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Characterization of the Influence of a Small Molecule Inhibitor on Ras-Related Proteins Interactions

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Characterization of the Influence of a Small Molecule Inhibitor on Ras-Related Proteins Interactions

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Abstract

The Ras superfamily of small G proteins are involved in cell-signaling processes that, if not regulated, may lead to cell multiplication, apoptosis inhibition, and tumorigenesis. They function as molecular switches, which through GTP/GDP exchange cycle, switch on or off cellular activities. Overexpression and/or hyperactivity of these proteins have been linked to many diseases including various cancers. CDC42, a member of the Rho subfamily of the Ras superfamily of small G proteins, participates in the regulation of many cellular processes including cell adhesion, mitosis, and cytoskeletal rearrangements. CDC42 binds to and activates many effector proteins including CDC42-activated kinase (ACK). Abnormal activities of CDC42/ACK have been associated with tumor development. It is paramount that small molecule inhibitors be developed to inhibit CDC42 interactions with its effector proteins leading to abnormal signaling activities. A small molecule, ZCL278, has been shown to interact with CDC42 and alter CDC42-stimulated overactivity. In this current work, we investigated the influence of ZCL278 on CDC42 wild type and CDC42F28L mutant as well as its influence on CDC42/ACK protein-protein interactions. Our In vitro binding assay data suggest that ZCL278 decreased binding capacity of CDC42 to ACK. ZCL278 was shown to lower the affinity of CDC42 for ACK by a nanomolar-to-micromolar jump in an extrinsic fluorescence titration experiment. Isothermal titration calorimetry experiments data showed that in absence of ZCL278, CDC42 bound to ACK with an apparent *Kd* value of 0.6 μM while in the presence of ZCL278 the apparent *Kd* peaked to 4 μM. Overall, our binding experiments suggested that 1) ZCL278 decreased the binding affinity of CDC42 to ACK, 2) ZCL278 bound to both CDC42 and CDC42F28L with micromolar affinity, 3) ZCL278 induced structural modifications in CDC42 and CDC42F28L upon binding, and 4) ZCL278 rendered CDC42 and CDC42F28L more thermally stable.

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List of abbreviations

CDC42	Cell division control protein 42 homolog
ACK	Activated CDC42 kinase
GST	Glutathione S-transferase
GDP	guanosine diphosphate
GTP	Guanosine triphosphate
Kd	Dissociation constant
ITC	Isothermal titration calorimetry
NMR	Nuclear magnetic resonance
sNBD	Succinimidyl 6-(*N*-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl) Amino) Hexanoate
SEC	Size exclusion chromatography
NBD	Nucleotide binding domain
GAP	GTPase-activating proteins
GEF	Guanine nucleotide exchange factor
GDI	Guanosine dissociation inhibitor
NRTK	non-receptor tyrosine kinase
SMI	small molecule inhibitor
IPTG	Isopropyl β- d-1-thiogalactopyranoside
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

CHAPTER ONE

Introduction

Ras Superfamily: function, structure, and division

The Ras superfamily are small guanosine triphosphatases (GTPases). They encompass more than 150 members of which the Ras proteins are the founding family [1]. They function as binary membrane-anchored timing switches cycling between an active and an inactive conformation. In the active conformation, these proteins switch on cell processes and switch them off in the inactive conformation [1] (figure 1); when active, Ras superfamily are bound to GTP (Ras-GTP) and bound to GDP (Ras-GDP) when inactive. The conversion from the GDP- to GTP-bound conformations (and vice versa) is assisted by regulatory proteins such as guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanosine dissociation inhibitors (GDIs) [2]. GEFs help to convert GDP-bound to GTP-bound Ras, while GAPs reverse this process by converting Ras-GTP back to Ras-GDP [2]. GDIs bind to GDP-bound Ras and keep them in the inactive conformation by preventing GDP/GTP exchange and their migration toward the cell membrane [2].

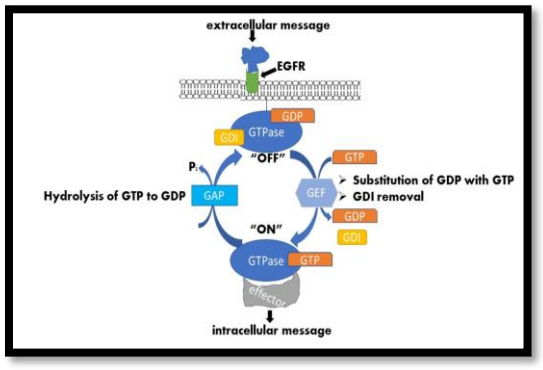

Fig.1. Cycle of Ras proteins. The cycle is regulated by GDIs which keep the Ras proteins in their inactive state and protect them upon synthesis. GEFs and GAPs activate and deactivate Ras proteins by enhancing their intrinsic nucleotide exchange and GTP hydrolysis capacity, respectively.

Ras superfamily cover more than 150 members of which Ras proteins are the founding family [3]. They are ubiquitously expressed in all eukaryotes [3]; they are found in mammals, plants, birds, insects, fungi, and yeast [4]. Based on sequence homology, they are divided into five families: Ras, Rho, Rab, Ran, Arf, and Rheb [3]. Beginning at the N-terminus, Ras superfamily have in common a set of five conserved GDP/GTP binding motif components [5] (figure 2). These binding motif components are: G1, GXXXXGKS/T; G2, T; G3, DXXGQ/H/T; G4, T/NKXD; and G5, C/SAK/L/T. Altogether, they constitute a ~20 KDa G domain, also called GTP-binding domain, that is conserved throughout the entire Ras superfamily [5]. This domain comprises five α-helices (A1–A5), six β-strands (B1–B6) and five loops (G1–G5) with G2 loop corresponding to switch I, G3 loop corresponding to switch II (figure 2). The G1 loop is found between the B1 strand and A1 helix with the motif X1X1X1GXXXXGK (S/T), where X1 can be leucine (L), valine (V) and isoleucine (I), and X can be any amino acid which lodges the α- and β- phosphate groups [6]. The G2 loop contains a conserved threonine that links the A1 helix and the B2 strand. This loop brings about the binding of Mg2+ through conserved amino acid residues. The G3 loop is found at the N-terminus of the A2 helix with the motif XXXXDXXGX. Its principal role is to bind Mg2+ and the γ-phosphate of GTP or GDP. The G4 loop comprises the motif XXX (G/A) (T/N) KXD and the G5 loop brings about the identification of the guanine base (figure 2).

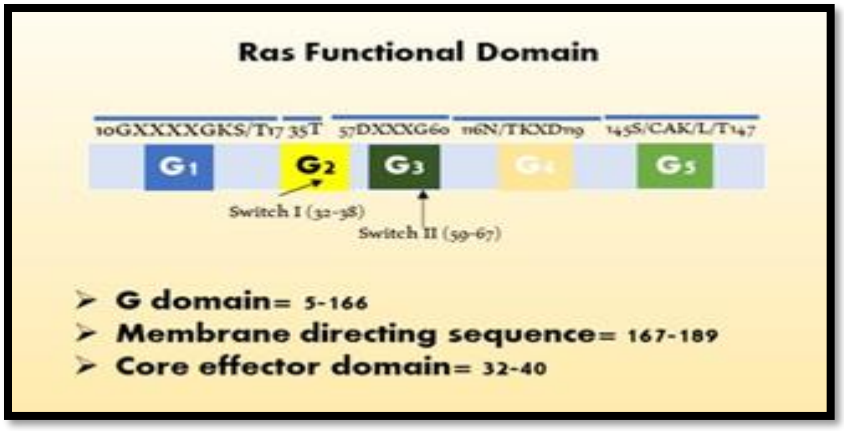

Fig.2. Ras functional domains.

The Ras superfamily of proteins show a high level of affinity for GDP and GTP; they exchange GDP with GTP and hydrolyze GTP to GDP at very slow speed [2]. Consequently, regulatory proteins such as GEFs and GAPs task to increase the rate of GDP/GTP exchange and GTP hydrolysis, respectively, to meet the requirements for fast reactions under physiological conditions [2]. In the same branch, small GTPases have similar and different GAPs and GEFs, whereas withing different branches they have structurally unique and mechanistically analogous GAPs and GEFs. Ras-GDP and Ras-GTP have similar structural conformations but show major structural differences in the switch I (residues 32-38) and switch II (59-67) regions. Ras-GTP show high level affinity for effector proteins.

The conformational modifications of the small GTPases allow them to be recognized by effector and regulatory proteins in their active state (Ras-GTP) and inactive state Ras (Ras-GDP) and bind them as a result. The switch I, found at the core effector domain, is crucial in direct binding to effector proteins. Small GTPases have been shown using a loaded-spring mechanism when binding to effector proteins [6]. A key hallmark of Ras superfamily is their post-translational modifications by lipids for translocation to the membrane [7] (figure 3). Effector proteins like GEFs and GAPs switch Ras on and off upon being recruited to inner membrane and positioned

in the vicinity of the Ras proteins where the GTP concentration is tenfold compared to that of the GDP. These lipid modifications include farnesyl and geranylgeranyl isoprenoid for most Ras and Rho proteins whose N-terminal contains the tetrapeptide motif CAAX (C for cysteine, A for aliphatic, X for any amino acid). This motif is the recognition sequence for farnesyltransferase and geranylgeranyltransferase I. These enzymes catalyze the covalent attachment of a farnesyl or geranylgeranyl isoprenoid, respectively, to the cysteine residue of the tetrapeptide motif. Rab proteins have C-terminal motifs such as CC, CXC, CCX, CCXX, or CCXXX with C for cysteine. These C-terminal motifs serve as recognition sequence for geranylgeranyltransferase II, which also catalyzes the addition of geranylgeranyl group [8]. Some Arf proteins have their N-termini modified by a myristate fatty acid. These modifications are paramount to the promotion of Ras proteins attachment to the cell inner membrane and their subcellular localization, which is key to fulfilling their biological functions [9]. While some members of the Ras superfamily do not undergo lipid modifications but are anchored to the membranes, some are neither lipid modified, nor membrane anchored [10].

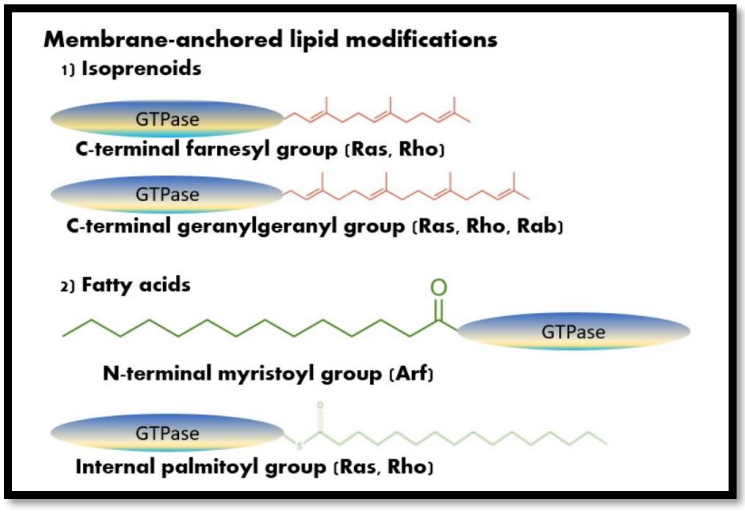

Fig.3. Ras proteins membrane-anchored lipid modifications. These modifications, mostly of lipidic nature, act as localization signaling sequence for a distinct Ras-like protein family.

Ras proteins are involved in numerous signaling transduction pathways. A well-studied Ras signaling pathway is the activation of Ras proteins by epidermal growth factor tyrosine kinase via the RasGEFSos [9]. Arf (ADP-ribosylation factor) and Reb (Ras-like proteins in the brain) proteins oversee gathering, piling, and targeting vesicles to the correct cellular locations. Arf proteins encompass up to 30 proteins with Arf1 being the most studied [10]. Arf-GTP binds to effector proteins including vesicle coat proteins [10]. Conformational discrepancies between the GDP- and GTP bound states bring about multiple changes in switch I and II regions and in the N-terminal region that facilitates the myristate moiety to interconnect with membranes in their active state. Reb proteins represent the largest family with up to 61 members [11]. Reb proteins regulate vesicle formation, movement, fusion, and vesicular cargo transport. They are found in distinct intracellular compartments according to their function in specific vesicle transport activities [12]. Reb proteins location depends on prenylation, and the various C-terminal sequences dictate their specificity [13].

Ran (Ras-like nuclear) proteins participate in the nucleus-cytoplasm transport of RNA and proteins; they are the most abundant Ras-related proteins in the cell [14]. Unlike other families of small proteins, Ran proteins activities are contingent on a Ran-GTP gradient [14]. Nuclear Ran-GTP binds to importin to bring about cargo delivery and binds to exportin-complexed cargo to facilitate cargo export [15]. The Rho proteins represent key modulators of actin restructuring [15]. Consequently, they participate in cell migration, wound healing, cell adhesion, cell polarity, membrane trafficking, and cytokinesis [15]. Rho proteins are greatly conserved in the entire eucaryotic kingdom. Among the 20 members identified the most notable and well-known are RhoA, Rac1, and CDC42 [16].

Rho proteins: functions, structure, regulation, divisions, and diseases

Rho proteins are characterized by the existence of a Rho GTPase-like domain [17]. Rho proteins are distinguished from other small GTPases by the structural component called the “Rho insert domain” found in between the fifth β-strand and the fourth α-helix in the small GTPase domain [17]. Typically, most Rho proteins are small (190-260 amino acids) and contain only a GTPase domain and short N- and C-terminal elongations. In the GTPase domain, they have about 30% amino acid sequence identity with Ras proteins and 40-95% sequence identity within the same family [20]. All Rho proteins have the sequence motif typically found in all GTP-binding proteins; they bind to GDP and GTP with great affinity; they cycle back and forth between active, GTP-bound and inactive, GDP-bound states. Moreover, most members are lipid modified at C-terminus post-translationally (figure 3). In conjunction with other C-terminal changes, the addition of the terminal lipids assists in their subcellular localization and anchorage to specific membranes, which is paramount for carrying out their functions.

Function-related information on Rho proteins has mostly stemmed from research conducted on RhoA, Rac1 and CDC42. They all share the growth-promoting and anti-apoptotic roles; they are also involved in the regulation of gene expression via the activation of molecular signals such as serum response factor, NF-κB, the stress-activated protein kinases and cyclin D1 [18,19]. All three work in unison to facilitate actin cytoskeleton reorganization; however, they have unique effects on cell shape and movement [20,21]. RhoA functions as a promotor of actin-myosin contractility and, consequently, the development of stress fibers and focal adhesions which regulate cell shape, anchorage, and motility. Rac1 facilitates actin polymerization and lamellipodia formation.

CDC42 promotes the formation of thin, finger-like cytoplasmic elongations called filopodia which consist of tight actin bundles and might participate in the recognition of the extracellular stimuli from the environment.

Like other Ras protein, the GDP- and GTP-bound conformations of the Rho proteins structurally vary mainly in two regions, switch I and II; this characteristic probably exists in all Rho proteins[22]. The conformational structure of the GTP-bound Rho proteins leads to an increase in the binding affinity for downstream effector proteins. Each Rho protein can recognize several effectors, and some effector proteins are recognized by several family members. Rho proteins interaction with downstream effectors regulates signaling pathways that control the many functions of Rho proteins. The great number of effectors identified for RhoA, Rac1, CDC42 and other family members underlines the functional complexity and diversity of these small GTPases [23].

The Rho family of proteins

Based on their amino acid sequence identity, structural motifs and biological roles, Rho proteins can be divided into six subfamilies that show close but distinguishable properties: 1) the RhoA-related subfamily (RhoA, RhoB and RhoC), 2) the Rac1-related subfamily Rac1 (and its splice variant Rac1b), Rac2, Rac3 and RhoG, 3) the CDC42-related subfamily CDC42 (and its brain-specific C-terminal splice variant G25K), TC10, TCL, Chp/Wrch-2 and Wrch-1, 4) the Rnd subfamily (Rnd1, Rnd2, and RhoE/Rnd3), 5) the RhoBTB subfamily, 6) the recently described Miro subfamily. Moreover, RhoD, Rif and TTF/RhoH do not belong to any of these subfamilies.

Rho-related proteins

RhoA, RhoB and RhoC show up to 85% amino acid sequence identity. They are all reported to participate in actin-myosin contractility and to interact with similar guanine nucleotide exchange factors (GEFs) and effectors. Nonetheless, they have several functional distinct differences which are likely largely due to the difference in their C-terminal last 15 amino acids, which control subcellular membrane anchorage. RhoA is found in the cytosol and to a certain extent attached to the plasma membrane; RhoB is anchored to the plasma membrane and endomembrane vesicles; RhoC is found in the cytosol and attached to perinuclear structures [24-26].

RhoB [27] differs from RhoA and is also subject to transcriptional regulation. RhoB mRNA and protein are stable and are upregulated by growth factors during mitosis G1 and S phases, which hints that RhoB participates in cell proliferation [28,29]. The prenyl group can be added to RhoB by either a geranylgeranyl or a farnesyl isoprenoid group and can be further modified by the palmitate fatty acid (fig. 3) [30].

In contrast to RhoA and RhoC, RhoB appears to have a growth-inhibitory effect [31,32]. It has been reported that the expression of RhoB is downregulated in tumors [33].

RhoC [34] has as well been linked to tumorigenesis. It is overexpressed in malignant pancreatic ductal carcinomas [35], inflammatory breast cancer tumors, and highly metastatic melanomas [36]. “Ectopic overexpression” of RhoC augments tumorgenicity and metastatic properties of tumor progenitor cells [35,36].

Furthermore, RhoC stimulates the expression of angiogenic factors in human mammary epithelial cells [35], which may promote tumor vascularization. Nonetheless, activated RhoC does not cause NIH 3T3 mouse fibroblasts transformation [37].

It has also been found that a GEF that binds to RhoA and RhoB but not to RhoC [38], and it has been reported that RhoC interacts better with Rho kinase, an important effector for the RhoA proteins, than does RhoA [39].

Rac1-related proteins

The entire Rac-related proteins family induces lamellipodia and membrane ruffles formation by interacting with the PIR121-Nap125-HSPC300-WAVE complex [40]. Rac1, Rac2 and Rac3 share up to 88% of sequence identity. These three subfamilies differ mainly in the C-terminal 15 residues. Rac1b, a splice variant of Rac1, brought about by alternative splicing, has an additional 19-residue segment located C-terminal to the switch II region.

Rac1b, majorly expressed in breast and colon cancers [41,42], has an enhanced intrinsic guanine nucleotide exchange rate and a diminished intrinsic GTPase activity [43,44]. Rac1b cannot bind to Rho guanine nucleotide dissociation inhibitor (RhoGDI) and displays, as result, increased attachment to the plasma membrane [44]. However, Rac1b is not capable of activating the JNK stress-activated kinase; however, it keeps the capacity to activate the NF-κB transcription factor [44].

Rac1 is ubiquitously expressed while Rac2 is expressed merely in hematopoietic cells, where it appears to have specified roles [45]. Decreased expression of Rac1 leads to embryonic mortality [46], while a lack of Rac2 leads to hematopoietic cell defects in mice but sustains a normal

development [47]. Downregulated, deleted, or inactivated Rac2 gene is linked to various defects in white blood cells [48-50]. Most of these inadequacies are probably due to the Rac2-specific activation of NADPH oxidase [51,52], and production of reactive oxygen species (ROS) in hematopoietic cells [53]. The generation of ROS is essential in both host defense machineries [47] and blood cell differentiation signaling mechanisms [50]. Those further anomalies in hematopoietic cell role observed with a merged Rac1 and Rac2 deficiency highlight the overlaying and unique functions of these related small GTPases in hematopoietic cells [51].

Rac3 is mostly expressed in brain but is upregulated once the fibroblasts are serum-stimulated [53]. Compared to Rac1, Rac3 is found more in the membrane [54]. Moreover, it has increased activity in breast cancers [54] and the *Rac3* gene is located near a portion of chromosome 17 that is oftentimes removed in breast cancers, which might cause its expression to be unregulated [55].

Vincent et al. first delineated *RhoG* as a "late-response gene" stimulated upon serum activation of fibroblasts, appearing to be a gene regulated once transcribed, which later participates in cell cycle regulation [56]. Activation of RhoG mutants lead to lamellipodia development, membrane ruffling, and formation of filopodia to some degree [57]. The signaling mechanism is unclear; some reports suggest that it controls its cellular influences via the activation of Rac1 and CDC42 [57]. Other reports suggest that the signaling mechanism is independent of Rac1 and CDC42 [58]. Nevertheless, a recent report suggests that Rac1 is activated by RhoG through the involvement of the DOCK180 RhoGEF [58].

CDC42-related proteins

The CDC42-related proteins all participate in filopodia formation, mostly via their interaction with Wiscott-Aldrich syndrome protein (WASP) or N-WASP [59,60] except for Wrch-1, which

has no interaction with either WASP or N-WASP [61]. Two isoforms of human and mouse CDC42 are produced via alternative splicing of the same gene [62,63]. Both isoforms have 191 amino acid residues that vary at residue 163 and in their C-terminal last ten amino acids.

The most studied isoform, placental CDC42 (CDC42p), has a ubiquitous expression, while the other isoform, brain CDC42 (CDC42b), is found only in the brain.

TC10 [64] and TCL are closely related and have roles that bear much similarity to that of CDC42 [65]. Filopodia generated by TC10 and TCL are more extended than those stimulated by CDC42 [66,67]. TC10 and TCL bind to most effectors of CDC42; however, some key structural contrasts exist between them [66,67]. While the C-terminal CAAX motifs of TC10 and TCL indicate that they can be modified either by farnesylation or geranylgeranylation, the C-terminus of CDC42 can only be geranylgeranylated. TC10 can also be palmitate-modified, which allows it to escape RhoGDI-1 recognition [68]. TC10 and TCL activities appear to be controlled by extracellular stimuli dissimilar from the ones that control CDC42.

No report yet on the regulation of TC10 and TCL by the Dbl family, which has been said to be the most frequent activators of Rho proteins [69]. It has instead been suggested that TC10 activation occurs thanks to the Rap GEF C3G once adipocytes have been stimulated by insulin [70]. Activated TC10 then regulates GLUT-4 migration to the plasma membrane and is essential for the differentiation of adipocytes [71]. Furthermore, the expression of TC10 and TCL are upregulated during the nerve regeneration process [72] and neurite cell differentiation [73]; and activation of TC10 and TCL promotes neurite extension. Two distinct isoforms of TCL have been found. One is characterized by the presence of an elongation of ten amino acid residues at the N-terminus [70].

The rat protein Chp (its human version is called Wrch-2) has been identified as a Pak2-serine/threonine-kinase-interacting protein in a yeast two-hybrid screen [74]. Similar to CDC42 activation, activation of Chp leads to its interaction with Pak and N-WASP and induces filopodia formation [74].

Chp varies from other Rho proteins by its lack of a conventional C-terminal CAAX prenylation sequence. However, the C-terminus is necessary for its biological functions [74].

Wnt-1 signaling pathway upregulates the gene that codes for Wrch-1 [75]. Wnt-1 binds to the "Frizzled cell-surface" receptor which caused the APC tumor suppressor to be deactivated and the transcription activity of β-catenin and transformation to be activated. Wrch-1 expression in mammary epithelial cells imitates the influences of Wnt-1 transformation on cell morphology [75] highlighting the importance of Wrch-1 signaling element in Wnt-dependent oncogenesis. To date, no known RhoGEFs, or GAPs, or GDIs have been reported as regulators of Chp and Wrch-1 proteins.

Nonetheless, the capacity of a dominant-negative S45N type of Chp to stop tumor necrosis factor α (TNF-α)-controlled signaling activity suggests that Chp GEFs may exist. Extra N-terminal and C-terminal sequences that flank the GTPase domains are found in both Chp and Wrch-1. Proline-rich sequences that may function as Src-homology 3 (SH3) domain recognition sequences are found in their N-terminal sequences. This indicates that proteins containing a SH3 domain could identify and mediate their roles. Likewise, the extra C-terminal sequences of these proteins may help recognize their roles and mediations from those related to CDC42.

The Rnd subfamily

The three Rnd-family proteins have a 45-49% sequence identity with RhoA. However, they display biochemical and functional characteristics that are different from those of RhoA members. They are GTPase activity deficient, they are unstable in both the GDP- and GTP-bound states [76], and RhoE/Rnd3 (known as Rho8) is essentially GTP-bound in vivo [77].

They also have a substitution that causes a decrease in the affinity for nucleotides. They are post-translationally farnesylated instead of geranylgeranylated [78]. They inhibit the stimulation of contractility, actin formation, and the focal adhesions generated by RhoA GTPase, which represents a key functional characteristic of RNd1 and RhoE/Rnd3 [79]. This was brought about by interacting and activating p190RhoGAP and for RhoE/Rnd3 at least, interacting and deactivating the RhoA effector ROCK1 [80]. Socius is another Rnd effector that could take part in this activity; however, it is unclear how this assists in the cycling activity of these proteins.

During the fetal development, Rnd1 is ubiquitously expressed; in embryos of Xenopus, its level of expression is very high in tissues where extensive morphogenetic modifications occur [79]. Nonetheless, in adults, Rnd1 is mostly expressed in the brain and the liver.

The most expression of RNd2 is seen in the testis, the brain, and liver [80]. Very little is known about its roles, but it has been reported to interacts with proteins such as Vps4-A, a protein that participates in endosome sorting [79], rapostlin, a protein that is involved in neurite branching [81], and MgcRacGAP, a RhoGAP that takes part in cytokinesis [82].

RhoE/Rnd3 has been identified as a protein that binds to p190RhoGAP which leads to the inhibition of RhoA through the mediation of Rnd. The mRNA of RhoE/Rnd3 is ubiquitously

present, however, the protein expression is very sparse [83]. The transformation of Madin-Darby canine kidney (MDCK) cells by Raf leads to the upregulation of RhoE/Rnd3 which regulates the stress fiber removal and multilayer development associated with this transformation.

Likewise, Ras transformation, in human epithelial cells, leads to an upregulation of RhoE/Rnd3 protein expression levels.

The stimulation of Platelet-derived growth factor (PDGF) by fibroblasts promotes RhoE/Rnd3 protein production which is linked to the loss of stress fibers and cell body rounding [84].

RhoD and Rif

RhoD and Rif share up to 48% of sequence similarity and have an extra N-terminal sequence; however, studies suggest that they might functionally diverge in their impacts on actin organization [85,86]. These two proteins have been however suggested to have similar roles in the management of actin organization [87].

RhoD has been reported to have implication in the regulation of two different cell activities associated to actin. First, it mediates the transport of early endosomes which is actin dependent [119] via its interaction with the diaphanous-related formin protein hDia2C, which leads to the activation of Src [88,89]. Second, it stimulates the dismantlement of actin stress fibers and destruction of focal adhesions and prevents cell motility [87]. It is unknown whether there is correlation between the two processes. Rif has a very wide expression and contributes to filopodia development despite its remote relation to CDC42 (43% identity).

Moreover, based on its effector domain sequence, Rif would not be expected to interact with CDC42 effectors that are involved in the reorganization of actin like N-WASP and WASP.

RhoH/TT

RhoH/TTF was initially discovered as a chimeric transcript from a chromosome 3:4 translocation [90]. It was subsequently named RhoH due to its expression in hematopoietic cells specifically. The *RhoH/TTF* gene is mutated or mixed with other genes in diseases like non-Hodgkin's lymphoma and multiple myeloma [90]. RhoH/TTF contains a Rho insert sequence, but shorter than that of other Rho GTPases. RhoH/TTF, like Rnd proteins, is deficient in the conserved residues that correlated with G12 and Q61 that are also found in other Rho GTPases and as a result is likely to be GTPase lacking, and consequently not controlled by GDP/GTP cycling [90].

The RhoBTB subfamily

RhoBTB subfamily of proteins have been discovered in a database search for Rho-linked GTPases in *Dictyostelium* [91]. These unusual Rho GTPases have different structures from the other Rho-family members and have major extra sequences downstream to the Rho GTPase domain. The extra C-terminal sequences have a tandem repeat of BTB domains and is deficient in the C-terminal CAAX prenylation signals. The BTB domain was originally discovered in the *Drosophila* transcriptional repressors Broad complex, Tramtrack and Bric-a-brac, and exists in as many as 200 human proteins [92].

In mammals, this family of GTPases includes three members: RhoBTB-1, RhoBTB-2, and RhoBTB-3. Both RhoBTB-1 and RhoBTB-2 contain the Rho insert sequence that differs from the Rho GTPases and other Ras superfamily proteins.

The GTPase domain of RhoBTB-3, nonetheless, is somewhat conserved and atypical of Rho domain. Therefore, it has been suggested that RhoBTB-3 should not belong to the Rho-family.

RhoBTB2 was discovered as a deleted gene (*DBC2*) independently. Whether or not it is mutated, it is expressed in many breast cancer cell lines and tissue samples [93].

Expression of the wild-type RhoBTB2 prevents formation of T47D human breast cancer cells [93]; however, this is not the case for the mutated versions found in breast cancers.

The Miro subfamily

The Miro subfamily of GTPases consists of two proteins: Miro-1 and Miro-2 [94]. These two proteins contain presumably two GTPase domains and two EF-hand motifs. The N-terminal GTPase domain shares high sequence similarity with that of other Rho GTPases. These two proteins Miro-1 and Miro-2 have been called atypical Rho proteins since they were discovered through a database search for proteins having the Rho domain [94].

Nonetheless, based on the divergence of their overall sequence from the other Rho GTPases, added to a deficiency in the Rho-specific insert sequences, these GTPases should not be part of Rho family [94,95].

Rho GTPases activities have been thought initially to be controlled by the shuttling between an inactive, GDP-bound version, and an active, GTP-bound version of the Rho GTPases. Nonetheless, it has been recently found that their activities are controlled by other ways. These other types of regulation appear to be essential for the less investigated Rho GTPases.

Regulation by nucleotide binding

The GDP/GTP cycle of Rho proteins is regulated majorly by the GEFs, the GAPs, and the GDIs [96]. The control mechanism of these three families of regulatory proteins, with some exceptions, has yet to be fully elucidated. The RhoGEF family is a very large family which

encompasses as many as 60 members which are part to the Dbl family of proteins [96] and about ten members which are part of a more recently discovered Dock family [97].

The RhoGAP family, a vast group, counts as many as 80 different members encoded in the human genome; and the way they are regulated are even less clear than those of the RhoGEFs. These proteins diverge a great deal in sequence and domain structure but show more sequence similarity in their RhoGEF and RhoGAP domain [98].

Regulation by lipid modification

Rho GTPase capacity to accomplish its functions depends very much on its anchorage to the membranes and their subcellular localizations.

These activities are controlled by C-terminal lipid group and sequences, and by binding to RhoGDIs as well. The level of the cellular dissemination of Rho GTPases indicates their importance in that location.

Contrary to RhoGEFs and RhoGAPs, the number of known RhoGDIs is very low. Three well-defined RhoGDIs have been identified: RhoGDI-1, RhoGDI-2, and RhoGDI-3; some other proteins have also been suggested to possess GDI activity towards Rho proteins [99,100]. The three most studied GDIs appear to differ in their specificity to bind Rho proteins. While RhoGDI-1 interacts with RhoA, Rac1 and CDC42 with high affinity, RhoGDI-3 binds with high affinity as well to RhoB and RhoG; they however seem to bind to RhoA and CDC42 with a lower affinity, but they do not bind to Rac1 and Rac2 [101]. RhoGDI-2 targets are yet to be found, but it does not readily bind to RhoA, Rac1 or CDC42 [102]. The concentration of RhoGDI-1 in the cell was compared to that of the target GTPases [103].

Interestingly, based on the stoichiometry of RhoGDI-1:GTPases, it was suggested that most Rho protein in the cell are bound to GDIs which leads to their inactivation, which suggest that binding of Rho to Rho-GDI represents a very powerful way to regulate RhoGTPases. Several proteins have been found to bind to RhoGDIs and possibly control their binding interaction with Rho proteins [104,105].

Most Rho GTPases have a terminal CAAX tetrapeptide sequences that are isoprenylated. Some Rho members have a C15 F isoprenoid, and some others have a more hydrophobic C20 geranylgeranyl isoprenoid tail [106,107]. The geranylgeranyl modification facilitates tighter association to the membrane; however, the sort of isoprenoid tail does not appear to provide functional uniqueness, except for RhoB [108-111].

The subcellular location of Rho GTPases is not merely determined by the addition of terminal lipid group. For instance, Ras proteins require a second C-terminal signal to be wholly functional. Some protein members are further modified by a palmitate at C-terminal cysteine residues right after the CAAX motif. Some members have sequences rich in lysine/arginine residues and some others have both sequence components. The addition of a palmitoyl group can also impact RhoGDI recognition [110]. For instance, RhoB palmitoylation disturbs RhoGDI-1 identification and impacts subcellular localization as a result [110]. The conventional CAAX motifs are still absent in members like Chp, RhoBTB, and Miro, shedding doubt on whether their C-terminal sequences have any relevance in subcellular localization [111].

This variety in the C-terminal sequences of these proteins helps a great deal in diversifying their roles [112]. RhoGDIs have key roles in the regulation of Rho subcellular localization.

For example, RhoG is located in the Golgi complex by RhoGDI-3 [113]. RhoA and RhoB are found at very different locations in the cell [114], probably due to their difference in GDI binding.

Protein kinase A can control the binding of RhoA to GDI by phosphorylating a C-terminal serine in RhoA which subsequently leads to the binding of RhoA to GDI [116]; similar binding patterns have been observed for other RhoA members. Downregulation of RhoGDI-2 is observed in many tumors; RhoGDI-2-tranfected metastatic cell lines show inhibition in cell metastasis [117].

Regulation by gene expression

A great number of Rho GTPases appear to be tightly controlled by their expression level in addition to them being regulated by GEFs, GAPs, and GDIs. The level of mRNA and proteins belonging to the Rho GTPases family such as RhoA, Rac1 and CDC42 are somewhat stable in most tissues, the expression level of most other Rho GTPases is not.

Several Rho proteins are only expressed in some tissues which is indicative of their specific roles in these cells. Moreover, among the less studied Rho GTPases, some appear to only be regulated transcriptionally which suggests that their gene expression is dictated by necessity.

CDC42: functions and structure

The CDC42 gene codes for a 21.3 KDa, 191 amino acids small GTPase [105,106]. Through alternative splicing, a single CDC42 gene results in two isoforms: isoforme 1 (known as placental isoform, CDC42p) [107] and isoform 2 (also called brain isoform, CDC42b) [108]. CDC42 is a member of the Rho family and is crucial to many cellular processes including establishment of cell polarity and morphogenesis [105].

It was originally identified as an arbitrator of cell division in *S. cerevisiae* (yeast) [105,106]. In humans, CDC42 modulates signaling pathways that oversee four main cell processes which are: cell polarity (which depends on the aysmetrical), transport and endocytosis, actin polymerization, and RNA processing [20].

It plays key roles in the development of bone formation, cardiovascular, immune, and nervous systems. Moreover, CDC42 is necessary for the development of the pancreas, blood cells, eye, and skin [109]. Structurally, CDC42 is a dimer with two homologous chains A and B (figure 4) [20]. Its domains include a P-loop domain which lodges the nucleoside triphosphate hydrolase and a small GTP binding domain [20]. A comparison of RhoA-GDP and RhoA-GTP crystal structures unveils that the conformational contrasts between GTP and GDP-bound configurations are limited predominantly to the switch I and switch II regions [21].

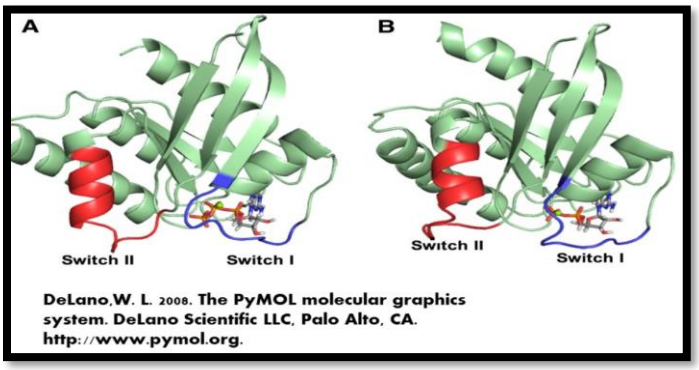

Fig.4. Structure of CDC42. CDC42 contains two homologous chains: A and B and two important regions: Switch I and II.

ACK Family Kinases

ACK1 (also called TnK2) is a 120 kDa protein [107] with an N-terminal end that contains a sterile alpha motif (SAM) domain, a kinase domain, an SH3 domain, and a CDC42/Rac-interactive domain (CRIB) [108,109] (figure 5). The C-terminal end of ACK1 contains important regions such as multiple proline-rich sequences, a clathrin-binding motif [54], a

ubiquitin binding domain [26], and a region that has a high homology with Mig6 [55]. The ACK family kinases are the only NRTKs that have a SH3 domain located C-terminally to the kinase domain. They are as well the only tyrosine kinases that contain a CRIB domain.

ACK1 is the first member of the ACK family. It was cloned from a human hippocampal expression library via its capacity to bind active CDC42 specifically and not Rac1 or RhoA [54]. The ACK1 encoding gene is found on chromosome 3q29 in humans which has been shown to be associated with prostate cancer development and is used as metastatic deterioration predictor in breast cancer [55].

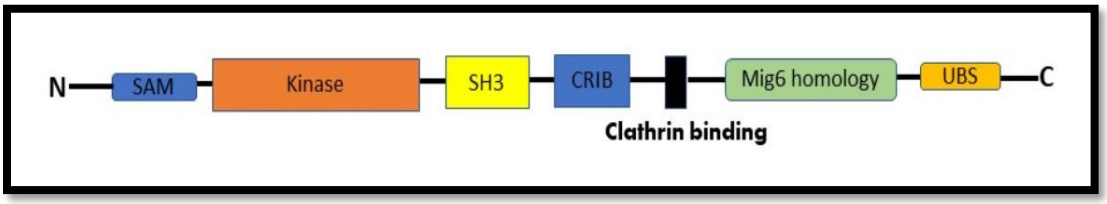

Fig.5. ACK1 domains arrangement. The ACK family kinases are the only NRTKs that have a SH3 domain located C-terminally to the kinase domain. They are as well the only tyrosine kinases that contain a CRIB domain.

In its downregulated conformation, ACK1 is autoinhibited by an intramolecular interaction between the Mig6 homology region (MRH) and the kinase domain [121]. Phosphorylation and activation of ACK1 can be promoted by several stimulatory signals such as phosphorylation of the MHR by receptors like EGFR, the attachment of the poly-proline containing regions to the SH3 domain, and the binding of GTP-CDC42 to the CRIB domain [123,124]. Upon activation, ACK1 phosphorylates and activates guanine nucleotide exchange factor Dbl, which in turn regulates CDC42 by promoting GDP/GTP exchange [125]. Activated ACK1 also phosphorylates downstream effectors such as p130Cas or WASP to regulate cell adhesion and migration [126,127].

Moreover, in its active conformation, ACK1 promotes tumor formation by the phosphorylation of Wwox (a tumor suppressor), Akt, Androgen receptor (a transcriptional activator) [128].

Rho GTPases in cancer

Rho proteins have been reported to participate in the development of pathological states including cell migration, invasion, and cell metastasis, inflammation, as well as wound repair in addition to their physiological roles. It has been reported that Rho GTPases assist in most steps leading to tumor initiation and progression which include the ability to acquire unrestricted proliferation ability, survival and evasion from apoptotic mechanisms, invasion into tissues and the establishment of metastasis.

Primary tumors, by and large, develop due to many mutations and epigenetic modifications which affect essential genes that eventually impact cell proliferation and survival.

In 15% of all human tumors exist mutations that activate K-Ras, N-Ras, and H-Ras. In contrast to the Ras family of proteins, Rho family of proteins undergo very little mutation in tumors while their level of expression and activity is often modified. For instance, various Rho GTPases are found upregulated in certain human tumors which include RhoA, RhoC, Rac1, Rac2, Rac3, CDC42, Wrch2/RhoV and RhoF [129,130].

Unrestrained cell proliferation associated with enhanced survival signaling activities allowing tumor cells to avoid apoptosis lead to tumorigenesis. Certain Rho GTPases trigger cell cycle progression and mediate gene transcription which could partly elucidate their propensity to oncogenicity [131]. The stimulation of tumor vascularization is paramount for tumors to develop and exceed certain size and malignant cells produce factors that facilitate the formation of new

blood vessels from pre-existing blood vessels in the vicinity. It is thought that some Rho GTPases can mediate pro-angiogenic factors release to enhance the formation of new blood vessels [132].

Epithelial cancer invasion begins when the epithelium has become defective and malignant cells have destroyed the membrane and penetrated the underlying stroma. This usually means the epithelial cell–cell contacts have become loose and addition of a more moving phenotype in an activity often called “epithelial to mesenchymal transition” (EMT). Invading epithelial cancer cells often have decreased expression of E-cadhenin which is the cell-cell adhesion protein and begin the expression of markers of mesenchymal nature like vimentin and N-cadherin [133]. Non-epithelial cancers migrate into solid tissues as well [134].

Invasion of cancer cells into tissues can be done either individually or collectively. It has been reported that single cells use two ways to migrate: mesenchymal and amoeboid migration. Cells undergoing mesenchymal migration possess an extended morphology and expand long protuberance at the front. Integrin-based adhesion and a solid traction are what drives the migratory movement in addition to the degradation of extracellular matrix (ECM) by transmembrane proteases [134]. On the contrary, amoeboid migration does not depend on the action of extracellular proteases and is assisted by cortical actomyosin-based contraction. When cells invade collectively, they move together through the extracellular matrix and keep cell–cell adhesions. The cell located at the front of the group produces the migratory traction needed for movement and the other cells are pulled passively. The ECM degradation by matrix metalloproteases (MMPs) is paramount for this sort of group migration [135]. To migrate into remote tissues, tumor cells have to penetrate the vascular or lymphatic system for proliferation in the new tissue.

The capacity of Rho GTPases to control the dynamics of cytoskeleton, cell adhesion and cell migration [136] highlights their key function in cancer cell invasion and metastasis.

The Rho family consists of subfamilies including RhoA, RhoB and RhoC proteins which are very conserved. While RhoB expression and activity are frequently diminished in human tumors, that of RhoA and RhoC are often enhanced in human tumors [137].

RhoA is involved in almost all levels of cancer progression. RhoA may play an important function during tumor proliferation and survival [137]. In normal epithelial cells, RhoA participates in the organization of epithelial polarity and junction assembly and function [138]. However, it also has impacts on the destruction of the epithelial during the progression of tumor. Rho action can be prohibited by adherins which can cause the phenotype to be more mobile [139].

Distinct GEFs and GAPs have impact on the actions of Rho proteins in different situations either assisting in the organization of the epithelia and polarity or in epithelial EMT [140].

RhoA is crucial for migration done according to the amoeboid and mesenchymal way. It is suggested that RhoA-ROCK signaling stimulates cortical contractility based on actomyosin resulting in amoeboid migration by blistering and in tail withdrawing in mesenchymal migration as well [138]. It was shown in a 3D *in vitro* invasion model done with co-cultures of SSC12 carcinoma cells, which have not been transformed in the mesenchymal mode that keep certain epithelial markers, that stromal fibroblasts have displayed a fibroblast directing cell in this version of group cell invasion [141]. This fibroblast cell produces the necessary pulling power and reshapes the matrix via MMPs.

It was found that many Rho GTPases were needed in the commanding fibroblast and the other tumor cells where a RhoA protein controls the myosin light chain (MLC) in the leading cell and mostly CDC42 and MRCK role in the following cells.

It has been further demonstrated that Rho GTPases can control MMPs synthesis, influencing matrix reshaping and tumor cell invasion. Moreover, Rho can stimulate MMPs expression or secretion [142].

RhoC has not been reported to have any transforming action contrary to RhoA. The contribution of RhoC in cancer seems to be limited majorly to metastasis [143]. Research conducted on melanoma metastasis found that RhoC gene expression was upregulated [144] and has as a result been suggested being a marker for low prognosis in different cancers[145].

Enhanced RhoC expression has been proposed to be the possible reason for the increase in invasion and metastasis stimulated by microRNA-10b overexpression in breast cancer [146]. Research on RhoC knock-out mice indicated that RhoC is not required for tumor initiation and embryogenesis yet is indispensable in metastasis.

There is an increase in RhoC expression during EMT in a colon cancer model and assists in migration induced by EMT, while RhoA expression levels decrease. Studies involving knocking down *RhoC* gene by RNAi reveal that RhoC is crucial for cell invasion in vitro. It is yet to clarify the mechanism by which RhoC stimulates invasion and metastasis or the reason for varying impacts from RhoA.

Studies largely done on chemical inhibitors show that RhoA and RhoC and their following target ROCK are necessary for cancer cell leaking and secretion [147]. Surprisingly, RhoC can

stimulate angiogenic factors synthesis in breast cancer which could assist in facilitating invasion into blood vessels and causing metastasis. Further work is necessary to clarify the probable assistance of Rho GTPases in the leaking of cancer cells.

RhoB expression often plummets in human tumors and its expression level usually goes down as a tumor becomes more aggressive, which is not the case for RhoA and RhoC [148]. RhoB has been suggested to function as a tumor suppressor as it becomes active as a result of the production of several stress stimuli including DNA damage or hypoxia. It has also been reported that RhoB prevents tumor development and metastasis and play keys roles in cell proapoptotic activity. RhoB knock-out mice grow well but develop increased carcinogen-induced skin tumor, which is in concurrence with a function of tumor suppressor carried out by RhoB [149].

RhoB also decreases cell invasion: for instance, it has been argued that RhoB works downstream of PKCτ to control cancer cell invasion in vitro [150] and reports also showed that it decreased invasion and metastasis induced by Ras proteins [151].

The suppression mechanism by which RhoB works to suppress tumor development and invasion is yet to be elucidated; its function in endosomal trafficking could play a role in that. RhoB controls proteins delivery which include growth factor receptors and the tyrosine kinase Src to cell compartments that are very specific [152]; this may have some effect on cell proliferation and invasion.

CDC42 and its very closely related Rho proteins such as RhoQ/TC10 and RhoJ/TCL constitute a unique Rho GTPases subfamily. There is an increase in the CDC42 expression level in some breast cancers [153], yet it is reported that the deficiency in CDC42 promotes liver cancer

formation in a liver-specific knock-out study [154]. This suggests that the participation of CDC42 in cancer development might be tissue specific. This could explain the various functions of CDC42 in adjusting cell polarity as well as cell cycle continuation.

CDC42 and RhoQ/TC10 promote cell transformation and assist in cell transformation caused by Ras [155]; CDC42 contribution to cell transformation is proposed to be a result of its influence on receptor trafficking and degradation [156]. RhoQ/TC10 is also involved in receptor trafficking, especially of the glucose transporter [157]. However, it is not known whether this helps in its contribution to cell transformation. Another way CDC42 could influence cell cycle continuation is by controlling chromosome separation during mitosis [158]. Among all Rho GTPases, only *CDC42* gene knockdown was determined to trigger "chromosome misalignment" during mitosis which caused cells to be multinucleated.

CDC42 participates in the organization of epithelial polarity. It also contributes to migratory polarity through binding to the Par3/Par6/aPKC polarity complex, which subsequently controls Rac via Tiam1 [159]. It is predicted that CDC42 prevents invasion by enhancing epithelial polarity, but also causes migration inversely.

Therapeutic strategies to target Rho-related cancers

Targeting the signaling pathways regulated by Rho proteins has gained much attention recently in the cancer drug discovery field, with strategies aiming at CDC42 which can also be potentially applied in neurodegenerative and infectious diseases [160-163]. Inhibition of CDC42-mediated pathways can be done using several strategies which include inhibition of the post translational lipid modification, inhibition of CDC42 regulatory proteins and CDC42 downstream effector

interactions, direct inhibition of effector kinases, and covalent irreversible inhibition of nucleotide exchanges assisted by GEF.

Tackling CDC42 nucleotide binding

Since the activity of Rho GTPases is mediated by the nucleotide they are bound to, a simple strategy would be to prevent GTP from binding to Rho GTPases. Many reports on the strategy aiming at inhibiting GTP from binding to Rho GTPases have detailed the challenges for the design and generation of small molecules being able to target the nucleotide binding sites of GTPases and successfully compete with the picomolar binding affinity of the nucleotide [164]. Despite all the adversity encountered, the use of this approach has led to the discovery of small molecule inhibitors like ML141 [163] (figure 6) and R-Ketorolac (figure 7) [165].

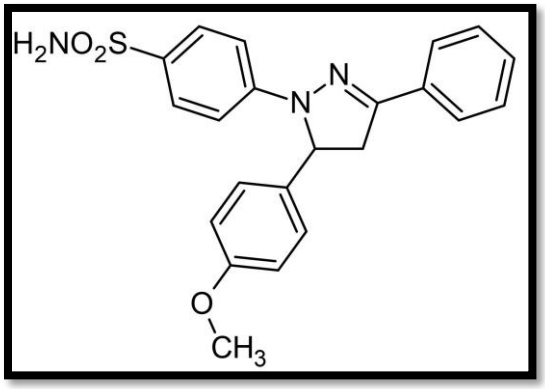

Figure 6. Structure of ML141

ML141 was discovered in an in-silico screening against key members of the Ras superfamily due to its capacity to diminish GTP binding to CDC42. ML141 was proposed to interact with an allosteric site of CDC42 to cause GTP dissociation. Nevertheless, ML141 does not have any

inhibitory effect on cell migration [165]. Likewise, R-Ketorolac works as allosteric inhibitor of GTP to CDC42 and Rac1 which is closely related to CDC42 [166].

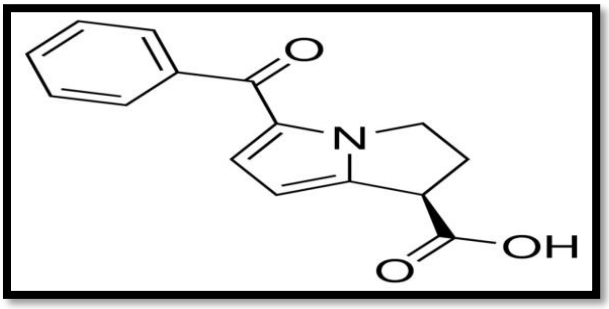

Figure 7. Structure of R-Ketorolac

Studies show that ovarian epithelial cancer cell lines that were treated with the small molecule R-Ketorolac displayed decreased migratory and invasive behaviors [167].

Tackling CDC42–GEF interactions

Targeting CDC42 can also be done through directly targeting specific CDC42–GEF complexes interfaces. This strategy has led to discovery of several small molecule inhibitors [168,169]. In this strategy, the biggest challenge arises from the overlaying interfaces between specific CDC42–GEF and the indiscriminate binding activity of the GEFs to Rho proteins [170,171], which leads to small molecule inhibitors lacking in potency and specificity. This has led to many small molecule inhibitors with pan or dual inhibitory properties towards Rho proteins like CDC42 and Rac1. Concerning pan Rho family small moelcule inhibitors, it can be suggested that designing drugs with a more promiscuous binding pattern can be more advantageous to overcome resistance to a treatment against a single Rho GTPase protein target, since many of the cell functions of Rho family GTPases depend on a defined equilibrium between several Rho family members [172]. However, investigating and evaluating the singular biological effects resulting from inhibition of CDC42 requires specificity and selectivity within the Rho proteins.

Tackling CDC42–GEF interfaces

CDC42-Intersectin (ITSN1) is the complex having been targeted the most [173]. ITSN1 is a GEF specific to CDC42 [174] and is also the RhoGEF that has undergone the most mutations in some cancers [175]. ZCL278 (figure 8) was identified as a small molecule inhibitor by a high throughput virtual screening where small molecules were designed to dock into the ITSN1 binding pocket of CDC42.

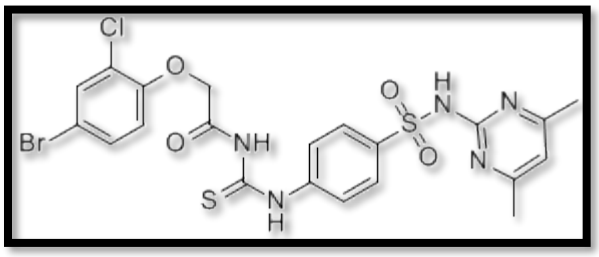

Fig. 8 Molecular structure of ZCL278

It was predicted that ZCL278 would H-bond with Thr35, Asn39 and Asp57 of CDC42 as well as hydrophobically interact with Val36 and Phe56. It is with low micromolar affinity that ZCL278 binds to CDC42 and was found to inhibit various cell activities mediated by CDC42 [175]. Despite its inhibitory prowess, ZCL278 was soon proved to be a partial agonist of CDC42 in some situations, and focus was shifted from ZCL278 to ZCL367 [176]. ZCL367 has enhanced potency and better selectivity in another screen assay conducted on A549 and PC3 cell migration [176]. It was also found that ZCL367 possesses select potency towards CDC42 (IC50 = 0.098 μM) over RhoA (IC50 = 29.7 μM) and Rac1 even if it is relatively much less potent (IC50 = 0.19 μM). Prevention of migration and proliferation has been demonstrated in studies involved lung and prostate cancers driven by EGFR and Ras [176]. Moreover, ZCL367 was shown in a lung cancer xenograft mouse model to reduce tumor development upon ZCL367 therapy and filopodia formation mediated by CDC42 was reduced in cell lines [176].

CASIN (figure 9) is another small molecule that interacts with CDC42-GDP with a ~300 nM affinity and prevents the dissociation of GDP catalyzed by the CDC42-Intersectin complex [177,178]. Docking studies reveal that CASIN interacts with inactive CDC42 at a site adjacent to the switch I where a Met45 residue would create a critical binding interaction: mutation of Met45 (M45E) disrupted CASIN ability to bind CDC42.

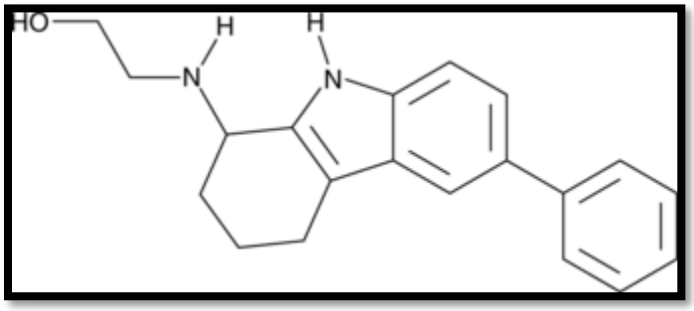

Fig. 9 Molecular structure of CASIN

It has been demonstrated that CASIN prevents the mobilization of F-actin and cell migration [179]. Lately, it has been found that CASIN prolonged murine lifespan by a period of four days, revealing an interesting optional route to inhibit CDC42 [180].

Targeting the nearest relative of CDC42, Rac1, has been very advantageous in the search for CDC42 inhibitors. NSC23766 (figure 10) is a small molecule produced initially to prohibit the interaction between Rac1-Trio and Rac1-Tiam1[181].

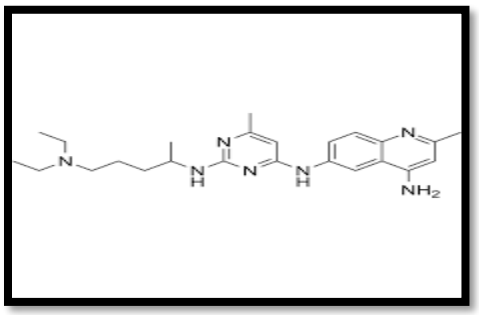

Fig. 10 Molecular structure of NCS23766

NSC23766 was discovered through in silico screening as a small molecule that bound at the GEF-recognition pocket of Rac1 which centers on Trp56 residue. Ehop-016 was another small molecule formed from NSC23766 with both molecules possessing a central pyrimidine ring with six members [182]. It was found to inhibit Rac1 with a 1.1 μM affinity; it was also found that Ehop-016 inhibits the activity of CDC42 at a 10μM concentration [183]. MBQ-167 was also discovered by the same group in virtual screening to discover a small molecule that is able to bind deeper into the NSC23766 binding pocket found on Rac1, through the exploitation of a H-bond formation with Asn39 residue. Asn39 is found in switch I of Rac1 and CDC42, which makes MBQ-167 a dual CDC42/Rac1 inhibitor with IC50 of 103 nM for Rac1 and an IC50 of 78 nM for CDC42. Characterization studies of the small molecule MBQ-167 led to a preclinical mouse model where it prevented the development of HER-2 type tumor and invasion in immunocompromised mice [184].

NSC23766 has been used to construct small molecules being able to target the CDC42-Dbs [182]. AZA1 (figure 11) is a small molecule that was reported to have the potential to inhibit Rac1–GEF complex formation [185].

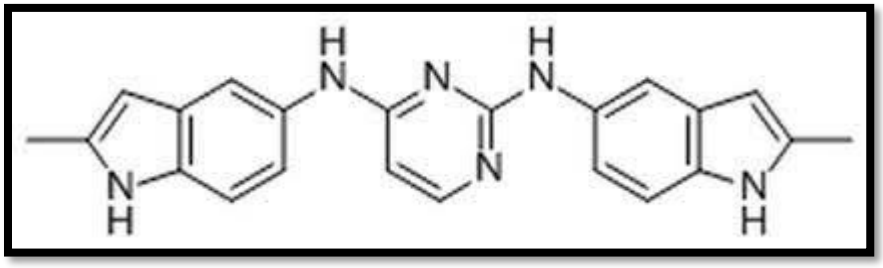

Fig. 11 Molecular structure of AZA1

It was shown that AZA1 was a dual inhibitor for Rac1 and CDC42, with the capacity to suppress both Rho GTPases activities [186].

The potency of AZA1 is relatively low, which suggests the existence of many bonds that are very flexible inside the small molecule [186]. AZA197, a more restrained small molecule, has recently been synthesized, which is found in the classic 'flat' small molecule chemical pocket [187].

Targeting the interaction of CDC42–Dbs led to satisfactory specificity, without any prohibition of Rac1 or RhoA and with a decreased nucleotide exchange for CDC42. AZA197 was shown to prevent proliferation, cell migration and invasion in both HT-29 and SW620, two colorectal cancer cell lines. AZA197 was further shown to reduce tumor development in a murine xenograft model using SW620 cells [187].

Tackling cysteine through irreversible inhibition

A completely different method of prohibiting CDC42–GEF interaction is the use of a cysteine residue next to but not within the interface of the two binding proteins (CDC42-GEF) that causes inhibition of nucleotide exchange catalyzed by GEF. A group of covalent, irreversible small molecule inhibitors have been made lately to target RhoA Cys107 residue [188]. DC-Rhoin04, the tightest binder, prevented RhoA nucleotide exchange with a IC50 of ∼3 μM [188]. All these molecules possess electron-deficient alkenes that can react with the nucleophilic cysteine. The cysteine of interest exists merely in Rho proteins and as a result brings about selectivity over the other members of Ras superfamily. It was revealed that the crystal structure of the DC-Rhoin04-RhoA complex contains a binding pocket, called the CLocK pocket, which expands from Cys107 residue and is stimulated by DC-Rhoin04 binding interaction. CDC42 Cys105 is thought to be DC-Rhoin04 site of action, permitting it to prevent the CDC42-intersectin binding.

It was shown that DC-Rhoin04 suppressed cell migration and invasion in the breast cancer cell line MDA-MB-231.

Small molecules inhibiting cysteine covalently possess the capacity to promiscuously bind to other Rho proteins with other cysteines located on off-target protein surfaces and display toxicity if the small molecule inhibitor is very sensitive or specificity deficient [189].

The off-target capacity to cross-react with the compound DC-Rhoin was probed and it was shown to have no inhibitory activity against Ras proteins, which lacks the Cys105/107 counterpart [188]. It is yet to be probed the timescale of DC-Rhoin target inhibition since this is very important for their success as prolonged inhibition can lead to cell toxicities [190]. Producing an irreversible inhibitor strategically targeting a cysteine has seen the most success in the targeting of K-Ras G12C mutations [191], which led the small molecules MRTX849 (figure 12) and AMG-512 to clinical trials [192]. These small molecules target an oncogenic GDP-bound, inactive K-Ras with a cysteine mutation [193]; however, wild type RhoA/CDC42 Cys105/107 can also be targeted by the DC-Rhoin small molecules. DC-Rhoin interacts with the inactive form and may as well bind the active form if the cysteine residue is exposed to the solvent. DC- molecule binds at the CLocK binding groove that is next to, yet distinct from the binding site that is targeted in K-RasG12C.

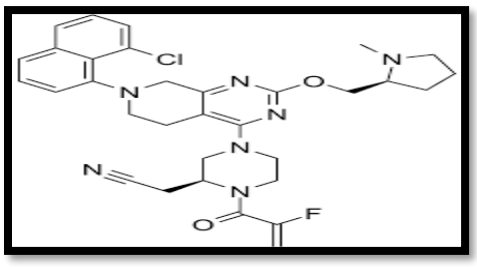

Fig. 12 Molecular structure of MRTX849

Tackling CDC42-effector interfaces

Another approach to target Rho proteins is to develop peptide or small molecules that can target CDC42-effector interfaces [194]. This approach takes advantage of the selectivity of the effector proteins for their target.

CDC42 binding peptides from a CIS display screening were selected and were used for the design of second-generation peptides which display low nanomolar binding affinity for CDC42. It was shown by mapping using NMR chemical shift that the second-generation peptides bind far from the switch regions yet displaying binding clash for a group of CDC42 effectors like ACK, WASP and PAK1. It was shown that binding shows a small difference between inactive CDC42 and active CDC42, which indicates that the peptide inhibitors promiscuously target both CDC42-GDP and CDC42-GTP [195].

Recently, peptides have been recognized as a fast-growing therapeutic method just in between small molecules and antibodies. Advancement in the integration of "non-canonical amino acids" to widen the advantageous virtues of peptides according to prior characterization work on protein–protein interfaces [196], "molecular grafting" [197] and new cellular means for delivery which include cell-penetrating peptides [198] and nanoparticles [199], which indicate some of the recent progress solving stability and delivery problems.

Moreover, new therapeutic strategies like nanobodies, "target-binding fragments of monoclonal antibodies", and PROTACs show the tendency to prevent or decrease cytosolic protein levels through degradation by proteosomes. While these strategies have not seen any application in the case of CDC42, nanobodies have been developed to selectively bind the GTP-bound form of RhoB, and consequently can be considered a new strategy to inhibit Rho proteins [200].

Tackling directly CDC42 effectors

CDC42 downstream effector proteins show many potential targets for drug design. Cancer mutational data have shown that, CDC42 kinase effector proteins like PAK1, PAK2, PAK4, MRCKα, MRCKβ and ACK which have been targeted directly, display the biggest level of modifications among the CDC42 effector protein. Nonetheless, despite their druggable properties, they are at a disadvantage since they present similar specificity and resistance problems that are also found when targeting other kinases [201].

Problems in finding selectivity for the protein kinases downstream of CDC42 majorly stem from the fact that the ATP binding site is structurally very conserved, since the majority of kinases have a common conserved Asp-Phe-Gly (DFG) motif which is able to exist in two different conformations [202]. Category I inhibitors impede the kinase in the 'DFG-in' active state and "compete" with ATP, creating key H-bonds with the "kinase hinge region". Category II inhibitors interact and maintain a kinase in its inactive 'DFG-out' state, filling both the ATP-binding pocket and a hydrophobic allosteric site developed in the inactive state. Allosteric inhibitors of type III and IV show promising options to reach kinase specificity.

Category III inhibitors interact with and bind at a site near the ATP groove with no contact with the "hinge region" and in a hydrophobic groove developed in the DFG-out state. Category IV inhibitors bind at a unique allosteric pocket, remotely from the catalysis site. Categories I, II and III inhibitors have been illustrated for effector kinases of CDC42.

The PAK family of proteins have been the object of much research; however, progress in developing PAK inhibitors has been obstructed by problems related to specificity and toxicity since they are ubiquitously expressed.

A loose ATP binding rift has been found in the PAK proteins [203], which may partially illustrate the issues for finding specific type I inhibitors. PF-3758309 binds at PAK4 ATP binding site, H- bonding with the hinge region and creating an important ionic interaction with Asp458 of the DFG motif through its dimethylamine group [204]. PF-3758309, nonetheless, failed phase I clinical trials because it does not provide dose-dependent efficacy since it was revealed to be a multi-drug transporter P-glycoprotein substrate [205].

KPT-9274, PAK4 Type II allosteric modulator, reached a Phase I clinical trial for its efficacy in advanced solid malignancies and non-Hodgkin's lymphoma. KPT-9274 is a “dual inhibitor” of PAK4 and nicotinamide phosphoribosyl transferase (NAMPT), which is a metabolic enzyme that catalyzes the first step in the pathway that leads to the biosynthesis of NAD+ from nicotinamide [206-208]. It was found that KPT analogues interacted with PAK4 by chemical proteomics, but impediment of NAD+ biosynthesis was also documented. It was revealed through a CRISPR-induced drug resistance study that NAMP is the main target for KPT-9274. NAD+ is an important proliferation metabolite and tumors rely on it to rapidly grow [209]. Dual inhibition of NAMPT and PAK4 signaling has found therapeutic use in many cancers due to cooperative actions [210].

KPT-924 hydrophobically interacts with DFG motif Phe193 and NAMPT Tyr. No structure of KPT-924-PAK4 complex exists, but it is suggested that it binds at the same location on PAK4. The capacity of KPT-9274 to impede kinase-independent functions of PAK4 as well may also lead to beneficial drug properties over the type I inhibitor PF-3758309.

There has also been some advancement in targeting other CDC42 kinase effector proteins, for instance, the small molecule inhibitors of MRCKα and MRCKβ, BDP8900 and BDP9066.

It was reported that through “structure-guided fragment elaboration”, BDP8900 and BDP9066 were discovered as stronger binders of the MRCK ATP-binding site than the initially discovered BDP5290 [211]. Hematologic cancers were reported to be the most sensitive to BDP9066 in a study consisted of screening up to 750 human cancer cell lines across 40 cancer types. It was also shown that BDP9066 had therapeutic property in skin cancer, for instance, skin papilloma growth was diminished upon tropical treatment of BDP9066 in a model of murine squamous cell carcinoma [212]. In summary, developing stronger and selective small molecules should facilitate the understanding of their potential inhibitory property towards MRCK in cancer [213].

There has not been much progress in the search for ACK inhibitors, and the ones that have been reported merely target the ATP-binding site [214]. The most studied and understood ACK inhibitor is AIM-100. Many molecules initially engineered to target other kinases also show inhibitory activity toward ACK as all those molecules aim at the very conserved ATP binding site. For instance, NVP-TAE684, a small molecule inhibitor of the oncogenic tyrosine kinase, NPM-ALK [215] also binds ACK with a 2 nM Kd [216]. However, NVP-TAE684 has not progressed due to potential toxicity issues.

The pathways of PAK and MRCK converge downstream on LIM kinases. Major challenge has been encountered when trying to find selective Type I and II inhibitors for LIMK1 and LIMK2, because of their 71% sequence identity in the kinase domain [217]. Nonetheless, various compounds have been designed and constructed which possess high binding affinities and promising pharmacokinetic virtues [218]. Several type I kinase inhibitors and allosteric compounds have been discovered for LIMK1 and LIMK2. It has been reported that a dual inhibitor of LIMK2 and ROCK, LX7101, has finished a Phase 1/2a research study in primary open-angle glaucoma and ocular hypertension, the data are not available yet.

Sulfonamide compound, the first Type III inhibitor known as LIMK2 ‘compound 22’, binds in a hydrophobic groove in the inactive DFG [219].

Inhibiting CDC42 anchorage to the membrane

Another strategy to target CDC42 is to prevent their anchorage to the membrane. CDC42 undergoes post translational modification at its C-terminus by a prenyl group; preventing this modification from occurring has been probed. The transfer of the prenyl group is catalyzed by type I geranylgeranyl transferase (GGTase I) and several small molecules with potential inhibitory activity toward this enzyme have been synthesized. Moreover, statins, which show inhibitory actions against the synthesis of a GGTase I substrate, geranylgeranyl pyrophosphate, have been probed as well. Nonetheless, the vast number of proteins that are natural substrates for the geranylgeranyl transferases causes unnecessary inhibition of other protein functions, which leads to severe side effects. This can illustrate why no GGTase small molecule inhibitors so far have found approval for clinical purposes [220].

Galanthamine, a natural analog of secramine A, prevents membrane attachment of geranylgeranylated CDC42 [221]. It was found that the inhibitory influence of Secramine A depends on RhoGDI-1, which serves as molecular chaperone that transports Rho GTPases from the cytosol to the targeted membranes.

Involvement of ACK1 and CDC42 in Cancer

ACK1 is involved in numerous stages and various cancers as studies conducted on the survival of cells transformed by v-Ras revealed that ACK1 was required for the survival of transformed cells [222].

A study of about 180 advanced-stage cancers revealed that up to 9% of the lung tumors and 14% of the ovary tumors displayed an overexpression of the *ACK1* gene on chromosome 3 [160]. ACK1 mRNA was found to be overexpressed in 42% of the aggressive lung tumors and 77% of metastatic hormone refractory prostate tumors [161]. Furthermore, overexpression of ACK1 generated various epithelial-to-mesenchymal transition (EMT) phenotypes in human breast cancer cell line MDA-MB-231. Mutational studies found that activation of ACK1 by a somatic point mutation led to EMT phenotypes in renal cancer cells [223].

As many as 41 mutations of CDC42 have been found in various cancers [224]. CDC42(G12V) was found in a melanoma biopsy as an oncogenic Ras mutation [225], CDC42(F28L) was shown to promote cell growth and malignant transformation of NIH 3T3 cell lines [226]; fast-cycling CDC42 mutants can exchange GDP for GTP yet still hydrolyze GTP and thereby promoting tumorigenesis. Some CDC42 mutants that cannot hydrolyze GTP (slow-cycling mutants) are kept in the active conformation and oftentimes prevent cell growth [226].

Influence of a Small Molecule on Ras-related proteins

The continuous necessity for small molecule inhibitors (SMIs) to tackle aberrant signaling processes triggered by Ras proteins in which ACK1 and CDC42 are directly involved makes them promising therapeutic targets. Based on the classic “GTPase cycle” model of Ras proteins, CDC42 alternates between an inactive GDP-bound state and an active GTP-bound state. The binding of GTP to CDC42 and the hydrolysis of GTP γ-phosphate to GDP are strictly modulated by regulatory proteins such as GEFs, GAPs, and GDIs [2,3]. Upon CDC42 activation, CDC42 arbitrates signaling cascades leading to the activation of more than 20 downstream effector proteins including protein and lipid kinases, scaffolding proteins, and cytoskeletal interacting

proteins bringing about modifications in cell processes such as cell polarity, adhesion, migration, growth, actin cytoskeleton formation, membrane trafficking and transcription. Each step of this cycle is tightly controlled [3].

Evidence shows that cell transformation occurs as a result of deregulation at either the GDP/GTP exchange level or at the GTP hydrolysis level of the GTPase cycle [167]. Cell transformation can also occur due to mutated enzymes involving in the migration and attachment of CDC42 to the cell membrane. Moreover, it may also stem from a mutated CDC42 that escapes the regulatory mechanisms, becomes overexpressed, and leads to anomalous activation of downstream effectors via abnormal signaling cascades. To solve this problem, drug candidates are designed with the 3-D structures of the wild type and mutated CDC42 in mind. Strategically, new SMIs can be designed and constructed that: 1) inhibit impaired specific GEF- or GAP-CDC42 complex formation, 2) hinder GTP-to-CDC42 binding, 3) impair CDC42-effector protein binding interactions, 4) restrict CDC42 from localizing to the cell membrane. To this end, SMI have been designed and constructed with varying level of success.

Among these SMIs, ZCL278, a 4-bromine-2-chlorophenol derivative (figure 8), has been so far very promising for its disruptive action on the binding of CDC42 with intersectin (ITSN1), a CDC42-specific GEF. ZCL278 can target CDC42 switch I binding pocket in which it binds to prevent CDC42- intersectin interaction. This CDC42-ZCL278 complex displays promising results in cancer treatment and ZCL278 shows itself to be very promising thanks to its high membrane permeability and low toxicity [175].

ZCL278 has been identified via high throughput in silico screening by Friesland et al. Molecular features of the CDC42-ZCL278 binding interaction remain lacking.

Nonetheless, using fluorescence spectrometry and surface plasmon resonance, studies revealed that there was direct binding between CDC42 and ZCL278 [175]. Furthermore, it was also shown that ZCL278 could bind to CDC42 via the switch I domain, which is the domain to which effector proteins bind to elicit intracellular responses [175].

Although ZCL278 was proven to be a promising CDC42 SMI, much remains to be known about their binding interaction such as the identification of key residues involving in this binding, the possibility that inactive CDC42 to bind to ZCL278, the identification of structural features of ZCL278 that promote its binding to CDC42, exploring the possibility of ZCL278 to bind to CDC42 downstream effector proteins such as ACK. Thus, our goal is to investigate the influence of ZCL278 on CDC42-ACK binding interaction and to characterize CDC42 wild type and CDC42F28L in the presence of ZCL278. To achieve that goal, *E. Coli* were used to express CDC42, CDC42F28L, and ACK peptide (504-545). Then, biochemical, and biophysical techniques such circular dichroism spectroscopy, fluorescence spectroscopy, *in vitro* binding assay, isothermal titration calorimetry, and GTP/GDP exchange assay were used to investigate the influence of ZCL278 on CDC42-ACK interaction and characterize the wild type and mutant CDC42.

References

1) Cherfils, Jacqueline, and Mahel Zeghouf. "Regulation of small gtpases by gefs, gaps, and gdis." Physiological reviews 93.1 (2013): 269-309.

2) Bos, Johannes L., Holger Rehmann, and Alfred Wittinghofer. "GEFs and GAPs: critical elements in the control of small G proteins." Cell 129.5 (2007): 865-877.

3) Wennerberg, Krister, Kent L. Rossman, and Channing J. Der. "The Ras superfamily at a glance." Journal of cell science 118.5 (2005): 843-846.

4) Trabalzini, Lorenza, and Saverio Francesco Retta. Ras Signaling-Methods and Protocols. Vol. 1120. Humana Press Inc.(USA), 2014.

5) Bourne, Henry R., David A. Sanders, and Frank McCormick. "The GTPase superfamily: conserved structure and molecular mechanism." Nature 349.6305 (1991): 117-127.

6) Schmidt, Anja, and Alan Hall. "Guanine nucleotide exchange factors for Rho GTPases: turning on the switch." Genes & development 16.13 (2002): 1587-1609.

7) Bernards, Andre, and Jeffrey Settleman. "GAP control: regulating the regulators of small GTPases." Trends in cell biology 14.7 (2004): 377-385.

8) Bishop, Anne L., and Alan Hall. "Rho GTPases and their effector proteins." Biochemical Journal 348.2 (2000): 241-255.

9) Repasky, Gretchen A., Emily J. Chenette, and Channing J. Der. "Renewing the conspiracy theory debate: does Raf function alone to mediate Ras oncogenesis?." Trends in cell biology 14.11 (2004): 639-647.

10) Herrmann, Christian. "Ras–effector interactions: after one decade." Current opinion in structural biology 13.1 (2003): 122-129.

11) Cox, Adirenne D., and Channing J. Der. "Ras family signaling: therapeutic targeting." Cancer biology & therapy 1.6 (2002): 599-606.

12) Seabra, Miguel C., and Christina Wasmeier. "Controlling the location and activation of Rab GTPases." Current opinion in cell biology 16.4 (2004): 451-457.

13) Wennerberg, Krister, Kent L. Rossman, and Channing J. Der. "The Ras superfamily at a glance." Journal of cell science 118.5 (2005): 843-846.

14) Nie, Zhongzhen, Dianne S. Hirsch, and Paul A. Randazzo. "Arf and its many interactors." Current opinion in cell biology 15.4 (2003): 396-404.

15) Pasqualato, Sebastiano, Louis Renault, and Jacqueline Cherfils. "Arf, Arl, Arp and Sar proteins: a family of GTP-binding proteins with a structural device for 'front–back'communication." EMBO reports 3.11 (2002): 1035-1041.

16) Pereira-Leal, José B., and Miguel C. Seabra. "Evolution of the Rab family of small GTP-binding proteins." Journal of molecular biology 313.4 (2001): 889-901.

17) Valencia, Alfonso, et al. "The ras protein family: evolutionary tree and role of conserved amino acids." Biochemistry 30.19 (1991): 4637-4648.

18) Pruitt, Kevin, and Channing J. Der. "Ras and Rho regulation of the cell cycle and oncogenesis." Cancer letters 171.1 (2001): 1-10.

19) Van Aelst, Linda, and Crislyn D'Souza-Schorey. "Rho GTPases and signaling networks." Genes & development 11.18 (1997): 2295-2322.

20) Hall, Alan. "Rho GTPases and the actin cytoskeleton." Science 279.5350 (1998): 509-514.

21) Schmitz, Arndt AP, et al. "Rho GTPases: signaling, migration, and invasion." Experimental cell research 261.1 (2000): 1-12.

22) Vetter, Ingrid R., and Alfred Wittinghofer. "The guanine nucleotide-binding switch in three dimensions." Science 294.5545 (2001): 1299-1304.

23) Bishop, Anne L., and Alan Hall. "Rho GTPases and their effector proteins." Biochemical Journal 348.2 (2000): 241-255.

24) Adamson, Peter, Hugh F. Paterson, and Alan Hall. "Intracellular localization of the P21rho proteins." The Journal of cell biology 119.3 (1992): 617-627.

25) Michaelson, David, et al. "Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding." The Journal of cell biology 152.1 (2001): 111-126.

26) Wang, L., Yang, L., Luo, Y. and Zheng, Y. (2003). A novel strategy for specifically downregulating individual Rho GTPase activity in tumor cells. J. Biol. Chem. 278, 44617-44625.

27) Chardin, P., Madaule, P. and Tavitian, A. (1988). Coding sequence of human rho cDNAs clone 6 and clone 9. Nucleic Acids Res. 16, 2717.

28) Jahner, D. and Hunter, T. (1991). The ras-related gene rhoB is an immediateearly gene inducible by v-Fps, epidermal growth factor, and platelet-derived growth factor in rat fibroblasts. Mol. Cell. Biol. 11, 3682-3690.

29) Zalcman, G., Closson, V., Linares-Cruz, G., Lerebours, F., Honore, N., Tavitian, A. and Olofsson, B. (1995). Regulation of Ras-related RhoB protein expression during the cell cycle. Oncogene 10, 1935-1945.

30) Adamson, P., Marshall, C. J., Hall, A. and Tilbrook, P. A. (1992b). Post-translational modifications of p21rho proteins. J. Biol. Chem. 267, 20033-20038.

31) Chen, Z., Sun, J., Pradines, A., Favre, G., Adnane, J. and Sebti, S. M. (2000). Both farnesylated and geranylgeranylated RhoB inhibit malignant transformation and suppress human tumor growth in nude mice. J. Biol. Chem. 275, 17974-17978.

32) Du, Wei, and George C. Prendergast. "Geranylgeranylated RhoB mediates suppression of human tumor cell growth by farnesyltransferase inhibitors." Cancer research 59.21 (1999): 5492-5496.

33) Adnane, Jalila, et al. "Suppression of rho B expression in invasive carcinoma from head and neck cancer patients." Clinical Cancer Research 8.7 (2002): 2225-2232.

34) Chardin, Pi, P. Madaule, and A. Tavitian. "Coding sequence of human rho cDNAs clone 6 and clone 9." Nucleic acids research 16.6 (1988): 2717.

35) Suwa, H., et al. "Overexpression of the rhoC gene correlates with progression of ductal adenocarcinoma of the pancreas." British journal of cancer 77.1 (1998): 147-152.

36) Van Golen, Kenneth L., et al. "RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype." Cancer research 60.20 (2000): 5832-5838.

37) Wang, L., Yang, L., Luo, Y. and Zheng, Y. (2003). A novel strategy for specifically downregulating individual Rho GTPase activity in tumor cells. J. Biol. Chem. 278, 44617-44625.

38) Arthur, William T., et al. "XPLN, a guanine nucleotide exchange factor for RhoA and RhoB, but not RhoC." Journal of Biological Chemistry 277.45 (2002): 42964-42972.

39) Sahai, Erik, and Christopher J. Marshall. "ROCK and Dia have opposing effects on adherens junctions downstream of Rho." Nature cell biology 4.6 (2002): 408-415.

40) Eden, Sharon, et al. "Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck." Nature 418.6899 (2002): 790-793.

41) Jordan, P., Brazao, R., Boavida, M. G., Gespach, C. and Chastre, E. (1999). Cloning of a novel human Rac1b splice variant with increased expression in colorectal tumors. Oncogene 18, 6835-6839.

42) Schnelzer, A., Prechtel, D., Knaus, U., Dehne, K., Gerhard, M., Graeff, H., Harbeck, N., Schmitt, M. and Lengyel, E. (2000). Rac1 in human breast cancer: overexpression, mutation analysis, and characterization of a new isoform, Rac1b. Oncogene 19, 3013-3020.

43) Fiegen, D., Haeusler, L. C., Blumenstein, L., Herbrand, U., Dvorsky, R., Vetter, I. R. and Ahmadian, M. R. (2004). Alternative splicing of Rac1 generates Rac1b, a self-activating GTPase. J. Biol. Chem. 279, 4743-4749.

44) Matos, P., Collard, J. G. and Jordan, P. (2003). Tumor-related alternative spliced Rac1b is not regulated by Rho-GDI and exhibits selective downstream signaling. J. Biol. Chem. 278, 50442-50448.

45) Didsbury, J., et al. "Rac, a novel ras-related family of proteins that are botulinum toxin substrates." Journal of Biological Chemistry 264.28 (1989): 16378-16382.

46) Sugihara, K., Nakatsuji, N., Nakamura, K., Nakao, K., Hashimoto, R., Otani, H., Sakagami, H., Kondo, H., Nozawa, S., Aiba, A. et al. (1998). Rac1 is required for the formation of three germ layers during gastrulation. Oncogene 17, 3427-3433.

47) Roberts, Andrew W., et al. "Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense." Immunity 10.2 (1999): 183-196.

48) Ambruso, Daniel R., et al. "Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation." Proceedings of the National Academy of Sciences 97.9 (2000): 4654-4659.

49) Kasper, Brigitte, et al. "Differential expression and regulation of GTPases (RhoA and Rac2) and GDIs (LyGDI and RhoGDI) in neutrophils from patients with severe congenital neutropenia." Blood, The Journal of the American Society of Hematology 95.9 (2000): 2947-2953.

50) Li, Baiyong, et al. "Role of the guanosine triphosphatase Rac2 in T helper 1 cell differentiation." Science 288.5474 (2000): 2219-2222.

51) Dorseuil, Olivier, et al. "The Rac Target NADPH Oxidase p67phox Interacts Preferentially with Rac2 Rather Than Rac1 (∗)." Journal of Biological Chemistry 271.1 (1996): 83-88.

52) Mizuno, Takakazu, et al. "Regulation of the superoxide-generating NADPH oxidase by a small GTP-binding protein and its stimulatory and inhibitory GDP/GTP exchange proteins." Journal of Biological Chemistry 267.15 (1992): 10215-10218.

53) Haataja, Leena, John Groffen, and Nora Heisterkamp. "Characterization of RAC3, a novel member of the Rho family." Journal of Biological Chemistry 272.33 (1997): 20384-20388.

54) Mira, J. P., Benard, V., Groffen, J., Sanders, L. C. and Knaus, U. G. (2000). Endogenous, hyperactive Rac3 controls proliferation of breast cancer cells by a p21-activated kinase-dependent pathway. Proc. Natl. Acad. Sci. USA 97, 185-189.

55) Morris, C. M., Haataja, L., McDonald, M., Gough, S., Markie, D., Groffen, J. and Heisterkamp, N. (2000). The small GTPase RAC3 gene is located within chromosome band 17q25.3 outside and telomeric of a region commonly deleted in breast and ovarian tumours. Cytogenet. Cell Genet. 89, 18-23.

56) Vincent, S. Y. L. V. I. E., P. H. I. L. I. P. P. E. Jeanteur, and Philippe Fort. "Growth-regulated expression of rhoG, a new member of the ras homolog gene family." Molecular and cellular biology 12.7 (1992): 3138-3148.

57) Gauthier-Rouvière, Cécile, et al. "RhoG GTPase controls a pathway that independently activates Rac1 and CDC42Hs." Molecular biology of the cell 9.6 (1998): 1379-1394.

58) Katoh, H. and Negishi, M. (2003). RhoG activates Rac1 by direct interaction with the Dock180-binding protein Elmo. Nature 424, 461-464.

59) Machesky, Laura M., and Robert H. Insall. "Scar1 and the related Wiskott–Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex." Current biology 8.25 (1998): 1347-1356.

60) Miki, Hiroaki, et al. "Induction of filopodium formation by a WASP-related actin-depolymerizing protein N-WASP." Nature 391.6662 (1998): 93-96.

61) Aspenström, Pontus, Åsa Fransson, and Jan Saras. "Rho GTPases have diverse effects on the organization of the actin filament system." Biochemical Journal 377.2 (2004): 327-337.

62) Marks, P. W. and Kwiatkowski, D. J. (1996). Genomic organization and chromosomal location of murine CDC42. Genomics 38, 13-18.

63) Nicole, S., White, P. S., Topaloglu, H., Beigthon, P., Salih, M., Hentati, F. and Fontaine, B. (1999). The human CDC42 gene: genomic organization, evidence for the existence of a putative pseudogene and exclusion as a SJS1 candidate gene. Hum. Genet. 105, 98-103.

64) Drivas, G. T., et al. "Characterization of four novel ras-like genes expressed in a human teratocarcinoma cell line." Molecular and Cellular Biology 10.4 (1990): 1793-1798.

65) Vignal, Emmanuel, et al. "Characterization of TCL, a New GTPase of the Rho Family related to TC10 and CDC42* 210." Journal of Biological Chemistry 275.46 (2000): 36457-36464.

66) Murphy, G. A., Solski, P. A., Jillian, S. A., Perez de la Ossa, P., D'Eustachio, P., Der, C. J. and Rush, M. G. (1999). Cellular functions of TC10, a Rho family GTPase: regulation of morphology, signal transduction and cell growth. Oncogene 18, 3831-3845.

67) Neudauer, C. L., Joberty, G., Tatsis, N. and Macara, I. G. (1998). Distinct cellular effects and interactions of the Rho-family GTPase TC10. Curr. Biol. 8, 1151-1160.

68) Michaelson, David, et al. "Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding." The Journal of cell biology 152.1 (2001): 111-126.

69) Schmidt, Anja, and Alan Hall. "Guanine nucleotide exchange factors for Rho GTPases: turning on the switch." Genes & development 16.13 (2002): 1587-1609.

70) Chiang, S. H., Hou, J. C., Hwang, J., Pessin, J. E. and Saltiel, A. R. (2002). Cloning and functional characterization of related TC10 isoforms, a subfamily of Rho proteins involved in insulin-stimulated glucose transport. J. Biol. Chem. 277, 13067-13073.

71) Nishizuka, M., Arimoto, E., Tsuchiya, T., Nishihara, T. and Imagawa, M. (2003). Crucial role of TCL/TC10beta L, a subfamily of Rho GTPase, in adipocyte differentiation. J. Biol. Chem. 278, 15279-15284.

72) Tanabe, Katsuhisa, et al. "The small GTP-binding protein TC10 promotes nerve elongation in neuronal cells, and its expression is induced during nerve regeneration in rats." Journal of Neuroscience 20.11 (2000): 4138-4144.

73) Abe, Tomoyuki, et al. "Small GTPase Tc10 and its homologue RhoT induce N-WASP-mediated long process formation and neurite outgrowth." Journal of Cell Science 116.1 (2003): 155-168.

74) Aronheim, Ami, et al. "Chp, a homologue of the GTPase CDC42Hs, activates the JNK pathway and is implicated in reorganizing the actin cytoskeleton." Current biology 8.20 (1998): 1125-1129.

75) Tao, Weikang, et al. "Wrch-1, a novel member of the Rho gene family that is regulated by Wnt-1." Genes & development 15.14 (2001): 1796-1807.

76) Nobes, Catherine D., et al. "A new member of the Rho family, Rnd1, promotes disassembly of actin filament structures and loss of cell adhesion." The Journal of cell biology 141.1 (1998): 187-197.

77) Foster, Rosemary, et al. "Identification of a novel human Rho protein with unusual properties: GTPase deficiency and in vivo farnesylation." Molecular and cellular biology 16.6 (1996): 2689-2699.

78) Guasch, Rosa M., et al. "RhoE regulates actin cytoskeleton organization and cell migration." Molecular and cellular biology 18.8 (1998): 4761-4771.

79) Wennerberg, Krister, et al. "Rnd proteins function as RhoA antagonists by activating p190 RhoGAP." Current Biology 13.13 (2003): 1106-1115.

80) Riento, K., Guasch, R. M., Garg, R., Jin, B. and Ridley, A. J. (2003). RhoE binds to ROCK I and inhibits downstream signaling. Mol. Cell. Biol. 23, 4219-4229.

81) Aoki, Junko, et al. "Rnd1, a novel rho family GTPase, induces the formation of neuritic processes in PC12 cells." Biochemical and biophysical research communications 278.3 (2000): 604-608.

82) Loirand, Gervaise, et al. "The Rho-related protein Rnd1 inhibits Ca2+ sensitization of rat smooth muscle." The Journal of physiology 516.Pt 3 (1999): 825.

83) Nishi, Miyuki, et al. "RhoN, a novel small GTP-binding protein expressed predominantly in neurons and hepatic stellate cells." Molecular brain research 67.1 (1999): 74-81.

84) Fujita, Hirotada, et al. "Rapostlin is a novel effector of Rnd2 GTPase inducing neurite branching." Journal of Biological Chemistry 277.47 (2002): 45428-45434.

85) Murphy, Carol, et al. "Endosome dynamics regulated by a Rho protein." Nature 384.6608 (1996): 427-432.

86) Ellis, S. and Mellor, H. (2000). The novel Rho-family GTPase rif regulates coordinated actin-based membrane rearrangements. Curr. Biol. 10, 1387-1390.

87) Gasman, Stéphane, Yannis Kalaidzidis, and Marino Zerial. "RhoD regulates endosome dynamics through Diaphanous-related Formin and Src tyrosine kinase." Nature Cell Biology 5.3 (2003): 195-204.

88) Preudhomme, C., et al. "Nonrandom 4p13 rearrangements of the RhoH/TTF gene, encoding a GTP-binding protein, in non-Hodgkin's lymphoma and multiple myeloma." Oncogene 19.16 (2000): 2023-2032.

89) Preudhomme, C., et al. "Nonrandom 4p13 rearrangements of the RhoH/TTF gene, encoding a GTP-binding protein, in non-Hodgkin's lymphoma and multiple myeloma." Oncogene 19.16 (2000): 2023-2032.

90) Li, Xiaoyu, et al. "The hematopoiesis-specific GTP-binding protein RhoH is GTPase deficient and modulates activities of other Rho GTPases by an inhibitory function." Molecular and cellular biology 22.4 (2002): 1158-1171.

91) Rivero, Francisco, et al. "The Dictyostelium discoideum family of Rho-related proteins." Nucleic acids research 29.5 (2001): 1068-1079.

92) Collins, Tucker, James R. Stone, and Amy J. Williams. "All in the family: the BTB/POZ, KRAB, and SCAN domains." Molecular and cellular biology 21.11 (2001): 3609-3615.

93) Hamaguchi, Masaaki, et al. "DBC2, a candidate for a tumor suppressor gene involved in breast cancer." Proceedings of the National Academy of Sciences 99.21 (2002): 13647-13652.

94) Fransson, Åsa, Aino Ruusala, and Pontus Aspenström. "Atypical Rho GTPases have roles in mitochondrial homeostasis and apoptosis." Journal of Biological Chemistry 278.8 (2003): 6495-6502.

95) Aspenström, Pontus, Åsa Fransson, and Jan Saras. "Rho GTPases have diverse effects on the organization of the actin filament system." Biochemical Journal 377.2 (2004): 327-337.

96) Schmidt, Anja, and Alan Hall. "Guanine nucleotide exchange factors for Rho GTPases: turning on the switch." Genes & development 16.13 (2002): 1587-1609.

97) Côté, Jean-François, and Kristiina Vuori. "Identification of an evolutionarily conserved superfamily of DOCK180-related proteins with guanine nucleotide exchange activity." Journal of cell science 115.24 (2002): 4901-4913.

98) Moon, S. Y. and Zheng, Y. (2003). Rho GTPase-activating proteins in cell regulation. Trends Cell Biol. 13, 13-22.

99) Anastasiadis, Panos Z., et al. "Inhibition of RhoA by p120 catenin." Nature cell biology 2.9 (2000): 637-644.

100) Degani, S., Balzac, F., Brancaccio, M., Guazzone, S., Retta, S. F., Silengo, L., Eva, A. and Tarone, G. (2002). The integrin cytoplasmic domain-associated protein ICAP-1 binds and regulates Rho family GTPases during cell spreading. J. Cell Biol. 156, 377-387.

101) Adra, C. N., Manor, D., Ko, J. L., Zhu, S., Horiuchi, T., van Aelst, L., Cerione, R. A. and Lim, B. (1997). RhoGDIgamma: a GDP-dissociation inhibitor for Rho proteins with preferential expression in brain and pancreas. Proc. Natl. Acad. Sci. USA 94, 4279-4284.

102) Zalcman, G., Closson, V., Camonis, J., Honore, N., Rousseau-Merck, M. F., Tavitian, A. and Olofsson, B. (1996). RhoGDI-3 is a new GDP dissociation inhibitor (GDI). Identification of a non-cytosolic GDI protein interacting with the small GTP-binding proteins RhoB and RhoG. J. Biol. Chem. 271, 30366-30374.

100) Takahashi, Kazuo, et al. "Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein." Journal of Biological Chemistry 272.37 (1997): 23371-23375.

101) Yamashita, T. and Tohyama, M. (2003). The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. Nat. Neurosci. 6, 461-467.

102) Zerial, Marino, and Heidi McBride. "Rab proteins as membrane organizers." Nature reviews Molecular cell biology 2.2 (2001): 107-117.

103) Weis, Karsten. "Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle." Cell 112.4 (2003): 441-451.

104) Li HY, Cao K, Zheng Y. Ran in the spindle checkpoint: a new function for a versatile GTPase. Trends Cell Biol. 2003 Nov;13(11):553-7. doi: 10.1016/j.tcb.2003.09.003. PMID: 14573347.

105) Etienne-Manneville, Sandrine, and Alan Hall. "Rho GTPases in cell biology." Nature 420.6916 (2002): 629-635.

106) Ridley, Anne J. "Rho family proteins: coordinating cell responses." Trends in cell biology 11.12 (2001): 471-477.

107) Shinjo, Katsuhiro, et al. "Molecular cloning of the gene for the human placental GTP-binding protein Gp (G25K): identification of this GTP-binding protein as the human homolog of the yeast cell-division-cycle protein CDC42." Proceedings of the National Academy of Sciences 87.24 (1990): 9853-9857.

108) Munemitsu, S., et al. "Molecular cloning and expression of a G25K cDNA, the human homolog of the yeast cell cycle gene CDC42." Molecular and cellular biology 10.11 (1990): 5977-5982.

109) Melendez, Jaime, Matthew Grogg, and Yi Zheng. "Signaling role of CDC42 in regulating mammalian physiology." Journal of Biological Chemistry 286.4 (2011): 2375-2381.

110) Boureux, Anthony, et al. "Evolution of the Rho family of ras-like GTPases in eukaryotes." Molecular biology and evolution 24.1 (2007): 203-216.

111) Jaffe, Aron B., and Alan Hall. "Rho GTPases: biochemistry and biology." Annu. Rev. Cell Dev. Biol. 21 (2005): 247-269.

112) Bos, Johannes L., Holger Rehmann, and Alfred Wittinghofer. "GEFs and GAPs: critical elements in the control of small G proteins." Cell 129.5 (2007): 865-877.

113) Riento, Kirsi, et al. "RhoE function is regulated by ROCK I-mediated phosphorylation." The EMBO journal 24.6 (2005): 1170-1180.

114) Rolli-Derkinderen, Malvyne, et al. "Phosphorylation of serine 188 protects RhoA from ubiquitin/proteasome-mediated degradation in vascular smooth muscle cells." Circulation research 96.11 (2005): 1152-1160.

115) Vlahou, Georgia, and Francisco Rivero. "Rho GTPase signaling in Dictyostelium discoideum: insights from the genome." European journal of cell biology 85.9-10 (2006): 947-959.

116) Lang, Paul, et al. "Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes." The EMBO journal 15.3 (1996): 510-519.

117) Gildea, John J., et al. "RhoGDI2 is an invasion and metastasis suppressor gene in human cancer." Cancer research 62.22 (2002): 6418-6423.

118) Manser, Edward, et al. "A non-receptor tyrosine kinase that inhibits the GTPase activity of p21 CDC42." Nature 363.6427 (1993): 364-367.

119) Teo, Mabel, et al. "The tyrosine kinase ACK1 associates with clathrin-coated vesicles through a binding motif shared by arrestin and other adaptors." Journal of Biological Chemistry 276.21 (2001): 18392-18398.

120) Shen, Feng, et al. "Activated CDC42-associated kinase 1 is a component of EGF receptor signaling complex and regulates EGF receptor degradation." Molecular biology of the cell 18.3 (2007): 732-742.

121) Zhang, Xuewu, et al. "Inhibition of the EGF receptor by binding of MIG6 to an activating kinase domain interface." Nature 450.7170 (2007): 741-744.

122) Van Der Horst, Edward Htun, et al. "Metastatic properties and genomic amplification of the tyrosine kinase gene ACK1." Proceedings of the National Academy of Sciences 102.44 (2005): 15901-15906.

123) Modzelewska, Katarzyna, et al. "ACK1 mediates CDC42-dependent cell migration and signaling to p130Cas." Journal of Biological Chemistry 281.49 (2006): 37527-37535.

124) Yang, Wannian, et al. "Activation of the CDC42-associated tyrosine kinase-2 (ACK-2) by cell adhesion via integrin β1." Journal of Biological Chemistry 274.13 (1999): 8524-8530.

125) Kato-Stankiewicz, Juran, et al. "Epidermal growth factor stimulation of the ACK1/Dbl pathway in a CDC42 and Grb2-dependent manner." Biochemical and biophysical research communications 284.2 (2001): 470-477.

126) Eisenmann, Kathryn M., et al. "Melanoma chondroitin sulphate proteoglycan regulates cell spreading through CDC42, ACK-1 and p130 cas." Nature cell biology 1.8 (1999): 507-513.

127) Mahajan, Nupam P., et al. "Activated tyrosine kinase ACK1 promotes prostate tumorigenesis: role of ACK1 in polyubiquitination of tumor suppressor Wwox." Cancer research 65.22 (2005): 10514-10523.

128) Nur-E-Kamal, Alam, et al. "Requirement of activated CDC42-associated kinase for survival of v-Ras-transformed mammalian cells." Molecular cancer research 3.5 (2005): 297-305.

129) Aronheim, Ami, et al. "Chp, a homologue of the GTPase CDC42Hs, activates the JNK pathway and is implicated in reorganizing the actin cytoskeleton." Current biology 8.20 (1998): 1125-1129.

130) Del Pulgar, Teresa Gómez, et al. "Rho GTPase expression in tumourigenesis: evidence for a significant link." Bioessays 27.6 (2005): 602-613.

131) Benitah, Salvador A., et al. "Rho GTPases in human cancer: an unresolved link to upstream and downstream transcriptional regulation." Biochimica et Biophysica Acta (BBA)-Reviews on Cancer 1705.2 (2004): 121-132.

132) Merajver, Sofia D., and Saad Z. Usmani. "Multifaceted role of Rho proteins in angiogenesis." Journal of mammary gland biology and neoplasia 10.4 (2005):

133) 291-298. Wheelock, Margaret J., et al. "Cadherin switching." Journal of cell science 121.6 (2008): 727-735.

134) Friedl, Peter, and Katarina Wolf. "Tumour-cell invasion and migration: diversity and escape mechanisms." Nature reviews cancer 3.5 (2003): 362-374.

135) Wolf, Katarina, et al. "Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion." Nature cell biology 9.8 (2007): 893-904.

136) Jaffe, Aron B., and Alan Hall. "Rho GTPases: biochemistry and biology." Annu. Rev. Cell Dev. Biol. 21 (2005): 247-269.

137) Abraham, Manoj T., et al. "Motility-related proteins as markers for head and neck squamous cell cancer." The Laryngoscope 111.7 (2001): 1285-1289.

138) Braga, Vania MM, and Alpha S. Yap. "The challenges of abundance: epithelial junctions and small GTPase signalling." Current opinion in cell biology 17.5 (2005): 466-474.

139) Wildenberg, Gregg A., et al. "p120-catenin and p190RhoGAP regulate cell-cell adhesion by coordinating antagonism between Rac and Rho." Cell 127.5 (2006): 1027-1039.

140) Labouesse, Michel. "Epithelium–Mesenchyme: A Balancing Act of RhoGAP and RhoGEF." Current Biology 14.13 (2004): R508-R510.

141) Gaggioli, Cedric, et al. "Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells." Nature cell biology 9.12 (2007): 1392-1400.

142) Lozano, Encarnación, Martha Betson, and Vania MM Braga. "Tumor progression: small GTPases and loss of cell–cell adhesion." Bioessays 25.5 (2003): 452-463.

143) Hakem, Anne, et al. "RhoC is dispensable for embryogenesis and tumor initiation but essential for metastasis." Genes & development 19.17 (2005): 1974-1979.

144) Clark, Edwin A., et al. "Genomic analysis of metastasis reveals an essential role for RhoC." Nature 406.6795 (2000): 532-535.

145) Kleer, Celina G., et al. "RhoC GTPase expression as a potential marker of lymph node metastasis in squamous cell carcinomas of the head and neck." Clinical cancer research 12.15 (2006): 4485-4490.

146) Ma, Li, Julie Teruya-Feldstein, and Robert A. Weinberg. "Tumour invasion and metastasis initiated by microRNA-10b in breast cancer." Nature 449.7163 (2007): 682-688.

147) Miles, Fayth L., et al. "Stepping out of the flow: capillary extravasation in cancer metastasis." Clinical & experimental metastasis 25.4 (2008): 305-324.

148) Huang, M., and G. C. Prendergast. "RhoB in cancer suppression." Histology and histopathology (2006).

149) Liu, Ai-Xue, et al. "RhoB is dispensable for mouse development, but it modifies susceptibility to tumor formation as well as cell adhesion and growth factor signaling in transformed cells." Molecular and cellular biology 21.20 (2001): 6906-6912.

150) Baldwin, R. M., D. A. E. Parolin, and I. A. J. Lorimer. "Regulation of glioblastoma cell invasion by PKCι and RhoB." Oncogene 27.25 (2008): 3587-3595.

151) Jiang, Kun, et al. "Akt mediates Ras downregulation of RhoB, a suppressor of transformation, invasion, and metastasis." Molecular and cellular biology 24.12 (2004): 5565-5576.

152) Sandilands, Emma, et al. "Src kinase modulates the activation, transport and signalling dynamics of fibroblast growth factor receptors." EMBO reports 8.12 (2007): 1162-1169.

153) Fritz, Gerhard, Ingo Just, and Bernd Kaina. "Rho GTPases are over-expressed in human tumors." International journal of cancer 81.5 (1999): 682-687.

154) Van Hengel, Jolanda, et al. "Continuous cell injury promotes hepatic tumorigenesis in CDC42-deficient mouse liver." Gastroenterology 134.3 (2008): 781-792.

155) Murphy, Gretchen A., et al. "Cellular functions of TC10, a Rho family GTPase: regulation of morphology, signal transduction and cell growth." Oncogene 18.26 (1999): 3831-3845.

156) Wu, Wen Jin, Shine Tu, and Richard A. Cerione. "Activated CDC42 sequesters c-Cbl and prevents EGF receptor degradation." Cell 114.6 (2003): 715-725.

157) Ridley, Anne J. "Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking." Trends in cell biology 16.10 (2006): 522-529.

158) Yasuda, Shingo, et al. "An essential role of CDC42-like GTPases in mitosis of HeLa cells." FEBS letters 580.14 (2006): 3375-3380.

159) Pinner, Sophie, and Erik Sahai. "PDK1 regulates cancer cell motility by antagonising inhibition of ROCK1 by RhoE." Nature cell biology 10.2 (2008): 127-137.

160) Rong, Zhouyi, et al. "Activation of FAK/Rac1/CDC42-GTPase signaling ameliorates impaired microglial migration response to Aβ42 in triggering receptor expressed on myeloid cells 2 loss-of-function murine models." The FASEB Journal 34.8 (2020): 10984-10997.

161) Barcia, Carlos, et al. "ROCK/CDC42-mediated microglial motility and gliapse formation lead to phagocytosis of degenerating dopaminergic neurons in vivo." Scientific reports 2.1 (2012): 1-13.

162) Aguilar, Byron J., Yi Zhu, and Qun Lu. "Rho GTPases as therapeutic targets in Alzheimer's disease." Alzheimer's research & therapy 9.1 (2017): 1-10.

163) Hong, Lin, et al. "Characterization of a CDC42 protein inhibitor and its use as a molecular probe." Journal of Biological Chemistry 288.12 (2013): 8531-8543.

164) John, Jacob, et al. "Kinetic and structural analysis of the Mg (2+)-binding site of the guanine nucleotide-binding protein p21H-ras." Journal of Biological Chemistry 268.2 (1993): 923-929.

165) Yamao, Masataka, et al. "Distinct predictive performance of Rac1 and CDC42 in cell migration." Scientific reports 5.1 (2015): 1-14.

166) Liu, Wei, et al. "Mobilization of hematopoitic stem/Progenitor cells by a CDC42 activity-specific inhibitor." Blood 112.11 (2008): 68.

167) Guo, Yuna, et al. "R-ketorolac targets CDC42 and Rac1 and alters ovarian cancer cell behaviors critical for invasion and metastasis." Molecular cancer therapeutics 14.10 (2015): 2215-2227.

168) Gray, Janine L., Frank von Delft, and Paul E. Brennan. "Targeting the small GTPase superfamily through their regulatory proteins." Angewandte Chemie International Edition 59.16 (2020): 6342-6366.

169) Müller, Paul M., et al. "Systems analysis of RhoGEF and RhoGAP regulatory proteins reveals spatially organized RAC1 signalling from integrin adhesions." Nature cell biology 22.4 (2020): 498-511.

170) Bagci, Halil, et al. "Mapping the proximity interaction network of the Rho-family GTPases reveals signalling pathways and regulatory mechanisms." Nature cell biology 22.1 (2020): 120-134.

171) Snyder, Jason T., et al. "Structural basis for the selective activation of Rho GTPases by Dbl exchange factors." Nature structural biology 9.6 (2002): 468-475.

172) Smith, William J., et al. "A CDC42 mutant specifically activated by intersectin." Biochemistry 44.40 (2005): 13282-13290.

173) Jeganathan, Niranjan, et al. "Rac1-mediated cytoskeleton rearrangements induced by intersectin-1s deficiency promotes lung cancer cell proliferation, migration and metastasis." Molecular Cancer 15.1 (2016): 1-17.

174) Russo, Angela, and John P. O'Bryan. "Intersectin 1 is required for neuroblastoma tumorigenesis." Oncogene 31.46 (2012): 4828-4834.

175) Friesland, Amy, et al. "Small molecule targeting CDC42–intersectin interaction disrupts Golgi organization and suppresses cell motility." Proceedings of the National Academy of Sciences 110.4 (2013): 1261-1266.

176) Aguilar, Byron J., et al. "Inhibition of CDC42–intersectin interaction by small molecule ZCL367 impedes cancer cell cycle progression, proliferation, migration, and tumor growth." Cancer biology & therapy 20.6 (2019): 740-749.

177) Liu, Wei, et al. "Rational identification of a CDC42 inhibitor presents a new regimen for long-term hematopoietic stem cell mobilization." Leukemia 33.3 (2019): 749-761.

178) Nguyen, Phuong, et al. "Rational targeting of CDC42 overcomes drug resistance of multiple myeloma." Frontiers in oncology (2019): 958.

179) Florian, Maria Carolina, et al. "Inhibition of CDC42 activity extends lifespan and decreases circulating inflammatory cytokines in aged female C57BL/6 mice." Aging cell 19.9 (2020): e13208.

180) Gao, Yuan, et al. "Rational design and characterization of a Rac GTPase-specific small molecule inhibitor." Proceedings of the National Academy of Sciences 101.20 (2004): 7618-7623.

181) Montalvo-Ortiz, Brenda L., et al. "Characterization of EHop-016, novel small molecule inhibitor of Rac GTPase." Journal of Biological Chemistry 287.16 (2012): 13228-13238.

182) Zeng, Rui-Jie, et al. "RAC1 inhibition reverses cisplatin resistance in esophageal squamous cell carcinoma and induces downregulation of glycolytic enzymes." Molecular oncology 13.9 (2019): 2010-2030.

183) Humphries-Bickley, Tessa, et al. "Characterization of a dual Rac/CDC42 inhibitor MBQ-167 in metastatic cancer." Molecular cancer therapeutics 16.5 (2017): 805-818.

184) Ferri, Nicola, et al. "Virtual screening approach for the identification of new Rac1 inhibitors." Journal of medicinal chemistry 52.14 (2009): 4087-4090.

185) Zins, Karin, et al. "A Rac1/CDC42 GTPase-specific small molecule inhibitor suppresses growth of primary human prostate cancer xenografts and prolongs survival in mice." PloS one 8.9 (2013): e74924.

186) Murphy, Natasha P., Helen R. Mott, and Darerca Owen. "Progress in the therapeutic inhibition of CDC42 signalling." Biochemical Society Transactions 49.3 (2021): 1443-1456.

187) Zins, Karin, et al. "Targeting Cdc42 with the small molecule drug AZA197 suppresses primary colon cancer growth and prolongs survival in a preclinical mouse xenograft model by downregulation of PAK1 activity." Journal of translational medicine 11.1 (2013): 1-16.

188) Sun, Zhongya, et al. "Covalent inhibitors allosterically block the activation of Rho family proteins and suppress cancer cell invasion." Advanced Science 7.14 (2020): 2000098.

189) Flanagan, Mark E., et al. "Chemical and computational methods for the characterization of covalent reactive groups for the prospective design of irreversible inhibitors." Journal of medicinal chemistry 57.23 (2014): 10072-10079.

190) Abdeldayem, Ayah, et al. "Advances in covalent kinase inhibitors." Chemical Society Reviews 49.9 (2020): 2617-2687.

191) Ostrem, Jonathan M., et al. "K-Ras (G12C) inhibitors allosterically control GTP affinity and effector interactions." Nature 503.7477 (2013): 548-551.

192) Hallin, Jill, et al. "The KRASG12C inhibitor MRTX849 provides insight toward therapeutic susceptibility of KRAS-mutant cancers in mouse models and patients." Cancer discovery 10.1 (2020): 54-71.

193) Canon, Jude, et al. "The clinical KRAS (G12C) inhibitor AMG 510 drives anti-tumour immunity." Nature 575.7781 (2019): 217-223.

194) Scott, Duncan E., et al. "Small molecules, big targets: drug discovery faces the protein–protein interaction challenge." Nature Reviews Drug Discovery 15.8 (2016): 533-550.

195) Tetley, George JN, et al. "The discovery and maturation of peptide biologics targeting the small G-protein CDC42: A bioblockade for Ras-driven signaling." Journal of Biological Chemistry 295.9 (2020): 2866-2884.

196) Goto, Yuki, Takayuki Katoh, and Hiroaki Suga. "Flexizymes for genetic code reprogramming." Nature protocols 6.6 (2011): 779-790.

197) Conibear, Anne C., et al. "Approaches to the stabilization of bioactive epitopes by grafting and peptide cyclization." Peptide Science 106.1 (2016): 89-100.

198) Wolfe, Justin M., et al. "Machine learning to predict cell-penetrating peptides for antisense delivery." ACS central science 4.4 (2018): 512-520.

199) Pal, Indrani, et al. "A peptide-nanoparticle system with improved efficacy against multidrug resistant bacteria." Scientific reports 9.1 (2019): 1-11.

200) Bery, Nicolas, et al. "A targeted protein degradation cell-based screening for nanobodies selective toward the cellular RHOB GTP-bound conformation." Cell chemical biology 26.11 (2019): 1544-1558.

201) Barouch-Bentov, Rina, and Karsten Sauer. "Mechanisms of drug resistance in kinases." Expert opinion on investigational drugs 20.2 (2011): 153-208.

202) Roskoski Jr, Robert. "Classification of small molecule protein kinase inhibitors based upon the structures of their drug-enzyme complexes." Pharmacological research 103 (2016): 26-48.

203) Zhang, Eric Y., Byung Hak Ha, and Titus J. Boggon. "PAK4 crystal structures suggest unusual kinase conformational movements." Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics 1866.2 (2018): 356-365.

204) Murray, Brion W., et al. "Small-molecule p21-activated kinase inhibitor PF-3758309 is a potent inhibitor of oncogenic signaling and tumor growth." Proceedings of the National Academy of Sciences 107.20 (2010): 9446-9451.

205) Bradshaw-Pierce, Erica Lynn, et al. "Tumor P-glycoprotein correlates with efficacy of PF-3758309 in in vitro and in vivo models of colorectal cancer." Frontiers in pharmacology 4 (2013): 22.

206) Aboukameel, Amro, et al. "Novel p21-activated kinase 4 (PAK4) allosteric modulators overcome drug resistance and stemness in pancreatic ductal adenocarcinoma." Molecular cancer therapeutics 16.1 (2017): 76-87.

207) Aboud, Omran Abu, et al. "Dual and specific inhibition of NAMPT and PAK4 by KPT-9274 decreases kidney cancer growth." Molecular cancer therapeutics 15.9 (2016): 2119-2129.

208) Neggers, Jasper Edgar, et al. "Target identification of small molecules using large-scale CRISPR-Cas mutagenesis scanning of essential genes." Nature communications 9.1 (2018): 1-14.

209) Yaku, Keisuke, et al. "NAD metabolism in cancer therapeutics." Frontiers in oncology 8 (2018): 622.

210) Mpilla, Gabriel, et al. "PAK4-NAMPT dual inhibition as a novel strategy for therapy resistant pancreatic neuroendocrine tumors." Cancers 11.12 (2019): 1902.

211) Unbekandt, Mathieu, et al. "A novel small-molecule MRCK inhibitor blocks cancer cell invasion." Cell Communication and Signaling 12.1 (2014): 1-15.

212) Unbekandt, Mathieu, et al. "Discovery of potent and selective MRCK inhibitors with therapeutic effect on skin cancer." Cancer research 78.8 (2018): 2096-2114.

213) Wilkinson, Simon, Hugh F. Paterson, and Christopher J. Marshall. "CDC42–MRCK and Rho–ROCK signalling cooperate in myosin phosphorylation and cell invasion." Nature cell biology 7.3 (2005): 255-261.

214) Mahajan, Kiran, and Nupam P. Mahajan. "ACK1 tyrosine kinase: targeted inhibition to block cancer cell proliferation." Cancer letters 338.2 (2013): 185-192.

215) Galkin, Anna V., et al. "Identification of NVP-TAE684, a potent, selective, and efficacious inhibitor of NPM-ALK." Proceedings of the National Academy of Sciences 104.1 (2007): 270-275.

216) Davis, Mindy I., et al. "Comprehensive analysis of kinase inhibitor selectivity." Nature biotechnology 29.11 (2011): 1046-1051.

217) Salah, Eidarus, et al. "Lessons from LIMK1 enzymology and their impact on inhibitor design." Biochemical Journal 476.21 (2019): 3197-3209.

218) Manetti, Fabrizio. "Recent advances in the rational design and development of LIM kinase inhibitors are not enough to enter clinical trials." European Journal of Medicinal Chemistry 155 (2018): 445-458.

219) Goodwin, Nicole C., et al. "Discovery of a type III inhibitor of LIM kinase 2 that binds in a DFG-out conformation." ACS medicinal chemistry letters 6.1 (2015): 53-57.

220) Storck, Elisabeth M., et al. "Dual chemical probes enable quantitative system-wide analysis of protein prenylation and prenylation dynamics." Nature chemistry 11.6 (2019): 552-561.

221) Pelish, Henry E., et al. "Secramine inhibits CDC42-dependent functions in cells and CDC42 activation in vitro." Nature chemical biology 2.1 (2006): 39-46.

222) Chua, Boon Tin, et al. "Somatic mutation in the ACK1 ubiquitin association domain enhances oncogenic signaling through EGFR regulation in renal cancer derived cells." Molecular oncology 4.4 (2010): 323-334.

223) Xiao, Xing-Hua, et al. "Regulating CDC42 and its signaling pathways in cancer: small molecules and MicroRNA as new treatment candidates." Molecules 23.4 (2018): 787.

224) Forbes, Simon A., et al. "COSMIC: somatic cancer genetics at high-resolution." Nucleic acids research 45.D1 (2017): D777-D783.

225) Hodis, Eran, et al. "A landscape of driver mutations in melanoma." Cell 150.2 (2012): 251-263.

226) Fidyk, Nancy, Jian-Bin Wang, and Richard A. Cerione. "Influencing cellular transformation by modulating the rates of GTP hydrolysis by CDC42." Biochemistry 45.25 (2006): 7750-7762.

CHAPTER TWO

Characterization of CDC42 and ACK interaction in the presence of the small molecule inhibitor ZCL2782

Abstract

The Ras superfamily of small GTPases are involved in cell-signaling processes that, if not regulated, may lead to aberrant cell multiplication, cell death inhibition, and tumor formation. They operate as molecular switches, which through GTP/GDP exchange cycles, switch on and off cellular processes. Overexpression of these proteins has been linked to many cancers. CDC42, a member of the Rho family of the Ras superfamily of small GTPases, is involved in the regulation of many cellular processes including cell adhesion, mitosis, and cytoskeletal rearrangement. It binds and activates many effector proteins including CDC42-activated kinase (ACK). Aberrant activities of CDC42-ACK interactions have been associated with tumor development and metastasis. It is of utmost importance that small molecule inhibitors be designed and constructed to inhibit CDC42 interactions with its effector proteins leading to abnormal signaling activities. A small molecule inhibitor, ZCL278, has been shown to interact with CDC42 and alter CDC42-stimulated overactivity. In this work, we investigated the influence of ZCL278 on CDC42-ACK interaction using techniques including fluorescence spectroscopy, *in vitro* binding assay, isothermal titration calorimetry, and circular dichroism spectroscopy. Our data suggest that ZCL278 overall perturbs CDC42/ACK interaction. Our In vitro binding assay data show that ZCL278 decreased the binding affinity between CDC42, and ACK. sNBD-CDC42 fluorescence titration experiments data suggest that ZCL278 lowered the binding affinity of CDC42 for ACK from 0.2 μM. to 1.6 μM.

Isothermal titration experiments data, consistent with our fluorescence quenching data, show that the presence of ZCL278 decreased CDC42-ACK binding affinity by interfering with CDC42-ACK binding interaction causing the apparent *Kd* value to increase from 0.6 μM to 4 μM..

Introduction

CDC42 belongs to the Rho family of the Ras superfamily of small GTPases [1]. It is involved in the modulation of many cellular activities including cell adhesion, mitosis, and cytoskeletal rearrangements [1]. CDC42 interacts with many effector proteins including ACK1 to carry out its functions [2]. ACK1 was originally identified as a non-receptor tyrosine kinase (NRTK) [3], and as such links receptor tyrosine kinases to effector proteins [3]. Inactive ACK1 is found in an autoinhibited state stabilized by a molecular interaction between its Mig6 homology region (MHR) and its kinase domain [5]. Through its interactions with effector proteins like AKT, AR (androgen receptor) and ER (estrogen receptor), ACK1 contributes to the development of many cancers including prostate and breast cancers and is found continually activated in many other cancers [6]. ACK can be activated by various ligands including EGF and insulin and can also be activated by CDC42 with which it interacts via its CRIB (CDC42/Rac Interactive Binding) region [7]. Abnormal activity and overexpression of CDC42 have been associated to cell proliferation and malignant transformation [8]. Aberrant activity of the CDC42-ACK signaling pathway contributes to prostate cancer by phosphorylating and activating AR and degrading tumor suppressor Wwox [9]. Consequently, attempting to block CDC42-ACK signaling pathway leading to cancerous and diseased states has been the object of much research for drug candidate targets. ZCL278 is a small molecule inhibitor reported to impede CDC42 dysregulated actions *in vitro* [10]. In our lab, ZCL278 has been used in characterization work involving CDC42 and CDC42T35A.

In this research project, we probed the influence of ZCL278 on protein-protein interactions involving CDC42 and ACK using biochemical and biophysical approaches. Our in vitro binding assay data suggest that ZCL278 decreased binding between CDC42 and ACK. Extrinsic fluorescence quenching data suggest that ZCL278 altered the binding affinity of CDC42 for ACK by an eightfold decrease. Isothermal titration calorimetry data was consistent with our fluorescence quenching results as they showed that the presence of ZCL278 modified CDC42-ACK binding affinity by a tenfold decrease.

Materials and Methods

Protein Expression

6His-CDC42 and GSTACK were expressed in pET-15b and pGEX vectors as 6His-tag and GST fusion proteins, respectively in the lab. GSTACK plasmid was a gift from Dr D. Owen from Cambridge University.

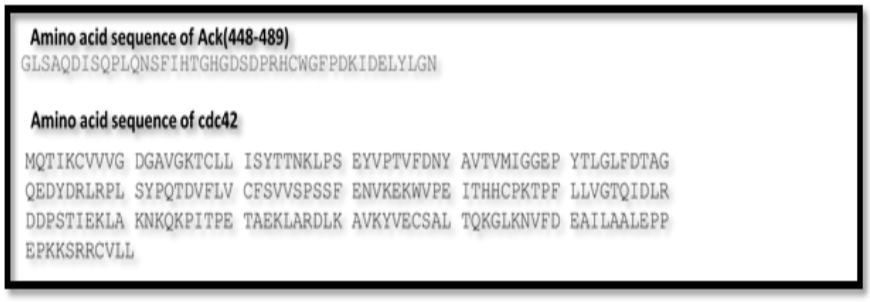

Figure 1. Amino acid sequence of ACK (448-489) and CDC42

Recombinant Protein Production

Escherichia coli BL21 cells were used to express recombinant proteins. Seed cultures expressing 6His-CDC42 and GSTACK were diluted in fresh 2TY growth medium (1:10) and grew to OD600 0.5-0.8, 0.7–0.9 at 37 °C respectively. Upon reaching the desired OD600, they were

induced with isopropyl-β-d-1-thiogalactopyranoside (IPTG) (0.5 mM) for 4 h (CDC42) and 5 h (GSTACK). Proteins were purified by affinity chromatography with Histrap HP (6His-CDC42) (GE Healthcare) and Gstrap 4B columns (Sigma-Aldrich) on the ÄKTA start protein purification system (GE Healthcare). GSTACK was eluted with 10 mM reduced glutathione (20 mM Tris, pH 8.0). Upon purification, 6His-CDC42 and GSTACK were dialyzed in 1x PBS pH 8.0 and pH 7.4 respectively. The GST moiety was cleaved with thrombin and removed via heat treatment. Protein concentrations were measured by using the extinction coefficient of each protein. Purity of proteins was verified with SDS-PAGE.

Heat Treatment

Previously designed in Dr Kumar and Adams' lab [11], the heat treatment consists of heating the thrombin-cleaved products (GST and peptide) until the GST moiety aggregates. Upon heating, the sample was centrifuged twice, and the supernatant (containing the soluble peptide) was kept and stored for follow-up experiments. Briefly, upon thrombin cleavage the sample was incubated at 80-90 °C for 25 minutes. It was then centrifuged twice for 10 minutes at 14K RPM in a microcentrifuge. The soluble supernatant was then frozen for downstream experiments and the aggregated pellet was discarded.

Nucleotide Exchange

CDC42 is synthesized in the GDP-bound inactive state. CDC42 must undergo nucleotide exchange (GDP/GTP exchange) for it to become active. The process involves the exchange of GDP for GTP in the presence of EDTA and an excessive amount of GTP analog (β, γ-Methyleneguanosine 5′-triphosphate) enough to competitively displace GDP from nucleotide biding pocket.

Briefly, GDP-CDC42 was mixed with 5 mM EDTA and GTP analog (1:10 ratio) and incubated on a rocker at 4 °C for 3 hours. GDP, EDTA and the excess unbound GTP were removed with a PD-10 desalting column upon incubation. The pooled protein was then detected with the Bradford reagent, combined into one sample to which was added 10 mM MgCl2, lyophilized, and stored for downstream experiments.

ZCL278 Stock Solution Preparation

ZCL278 (figure 3) was purchased from ApexBio Technology (cat #A8300) and prepared in 100% DMSO. It was then aliquoted (20 mM aliquots) and stored at -20 °C.

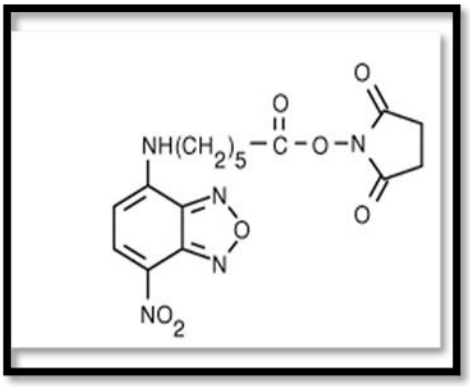

Figure 2. Chemical structure of sNBD

Intrinsic GTP Hydrolysis Assay

GTPases constitute a large group of hydrolase enzymes binding to the nucleotide guanosine triphosphate (GTP) and hydrolyzing it to guanosine diphosphate (GDP).[1] The GTP binding and hydrolysis occur in the highly conserved P-loop G domain, a protein domain common to many GTPases. In this experiment, we aimed at studying the impact of ZCL278 on the CDC42 intrinsic GTP hydrolysis in the presence of ACK.

GTP hydrolysis experiments were conducted according to the kit manufacturer (Promega). Experiments probing the intrinsic GTPase activity of CDC42 were conducted on CDC42 alone, CDC42/ACK, CDC42/ZCL278, and CDC42/ZCL278/ACK. All experiments were conducted in triplicates. In all experiments, an equimolar concentration (5 μM) CDC42 and ACK, and 20 μM of ZCL278 were used.

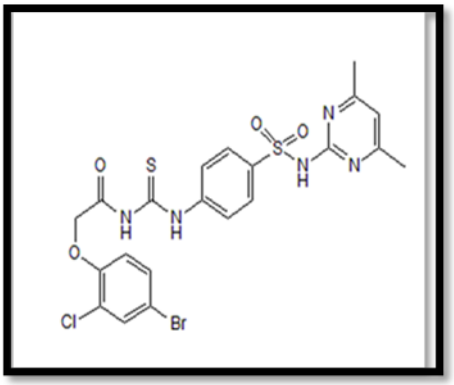

Figure 3. Chemical structure of ZCL278

In Vitro Binding Assays

In vitro binding assays are affinity-based assays which consist of binding either two purified proteins together or two proteins from a crude protein lysate. The protein-protein complex is then purified via affinity-based purification since one of the proteins bound together carries an affinity tag. This technique is used to determine the physical interaction between two proteins. However, in our current work it was used to study the influence the small molecule inhibitor ZCL278 on the formation of CDC42-ACK complex. Briefly, equimolar concentrations of CDC42 and GSTACK (200 μM each) were incubated in the absence and presence of 50 μM ZCL278 in 20 mM Tris, 100 mM NaCl, 10 mM MgCl2 for 3 h at 4°C. The mixture was then purified using GSTrap 4B columns on the ÄKTA start protein purification system. The bound complex GSTACK-CDC42 was eluted with 10 mM reduced glutathione (in 20 mM Tris, pH 8.0).

Ten percent of 100 % trichloroacetic acid (TCA) solution was added to precipitate the complex. After TCA precipitation, 8M urea was added to the protein sample and was analyzed on a 15% SDS-PAGE.

Intrinsic Fluorescence Quenching

Fluorescence titration of CDC42 and ACK by ZCL278 was carried out by monitoring the change of fluorescence intensity of tryptophan of CDC42 and ACK upon ZCL278 binding. Fluorescence measurements were collected using a fluorescence Spectrophotometer F-2500 (Hitachi) equipped with a slit width set to 2.5 nm. Measurements of intrinsic fluorescence of CDC42 and ACK were performed using an excitation wavelength of 280 nm and emission intensity was recorded from 300 nm to 450 nm. Both titration experiments were performed using a fixed concentration of CDC42 (10 µM) and ACK (10 µM) placed in a quartz cuvette which was titrated by the addition of 15 µM ZCL278 at each titration. All experiments were conducted in triplicate in 1X PBS, 10 mM Mgcl2, pH 8.0. Samples in the cuvette were mixed after each titration before taking the reading.

Extrinsic Fluorescence Quenching

To better understand the molecular details on CDC42 regulation and its interaction with effector proteins, fluorescence spectroscopic assays were performed. CDC42 was specifically labeled with a fluorescent group, succinimidyl 6-[(*7*-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]hexanoate (sNBD) (figure 2) which served as an extrinsic fluorophore for CDC42 binding interactions with effector proteins [12]. Here, it was used to monitor the influence of ZCL278 on the CDC42-ACK binding interaction. The labeling process was performed as previously described [13]. Briefly, CDC42 was incubated with sNBD at a 1:15 ratio for 1 h at room temperature on a rocker.

Unbound sNBD was removed by centrifugation, dialysis, and desalting with a PD-10 column. SNBD, with excitation and emission wavelength of 488 and at 545 nm, respectively [12], is covalently attached to Lysine150 on CDC42 [12] (figure 4). Once labeled, sNBD-CDC42 complex displays fluorescence change upon binding to an effector protein, which can be used to monitor the binding interaction between CDC42 and ACK [12]. Consequently, a control experiment was performed as follows :70 nM of sNBD-CDC42 was titrated with an increasing amount of ACK. The final concentration of ACK in the cuvette varied from 0 nM to 2 μM. Then 5 μM of sNBD-CDC42 incubated with 5 μM of ZCL278 was titrated with 2.5 mM ACK at a time as the continuous quenching of the fluorescence was monitored.

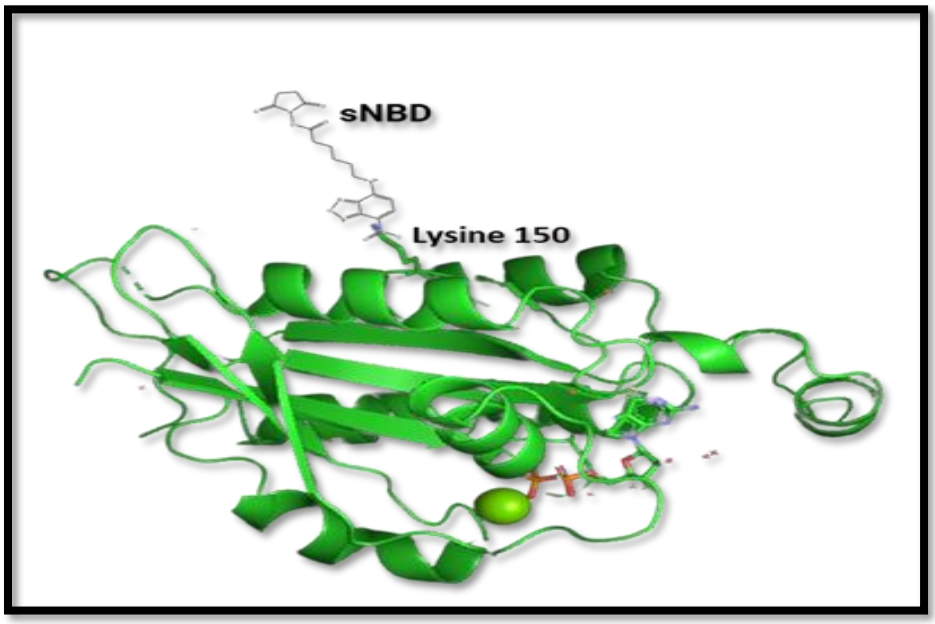

Figure 3. sNBD covalently attached to CDC42 at Lysine 150

The *Kd* of both experiments were calculated by nonlinear least squares and fit to the equation:

$$F = F_i + F_f\left[\frac{(K_D + L_t + R_t) - \sqrt{(K_D + L_t + R_t)^2 - 4R_tL_t}}{2R_t}\right]$$

Where F represents the change in fluorescence over the initial fluorescence (ΔF/Fo); Fi represents the initial value of ΔF/Fo; Ff represents the final value of ΔF/Fo; Lt is the total concentration of ACK, and Rt is the total concentration of CDC42. The fluorescence intensity was measured using a SLM 8000c spectrofluorometer operated in the photon-counting mode. All experiments were performed in triplicates in 20 mM Tris, 100 mM NaCl, 10 mM Mgcl2, pH 8.0.

Isothermal Titration Calorimetry (ITC)

ITC is a technique used to quantitatively study biomolecular interactions. It consists of binding two biomolecules where one is placed in the syringe and the other one in the cell. It operates by measuring the amount of heat released or absorbed during the binding experiment.

By measuring the heat transfer, ITC provides the thermodynamic profile of the molecular interaction consisting of the binding constants (K_d), reaction stoichiometry (n), enthalpy (ΔH) and entropy (ΔS). In our lab, this technique was used to probe the influence of ZCL278 of thermodynamic profile of CDC42/ACK interaction. The experiment was performed in a MicroCal iTC200 at 25 °C. Lyophilized CDC42 and ACK were dissolved in 10 mM phosphate buffer pH 8.0, 100 mM NaCl, 10 mM MgCl2, 0.5 mM TCEP, 1 % DMSO. A concentration of 16 μM of CDC42 (placed in the cell) was titrated by 181 μM of ACK placed in the syringe. The CDC42-ZCL278 complex for formed by incubating 16 μM CDC42 with 33 μM ZCL278 right before the experiment. The CDC42-ZCL278 complex was placed in the cell and was titrated with 181 μM ACK placed in the syringe. A volume of 1 μL of 100% DMSO was added to the ACK sample to prevent buffer mismatch. The titration consisted of 30 injections with 1.3 μL of the titrant (ACK) injected at each injection. A blank run was performed in which CDC42 was replaced with the buffer and was titrated by ACK.

Another blank run was performed in which ZCL278 was added to the buffer placed in the cell with no CDC42 and was titrated by an ACK sample in which was added an appropriate amount of DMSO to avoid buffer mismatch. The data were fitted to an equation describing binding at a single site, using the Origin software supplied to provide estimates of the thermodynamic profile of the interaction including K_a, ΔH, and the apparent stoichiometry of interaction.

Far-UV Circular Dichroism (CD)

Far-UV CD calculations were performed using a Jasco J-1500 CD spectrometer. Conformational modifications in the secondary structure of individual proteins (CDC42, ACK), protein-small molecule complexes (CDC42-ZCL278, ACK-ZCL278), and protein-protein-small molecule complexes (CDC42-ACK, ACK-CDC42-ZCL278) were analyzed in the Far-UV region between 190 to 250 nm. A concentration of 50 μM for each protein (CDC42, ACK) was used in all experiments which were performed in PBS, 0.27mM KCl, 0.18mM KH2PO4, 1.5mM Na2HPO4, 13.7mM NaCl, 10 mM MgCl2, pH 8.0 in a 1-mm pathlength quartz cuvette. The scan speed, band width and data pitch were respectively set to 50nm/min, 1.00nm/min and 0.1nm/min. All scans were performed in triplicate and averaged to obtain the final CD spectra. All individual spectrum was subtracted from the resulting complex spectra so the final difference spectrum could be obtained.

Results

ZCL278 Influence on non-complexed CDC42 and ACK fluorescence quenching

Before ACK/CDC42 interaction experiments were performed, it was important to ascertain that ZCL278 only binds to CDC42 and not to ACK.

To this end, a tryptophan fluorescence quenching experiment involving CDC42 and ACK individually in the presence of ZCL278 was conducted. As can be shown from figure 4A, ACK tryptophan fluorescence remained unchanged during the titration, which can be explained by the fact the switch I region in the CDC42 has very little similarity to the ACK CRIB region which is where these two proteins interact. However, ZCL278 quenched CDC42 tryptophan fluorescence and caused CDC42 tryptophan maximum fluorescence absorption to shift from 320 when tryptophan finds itself a non-polar environment to 355 nm when it is now exposed to polar environment as can be seen on figure 4B. Overall, figure 3 shows that ZCL278, by binding to CDC42, caused structural modifications in CDC42 leading tryptophan from being buried in an hydrophobic microenvironment to being exposed to the outer hydrophilic environment of the protein indicating a binding interaction. ZCL278 quenching action on CDC42 suggests the formation of hydrogen bonds between CDC42 Thr35 and NH groups of ZCL278. Once CDC42 Thr35 H-bonds with ZCL278 NH groups, given the importance of Thr35 in the protein stability, it undergoes conformational changes which end up changing tryptophan microenvironment, thereby quenching its fluorescence intensity. This is a very useful technique used in protein biochemistry to monitor protein-protein interaction (provided that one protein is tryptophan-free) and protein-SMI interaction. Our fluorescence quenching data for CDC42 are consistent with those published by Friesland et al. [10] where they quenched tryptophan fluorescence with ZCL278 to determine the binding affinity of CDC42-ZCL278 binding interaction.

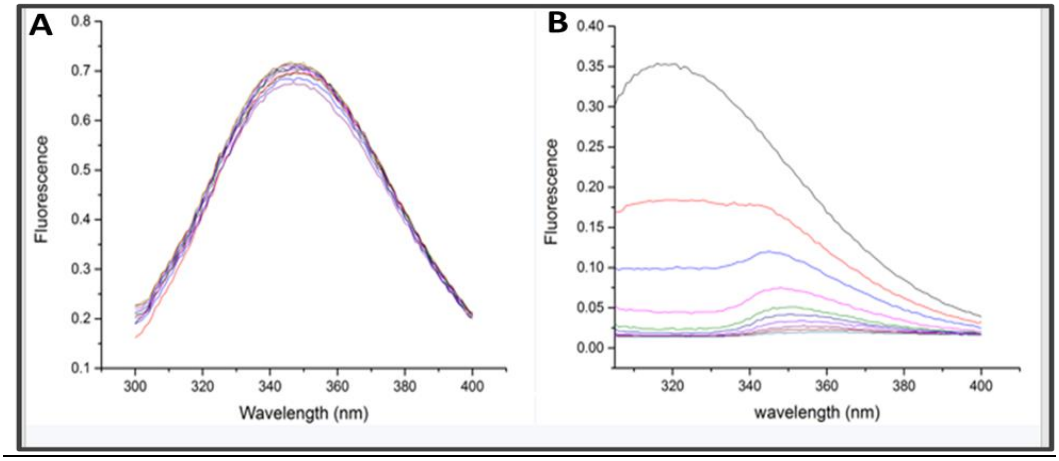

Figure 4. Tryptophan Fluorescence quenching of ACK and CDC42 by ZCL278. A) ACK tryptophan fluorescence remained unchanged B) CDC42 tryptophan maximum fluorescence absorption shifted from 320 nm to 355 nm.

ZCL278 influences CDC42:ACK binding

Since CDC42 binds to ACK with nanomolar affinity [10], an *in vitro* binding assay was conducted to probe the direct impact of ZCL278 on the CDC42-ACK interaction. As it is shown in figure 5A, in the presence of ZCL278 (+ZCL278) (lane 3) there is less CDC42 compared to the one with no ZCL278 (lane 2). Using the UN-SCAN-IT gel 7.1 software, figure 5B depicts graphically how CDC42-ACK binding decreased in the presence of ZCL278. During the incubation of CDC42 with GSTACK, only 15% of the CDC42 binds to GSTACK. However, up to 10% of CDC42-ZCL278 binds to GSTACK. This decrease in binding suggests that when ZCL278 is incubated with CDC42, key hydrophobic and ionic interactions occur between CDC42 Phe28 and the aromatic rings of ZCL278, and between Thr35, Thr43 and ZCL278 NH groups. As a result, since these key residues in CDC42 such as Thr35 and Thr43 and the aromatic rings are also important for non-covalent interactions between CDC42 and ACK, ACK can no longer bind to CDC42 effectively due to the “unavailability” of these key residues to establish the bonds, which leads to a reduced binding.

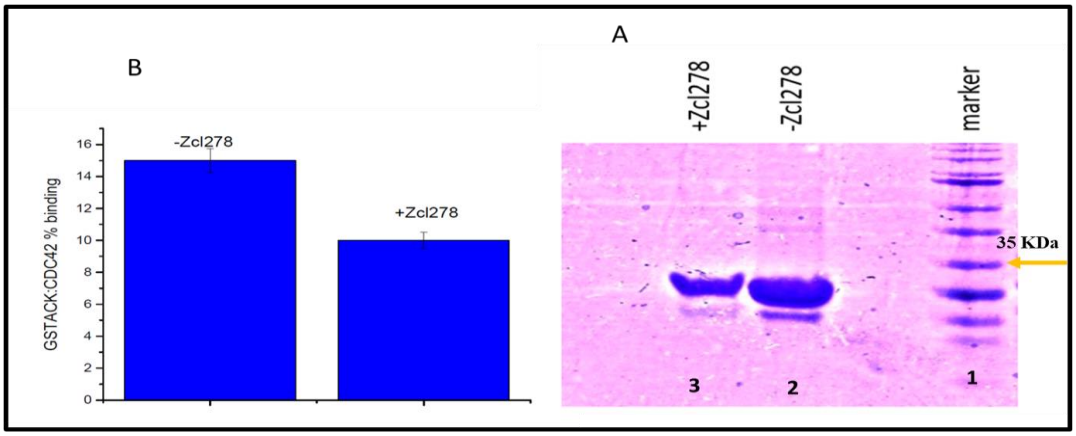

Figure 5. A) In-vitro binding assays comparing the binding affinities between GST-ACK/CDC42 in the presence/absence of ZCL278. In the presence of ZCL278 (lane 3), there is a decreased binding of CDC42 (20KDa) to GST-ACK (31KDa) characterized by a scant amount of CDC42 in the +ZCL278. The concentration of ZCL278 used was 50 µM. B) A graphical depiction of the In vitro binding assays using UN-SCAN-IT gel 7.1 software.

Fluorescence spectroscopy shows reduced CDC42:ACK binding affinity in the presence of ZCL278

As stated earlier, CDC42-ACK binding affinity is in the nanomolar range [29,30]. It has been reported that sNBD fluorescent property can be used to monitor CDC42 interactions with its effectors [12]. To probe a possible influence of ZCL278 on the CDC42-ACK binding affinity, we conducted fluorescence spectroscopy using an extrinsic fluorescent reporter group covalently attached to the side chain amine of Lys150 on CDC42 (figure 2). Figure 6 depicts a sNBD-CDC42-ACK binding control experiment which shows a binding affinity (K*d*) value of 200 nM (figure 6). In the presence of ZCL278 the K*d* value increased to 1.6 µM as shown in fig 5B. The Kd value for our CDC42-ACK binding experiment is in the nanomolar range consistently with published Kd values for CDC42 and ACK [13]. This decrease in CDC42-ACK binding affinity suggests that the switch I is unavailable since ZCL278 occupies it by forming CDC42-ZCL278 complex, and that key residues such as Thr35 and Thr43 as well as several hydrophobic residues

like Phe28 located in the switch I are no longer available for non-covalent interactions between CDC42 and ACK.

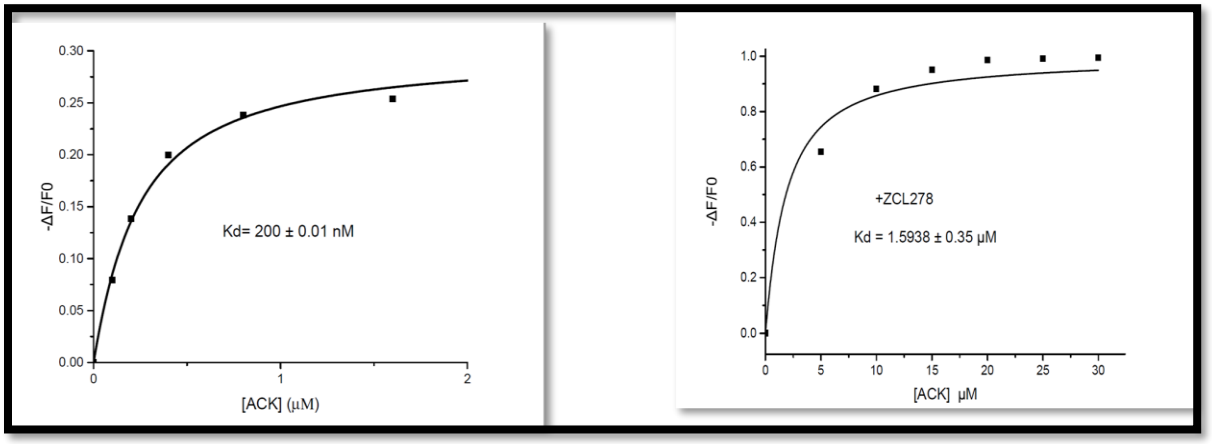

Figure 6. sNBD fluorescence titration of CDC42 with ACK. (A) In the absence of ZCL278, CDC42 binds to ACK with a Kd value of 200 nM. (B) In the presence of ZCL278, the binding affinity between CDC42 and ACK diminished with an increased Kd value of 1.6 μM.

ZCL278 decreased CDC42-ACK binding affinity in isothermal titration calorimetry

ITC was used to monitor the influence of ZCL278 on the CDC42-ACK protein-protein interaction. Experiments first involved the titration of CDC42 with ACK, an apparent Kd value of 600 nM was calculated. In the second set of experiments, CDC42-ZCL278 complex was titrated with ACK and a Kd value of 4 μM, nearly 7-fold increase of the Kd calculated for the experiments in the absence of ZCL278 as shown in figure 7. The Kd value for our ITC from the CDC42-ACK binding experiment is very close to the one we got from our fluorescence quenching experiment. This reduced binding affinity between CDC42 and ACK in the presence of ZCL278 is consistent with our previous data and suggests that the switch I is occupied by ZCL278, and several key residues in the switch I are longer available to establish non-covalent interactions between CDC42 and ACK.

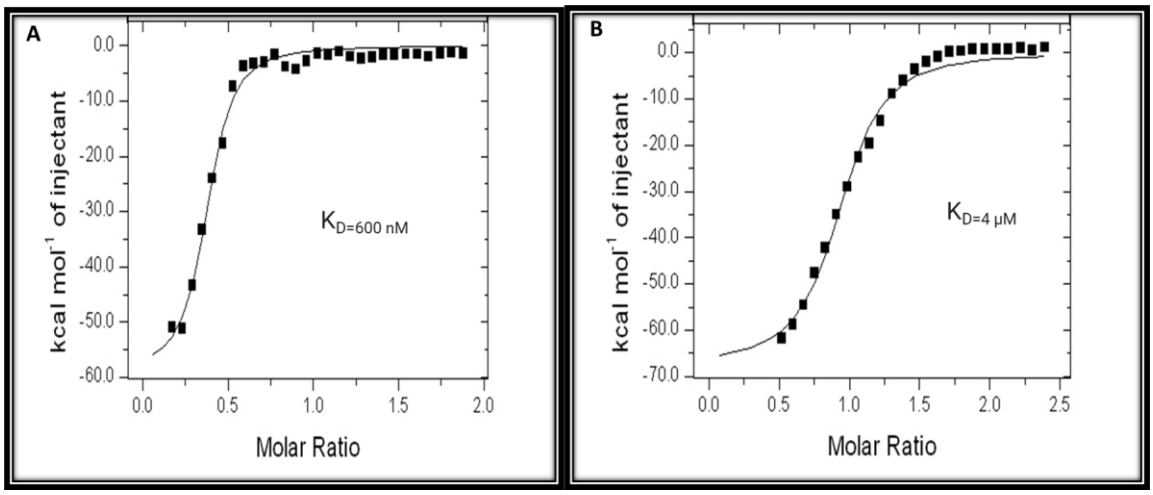

Figure 7. Isothermal titration calorimetry of CDC42 with ACK. (A) In the absence of ZCL278, CDC42 binds to ACK with a Kd value of 600 nM. (B) In the presence of ZCL278, the binding affinity between CDC42 and ACK diminished with an increased Kd value of 4 µM.

CD spectroscopy revealed major structural changes caused by ZCL278 to CDC42-ACK interactions

Far-UV circular dichroism (CD) is a technique used to investigate the secondary structure of chiral macromolecules such as proteins or non-chiral molecules in a chiral environment. To this end, far-UV CD was used to probe structural modifications that occurred in the CDC42 secondary structure upon ZCL278 and ACK binding to CDC42. Far-UV CD spectra of CDC42 (figure 8A) shows a negative peak at around 210 nm along with a deeper peak at 220 nm, which is characteristic of secondary structure with an α-helix prevalence. Upon binding of ZCL278, changes in the structure of CDC42 occurred which led to a change from an α-helix dominated secondary structure to a more disordered structure (figure 8B) and even more disordered upon ACK binding to the ZCL278-CDC42 complex (figure 8C). However, a secondary structure with a prevalence of β-sheets was observed upon ACK binding to CDC42 (figure 8D) suggesting that ZCL278 binding to CDC42 really interferes with CDC42-ACK interaction by causing such contrasted secondary structural modifications between CDC42-ACK and ZCL278-CDC42-ACK

complexes. Figures 8E and F show that ACK adopts a random coil secondary structure in the presence and absence of ZCL278. Thr35 is key in maintaining the stability of CDC42. When ZCL278 NH groups H-bond with Thr35, it apparently destabilizes CDC42 overall structure which most likely caused the secondary structure to change from a well-defined secondary structure to a random coil. The binding of ACK to the CDC42-ZCL278 complex leads to the disappearing of the α-helix portion and to more disordered structure, which suggests that we are dealing with 3-component complex with no defined secondary structure. The lack ofinfluence of ZCL279 showed in the ACK CD spectrum suggests that ZCL278 did not interact with ACK and as a result has no influence on its secondary structure. It also suggests that the CRIB region in ACK has no affinity for ZCL278.

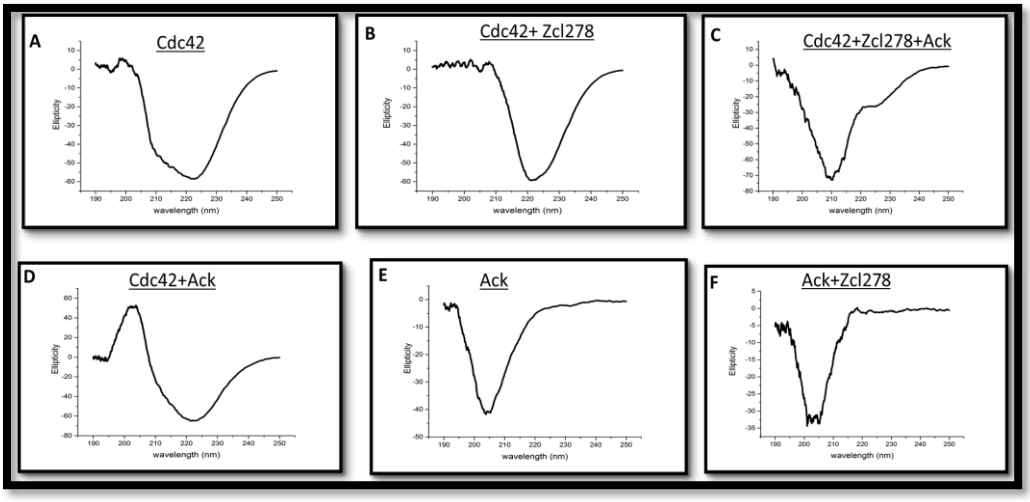

Figure 8. Circular dichroism difference spectral data for CDC42 in the presence and absence of ZCL278 and ACK. Binding of ACK and ZCL278 to CDC42 induced major secondary structure differences.

Discussions

ZCL278, as displayed in our data, impeded CDC42-ACK interaction and caused CDC42 structural modifications upon binding. This suggests that ZCL278 must be probed and explored further on downstream proteins like Wasp and PAK, and other proteins taking part in regulating

the cycle including GEFs and GAPs for acquisition of detailed molecular knowledge regarding the strength and selectivity of the drug candidate. Data gathered from the *in vitro* binding assay indicated that ZCL278 incubation with CDC42 led to 5 % decrease in the capacity of ZCL278-CDC42 complex to bind to ACK. Given CDC42 binds to ACK with low nanomolar affinity and binds to ZCL278 with low micromolar affinity, this is quite significant. In addition, given the concentration of CDC42 in the experiment was much higher relative to that of the ZCL278, a reduction of 5% in the binding capacity is quite noteworthy. The incubation of ZCL278 with CDC42 caused the binding affinity to decrease tenfold based on data from fluorescence spectroscopy and isothermal titration experiments. Protein-protein interactions and protein-protein-SMI experiments are usually laden with issues. These problems range from small molecule is not water soluble, protein aggregates in the appropriate solvent of the small molecule, to adding disulfide bond breaker like TCEP to optimize binding which in turn disturbs the complex formation and even changes the sample pH. Therefore, accurately determining the Kd from fluorescence quenching and isothermal titration calorimetry experiments was very difficult. However, the closeness of Kd values obtained from triplicate experiments increases the accuracy of our data. Nonetheless, structural modifications should be done on ZCL278 to make it more hydrophilic so it can behave better in aqueous environment which is a better mimic of the cytosol.

The interaction between CDC42 and ACK determined by these key residues, Ala41, Val42, Thr43, Gly47, Thr52, Phe56, and Leu174, with Ala41, Val42, Thr43, Gly47 in the switch I region [1]. It was predicted that ZCL278 would H-bond with Thr35, Asn39, and Ap57 of CDC42 (figure 1) and hydrophobically interact with Val36 and Ph56. It is most likely that ZCL278 disturbs CDC42-ACK interaction by H-bonding with Thr35 and Thr43 and hydrophobic bonds

with Ala41, Val42, Gly47 in the switch I, which in turn disturbed the CDC42-ACK binding complex formation. More probing and exploratory work should be conducted on ZCL278 including NMR spectroscopy and molecular dynamics simulation, which should reveal to be paramount in unveiling molecular details explaining ZCL278 interference with CDC42-ACK interactions. Co-transfecting CDC42 and ACK into Cos-alpha cell lines and studying their transgenic expression and interaction in the absence and presence of ZCL278 should be particularly useful. That would unveil key knowledge on how ZCL278 influences the binding of CDC42 to ACK in eucaryotic cells, which would a better reflection of what would happen in human cell scenario, which could provide important insights into the efficacy of ZCL278 as a drug in humans. So far, no in vitro research involving CDC42, and ACK has been conducted. So, studying the transgenic expression of these two genes in vitro in relation to ZCL278 would reveal much.

Conclusion

Overall, our data suggest that ZCL278 interferes with the CDC42-ACK binding interaction. It does so most likely by binding to CDC42 switch I region which in turn interferes with CDC42-ACK binding. Much remains to be known about ZCL278 interference with the direct binding of CDC42 to ACK. Despite major disadvantages that ZCL278 suffers from, it has helped showed once again the necessity of finding small molecules inhibitors more potent and more selective to specifically target Ras superfamily of proteins individually, which will ultimately aid in tackling more effectively Ras-driven cancers.

References

1) Cherfils, Jacqueline, and Mahel Zeghouf. "Regulation of small gtpases by gefs, gaps, and gdis." Physiological reviews 93.1 (2013): 269-309.

2) Bos, Johannes L., Holger Rehmann, and Alfred Wittinghofer. "GEFs and GAPs: critical elements in the control of small G proteins." Cell 129.5 (2007): 865-877.

3) Blume-Jensen, Peter, and Tony Hunter. "Oncogenic kinase signalling." Nature 411.6835 (2001): 355-365.

4) Sicheri, Frank, Ismail Moarefi, and John Kuriyan. "Crystal structure of the Src family tyrosine kinase Hck." Nature 385.6617 (1997): 602-609.

5) Modzelewska, Katarzyna, et al. "ACK1 mediates CDC42-dependent cell migration and signaling to p130Cas." Journal of Biological Chemistry 281.49 (2006): 37527-37535.

6) Yang, Wannian, et al. "Activation of the CDC42-associated tyrosine kinase-2 (ACK-2) by cell adhesion via integrin β1." Journal of Biological Chemistry 274.13 (1999): 8524-8530.

7) Kato-Stankiewicz, Juran, et al. "Epidermal growth factor stimulation of the ACK1/Dbl pathway in a CDC42 and Grb2-dependent manner." Biochemical and biophysical research communications 284.2 (2001): 470-477.

8) Eisenmann, Kathryn M., et al. "Melanoma chondroitin sulphate proteoglycan regulates cell spreading through CDC42, ACK-1 and p130 cas." Nature cell biology 1.8 (1999): 507-513.

9) Mahajan, Nupam P., et al. "Activated tyrosine kinase ACK1 promotes prostate tumorigenesis: role of ACK1 in polyubiquitination of tumor suppressor Wwox." Cancer research 65.22 (2005): 10514-10523.

10) Friesland, Amy, et al. "Small molecule targeting CDC42–intersectin interaction disrupts Golgi organization and suppresses cell motility." Proceedings of the National Academy of Sciences 110.4 (2013): 1261-1266.

11) Sakhel, Beatrice, et al. "Simplification of the purification of heat stable recombinant low molecular weight proteins and peptides from GST-fusion products." Journal of Chromatography B 1172 (2021): 122627.

12) Leonard, David A., et al. "Use of a fluorescence spectroscopic readout to characterize the interactions of CDC42Hs with its target/effector, mPAK-3." Biochemistry 36.5 (1997): 1173-1180.

13) Elliot-Smith, Andrea E., et al. "Specificity determinants on Cdc42 for binding its effector protein ACK." Biochemistry 44.37 (2005): 12373-12383.

CHAPTER THREE:

Characterizing CDC42 and CDC42F28L in the presence of the small molecule inhibitor ZCL278

Abstract

The Ras superfamily of small GTPases are involved in cell-signaling processes that, if not regulated, may lead to continued cell multiplication, inhibited apoptosis, and cell transformation. They work as molecular controllers, which via GTP/GDP exchange cycles, modulate cellular activities by switching them on and off. Many mutations have been identified in Ras proteins which prevent GTP hydrolysis and irreversibly keep the GTPase trapped in the 'on' (GTP-bound) state, and transformed several cell types and have been found in up to 40% of all human tumors. Other Ras-related GTPases, including members of the Rho subfamily, have been involved in cell growth regulation. Particularly, CDC42 is one those Rho members that has recently received a great deal of attention. CDC42 is involved in the regulation of many cellular processes including cell adhesion, mitosis, and cytoskeletal rearrangements. Evidence shows that CDC42 hyperactivation leads to cell transformation as revealed in studies conducted on the 'fast-cycling' CDC42 mutants which are able of constitutive nucleotide exchange, but still exhibit full GTP hydrolytic activity [1]. One example of this so-called 'fast-cycling' CDC42 mutant is CDC42F28L. The CDC42F28L variant can do GDP/GTP exchange rapidly, thereby greatly increasing the cytoplasmic pool of activated CDC42 resulting in oncogenic activities. It is imperative that the biophysical parameters of this mutant be studied so that drugs can be designed to stop its oncogenic behavior. ZCL278 is a selective inhibitor of CDC42. It binds directly to CDC42 and disturbs its functions. This work aimed at characterizing CDC42 and CDC42F28L in the presence and absence of ZCL278.

Overall, data from our experiments show that ZCL278 bound to both CDC42 and CDC42F28L similarly with micromolar affinity, and that ZCL278 induced conformational modifications in CDC42 and CDC42F28L upon binding and rendered CDC42 and CDC42F28L more thermally stable.

Introduction

The Ras superfamily of G proteins is implicated in several cellular activities such as cell division, protein trafficking, cytoskeletal rearrangements, and secretion. These GTPases are regulated by shuttling between the biologically active GTP form and the inactive GDP-bound form [1, 2]. Due to their intrinsic GTP hydrolysis capacity, these small GTPases hydrolyze GTP to GDP. This GDP-GTP cycle is controlled by a variety of regulatory proteins such as GDI, GEF, and GAPs [3-5]. The Ras proteins have been the object of considerable investigation due to their potential oncogenic capacity. Oncogenic Ras proteins induce cell transformation as a result of either a constitutively active GTPase or an increase in the rate of GTP/GDP shuttling, which ultimately leads to an increased downstream kinases activity. Several mutations in Ras proteins that inhibit their GTPase activity have been involved in several cancers [6-8]. For instance, a mutant protein which results from the replacement of phenylalanyl 28 with leucine (CDC42F28L) in CDC42 wild-type causes cell transformation due to the increased rate of cycling between the active and inactive forms of the mutant protein [7]. The CDC42F28L mutation which led to cell transformation is comparable to the one induced by the oncogenic protein Dbl, which catalyzes fast GTP/GDP exchange in CDC42. Phenylalanyl 28 mainly stabilizes the nucleotide by strongly interacting with the aromatic ring of the attached nucleotide [10,13,14].

Since ZCL278, a selective inhibitor of CDC42, was shown in our previous binding experiments to influence CDC42/ACK interaction, we characterized CDC42 and CDC42F28L in the absence and presence of ZCL278 in this current work. DSC data suggest that ZCL278 made CDC42 and CDC42F28L more thermally stable while heat-induced denaturation data did not reveal any noticeable change in the denaturation pattern caused by heat in the presence and absence of ZCL278. Fluorescence quenching data suggest that there was no change in the quenching pattern of CDC42 and CDC42F28L in the absence and presence of ZCL278. Urea-induced denaturation showed no difference in the denaturation pattern of CDC42 in the presence and absence of ZCL278. While in the presence of ZCL278, CDC42F28L showed a two-state denaturation process, CDC42F28L showed a three-state denaturation in its absence. Our intrinsic GTPase nucleotide exchange data showed that ZCL278 had no influence on the CDC42 and CDC42 intrinsic GTP/GDP exchange.

Materials and Methods

Protein constructs

CDC42 and CDC42F28L were expressed in pET-15b vectors as 6His-tag fusion proteins.

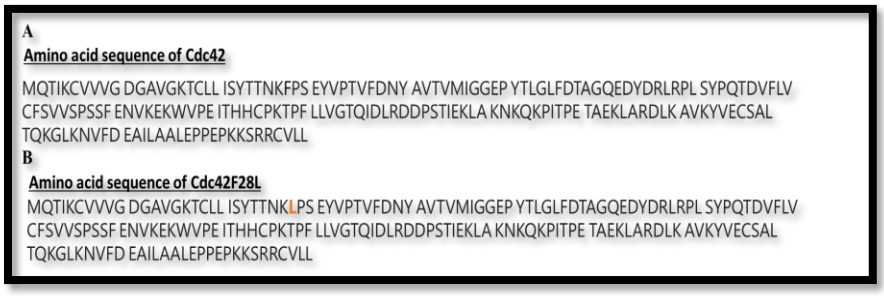

Figure 1. Amino acid sequence of CDC42 (A) and that of CDC42F28L (B) with a single point mutation in red.

Recombinant protein expression

Escherichia coli BL21 cells were used to express recombinant proteins. Seed cultures expressing CDC42 and CDC42F28L were diluted in fresh 2TY growth medium (1:10) and grew to OD600 0.5-0.8 at 37 °C. Upon reaching the desired OD600, they were induced with isopropyl-β-d-1-thiogalactopyranoside (IPTG) (0.5 mM) for 4 hours. Proteins were purified by affinity chromatography with Histrap FF column (GE Healthcare) on the ÄKTA start protein purification system (GE Healthcare). Upon purification, CDC42 and CDC42F28L were dialyzed in PBS pH 8.0. Protein concentrations were measured by using the extinction coefficient of each protein. Purity of proteins was verified with SDS-PAGE.

Nucleotide exchange

CDC42 as well as CDC42F28L is overexpressed in its GDP-bound, inactive state. CDC42 then undergoes nucleotide exchange (GDP/GTP exchange) using GTP analog (β, γMethyleneguanosine 5′-triphosphate) (10X, CDC42; 20X, CDC42F28L) enough to competitively displace GDP from nucleotide biding pocket. Briefly, GDP-CDC42 or GDP-CDC42F28L were mixed with 5 mM EDTA and GTP analog (1:10 or 1:20 ratio) and incubated on a rocker at 4 °C for three hours for CDC42 and six hours for CDC42F28L. GDP, EDTA and the unbound GTP were removed with a PD-10 desalting column upon incubation. The pooled protein was then detected with the Bradford reagent, combined into one sample to which was added 10 mM MgCl2, lyophilized, and stored for downstream experiments.

ZCL278 solution preparation

ZCL278 was purchased from ApexBio Technology (cat #A8300) and prepared in 100% DMSO. It was then aliquoted and stored at -20 °C.

Intrinsic fluorescence quenching

Fluorescence measurements were collected using Jasco J-1500 CD spectrometer. Measurements of intrinsic fluorescence of CDC42 and CDC42F28L in the presence of ZCL278 were performed at a fixed excitation wavelength (280 nm) and emission intensity was recorded from 300 to 450 nm. All titration experiments were performed using a fixed concentration of CDC42 (2 µM) and CDC42F28L (2 µM) placed in a quartz cuvette which was titrated by the addition of 20 µM ZCL278 (1: 10 ratio) at each titration. ZCL278 showed absorbance at around 320 nm, so correction was made by subtracting the ZCL278 fluorescence absorbance from those of the proteins. All experiments were conducted in triplicate in PBS, 10 mM MgCl2, pH 8.0. Samples in the cuvette were mixed by pipetting up and down after each titration and a 5-minute delay was taken for sample equilibrium before taking the next reading. The excitation/emission bandwidth and the data pitch were set to 5.0 nm/min, 10.0 nm/min, 1.0 nm/min, respectively. The Kd values and fitting models were determined with the Hill equation on the Origin software.

Far-UV Circular Dichroism

Circular dichroism (CD) calculations were performed using a Jasco J-1500 CD spectrometer. Conformational modifications in the secondary structure of individual proteins CDC42 and CDC42F28L in the absence and presence of ZCL278 (10 µM) were analyzed in the Far-UV region between 190 to 250 nm. A concentration of 50 µM of each protein (CDC42, CDC42F28L) was used in all experiments which were performed in PBS, 0.27mM KCl, 0.18mM KH2PO4, 1.5mM Na2HPO4, 13.7mM NaCl, 10 mM MgCl2, pH 8.0 in a 1-mm pathlength quartz cuvette. The scan speed, band width, and data pitch were respectively set to 50nm/min, 1.00nm/min and 0.1nm/min. All CD measurements were performed in triplicate and all scans were accumulated three times to obtain the final averaged CD spectra.

All individual spectrum was subtracted from the resulting complex spectra so the final difference spectrum could be obtained.

Differential scanning calorimetry

Heat-induced denaturation of CDC42 and CDC42F28L in the presence of ZCL278 was performed with MicroCal DSC (Malvern). CDC42 and CDC42F28L (50 µM) was dialyzed in PBS, 10 mM MgCl2, pH 8.0, at 4°C. Samples were heated from 25 to 90 °C using a scan rate of 1.5 °C/min. Buffer samples without protein were run prior to the experiment to serve as baseline and were subtracted from the protein scan. All DSC experiments were done in triplicate. The melting curve was constructed, and the Tm value determined by the DSC machine.

Urea denaturation using CD spectroscopy

Urea denaturation measurements were performed using CD spectroscopy with the Jasco J-1500 CD spectrometer which is equipped with a cell holder capable of rotation. This instrument is also equipped with an automatic titration unit suitable for urea denaturation. CDC42 and CDC42F28L was dialyzed in PBS, 0.27mM KCl, 0.18mM KH2PO4, 1.5mM Na2HPO4, 13.7mM NaCl, 10 mM MgCl2, pH 8.0 in a 1-mm pathlength quartz cuvette. CDC42 or CDC42F28L (50 µM), with or without ZCL278 (10 µM), was placed in the cell, and urea (8M) was placed in the syringes. The scan speed, band width, and data pitch were respectively set to 50 nm/min, 1.00nm/min and 0.1nm/min.

The titration was set up into 40 injections with 0.2 µM of urea added at each titration. All measurements were performed in triplicate. Denatured fractions were plotted as a function of urea concentration.

Fractions of unfolded proteins were calculated using the equation:

$$Fd = \frac{MEx - MEi}{MEF - MEi}$$

where Fd is the fraction of unfolded protein, MEx is the second absorbance of the folded protein, MEi is the first absorbance of folded protein, and MEF is the last absorbance of the unfolded protein. The curves were constructed by plotting the fraction of unfolded protein as a function of urea concentration.

Temperature denaturation using CD spectroscopy

Temperature denaturation experiments were done by CD spectroscopy using Jasco J-1500 CD spectrometer equipped with a variable temperature cell holder. CDC42 or CDC42F28L (50 µM) was dialyzed in PBS, 0.27mM KCl, 0.18mM KH2PO4, 1.5mM Na2HPO4, 13.7mM NaCl, 10 mM MgCl2, pH 8.0 in a 1-mm pathlength quartz cuvette. Experiments were done with CDC42 or CDC42F28L in the presence and absence of ZCL278 (10 µM). The scan speed, band width, and data pitch were respectively set to 50 nm/min, 1.00nm/min and 0.1nm/min, and temperature was raised from 25 to 90 °C with a scan speed of 1.5°C/min, and a temperature interval of 5 °C. Denatured fractions were plotted as a function of temperature. Fractions of unfolded proteins were calculated using the equation:

$$Fd = \frac{MEx - MEi}{MEF - MEi}$$

where Fd is the fraction of unfolded protein, MEx is the second absorbance of the folded protein, MEi is the first absorbance of folded protein, and MEF is the last absorbance of the unfolded protein. The curves were constructed by plotting the fraction of unfolded protein as a function of temperature.

Intrinsic GTP hydrolysis assay

Intrinsic GTP hydrolysis assays were performed to probe the intrinsic GTPase activity of CDC42 and CDC42F28L in the presence of ZCL278, and were performed according to the kit manufacturer, Promega. Briefly, GDP-CDC42 and GDP-CDC42F28L (3.5 μM each) (with or without ZCL278 (5 μM)) was added to a GAP buffer (from kit) and was mixed with 5 μM of GTP and 1 mM DTT to initiate the GTP hydrolysis. After 3 hours for CDC42 and 0.5 hour for CDC42F28L, reagents from the kit was added to the reaction mix and was incubated for 30 minutes upon which time a detection reagent was added to the mix and was incubated for 10 more minutes after which period the luminescence was measured using the Synergy H1 Hybrid Multi-Mode Reader.

Results

ZCL278 binds to CDC42 and CDC42F28L with micromolar affinity

In protein biochemistry, fluorescence spectroscopy is used to monitor protein (un)folding, and to probe protein conformational modifications and binding interactions. To calculate the binding affinity of ZCL278 to CDC42 and CDC42F28L, a tryptophan fluorescence quenching experiment was performed. ZCL278 caused tryptophan fluorescence quenching in both proteins in a similar manner as shown in figure 1.

Curve fits and Kd values for CDC42 and CDC42F28L were calculated using the equation already described in the method section. Molecular dynamics simulation data from our lab showed that ZCL278 most likely binds to CDC42 at the switch I region by establishing non-covalent interactions between CDC42 Thr35 and ZCL278 NH groups. Each titration causes intermolecular changes leading to conformational changes that trigger little changes in tryptophan residue microenvironment resulting in fluorescence quenching. The titration was carried out until the fluorescence was quenched. These close kd values highlight the very little structural differences between CDC42 and CDC42F28L since Ph28 is not essential for CDC42 overall secondary structure. Various binding experiments involving the direct binding of a small molecule inhibitor to a Ras protein provided Kd values in the low micromolar range, which is consistent to our Kd values that are also in the low micromolar range. This low binding affinity trend highlights the challenges in finding a potent small molecule inhibitor that can bind Ras proteins with binding affinity in the low nanomolar range. With such tight binding, this small molecule inhibitor will be more likely able to compete with Ras-effector tight binding interactions which will result in a small molecule inhibitor that will more likely become a drug that can effectively tackle Ras-related cancers.

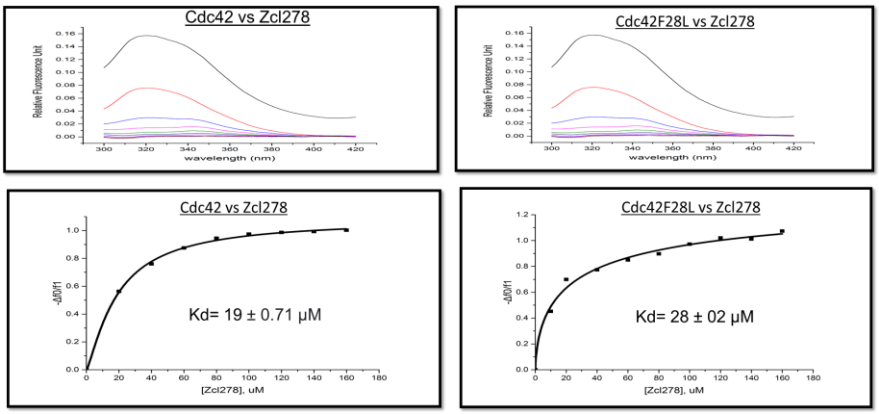

Figure 2. Fluorescence titration of CDC42 and CDC42F28L with ZCL278. Both proteins bind to ZCL278 with micromolar affinity.

Far-UV CD shows ZCL278 caused structural changes in CDC42 and CDC42F28L

Far-UV circular dichroism is a technique used to investigate the secondary structure of optically active macromolecules such as proteins. To this end, far-UV CD was used to explore structural modifications that could occur in the CDC42 secondary structure as a result of the introduction of the single point mutation and upon binding of CDC42 and CDC42F28L to ZCL278. Far-UV CD spectra of CDC42 (figure 3A) shows a negative peak at around 210 nm along with a deeper peak at 220 nm, which is characteristic of secondary structure with an α-helix prevalence. Figure 3B shows that the secondary structure of the mutant remained nearly unchanged with, however, contrary to the wild-type spectra, the mutant spectra show a steeper positive peak at 210 nm and a deeper negative peak at 210 nm. This is characteristic of a secondary structure with a dominance of α-helix and some β-sheets. Upon binding of ZCL278, changes in the structure of CDC42 occurred which led to a shift from α-helix to a disordered secondary structure with some α-helix (figure 3C). The same trend is observed in figure D where the addition of ZCL278 changed the CDC42F28L secondary structure dominated by α-helix- to a possible combination of β-sheets and disordered segments.

Molecular dynamics simulation data gathered from our lab (unpublished data) and by Friesland et al. suggest that ZCL278 most likely binds to CDC42 at the switch I region by establishing non-covalent interactions between CDC42 Thr35 and ZCL278NH groups. Through these non-covalent interactions, ZCL278 induced conformational changes in CDC42 secondary structure which result in a more disordered structure in both wild-type and mutant. The mutation caused the CDC42F28L variant to have a lower percentage of α-helix which can be shown by the negative peak which tends more towards the 210 nm mark than the 220 nm mark.

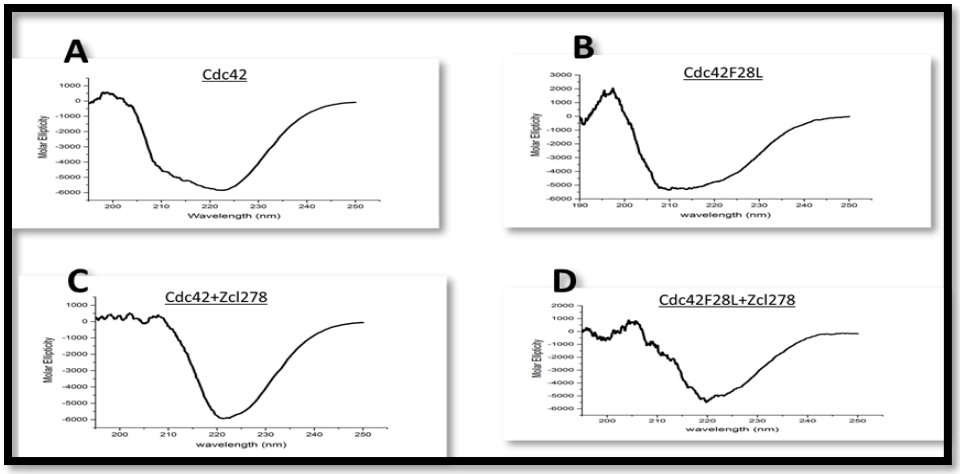

Figure 3. Far-UV CD of CDC42 and CDC42F28L in the presence of ZCL278. Both proteins underwent changes from an α-helix- to a β-sheet-dominated secondary structure upon binding to ZCL278.

CDC42 and CDC42F28L became more thermally stable in the presence of ZCL278 in DSC

DSC is a technique used to probe the thermal stability of macromolecules such as proteins. In our case, we investigated the thermal stability of CDC42 compared to CDC42F28L as well as their thermal stability in the presence of ZCL278. Overall, the apparent Tm of CDC42, 62 ± 0.5 °C, remained seemingly unchanged compared to that of CDC42F28L, 64 ± 0.8 °C (figure 4). However, both wild-type and mutant became thermally more stable in the presence of ZCL278. The increase in the thermal stability of the wild-type and the mutant would suggest that the small molecule inhibitor shields CDC42 and CDC42F28L switch I region by establishing non-covalent bonds (ionic and hydrophobic) between CDC42 Thr33 and ZCL278 NH groups. The Tm obtained in the DSC experiment is consistent with Tm published from our lab [15]. The Tm obtained for the CDC42F28L thermal denaturation is very close to that of the wild type. This is not surprising and would suggest that the mutation does not cause much change in CDC42 secondary structure.

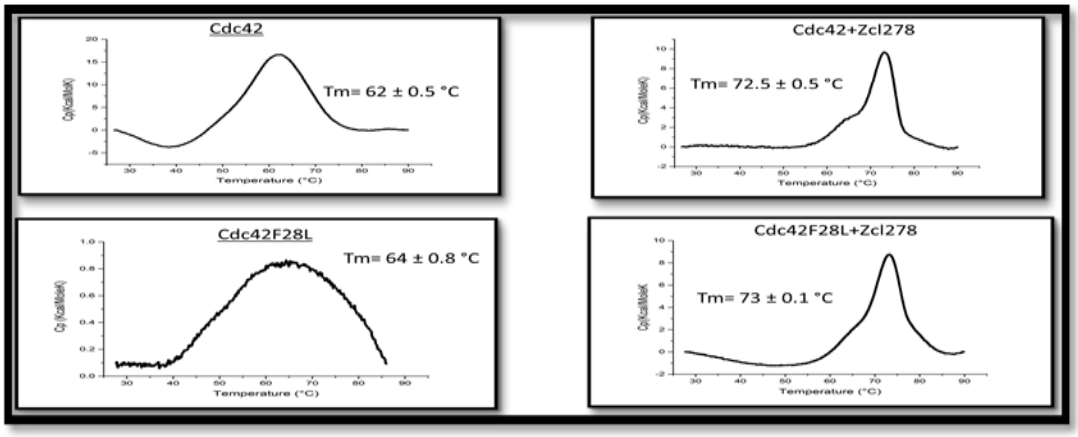

Figure 4. DSC thermograms of CDC42 and CDC42F28L in the presence of ZCL278. The apparent Tm of CDC42 remained unchanged compared to that of CDC42F28L while wild-type and mutant became thermally more stable in the presence of ZCL278.

CD spectroscopy shows a two-state urea-induced unfolding of CDC42F28L in the presence of ZCL278

Urea-induced unfolding using CD spectroscopy is a technique used to monitor the structural changes occurring in the secondary structure of a protein in the presence of an increasing amount of urea. In our case, we monitored the unfolding of CDC42 and CDC42F28L in the presence of an increasing concentration of urea using CD spectroscopy. A 3-state unfolding process was observed. For CDC42 in the absence and presence of ZCL278, in the first state CDC42 remained folded from 0 to 2.4 µM of urea. A second state was observed between 2.4 and 5. 8 µM, after which the protein completely unfolded and stayed so until the addition of last urea injection (figure 5). A 2-state unfolding process was observed for CDC42F28L in the presence of ZCL278. CDC42F28L secondary structure remained folded from 0 to 2.4 µM, starts unfolding from 2.4 to 5.8 µM, and completely unfolded and remained so from 6 to 8 µM. Surprisingly, in the presence of ZCL278 the protein appeared to have already been in an “intermediate state” right at the onset of the titration and remained in that state from 0 to 5.8 µM, then completely unfolded from 5.8 to 8 µM. Urea-induced denaturation based on CD spectroscopy did not suggest an impact of the

small molecule inhibitor on the denaturation of wild-type. It is unclear why this is the case, and more research should be done to unveil the reason for the ineffectiveness of the small molecule inhibitor in protecting CDC42 from being denatured by urea. Interestingly, CDC42F28L variant displays a two-state urea-induced denaturation in the presence of ZCL278. This result would suggest that the drug destabilizes the variant and causes it to denature faster by urea. This is a bit contradictory to what we have seen so far, so more research needs to be done towards elucidating those data.

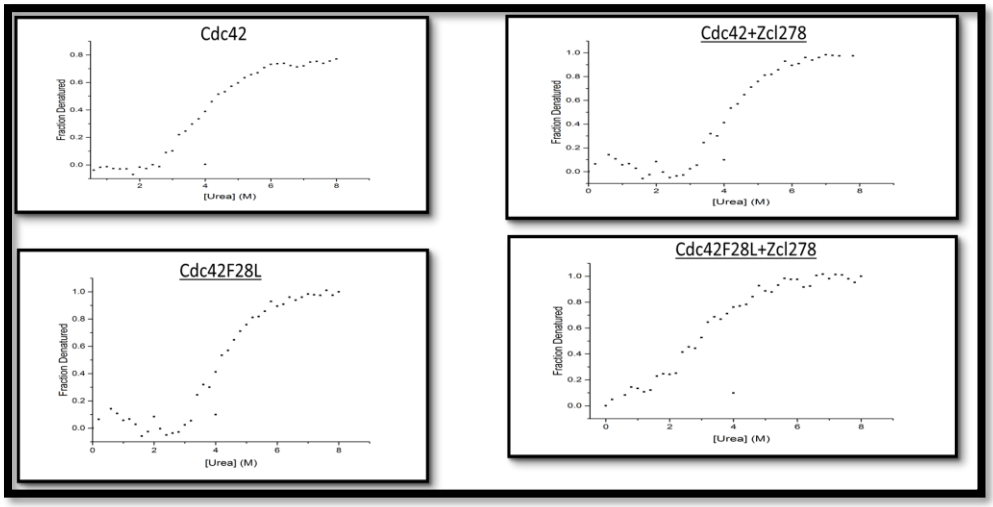

Figure 5. Urea-induced unfolding of CDC42 and CDC42F28L in the presence or absence of ZCL278. No significant difference was observed in the urea-induced unfolding of CDC42 in the presence of ZCL278. In the presence of ZCL278, a 2-step unfolding was observed for CDC42F28L compared to a 3-step unfolding observed in the absence of ZCL278.

CD spectroscopy shows no change in heat-induced unfolding of CDC42 and CDC42F28L in the presence of ZCL278

Temperature-induced unfolding using CD spectroscopy is a technique used to monitor the structural changes occurring in the secondary structure of a protein by heating the sample. In our case, we monitored the unfolding of CDC42 and CDC42F28L in the presence of ZCL278 by heating the protein samples from 25 to 90 °C using CD spectroscopy. A 3-state unfolding process was observed for both proteins (wild-type and mutant) in the absence and presence of

ZCL278. The proteins remained folded from 25 to 45 °C, started unfolding from 45 to 73 °C, then completely unfolded from 73 to 90 °C. These heat-induced denaturation experiment data suggest that the wild type and the mutant are heat-denatured in a similar pattern and that the SMI has no influence on CDC42 and CDC42F28L heat-induced denaturation. Those denaturation experiments either by urea or by heat are done based on CD spectroscopy at 220 nm. This wavelength is where the negative peak is at for a CDC42 or CDC42F28L in a CD spectrum, which indicates the prevalence of alpha helix in the secondary structure of the wild type and the mutant. Whether or not the selection of this wavelength has to do with the incapacity of the experiment to reveal the impact of the SMI on the proteins, that is beyond my knowledge and perhaps urea- and heat-induced denaturation experiments based on fluorescence spectroscopy would reveal more about the influence of the small molecule inhibitor on the CDC42 and CDC42F28L variant denaturation experiments. Perhaps this has to with the way the circular dichroism displays or records protein denaturation. I really do not know.

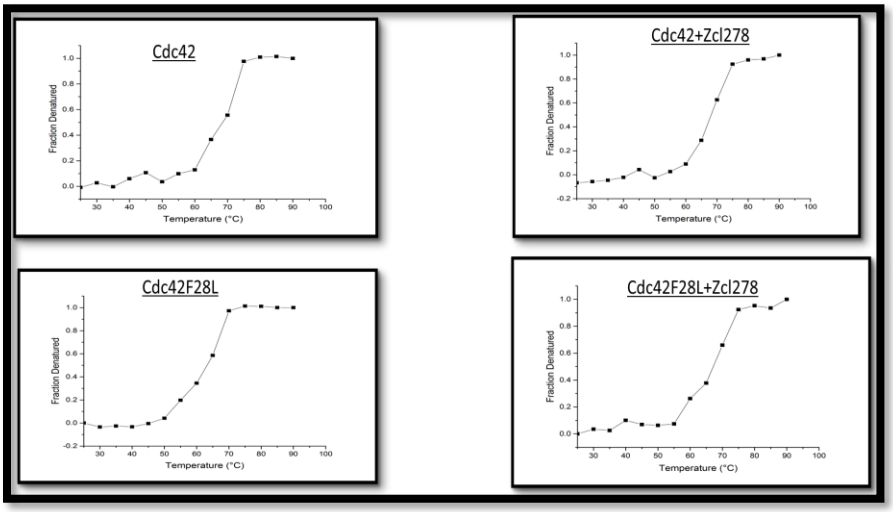

Figure 6. Temperature-induced unfolding of CDC42 and CDC42F28L in the presence or absence of ZCL278. No significant difference was observed for either protein in the presence of ZCL278.

Intrinsic GTP hydrolysis of CDC42 and CDC42F28L remained unchanged in the presence of ZCL278

The GTPase-Glo assay kit is used to monitor the intrinsic, GAP-induced, or GEF-induced GTP hydrolysis activity of a GTPase by measuring the amount of GTP left at the end of the hydrolysis which is converted to ATP and is used to produce light (luminescence) by luciferase. A high luminescence intensity means a slow-acting GTPase which hydrolyzes few GTP molecules which results in a large amount of GTP left at the end of the reaction to be converted into ATP, then eventually light (more light). A low luminescence signal means a fast-acting GTPase which hydrolyzes a large amount of GTP resulting in very few molecules left at the end of the reaction to be converted into ATP, then light (less light). In our case, we studied the influence of the small molecule ZCL278 on CDC42 and CDC42F28L intrinsic GTP hydrolysis activity.

No significant difference was observed for CDC42 and CDC42F28L GTP hydrolysis activity in the presence of ZCL278 (figure 7). The apparent lack of influence of ZCL278 on CDC42/CDC42F28L intrinsic GTP/GDP exchange would suggest that ZCL278 does not bind at the nucleotide binding site, as it is already known, and therefore has no impact on the GTP/GDP exchange. It could also suggest the incapacity of ZCL278 to compete with the high affinity between GTP and CDC42 and CDC42F28L.

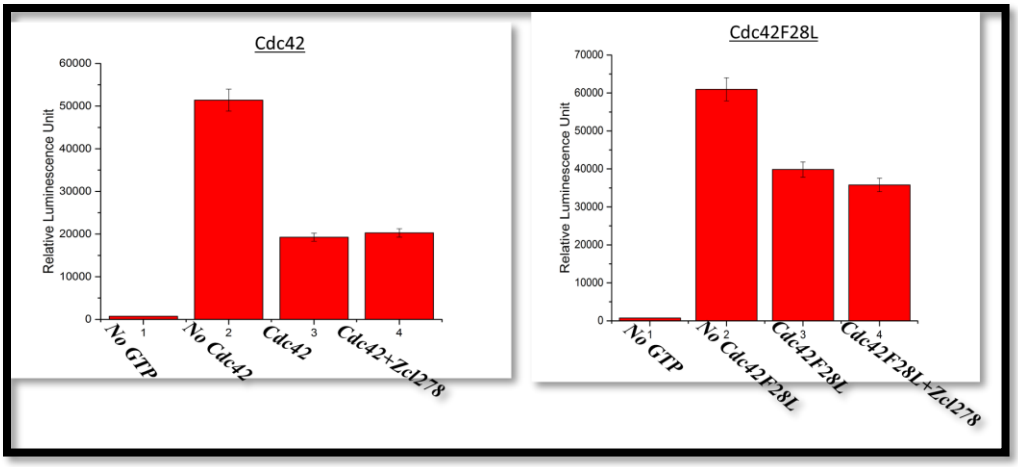

Figure 7. Intrinsic GTP hydrolysis of CDC42 and CDC42F28L in the presence of ZCL278. Bar 1 represents the relative luminescence intensity of a negative control (no GTP); bar 2 represents the positive control (GTP with no GTPase). Bars 3 and 4 represent the GTPase activity of CDC42 and CDC42F28L in the presence of ZCL278. No significant difference was observed in the GTPase activity of either protein in the presence of ZCL278. This figure is not comparing GTP hydrolysis activity of CDC42 and that of CDC42F28L which take place in different timescales.

Discussions and Future studies

Data collected from CD spectroscopy experiments show that ZCL278 changed the secondary structure of CDC42 and CDC42F28L with a prevalence of alpha helix into a random coil. The Conformational modification may stem from the fact that the H—bond interactions between Thr35 of CDC42 and the NH groups of ZCL278, which causes a destabilization of CDC42 structure since Thr35 residue has a crucial function in CDC42 conformational stability. Moreover, through hydrophobic interaction between CDC42 Ph28 and the bulky rings of ZCL278, ZCL278 can cause alterations in the 3D structure of CDC42 since, like Thr35, Phe28 contributes to the overall structural stability of CDC42.

Through its interaction with Phe28, Thr35, Thr43, Ala41, Val42, Gly47, ZCL278 has shielding action on the switch I, Thr35 in particular, which is structurally very crucial for CDC42 stability,

which led to an increased proteins stability as the DSC data show. There was not much difference in the Heat- and urea-induced denaturation based on CD spectroscopy for CDC42 in the presence and absence of ZCL278, except for a two-step urea-induced denaturation of CDC42F28L variant compared to a three-step urea-induced denaturation for CDC42. More investigative work like NMR and molecular dynamics simulation need to be explored which should unveil potential mechanisms behind those findings. GTPase assays consisting of CDC42 and CDC42F28L intrinsic nucleotide exchange did not display any noteworthy change in the nucleotide exchange pattern either in the absence or presence of ZCL278. This results most likely from the binding of ZCL278 to the switch I, which contributes very little in nucleotide exchange.

Conclusion

In summary, data from our fluorescence spectroscopy experiments show that ZCL278 bound to both CDC42 and CDC42F28L with micromolar affinity and induced a red shift in both proteins similarly. CD spectroscopy data showed that ZCL278 induced structural modifications in CDC42 and CDC42F28L upon binding, and DSC data showed that ZCL278 rendered CDC42 and CDC42F28L more thermally stable. Urea-induced unfolding based on CD spectroscopy suggests that in the presence of ZCL278 CDC42F28L unfolded in two stages. No noticeable change in the heat-induced unfolding of CDC42 and CDC42F28L based on CD spectroscopy was observed. Intrinsic GTPases activity assay showed that ZCL278 had no influence on the intrinsic GTPases activity of CDC42 and CDC42F28L.

More studies including NMR spectroscopy studies need to be conducted to unveil molecular details related to the way ZCL278 influenced some biophysical parameters of CDC42 and CDC42F28L and why it had no influence on some other biophysical parameters. These data will

be proved very useful in designing effective drugs with high and selective potency to combat

Ras-triggered cancers.

References

1) Segev, Nava. "GTPases in intracellular trafficking: an overview." Seminars in cell & developmental biology. Vol. 22. No. 1. Academic Press, 2011.

2) Conklin, B. R., et al. "Recombinant Gq alpha. Mutational activation and coupling to receptors and phospholipase C." Journal of Biological Chemistry 267.1 (1992): 31-34.

3) Hart, M. J., et al. "Nature354, 311− 314." (1991).

4) Horii, Y., et al. "A novel oncogene, ost, encodes a guanine nucleotide exchange factor that potentially links Rho and Rac signaling pathways." The EMBO Journal 13.20 (1994): 4776-4786.

5) Farr, Christine J., et al. "Analysis of RAS gene mutations in acute myeloid leukemia by polymerase chain reaction and oligonucleotide probes." Proceedings of the National Academy of Sciences 85.5 (1988): 1629-1633.

6) Shinohara, Nobuo, and Tomohiko Koyanagi. "Ras signal transduction in carcinogenesis and progression of bladder cancer: molecular target for treatment?." Urological research 30.5 (2002): 273-281.

7) Feltham, J. L., et al. "Biochemistry36, 8755− 8766." (1997).

8) Pai, Emil F., et al. "Structure of the guanine-nucleotide-binding domain of the Ha-ras oncogene product p21 in the triphosphate conformation." Nature 341.6239 (1989): 209-214.

9) Pai, Emil F., et al. "Refined crystal structure of the triphosphate conformation of H-ras p21 at 1.35 A resolution: implications for the mechanism of GTP hydrolysis." The EMBO journal 9.8 (1990): 2351-2359.

10) Schlichting, Ilme, et al. "Proton NMR studies of transforming and nontransforming H-ras p21 mutants." Biochemistry 29.2 (1990): 504-511.

11) Lin, R., et al. "A novel CDC42Hs mutant induces cellular transformation." Current Biology 7.10 (1997): 794-797.

13) John, Jacob, Matthias Frech, and Alfred Wittinghofer. "Biochemical properties of Ha-ras encoded p21 mutants and mechanism of the autophosphorylation reaction." Journal of Biological Chemistry 263.24 (1988): 11792-11799.

14) Friesland, Amy, et al. "Small molecule targeting CDC42–intersectin interaction disrupts Golgi organization and suppresses cell motility." Proceedings of the National Academy of Sciences 110.4 (2013): 1261-1266.

15) Chandrashekar, Reena, et al. "A switch I mutant of Cdc42 exhibits less conformational freedom." Biochemistry 50.28 (2011): 6196-6207.

CHAPTER FOUR: Conclusion and Future studies

Thus far we have discussed several strategic approaches by which Ras-induced cancers can be tackled. One of them consists of interfering with the direct interaction between Ras proteins and their downstream effectors. There are three ways that Ras-effector complexes can be targeted: 1) targeting Ras proteins to change their conformational structures so they can no longer bind the effectors effectively, 2) targeting the effectors to induce conformational changes so they can no longer interact with Ras proteins, 3) targeting the Ras-effector complex. In this project, we explored the strategy consisting of tackling the Ras protein directly and observing how Ras-effector interaction differs from Ras-SMI-effector interaction. This is however a very difficult approach given the tight binding between Ras proteins and their effectors, and the large protein-protein binding surface between the binding partners [1], which has presumably halted any progress in finding an effective small molecule inhibitor of Ras-effector complexes. This pattern is no different from that of our findings even though we are dealing with Rho proteins. Our binding experiments data suggest 1) a mere 5% decrease in CDC42-ACK binding in the presence of ZCL278 even at a 50 µM concentration (protein concentration used was 200 µM), 2) approximately a tenfold decrease in the binding affinity in the presence of 10 µM ZCL278 while the concentration of CDC42 was 18 µM. Other notable direct Ras protein small molecules inhibitors include ZCL367 which is more potent and more selective than ZCL278, CASIN which binds CDC42-GDP with a 300nM affinity, NSC23766 which binds Rac1, a very close relative of CDC42, with a 1.1 µM affinity, AZA1 and AZA197 which bind Rho proteins with low affinity [2]. Although very little data relative to direct inhibition of the Ras-effector interactions are available, through observation of the structure of those small molecule inhibitors (figure 1), it can be predicted that the same low binding pattern will be observed, meaning the small

molecules will not be able to compete with the effectors for binding to Ras proteins because of their high binding affinity, which has been the issue in finding potent Ras proteins SMIs.

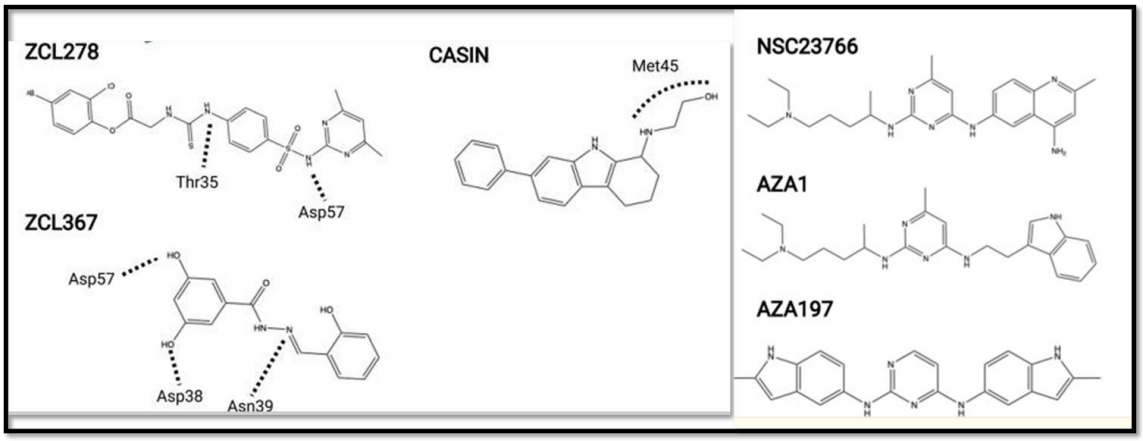

Figure 1. Comparison of the structures of several Rho protein SMIs. There is very close structural resemblance between the ZCLs as they both target Ras protein switch I region. CASIN targets a different region which is the nucleoside binding pocket, hence its dissimilar structural resemblance compared to that of the ZCLs. NCS23766 and the AZAs are both Rac1 direct inhibitors, which explains their similar structure and some structural difference from the structures of the other two groups. Rac1 is the closest relative of CDC42.

Another approach to tackle Ras-related cancers is to target Ras protein-effector interactions by designing a small molecule inhibitor that can directly bind to the effectors. Such strategy has led to the discovery of multiple inhibitors including rigosertib (figure 2) [3].

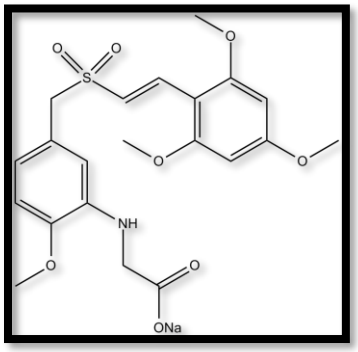

Figure 2. Structure of Rigosertib.

This drug is presently in phase III clinical trials treating myelodysplastic syndromes. Even though our goal was not to use ZCL278 to target the CRIB region of ACK, we nonetheless conducted binding experiments between ZCL278 and ACK which ended up showing no binding between these two, and by comparing the structure of ZCL278 to that of rigosertib, one can easily see there is immense structural differences (figure 3).

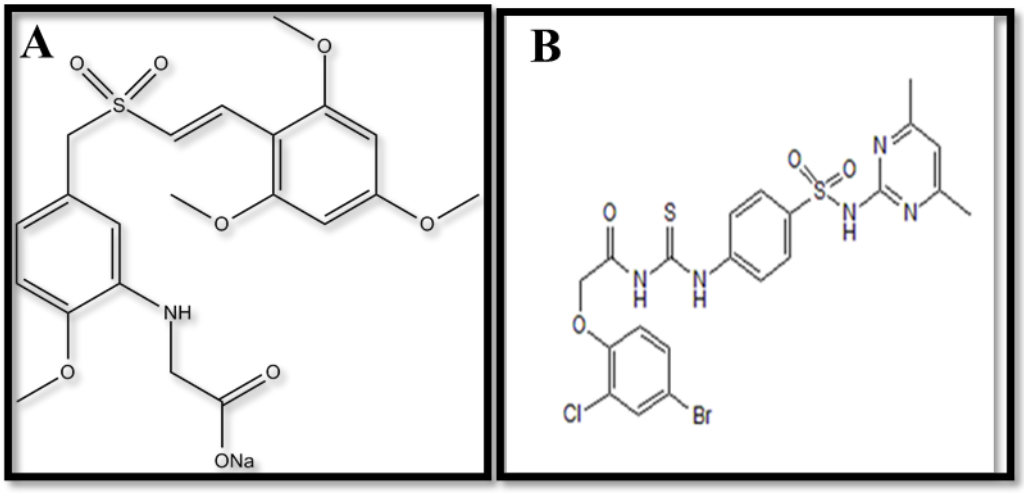

Figure 3. Structure of Rigosertib (A) and ZCL278 (B)

Other small compounds have been reported to target Ras-effector interactions by binding directly to the complex [4]. However, it was never proven that those compounds clearly bind to Ras proteins or to the effectors or to the complex [4]. Another approach in the search of Ras-related cancers therapy is to target the GTP/GDP cycle. Ras proteins shuttle between three distinct states: a GDP-bound state, a transient nucleotide-free state, and a GTP-bound state that binds directly to effector proteins. NMR spectroscopic studies showed that Ras displays distinct conformational structures upon GTP binding [5]. In the inactive state, the switch I region adopts an open conformation which facilitates nucleotide exchange and prevents effector binding. In the transient state, the switch I has a closed conformation over the nucleotide that facilitates effector

binding and GTP hydrolysis. When Ras proteins are in the nucleotide-free state, it has two conformations: a T conformation and a R conformation. In the T conformation, Ras is catalytically impotent whereas in the R conformation Ras is active [5].

It has been reported that inhibitors have been discovered which target different activation conformations of Ras proteins [6], which led to the discovery of small molecule inhibitors like Zn-cyclen [6], thiol-reactive compounds [7], and ARS-1620 (figure 4) [8].

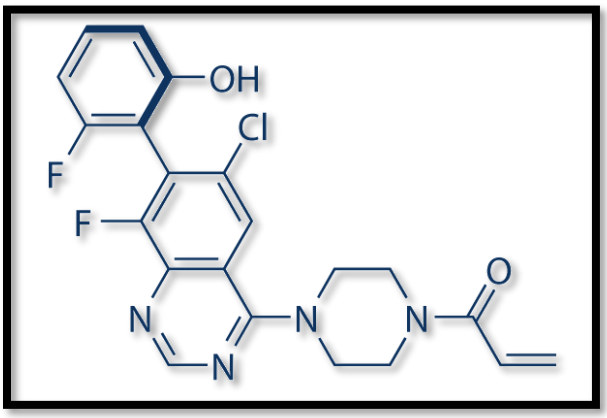

Figure 4. Structure of ARS-1620

Structurally, these small molecules are very different and more complex than ZCL278, which suggests why the intrinsic CDC42 GTP/GDP exchange assay that we conducted did not result in any change in the presence of ZCL278. Although this is a very difficult approach given the picomolar affinity with which Ras proteins bind to GTP and GDP, it has remained a very promising strategy in the search for Ras-related cancers treatment thanks to new structural details related to GTP-, and GDP- bound states of the Ras proteins that are revealed every year.

Direct binding interaction experiments between Ras superfamily of proteins and small molecules inhibitors have been attempted and there has been interesting progress so far.

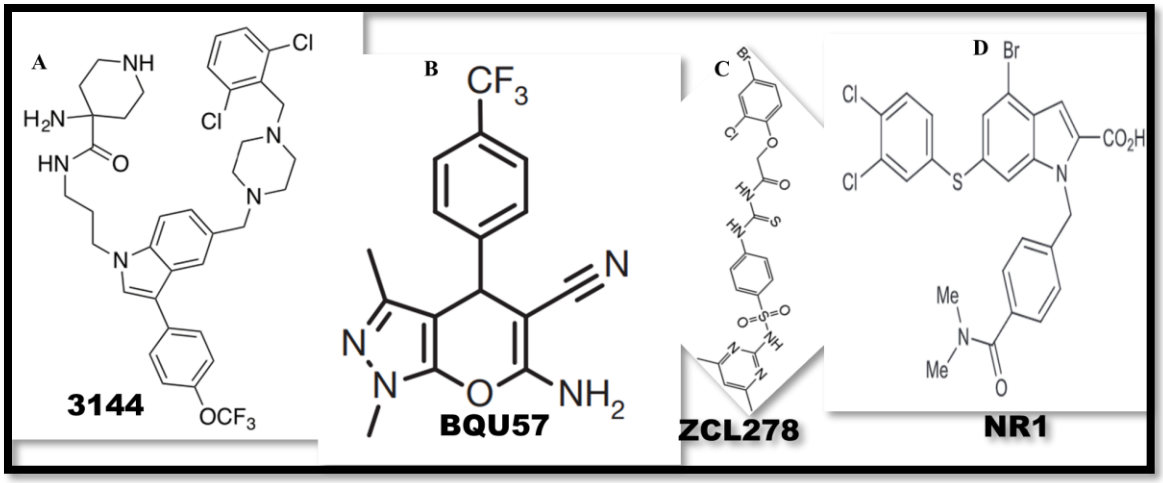

Figure 5. Structural comparison of four SMIs that bind to Ras superfamily of proteins. Although the GTP binding domain of Ras proteins are very conserved and all these SMIs target the switch I region, they are quite different in size, shape, potency, and selectivity.

A small molecule called compound 3144 (figure 5A) has been found to bind KRAS with an 18 μM binding affinity [9]. This compound showed metabolic stability in liver microsomes and anti-tumor activities in xenograft mouse cancer models [9]. BQU57 (figure 2B) is another small molecule inhibitor that has been shown to preferentially bind to Ral GTPase over Ras and Rho proteins with a binding affinity of 7.8 μM, thereby inhibiting the development of tumors [10]. Another small molecule inhibitor called NR1 (figure 2D) has been reported to directly bind to Rheb at the switch II region and selectively prevents mTORC1 activation [11]. Although these small molecule inhibitors are different in size and shape from ZCL278, they all however bind to Ras proteins with low binding affinity regardless of their binding site. This pattern remains similar for many Ras small molecule inhibitors, which highlights the challenge of finding a potent Ras inhibtors given the strong Ras-effector binding affinity.

ZCL278 was intended to be a CDC42-GEF complex formation inhibitor, more specifically, CDC42-ITSN1 complex inhibitor. However, it was used in our lab to influence the CDC42-ACK complex formation based on the GTPase-effector interaction inhibition strategic approach.

ZCL278 as our data show interfered with CDC42-ACK interaction and modified CDC42 and CDC42F28L structures upon binding, which suggests that ZCL278 must be investigated on more effector proteins like Wasp and PAK, and regulatory proteins like GEFs and GAPs to gain more detailed information related to its selectivity and potency.

The *in vitro* binding assay data show that ZCL278 caused about 5 % decrease in CDC42-ACK binding. This is not much but promising given CDC42 binds ACK with nanomolar affinity and ZCL278 with micromolar affinity. Moreover, given the concentration of CDC42 used in the experiment (200 μM) was much higher compared to that of the ZCL278 (50 μM), a 5% decrease in the binding is significant. ZCL278 caused approximately a tenfold increase in the Kd values in fluorescence spectroscopy (FS) and ITC experiments. For difficulties associated with protein-protein interactions and protein-protein-small molecule interactions such as small molecule's insolubility in protein solvent, protein aggregation in small molecule solvent, addition of disulfide bond breaker to optimize binding which in turn may interfere with the complex formation and even changes the solution pH, it was particularly hard to determine accurately the Kd values from FS and ITC. Nonetheless, the Kd values from our FS and ITC are close enough and are obtained from triplicate experiments which increase our data reliability. Consequently, improvements can be made on ZCL278 by making it more hydrophilic so that binding experiments involving ZCL278 can be done in an environment more like the cytoplasmic environment.

CDC42-ACK interaction is specifically determined by these seven residues, Ala41, Val42, Thr43, Gly47, Thr52, Phe56, and Leu174, with Ala41, Val42, Thr43, Gly47 in the switch I region [9]. ZCL278 was predicted to make H-bonds with Thr35, Asn39, and Ap57 of CDC42 (figure 6) and hydrophobic interactions with Val36 and Ph56. ZCL278 most likely interfered

with CDC42-ACK interaction by making H-bonds with Thr35 and Thr43 and hydrophobic bonds with Ala41, Val42, Gly47 in the switch I, which in turn impeded the CDC42-ACK binding interaction.

More investigative work on ZCL278 including NMR spectroscopy will be paramount in unveiling molecular details explaining ZCL278 interference with CDC42-ACK interactions.

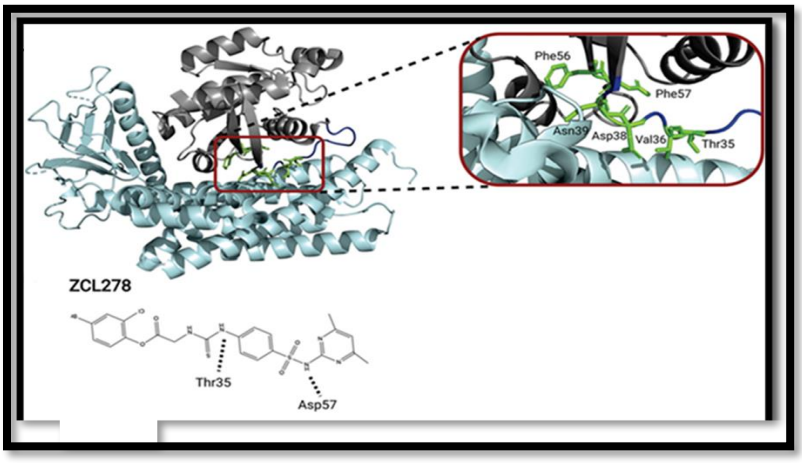

Figure 6. Prediction of intermolecular interactions between ZCL278 and CDC42.

CD spectroscopy data show that ZCL278 upon binding to CDC42 and CDC42F28L changed their secondary structure dominated by alpha helix into a random coil. The Conformational modification may be due mostly likely to the H—bond interactions between CDC42 Thr35 and ZCL278 since Thr35 plays a key role in CDC42 conformational stability. Moreover, through hydrophobic interaction between CDC42 Ph28 and ZCL278, ZCL278 can alter the 3D structure of CDC42 since Phe28 contribution to CDC42 overall structural stability is crucial and is done via hydrophobic interaction between CDC42 Phe28 and the guanine ring of the nucleotide. ZCL278 through its interaction with Phe28, Thr35, Thr43, Ala41, Val42, Gly47, shields the switch I particularly Thr35 which is structurally very important for CDC42 stability which made

it more thermally stable as indicated by the DSC data. Heat- and urea-induced denaturation based on CD spectroscopy did not show much ZCL278 influence on the CDC42 and CDC42F28L denaturation except for a two-step urea-induced denaturation of CDC42F28L compared to a three-step urea-induced denaturation for CDC42. More investigation needs to be conducted to shed more light on this.

CDC42 and CDC42F28L intrinsic nucleotide exchange did not show any difference in the absence or presence of ZCL278; this is due mostly to the fact that ZCL278 binds in the switch I which plays very little role in nucleotide exchange. Targeting signaling pathways modulated by Rho family members has received increasingly much attention in drug discovery since this approach has potential applications not only in cancer but also in neurodegenerative [10-12] and infectious diseases [13]. There exist several ways in which inhibition of pathways controlled by CDC42 has been approached, and one of them consists of targeting the CDC42-GEF interface, and the complex that has been targeted the most is the CDC42-Intersectin complex (ITSN1).

ITSN1 is a CDC42 specific GEF [14,15] which is among the several RhoGEFs that are frequently mutated in some types of cancer [16,19]. ZCL278 was docked into the ITSN1 binding groove of CDC42 and showed promising results in *in vitro* studies with its anticancer properties [20], and research conducted in our lab suggests that ZCL278 interfered with CDC42-ACK direct interaction. Nonetheless, ZCL278 suffers from several disadvantages: 1) ZCL278 was shown to be a partial agonist of CDC42 under certain circumstances; 2) ACK binds CDC42 with nanomolar affinity while ZCL278 binds CDC42 with micromolar affinity; consequently, the amount of ZCL278 sufficient to competitively prevent ACK or any other effector proteins from binding CDC42 will be probably too high and might even be cytotoxic; 3) ZCL278 is highly hydrophobic and very membrane-permeable, which is a poor feature for a drug candidate

according to the Lipinski's rules [18]. The discovery of the ZCL small molecule family has led to another SMI called ZCL367 which had more selectivity potency towards CDC42 (IC50 = 0.098 μM) than ZCL278 [2].

ZCL367 was shown to inhibit migration and proliferation in lung and prostate cancer driven by EGFR and Ras [2]. Moreover, a lung cancer xenograft mouse model treated with ZCL367 displayed decreased tumor growth [2]. Overall, the discovery of the ZCL SMI family has shed more light on the Ras-related drug discovery area, particularly on SMIs targeting CDC42. It has shown once again the structural and biological challenge faced in Ras-related proteins therapeutic research [2]. With more research being done in Ras-driven cancer drug discovery, we hope that the wealth of data being yearly published will contribute to the development of more potent SMIs with high selectivity for individual Ras family members which will help tackle cancer more drastically.

References

1) Orgován, Zoltán, and György M. Keserű. "Small molecule inhibitors of RAS proteins with oncogenic mutations." Cancer and Metastasis Reviews 39.4 (2020): 1107-1126.

2) Murphy, Natasha P., Helen R. Mott, and Darerca Owen. "Progress in the therapeutic inhibition of Cdc42 signalling." Biochemical Society Transactions 49.3 (2021): 1443-1456.

3) Athuluri-Divakar, Sai Krishna, et al. "A small molecule RAS-mimetic disrupts RAS association with effector proteins to block signaling." Cell 165.3 (2016): 643-655.

4) Kato-Stankiewicz, Juran, et al. "Inhibitors of Ras/Raf-1 interaction identified by two-hybrid screening revert Ras-dependent transformation phenotypes in human cancer cells." Proceedings of the National Academy of Sciences 99.22 (2002): 14398-14403.

5) Spoerner, Michael, et al. "Conformational states of Ras complexed with the GTP analogue GppNHp or GppCH2p: implications for the interaction with effector proteins." Biochemistry 44.6 (2005): 2225-2236.

6) Spoerner, Michael, et al. "A novel mechanism for the modulation of the Ras-effector interaction by small molecules." Biochemical and biophysical research communications 334.2 (2005): 709-713.

7) Ostrem, Jonathan M., et al. "K-Ras (G12C) inhibitors allosterically control GTP affinity and effector interactions." Nature 503.7477 (2013): 548-551.

8) Janes, Matthew R., et al. "Targeting KRAS mutant cancers with a covalent G12C-specific inhibitor." Cell 172.3 (2018): 578-589.

9) Welsch, Matthew E., et al. "Multivalent small-molecule pan-RAS inhibitors." Cell 168.5 (2017): 878-889.

10) Yan, Chao, et al. "Discovery and characterization of small molecules that target the GTPase Ral." Nature 515.7527 (2014): 443-447.

11) Mahoney, Sarah J., et al. "A small molecule inhibitor of Rheb selectively targets mTORC1 signaling." Nature communications 9.1 (2018): 1-12.

12) Elliot-Smith, Andrea E., et al. "Specificity determinants on CDC42 for binding its effector protein ACK." *Biochemistry* 44.37 (2005): 12373-12383.

13) Hickman, Suzanne E., and Joseph El Khoury. "TREM2 and the neuroimmunology of Alzheimer's disease." Biochemical pharmacology 88.4 (2014): 495-498.

14) Neumann, H., M. R. Kotter, and R. J. M. Franklin. "Debris clearance by microglia: an essential link between degeneration and regeneration." Brain 132.2 (2009): 288-295.

15) Hong, Lin, et al. "Characterization of a CDC42 protein inhibitor and its use as a molecular probe." Journal of Biological Chemistry 288.12 (2013): 8531-8543.

16) Snyder, Jason T., et al. "Structural basis for the selective activation of Rho GTPases by Dbl exchange factors." Nature structural biology 9.6 (2002): 468-475.

17) Smith, William J., et al. "A CDC42 mutant specifically activated by intersectin." Biochemistry 44.40 (2005): 13282-13290.

18) Russo, Angela, and John P. O'Bryan. "Intersectin 1 is required for neuroblastoma tumorigenesis." Oncogene 31.46 (2012): 4828-4834.

19) Jeganathan, Niranjan, et al. "Rac1-mediated cytoskeleton rearrangements induced by intersectin-1s deficiency promotes lung cancer cell proliferation, migration and metastasis." Molecular cancer 15.1 (2016): 1-17.

20) Friesland, Amy, et al. "Small molecule targeting CDC42–intersectin interaction disrupts Golgi organization and suppresses cell motility." Proceedings of the National Academy of Sciences 110.4 (2013): 1261-1266.

21) Benet, Leslie Z., et al. "BDDCS, the rule of 5 and drugability." Advanced drug delivery reviews 101 (2016): 89-98.

CHAPTER FIVE: Experimental issues and solutions

Purification of a 4-KDa peptide: Problems and solution

ACK1, also known as TNK2, is a 120 KDa protein consisting of multiple domains including an a SH3 and CRIB (CDC42-Rac interactive binding) domain [1]. This CRIB domain is commonly shared among the CDC42 downstream effectors including members of the PAK family, and the ACKs. CDC42 binds to the CRIB domain through the switch I and activates ACK1, which is one of the three known ways ACK1 is activated [2]. Human ACK1 is majorly different from bacterial ACK1 and purifying a biologically active ACK1 in E coli is simply impossible not only for the difficulties encountered when purifying such a big protein from E coli since an affinity tag would have to be attached to its end for purification purposes, but also for the complicated post-translational modifications ACK1 is subject to once synthesized [3]. A simple way to express and conveniently purify ACK1 is to express the domain of interest (CRIB domain) and attach a GTS tag at its end. Attaching a GST tag not only helps solubilize the peptide [4], but it allows purification using a GST affinity column as well. However, this GST tag can create potential problems when it is time to remove it with thrombin and to separate the cleaved ACK from the cleaved GST for downstream experiments. And this was where the most problems were encountered. The thrombin cleavage was always successful; however, it was always difficult to obtain ACK sample without some remaining GST, which would end up negatively influencing downstream experiments. And even if it was thought that ACK was present in the sample, seeing it on the gel was a headache since ACK is a very small peptide that is barely seen on a gel even at 15% acrylamide. After many attempts trying to separate the thrombin-cleaved products using affinity chromatography with the GST tag, it was decided that it could be easier to use size exclusion chromatography.

After many attempts, it was found that 1) the ACK is quickly diluted once loaded on the size exclusion column, 2) the presence of just one tryptophan residue in ACK caused the UV signal on the FPLC machine to be barely visible. Therefore, another method had to be found to successfully separate the cleaved GST tag from cleaved ACK and to see the 4-KDa ACK on the SDS-PAGE. That is when a method developed in Dr Kumar's and our lab significantly changed everything. This method is referred to as "heat treatment"; it consists of heating the thrombin-cleaved products up to 80 ℃ [3]. This high temperature causes the GST tag to aggregate while the peptide, due to its high thermal stability, stays in its soluble state. Then, the sample is centrifuged twice, the pellet is disposed of, and the supernatant which contains the soluble ACK is carefully removed and placed in a new tube. A tris-tricine gel is run to better visualize the peptide on the gel [4,5]. The heat treatment has proved itself to the best solution to purify ACK. Tris-tricin gel was also very helpful since I just could not see my peptide on the gel unless tris-tricine gel was used.

In vitro binding assay: Problems and solution

An in vitro binding assay is an affinity-based assay which consists of binding either two purified proteins together or two proteins from a crude protein lysate. The protein-protein complex is then purified via affinity-based purification since one of the proteins bound together carries an affinity tag. This technique is used to determine the binding interaction between two proteins. Our in vitro binding assay was done between purified GSTACK and CDC42. The issues that were encountered the most with that essay was that it was always hard to see CDC42 on the SDS-PAGE since not all the CDC42 bound to GSTACK, and the amount that bound was almost always diluted during elution.

This dilution issue was even worse when trying to use size exclusion chromatography to solve this problem since size exclusion chromatography, due to the size of the column (120 mL) is notorious for extreme sample dilution, leading to huge loss of protein. To circumvent this problem, it was decided to concentrate the eluates which contained the GSTACK-CDC42 complex, but due to long periods of centrifuging, much of the protein was lost, and nothing could be seen on the gel. Another technique that successfully helped was the "TCA precipitation", which consists of aggregating and precipitating the GSTACK-CDC42 complex using 10% Trichloroacetic acid (TCA) [6]. The precipitated complex is then washed with acetone to remove any remaining TCA and an appropriate amount of 8M urea and sample dye were added to the precipitate which was then heated for 1-3 minutes and was loaded on SDS-PAGE.

Isothermal titration calorimetry: Problems and solution

ITC is a technique used to quantitatively study biomolecular interactions. It consists of binding two biomolecules where one is placed in the syringe and the other one in the cell. It operates by measuring the amount of heat released or absorbed during the binding experiment. By measuring the heat transfer, ITC provides the thermodynamic profile of the molecular interaction consisting of the binding constants (Kd), reaction stoichiometry (n), enthalpy (ΔH) and entropy (ΔS) [7]. There can be multiple sources of heat in an ITC: one source of heat is dilution. Whenever there is dilution, there also heat that is released which comes from the ionic bonds destruction and H-bonds formation between the solute and water since most if not all ITC experiments are conducted in aqueous solutions. Another source of heat is the binding interaction between the two molecules being studied. This heat results from the intermolecular and atomic rearrangements due to conformation change that occurs upon binding of the molecules.

Based on these heat sources, the more molecules there are in the cell, the more heat sources there are, the harder it is going to be to calculate the actual heat coming from the binding of the two molecules of interest. Our ITC experiment involved ACK, CDC42, DMSO, TCEP, so there were four different molecules in our ITC cell that were individually generating dilution heat and possibly binding heat, and due to storage and cost reduction purposes, it was unsound to add the DMSO and the TCEP to the working 1-Liter dialysis buffer. Therefore, DMSO and TCEP were added by pipetting the appropriate amounts of DSMO and TCEP directly to the samples to prevent buffer mismatch and due to inaccuracy associated with pipetting small volumes, it was very difficult to match the buffer contents in the syringe and the cell, and that was the main source of our issues. Another potential source of issues was the fact that DMSO is very reactive and tends to react with buffer components, hence causes binding heat generation [8]. It was also reported that DMSO significantly decreased binding affinity when used in binding interaction [9]. Consistent with this finding, it was also reported in protein characterization studies, that the presence of DMSO, even when used at low percentage, caused drastic differences in proteins behaviors, and altered the binding affinities of binding interactions [10]. Another problem was the addition of TCEP, a disulfide bond breaker, to prevent dimerization induced by concentrated ACK. This component significantly changed the pH of buffers which led to a buffer mismatch. The way to circumvent this problem is to add the TCEP in the dialysis buffer, but this is not cost effective since TCEP is relatively expensive and to reach the appropriate concentration (5 mM) in a one-liter dialysis buffer, you have to add almost the entire content of the bottle. At best, we ended up having consistent data from our ITC experiments conducted in triplicate, but it is essential that these data be interpreted and understood in the context of these experiments and the potential sources of inaccuracy that were nevertheless necessary for this experiment to

successfully take place. Some of these issues were successfully dealt with; for instance, dilution heat inaccuracy was significantly minimized by dialyzing both proteins in the same buffer and by diluting ZCL278 stock solution (dissolved in 100% DMSO) in the working dialysis buffer. Nonetheless, the heat generated by the promiscuous binding of DMSO to the buffer salts and the heat from the binding of TCEP to ACK S-S bonds were hard to account for. This can be seen in the shift of the baseline of the binding isotherm. It was speculated that this baseline shift could be fixed by increasing the delay time between injections or at least the first six injections, but this never worked, but can still be tried in future studies in similar scenarios. It is always recommended to run control experiments by removing one or more components of the sample and do buffer subtraction. However, even when one of these components is removed, at least two more components remain in the sample, and are interacting with each other, which greatly increased the complication level of the experiment.

2D NMR spectroscopy: Problems and solution

NMR spectroscopy, in the field of protein NMR spectroscopy, is used to collect data about the structure and the dynamics of proteins. Protein NMR spectroscopy research involves multiple phases including expressing your protein of interest in a medium (M9) that will allow the incorporation of isotope in the protein of interest, in our case it was the N15 isotope, purifying the N15-labeled CDC42, preparing the sample in the correct buffer, collecting the data, and interpreting the data.

In a protein NMR spectroscopy, problems can arise at any of the phases of the experiment. The problems that were encountered in our case were mostly at the protein expression level and at the measurement level.

The yield of CDC42 in *E. coli* was always low in the M9 media since it is not as rich as other media like LB. So doing large scale production (up to 10 Liters) of the labeled protein was almost a necessity. It is important to note at least in my case, that the cells grew as fast as they did for expression done in LB media. The problem was mainly the low yield which basically impeded progress in the NMR work. But data were still gathered since we were using the Bruker 700 MHZ magnet probe (figure 1) Things worsened when the 700 MHZ-magnet probe could not be used any more, and since we started using a Bruker 500 MHZ, a concentration of 0.3-0.5 mM was needed. This was a major problem since it was never easy to get to such concentration. And the few times, we got it, the protein ended up aggregating (figure 2). I speculated that one way to solve this problem was to express massively enough protein so a desired concentration can be achieved in enough volume to prevent aggregation. This plan was being pursued until the 500 MHZ magnet broke down. So whether or not it would have worked remains unknown.

References

1) Prieto-Echagüe, Victoria, and W. Todd Miller. "Regulation of ack-family nonreceptor tyrosine kinases." Journal of signal transduction 2011 (2011)

2) Manser, Edward, et al. "A non-receptor tyrosine kinase that inhibits the GTPase activity of p21CDC42." Nature 363.6427 (1993): 364-367.

3) Macek, Boris, et al. "Protein post-translational modifications in bacteria." Nature Reviews Microbiology 17.11 (2019): 651-664.

4) Schägger, Hermann. "Tricine–sds-page." Nature protocols 1.1 (2006): 16-22.

5) Koontz, Laura. "TCA precipitation." Methods in enzymology. Vol. 541. Academic Press, 2014. 3-10.

6) Schäfer, Frank, et al. "Purification of GST-tagged proteins." Methods in Enzymology. Vol. 559. Academic Press, 2015. 127-139.

7) Velázquez-Campoy, Adrián, et al. "Isothermal titration calorimetry." Current protocols in cell biology 23.1 (2004): 17-8.

8) Brayton, C. Flagg. "Dimethyl sulfoxide (DMSO): a review." The Cornell Veterinarian 76.1 (1986): 61-90.

9) Cubrilovic, Dragana, and Renato Zenobi. "Influence of dimehylsulfoxide on protein–ligand binding affinities." Analytical chemistry 85.5 (2013): 2724-2730.

10) Tjernberg, Agneta, et al. "DMSO-related effects in protein characterization." Journal of biomolecular screening 11.2 (2006): 131-137.