, and interaction with other proteins. Moreover, it is not known what specific protein residues are affected by the small molecule binding. [13]

In this study, computational, biophysical, and biochemical assays were performed explain the unanswered questions about the mechanisms of ZCL278 binding to Cdc42. Results from this study show a considerable binding affinity of the drug to the protein. They show that ZCL278 makes the protein more stable and resistant to denaturation. Potential amino acids involved in the

binding were also identified. Effect of the small molecule on Cdc42 GTP hydrolysis was also tested. A Cdc42 variant, T35A was used as a comparison to the wild type. This mutant is located in the Switch 1 region and previous studies from our lab have shown that this it leads to the loss of conformational freedom in the region. [31] These studies highlight ZCL278 as a potential Cdc42 inhibitor that can be used to block Cdc42 abnormal activity and reduce its negative effects in the cell.

II.3 Materials and Methods

Growth, Expression and Purification

Histidine tagged Cdc42 WT and T35A constructs were cloned into a pET-15b vector and over expressed in *E. coli*. One mL of a stored glycerol stock was inoculated in 100mL of LB broth media with 100uL of ampicillin and incubated at 37°C overnight. The 100 seed culture were then added to 2L of LB media and 2mL ampicillin. The growth was incubated and monitored on a shaker at 37°C. A 600nm OD reading of 0.4–0.6 was achieved before the culture was induced by 1mM isopropyl β-D-thiogalactopyranoside (IPTG). After 5 hours of incubation, cells were harvested by centrifugation at 6500rpm for 30 minutes. The pellets were then washed in 25mM Tris, 50mM NaCl, 10mM $MgCl_2$, P.H 8.0 and stored at -80^0C. The his-tagged Cdc42 WT and T35A were purified by affinity chromatography on AKTA Start FPLC. Gel electrophoresis, LC-UV MS and MALDI-TOF were used to verify the molecular weight and purity of the proteins.

Nucleotide Exchange

To activate Cdc42 WT and T35A, a nonhydrolyzable GTP analog (GMPPCP, Sigma) was attached. The protein first was dialyzed in 25mM Tris, 50mM NaCl, P.H 8.0. It was then incubated for 2 hours at 4°C with 5mM EDTA and a 5:1 molar ratio of GMPPCP:protein. Prior to desalting, the PD-10 column (GE Life Sciences) was equilibrated with the buffer. 5mL of the protein was

then added to the column and one mL fractions were collected and tested for protein presence by a Bradford assay. After all the protein was collected, 10mM of $MgCl_2$ was added.

Molecular Dynamic Simulations

The simulations were carried out in collaboration with the Moradi group at the University of Arkansas. The crystal structure of wild type (WT) Cdc42 (pdb id: 1AN0) was used as the starting structure. After removing crystal waters, appropriate protonation states for all residues were assigned and hydrogens were added using the MOE (Molecular Operating Environment) software. ZCL278 was built in MOE and docked into a site between the Switch 1 and 2 regions on Cdc4 using the induced fit docking protocol in MOE. As a first step, all possible binding sites on Cdc42 were identified using MOE. Of the 13 sites identified, eight were chosen which all contained the Switch 1 region, and ZCL278 was docked. Wall constraints were placed around the small molecule to restrict conformational search space. After several iterations of refinement, 50 docking poses were generated and 5 conformationally distinct poses were selected for the MDS. The complex was solvated and modeled for the MDS using the CHARMM-GUI web interface. The final model of about 48,000 atoms consisting of one protein molecule, one GTP, one ZCL278, one Mg^{2+}, 0.15M NaCl, and about 15,000 water molecules, was simulated using the NAMD 2.11 software. Each pose was energy minimized for 10,000 steps, then equilibrated for 5 ns in the presence of harmonic restraints on the protein backbone and side chain heavy atoms and production runs were performed for 200 ns for each pose. The same protocol was repeated for the T35A variant resulting in 50 poses, 5 of which were selected for MDS similar to Cdc42(WT).

All simulations were performed under periodic boundary conditions. CHARMM force field parameters were used for the protein, water, GTP and Mg^{2+}, and CHARMM general force field (CGenFF) parameters were used for ZCL278. The most stable structure poses from MDSs

were retained for further analyses. MDSs for the Cdc42(WT)-ZCL278 complex and the Cdc42(T35A)-ZCL278 complex were compared to see how the small molecule interacted with the Switch 1 region of the T35A variant with reduced backbone dynamics at the binding surface versus that of Cdc42(WT). ZCL278 relative binding free energies to both Cdc42(WT) and Cdc42(T35A) were calculated using the MDS data (the last 100 ns) and the MM/PBSA method for each pose.

Small Molecule Fluorescence Titration

Cdc42 was dialyzed in a buffer consisting of 25mM Tris, 10 mM $MgCl_2$ and 50 mM NaCl. Various aliquots of ZCL278 were added to a quartz cuvette containing 0.2mg/mL of the protein solution and incubated for 5 min before each fluorescent measurement on the Jasco 1500 CD Spectrophotometer. An excitation wavelength of 280 nm and an emission of 300-400nm was measured after each addition. By looking at the emission peak of 350nm, a titration curve was fitted by a non-lineal fitting model in Origin and the Kd was obtained.

Isothermal Titration Calorimetry (ITC)

The binding between Cdc42 and ZCL278 was measured by ITC using a MicroCal iTC200 (Malvern Inc.) At 25^0C, the sample cell contained CDC42-GMPPCP at concentrations in the range of 10–20 uM with 3.4-6.7% DMSO. In the syringe, ZCL278 at 100–200 uM was loaded. ZC278 was dissolved in DMSO at an original concentration of 3mM before being dissolved in buffer to desired concentration. For each sample, a speed of 750 rpm was maintained while performing 30 titrations. A control of the experiment was obtained by titrating ZC278 into buffer alone and relevant subtractions were made. The resulting ITC graphs fitted by using the manufacturer provided Microcal Origin software to calculate the Kd and other thermodynamic features.

Differential Scanning Calorimetry (DSC)

DSC scans performed using Microcal PEAQ-DSC. (Malven, Inc). To run the sample, 1mg/mL of Cdc42 was dialyzed in 50 mM Tris, 10 mM $MgCl_2$ and 50 mM NaCl PH 8.0 and added to the sample spot on the machine. The same buffer was added in the reference cell. The sample was heated from 25 to 85 °C at with a rate of 60°C/per hour. Buffer subtraction and analysis of the resulting scans was done by the manufacturer-provided software.

Temperature Denaturation

Temperature denaturation studies were performed by characterizing circular dichroism on Jasco 1500. The variable temperature measurement program was used on the machine. For each reading, 0.5 mg/ml of Cdc42 were used in the presence and absence of ZCL278. Cdc42 was first dialyzed in 50 mM Tris, 10 mM $MgCl_2$ and 50 mM NaCl at PH of 8.0. In a quartz cuvette, Cdc42 was placed with either 0.7% DMSO or 50uM ZCL278 and the temperature was set from 25 to 90 °C in steps of 0.5 °C/min and wavelength range of 190 to 250 nm. After completion, the molar ellipticity of the readings at 208nm from each temperature were calculated and fitted into Origin to calculate the midpoint corresponding to the Tm of the protein.

Chemical Denaturation

Urea denaturation experiments on Cdc42 in absence and presence of ZCL278 were measured on the Jasco 1500 CD Spectrophotometer. Using a built-in automatic titration function, the denaturant was added to the protein, then CD and fluorescence readings were automatically taken before adding more. 0.5mg/ml of Cdc42 and T35A in the presence and absence of ZCL278 were used. Chemical denaturation of the protein was measured using fluorescence intensity at 350nm. The fraction of denatured protein was calculated using the equation $F_d=(F_x-F_o)/(F_f-F_o)$. F_d is the fraction of the denatured protein, F_x is the absorbance intensity taken at each concentration,

F_o is initial reading before denaturant, and F_f is the final reading. The denaturation curves were created by plotting the fraction of denatured protein vs. urea concertation. The midpoint values (C_m) of Cdc42 WT and T35A were calculated in the presence and absence of ZCL278. The equilibrium constant $K_d=F_d/(1-F_d)$ and the Gibbs free energy of protein unfolding $\Delta G=-RT\ln(K_d)$ were also calculated. R is the gas constant and T is absolute temperature.

Limited Proteolytic Digestion

Limited digestion was performed using chymotrypsin (Sigma) in a buffer containing 50 mM Tris, 10 mM $MgCl_2$ and 50 mM NaCl at a PH of 8.0. The chymotrypsin digestion was performed at an enzyme to protein ratio of 5:1. The protease activity was stopped using TCA precipitation by adding 20% TCA, centrifuging at 13K rpm for 5 minutes, washing the pellet with acetone and dissolving the sample in 30uL of 8M urea and 15uL of SDS sample buffer (100 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 200 mM β-mercaptoethanol, and 0.2% bromophenol blue). Digestion was stopped at time intervals of 0, 30, 60 and 90. Limited proteolytic cleavage was measured by measuring the 21-kDa band on a 20% SDS-PAGE gel of the protein. The analysis of the gel was done by UN-SCAN-IT.

GTPase Hydrolysis Assay

GTPase Hydrolysis assays were performed using the ATPase/GTPase Activity Assay kit from Sigma Aldrich. Triplicates were used. At 25^0C, 10uL of 0-10uM of Cdc42 were used with and without ZCL278 in a buffer of 50 mM Tris, 10 mM $MgCl_2$ and 50 mM NaCl at a PH of 8.0. Concentration increments were made in triplicates and after adding protein and buffer, 30uL of 50uM GTP was added to the 10uL solution to start the reaction. The reaction was incubated for 45 minutes and stopped by adding the color reagent from the kit. After another incubation of 30 minutes, the absorbance of the samples in the 96 well plate was measured. A buffer control and

GTP-only control were used and subtracted accordingly while processing the data in Microsoft excel.

II.4 Results

Molecular Dynamics Simulations Show Tight Binding of ZCL278 in the Switch I region of Cdc42

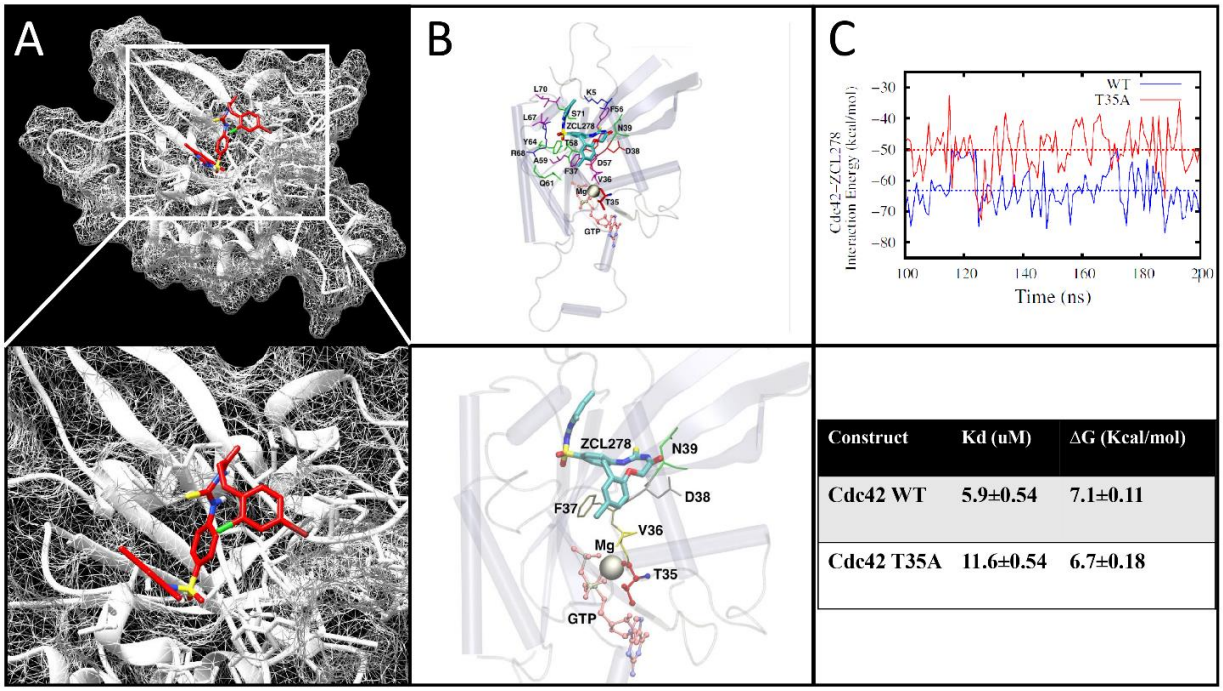
To understand the binding between Cdc42 and ZCL278, we started with molecular dynamics simulations. These simulations are crucial in evaluating multiple binding poses and models to learn how the small molecule interacts with the protein. Relative binding energies and thermodynamics parameters of the binding can be approximated and compared to the experimental values. These results can then be compared to the experimental results later.

In collaboration with Dr. Mahmoud Moradi's group at the University of Arkansas, we developed structure poses for the Cdc42 WT and Cdc42 T35A mutant bound to ZCL278. Figure 1A and 1B shows the binding positions of Cdc42 and ZCL278 along with the residues involved. In Figure 1C, the interaction energies and binding affinities for both protein constructs are shown.

The crystal structure of Cdc42 (PBD: 1AN0) was used to as a starting point to perform MDS. Using the MOE software, the T35A mutant and ZCL278 were built, and the molecule was docked into a site between the Switch 1 and 2 regions on Cdc42 WT and T35A using an induced fit docking model. 50 docking poses of Cdc42 WT and T35A were generated and 5 conformationally distinct poses for each were selected for the simulations. A complex of one protein molecule, GTP, ZCL78, on Mg^{2+} and 0.15M NaCl plus 15000 water molecules was used for simulations in NAMD 2.11. After MDS, the most stable structure poses from were retained for further analysis. Compared to Cdc42 WT, the small molecule interacted with the Switch 1 region

of the T35A mutant with more reduced backbone dynamics at the binding surface. The binding affinity of the most stable Cdc42 pose was 5.9uM for WT and 11.6uM for T35A.

ZCL278 binding free energies to both Cdc42(WT) and Cdc42(T35A) were calculated using the MDS data (the last 100 ns) and the MM/PBSA method for each pose. and were compared to experimentally determined binding energies from fluorescence spectroscopy (Figure 1C). The experimentally determined values of ΔG are consistent with those from MDS. The binding free energy was slightly increased for the T35A variant from both experimental and MDS calculations, which is also reflected in the interaction energies shown (Figure 1C) for the most stable poses of WT and T35A, suggesting that the dynamics of the Switch 1 region in T35A might foster a less spontaneous interaction with ZCL278.



Construct	Kd (uM)	ΔG (Kcal/mol)
Cdc42 WT	5.9±0.54	7.1±0.11
Cdc42 T35A	11.6±0.54	6.7±0.18

Figure 1. Molecular Dynamics Simulations of the binding of ZCL278 to Cdc42 and Mutant. A. Top: figure of Cdc42 WT binding to ZCL278. Bottom: Surface of Cdc42 binding to ZCL278 zoomed in. B. Amino acid residues involved in binding of ZCL278 to Cdc42. The

ITC and Fluorescence Titration Show a High Binding Affinity Between WT and ZCL278

The small molecule's binding affinity to Cdc42 was confirmed experimentally using two different methods. In the study by Friesland et. al, the binding affinity of Cdc42 to ZCL278 was calculated to be around 6.4uM and 11.4uM using fluorescence titration and surface plasmon resonance. [27] To study the binding, a fluorescence titration experiment was first done by reading tryptophan fluorescence at 350nm after each titration of 3mM ZCL278 to 0.5mg/mL of Cdc42. A fit curve of the results generated a binding affinity of 5.88uM for Cdc42. (Figure 2B) Next, Isothermal Titration Calorimetry was used and 150uM of ZCL278 was titrated to 10uM of Cdc42 and binding resulted in a Kd of 7.75uM for WT. (Figure 2A). The Kd numbers obtained in our studies are within the same range of the MDS and of the published data. This data also shows that ZCL278 has one of the highest binding affinity to Cdc42 when compared to other small molecules.

[23] [26]

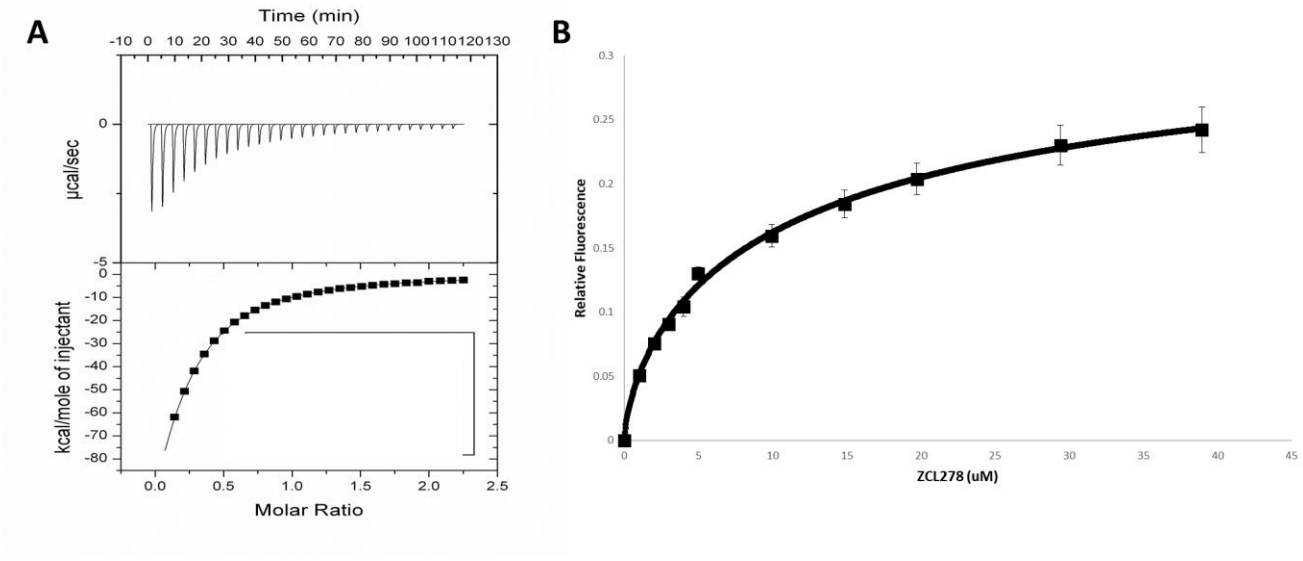


Figure 2. Binding affinity of ZCL278 to Cdc42. A. Isothermal titration calorimetry of ZCL278 to Cdc42. B. Fluorescence titration of ZCL278 to Cdc42. The Kd calculated for ITC is 7.75uM while the Kd for FL titration is 12.6uM.

ZCL278 Increases Cdc42's Stability Against Chemical and Enzymatic Denaturation

Previous studies have shown that ZCL278 has a significant effect on various Cdc42 related effects in different types of cells. [27] However, molecular details of the small molecule interaction with Cdc42 are still unknown. In this study, the small molecule's effect on the structural stability of cdc42 was investigated. Structural stability of the protein is important since it affects the protein's activity and function as well as interaction with effectors. To test the stability, chemical denaturants were added to the protein in the presence and absence of the small molecule. [32]In addition, the T53A mutant was also tested to investigate whether this residue plays a role in the stability of the protein and the binding of the small molecule. Increasing concentrations of 8M urea were added to Cdc42 and change in Tryptophan fluorescence was recorded and plotted. The results

of this study show that the presence of ZCL278 causes an increase in the amount of chemical denaturant needed to unfold the Cdc42 WT. On the other hand, ZCL278 did not have a similar effect on the T35A mutant. (Figure 3C) When the energies of denaturation are compared, ZCL278 bound Cdc42 required more energy to unfold while the mutant requires almost the same energy. (Figure 3A)

In the Switch 1 region, there are two tryptophan residues and two phenylalanine residues. These residues are susceptible to cleavage by chymotrypsin. [8] If the small molecule binds in the Switch 1 region as hypothesized by MDS, the small molecule binding should protect the protein from limited chymotrypsin digestion. Cdc42 was subjected to limited digestion with an enzyme to protein ratio of 1 to 4. Results were then collected in limited time intervals and digestion was stopped. The results were then run on an SDS-PAGE gel and scanned. Intensity values were quantified using UN-SCANNIT software. Results show that, when bound to the small molecule, the Cdc42 protein becomes more resistant to chymotrypsin digestion. These results further show that the small molecule binds to the Switch 1 region and stabilizes the protein.

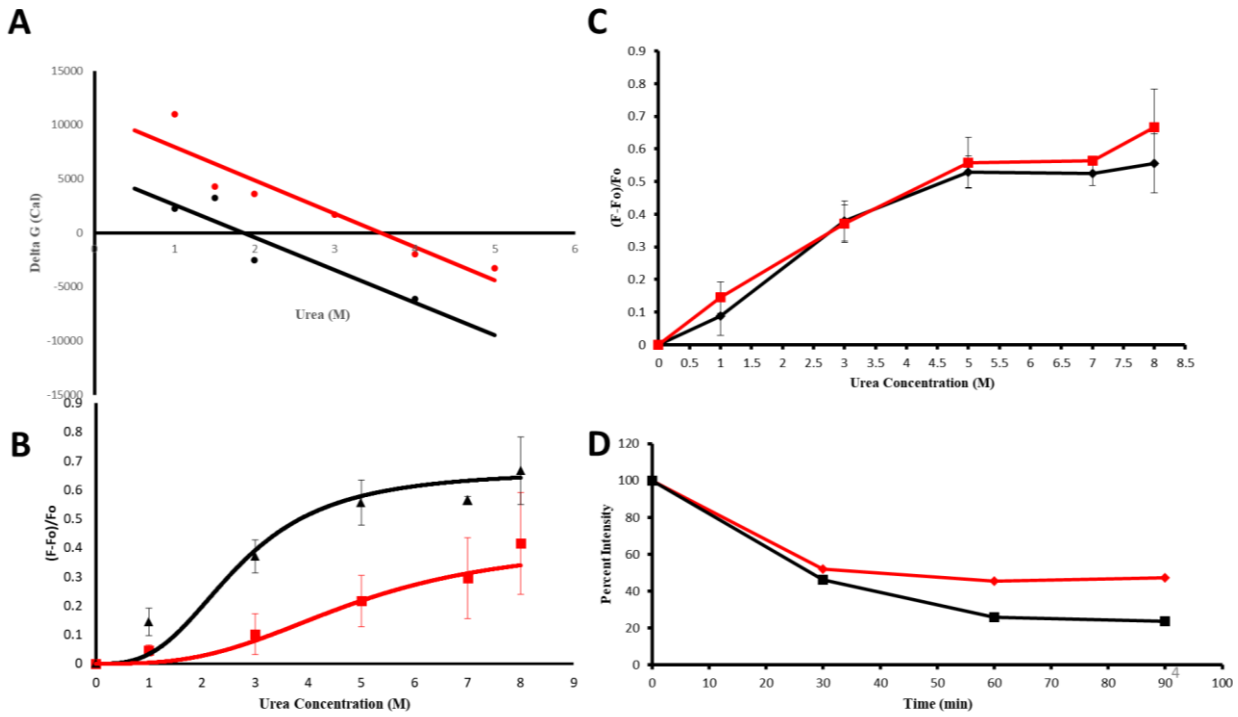


Figure 3. Cdc42 stability in the absence (black) and presence (Red) of ZCL278. A. Delta G plots from Cdc42 urea denaturation. B. Urea denaturation of Cdc42 WT in the presence and absence of the small molecule. (Cm of Cdc42 alone is 3.66M and 4.85M for Cdc42+ZCL278) C. Urea denaturation of Cdc42 T35A in the presence and absence of the small molecule. (Cm=4.4M) D. Limited chymotrypsin digestion of Cdc42 in the presence and absence of ZCL278. All samples had DMSO as a control.

ZCL278 Makes Cdc42 Resistant to Temperature Denaturation

To further test the small molecule's effect on Cdc42's stability, temperature denaturation studies were carried out. On the Jasco 1500 spectrophotometer, the temperature of Cdc42 was gradually increased and a circular dichroism reading was taken at each step. The molar ellipticity change at 208nm was recorded and plotted against temperature in Origin to visualize changes in protein's helical structure as the temperature increases. Results show a slight in temperature resistance of Cdc42 when bound to ZCL278. The unfolding temperature goes from 60^0C for Cdc42 alone to 85^0C when bound to ZCL278. Differential scanning calorimetry (DSC) is another technique that can be used to study the thermodynamics of a protein's unfolding as the temperature increases. [33] DSC results show a slight increase in Tm when ZCL278 is added to ZCL278.

(57.8^{0}c±2.7e-2 vs 59.3^{0}C±2.7e-2). Taken together, these results suggest that ZCL278 shields the protein from thermal as well as enzymatic and chemical denaturation, highlighting the need for further investigation of this small molecule's influence on Cdc42.

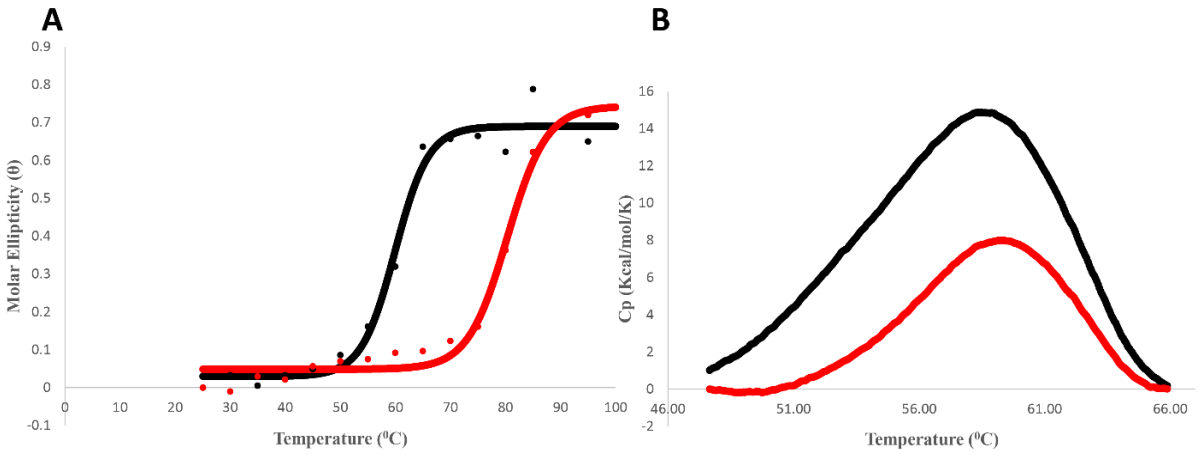


Figure 4. Thermal Stability of Cdc42 in the presence (Red) and absence (Black) of the small molecule. A. Thermal denaturation profile from CD readings at 208 nm. Tm of 59.94^{0}C for Cdc42 and 80.31^{0}C with ZCL278 added. B. Differential scanning calorimetry of Cdc42 with (red) and without (black) ZCL278. Tm of 57.8^{0}c±2.7e-2 for Cdc42 and 59.3^{0}C±2.7e-2 with ZCL278 added.

Cdc42 Intrinsic Hydrolysis is Inhibited by ZCL278

As GTPases, Cdc42 proteins function as molecular switches through GTP hydrolysis. [22] The cellular GTP hydrolysis is facilitated by RhoGAPs, since Cdc42 intrinsic hydrolysis is very low. [34] In this experiment, the effect of ZCL278 on the intrinsic activity of Cdc42 was investigated. GTP hydrolysis occurs in the Cdc42 catalytic domain and facilitated by the Switch 1 and Switch 2 residues as illustrated in Figure 2. [35][36][37]. We explored effects of the binding of the small molecule into this region on hydrolysis. To measure this, a concentration dependent hydrolysis assay was performed. Hydrolysis was measured at increasing concentrations of Cdc42. After incubation with GTP, hydrolysis was stopped, and the amount of the released phosphate was measure using fluorescence. The data shown in figure 5 shows that ZCL278 slightly affects Cdc42

intrinsic GTP hydrolysis. The level of hydrolysis dropped when ZCL278 was added. This finding shows that Cdc42 activity and function is inhibited by ZCL278. It also confirms that ZCL278 may bind in or near the regions involved in the hydrolysis.

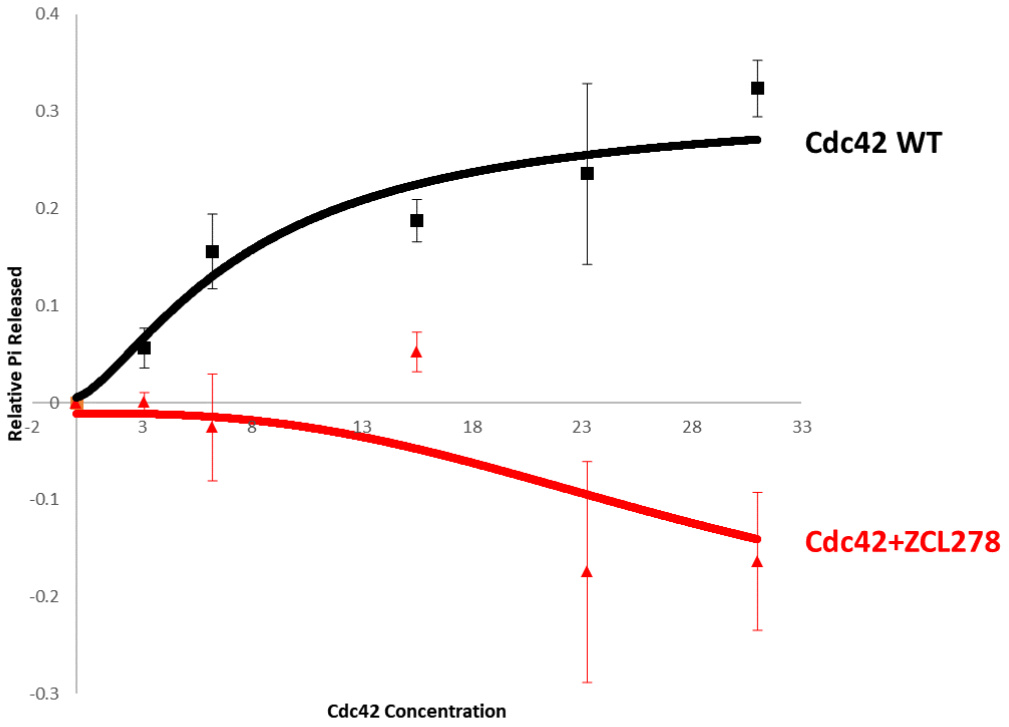


Figure 5. Effects of ZCL278 on Cdc42 intrinsic GTPase activity. Increasing concentration of Cdc42 in the presence (Red) and absence (black) on the small molecule (ZCL278).

II.5 Summary and Discussion

Cdc42 plays a major role in many cell processes such as cytoskeleton control to motility and survival. This protein is crucial in cancer drug discovery since it regulates several cell processes and responsible for cancer progression. [16] Approaches targeting Cdc42 have not been sufficient in understanding/finding a suitable Cdc42 inhibitor. The main reason comes from a lack of understanding of the interaction between ZCL278 and the protein.

To design and discover better drugs, we need to understand the effects of these drugs on the protein's stability, structure, function. A detailed characterization of the binding of the drug to the protein is needed. Detailing what regions are affected and what residues are involved.

In this study, we used computational, biochemical, and biophysical methods to characterize the interaction between ZCL278 and Cdc42. We used, CD, DSC, and fluorescence spectroscopy to show that the protein's stability is enhanced by the small molecule. It is made resistant to chemical, temperature and enzyme denaturation. Using several binding techniques, we calculated an average binding affinity of Cdc42 to ZCL278 of 8.9uM. This is a moderate affinity of the protein, but a drug candidate will need a better affinity. Our results were also consistent with MDS and published data.

The simulations also showed that the protein binds in the Switch 1 region. It is important to note that the simulated binding is opposite of the poses proposed in the Friesland paper. But since we used high level dynamics evaluating multiple poses, we are confident that our proposed binding model is more accurate. This is compared to the docking mechanism used in the paper without simulations. Also, the simulations and mutation studies have shown us that the small molecule binding more likely affects the Switch 1 and Switch 2 regions in a pocket surrounded by

amino acids T35, V36, F37, D38, N39, F56, D57, T58, Y64, L67 and L70. This small molecule also affects Cdc42 GTPase function.

These findings show that ZCL278 is a promising Cdc42 inhibitor. It affects the protein's stability, structure, and GTPase activity. One thing to note is that ZCL278 has a low solubility in water and buffer. It needs to be dissolved in 100% of DMSO before being added to buffer (Resulting in 1% DMSO in buffer on average). This interferes with multiple biophysical and biochemical assays and hinders its ability as a drug candidate since DMSO is toxic.

Furthermore, the affinity of 8.9uM is not high enough for a drug. Henceforth, we are using information from this design better Cdc42 inhibitors. This binding information will be used to modify the molecular design and incorporate functional groups that render the protein soluble without affecting the inhibitor's effect on the protein.

Using ChemSpace, a library of over one billion small molecules was searched for similar crucial functional groups as ZCL278. The Swiss Institute of Bioinformatics tools was then used to compare the absorption, distribution, metabolism, and excretion (ADME) of the molecules and Autodeck Vina used to test the binding of those small molecule to Cdc42.[28][29] The SwissADME tool was also used to simulate and compare the small molecules solubility in water. Using these tools and other information in the next chapters, we have come up with small molecule candidates that have a similar affinity with higher solubility values than ZCL278. those findings are presented in Chapter 4 of this study. These results will be valuable for the next phase of discovering better Cdc42 inhibitors.

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Chapter III. One Step Purification of an Active Pak1 Derived Peptide

III.1 Abstract

PAK proteins are important serine/threonine kinases in cancer growth and progression pathways. The PAK proteins bind to multiple effectors and they induce various processes in the cell such as cycle progression, proliferation, and survival. Research has proven important to study PAK interactions with other proteins because of the abnormalities in these interactions that result in diseased cell states. Because of its complex structure and size (60.65Kda), studies using active derivatives with smaller sequences have been used. In this study, a biologically active PAK derivative called PBD46 is used. This derivative isolated in 1998 by Oswald et. al is purified using a GST construct. This GST bound peptide is usually expressed in E Coli and purified through two or three rounds of chromatography. This process takes more than 5 days and results in low protein yield and purity. In this study, we present a quicker and more efficient method to purify PBD46. Using techniques from a recently published peptide purification method, we performed heat treatments on the proteins and experimented with conditions that could separate the cleaved GST from PBD46. The obtained PBD46 was at a purity higher than the conventional method and it took less steps and a shorter time to isolate it. After isolation, we tested PBD46's structural integrity and stability compared to PBD46 purified using conventional methods. Various biochemical and biophysical assays showed that the two peptides were the same. The interaction of the peptide to other proteins was also studied; purified PBD46 showed a tight binding to active Cdc42. Using this method, another Cdc42 binding peptide was also successfully isolated. In summary, we here highligh a method that will help to purify PBD46 peptides easily, quickly, and more efficiently to study PAK effector binding. This method can be scaled to produce more protein for larger assays, and it can also be modified to synthesize other peptides.

III.2 Introduction

PaK1 was discovered as an effector of Rho GTPases in the rat brain, it was later found to be overexpressed in ovarian cancer. [1][2] Since then, major interest in research for PAKs has increased. PAK1 is now regarded as one of the majorly overexpressed genes in many mammalian cancers. [3] Pak1 is part of a family of 6 different kinases (Pak1-6) that are divided in 2 different groups; group 1 and group 2. [4] Studies show that Pak1 is involved in cell polarity, invasion, and migration. [5][2][6] It is also involved in cell division, proliferation, and survival. [7][8][9] In 1995, different research groups demonstrated that group 1 Paks (Pak1-3) act as effectors of Cdc42 and Rac GTPases while group 2 kinases do not require active Cdc42/Rac to be activated. [10][11][12] The PAK interaction with Cdc42 and Rac is important as it is crucial for cytoskeletal organization and dynamics as well as cell migration. [13] PAKs also interact with many other proteins involved in major cell processes. As kinases, they are involved in phosphorylation and activation of a multitude of proteins. Some examples are the PAK1 phosphorylation of Erk involved in cell migration and Etk/Bmx involved in tumorigenesis. [14][15] In addition to cancers, PAK proteins are also involved in the cardio-vascular diseases and neurological disorders. [16]

In 1998, Adam et. al found that PAK1 had an important role in human breast cancer cell invasion after finding a link in ErbB2 growth factor receptor signaling. [2] Subsequent studies established Pak1 and other Paks as major regulators in the cell affecting different major pathways. So far PAKs are thought to be involved in cancer related processes such as cell-cycle progression, angiogenesis, metastasis, invasion, cell survival proliferation and more. [3] In addition to affecting proteins in the cytoplasm, PAKs are also localized in the nucleus where they affect some proteins involved in human cancers. One example is the PAK1 phosphorylation of integrin linked kinase (ILK) that results in the protein's localization in the cell and insteraction with chromatin. [17]

Consequently, many drug discovery approaches target PAKs for therapeutic purposes. There have been chemical inhibitors, peptides and RNAi targeting PAKs directly using its structural and functional features. [3][16] Other examples include using the autoinhibitory domain of PAK as a kinase inhibitor [18] Instead of inhibiting PAK directly, many approaches target PAK's interaction with different effector proteins. Many compounds and approaches have been used to target PAK protein-protein interactions (PPIs). One of the approaches is targeting Cdc42 interaction with PAK1. This lab is currently studying the effect of a Cdc42 inhibitor, ZCL278 on the PAK1 interaction with Cdc42. [19]

PAK's role in various pathways and cell processes along with its involvement in different diseases make it a valuable target to study and inhibit. However, the PAK's structure and size make it difficult to isolate and study as a whole protein. PAK1, for example, has three different domains; a kinase domain in the C-terminal region that carries out the kinase activity, a binding domain known as the CRIB (Cdc42/Rac interactive binding) region that binds to other proteins and finally an auto-inhibitory domain that suppresses kinase activity. In its regular form, PAK1 is a homodimer in inactive conformation. The kinase domain of one subunit of the complex binds to an autoinhibitory domain of the other. [20] [3] Structural determination as well as protein isolation and purification of the full protein have proven to be difficult. However, depending on what part of the protein being studied, one of the three parts of the proteins can be individually, isolated, synthesized and studied. [21]

The CRIB domain is common in proteins that bind to activated Rho GTPases like Cdc42 and Rac. GTP bound Cdc42 binds to the CRIB region of Pak1 and activates the protein. This activation leads to a cascade of reactions that result in regulation of the cell-cycle progression, proliferation, and survival. [22][3]. Our laboratory is interested in the interaction between the two

proteins and how it can be targeted by small molecules to regulate these effects. But to target these proteins, the CRIB region needs to be isolated. In 1998, the research group led by Oswald et al. synthesized a plasmid containing the 46 main amino acids of the CRIB region. These residues were identified as a minimal-binding domain of PAK to Cdc42. The group named fragment PBD46, a protein binding domain with 46 residues. [23] PBD46 has been an excellent model to study PAK protein's effects on Cdc42. Preliminary binding studies found that PBD46 binds to Cdc42 with an affinity of 20 nM. [1] [24] In this laboratory, we were able to find that PBD46 affects intrinsic Cdc42 GTP hydrolysis [21]. However, when purifying PBD46, the steps involved are numerous, the efficiency of purification is very low, and purity is not guaranteed.

In the lab, the PBD46 plasmid used is bound to GST for affinity chromatography. It is expressed as a GST fusion protein from pGEX-2T plasmid in the E. coli strain BL21 (DE3) cells. When purifying it, the peptide is first made in a bacterial glycerol stock and then cultured overnight in a small seed culture which is then transferred into a bigger culture. After harvesting and centrifuging the sample, the pellet is the lysed, sonicated, centrifuged, and filtered and then ran through a GSTrap FF 5 mL column (GE Healthcare) and eluted with 20mM glutathione containing buffer. After this step, the protein is dialyzed overnight and 20units of thrombin per mg of protein is added afterwards to separate the GST. The protein is then again run through the GST column and collected while the GST stays in the column and eluted later. After this step, the purity of the protein is checked and if not pure, the protein is run through a size exclusion column which separates the protein by size and is more accurate than affinity chromatography. (Figure 1) There are a few problems with this purification procedure. First, with the many steps involved, PBD46 purification can take up to a week to finish. Secondly, because of the steps involved, the protein yield is very low; a culture of 4 litters of media and bacteria makes only 1mg of protein. And

lastly,as shown on Figure 1, if no size exclusion is performed, the protein is usually not fully pure, an extra step that adds another day to the procedure is needed to further purify the peptide.

Our lab needed an efficient, quicker, and more accurate way to purify PBD46. A recent study published in collaboration with Kumar et. al presented a new method to purify GST bound peptides.[25] [26] Using the same approach, a new method to purify PBD46 was developed. The results of this method show no decrease in purity and an increase in efficiency. This method reduces PBD46 purification time by more than half while maintaining purity and improving protein yield. Different conditions, heat and incubation times were tried to insure the best results. The purity and yield of the protein was checked at every step and the purified protein's stability was characterized by CD, Fluorescence, DSC and other biochemical and biophysical techniques. In this study, we found a quicker, purer, and more efficient way to purify PBD46. This method will be used to purify other Cdc42-binding as well.

III.3 Materials and Methods

Protein Growth and Culture

PBD46 was designed, grown and expressed as a GST-fusion protein from the pGEX-2T vector in the BL21 strain of E.Coli. Using LB medium with 0.1% ampicillin, the bacteria was first grown in a 100mL seed culture overnight. The next day, the seed culture was inoculated to a 1L culture and grown at 37^0C to an OD of 0.8-1.0 at 600nm. The culture was then induced with 1 mM IPTG for 4.5 hours. To harvest the cells, they were centrifuged at 6,000 rpm for 30 minutes at 4 °C and washed with 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2mM KH_2PO_4; pH 7.4).

Affinity Purification of GST-PBD46 on FPLC

The PBD46-GST pellet was resuspended in 30mL of lysis buffer (1xPBS, 30mg lysozyme, 1X Halt Protease Inhibitor cocktail (Thermo Scientific), 1.25mM PMSF and a pinch of DNAse). The sample was then sonicated using the Sonifier 150 from Branson Ultrasonics. The mixture was then centrifuged at 18,500 rpm for 30 minutes and passed through a 0.2 uM filter. The protein sample was then loaded onto a prepacked GSTrap column (GE Healthcare) using Akta Start FPLC (GE Healthcare). The protein was eluted with an elution buffer (20 mM Tris and 10 mM reduced glutathione at pH 8.0) and dialyzed in 1x PBS.

To separate the protein from GST, a thrombin cleavage was performed overnight using 150 units of thrombin per ug of protein. The separated mixture was stopped using the 1X Halt Protease Inhibitor cocktail (Thermo Scientific). It was then run on the GSTrap column and the protein was collected while the gst was trapped on the column and eluted later. The protein was then checked for purity using 20% SDS-PAGE and the concentration was checked using Nanodrop Lite (Thermo Scientific). To further purify the protein, it was run on the HiPrep Sephacryl S-100 HR (GE Healthcare) size exclusion column using the Akta FPLC.

Thrombin Cleavage and Heat Treatment

To separate the GST tag from PBD46, it was first subjected to thrombin cleavage. Using one thrombin unit per 150ug of protein, the mixture was left overnight (15 hours) to ensure complete cleavage. To stop the cleavage, 1X Halt Protease Inhibitor cocktail (Thermo Scientific) was added to the mixture. The heat treatment experiment was set up with various temperatures (25, 30, 50, 70, and 90^0C). After a 30-minute incubation, the protein was centrifuged twice at 14000rpm for 10 minutes. The protein concentration was then calculated using the Nanodrop Lite

(Thermo Scientific) . Size and purity were measured using LC-UV MS, MALDI-TOF and SDS-PAGE.

Circular Dichroism

Jasco 1500 CD spectrophotometer was used to measure circular dichroism. At 25^0C, 0.1 mg/ml of PBD46 dialyzed in 50 mM Tris, 10 mM $MgCl_2$ and 50 mM NaCl at PH 8.0 was put into the quartz cuvette. CD measurements of 250 -190 were taken at 5 nm/min. The spectra was then converted to molar ellipticity and graphed.

Fluorescence

The PBD46 peptide was dialyzed in 50 mM Tris, 0.5 mM MgCl2 and 50 mM NaCl at PH 8.0. At 250C, 500uL of 0.1mg/mL PBD46 was added to a quartz cuvette and a fluorescent measurement was taken on the Jasco 1500 CD Spectrophotometer. The protein was excited at 280 nm, and the emission range of 300-400nm. The data was exported and analyzed on excel and Origin.

LC-UV MS

To measure the protein's size and purity, LC-UV-MS was performed for PBD46 using high resolution Shimadzu-IT-TOF MS coupled with Shimadzu HPLC-UV. The sample was passed through a Bio wide Pore C8 reverse column (4.6 mm x 15 cm, 5 um) (Supelco, St. Louis, MO) A solvent rate of 0.7 ml/min was run on the column using a 0.1% formic acid/ acetonitrile gradient of 0-50% over 40 minutes. The ESI MS was operated between m/z 300-1700 in positive ion mode at optimum default settings for the column flow of 0.7 ml/min. The resulting mass was analyzed using the manufacturer provided software. [25]

MALDI-TOF

To analyze the purified peptide sequence, 1μg of the PBD46 peptide was dissolved in 25 mM ammonium bicarbonate and digested using 20 ng of trypsin for 24 hours at 37 °C. The trypsin digest was then desalted and concentrated using "ZIPTM" C18 pipette tips. The eluted sample were mixed with an equal volume of saturated α-cyano-4-hydroxycinnamic acid in 34% acetonitrile and 0.1% formic acid, spotted onto a stainless steel MALDI target, and analyzed by MALDI-TOF MS. The MALDI-TOF MS analysis was performed in the m/z range of 500–5000 using a Bruker Ultra-Flex II MALDI-TOF mass spectrometer in positive ion mode. [25]

Differential Scanning Calorimetry

To study PBD's temperature induced unfolding, DCS experiments were performed using Microcal PEAQ-DSC. 250uL of 1mg/mL of PBD46 were added to the sample cell and the 250uL of the buffer (50 mM Tris, 10 mM $MgCl_2$ and 50 mM NaCl, PH 8.0) in the reference cell. The sample was then heated from 25 to 85 °C at a rate of 60°C/per hour. Buffer subtraction and analysis of the resulting thermograms was done by the manufacturer-provided software.

SNBD Fluorescence Titration

Extrinsic fluorescence was performed by attaching a fluorescent label named sNBD (succidinimidyl 6-[(7-nitrobenz-2- oxa-1,3-diazol-4-yl)amino]hexanoate). [24]To attach the label to the protein, it was incubated with Cdc42 using a ratio of 12:1 (fluorophore: protein). The mixture was then incubated for 3 hours, centrifuged at 4500rpm for 5 minutes and then dialyzed overnight in 50 mM Tris, 10 mM $MgCl_2$ and 50 mM NaCl at PH of 8.0 to remove unbound SNBD. UV Vis and Fluorescence spectroscopy of the protein and buffer were taken at every step to ensure that excess sNBD was successfully removed.

Purified PBD46 in 50 mM Tris, 10 mM $MgCl_2$ and 50 mM NaCl at PH of 8.0 was titrated in 100nM of active Cdc42 for extrinsic fluorescence studies. Starting with 500uL of Cdc42 in a quartz cuvette, 5 uLs of PBD46 were added at each titration and a fluorescence reading was taken with the excitation of 488nm and an emission spectrum of 500-600 nm. After titration, readings of the peak at 550nm were taken and plotted against the concentration of added PBD46. A non-linear fitting model of the equation below was used to create a fitting curve and calculate the protein's binding affinity.

$F=Fi + Ff\ [(KD + Lt + Rt) - \sqrt{}\ ((KD + Lt + Rt)^2 - 4RtLt)]/2Rt$ where F is $\Delta F/Fo$ (Change in fluorescence reading over initial fluorescence), Ff is the final F value, Lt is the concentration of PBD, and Rt is the total of Cdc42. [27]

2D HSQC NMR Spectroscopy

One mL of PBD46-GST glycerol stock was grown in 10mL of LB for 5 hours. The growth was then centrifuged at 4500rpm for 10 min and then washed with the NMR buffer (10 mM NaH_2PO_4, 5 Mm $MgCl_2$, and 25 mM NaCl, 1mM NaN_3 at PH:5.5). This mixture was then dissolved in 100mL of M9 media (95.5 mM KH_2PO_4, 57.4mM K_2HPO_4, 63.4mM Na_2HPO_4,13.8 mM K_2SO_4, 9.35 mM NH_4Cl, 0.1 mM $CaCl_2$, and 0.4% glucose with a vitamin mixture of 0.4 mg each of pantothenoic acid, choline chloride, folic acid, myo-inositol, nicotinamide, pyridoxyal hydrochloride, and riboflavin and 25 mg/L thiamine). The 100mL seed culture was grown overnight at 37^0C and added to a 2L LB media flask the next day. The culture was monitored and stopped at O.D of 0.8-1.0. The harvest and affinity purification of these proteins was the same as the procedure for unlabeled samples described above. PBD46 was dialyzed in NMR buffer and concentrated to 0.3-0.5mM. At 25^0C, 450uL was added to 50mL of D_2O and the sample was loaded

into the NMR machine. The HSQC of the PBD46 were run on a Bruker Avance 500 MHz NMR. The 2D HSQC spectra were visualized, processed, and analyzed using Topspin and Sparky.

III.4 Results

Purification of GST fused pak1 derivative using affinity and size exclusion chromatography

Purification of PBD46 was first performed using affinity and size exclusion chromatography. The protein was expressed in E. Coli with a GST tag and a thrombin cleavage site. In the conventional method used in the lab. A two-step GST affinity chromatography purification is performed after expression, lysis, centrifugation, and filtration of the bacterial cells to isolate proteins. In other cases, an additional size exclusion chromatography is performed to get a purer protein.

This is a well-established technique that separates and purifies the protein from the GST. However, when dealing with small peptides, it is hard to separate these proteins and get a high purity. Moreover, further methods to increase the purity greatly lower the yield. For example, using the method described above, we get 1mg per 1.5L. And it takes a day for expression, a day for affinity chromatography, another day for the second affinity chromatography, and if needed a fourth day for size exclusion chromatography. The chromatograms in Figure 1 highlight the steps involved in this procedure. The first two steps produce a purity of 68.6% and an additional size exclusion add 91.2% in purity, but the extra steps greatly reduce the final protein yield. While this purification method is well established but PBD46, the yield is too low to perform experiments. A better method is needed to improve yield.

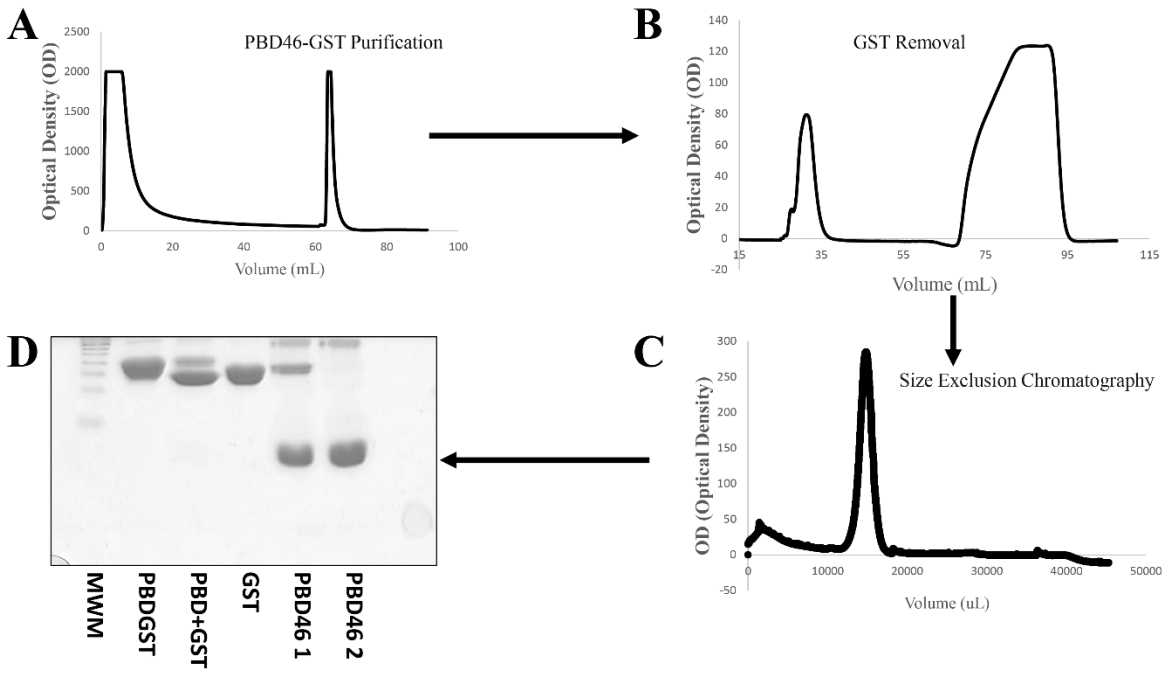

Figure 1. Conventional three-step chromatography method for PBD46 purification. A. First affinity chromatography to isolate PBD46GST. B. Second affinity purification after thrombin cleavage to separate GST from PBD46. C. Size exclusion chromatography to remove any impurities left. D. SDS-PAGE showing results from every step.

Separation of GST From the Pak1 Derivative Generates a Pure Protein at High Temperature

To separate PBD46 from GST, a new method using heat treatment was used. As the protein is heated more, GST should aggregate and separate from PBD46. In this method, the protein was first cleaved by thrombin overnight and different samples were incubated at different temperatures for 30 minutes. After stopping the cleavage by a protease inhibitor, the samples were incubated at 30, 50, 70 and 90 degrees Celsius. After heating, the samples were centrifuged at 13,000 rpm for 10 minutes to remove GST. A second centrifugation at the same speed and time was performed to remove excess GST. After, an SDS-PAGE of the supernatant and the pellet were run. The pellet was suspended in 8M urea before adding the sample dye. As seen in Figure 2, GST starts getting out of the solution at 50 degrees and by 90 degrees, the GST is pulled out of the solution completely. This data shows that PBD46 can be purified from GST successfully using this method.

We have decided to go with the temperature of 70^0C because it shows the best purity with complete separation. We have created and established a method that can be scaled to make more proteins.

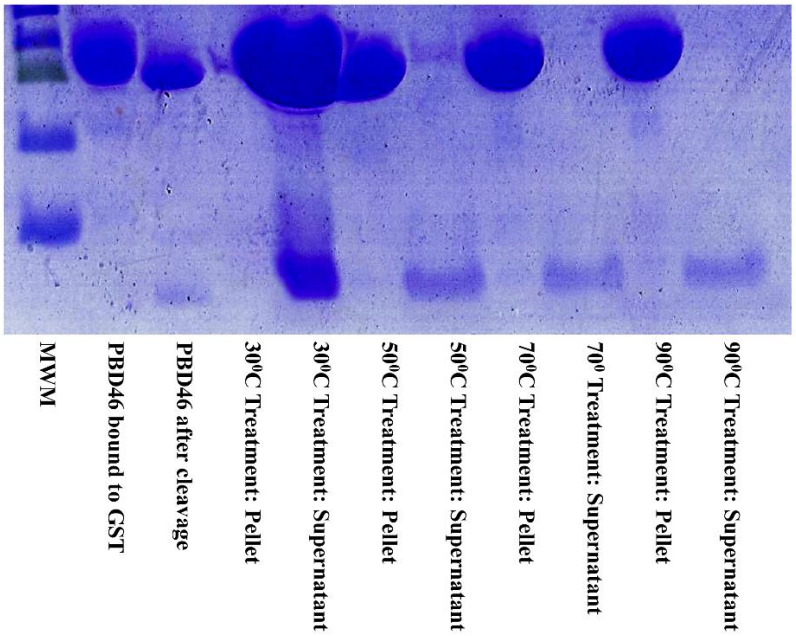

Figure 2. Purification of GST bound PBD46 by heat treatment. The sample was heated at various temperatures for 30 minutes after thrombin cleavage. The aggregated GST was then removed by a two-step centrifugation process.

Purity, Mass and Sequence of the Heat Purified PBD46

The results from SDS PAGE showed that the heat purified pbd46 has a size of 10Kd. This is consistent with the pbd46 purified using affinity chromatography. With 46 amino acids, PD46 should have a size of 5Kd. However, it has been reported that PBD46 forms a doublet that shows up at 10 Kd. [28] To further verify the proteins size and purity, LC-UV MS was performed on the peptide. Using the Shimadzu HPLC-UV, the size of the peptide was once again confirmed to be 10Kd with no impurities in the sample. Furthermore, MALDI-TOF was performed to look at the purified peptide sequence. The tryptic peptides measured by MALDI were an exact match to the PBD sequence. These results show that the heat purified peptide has the same size and sequence of the conventionally purified peptide. However, the purity of the heat purified PBD46 is 5%

higher than the conventionally purified one. While the size and sequence of the peptides are the same, more assays are needed to compare the structural integrity of the two peptides.

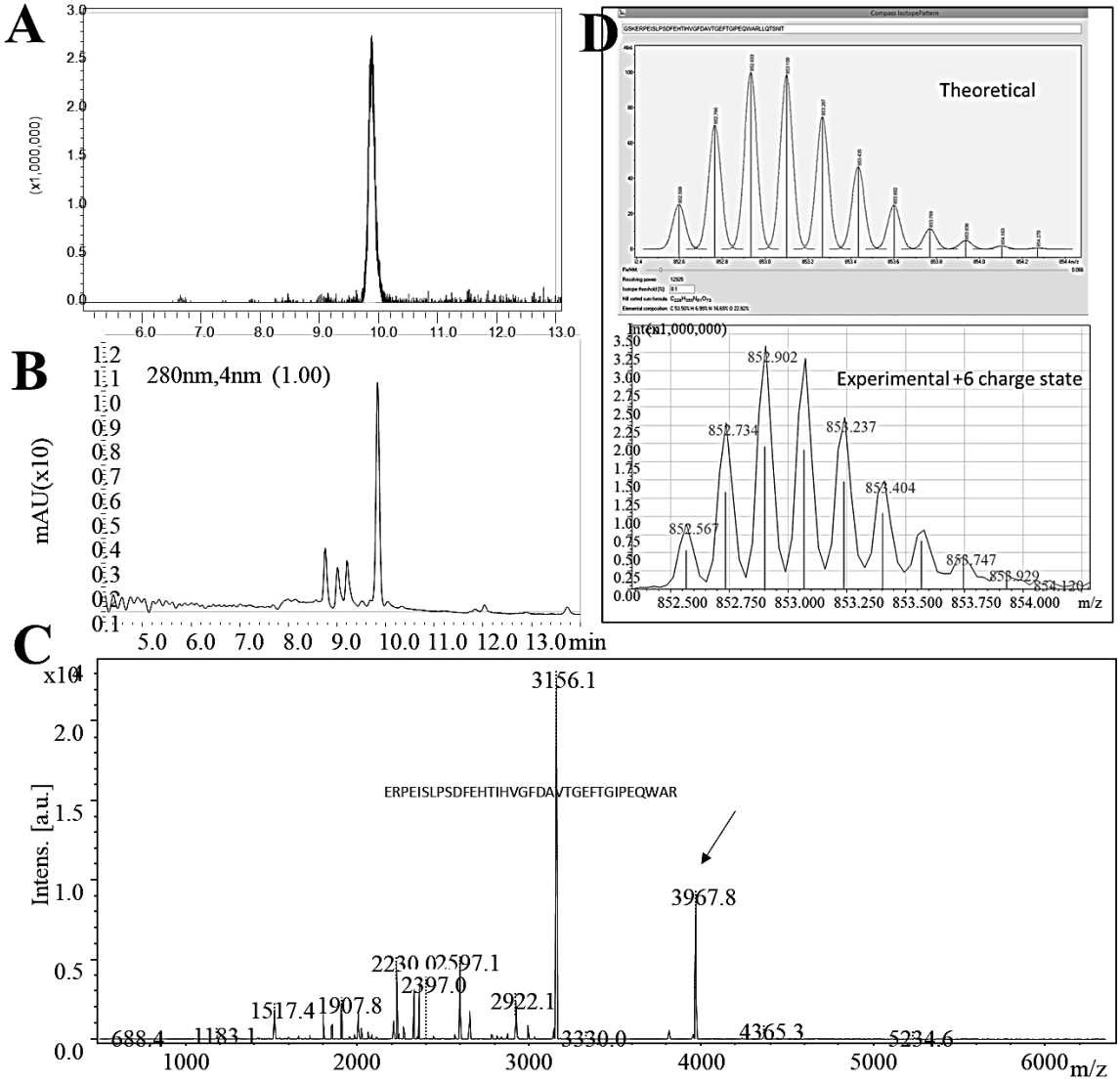

Figure 3. Purity, size, and Identity of PBD46 by mass spectrophotometry. A. LC-Mass spectrophotometry of PBD46. B. LC-UV spectrophotometry. C. MALDI-TOF of PBD46 triptic peptides to confirm the peptide sequence. D. The predicted vs observed MS charge distribution.

Characterization of the Stability, Structure and Folding Pattern of the Heat Purified PBD46

Using multiple spectroscopic methods, the stability, structure, and folding pattern of the heat purified peptide were investigated and compared to the conventionally purified peptide. The thermal stability was investigated and the denaturation profile of the heat purified PBD46 was compared to the conventionally purified one using DSC. As seen on Figure 4A, the Tm of the conventionally purified method (41.6 ◦C) is around the same as the Tm from the heat purified PBD46 (41.3 ◦C). The folding and secondary structure patterns of the two PBD46 samples was also measured compared. Circular dichroism was used to look at the secondary structure and fluorescence spectroscopy was used to look at the folding pattern. The heat purified PBD46 showed no difference in CD and FL and even absorbance spectra. These results mean that we have synthesized PDB46 with same structure, sequence, and denaturation profile. Oswald et. al remarked that this peptide does not only have the same structure and folding pattern, but it is also as biologically active as the CRIB domain and can bind to active Cdc42. [29]

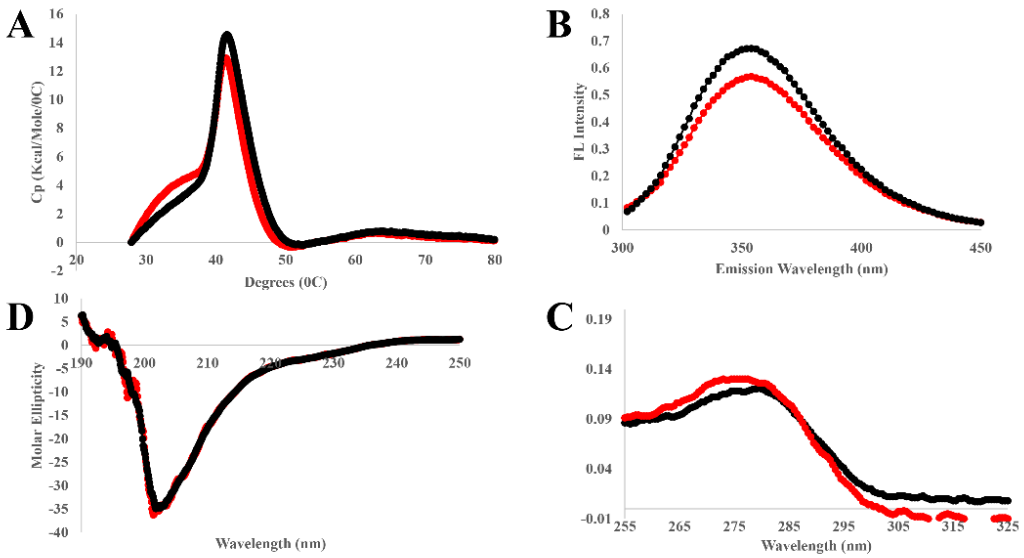

Figure 4. Spectroscopic and calorimetric assays comparing the heat-treated peptide (red) with the conventionally purified peptide (black). A. Differential scanning calorimetry. B. Fluorescence spectroscopy. C. Absorbance spectroscopy. D. Circular dichroism.

PBD46 binds to Cdc42 with high affinity

An important protein interaction in the PAK1 pathway is binding to Cdc42. When PAK binds to Cdc42 in the CRIB region, the PAK is activated. This activation is crucial for cytoskeletal organization and dynamics as well as cell migration. [13] In order to test the functionality of the purified PBD46, the binding between Cdc42 and PBD46 was investigated. In this experiment, a covalently bound extrinsic fluorophore sNBD was used. sNBD binds to lysine 150 on Cdc42 and can be excited at 488 nm with an emission range of 545nm.[27] Therefore, this fluorophore can be explored to measure the binding of another protein since the excitation and emission spectra are outside of tryptophan fluorescence. To covalently bind sNBD to Cdc42, they were incubated together at room temperature for 3 hours at a ratio of 12:1 (Fluorophore:Protein). Then, a series of centrifugation and dialysis were performed to get rid of unbound sNBD. Bonding studies were performed by titrating PBD46 to Cdc42. After the experiment, a nonlinear fitcurve was generated in origin and a binding affinity of 83.26nM was obtained. This shows that the purified PBD46 by heat treatment is active and binds to Cdc42.

HSQC Backbones of Purified PBD46

PBD46 was expressed in M9 media to produce a 15N labeled, GST bound peptide. After affinity chromatography and thrombin cleavage, one half of the protein was separated by the the heat treatment method and the other by the conventional affinity chromatography method. This experiment wanted to test if the heat treatment of the peptide affects any backbone dynamics. Two dimensional $^{1}D^{15}N$ NMR is a great way to measure these dynamics. As shown in Figure 5B, the backbone dynamics of both peptides remained the same. There are no shifts in the backbone residue peaks.

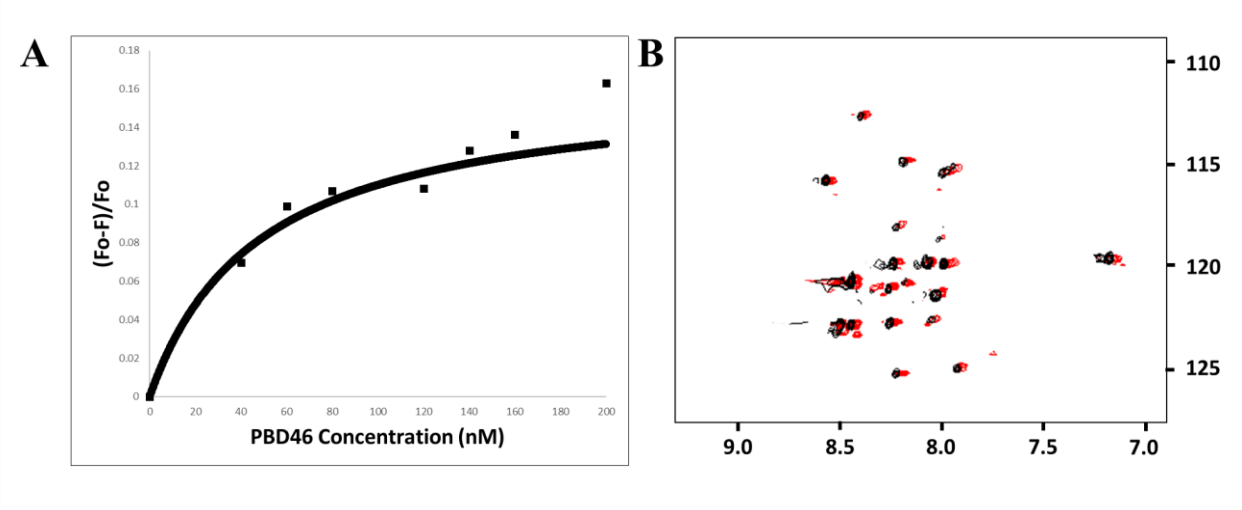

Figure 5. A. Characterization of PBD46-Cdc42 binding to Cdc42 though fluorescence titration of the peptide to an sNBD-labeled Cdc42. B. HSQC spectra of heat treated PBD46 (red) on top of the conventionally purified PBD46 (black)

Heat Treatment of Cdc42-Binding, GST-Bound Peptides

As shown in our previous publication, this method can work on other peptides. [25] There are a multitude of Cdc42 binding peptides used to study the interaction of Cdc42 with other kinases. One example in the peptides helping to study the crucial interactions between Cdc42 and Ack as well as WASP. [30][31][32] GST constructs of these two proteins have previously been designed. Since our laboratory works with these peptides, the heat treatment method was used on these proteins. Thrombin treated Ack was separated from GST using the same method. (Figure 6A) To confirm that this method only works for peptides and not large proteins, we attempted to purify GST bound Cdc42 (21Kda). As shown in Figure 6B, thrombin treated Cdc42 was not able to be separated from GST under the same temperature and incubation conditions.

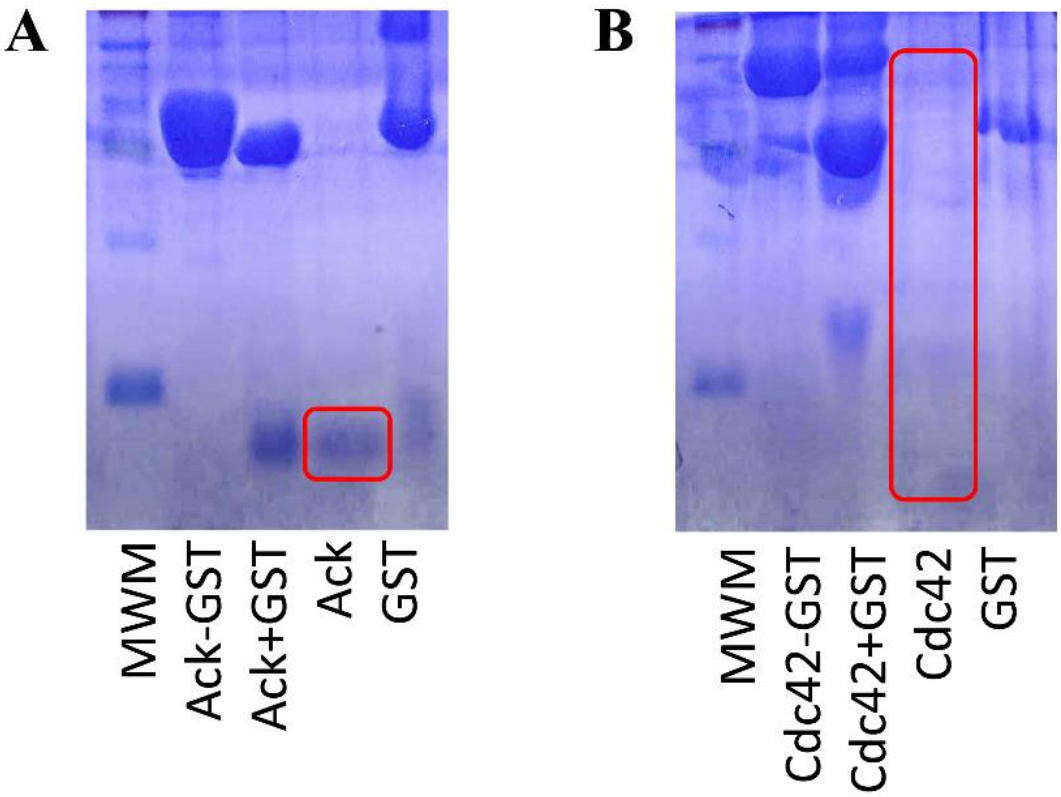

Figure 6. One step heat purification Method on GST bound Ack (A) and Cdc42 (B).

III.5 Summary and Discussion

The Pak1 are important proteins in cell functionality. They play important roles in various cancers. [33] Pak1, the most common and important of the PAKs binds to Cdc42 and activates a cascade of pathways that lead to different cancers, Alzheimer's disease and more. [34] To study PAK1, a biologically active peptide is used. This is taken from a CRIB region responsible for binding effector proteins. This section is important to study the interaction between Cdc42 and PAK1. This peptide is usually purified by affinity chromatography by binding it to GST. This procedure has been used for decades years and it has been consistent but it generates very low protein yields.[29] Additionally, the conventional method takes 5 days and generates a peptide with low yield and quality. New purification methods that are faster and that generate higher yields

are needed. This lab set out to find a solution by studying new methods used to purify peptides. We tried different methods and we chose one that uses heat treatment to purify peptides. [25] This study is important because studying the interaction requires pure proteins and some of the binding assays such as ITC require high amounts of the peptide. Improving this purification method will help all researchers working on these proteins in the field to get better results faster.

In this study, we performed a quick and effective purification of PBD46 via heat treatment. The heat aggregates GST and it is then centrifuged out. What we have left is pure PBD46. We explored this by trying different temperatures and incubation times and we decided that the best conditions are 30-minute incubations in 70 degrees Celsius. When compared to the protein purified using affinity chromatography, the protein showed a higher purity and yield. We also looked at the denaturation profile and secondary structure of the peptides. It shows that they have the same structure and denaturation profile. We also explored how this protein binds to Cdc42 to make sure it is active. Results show a binding of the heat-treated peptide to Cdc42. Looking at 2D NMR experiments, the new PBD46 overlay does not show any shift in the NMR resonance of the residues. These results all show that the backbone structure and function is not affected by the heat through this new method of purification. This eliminated the additional chromatography steps and cuts time by less than half. In addition, the purity of this protein is even higher that the conventional method. The yield is higher as well.

There are other Cdc42 binding peptides being studied at the moment. In our lab, Ack is another peptide used to study Cdc42 interactions. This peptide was also successfully purified using the new method. Results from this method will help researchers around the world to study Cdc42 interactions faster and more efficiently. This method can also be used to purify other peptides and even scaled up for industrial productions.

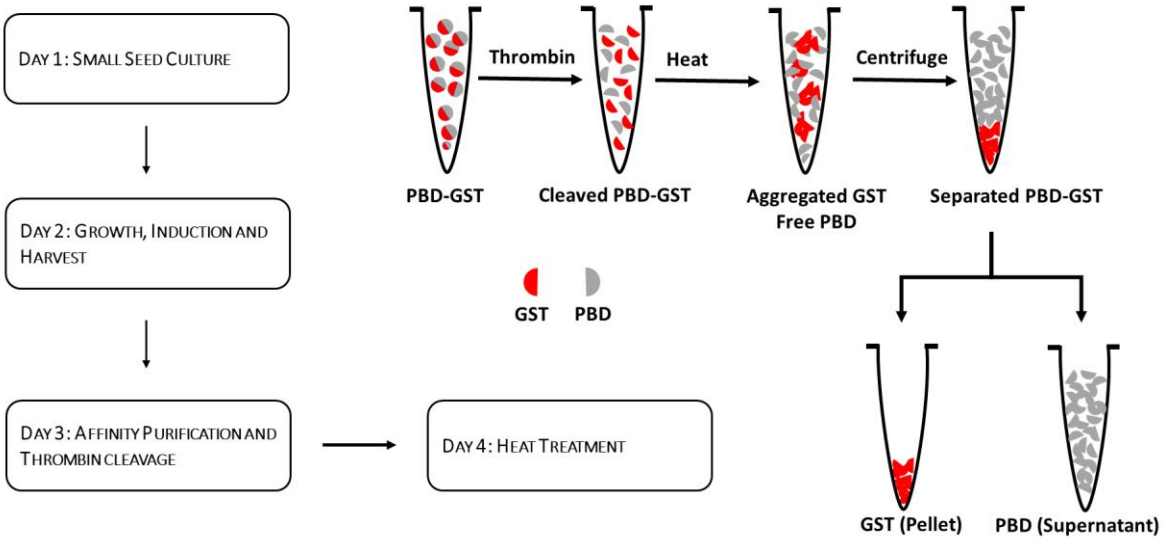

Figure 7. Overview of the purification of small peptides using the heat treatment method.

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Chapter IV. Small Molecule Binding to Cdc42 Inhibits its PAK1 Interaction

IV.1 Abstract

Protein interactions involving Ras GTPases are crucial in regulating major cell pathways. Because of the complexity of Ras GTPase regulation, their high affinity to GTP and their dynamic nature, targeting these GTPases directly has been problematic in the last decades. That is why some researchers have moved to targeting their interaction with their downstream effectors. While the KRas GTPase PPI inhibitors have gained success lately, inhibition of PPIs involving Rho GTPases has not been explored. In this chapter, we studied the interaction of Cdc42, an important Rho GTPase, and its interaction with PAK1, a serine/threonine kinase involved in many mammalian cancers. A novel small molecule was used study the inhibition of this interaction. Using computational and biophysical assays, the binding of a PAK1 derived peptide to Cdc42 was studied. The results of this study show that ZCL278 inhibits PAK1 binding to Cdc42 and reduces PAK1's effects on Cdc42 activity. We also show that ZCL278 binds in a pocket between Switch 1 and Switch 2 regions of Cdc42 and the CRIB region of PAK1. While successful, ZCL278 is not highly effective in binding to Cdc42 and inhibiting PAK1 binding. Using our results, we here present novel small molecules that might address the ZCL278 shortcomings and serve as candidates for better inhibitors of Cdc42 PPIs.

IV.2 Introduction

Ras GTPase interactions continue to be explored because of their involvement in various cell pathways and diseases. Compared to other major oncogenes, Ras GTPase induced conditions have been harder to treat and the Ras GTPase drug discovery field has not generated any successful drug. [1][2] [3] This is mainly because of their structure and dynamic nature, their high affinity to GTP and GDP as well and the complexity of their regulation. However, small molecules targeting protein-protein interactions of Ras proteins have shown great potential in recent years. [4][5] In this study, PPIs involving Cdc42, a protein in the family of Rho GTPases, are investigated as a model for Ras protein signaling. Cdc42 is a GTPase involved in multiple cell processes such as polarity, motility, invasion and proliferation. [6] In the cell, Cdc42 regulates migration and adhesion and is therefore involved in cancer progression. Cdc42 malfunction is observed in various cancers such as breast, colon and prostate [6] Understanding how this protein operates and binds to others will give us more insights in Ras protein mechanism and inhibition.

As a GTPase protein, Cdc42 needs to be activated to bind to effectors and activate other proteins. To achieve this, Cdc42 is converted from GDP bound form to GTP bound form. This switch between active and inactive forms of Cdc42 is regulated by different effector proteins. The guanine exchange factors, (GEFs) regulate the exchange between GTP and GDP bound forms of the protein while GTP activating proteins (GAPs) catalyze the GTP hydrolysis activity. Guanine dissociation inhibitors (GDI) prevent the GDP / GTP exchange by sequestering Cdc42 into the cytosol. [7] [8] In addition to the GTP binding site, the Cdc42 structure also has other features that are important to its function. In terms of PPIs, there are two important regions, Switch 1 (residues 28–40) and Switch 2 (residues 57–74) that are responsible to binding various effector proteins.

Switch 1, in particular, is responsible for binding to many major downstream effectors of Cdc42 to transmit signals and affect various cell processes.[9]

The effectors that bind to the Switch 1 region have a similar conserved binding sequence region known as the CRIB region. The CRIB region has a protein sequence of XISXPXXXXFXHXXHVGXD. This sequence is found in many Cdc42 and Rac1-binding proteins. Some of the proteins with a CRIB region are ACK, WASP, and PAK proteins. [10] [11] When Cdc42 binds to ACK, ACK is activated and activates pathways involving control of intracellular trafficking events affecting processes linked to growth arrest and differentiation. [12] In the case of wasp, the binding of Cdc42 results in to control of actin polymerization and filopodia growth. [13] The most abundant effector of Cdc42 is PAK1, Cdc42 binds and activates PAK1 which in turn activates and phosphorylate different proteins.

PAK is a large family of protein kinases involved in different cell processes. There are 2 main families of PAK kinases. Family 1 composed of PAKs 1-3 is activated by Cdc42/Ras GTPases and has an autoinhibitory domain. Family 2 of PAK 4-6 does not have an autoinhibitory domain and does not need Cdc42/Rac activation. Of all PAK proteins, PAK1 is the mostly expressed in human cancers. Studies show that PAK1 is involved in cell polarity, invasion, migration and more. [14][15][16] It is also involved in cell division, proliferation, and survival. [17][18][19] Consequently, PAK1 is affected in a large number of human cancers. To be activated, PAK1 binds active Cdcd42 or Rac. The Switch 1 region of Cdc42 binds to PAK1 in the CRIB region and activated PAK1 can now phosphorylate different proteins. Studies by Yong Jae Shin et al have found that residues 75 to 83 in CRIB region of PAK are involved in Cdc42 binding. [11] [20] In the Switch 1 region, PAK1 binds to residues 40 to 46 of Cdc42. [21]

Because of the role of Cdc42-PAK1 interaction in cell processes and its involvement in cancer, there has been great interest in targeting and inhibiting this interaction for therapeutic purposes. However, while inhibitor design targeting PAK1 has been going on for years, there remains to be an effective inhibitor of Cdc42-PAK1 interaction. [22] Since peptides and other macromolecules have not been effective in inhibiting PPIs, small molecules may be ideal candidates since they can occupy small binding pockets between the two proteins. [4]

In general, Small molecules interfere with PPIs through three different methods, orthosteric inhibition, allosteric regulation, and interfacial binding. Orthosteric inhibition involves molecules competing with interacting partners. In allosteric regulation, the small molecules bind at targets different from the interaction and induce dynamic changes that affect the interaction. Lastly, interfacial inhibition involves the small molecule binding to the already bound protein complex and locking it in an active state. [4] In Cdc42 pathways, the protein interaction is targeted by modeling the interaction using a structure-based drug design then performing a compound lead simulation to find hits which are then tested in the laboratory against proteins and cells for activity. Using these approaches, a few molecules targeting Cdc42 PPIs have been designed. [8] One of the recent promising molecules is ZCL278. [23]

ZCL278 is a small molecule inhibitor of Cdc42-intersectin binding. This small molecule was designed based of the binding interface between the two proteins. [23]Through computer modeling and high throughput testing of several molecules, ZCL278 was chosen as the best candidate. When tested in cells, ZCL278 inhibited several Cdc42 related cell processes like microspike formation and neuronal branching. The inhibitor also reduced Cdc42 activity in cells. In the study, the group showed that ZCL278 was specific to Cdc42 and not Rac, which is another Rho GTPase. [23]

Further studies testing ZCL278 have confirmed that it is an efficient Cdc42 inhibitor. [24] [25] In our lab, we have shown that ZCL278 inhibits Cdc4 and affects its stability and structure. It also inhibits intrinsic Cdc42 GTP hydrolysis. Through MDS and other assays, we have shown that the Switch 1 region might be involved in the ZCL278 binding. (See chapter 2)

However, the molecular details of ZCL278 interaction with Cdc42 has not been fully explored. Furthermore, the effects of this inhibitor on Intersectin and PAK1 binding have not been studied. It is not known whether ZCL278 is an orthosteric, allosteric, or interfacial binding inhibitor [24] In this study, we used ZCL278 to investigate its effect on Cdc42 binding to a PAK1 fragment of 46 amino acids, PBD46. This fragment comprises of amino acids from the CRIB region that are responsible for Cdc42 binding. Using computational models, in vitro binding assays, fluorescence titration studies, isothermal titration, and hydrolysis assays, we investigated the effect of ZCL278 on Cdc42-PBD46 interaction.

IV.3 Materials and Methods

Growth, Expression and Purification

Histidine tagged Cdc42 WT constructs were cloned into a pET-15b vector and over expressed in *E. coli*. One mL of a stored glycerol stock was inoculated in 100mL of LB broth media with 100uL of ampicillin and incubated at 37°C overnight. The 100 seed culture were then added to 2L of LB media and 2mL ampicillin. The growth was incubated and monitored on a shaker at 37°C. A 600nm OD reading of 0.4–0.6 was achieved before the culture was induced by 1mM isopropyl β-D-thiogalactopyranoside (IPTG). After 5 hours, cells were harvested by centrifugation at 6500rpm for 30 minutes. The pellets were then washed in 25mM Tris, 50mM NaCl, 10mM $MgCl_2$, P.H 8.0 and stored at -80^{0}C. The his-tagged Cdc42 WT was purified by

affinity chromatography on AKTA Start FPLC. Gel electrophoresis, LC-UV MS and MALDI-TOF were used to verify the molecular weight and purity of the proteins.

Nucleotide Exchange

To activate Cdc42, a nonhydrolyzable GTP analog (GMPPCP, Sigma) was attached. The protein first was dialyzed in 25mM Tris, 50mM NaCl, P.H 8.0. It was then incubated for 2 hours at 4°C with 5mM EDTA and a 5:1 molar ratio of GMPPCP:protein. Prior to desalting, the PD-10 column (GE Life Sciences) was equilibrated with the 1x PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2mM KH_2PO_4; pH 7.4) prior to adding 5ml of protein. One mL fractions were collected and tested for protein presence by a Bradford assay. After all the protein was collected, 10mM of $MgCl_2$ was added.

PBD46 Growth and Culture

PBD46 was expressed as a GST-fusion protein from pGEX-2T in the E. coli strain of BL21. Using LB medium with 0.1% ampicillin, the bacteria was first grown in a 100mL seed culture overnight. The next day, the seed culture was inoculated to a 1L culture and grown at 37^0C to an OD of 0.8-1.0 at 600nm. The culture was then induced with 1 mM IPTG for 4.5 hours. To harvest the cells, they were centrifuged at 6,000 rpm for 30 minutes at 4 °C and washed with 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2mM KH_2PO_4; pH 7.4).

Affinity Purification of GST-PBD46 on FPLC

The PBD46-GST pellet was resuspended in 30mL of lysis buffer (1xPBS, 30mg lysozyme, 1X Halt Protease Inhibitor cocktail (Thermo Scientific), 1.25mM PMSF and a pinch of DNAse). The sample was then sonicated using the Sonifier 150 from Branson Ultrasonics and centrifuged at 18,500 rpm for 30 minutes and passed through a 0.2 uM filter. The protein sample was then loaded onto a prepacked GSTrap column (GE Healthcare) using Akta Start FPLC (GE Healthcare).

The protein was eluted with an elution buffer (20 mM Tris and 10 mM reduced glutathione at pH 8.0) and dialyzed in 1x PBS.

Thrombin Cleavage and Heat Treatment

To separate the GST tag from PBD46, it was first subjected to thrombin cleavage. Using one thrombin unit per 150ug of protein, the mixture was left overnight (15 hours) to ensure complete cleavage. To stop the cleavage, 1X Halt Protease Inhibitor cocktail (Thermo Scientific) was added to the mixture. The heat treatment experiment was set up with various temperatures (25, 30, 50, 70, and 90^0C). After a 30-minute incubation, the protein was centrifuged twice at 14000rpm for 10 minutes. The protein concentration was then calculated using the nanodrop Nanodrop Lite (Thermo Scientific). Size and purity were measured using LC-UV MS, MALDI-TOF and SDS-PAGE.

Isothermal Calorimetry

The binding between Cdc42 and PBD46 was measured by ITC using a MicroCal iTC200 (Malvern Inc.) At 25^0C, the sample cell contained CDC42-GMPPCP at concentrations in the range of 10–20 uM with 0.1-0.5% DMSO. In the syringe, PBD46 at 200–300 uM was loaded. ZC278 was dissolved in DMSO at an original concentration of 3mM and added to Cdc42 to achieve a concentration of 100uM. For each sample, a speed of 750 rpm was maintained while performing 30 titrations. A control of the experiment was obtained by titrating PBD46 into buffer alone and relevant subtractions were made. PBD46 experiments were performed with and without ZCL278. The resulting ITC graphs fitted by using the manufacturer provided Microcal Origin software to calculate the Kd and other thermodynamic features.

GTPase Hydrolysis Assay

GTPase Hydrolysis assays were performed in triplicates using the ATPase/GTPase Activity Assay kit from Sigma Aldrich. At 25^0C, 10uL of 0-50uM of Cdc42 were used with and without ZCL278 in a buffer of 50 mM Tris, 10 mM $MgCl_2$ and 50 mM NaCl at a PH of 8.0. After adding protein and buffer, 30uL of 50uM GTP was added to the 10uL solution to start the reaction. 100uM of ZCL278 and 10uM PBD46 were added in each sample accordingly. The reaction was incubated for 45 minutes and stopped by adding the color reagent from the kit. After another incubation of 30 minutes, the absorbance of the samples in the 96 well plate was measured. A buffer control and GTP-only control were used and subtracted accordingly while processing the data in Microsoft Excel.

sNBD Binding Attachment

To attach a fluorescent label, sNBD was used. To attach the label to the protein, it was incubated with Cdc42 using a ratio of 12:1 (fluorophore: protein). The mixture was then incubated for 3 hours, centrifuged at 4500rpm for 5 minutes and then dialyzed overnight in 50 mM Tris, 10 mM $MgCl_2$ and 50 mM NaCl at PH of 8.0 to remove unbound sNBD. The next day, the protein was run through a 0.2uL filter and a PD10 gravity desalting column. UV Vis and Fluorescence spectroscopy of the protein and buffer were taken at every step to ensure that excess sNBD was successfully removed.

Fluorescence Titration

At 25^0C, 200uM of Purified PBD46 in 50 mM Tris, 10 mM $MgCl_2$ and 50 mM NaCl at PH of 8.0 was titrated in 0.1mg/mL of active Cdc42. Starting with 500uL of Cdc42 in a quartz cuvette, 5 uLs of PBD46 were added at each titration and a fluorescence reading was taken with the excitation of 488nm and an emission spectrum of 500-600 nm. After titration, readings of the peak

at 550nm were taken and plotted against the concentration of added PBD46. A non-linear fitting model of the equation below was used to create a fitting curve and calculate the protein's binding affinity. $F=Fi + Ff [(KD + Lt + Rt) - \sqrt{((KD + Lt + Rt)^2 - 4RtLt)}]/2Rt$ where F is $\Delta F/Fo$ (Change in fluorescence reading over initial fluorescence), Ff is the final F value, Lt is the concentration of PBD, and Rt is the total of Cdc42. [26]

Small Molecule Virtual Screening and Pharmacokinetics Simulations

A library of over one billion molecules from Chem Space (chem-space.com) was used to search for small molecules similar to ZCL278 in terms of structure, size and functional groups. The resulting molecules were then ranked using Lipinski rule of five, Fsp3, polar surface area and more. [27] Furthermore, the ADMET (absorption, distribution, metabolism, and excretion) and PK (pharmacokinetics) properties were used to find drug like molecules that are potential Cdc42 inhibitors. [28] The resulting molecules were also compared to ZCL278 using the parameters stated above. Blind and induced fit docking studies were then performed using PyRx, CB Dock and Autodock Vina virtual screening suites to measure the resulting molecules affinity to Cdc42 and compare them to that of ZCL278. [29]

IV.4 Results

Simulation and Docking experiments show that ZCL278 binds in a pocket between PAK1 and Cdc42

To study the interaction, we started by examining the ZCL278-bound Cdc42 computed in Chapter 1. (from PDB 1AN0). To examine the complex, a comparison of our model with a PBD46-bound Cdc42 (PDB 1E0A) was done. In chimera UCSF, the two Cdc42 molecules were merged. The objective was to examine where ZCL278 is in relation to the PBD46 binding pocket. As seen in Figure 1A, ZCL278 is docked right between the two proteins. Residues from Cdc42 in the switch 1 and switch2 region (see figure 1C) and residues on PBD46 in the CRIB region seem to be involved in ZCL278 binding.

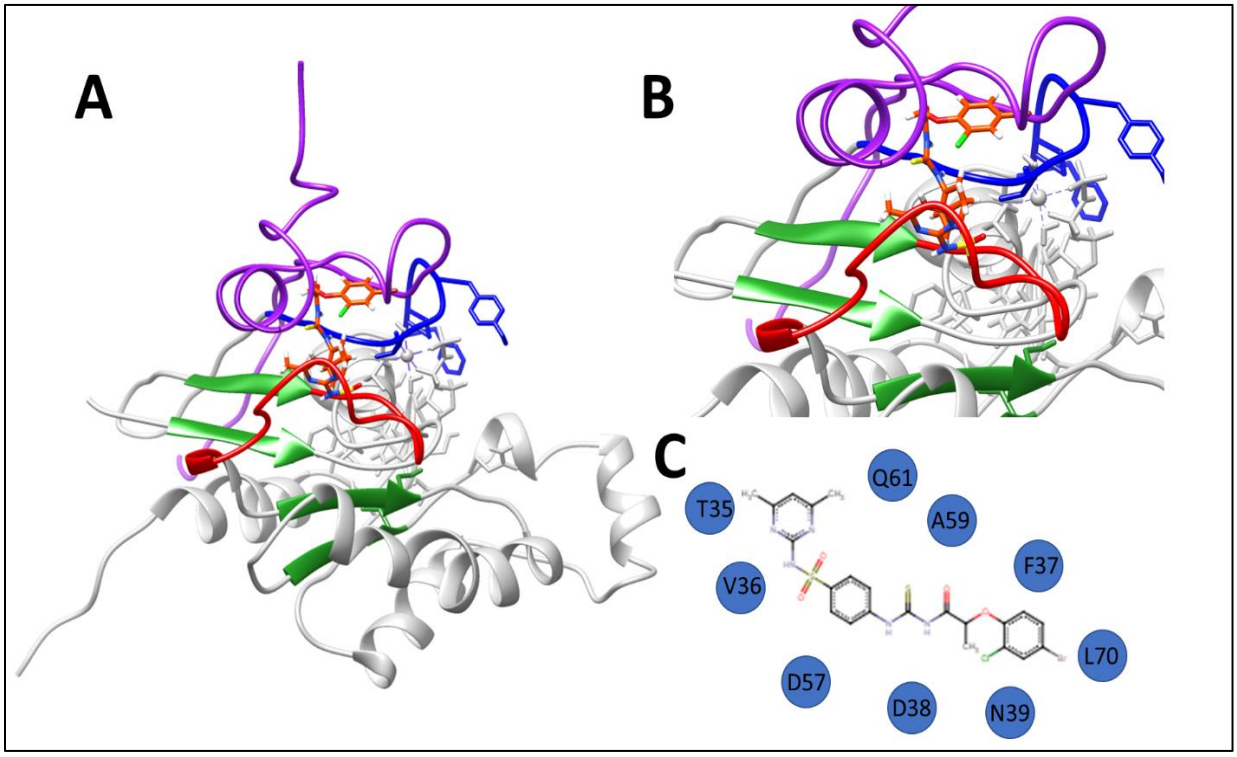


Figure 1. Modeling and docking of Cdc42-GDP to PBD46 and how it is affected by the binding of ZCL278. The Cdc42 bound to ZC278 using docking in MOE (PDB 1AN0) was merged with Cdc42 bound to PBD46 (PDB 1E0A) A. The Cdc42-GDP-ZCL278-PBD46 complex. B. A close up view of the binding interface. C. An overview of Switch 1 and Switch 2 residues involved in the binding.

sNBD binding studies show a lowered affinity of PBD46 to Cdc42 when bound to ZCL278

To further study the interaction between PBD46 and Cdc42, a fluorescence titration experiment was performed. Since PBD46 and Cdc42 both have tryptophan residues, it is not possible to measure titration by reading tryptophan fluorescence. A different fluorophore that emits light at a different wavelength is needed on one of the proteins. In our studies, we chose sNBD. sNBD binds to Cdc42 in a covalent manner at Lysine 150. [26] When bound to Cdc42, sNBD can be excited at 488nm and emit light at a range of 500-600nm. [26] To bind Cdc42 to sNBD, a multistep process is performed to make sure that unbound sNBD is removed. First, the protein is incubated with sNBD at a ratio of 1 to 12 for 3 hours then the mixture is centrifuged and dialyzed overnight. It is then filtrated and passed through gravity desalting column to remove any unbound molecules left. (Figure 2A for procedure). At every step, the fluorescence of the buffers and protein were taken to make sure that unbound sNBD is removed completely. (Figure 2B, 2C) Absorbance can be used too since sNBD absorbs light at 400-530nm. (Figure 2E, 2F). After proper tagging of sNBD to Cdc42. Concentrated PBD46 is titrated to sNBD-Cdc42 while measuring the fluorescence at 545nm. This is done in the presence and absence of the small molecule. As seen in Figure 2D, the binding of the Cdc42 to PBD46 decreases with the addition of the small molecule. This data further confirms that ZCL278 is binding to Cdc42 and preventing PBD36 to bind. It is an orthosteric inhibitor. The Cdc42 WT affinity to Cdc42-sNBD was measured to be 83.26nM while the ZCL278 bound Cdc42 produced an affinity of 145.6nM.

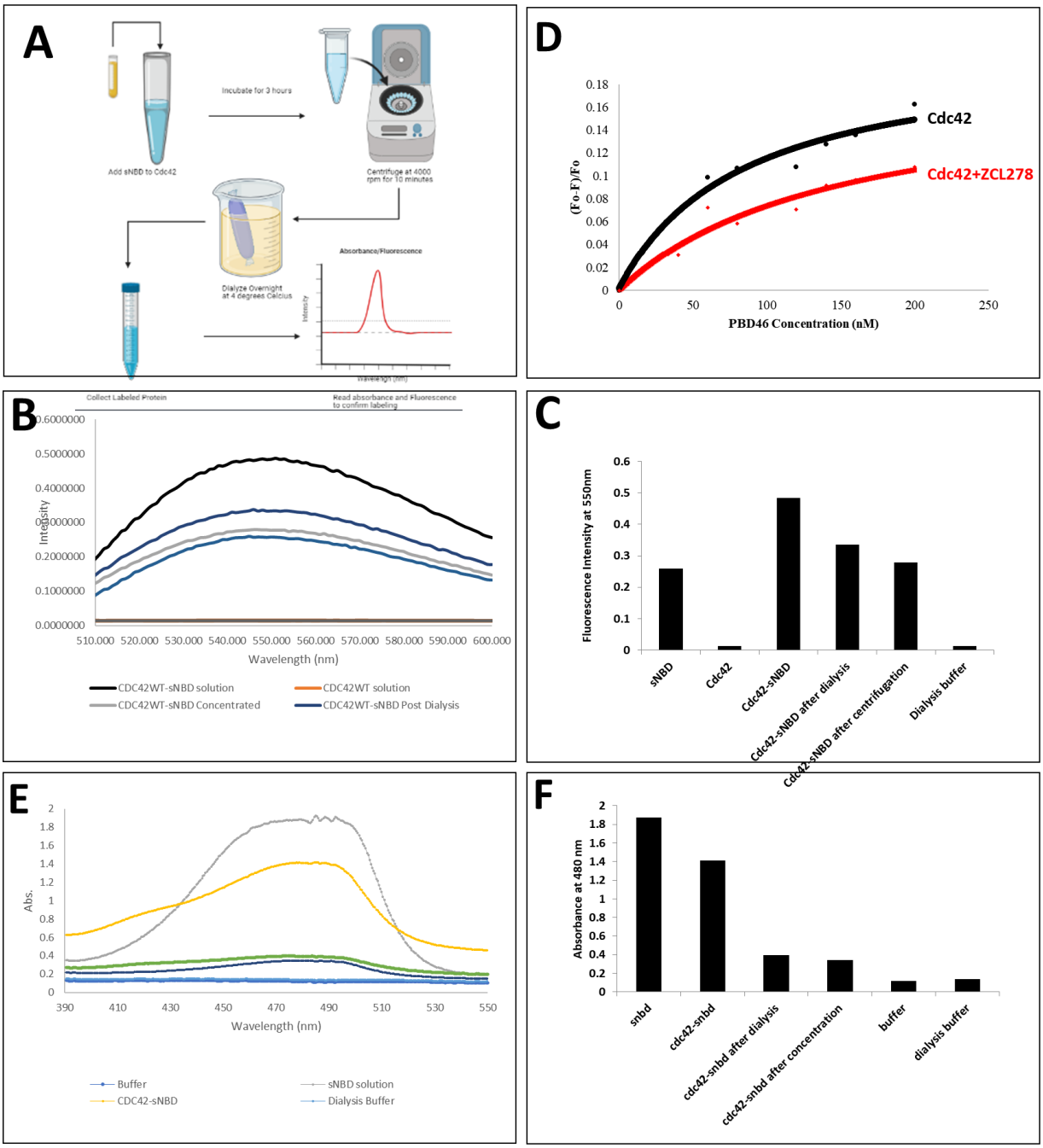


Figure 2. Cdc42 Fluorophore labeling and Fluorescence titration. A. An overview of the sNBD labeling process of Cdc42. Made using biorender.com. B and C. Confirmation of Cdc42 sNBD labeling using fluorescence. D. Fluorescence titration of PBD46 to Cdc42-sNBD in the absence (black) and presence (red) of ZCL278. E and F. Confirmation of Cdc42 sNBD labeling using absorbance.

PBD46-induced intrinsic Hydrolysis GTPase activity is slightly prevented by ZCL278

A study was performed in this lab which showed that intrinsic Cdc42 hydrolysis is inhibited by PBD46.The study showed that 10uM of PBD46 was enough to inhibit Cdc42 intrinsic activity (10uM). [30] We performed GTP hydrolysis studies, at increasing Cdc42 concentration to measure the effect of the peptide on GTP hydrolysis. In Figure 4, the addition of Cdc42 in the reaction increases the hydrolysis. When PBD46 is bound, the hydrolysis reduces. However, when ZCL278 is added to Cdc42 before the addition of PBD46, the hydrolysis is not inhibited as much. This result shows that ZCL278 prevents PBD46 from inhibiting Cdc42 GTPase activity.

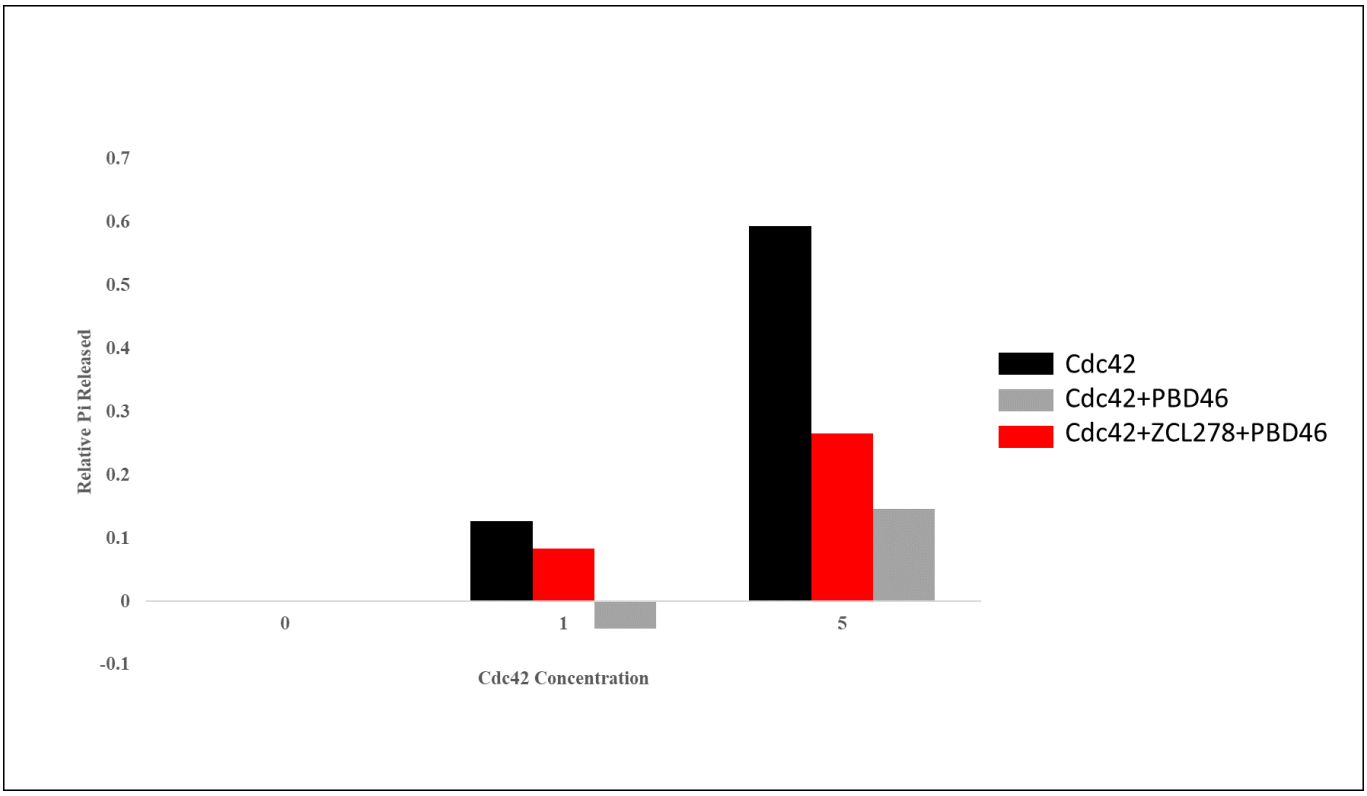
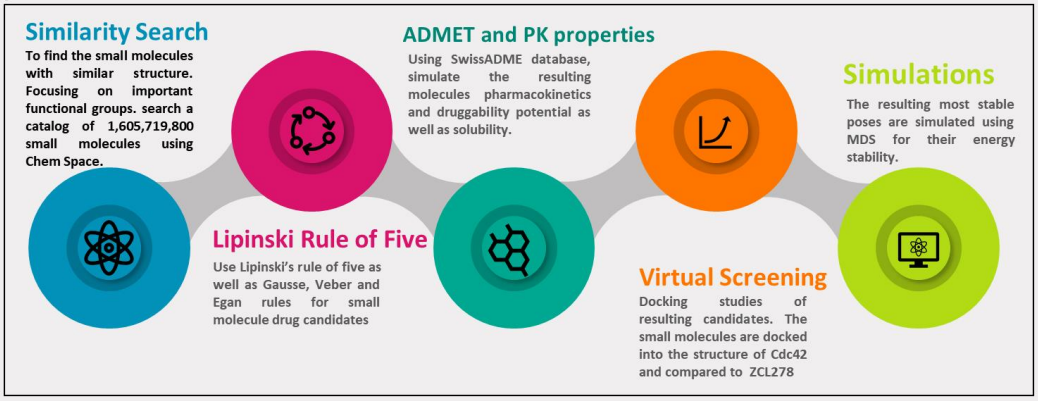


Figure 4. Cdc42 intrinsic hydrolysis assay in the presence of PBD46 (grey) and ZCL278+PBD46 (red)

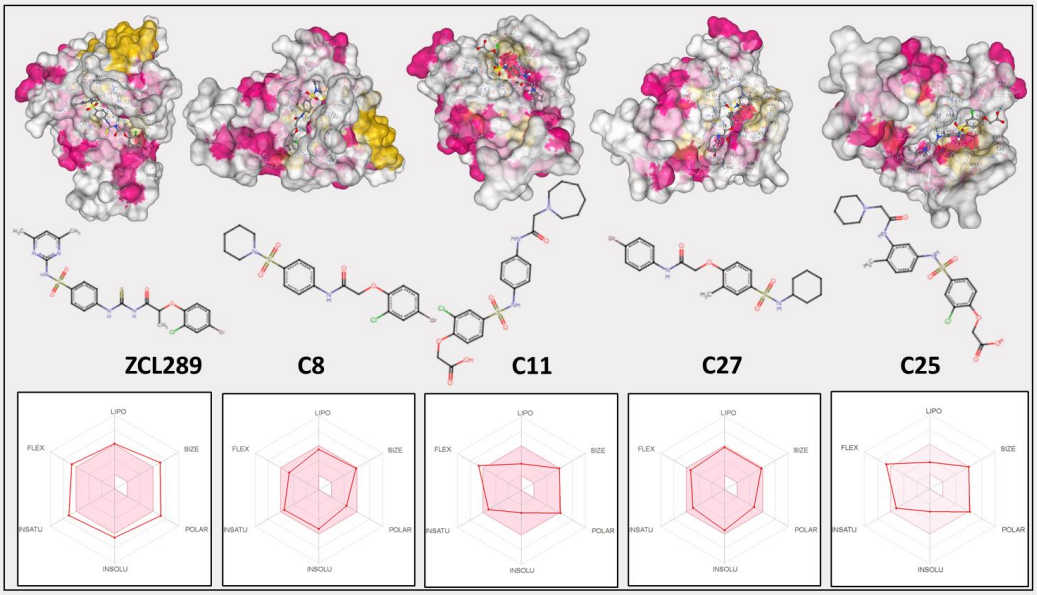
Discovery of Novel Small Molecule Inhibitors of Cdc42 PPIs

The results from this chapter and Chapter 2 provide insight into how small molecules bind to Cdc42. It shows how these small molecules affect the protein's structure, activity, function, etc. While ZCL278 is a good inhibitor, it cannot advance to the next step of drug discovery since it is not soluble in water, has low affinity for a drug candidate, and has minimal effect major cell functions. The goal of these experiments was to not only study the effect of the small molecule but to use the information to design better Cdc42 inhibitors. Using online and offline software, we designed small molecules that are going to serve as the next target for Cdc42 drug discovery. Starting with ZCL278, we used a database with libraries of 1,605,719,800 molecules, to search for small molecules similar to ZCL278 in terms of structure, size and functional groups. After getting 2000 results, these molecules were ranked through the ADMET (absorption, distribution, metabolism, and excretion) and PK (pharmacokinetics) properties to find drug like molecule that are potential Cdc42 inhibitors. [28] Furthermore, Lipinski rule of five, Fsp3, polar surface area and more were used as parameters to further filter these molecules. [27] These molecules were also compared to ZCL278. In the end, we found 46 highly soluble, ZCL78-like molecules with better drug capabilities that were selected for further testing. The next phase of this study is virtual screening of these small molecule to compare their affinity to Cdc42 to that of ZCL278. The summary of the procedure along with our best small molecule candidates are illustrated in Figure 5 A and B. While visually similar to ZCL278 with similar functional groups, these small molecules score higher on all pharmacokinetics tests.

A



B



C

Compound	Affinity – Induced Fit Docking (Kcal/mol)	Affinity – Blind Docking (Kcal/mol)	Log P	ESOL Solubility (mg/ml)
ZCL278	7.5	8.5	4.11	0.000164
C8	7.1	7.8	3.37	0.00237
C11	6.9	7.5	3.61	0.0024
C27	7.1	8.0	2.17	0.458
C25	7.3	8.2	2.47	0.0354

Figure 5. Novel small molecule discovery through similarity search and pharmacokinetics screening. A. An overview of our process. B. ZCL278 compared with 4 other novel molecules that are candidates for Cdc42 inhibition. For each molecule, plots of Lipophilicity (LIPO), Size (SIZE), polarity (POLAR), insolubility (INSOLU), insaturation (INSATU) and flexibility (FLEX) are represented in the bottom squares. C. Binding affinity results from blind and induced fit docking studies of the candidate molecules to Cdc42.

IV.5 Summary and Discussion

Recent studies have shown that targeting downstream overactive interactions of Ras GTPases can reduce the negative effects in the cell. [4] One example of an interaction that has bed extensively targeted with success is the Ras-Raf interaction. [31] Recently, an inhibitor of KRas-SOS inhibitor was introduced and is undergoing clinical trials at the moment. [32] This further validates that targeting Ras GTPase effector interaction can be beneficial over direct targeting. This is mainly due to the complex and redundant regulation of GTPases coupled with their high affinity to GTP and their dynamic nature. [33] Compared to their Ras subfamily, small molecule targeting of the Rho GTPase PPIs has not been explored. In our study, we investigated PPIs of Cdc42 and PBD46, an active derivative of PAK1. We also investigated how these interactions can be targeted by small molecules. [34][35] PAKs is a large family of serine/threonine kinases that play a role in many cell processes and involved in various types of cancer.[36] In this study, the interaction of Cdc42 to PAK1 was studied and inhibited.

In our experiments, we used a novel Cdc42 inhibitor, ZCL278, to study how it affects the PPI between Cdc42 and PBD46. The peptide was purified using a heat treatment method described in Chapter III. [37] Computational, biochemical, and biophysical studies were used to study the effect of ZCL278 on the interaction. Using software, ITC, fluorescence titration and hydrolysis assays, this study showed that ZCL278 may influence the affinity of PBD46 to Cdc42 and reduces the effects of Cdc42 activity. A computational model showed that ZCL278 occupies a pocket between the Switch 1 and Switch 2 regions on Cdc42 and CRIB region of PBD46.

While ZCL278 inhibits the interaction, more details are needed to qualify it as a potential drug candidate. Furthermore, its affinity for Cdc42 is moderate. Moreover, this small molecule is not 100% soluble in water. DMSO is needed to dissolve the molecule which interferes with the

biophysical assays. DMSO is also toxic to cells and animals at a concentration of 1% or higher. A better molecule will need to be designed to further explore the inhibition of these PPIs. Using results from this study and the previous chapter, we designed and tested small molecules that could advance the Cdc42 PPI inhibition field. These small molecules are more soluble and have better drug capabilities than ZCL278. Using induced-fit and blind docking, five molecules with an affinity for Cdc42 comparable to ZCL278 we identified. Further studies of these small molecules effects on Cdc42 will yield novel small molecules to study as drugs targeting Cdc42 PPIs.

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Chapter V. Summary, Conclusions, and Future Perspectives

Drug discovery approaches involving Ras GTPases still remain relevant in cancer drug research. This interest stems from Ras GTPases' involvement in more than a quarter of human cancers. [1][3]In addition, the Ras superfamily with 167 members is not only involved in cancers but also in many other diseases. These proteins are involved in regulation of all major cell processes. [2] Recently, there have been new discoveries and new approaches that look promising in targeting Ras GTPases. [3] However, most of these major discoveries focus on the Ras subfamily of these GTPases, i.e., HRas, KRas and NRas. Currently, there are at least 10 clinical trials targeting mutations of these GTPases. [3] While understanding of the Ras subfamily has advanced greatly in recent years, understanding of Rho GTPases has been left behind. Rho GTPases are some of the most abundant GTPase signaling and they are involved in many cell processes and found in various diseases. [4] Targeting aberrant Rho GTPases has not been explored extensively and there have not been successful drug candidates targeting these proteins.

Among Rho GTPases, Cdc42 is crucial for cell cycle regulation, polarity and, migration. [5] [6][7] Deregulation, overexpression and improper activation of this protein has been observed in many types of cancers. [8] However, details of small molecule targeting of these proteins has not been extensively investigated. There have been some targeting endeavors targeting Cdc42, but many have failed. [9] The successful molecules have only shown effects in the cells but details of how they work on the protein level are still lacking. It is important to study the interaction of Cdc42 with these small molecules to improve drug discovery efforts not only for Cdc42 but also for Rho GTPases in general.

In this work, we characterized the interaction of Cdc42 with a promising inhibitor, ZCL278. This is important because this interaction will reveal the small molecule's effects on the protein's

structure, stability, and function. It will help us understand the regions and residues that can be targeted by other molecules in the future. We also studied the interaction of this protein with other effectors and how this interaction can be interrupted by the small molecule. To do this, we used computational, biochemical, and biophysical techniques to characterize the interactions. These methods have shown us that the small molecule binds and stabilizes the protein making it resistant to denaturation. Cdc42 is a dynamic protein with moving Switch 1 and Switch 2 regions (Figure 2). [10] When the small molecule binds, the protein becomes less dynamic, and this makes it less reactive to effectors. This makes an overactive protein lee reactive and reduces its negative effects in the cell. We found that ZCL278 binds to Cdc42 in the Switch 1 and Switch 2 regions. These results show that this protein might interrupt not only the effectors but also the binding of the nucleotide and this should further affect Cdc42 activity. It then makes sense that our studies also show that the small molecule reduces the GTPase function of the protein. Cdc42 binding experiments with Pak1 show that ZCL278 interrupted this interaction. This is significant because there are more than one PAKs, and even more Cdc42 effectors with the same CRIB region. [10] These results show that ZCL278 does not just interrupt the binding of PAK1 but also PAK1-PAK6, Ack, WASP and others. [10] Our study confirmed the small molecule's effects on multiple Cdc42 effectors, not just PAK. This was achieved by using a universal CRIB peptide.

When studying the CRIB region throughout our interaction experiments, it became difficult to purify the peptide. Our experimentation led us to a new method to efficiently purity PBD46. Collaborating with Dr. Kumar's group, we greatly improved the yield and purity of this peptide and reduced the purification time by days. [11] In this method, a heat treatment was used to find the optimal temperature that aggregates the cleaved GST while leaving the peptide intact. This method has been efficient in not only purifying PBD46 but also other Cdc42 binding peptides such

as Ack1. Studies also showed that this method can be used to purify other peptides such as Chromo-domain 2 (CD2) and a heparin binding (HB) peptide. [11] This method can subsequently be scaled in an industrial setting to advance the studies of these interactions quickly and cheaply.

While ZCL278 has been a good inhibitor, there are some limitations that need to be improved before this drug candidate can be used in animals and human subjects. First, this small molecule is insoluble in water, DMSO needs to be added to dissolve it. DMSO has been problematic in most of our biophysical and biochemical assays since it reacts with buffer components and interferes with spectroscopic readings. For proper analysis, multiple control samples with DMSO alone were needed for subtraction in each experiment. Moreover, the percent level of the DMSO used in these assays can be toxic to cells or patients. A better molecule that is soluble in water needs to be designed. Second, even though a binding affinity of 8.6uM is good, it needs to be improved before it can advance to the next drug discovery stage. Binding affinity of normal drugs usually falls in the nanomolar range.[11] Another limitation of ZCL278 is its inefficient inhibition of Cdc42 function and Cdc42-related cell processes. Although there is an effect, the effect is minimal. [12] A better small molecule to address those shortcomings is needed.

Using the results from this study, we performed computer-aided drug discovery processes to design better molecules that might address ZCL278's deficiencies. Using online and offline computational tools, small molecules with the same Cdc42-binding groups were discovered from a large library of small molecules. These are smaller, more soluble small molecules with better ADMET and Lipinski factors than ZCL278. (Figure 5, chapter IV) [13][14] Furthermore, our binding models show that these small molecules have the potential to bind to Cdc42 better than ZCL278. In the future, these small molecules will be compared to ZCL278 in cell studies and other biochemical and biophysical assays performed here. We hypothesize that the small molecules

presented here will generate a better Cdc42 inhibitor and help advance the Cdc42 drug discovery research to the next phase.

This study is an important contribution to the discovery of Rho GTPases protein-protein interaction inhibitors. Using various methods, we have shown that a small molecule binds and stabilizes Cdc42 rendering it ineffective to cause various disease-causing cell processes. Although this small molecule was efficient, it needs more improvement before it can advance as a drug candidate. In this study, potentially better small molecules were identified. Subsequent studies should also involve small molecule experiments on mammalian and cancer cells by testing their effects on various Cdc42-caused cell effects such as migration, wound healing, and microspike formation. [12] After successful studies and iterations, the resulting small molecules should advance from cells to animals.

V.1. References

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