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ABSTRACT

KRAS mutations account globally for about one million deaths per year, but there is as yet no approved drug against KRAS. K-Ras proteins are organized into di-/oligomeric nanoscale signaling complexes, known as nanoclusters on the plasma membrane. In this thesis, I describe two targeting approaches to indirectly inhibit the oncogenic activity of K-Ras. First, we designed inhibitors that block the interaction between the trafficking chaperone PDE6D and K-Ras, thus blocking membrane localization of K-Ras. Secondly, we showed how the major folding chaperone Hsp90 and its co-chaperone Cdc37 affect K-Ras signaling. We then went on to develop and test novel inhibitors against the interface between Hsp90 and Cdc37.

K-Ras requires several post-translational modifications, such as farnesylation, for proper organization on the plasma membrane. Interaction of farnesylated K-Ras with the trafficking chaperone PDE6D results in its effective solubilization and translocation in the cytosol. Available PDE6D inhibitors are easily dislodged from PDE6D through the GTP-ARL2 unloading mechanism, consequently limiting the overall cellular potency of these inhibitors. To address this problem, we improved the compound design to withstand the ejection mechanism and added a cell penetration group to increase the bioavailability.

The second part connects to our previous finding, showing that the inhibition of Hsp90/Cdc37 by conglobatin A selectively blocks the activity of K-Ras but not H-Ras, and inhibits stemness activity of cancer cells. However, the exact mechanism by which Hsp90/Cdc37 inhibition blocks the activity of K-Ras remained unclear. Here, we discovered that the inhibition of Hsp90 downregulates the K-Ras nanocluster modulator galectin-3 by inhibiting HIF-1α. Decreased expression levels of galectin-3 and Hsp90-clients B- and C-Raf jointly contributed to selectively disrupt K-Ras membrane nanoclusters, thus blocking the oncogenic activity of K-Ras.

In order to identify novel Hsp90/Cdc37 inhibitors, we first selected compounds from a computational screening and then validated their ability to interrupt the Hsp90/Cdc37 complex in a split *Renilla* luciferase assay. Finally, we discovered two compounds that inhibited the Hsp90/Cdc37 complex formation. By assessing these compounds in cellular assays, we confirmed their K-Ras membrane organization disrupting activity and the impairment of the signaling pathways downstream of K-Ras. Furthermore, these compounds also decreased K-Ras dependent cancer cell proliferation in 2D monolayers, 3D spheroid growth and microtumor formation. Taken together, the work of this thesis has led to the development and characterization of novel small molecule inhibitors that indirectly target K-Ras. Our findings may form the basis for the development of future therapeutic agents against K-Ras dependent human diseases.

KEYWORDS: drug-development, K-Ras, Hsp90, Cdc37, nanoclustering, PDE6D

ABSTRAKT (Swedish Abstract)

KRAS-mutationer står globalt för cirka en miljon dödsfall per år, men det finns ännu inget godkänt läkemedel mot KRAS. K-Ras-proteiner är organiserade i di- / oligomera signalkomplex i nanoskala, kända som nanokluster på plasmamembranet. I denna avhandling beskriver jag två olika metoder för att indirekt hämma den onkogena aktiviteten hos K-Ras. Först designade vi hämmare som blockerar interaktionen mellan den intracellulära transport chaperonen PDE6D och K-Ras, för att blockera membranlokalisering av K-Ras. Som andra visade vi hur den huvudsakliga proteinvecknings chaperonen Hsp90 och dess co-chaperon Cdc37 påverkar K-Ras-signalering. Vi fortsatte sedan med att utveckla och testa nya hämmare mot samspelmellan Hsp90 och Cdc37.

För korrekt organisering på plasmamembranen kräver K-Ras flera post-translationell modifieringar, såsom farnesylering. Interaktionen mellan den farnesylerade K-Ras och chaperonen PDE6D resulterar i effektiv nedbrytning av K-Ras och dess translokering i cytosolen. Tillgängliga PDE6D-hämmare lösgörs lätt från PDE6D genom en GTP-ARL2- avstötningsmekanism. Detta har till följd att den totala cellulära effekten hos dessa hämmare begränsas. För att lösa detta problem förbättrade vi föreningens (hämmarens) design, så att den bättre kan motstå avstötnings-mekanismen. Dessutom ökade vi dess biologiska tillgänglighet genom att lägga till en kemisk grupp, som ökar hämmarens förmåga att penetrera cellmembranen.

Den andra delen ansluter till våra tidigare forskningsresultat som visar att blockering av Hsp90/Ccd37 med conglobatin A blockerar selektivt K-Ras aktivitet utan att påverka H-Ras aktivitet, samtidigt som den inhiberar stamcellsförmågan i cancerceller. Den exakta mekanismen genom vilken Hsp90 / Cdc37-inhibering blockerar K-Ras aktivitet förblev emellertid oklar. I denna avhandling upptäckte vi att hämningen av Hsp90 nedreglerar galectin-3, en nanoklustermodulator för K-Ras, genom att hämma HIF-lα. Minskade expressionsnivåer av galectin-3 och Hsp90-klienterna B- och C-Raf bidrog gemensamt till att selektivt hindra K-Ras från att bilda membran-nanokluster, som i sin tur ledde till att den onkogena aktiviteten hos K-Ras blockerades.

För att identifiera nya Hsp90 / Cdc37-hämmare valde vi först ut föreningar genom datorscreening och validerade sedan deras förmåga att avbryta Hsp90 / Cdc37-komplexet med hälp av ett delat *Renilla*-luciferastest. Vi upptäckte slutligen två föreningar som hämmade komplexbildningen av Hsp90 / Cdc37. Genom att utvärdera dessa föreningar i cellulära analyser, bekräftade vi deras förmåga att hindra K-Ras-membranorganisation och försämring av signalvägarna nedströms från K-Ras. Dessa föreningar minskade dessutom också K-Ras-beroende tillväxt av cancerceller i 2D-monolager, 3D-sfäroid tillväxt och mikrotumörbildning. Sammantaget har arbetet i denna avhandling lett till utveckling och karakterisering av nya småmolekylära hämmare som indirekt riktar sig mot K-Ras. Våra resultat kan ligga till grund för utvecklingen av framtida terapeutiska medel mot K-Ras-beroende sjukdomar.

NYCKELORD: läkemedelsutveckling, K-Ras, Hsp90, Cdc37, nanokluster, PDE6D

LIST OF THE ORIGINAL PUBLICATIONS

This thesis is based on publications, which are referred to in the text by their Roman numerals:

I. **Siddiqui FA**, Alam C, Rosenqvist P, Ora M, A Sabt, Manoharan GB, Bindu L, Okutachi S, Catillon M, Taylor T, Abdelhafez OM, Lonnberg H, Stephen AG, Papageorgio AC, Virta P, Abankwa D. PDE6D inhibitors with a new design principle selectively block K-Ras activity in cancer cells. ACS-Omega. 2019 Dec 23; doi: 10.1021/acsomega.9b03639; PMID: 31956834.

II. **Siddiqui FA***, Parkkola H*, Manoharan GM and Abankwa D. Medium-Throughput Detection of Hsp90/Cdc37 Protein-Protein Interaction Inhibitors Using a Split Renilla Luciferase-Based Assay. SLAS Discovery. 2019 Oct 29; doi 10.1177/247255219884033; PMID: 31662027. (*contributed equally)

III. **Siddiqui FA**, Parkkola H, Vukic V, Oetken-Lindholm C, Jaiswal A, Kiriazis A, Aittokallio T, Salminen T and Abankwa D. Novel small molecule Hsp90/Cdc37 interface inhibitors indirectly target K-Ras signaling (Communications Biology, in revision)

Other publications not included in the thesis

IV. Ahearn IM, Court HR, **Siddiqui FA**, Abankwa D, Philip MR. NRAS is Unique Among RAS Proteins in Requiring ICMT for Trafficking to the Plasma Membrane. 2020 (submitted)

V. Parkkola H, **Siddiqui FA**, Oetken-Lindholm C and Abankwa D. FLIM-FRET analysis of Ras nanoclustering and membrane-anchorage. Ras Activity and singnaling: Methods and Protocols; 2020. (submitted)

ABBREVIATIONS

ARL	Arf like Arl protein
ARF	ADP ribosylation factors
ATCC	American type culture collection
ATP	Adenosine triphosphate
CaM	Calmodulin
CAMKii	Ca^{2+}/calmodulin dependent protein kinase II
Cdc37	Cell division cycle 37
CDK4	Cyclin-dependent kinase 4
CEA	Carcinoembryonic antigen
CK2	Protein kinase CK2
CRL	C-terminal *Renilla* luciferase
CSCs	Cancer stem cells
DMSO	Dimethylsulfoxide
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FTI	Farnesyl transferase inhibitor
FLIM	Fluorescence lifetime imagining
Fzd8	Frizzled-8
FRET	Fluorescence resonance energy transfer
GAP	GTPase activating proteins
GEF	Guanine nucleotide exchange factor
Gal1	Galectin-1
Gal3	Galectin-3
GGT1	Geranylgeranyl transferase I
GDP	Guanosine diphosphate
GRB2	Growth factor receptor-bound protein 2
GRP94	Glucose-regulated protein 94
GTP	Guanosine 5' triphosphate
GTPase	Guanosine triphosphatase
HEK	Human Embryonic Kidney
HRAS	Harvey rat sarcoma

Hsp70	Heat shock protein 70
Hsp90	Heat shock protein 90
HTS	High Throughput Screening
HVR	Hypervariable region
KRAS	Kirsten rat sarcoma
MAPK	Mitogen Activated Protein Kinase
mTOR	Mechanistic target of rapamycin
NF-AT	Nuclear factor of activated T cells
NRL	N-terminal *Renilla* luciferase
PADC	Pancreatic ductal adenocarcinoma
PBR	Polybasic region
PBS	Phosphate buffered saline
PDE6D	Non catalytic δ-subunit of phosphodiesterase 6, prenyl binding protein
PI3K	Phosphoinositide 3-kinase
PKD1	Protein kinase D1
PPI	Protein-protein interface
PROTACs	Proteolysis targeting chimeras
Raf	Rat-1fibroblast kinase
Ras	Rat sarcoma (protein)
Rab	Ras-like proteins in brain
Rap	Ras related protein
RBD	Ras-binding domain
Rheb	Ras homologue enriched in brain
Rho	Ras homologus
RT	Room temperature
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulfate
S6K	Ribosomal S6-kinase
siRNA	Small interfering ribonucleic acid
SOS1	Son of sevenless 1
TNF	Tumor necrosis factor
TRAP1	TNF receptor associated protein 1 (Heat shock protein 75)
WHO	world health organization

1. INTRODUCTION

According to the World Health Organization, cancer is the second leading cause of death worldwide. In 2018 approximately 9.6 million deaths were caused by cancer. Cancer arises from the transformation of normal cells to tumor cells by mutations in one or more gene (Futreal et al., 2004). Mutations in *RAS* genes are associated with approximately 3.4 million new cancer cases each year worldwide. The formation of cancer is a multi-stage process from a pre-malignant lesion to a malignant cancer (Curtius et al., 2017). Both normal and cancer cells need to coordinate multiple networks of intracellular processes for their cellular growth and proliferation. These processes include a vast array of protein-protein interactions needed to elicit changes in cell metabolism, multiple signaling cascades, trafficking of biomolecules and plasma membrane organization of proteins. Deregulation of any of these processes may contribute to cancerogenesis.

Mutations in *RAS* genes accounts for approximately 19% of the total cancer burden (Prior et al., 2020). The *KRAS* gene is the most frequently mutated oncogene in cancer. K-Ras farnesylation is important for its proper plasma membrane anchorage. Farnesylated K-Ras interacts with the trafficking chaperone PDE6D, which facilitates K-Ras translocation to the plasma membrane. On the plasma membrane, a number of proteins, including the nanocluster scaffold galectin-3, regulate K-Ras activity.

My thesis work identified two different types of compounds that block the trafficking or membrane organization of oncogenic K-Ras. First, we have developed and characterized novel compounds that specifically block the interaction between K-Ras and trafficking chaperone PDE6D. In the second part, we described how inhibition of the protein interface of Hsp90/Cdc37 indirectly inhibited K-Ras nanoclustering and then identified novel Hsp90/Cdc37 interface inhibitors.

2. REVIEW OF THE LITERATURE

2.1 Overview of structure and function of Ras proteins

The Ras proteins belong to a large superfamily of small GTP-binding, low molecular weight proteins (21 to 25 kDa) (Bos, 1997). The Ras superfamily is further divided into several subfamilies depending on the degree of sequence conservation, like the Rho family, the ARF family and the Rab family (Colicelli, 2004; Takai et al., 2001). They all share a number of common features that are exemplified by Ras proteins.

Ras proteins cycle between a GTP-bound active state and a GDP-bound inactive state (Herrmann et al., 1996). This cycle of wild type Ras is regulated by guanine-nucleotide exchange factors (RasGEFs) and GTPase activating proteins (GAP) (Boon et al.) (Figure 1). Inactive Ras proteins turn to the GTP-bound state through the action of GEFs (Bos et al., 2007; Cherfils and Zeghouf, 2013). Active GTP-bound Ras interacts with downstream effectors to stimulate downstream cytoplasmic signaling cascades. Inactivation of Ras is mediated by the hydrolysis of GTP, which is accelerated by the GAP proteins (Herrmann et al., 1996). Ras proteins play crucial roles in the regulation of cell differentiation, cell growth and proliferation (Simanshu et al., 2017).

The H-Ras, K-Ras and N-Ras isoforms are 188 to 189 amino acids long and share 82 to 90% sequence identity. The G domain comprises 164 amino acids where the first 80 amino acids are identical, while the next 85 or 84 residues show 95% sequence identity. H-Ras, K-Ras and N-Ras isoforms differ in their carboxy-terminus, also known as the hypervariable region (HVR), which is only 25 amino acids long (Cox and Der, 2010). The *KRAS* gene encodes two splice variants K-Ras4A and K-Ras4B resulting from alternative splicing at the 4th exon. If *KRAS* is mutated, both K-Ras4A and K-Ras4B variants become oncogenic (Tsai et al., 2015). Both splice variants have different C-termini, thus K-Ras4A and K-Ras4B have different properties for membrane anchoring.

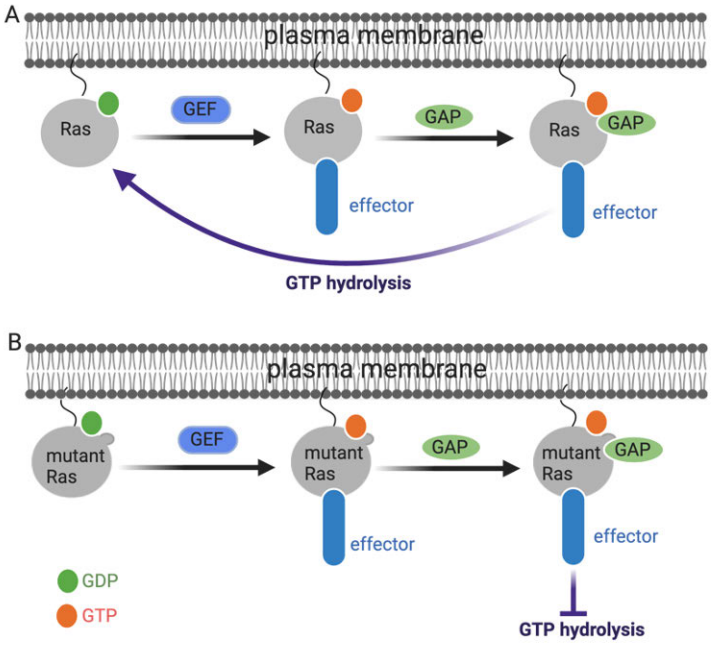


Figure 1. Ras GTPase cycle: (A) GDP bound Ras is in an inactive state, a GEF facilitates the release of GDP to be replace by GTP, conformational changes occur in Ras in GTP bound, allowing binding of downstream effectors. GAP proteins assist the stimulation GTP hydrolysis by Ras becomes inactive in its GDP-bound state (B). However, GAPs cannot assist in the hydrolysis on mutant Ras, consequently, mutant Ras remains predominantly in the GTP-bound active state

For proper anchorage on the plasma membrane, Ras proteins require several post-translational modifications on the hypervariable region (HVR) of the C-terminus CAAX box (Figure 2). The CAAX box contains, C for cysteine; A for aliphatic amino acid like alanine, isoleucine, leucine, proline and valine and X for any amino acid (Willumsen et al., 1984a; Willumsen et al., 1984b). Farnesyl-transferase adds a 15-carbon farnesyl group to the Ras CAAX box cysteine if X is any amino acid other than leucine (Hancock et al., 1989; Jackson et al., 1990). However, if X is leucine, or isoleucine or phenylalanine, Ras is instead geranylgeranyled by the geranylgeranyl-transferase. Methionine allows prenylation by both prenyl-transferases (farnesyl-transferase and geranylgeranyl-transferase), which is the case for N- and K-Ras4A/B but not H-Ras. On the endoplasmic reticulum, three terminal amino acids on the Ras are removed by the Ras-converting enzyme 1 (RCE1). After that, isoprenylcysteine carboxymethyltransferase (ICMT) adds the methyl group on the C-terminal prenylcysteine, which neutralizes the negative charge of the C-terminus

(Freije et al., 1999; Gutierrez et al., 1989; Otto et al., 1999). Moreover, Ras proteins, (H-Ras, N-Ras and K-Ras4A) but not K-Ras4B, are palmitoylated on one or two cysteines in the HVR by a palmitoyl-transferase. These are the most important post-translational modifications for Ras, since they make Ras lipophilic, thus facilitating its association with the plasma membrane, which is necessary for Ras activity.

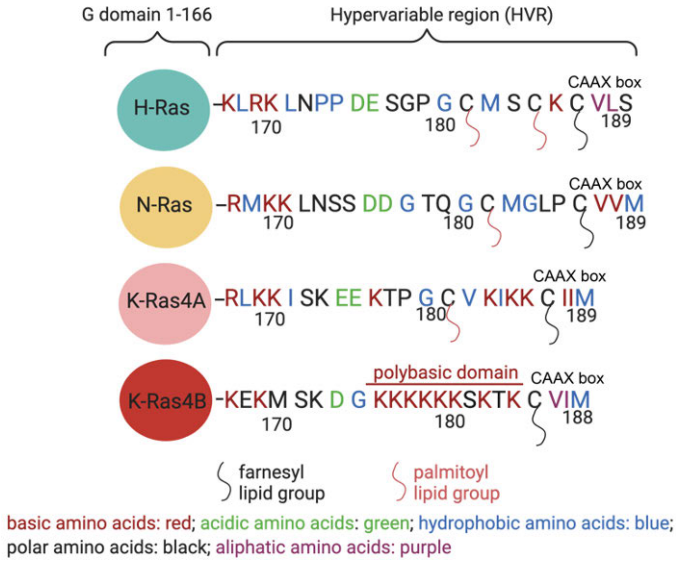


Figure 2. Ras protein sequence identity: All oncogenic Ras isoforms consist of 188 or 189 amino acids and show 82 to 90% sequence identity. Amino acids 1 to 166 form the G-domain that contains the nucleotide-binding region and the effector-binding region. Amino acids 167 to 189 represent the hypervariable region. This region has several post-translational modifications like farnesylation and palmitoylation.

H-Ras, K-Ras and N-Ras are oncoproteins as their genes frequently acquire activating mutations in various cancers (Downward, 2003). *KRAS* mutations are important oncogenic drivers in pancreatic, lung and colon cancers (Prior et al., 2012; Waters and Der, 2018). Missense mutations in the *KRAS* gene constitutively activate the protein, making K-Ras the most common single mutationally activated oncoprotein (Downward, 2003; Hancock, 2003).

Missense mutations in Ras at residues 12, 13 or 61 render it GAP insensitive, resulting in permanent GTP-bound active state of Ras, which in turn leads to persistent stimulus-independent activation of downstream effector signaling

(Baines et al., 2011; Prior et al.). The binding affinity of GTP to Ras is in the picomolar range, which is quite remarkable, considering the usual nucleotide binding affinity range from nanomolar to micromolar (μM) (Agola et al., 2012). As a result of this, attempts to target the GTP-pocket have not been successful (Baines et al., 2011).

2.1.1 Upstream regulation of Ras activity

Receptor tyrosine kinases (RTKs), including epidermal growth factor receptors (EGFRs), activate Ras proteins upon stimulation by extracellular growth factors. Binding of growth factors to the extracellular region of RTKs drive its dimerization or oligomerization and thereby its activation (Schlessinger, 2000). In response to growth factors, one receptor in the dimer or oligomer then phosphorylates one or more of the nearby receptors. Phosphorylated RTKs then serve as a site for the recruitment of signaling adaptors such as GRB2 or Shc (Ullrich and Schlessinger, 1990) (Figure 3). Ultimately, this mechanism leads to the recruitment of Ras GEFs to the plasma membrane. GEFs subsequently stimulate the release of GDP from Ras and allow the binding of GTP to Ras. Thus, in response to activated RTKs, Ras transmits signals to its downstream effectors to promote cell proliferation, growth and cell metabolism (Hancock, 2003). Therefore, deregulation of Ras signaling not only due to its mutations, but also by aberrant upstream regulation is associated with cancer hallmarks (Hanahan and Weinberg, 2011).

Previous studies suggested that Ras-GRF1, a nucleotide exchange factor, activates only H-Ras but not K-Ras4B (hereafter K-Ras) and N-Ras. Whereas Ras-GRF2 is responsible for the activation of K-Ras and N-Ras but not H-Ras (Clyde-Smith et al., 2000; Matallanas et al., 2003). Son of Sevenless (SOS) encodes the best studied GEF that is able to exchange GDP to GTP in all three Ras isoforms (Jaumot et al., 2002). GTP-loaded activated Ras binds to its downstream effectors, including Raf. All isoforms of Ras recruit or activate the effectors but their potency towards particular effectors appear different (Hancock, 2003; Plowman and Hancock, 2005). For example, K-Ras is more potent than H-Ras towards C-Raf in the induction of kinase activity and recruitment to the plasma membrane (Hood et al., 2019; Yan et al., 1998). H-Ras on the other side showed more potency in the activation of PI3K (Yan et al., 1998).

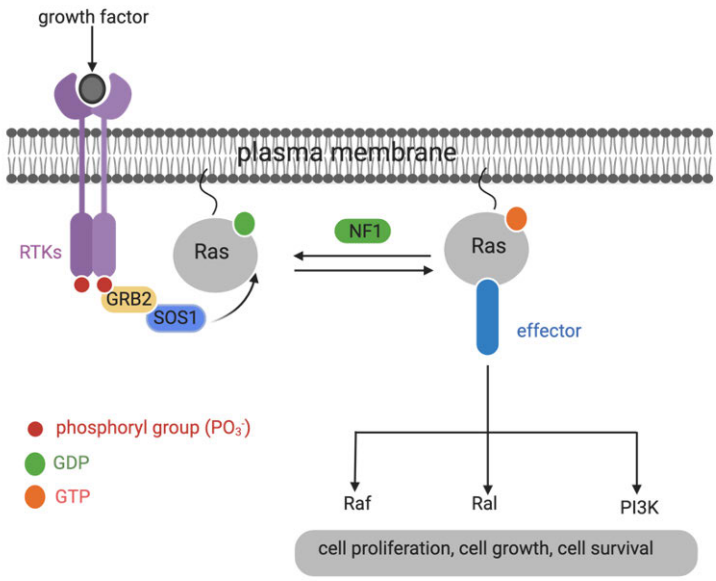


Figure 3: Regulation of Ras activation: Upon stimulation of RTKs by growth factors, phosphorylated RTKs act as the docking site for several proteins that contain the SH2 domain, thus recruit the adaptor protein like GRB2. GRB2 further recruits SOS1 (GEF) onto the plasma membrane where SOS1 catalyzes the exchange of Ras-GDP to Ras-GTP. GTP-bound activated Ras recruits its downstream effectors to transmit signals.

Likewise, K-Ras more potently activates Rac-dependent signaling than H-Ras (Cerione and Zheng, 1996; Hyvonen et al., 1995). Furthermore, H-Ras and K-Ras trigger more stimulation of NF-κB signaling compared to N-Ras (Millan et al., 2003). In contrast, only mutant K-Ras, but not H-Ras, potently drives cancer stem cell (CSC) properties in colorectal cancer (Moon et al., 2014; Wang et al., 2015a). K-Ras mutation induces the expression of CD44, CD133, CD166, which are markers of CSC (Moon et al., 2014).

In normal cells, GTP bound Ras needs to be deactivated to control Ras signaling networks. The Ras protein has a very weak intrinsic GTPase activity. Therefore, GAP proteins are required for the hydrolysis of GTP. Once GAP proteins convert Ras-GTP to Ras-GDP, inactive GDP-bound Ras cannot recruit its downstream effectors anymore. GAP proteins have a Ras GAP domain and a catalytic domain for GTP hydrolysis. The human genome contains 14 predicted RAS GAP genes (Bernards and Settleman, 2005). All these proteins contain a Ras GAP domain and have Ras GAP activity, except for IQGAP. IQGAP contains a Ras GAP domain but one amino acid is different in the catalytic domain. Consequently, they do not

exhibit the GTP-hydrolysis function (Brill et al., 1996; Weissbach et al., 1994). Neurofibromin, a Ras GAP encoded by the *NF1* gene, is the most widely studied in cancer (Cichowski and Jacks, 2001). Other Ras GAPs such as DAB2IP, RASAL2, RASA1, RASAL1 are also known tumor suppressors capable of inhibiting the oncogenic activity of Ras (Maertens and Cichowski, 2014).

KRAS mutation on codon 13 appear most frequently in gastrointestinal cancers (Segelov et al., 2016). The structure of K-Ras protein, harboring G13 mutations differs from other mutated proteins. K-RasG13 mutated proteins have lower GAP-stimulated GTPase activity than wild type protein but have higher GAP stimulated GTPase activity than other K-Ras mutated proteins, like codon 12 and 61 variants (Hunter et al., 2015). Therefore, K-RasG13 mutations may be less oncogenic than K-RasG12 and K-RasG61 mutations. However, Rabara et al. reported that the K-RasG13 mutation is associated with co-mutations in the Ras pathway in gastric cancer (Rabara et al., 2019). They also reported that approximately 12% of K-RasG13 mutated cancer patients and 50% of K-RasG13 mutated cancer cell lines also harbor NF1 co-mutations. This NF1 mutation present exclusively on K-RasG13 but not K-RasG12 and K-RasG61, thus K-RasG13 cells may benefit from the NF1 mutation to fully activate Ras signaling pathway. Other Ras GAPs such as DAB2IP, RASAL2, RASA1, RASAL1 are known tumor suppressors capable of inhibiting the oncogenic activity of Ras (Maertens and Cichowski, 2014).

Mutations in Ras keep Ras in GTP-bound active state, since GAPs cannot stimulate GTP hydrolysis on the mutant Ras (Scheffzek et al., 1997). Consequently, GTP loaded Ras continuously transmits downstream signals for cancer cell proliferation, growth and survival. Scheffzek et al. proposed that a mutation in Ras at position 12 or 13 creates a steric hindrance that prevents GAP's arginine finger from entering the GTPase site on Ras consequently blocking the hydrolysis of GTP (Scheffzek et al., 1997). Also, mutations at position 61 impair the GTP hydrolysis since glutamine 61 is part of the GTP hydrolysis mechanism. Some other mutations like A146, T158A, R164Q and K176Q permit the dissociation of GDP, which results in the eventual accumulation of Ras-GTP without the action of a GEF (Simanshu et al., 2017).

2.1.2 Downstream effectors of Ras

Raf kinases (A-Raf, B-Raf, C-Raf) and PI3K are the most studied downstream effectors of Ras. These kinases are major components of the MAPK and

PI3K/mTOR pathways, respectively (Desideri et al., 2015; Downward, 2003). Activated Raf kinases phosphorylate MEK1 at position Ser218, Ser222 and MEK2 at Ser222, Ser226. The conserved KDD motif of MEK1, Lys97, Asp190, Asp208 (K97, D190, D208) and MEK2; Lys101, Asp194 and Asp212 (K101, D194, D212) in the kinase domain, promote MEK1/2 dual specificity threonine/tyrosine protein kinase catalytic activity, which triggers the activation of ERK1/2 (Wu and Park, 2015). Phosphorylated ERK kinases translocate into the nucleus where they activate multiple of transcriptional genes like FOS, MYC, ELKI, ETS1 and DUSP1 (Ünal et al., 2017) (Figure 4).

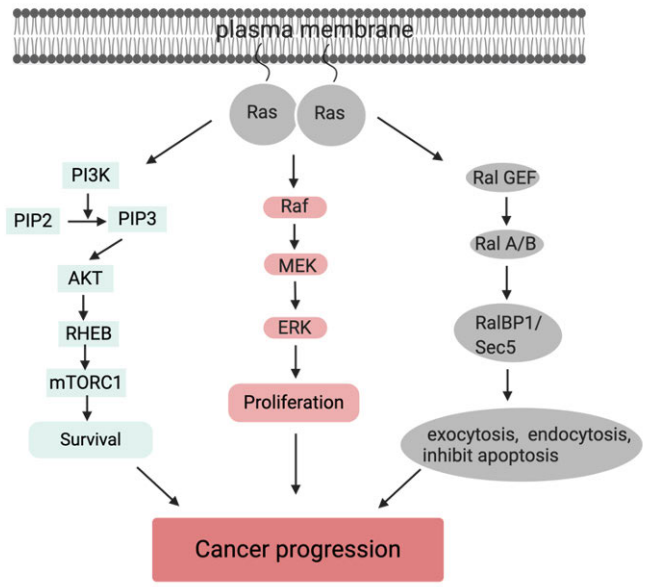


Figure 4: Signaling pathways downstream of Ras: Ras triggers MAPK, PI3K/mTORC1 and RAL-GEF pathways to stimulate cell proliferation, cell survival, exocytosis, actin organization, endocytosis and inhibition of apoptosis.

By contrast, PI3K phosphorylates the lipid phosphoinositide (4,5) bisphosphate (PIP2) and converts it to phosphoinositide (3,4,5) triphosphate (PIP3). PIP3 on the membrane recruits many other proteins like the kinases AKT and PDK1 (Vanhaesebroeck et al., 2001). All three Ras isoforms activate PI3Ks/AKT. GRB2 binds and activates SOS, which then activates Ras and this activates p110 (a subunit of PI3K) (Figure 3). AKT is a major component that controls extensive cellular processes like inhibition of autophagy, cell metabolism, cell survival and

cell proliferation (Andjelkovic et al., 1997; Castellano and Downward, 2011; Hers et al., 2011).

Like Raf and PI3K, Ral proteins are also key downstream effectors of the Ras. The Ras-Ral pathway regulates vesicular transport and actin organization (Gentry et al., 2014). RalA and RalB are two isoforms of Ral, that interact with various downstream effectors. Both isoforms are associated with distinct roles in regulation of vesicular trafficking, tumor formation and metastasis. Ras proteins interact with the RalGEF, thus activating Ral (Goldfinger and Michael, 2017). In addition, RalGEF interacts with various other downstream partners such as RalBP1, Sec5 and Filamin. Interaction with these proteins regulate the gene expression for regulation of exocytosis, actin organization, endocytosis and inhibition of apoptosis (Rodriguez-Viciana and McCormick, 2005).

2.1.3 Ras nanocluster signaling complexes

In the plasma membrane Ras proteins are tightly clustered in non-random proteo-lipid complexes, called nanocluster (Murakoshi et al., 2004; Zhou and Hancock, 2015); Prior et al., 2003). Approximately 40% of Ras proteins are arranged as immobile nanoclusters, while the remaining 60% Ras proteins are arrayed as monomer on the plasma membrane.(Plowman et al., 2005; Prior et al., 2003; Tian et al., 2010). Ras nanocluster contain around 6 to 8 proteins with a radius of approximately 9 nm. The lifetime of Ras nanoclusters is in the range of 0.1-1 s indicating that Ras nanoclusters are continuously forming and disassembling (Plowman et al., 2005; Zhou and Hancock, 2015). High expression of galectin-3 (Gal3) increases the K-RasG12V nanocluster formation and signaling output (Shalom-Feuerstein et al., 2008). Similarly, dimers of galectin-1 (Gal1) stabilize H-RasG12V nanocluster on the plasma membrane (Figure 5) (Blazevits et al., 2016). Ras nanoclusters recruit and activate downstream effectors such as Raf and therefore act as signaling platforms whereas Ras monomers fail to activate Raf and signaling networks (Murakoshi et al., 2004; Plowman et al., 2008; Shalom-Feuerstein et al., 2008; Tian et al., 2007). Moreover, MAPK quantal output from the plasma membrane increases when the Ras nanoclustering increases (Cho and Hancock, 2013).

K-Ras nanoclustering is regulated by endogenous proteins. These nanocluster scaffold modulators include Gal3, caveolae and nucleophosmin (Ariotti et al., 2014; Inder et al., 2009; Shalom-Feuerstein et al., 2008).

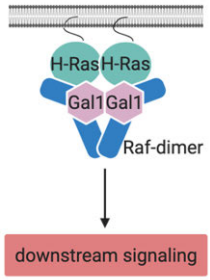


Figure 5: H-Ras nanocluster model: Galectin-1 dimer further stabilizes Raf dimer bound to H-Ras to form a stable nanocluster.

Interestingly, Gal1 regulates only H-Ras but not K-Ras nanoclustering (Belanis et al., 2008; Guzman et al., 2014). Galectins are small molecular weight proteins formed by two antiparallel β sheets, that bind specifically to β-galactosidase carbohydrates through highly conserved carbohydrate binding sites (Lopez-Lucendo et al., 2004). Recently, our group proposed an H-Ras nanoclustering model where Gal1 functions as a nanocluster scaffold modulator for H-RasG12V (Blazevits et al., 2016) (Figure 5). Our group showed that Gal1 does not directly bind to H-Ras but instead binds to the Ras binding domain (RBD) of effectors like Raf. Therefore, we concluded that dimers of Gal1 form bridges between two Raf dimers, resulting in a stable H-Ras nanocluster (Blazevits et al., 2016). This model endorses the previous findings that Raf-dimer inducing drugs increase Ras nanoclustering (Cho et al., 2012a). In analogy to the H-Ras nanoclustering model and previous findings, it is plausible to assume that Gal3 units form similar bridges between Raf proteins stabilize K-Ras nanoclusters.

Gal3 is the member of β-galactosidase-binding lectins, encoded by the LGALS3 gene, and characterized by a carbohydrate-recognition domain (Argueso and Panjwani, 2011). Gal3 is translocate in the cytoplasm, interacts with cell survival associated proteins, including B-cell lymphoma (Bcl-2) (Dong et al., 2018). In the nucleus Gal3 interacts with heterogenous ribonucleoproteins (hnRNPs) and thus plays roles in the mRNA splicing of several genes that are associated with metabolic processes, translational and transcriptional regulation (Fritsch et al., 2016). Overexpression of Gal3 also impairs RasGAP activity (Elad-Sfadia et al., 2004). Gal3 contributes to inhibition of apoptosis and promotes metastasis and neoplastic transformations (Dumic et al., 2006; Nakahara et al., 2005).

Recent data suggest that the dimerization/ oligomerization of K-Ras is required for the activation of downstream signaling (Chen et al., 2016). Super-resolution microscopy shows that the concentration of mutant K-RasG12D is directly proportional to formation of dimers or oligomers. That is, the amount of dimers and oligomers increase as the concentration of mutant K-RasG12D increases in the cell. Moreover, MAPK is not activated until the concentration of K-RasG12D reaches a threshold value needed for mutant K-RasG12D dimerization (Nan et al., 2015).

Higher expression of K-Ras4B than K-Ras4A suppressed growth and MAPK signaling in cultured cells (Zhang et al., 2001). Similarly, wild-type K-Ras suppresses the oncogenic activity of mutant K-Ras in hematopoietic cells but wild-type N-Ras does not show this function (Kong et al., 2016). In analogy to these experiments, Ambrogio et al., showed that Cre-induced tumors grow faster in animals model expressing mutant K-Ras when the wildtype allele is deleted (Ambrogio et al., 2018). The mechanism of growth inhibition by wildtype K-Ras might be due to the association of distinct signaling properties of wildtype and mutant K-Ras or may due to inactivating hetero-dimerization of wildtype and mutant K-Ras. However, NMR studies demonstrated that GTP bound K-Ras does not form stable heterodimers with GDP bound K-Ras (Lee et al., 2020; Muratcioglu et al., 2015).

In conclusion, MAPK output from the plasma membrane increases when Ras nanoclustering increases (Cho and Hancock, 2013) Moreover, Disruption in Ras nanoclustering impairs cellular signaling. Given that Ras proteins have been considered a major drug target and Ras proteins activate their signaling network only when present in nanoclusters (Plowman et al., 2008; Tian et al., 2007), inhibition of Ras nanocluster formation may offer an alternative strategy to target oncogenic K-Ras.

2.1.4 Deregulated Ras in human diseases

Mutations leading to hyperactivation of Ras have been associated with cancer progression, RASopathies and neurological abnormalities including neurocognitive impairment, hypotonia and macrocephaly (Rauen, 2013; Simanshu et al., 2017) . Collectively, pathologies due to mutations in RAS account for millions of deaths per year worldwide (Simanshu et al., 2017).

RASopathies comprise a group of syndromes, caused by the hyperactivation of Ras signaling due to germline mutations in the regulators of Ras, or Ras itself (Rauen, 2013). Neurofibromatosis type 1 (NF1) is the most common syndrome caused by hyperactivation of Ras (Cawthon et al., 1990). General phenotypic hallmarks of RASopathies include dark pigmented skin, hypertension, cardiovascular diseases, brain malformations, deficiency in vitamin D and skeletal abnormalities (Boyd et al., 2010). NF1 is caused by mutation in NF1 gene. Neurofibromin which is RasGAP is encoded by NF1 gene. A loss of function of neurofibromin results in hyperactivation of Ras (Brems et al., 2009; Rauen, 2013). People with NFI have a higher risk of developing malignancies. The most common RASopathies are listed below (Table 1).

Table 1: List of RASopathies and their cause

RASopathy	**cause**	**characteristics**
Neurofibromatosis type 1	Mutation in NF1 gene, which encodes RasGAP	Cardiovascular diseases, brain malformations (neural tumors), deficiency in vitamin D, bone deformities and skeleton abnormalities
Legius syndrome	mutations in SPRED1 gene	Developmental delay, learning disabilities
Noonan Syndrome	Mutation in PTPN11, SOS1, RAF1, KRAS	Broad forehead, congenital cardiac defect, reduced growth, bleeding disorder
Capillary Malformation–Arteriovenous Malformation Syndrome	Mutation in RASA1, which encodes inactivation of RasGAP (p120-RasGAP)	Deficiency in capillaries formation, malformations look like multiple small, round, pink or red spots on the skin
Costello syndrome	Mutation in HRAS	Developmental delay, unusually flexible joints, and distinctive facial features including a large mouth with full lips, heart problems

2.2 K-Ras in oncogenesis

Many oncogenes have been discovered that drive the progression and maintenance of cancer (Vicente-Duenas et al., 2013). Genetic alterations may lead to constitutive activation of oncogenes, triggering their increased expression and/or oncogenic signaling, as is the case with K-Ras, which becomes oncogenic after acquiring mutations (Baines et al., 2011). Mutated *KRAS* is known to be associated with poorer prognosis for patients in many types of cancer (Buscail et al., 2020; Christensen et al., 2020). In light of this, the study of *KRAS* mutations or deregulated K-Ras pathways may provide important clues that could be exploited for therapeutic strategies.

2.2.1 Role of oncogenic KRAS in cancer

Wild type K-Ras protein usually promotes cell cycle progression, cell proliferation and cell growth. However, if wild type K-Ras increases to an abnormal level, it can also induce apoptosis, cell growth arrest and replicative senescence (Jancik et al., 2010). Mutations in *KRAS* confer the oncogenic properties and are thus involved in the development of many types of human cancers (Kranenburg, 2005; McCoy et al., 1983). It has been observed that the wild type *KRAS* allele was absent in both human and mouse tumors. This indicates that loss of the wild type allele may promote the transformation of normal cells by one copy of mutant *KRAS* (Hegi et al., 1994). In fibroblasts, mutated K-Ras protein increased the expression of metalloproteinase 2 (MMP2) in the matrix and enhanced the invasion of cancer cells (Liao et al., 2003). A major role for MMPs in cancer progression is the degradation of extracellular matrix (ECM), thus allowing the cancer cells to migrate out from the primary tumor to form metastasis.

Cancer cells adapt to the microenvironment by metabolic reprograming to maintain cell survival and to meet the needs of macromolecule synthesis (Kerr et al., 2016; Robey et al., 2015). In hypoxic conditions, normal cells utilize the process of aerobic glycolysis rather than oxidative phosphorylation (OXPHOS) to provide energy. However, even during normoxia, cancer cells utilize aerobic glycolysis instead of OXPHOS to provide energy and macromolecules synthesis. This process is called the Warburg effect (Gasparre et al., 2013; Olivares and Vasseur, 2016; Warburg, 1956). Oncogenic K-Ras adjusts the metabolic changes in pancreatic ductal adenocarcinoma (PADC). *KRAS* mutations increase the

expression levels of glycolytic enzymes like hexokinase 1 and 2, glucose transporters, lactose dehydrogenase, and pyruvate kinase M2 (Iqbal et al., 2013; Seton-Rogers, 2015; Ying et al., 2012). Mutant K-Ras also alters the synthesis of macromolecules like nucleic acids, proteins and fatty acids by increasing the glucose uptake and redirecting the glycolysis to the pentose phosphate pathway (Sousa and Kimmelman, 2014; Ying et al., 2012).

2.2.2 *KRAS* mutations in human cancer

Table 2: Frequency for *KRAS* mutations in human cancer

Cancer type	*KRAS* (%)	*HRAS* (%)	*NRAS* (%)	Total (%)
Pancreas	88	0	0.4	88.4
Colon	50	0.5	4.2	≈ 55
Small intestine	26	0	1	27
Biliary duct	26	0	2	28
Lungs	36	0.8	1.6	38.4
Endorine	14.9	14.2	43.9	73
Gynaecological	37.1	4.4	5.3	46.8
Urinary tract	5	10	1	16
Skin (melanoma)	1.6	1.2	17	19.8

KRAS is the most frequently mutated among RAS isoforms and comprises 75% of total RAS mutations in cancer (Prior et al., 2020). Pancreatic cancer is on the top with approximately 88% of *KRAS* mutations. Further, *KRAS* mutations are present in 50 % of colon cancer and about 32% in lung cancers. *KRAS* mutations are also found in many other cancers like biliary tract, small intestine, breast cancer, liver cancer, cervical cancer, bladder cancer and myeloid cancer (Prior et

al., 2020; Pylayeva-Gupta et al., 2011; Singh et al., 2015) **(Table 2)** (Prior et al., 2020).

2.2.3 Oncogenic K-Ras induces dedifferentiation and drives cancer cell stemness

Breast cancer is the major cause of cancer-related deaths among women. Breast and colorectal cancer stem cells (CSCs) have been shown to be the driving force behind primary tumorigenesis, metastasis and drug resistance (Fearon and Wicha, 2014; Kreso and Dick, 2014). CSCs are present at low numbers but play a crucial role in metastasis and relapse of cancer (Pang et al., 2010; Schepers et al., 2012). Mutant K-Ras protein is known to drive CSC formation (Mani et al., 2008; Nash et al., 2010) (Figure 6). Using culture model of retinoic acid (RA)-induced stem cell differentiation to endoderm, only mutant (G12V) K-Ras but not H-Ras or N-Ras, initiate or promoted tumor like CSC properties (Quinlan et al., 2008; Wang et al., 2015a). CSCs have been identified for many types of cancer and are considered critical targets for cancer treatment.

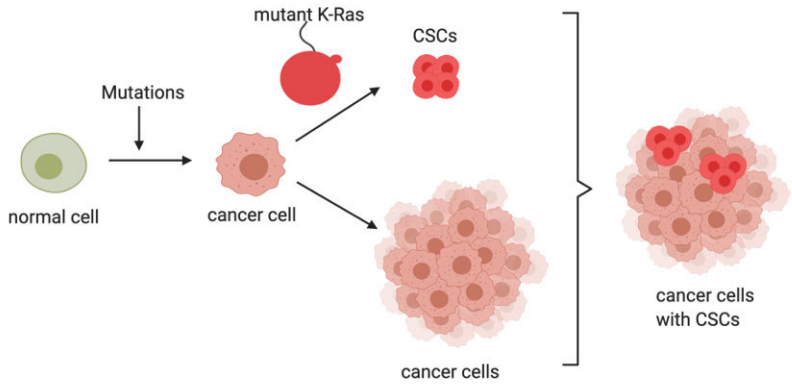


Figure 6: Oncogenic K-Ras promotes CSC: Mutations in normal cells transform them to cancer cells. Mutation in K-Ras protein promote the CSC stemness properties in these transformed cells.

The oncogenic ability of K-Ras, which facilitates the initiation and progression of cancer, depends on many factors (Li et al., 2018a). The first main factor is the expression level of K-Ras and its relative activation state. The activation state of K-Ras depends upon the capacity of K-Ras to remain GTP bound. Both mutant

K-Ras and EGFR signaling in coordination mediate the development of pancreatic cancer (Ardito et al., 2012; Navas et al., 2012). Mutant K-Ras initiates the transcription of the EGFR and its ligands like EGF by the activation of NF-κB signaling through serine/threonine kinase, protein kinase D1 (PKD1) (Liou et al., 2016).

Our group showed that previously established CSCs drug target specifically K-Ras but not H-Ras signaling, suggesting that K-Ras drives the stemness in CSCs (Najumudeen et al., 2016). Moreover, in colon cancer, oncogenic K-Ras enhances the stem cell like properties that originate in adenoma to carcinoma through MAPK signaling pathway (Le Rolle et al., 2016; Moon et al., 2014). In pancreatic cancer oncogenic K-Ras is responsible for enhancement of CSC properties through the PI3K/AKT/mTOR pathways (Matsubara et al., 2013).

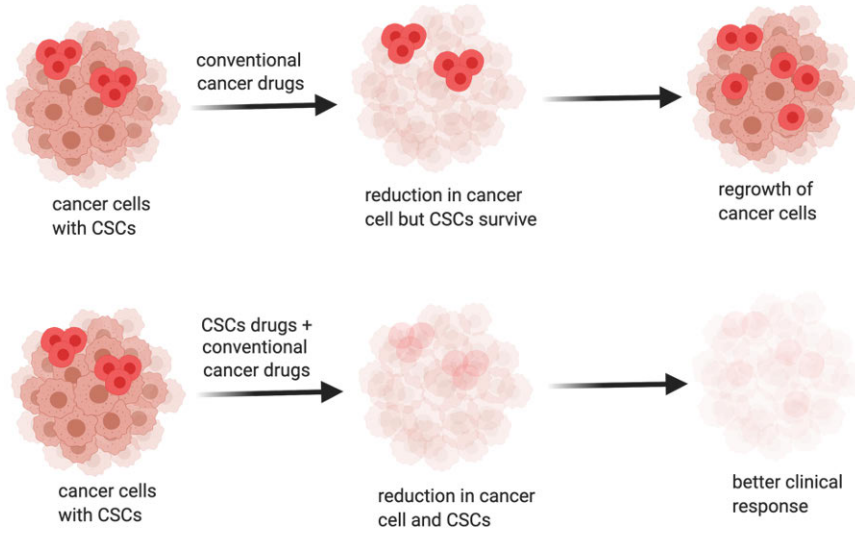


Figure 7: Hypothetical effect of CSC drugs on cancer treatment. Conventional cancer drugs target bulk cancer cells without affecting CSCs. Surviving CSCs reform the tumor (top). Conventional cancer drugs combined with CSCs drugs may give a better response in curing cancer (bottom)

Deficiency of K-Ras leads to embryonic lethality in mice, thus it may be concluded that K-Ras performs specific functions in embryonic development that cannot be compensated by H-Ras or N-Ras (Johnson et al., 1997; Plowman et al., 2003). Moreover, tumors driven by K-Ras are more metastatic than tumors driven by H-

Ras (Wong et al., 2013). Taken together, these data suggest that K-Ras plays a unique role in tumor biology and embryonic development. K-Ras and H-Ras have different capacities to initiate tumors, for example K-Ras has the ability to suppress Frizzled 8 (Fzd8) mediated non-canonical Wnt/Ca^{2+} signaling (Wang et al., 2015a). Fzd8 is a member of Frizzled receptor family which could activate canonical or non-canonical Wnt pathway. NF-AT and CAMKii are two main downstream effectors of non-canonical Wnt/Ca^{2+} signaling (Krebs, 1998; Rao et al., 1997). NF-AT is the transcription factor, which regulates multiple genes like TNF that are associated with tumor progression.

Farnesylation of K-Ras and the polybasic region at the C-terminal HVR region facilitate the interaction with the plasma membrane. K-Ras plasma membrane interaction alters when K-Ras is phosphorylated at Ser 181 by protein kinase C (PKC) in the polybasic region (Bivona et al., 2006). Furthermore, phosphorylated K-Ras not able to form K-Ras nanoclusters, thus resulting in a reduction in C-Raf plasma membrane recruitment (Plowman et al., 2008).

In the absence of growth factors calmodulin (CaM) binds to K-Ras at the positively charged HVR and prevents the association with the plasma membrane (Sperlich et al., 2016). Binding of CaM to K-Ras also inhibits its phosphorylation at Ser181 because this interaction sterically blocks the PKC phosphorylation site (Villalonga et al., 2001; Villalonga et al., 2002). As a result, non-phosphorylated K-Ras fails to activate the PI3K/AKT pathway, but in the presence of growth factors, CaM is unable to bind to K-Ras, therefore, allowing PKC to phosphorylates and activate K-Ras (Alvarez-Moya et al., 2010). Thus, under unfavorable conditions (less growth factors) K-Ras phosphorylation is required to drive cell proliferation and growth.

Therefore, binding of K-Ras to CaM is inhibited by phosphorylation of Ser181 by PKC. Moreover, enhanced phosphorylation of K-Ras by the activation of PKC inhibits the interaction of K-Ras and CaM, thus activating the non-canonical Wnt/Ca^{2+} pathway, which impairs K-Ras mediated stemness and tumorigenicity (Wang et al., 2015a). Therefore, blocking the K-Ras and CaM interaction may provide an additional approach to selectivity target K-Ras.

2.2.4 Therapeutic strategies for targeting oncogenic K-Ras

For more than three decades, K-Ras was considered undruggable. However, in recent years researchers developed an approach that directly targets the *RAS*

mutant tumors. Besides, many other than direct strategies are explored to target oncogenic K-Ras. For instance, targeting K-Ras maturation during post-translational modifications, disruption of K-Ras nanoclustering, inhibiting the interaction of K-Ras and its downstream effectors, inhibiting intracellular localization or trafficking of K-Ras and inhibition of the interaction of K-Ras and GEFs.

Direct targeting of oncogenic KRAS

The Shokat group developed the first small molecules that covalently bind to K-RasG12C in the GDP bound state. Inhibitor binding is dependent on the mutant cysteine, and hence does not bind to wild type K-Ras (Ostrem et al., 2013). These compounds block the nucleotide exchange and inhibit the K-RasG12C association with Raf (Ostrem et al., 2013). Several other, direct K-RasG12C covalent inhibitors like AMG-510 (Sotorasib) and ARS-1620 were also developed (Canon et al., 2019; Janes et al., 2018). Recently, AMG-510 showed promising anticancer activity in particular in lung and less in colorectal cancer patients harboring K-RasG12C mutation (Hong et al., 2020).

Inhibiting Ras activation

Another interesting strategy for Ras inhibition is to target protein-protein interactions. Some studies have reported that disrupting the interaction of Ras/SOS1 by small molecules inhibit the SOS1 mediated nucleotide exchange and thereby inhibit the activation of Ras (Maurer et al., 2012). They discovered that DCIA bind to Ras near nucleotide binding region, thus inhibits SOS mediated nucleotide exchange, inhibiting the activation of Ras (Maurer et al., 2012). Thus, compounds that block interaction of Ras and GEF could keep the GDP-bound inactive Ras state.

Targeting downstream effectors of Ras

Further, disruption of downstream effector proteins like Raf could be prevent the hyperactivation of Ras signaling. Most efforts were focused on finding Raf and MEK inhibitors. To date, more than 30 different mutations in the BRAF gene have been identified. The most common is the substitution of valine (V) by glutamate (E) at codon 600 (B-Raf^{V600E}), activating MEK and ERK signaling, thus promoting cell survival and proliferation (Spathis et al., 2019). The frequency of BRAF mutations varies widely; they are present in approximately 80% melanomas, 5% in colorectal carcinoma, and 1% to 3% of lung carcinomas (Davies et al., 2002). Mutations in BRAF play a prominent role in many cancers. and several B-Raf

inhibitors have been developed. Vemurafenib (PLX4032) and dabrafenib are clinically approved B-Raf inhibitors. Vemurafenib inhibits all three isoforms of Raf, effectively impairing MEK/ERK signaling in B-Raf^{V600E} melanoma cell lines (Bollag et al., 2010; Poulikakos et al., 2011). In 2015 Peng et al. reported that the B-Raf inhibitor, LY3009120, inhibits all isoforms of Raf, including B-Raf and C-Raf homodimers and heterodimers (Peng et al., 2015). Moreover, LY3009120 does not induce the paradoxical activation of MEK/ERK pathway in BRAF wild type cells. This property was missing in the previous Raf inhibitor, vemurafenib (Peng et al., 2015). Examples of other Raf inhibitors are sorafenib (also a multi kinase inhibitor) and PLX-4720, which inhibit the kinase activity of B-Raf and C-Raf, but induce the dimerization of Raf (hetero or homodimer) (Adnane et al., 2006). Another inhibitor, PLX7904, inhibits both B and C-Raf without inducing their dimerization, thus does not induce paradoxical activation of MEK/ERK pathway. PLX7904 also showed more potency towards mutant B-Raf^{V600E} (Zhang et al., 2015). Trametinib and binimetinib are inhibitors of MEK1/MEK2, thus inhibit the MAPK pathway, which are in clinical trials (geneca.fi).

Inhibiting Ras post-translational modifications and trafficking of K-Ras

Localization of RAS to the inner plasma membrane is necessary for the subsequent activation of RAS and further signal transduction. For proper anchoring on the plasma membrane, K-Ras requires several post-translational lipid modifications in the HVR. These include farnesylation by FT of the cysteine residue on CAAX C-terminus, followed by the cleavage of AAX and finally methylation of the cysteine residue. For other Ras proteins (excluding K-Ras4B), an additional step of palmitoylation of cysteine is necessary (Hancock et al., 1991; Hancock et al., 1990). The lipid modification of Ras, which renders it hydrophobic, takes place in the endoplasmic reticulum. The hydrophobic farnesylated Ras is then trafficked to the plasma membrane.

Blocking this lipid modification can potentially bar the recruitment of Ras to the plasma membrane. The most common inhibitors designed for this purpose have been farnesyltransferae inhibitors like tipifarnib, lonafarnib, L-778123 and FTI-277 (Wang et al., 2017). Unfortunately, inhibition of farnesyl-transferase failed to target mutated K-Ras and N-Ras, because both of these proteins are alternatively geranylgeranylated by geranylgeranyltransferase, which maintains their plasma membrane localization (Lerner et al., 1997). Accordingly, combined inhibition of farnesyl-transferase and geranylgeranyltransferase reduced K-Ras driven lung tumorigenesis in mice but also led to high toxicity (Liu et al., 2010).

Phosphodiesterase delta (PDE6D) of PDE6 solubilizes Ras for the translocation through the cytoplasm. To block the association of farnesylated K-Ras with PDE6D, a new compound, deltarasin, designed, which successfully decreases cancer cell survival in vitro and in mouse experiments. A detailed description of this novel target follows in next chapter.

Targeting K-Ras nanoclustering

Evidence from previous studies suggests that nanoclustering of K-Ras plays a critical role in K-Ras signaling output (Cho and Hancock, 2013) Inhibition of K-Ras nanoclustering is an innovative strategy to block oncogenic activity of K-Ras. Our group showed that some CSC drugs block K-Ras signaling via inhibition of K-Ras nanocluster formation on the plasma membrane (Najumudeen et al., 2016). Our group identified ophiobolin A and conglobatin A as novel candidates of CSCs inhibitors. Both ophiobolin A and conglobatin A selectively disrupted the oncogenic K-Ras membrane organization. Ophiobolin A disrupted the K-Ras nanoclustering by inhibiting CaM (Najumudeen et al., 2016).

Cho and colleagues use high-content imaging based screening to identify Ras membrane organization inhibitors. By using this method they identify staurosporine that inhibits the K-Ras nanocluster formation by disrupting phosphatidylserine (PS) distribution (Cho et al., 2012b). Recently, Yurugi et al demonstrated that natural compound rocaglamide, an inhibitor of prohibitins selectively disrupted K-Ras nanoclustering but not H-Ras and N-Ras (Yurugi et al., 2020). They discovered that plasma membrane associated prohibitins directly interact with K-Ras, PS and phosphatidic acids. Rocaglamide disrupts the K-Ras interaction with prohibitins, PS and phosphatidic acids, thus inhibiting K-Ras nanoclustering and tumor growth (Yurugi et al., 2020).

In addition, K-Ras can be targeted by using specific interactors, such as a monobody. The monobody NS1 binds with high affinity to K-Ras and H-Ras in the GDP- and GTP-bound state (Spencer-Smith et al., 2017). NS1 disrupts K-Ras and H-Ras nanoclustering and thus, inhibited the activation Raf. Inhibition of these critical components of Ras/Raf signaling cascades inhibited the Ras mediated transformation (Spencer-Smith et al., 2017).

2.3 Trafficking chaperone PDE6D regulates localization of K-Ras

2.3.1 Molecular structure and function of PDE6D

PDE6D is a trafficking chaperone of farnesylated proteins. PDE6D also known as PDEδ is the fourth subunit of the rod-specific cyclic guanosine monophosphate (cGMP) phosphodiesterase 6 (PDE6) (Gillespie et al., 1989). PDE6 is a unique member of a large family of PDE proteins that have two catalytic subunits, PDEα and PDEβ. Moreover PDE6 also has two inhibitory subunits (γ) (Gillespie et al., 1989; Li et al., 1990) PDEαβ is a dual post-translational modified protein where PDEα is farnesylated and PDEβ is geranylgeranylated (Qin et al., 1992). PDE6 is expressed only in photoreceptor but in addition to the retina PDE6D is also expressed in other tissues (Florio et al., 1996; Marzesco et al., 1998). Studies from a HeLa cDNA library screening suggested that PDE6D interacts with Rab13 (Marzesco et al., 1998). Moreover, PDE6D also interacts with ARF like small GTPase proteins (ARL2 and ARL3), which play a role in the vesicle transport system (Linari et al., 1999). Furthermore, small GTPases Ras, Rap and RHEB also interact with PDE6D (Hanzal-Bayer et al., 2002; Nancy et al., 2002). PDE6D client proteins are modified by farnesyl (15 Carbon) or geranylgeranyl (20 carbon)(Wright and Philips, 2006).

Approximately, 2% of all proteins are farnesylated, thus PDE6D modulates several cellular processes by facilitating the trafficking of its client proteins (Florio et al., 1996). Therefore, PDE6D inhibition may affect a number of clients that are relevant in cancer, such as K-Ras and other farnesylated proteins like Ras homolog enriched in brain (RHEB) and Ras-associated protein (Rap) (Papke et al., 2016). For instance, overexpression of RHEB is associated with cancer metastasis and poor prognosis (Liu et al., 2018). RHEB activates the mammalian target of rapamycin complex 1 (mTORC1), which controls the cell growth and metabolism (Yang et al., 2017). Another small GTPase, Rap1 is important for cell adhesion and cellular migration (Zhang et al., 2017). Thus, PDE6D regulates the activity of various farnesylated proteins by facilitating their diffusion in the cytosol.

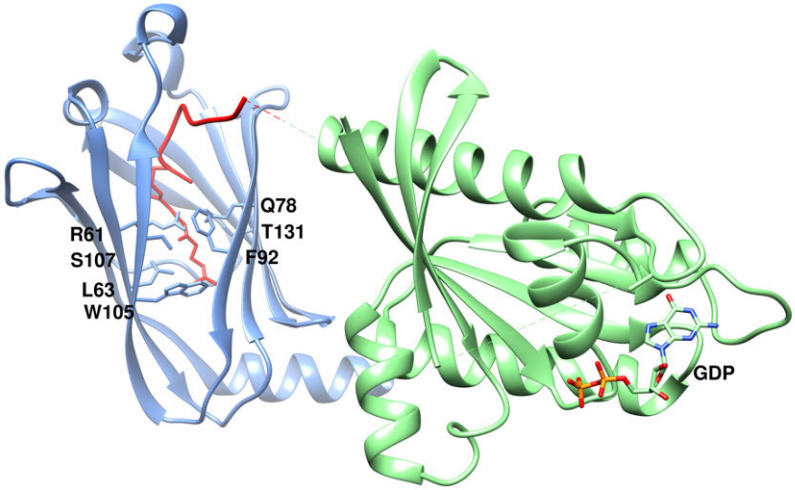


Figure 8: Crystal structure of farnesylated-K-Ras (Ras-F-Me) and PDE6D (PDB code 5tb5). The green ribbon represents GDP bound K-Ras, cyan ribbon represents the PDE6D. HVR and the farnesyl tail of K-Ras in the pocket of PDE6D is shown as solid red. The carboxy methyl group of C185 binds to prenyl pocket of PDE6D residues W90, F92 and I109. R61 and Q78 of PDE6D provide the polar environment for the carboxymethyl group of C185 (Dharmaiah et al., 2016).

PDE6D possesses a hydrophobic pocket between two β sheets propellers for binding of the farnesyl chain (Hanzal-Bayer et al., 2002). Studies demonstrate that PDE6D binds to its clients via its prenyl-binding pocket but this interaction depends on both prenylation and carboxymethylation of client proteins (Cook et al., 2000; Dharmaiah et al., 2016) (Figure 8). Thus, it enhances the solubilization for instance of K-Ras so that farnesylated K-Ras easily translocates in the aqueous cytoplasmic environment (Schmick et al., 2014). The carboxy-methyl group present on the farnesylated C185 of K-Ras plays an important role in the interaction between PDE6D and K-Ras. Disturbance of binding between PDE6D/K-Ras was observed in absence of methyl group, which caused the charge repulsion effect of the carboxylic acid group and thereby diminished the hydrophobic interaction of farnesylated C185 Ras (Dharmaiah et al., 2016).

Palmitoylation of H-Ras and N-Ras at the Golgi complex is necessary for their plasma membrane trafficking (Goodwin et al., 2005). Under normal conditions palmitoylated H-Ras does not depend on PDE6D for localization to the plasma membrane, whereas K-Ras depends on PDE6D for the localization (Chandra et al., 2011). This may be because of the dual palmitoylation of H-Ras, and the fact that palmitoylation blocks the access to the prenyl pocket of PDE6D (Dharmaiah

et al., 2016). Studies also suggested that unlike K-Ras4B, K-Ras4A is not able to bind PDE6D (Dharmaiah et al., 2016; Tsai et al., 2015). This may be because upstream of the prenylated cysteine two lysine residues probably interfere with the interaction between depalmityolated K-Ras4A and PDE6D.

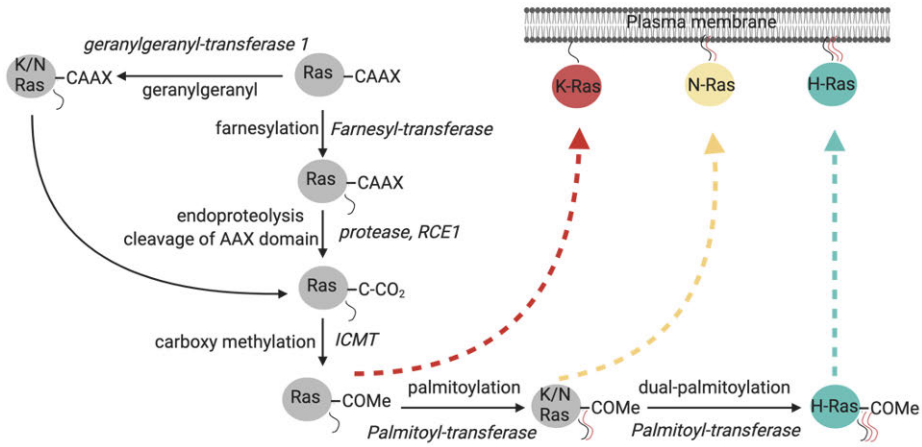


Figure 9: Post-translational modification required for Ras plasma membrane localization: First Ras proteins are farnesylated by farnesyl-transferase, alternatively K-Ras and N-Ras are geranylgeranylated by geranylgeranyl-transferase 1. Ras proteins go through the endoproteolytic cleavage of the AAX residues by the protease, RCE1. Next, Ras proteins are carboxymethylated by isoprenylcystein carboxymethyltransferase (ICMT). H-Ras and N-Ras are palmitoylated by palmitoyl-transferase

2.3.2 PDE6D regulates K-Ras localization and signaling

For K-Ras membrane anchorage the farnesyl and methyl groups are inserted in the bilayer of the plasma membrane and the polybasic region of the HVR interacts with the inner leaflet of the plasma membrane especially on the acidic phospholipids such as phosphatidylserine or phosphatidylinositol (Roy et al., 2000; Zhou et al., 2015). K-Ras spontaneously dissociates from the plasma membrane and in addition K-Ras is also lost from the plasma membrane by the process of endocytosis (Schmick et al., 2014; Willumsen et al., 1984a). K-Ras dissociates rapidly from endocytic vesicles as they lose their surface charge. This results in K-Ras molecules being released in the cytoplasm but this requires trafficking chaperones for it to move in the cytoplasm. In order for K-Ras to translocate in the aqueous cytoplasmic environment, the hydrophobic farnesyl moiety of K-Ras interacts with the solubilization factor PDE6D in the cytoplasm (Chandra et al., 2011) (Figure 10). PDE6D binds to farnesylated Ras proteins but

this binding does not depend upon the nucleotide state of the Ras proteins (Hanzal-Bayer et al., 2002; Nancy et al., 2002). Inhibition or knockdown of PDE6D decreases K-Ras on the plasma membrane, thus PDE6D is crucial for the maintenance of K-Ras activity as well (Schmick et al., 2015) Another protein is required that allosterically displaces K-Ras from PDE6D. The GTPase ARL2, when in the active GTP-bound state, binds to an allosteric site of PDE6D, thus initiating a conformational change so that farnesylated K-Ras is unloaded from PDE6D (Ismail et al., 2011). After unloading of K-Ras from PDE6D by the activity of ARL2 on the perinuclear membranes, K-Ras is trapped on the negatively charged recycling endosome. From here, K-Ras returns back to plasma membrane through vesicular transport system (Figure 10).

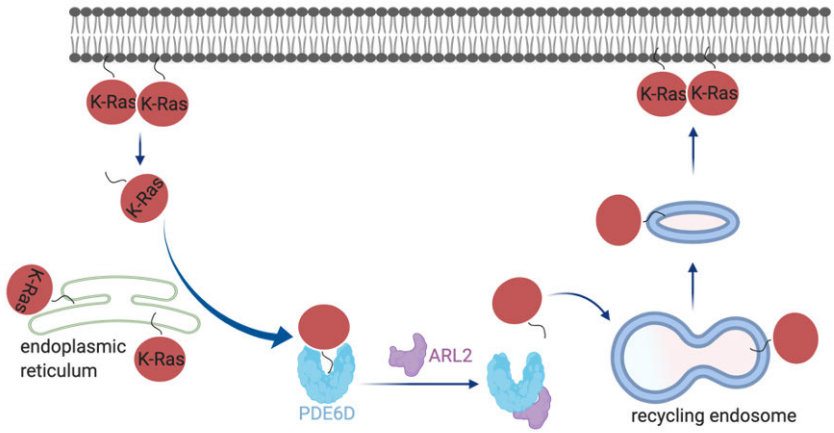


Figure 10: Trafficking of K-Ras: K-Ras spontaneously dissociates from the cellular membrane. K-Ras diffusion in the cytoplasm is facilitated by PDE6D, which solubilizes the lipid-modified, hydrophobic C-terminus of K-Ras. ARL2-GTP induces the ejection of K-Ras in the perinuclear region. In the perinuclear region K-Ras is trapped on recycling endosomes after which it is transported to the plasma membrane via vesicular transport.

2.3.3 Existing inhibitors of PDE6D block K-Ras signaling

Inhibition of PD6D/K-Ras interaction decreased the K-Ras concentration on the plasma membrane, thus blocking K-Ras signaling (Papke et al., 2016). It is shown that knock-down of PDE6D or its inhibition by small molecules like Deltarasin, Deltazinone 1, Deltasonamide 1 and 2, redistributes K-Ras to all endomembranes rather than the plasma membrane (Schmick et al., 2015). These, studies suggest that the interaction of K-Ras with PDE6D is necessary to solubilize K-Ras.

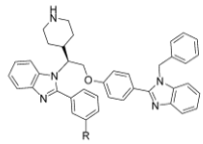
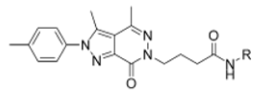
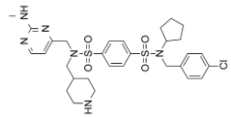
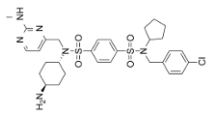
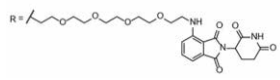
Inhibition of PDE6D by a small molecule blocks the K-Ras signaling in many *KRAS* mutant malignancies like colorectal, lung and pancreatic cancer (Klein et al., 2019; Leung et al., 2019).

Researchers from the Max-Planck Institute in Dortmund developed the first PDE6D inhibitor Deltarasin. Deltarasin has high binding affinity to PDE6D (K_D = 38 nM) (Zimmermann et al., 2013) and binds to the prenyl-binding pocket of PDE6D. They showed that Deltarasin inhibits the interaction of PDE6D/K-Ras which results in the delocalization of K-Ras. Moreover, inhibition of PDE6D/K-Ras interaction by Deltarasin inhibits cancer cell proliferation and blocks MAPK signaling. A major drawback of this compound is its high unspecific cell toxicity that may be because of off-target effects.

After a few years the same groups developed the next generation of compounds that were chemically different to Deltarasin, resulting in Deltazinone 1 (Papke et al., 2016). Similar to Deltarasin, Deltazinone 1 also binds to the prenyl-binding pocket of PDE6D with higher affinity (K_D = 8 nM). Therefore, inhibits PDE6D/K-Ras interaction and decreased cell proliferation. Deltazinone 1 downregulates the ERK phosphorylation in K-Ras dependent Panc-Tu-1 cells but it requires longer incubation time than Deltarasin. This indicates that Deltarasin has a good interaction with PDE6D as compared to Deltazinone 1. However, both compounds showed the similar binding affinity for PDE6D in in vitro assay. Therefore, less cellular activity of Deltazinone 1 may be because of ejection from PDE6D by ARL2 (Papke et al., 2016). Moreover, they also found that Deltazinone 1 rapidly metabolized in mice and therefore, it is not suitable for animal studies (Papke et al., 2016).

Waldmann group developed third generation compounds known as Deltasonamide 1 and 2 with binding affinities in the pM range. Both compounds Deltarasin and Deltazinone 1 have a binding affinity between 8 to 38 nM but they show their cellular activity at 5 to 20 μM concentrations. They explain these findings by the ejection of these PDE6D inhibitors by the release factor ARL2 (Martin-Gago et al., 2017). As compared to Deltarasin and Deltazinone 1, Deltasonamide could not be ejected from PDE6D. This decrease in release by ARL2 is because of the formation of additional hydrogen bonding in the pocket by Deltasonamide.

Table 3: PDE6D inhibitors show the difference between PDE6D binding and cellular activity

Inhibitor	Structure	PDE6D binding	Cellular activity in KRAS mutant cells
Deltarasin		38 nM	5 µM
Deltazinone		8 nM	5 µM
Deltasonamide 1		203 pM	5 µM
Deltasonamide 2		385 pM	5 µM
PROTAC 3		64.3 nM	1 µM

Proteolysis targeting chimeras (PROTACs) are emerging tools not only in cancer but also other human diseases. PROTACs are used to degrade proteins that play key roles in tumor development and metastasis. PROTACs are heterobifunctional molecules, consisting of two ligands connected by a linker.

One ligand binds to the target protein and another ligand to an E3 ubiquitin ligase (Khan et al., 2020). Thus the E3 ligase is recruited to the target protein and induces its degradation by the ubiquitin-proteasome system (Khan et al., 2020). The Waldmann group developed the first PROTACs for targeting PDE6D (Figure 11). These molecules efficiently and selectively degrade PDE6D by inducing proteasomal degradation at low concentration of 1 µM (Winzker et al., 2020). Unlike previous generations of PDE6D inhibitors, PROTACs have unique features. PROTACs do not need to bind the target protein permanently. They can be recycled after degradation of the target protein, thus they may act catalytically. Therefore, PROTACs based molecules may be a promising tool to gain new insight into PDE6D biology.

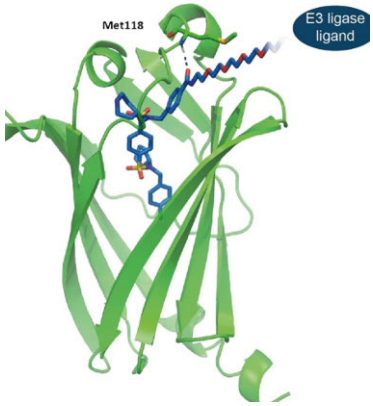


Figure11: Design of PDE6D PROTAC probes. Visualization of the PDE6D PROTAC 3 in the prenyl pocket of PDE6D based on computational modelling (PDB ID: 5ML3) (Winzker et al., 2020)

2.4 The molecular chaperone Hsp90

In our previous study, we identified that conglobatin A selectively inhibits K-Ras nanoclustering. But how conglobatin A inhibits K-Ras signaling was unclear. Other have shown that conglobatin A disrupts the interaction between the heat shock protein 90 (Hsp90) and its co-chaperone Cdc37, thus inducing apoptosis and inhibiting cancer cell proliferation (Huang et al., 2014). The molecular chaperone Hsp90 assists client protein folding, maturation and thus stabilization of its client proteins. Hsp90 client proteins not only depend on Hsp90 but also on co-chaperones for their maturation. The main role of co-chaperones is to deliver client proteins to the Hsp90 chaperone machinery, regulation of the Hsp90 ATPase cycle and stabilization of various Hsp90 conformational states. Many Hsp90 client proteins are involved in various human diseases like cancer and neurodegenerative diseases. A list of Hsp90 client proteins (Hsp90 interactors) is available on (https://www.picard.ch/downloads/Hsp90interactors.pdf)

There are five isoforms of Hsp90 discovered to date, which are encoded by different genes. Hsp90α and Hsp90β are two different cytoplasmic isoforms of Hsp90, which are encoded by two distinct genes. The functional difference between these two isoforms is still unclear (Whitesell and Lindquist, 2005). In the endoplasmic reticulum, there is an isoform of Hsp90 known as glucose-related protein (GRP94) and in mitochondria there is TNF receptor-associated protein 1

(TRAP1). Hsp90N is a membrane associated isoform of Hsp90 (Powers and Workman, 2007). Intracellular protein levels of Hsp90 are high and make up about 1 to 2% of total proteins (Mahalingam et al., 2009). Expression of Hsp90 is higher in cells upon nutrient deprivation, heat shock and hypoxia, the latter of which is associated with the tumor microenvironment. Thus, it may be concluded that a higher expression of Hsp90 is essential for the survival of cancer in unfavorable conditions (Whitesell and Lindquist, 2005).

2.4.1 Molecular structure and function of Hsp90

Hsp90 has three domains, the N-terminus has the ATP binding pocket, the middle domain has the ATP hydrolysis regulating domain, this middle domain is also responsible for Hsp90 client maturation and the C terminal domain is for the dimerization of Hsp90 (Wandinger et al., 2008; Whitesell and Lindquist, 2005). Another important role of the C-terminus of Hsp90 is to regulate the ATPase activity of the N-terminus and the recruitment of co-chaperones. The C-terminal domain contains a Met-Glu-Glu-Val-Asp (MEEVD) motif for the binding of co-chaperones like protein phosphatase 5 (PP5), Hsp90/Hsp70 organizing protein (HOP), peptidyl-proly-isomerase (PPIase) that contains tertratricopeptide repeat (TPR) domains (Buchner, 1999).

The chaperonin function of Hsp90 is dependent on the assistance of several co-chaperones, which are required for ATPase cycle regulation, conformational changes, the selection and binding with specific client proteins, and the subsequent dynamics and activation of Hsp90 (Taipale et al., 2010) (Figure 12). The co-chaperone HOP/Sti1 is believed to be involved in the early stages of the Hsp90 functional cycle and to help in recruiting Hsp70-bound client proteins, such as steroid hormone receptors. HOP/Sti1 binds and stabilizes the open conformation of Hsp90 and thus inhibits its ATPase activity (Chen and Smith, 1998). In 2012, the Lindquist group systematically and quantitatively studied the interactions of the co-chaperone cell division cycle 37 (Cdc37) with many of the Hsp90 clients, including most human kinases, transcription factors and E3 ligases (Taipale et al., 2012). Approximately 60% of all cellular kinases interact with Cdc37 (Taipale et al., 2012). Furthermore, all the interactions between Hsp90 and kinases were blocked after Cdc37 knockdown (Taipale et al., 2010). These results indicated that Cdc37 is a highly specialized co-chaperone that assists Hsp90 in recognizing its client kinases, cell division control proteins and receptors (Li et al., 2018b).

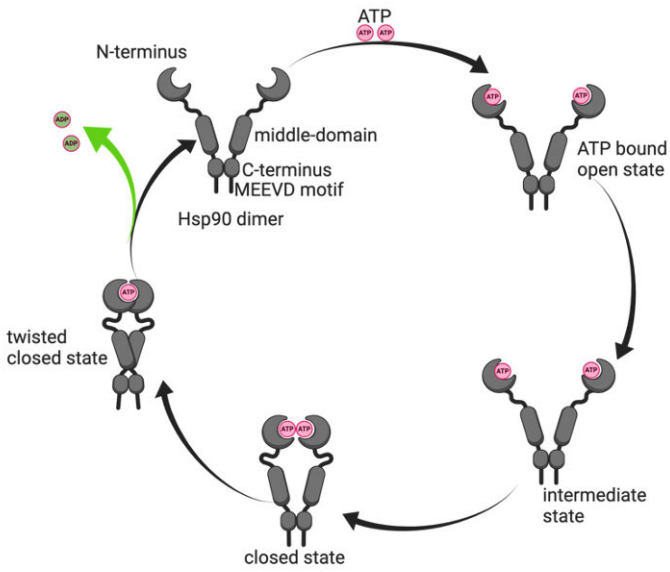


Figure 12: Model for Hsp90 conformational cycle: The Hsp90 dimer transmits through different conformation. In the open state, Hsp90 dimer remains in most of the time (Schopf et al., 2017). This allows the chaperone machinery to interact with Hop-Hsp70-Hsp40-client or Cdc37 and ATP. In the intermediate state, co-chaperones and client translocate on the Hsp90 chaperone machinery. In the closed state, Hsp90 releases Cdc37 from the machinery. In the twisted closed state Hsp90 proceeds through the ATPase cycle for the maturation of clients

To perform the chaperone function of Hsp90, ATP loading is necessary at the N-terminal ATP pocket (Prodromou et al., 2000). Cdc37 binds to the N-terminus of Hsp90, thus functions as an adaptor in the recruitment of client kinases to the Hsp90 chaperone machinery. Immature kinases bind to the N-terminus of Cdc37, while the middle of Cdc37 binds with the N-terminus of the open state of Hsp90. After that, Cdc37 translocates to the middle domain of Hsp90. After binding of ATP to the N-terminal ATP pocket, Hsp90 closes the ATP pocket lid. Binding of serine/threonine PP5 at position MEEDV motif is necessary for the proper functioning of Cdc37 and maturation of kinases (Schopf et al., 2017). PP5 removes the phosphate from Cdc37 and releases the Cdc37 from the Hsp90 chaperone machinery (Oberoi et al., 2016) (Figure 13)

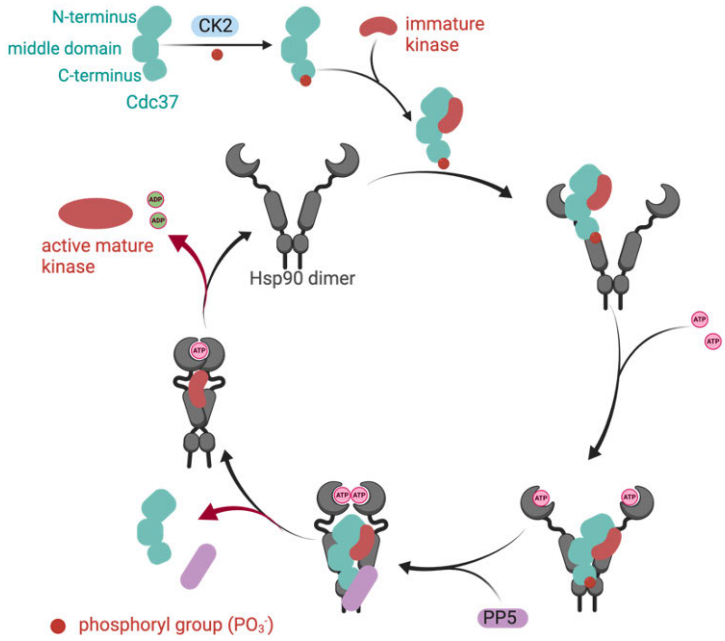


Figure 13: Hsp90 kinase chaperone cycle: Protein kinase CK2 phosphorylates Cdc37 on its C-terminus. Phosphorylated Cdc37 recognizes and delivers kinases to Hsp90 in the open state. Kinases bind to the N-terminus of Cdc37 and the middle domain of Cdc37 binds to the N-terminus of Hsp90. After that Cdc37 translocates to the middle domain of Hsp90 and ATP binds to N-terminal ATP pocket. Next, Hsp90 turns to a closed state and the binding of PP5 to the MEEVD motif of Hsp90 completes the function of Cdc37. Before, ATPase activity Cdc37 and PP5 release from the machinery. The client kinase matures and is released after hydrolysis of ATP.

ATP hydrolysis results in kinase folding and changes the Hsp90 conformation to the open state and release of Cdc37 (Verba et al., 2016). Cdc37 regulates the conformationally coupled ATPase mechanism of Hsp90, arresting the chaperone cycle in the client loading phase, prior to Hsp90's ATP-dependent N-terminal dimerization (Roe et al., 2004). Therefore it can be concluded that after the recognition and recruitment of client kinases, Cdc37 translocates from the N-terminal domain of Hsp90 to the middle domain. After translocation of Cdc37 to the middle domain of Hsp90, the ATP binding pocket is again available for the binding of nucleotides (Siligardi et al., 2004).

2.4.2 Hsp90 regulates multiple cellular processes

EGFR is a client of Hsp90. Maturation of EGFR depends upon the Hsp90 chaperone cycle. EGFR can be activated when a ligand like growth factor binds to it. Once EGFR is phosphorylated it initiates multiple signaling cascades like PI3K/AKT/mTOR and MAPK pathways. Hyperactivation or overexpression of EGFR or RTK promotes to cancer because RTK regulates multiple signaling pathways that are responsible for cell proliferation (Scaltriti and Baselga, 2006).

Human Epidermal Growth Factor Receptor 2 (HER2) also called ERBB2 is a member of the ERBB family of receptor tyrosine kinases, and is another client of Hsp90. HER2 proteins are structurally related to EGFR. It forms dimer with other members of ERB family, which results in autophosphorylation and activation of many signaling pathways such as PI3K/AKT, Ras/Raf/MAPK (Citri et al., 2004). Inhibition of Hsp90 results in degradation of HER2 and thus attenuates the HER2 dependent signaling pathways (Citri et al., 2004).

PI3K/AKT pathway activates many protein that have prosurvival effect like nuclear factor-κB (NF-κB). Moreover, AKT suppresses the activity of proapoptotic proteins such as Bad protein (Jiang and Liu, 2008). Mutations in the PI3K/AKT pathways increase cell proliferation and prevent cell apoptosis. AKT is client of Hsp90 and therefore, Hsp90 chaperone cycle responsible for the maturation of AKT. Inhibition of Hsp90 decreases the expression of AKT and blocks the AKT pathway (Sato et al., 2000). Thus, Hsp90 indirectly regulates the PI3K/AKT pathway. Raf-ERK is downstream of Ras signaling is also important signaling pathways for cell proliferation (Grbovic et al., 2006). B-Raf and C-Raf are other clients of Hsp90. Inhibition of Hsp90 decreases Raf kinase levels, thereby, blocking ERK signaling (Banerji et al., 2005).

Cyclin-dependent kinase 4 (CDK4) monitor the cell cycle check points and regulate the cell division cycle (Malumbres and Barbacid, 2001). Retinoblastoma (Rb) protein and the transcription factor EF2 regulate the G1/S cell cycle stage. Active non-phosphorylated form of Rb binds to EF2 and thereby inhibits EF2 function like cell proliferation or progression of cell cycle. When Rb is phosphorylated by CDK4, Rb not able to inhibits the transcriptional function of EF2 (Malumbres and Barbacid, 2001). CDK4 is a client kinase of Hsp90, therefore inhibition of Hsp90 leads to proteasomal degradation of CDK4 by and allows Rb to remain in its active unphosphorylated form (Srethapakdi et al., 2000).

Apoptosis plays a key role to control the population of unwanted cells. The blockage of apoptotic allows cancer cells to survive longer which give more time for the accumulation mutation. Thus, promote deregulated cell proliferation, stimulate angiogenesis, tumor progression (Hassan et al., 2014).

Bcl-2 and Bcl-xL are two important proteins that inhibit apoptosis and act as prosurvival factors (Kooijman, 2006). Apoptosis can be inhibited by the activation of insulin-like growth factor-1 (IGF-1) signaling. Activation of the IGF-1 pathway increases the expression of Bcl-2 and Bcl-xL Moreover, IGF-1, Bcl-2 and Bcl-xL are the clients of Hsp90. Furthermore, the IGF-1 receptor inactivates the proapoptotic protein known as Bad by the phosphorylation. Phosphorylated Bad dissociates from the Bcl-xL, thus Bcl-xL exhibits its antiapoptotic activity (Nielsen et al., 2004). IGF-1 receptor activation inhibits the activity of caspases and proteases by the phosphorylation. These caspases and proteases execute the apoptotic cascade (Kooijman, 2006).

Proper angiogenesis is required for the supply of nutrients and oxygen in a growing tumor. Vascular Epidermal Growth Factor (VEGF) signaling stimulates the angiogenesis for the formation of blood vessels to grow into the tumor. VEGFR 1 and 2 are clients of Hsp90 chaperone machinery. Inhibition of Hsp90 blocks the VEGF signaling, thus impairs the process of angiogenesis (Sanderson et al., 2006). Moreover, transcription factor hypoxia-inducible factor-1α (HIF-1α), which is a client of Hsp90 positively regulates the expression of VEGF (Isaacs et al., 2002). HIF-1 is a heterodimeric protein, which is composed of two subunits known as HIF-1α and HIF-1β. HIF-1 is overexpressed in many types of cancers including breast, lung, colon and prostate cancers (Zhong et al., 2001). HIF-1α is upregulated in low oxygen conditions during the rapid development of tumor. Higher expression of HIF-1α regulates the expression of multiple genes which are associated with cell metabolism, angiogenesis and cell survival (Semenza, 2002).

Similar to the higher expression of Hsp90 in many cancer, Cdc37 is also overexpressed in prostate and hepatocellular cancer (Stepanova et al., 2000; Wang et al., 2015b). Therefore, both Hsp90 and Cdc37 play a combined role in the survival and maintenance of cancer cells. There are many mutant proteins which are overexpressed in cancer cells. Hsp90 plays important role in the maturation of mutant or overexpressed proteins and thereby, survival of cancer cells (Schopf et al., 2017). Hsp90 involved in maturation of series of proteins like oncoproteins, kinases, transcription factors, receptor kinases. Many of these proteins are involved in tumor cell proliferation and growth (Vartholomaiou et al., 2016).

Cancer cells increase their dependence on Hsp90, if there is high level of mutant destabilized oncoproteins. Higher expression of Hsp90 is associated with tumor aggressiveness and poor prognosis (Pick et al., 2007; Wang et al., 2013). Mutant p53 also interacts with Hsp90 for its maturation which leads to an accumulation of dysfunctional p53 in cancer cells (Deb et al., 1999; Whitesell et al., 1998). Blocking of interaction between Hsp90 and mutant p53 decreased tumor growth and increased survival in mouse model (Alexandrova et al., 2015). As compared to normal cells, *KRAS* mutant cancer cells are more sensitive to Hsp90 inhibition (Park et al., 2016). This confirms the important role of Hsp90 in regulation of cancer progression. As discussed above, Hsp90 matures many of its kinases which involved multiple signaling pathways such as PI3K/AKT/mTOR and MAPK pathways. Inhibition of Hsp90 attenuates these important signaling pathways and thereby inhibits cancer cell proliferation and growth (Mielczarek-Lewandowska et al., 2020). As compared to healthy persons significantly higher levels of Hsp90 were detected in melanoma patients (Tas et al., 2017). Higher levels of Hsp90 in serum might also be considered as cancer marker of melanoma progression. Extracellular Hsp90 was also involved in angiogenesis and cell motility (Hance et al., 2014).

2.4.3 Types of Hsp90 inhibitors

N-terminal ATP pocket Hsp90 inhibitors

Most Hsp90 inhibitors have been developed to inhibit Hsp90 chaperone function by binding to Hsp90 at the N-terminal ATP pocket (Neckers, 2003). The benzoquinone ansamycins, such as geldanamycin (GA) was the first identified and its derivative 17-allyamino-geldanamycin (17AAG), was the first developed Hsp90 N-terminal ATP pocket inhibitor (Whitesell et al., 1994). The binding of GA on the N-terminal ATP pocket arrests the catalytic cycle of Hsp90 in the ADP-bound conformation, inactivating chaperone activity, which results in the ubiquitination and proteasomal degradation of client proteins (Blagg and Kerr, 2006). Drugging with GA results in degradation of Hsp90 client proteins, thus, impairs cancer promoting signaling (Workman et al., 2007). Absence of ATP-binding stabilizes the open conformation of Hsp90 and therefore blocks the Hsp90 chaperone cycle. Analysis from the previous studies suggested that GA binds to the same place where ATP binds (Roe et al., 1999). Other than toxic nature of N-terminal Hsp90 ATP pocket inhibitors, these type of inhibitors also induce heat shock response by heat shock factor 1 (HSF1), thus induce drug resistance. Under normal physiological conditions HSF1 is predominantly present

Table: 4: Types of Hsp90 inhibitors

Hsp90 inhibitor	Hsp90 inhibition mechanism
Geldanamycin	N-terminal ATP competitor
17-AAG	N-terminal ATP competitor
Luminespib	N-terminal ATP competitor
EGCG	Allosteric, C-terminal nucleotide pocket inhibitor
Novobiocin	Allosteric, C-terminal nucleotide pocket inhibitor
Celastrol	Allosteric, C-terminal Hsp90/Cdc37 interaction inhibitor
Withaferin A	Allosteric, C-terminal Hsp90/Cdc37 interaction inhibitor
Conglobatin A	N-terminal Hsp90/Cdc37 inhibitor
DDO-5936	N-terminal Hsp90/Cdc37 inhibitor

in the cytoplasm as monomers. Both Hsp90 and Hsp70 interact with HSF1 to retain it in the cytoplasm, thus suppress its activity (Abravaya et al., 1991). But in stressed conditions Hsp90 and Hsp70 interact with Hsp90 client proteins for their maturation/folding, thus release the HSF1. Therefore, during the heat shock response, HSF1 translocates into the nucleus to form a trimer. In the nucleus the trimer of HSF1 binds to heat stress elements (HSEs) to target chaperone promoter genes, thus increasing the expression levels of Hsp40, Hsp90 and Hsp70 (Kroeger and Morimoto, 1994). Upregulation of heat shocks proteins by the N-terminal ATP pocket inhibitors make it drug resistance. This may explain why N-terminal ATP pocket inhibitors become inefficient, as their application leads to higher levels of their target Hsp90.

C-terminal allosteric Hsp90 inhibitors

A second nucleotide-binding pocket is present on the C-terminus of Hsp90. The C-terminal domain is responsible for the dimerization of Hsp90 (Soti et al., 2003). Removal of the C-terminal domain significantly blocks the ATPase activity of N-terminal of Hsp90, which show importance of C-terminus in Hsp90 chaperone machinery (Goode et al., 2017; Whitesell and Lindquist, 2005).

A first C-terminal inhibitor was novobiocin, a natural compound. Novobiocin binds the nucleotide binding pocket of the C-terminus and thereby, inhibits the dimerization of Hsp90. Interestingly, compounds like GA do not bind to this pocket because both novobiocin and GA require different amino acids for its binding (Marcu et al., 2000). Like novobiocin, epigallocatechin-3-gallate (EGCG), which is an extract from green tea binds to the C-terminal nucleotide pocket, thus inhibiting Hsp90 chaperone activity.

Protein-protein interface inhibitors

Cdc37 is a co-chaperones that delivers kinases to the open state of Hsp90. Another class of Hsp90 inhibitors inhibits the interaction between Hsp90 and Cdc37. conglobatin A also known as FW-04-806, inhibits the Hsp90 function through binding of the N-terminus of Hsp90 and blocks the Hsp90/Cdc37 interaction (Huang et al., 2014). Hsp90/Cdc37 inhibitors also result in the degradation of many oncogenic kinases like AKT, B-Raf, C-Raf and HER2 (Polier et al., 2013). Inhibition of p23, which is also a co-chaperone of Hsp90, results in many client proteins destabilization. The natural compound gedunin binds to p23, inhibits the binding to Hsp90, which results in the degradation of steroid hormones or hormone receptors (Chadli et al., 2010; Patwardhan et al., 2013). Hsp90/Cdc37 interactor inhibitor DDO-5936 shows low toxicity and does not initiate heat shock response (Wang et al., 2019). DDO-5936 binds to Hsp90 on the Glu47, which is the critical residue for the binding of Cdc37. Inhibition of Hsp90/Cdc37 by DDO-5936 results in decrease expression of CDK4 and inhibit the cell proliferation (Wang et al., 2019).

3. AIMS OF THE STUDY

K-Ras is a major drug target because of its prominent role in cancer progression. Here, we describe the development of experimental drugs that indirectly target K-Ras but not H-Ras activity. We previously established that selectively inhibiting K-Ras enables anti-CSC activity. We focus on developing inhibitors against two chaperones, which afford this K-Ras selectivity.

The first one is the trafficking chaperone PDE6D, which interacts with farnesylated K-Ras and helps trafficking of K-Ras inside cells. Current PDE6D inhibitors developed by others lack resilience against ARL2 ejection and have poor, cell penetration ability. The second chaperone is the Hsp90/Cdc37 complex, which is targeted at its protein-protein interface by conglobatin A, a natural product with exceptionally low toxicity. Interface inhibitors are very difficult to identify. We therefore first aimed at establishing an assay that would allow us to find small molecule functional analogues of conglobatin A. Furthermore, in order to properly apply these compounds, we wanted to understand the mechanism of K-Ras but not H-Ras selectivity, from which we wished to derive mechanism-based biomarkers that could direct the testing and application of novel Hsp90 inhibitors.

Therefore, the specific aims of this thesis were

1. Development of novel PDE6D inhibitors with better resilience and cell penetration properties to target K-Ras
2. Development of an assay that identifies Hsp90/Cdc37 protein-protein interface inhibitors
3. Identification of novel Hsp90/ Cdc37 interface inhibitors and their mechanism of K-Ras selectivity

4. MATERIALS AND METHODS

A more detailed description of the methods and reagents can be found in original publications (I–III)

Cell lines

Cell line	Description	publication
HEK293 EBNA, HEK 293 cells expressing EBNA-1 gene	Embryonic kidney	I, II, III
MDA-MB-231	Breast adenocarcinoma	I, III
SKBR3	Breast adenocarcinoma	III
MIA PaCa-2	Pancreatic carcinoma	III
HCC-44	Non-small lung carcinoma	III
Hs 578T	Breast adenocarcinoma	II
HT-29	Colorectal adenocarcinoma	II
NCI-H358	Non-small lung carcinoma	II
HCT116	Colon carcinoma	II
A-375	Skin melanoma	II

Reagents

Reagent	Application	publication
jetPRIME	Cell culture	I, III
FuGENE HD	Cell culture	I, III
Lipofectamine RNAiMAX	Cell culture	I
DMEM	Cell culture	I,II, III
RPMI-1640	Cell culture	I, III
FBS	Cell culture	I, II, III
L-glutamine	Cell culture	I, II, III
DMSO	Cell culture	I, II, III
B27	Cell culture	I , III
Epidermal growth factor (EGF)	Cell culture	I, III
Fibroblast growth factor (FGF)	Cell culture	I, III
Paraformaldehyde (PFA)	FLIM-FRET	I, III
Mowiol 4-88	FLIM-FRET	I, III
Fluorescein	FLIM-FRET	I, III

Matrigel	CAM-assay	III
AlamarBlue	2D, 3D culture	I, III
RIPA buffer	Western blot	I; III
Prestained protein ladder	Western blot	I, III
Nitrocellulose membrane	Western blot	I, III
BSA	Western blot	I, III
ECL Western blotting substrate	Western blot	I, III
BCA protein assay kit	Western blot	I, III
Renilla luciferase assay kit	Split *Renilla* assay	II, III
Hsp90 N-terminus assay kit	ATP-binding site competition	III
HEPES buffer	Cytoplasmic and nuclear extract	III
MethoCult H4100	3D culture	III

Plasmids and siRNAs

Plasmid and siRNA	**publication**
pmGFP-K-RasG12V and pmCherry-K-RasG12V	I, III
pmGFP-H-RasG12V and pmCherry-H-RasG12V	I, III
mCit-Rheb and mCherryPDE6D	I
pcDNA3.1(+)-NRL-Hsp90 and pcDNA3.1(+)-Cdc37-CRL	II
pcDNA3.1(+)-NRL and pcDNA3.1(+)-CRL	II
pGL4.74	II
pcDNA3.1(+)-NRL-N-Hsp90	III
pcDNA3.1-galectin-1 and pcDNA3.1-galectin-3	III
siRNA-Hsp90 and siRNA-Cdc37	III
siRNA-galectin-3 and siRNA-HIF-1α	III
siRNA-KRAS, siRNA-HRAS and siRNA-PDE6D	II

Antibodies

Antigen	**manufacturer**	**publication**
PDE6D	Santa Cruz Biotechnology	I
Actin	Sigma-Aldrich	I, III
HER-2	ThermoFisher Scientific	III
Hsp70	ThermoFisher Scientific	III
Hsp90	Santa Cruz Biotechnology	III

Cdc37	Santa Cruz Biotechnology	III
Akt	Cell signaling Technology	III
ERK1/2	Cell signaling Technology	III
pERK1/2	Cell signaling Technology	III
HIF-1α	Novus Biologicals	III
C-Raf	Santa Cruz Biotechnology	III
Galectin-3	Santa Cruz Biotechnology	III
Anti-mouse	Santa Cruz Biotechnology	I, III
Anti-rabbit	R&D System	I, III

Inhibitors

Inhibitor	**publication**
Deltarasin	I
FTI-277	I, III
ARS-1620	I
Vermurafenib	I
Geldanamycin	II, III
17-AAG	II, III
Luminespib	II, III
Novobiocin	II, III
EGCG	II, III
Celastrol	II
Withaferin	II, III
Conglobatin A	II, III
Platycodin D	II
3-(2-Pyridyl)-5-(4-pyridyl)-1,2,4-triazole	II
Salinomycin	III
CAY10685	III
Compactin	III
Bortezomib	III
x6506, x1540 and other screening compounds	III
Elaiophylin , conglobatin B and C (BioAustralis)	

Experimental Procedures

Name	**publication**
K-Ras/HRas nanoclustering Fluorescence Lifetime Imaging (FLIM-FRET)	I, III
K-Ras/PDE6D and PDE6D/Rheb interaction (FLIM-FRET)	I
Confocal microscopy	I
SPR binding and inhibition assay	I
Sphere formation assay	I, III
2D cell proliferation	I, III
Western blotting	I, III
Nuclear and cytoplasmic extract	III
RT-qPCR	I
Computational docking	I, II
FA assay	I
Hsp90/Cdc37 interaction (split *Renilla* luciferase assay)	II, III
Hsp90 N-terminus ATP-binding site competition assay	III
Chick chorianllantoic membrane (CAM) assay	III

5. RESULTS AND DISCUSSION

In this thesis, I identified small molecule inhibitors that selectively target K-Ras by inhibiting two different chaperones of K-Ras. First, we developed compounds that inhibited the activity of trafficking chaperone PDE6D. Only proteins that are farnesylated, but not in addition palmitoylated can be clients of PDE6D, thus inhibition of PDE6D selectively blocks the activity of K-Ras but not H-Ras. Compounds that selectively inhibit K-Ras but not H-Ras membrane organization have the potential to impair cancer cell stemness properties such as tumorosphere formation (Najumudeen et al., 2016; Posada et al., 2017). Current PDE6D inhibitors show limited cellular activity because of the ejection of compounds from PDE6D by the action of ARL2. Here, we developed inhibitors that have a chemical spring moiety to provide resistance against ARL2 ejection and in addition feature a cell penetration group to increase bioavailability.

Secondly, our group previously discovered that conglobatin A selectively blocks K-Ras membrane organization. Moreover, conglobatin A was known to inhibit the interaction between Hsp90/Cdc37, thus reducing cell proliferation and inducing apoptosis (Huang et al., 2014). Thus, we concluded that conglobatin A blocks the activity of K-Ras by inhibiting Hsp90. This is in agreement with observations by others showing that inhibition of Hsp90 affects K-Ras signaling (Azoitei et al., 2012; Park et al., 2016). However, the exact mechanism is not clear. Therefore, here we elaborated how inhibition of the Hsp90/Cdc37 complex blocks K-Ras signaling. Finally, using a split *Renilla* luciferase assay, we identified novel small molecule protein-protein interface inhibitors that disrupt the Hsp90/Cdc37 interaction.

5.1 Design of novel PDE6D inhibitors with selective inhibition of K-Ras (I)

5.1.1 Structure and design of novel PDE6D inhibitors

ALR2-mediated ejection from the PDE6D prenyl-binding pocket and intracellular penetration were the main problems of the existing PDE6D inhibitors. To solve this problem, we designed a first-generation of compounds with three moieties (I: Figure 1A). Each moiety had its own function. For the top, we chose the generic,

heterocyclic coumarin ring. This moiety tolerates forming pi-stacking and hydrogen bonding with PDE6D. To deal with the ejection problem, we added a flexible hexyl-linker in the middle part, which acts as a buffering spring to provide flexibility against ejection by ARL2. On the bottom, we added a phosphodiester moiety, protected by the previously developed cell penetration group (Hecker and Erion, 2008; Kiuru et al., 2013). We designed and synthesized these novel inhibitors with our collaborators Dr. Mikko Ora and Prof. Pasi Virta, from the University of Turku. We named this compound Deltaflexin-1. We also synthesized several compounds for control experiments that allowed us to show the importance of each moiety (I: Figure 1A, S1A).

First, we validated our first-generation compounds by surface plasmon resonance (SPR) assay in vitro. In this assay, farnesylated and carboxymethylated K-Ras (K-Ras-F-Me) was captured on the sensor chip and binding of PDE6D was determined in the presence of the compounds. Interestingly, our model compound Deltaflexin-1 inhibited K-Ras and PDE6D binding very potently at micro-molar concentrations. The binding affinity value was only 2.6-fold higher than that of the known PDE6D inhibitor, Deltarasin (I: Figure 1B, C, Table 1). Moreover, compound **17**, in which the hexyl-linker was replaced by a methyl cyclohexylmethyl linker also had similar binding affinity as Deltaflexin-1, suggesting that this compound is a good control for cellular assays to test the ejection by ARL2. Furthermore, compound **14**, which lacked the bottom phosphodiester- and cell penetration-group, showed poor binding activity (I: Figure 1C, Table 1). Compound **19**, which had a bottom phosphodiester group but without the cell penetration group also showed poor PDE6D binding. Therefore, together with compound **17**, compound **14** and **19** were also good controls for cellular experiments.

Next, we docked Deltaflexin-1 and Deltasonamide derivative compound 8 (Martin-Gago et al., 2017) with the crystal structure of PDE6D. Interestingly, Deltaflexin-1 bound to PDE6D in a similar fashion as Deltasonamide i.e., three out of seven binding positions of Deltaflexin-1 and Deltasonamide were in common. Phosphodiester which is present in the bottom moiety of Deltaflexin-1 forms the hydrogen bonds with Arg61 and Gln78 at the base of the prenyl-binding pocket of PDE6D (I: Figure 1D, Figure SI D,E). Moreover, the top moiety interacts with Glu88, Ala112 and Met117 of PDE6D. We also computationally docked Deltaflexin-1 into PDE6D in the open and closed (ARL2-bound) state. There was a steric hindrance between the protected phosphodiester at the bottom of the

pocket at Arg61. The presence of the flexible hexyl-linker may facilitate the protected phosphodiester bottom moiety of Deltaflexin-1 to escape this hinderance.

5.1.2 Deltaflexins block intracellular PDE6D/K-Ras interaction and K-Ras membrane organization

To test the intracellular disruption between PDE6D/K-Ras, we used a Fluorescence Lifetime Imaging Microscopy-Fluorescence Resonance Energy Transfer (FLIM-FRET) assay. We tagged mGFP to K-RasG12V, which acts as donor fluorophore and mCherry to PDE6D which acts as acceptor fluorophore. Upon interaction, a donor fluorophore may transfer energy to an acceptor fluorophore, which results in decrease in lifetime of donor fluorophore. Interaction of these two tagged proteins (mGFP-KRasG12V and mCherry-PDE6D) in HEK293 EBNA, hereafter HEK cells, a decrease of the lifetime of the donor fluorophore is observed, which corresponds to an increase in FRET efficiency. Decrease in FRET efficiency, directly corresponds to an inhibition of the interaction between the two proteins. Interestingly, similar to Deltarasin, Deltaflexin-1 significantly inhibited the interaction between PDE6D/K-Ras (I: Figure 2A). Moreover, control compound 17 in which the flexible hexyl-linker was replaced by a methyl-cyclohexylmethyl linker was not able to inhibit the interaction in cells. However, this compound inhibited the in vitro PDE6D/K-Ras interaction in the SPR assay (I: Figure 1B,C, Table 1). Therefore, we concluded that the hexyl linker plays an important role to keep the inhibitory function of the novel compounds. To support the role of the hexyl linker, we shortened the linker to 4 carbons (methyl linker) in compound 20. Interestingly, similar to compound 17, compound 20 was not able to block the interaction (I: Figure 2A). Compounds without cell penetration group like 14 and 19 also did not impair the interaction between PDE6D/K-Ras (I: Figure 2A). This may be because they did not penetrate into cells. RHEB is another farnesylated client of PDE6D involved in the activation of the mTOR signaling pathway (Chandra et al., 2011). Similar to the PDE6D/K-Ras interaction, only Deltaflexin-1 and other compounds which have a hexyl linker and the cell penetration group disrupted the interaction between PDE6D and RHEB (I: Figure S2B).

Blocking the interaction of PDE6D/K-Ras inhibits the correct localization of K-Ras on the plasma membrane. The inhibition of PDE6D impacts more on the localization of K-Ras than on H-Ras (Schmick et al., 2014). To study the effect on

membrane organization of Ras, we did the cellular FLIM-FRET assay. We tagged mGFP-K-RasG12V or mGFP-H-RasG12V for donor and mCherry-K-RasG12V or mCherry-H-RasG12V for acceptor fluorophore. A decrease in FRET is observed if there is a disruption in Ras membrane organization (Berney and Danuser, 2003; Najumudeen et al., 2015). Indeed, treatment with Deltaflexin-1 impaired K-Ras membrane organization but not H-Ras. By contrast, treatment with an FTI selectively inhibited the membrane organization of H-Ras more than K-Ras, because K-Ras can be alternatively prenylated by geranylgeranyl transferase 1 (Lerner et al., 1997; Sun et al., 1999) (I: Figure 2B,C). Unlike Deltaflexin-1, Deltarasin inhibited membrane organization of both K-Ras and H-Ras. However, silencing of PDE6D decreased membrane organization of only K-Ras but not H-Ras, consistent with the fact that PDE6D regulates the trafficking of K-Ras more than that of H-Ras. Moreover, these results also suggested that the higher inhibition effect of Deltarasin on cancer cell proliferation were most probably due to off-target effects. Moreover, similar to previous results other control compounds 19, 14 and 17 did not show inhibitory activity towards K-Ras or H-Ras membrane organization. Thus, the flexible hexyl linker plays a critical role to enable tolerance against ARL2 ejection.

5.1.3 Deltaflexins inhibit oncogenic K-Ras-dependent cell proliferation and sphere formation

To examine the selectivity of novel compounds on 2D cell proliferation and tumorosphere formation (3D). Tumorosphere formation is clonogenic, anchorage independent and serum free tumorosphere growth in 3D culture, is a first line measure of the cancer cell stemness properties (Dontu et al., 2003). For these studies, we chose mutant KRAS cell lines HCT116 ($KRAS^{G13D}$), MDA-MB-231 ($KRAS^{G13D}$); HT29 (wildtype KRAS) and Hs 578T ($HRAS^{G12D}$). Deltaflexin-1 significantly inhibited cell proliferation of K-Ras driven colorectal cancer cells (HCT116) with an IC_{50} =11 μM but it had less effect on wildtype HT29 (IC_{50} = 40 μM) (I: Figure 3A,B). A similar activity of Deltaflexin-1 was also seen in breast cancer cell lines MDA-MB-231 and Hs 578T. Deltaflexin-1 decreased proliferation of MDA-MB-231 with a potency of IC_{50} = 7.2 μM but it showed less effect on HRAS mutant Hs 578T (IC_{50} = 21 μM) (I: Figure S3A,B,G).

Deltaflexin-1 impaired tumorosphere growth only in K-Ras mutant MDA-MB-231 cells but not H-Ras mutant HT-29 cells (I: Figure 2C,D). Treatment of Deltaflexin-1 significantly decreased tumorosphere formation only in K-RasG12V

overexpressing HEK cells but not mutant H-RasG12V overexpressing cells after knock down of PDE6D. (I: Figure S3 H-J). Similarly, Deltaflexin-1 derived control compounds 17 and 20 without hexyl and short linker, respectively, had no significant effect on tumorosphere formation (I: Figure 3C,D). From these results we concluded that compounds which have a hexyl linker and cell penetration group were able to inhibit K-Ras derived cancer cell proliferation and tumorosphere formation.

5.1.4 Improved design of second generation Deltaflexin compounds

After obtaining promising results from Deltraflexin-1 experiments, we developed a next generation of compounds. In the second generation we inserted a terephthalic acid moiety in place of the potentially toxic coumarin ring. This replacement generates compounds with some degree of similarity with bis-sulfonamide Deltasonamide derivative (compound 8) (I: Figure 4A) (Martin-Gago et al., 2017). We also changed the bottom moiety to a pharmacologically more stable 2-methyl-substituent on the S-acyl cell penetration group. Compound **23** (Deltaflexin-2) and its analogous compound **22** had improved binding affinity as compared to Deltaflexin-1, (Kd value only 2.92 μM) in SPR assay (I: Figure 4B, Table 1, Figure S4A, Table S1).

Computational docking data suggested that our 2nd generation compounds bind to similar positions on the PDE6D pocket as Deltaflexin-1 (I: Figure S4B). Interestingly, additional hydrogen bonding to Ser 115 by both compounds **22** and **23** was observed. Moreover, one more hydrogen bonding with Glu88 only by compound **23** provided extra strength for binding to PDE6D (I: Figure S4B).

Next, we tested the 2nd generation compounds in cellular assays. Compound **23** inhibited the PDE6D/K-Ras interaction in similar manner as Deltaflexin-1 as measured by using FLIM-FRET, but performed better than compound **22** (I: Figure 4C). Intriguingly, both compounds **22** and **23** decreased K-Ras membrane organization in a dose-dependent manner but compound **24** and **25** which have a 4n-methylen-linker failed to show this activity (I: Figure 4C). Furthermore, 2nd generation active compounds not inhibited H-Ras membrane organization (I: Figure S4C). Thus, similar to Deltaflexin-1, 2nd generation compounds selectively blocked the activity of K-Ras. Intriguingly, Deltaflexin-2 reduced 70% of sphere formation as compared to ARS-1620 in KRAS^{G12C} mutant NCI-H358 lung cancer

cells. Moreover, Deltaflexin-2 had no activity against B-RafV600E mutant A375 lung cancer cells (I: Figure 4E). These results confirmed that similar to Deltaflexin-1, Deltaflexin-2 also significantly inhibited spheroid growth in a K-Ras dependent manner (I: Figure 4E).

Previous PDE6D inhibitors need further improvements in cellular availability and activity, which can be seen by the different in vitro and cellular activities. These inhibitors demonstrate an approximately 1000-fold lower cellular activity in proliferation assays as compared to in vitro inhibition data. There was no big difference between in vitro and the cellular activity of Deltaflexin compounds, an advancement, which may be attributed to the presence of the cell penetration group and flexible hexyl linker. Furthermore, Deltaflexin compounds clearly act on-target in cells and thereby, selectively inhibit K-Ras activity. On the other hand, Deltarasin blocked the activity of K-Ras and H-Ras to an equal magnitude in the Ras membrane organization assay which may probably be because of the off-target activity of Deltarasin.

Deltaflexin-1 inhibited proliferation of mutant *KRAS* cancer cells at concentrations that were only 7 to 11-fold higher than the in vitro K_d determined in the K-Ras/PDE6D interaction SRP assay. However, as compared to Deltaflexin-1, Deltasonamide 2 had a cellular activity 650 to 1300 -fold lower than in vitro activity. This means that Deltaflexin-1 had a similar in vitro and cellular inhibitory activity. The better activity of Deltaflexin-2 as compared to Deltasonamides may be due to the presence of the one ring instead of two ring system allowed more flexibility of the top moiety. Moreover, both compounds **22** and **23** form additional hydrogen bonds with Ser 115. Furthermore, presence of the flexible hexyl linker provided resistance against ARL2 ejection. Deltaflexin-1 and -2 compounds reduced sphere formation efficacy in K-Ras derived cancer cells but Deltarasin inhibited tumorosphere growth in both *KRAS* and *HRAS* mutant cells. In conclusion our novel PDE6D inhibitors inhibit cancer cells by selectively blocking the activity of K-Ras.

5.2 Development of a split *Renilla* luciferase assay to identify Hsp90/Cdc37 protein-protein interface inhibitors (II)

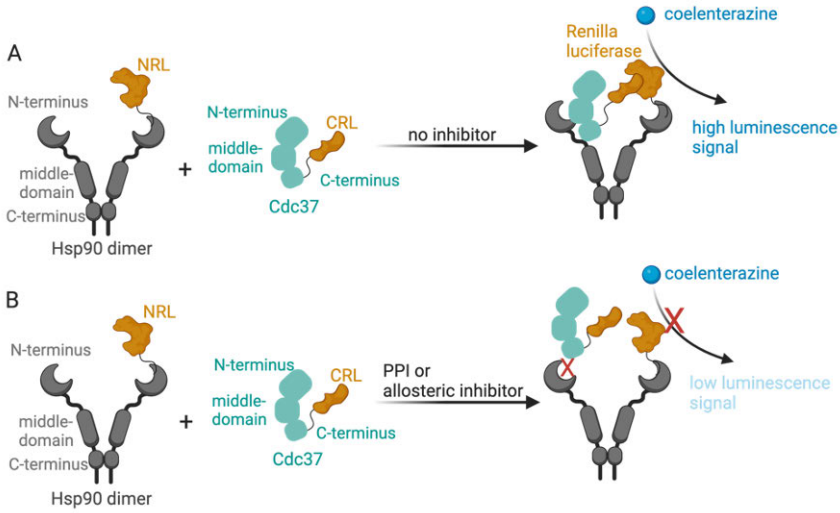


Figure 14: Schematic representation of the split *Renilla* luciferase assay for the detection of Hsp90/ Cdc37 complex inhibitors. In the reaction mixture, the NRL-Hsp90 interacts with the Cdc37-CRL fragment, which results in the formation of functional *Renilla* luciferase that is able to produce a high luminescence signal in the presence of coelenterazine (A). In the presence of a Hsp90/ Cdc37 protein-protein interface inhibitor or allosteric C-terminal inhibitor, NRL-Hsp90 is not able to interact with Cdc37-CRL, resulting in non-functional *Renilla* luciferase, leading to a loss of signal (B).

Currently, several Hsp90 inhibitors are available but none of them has reached the clinic, so far. N-terminal ATP competitive inhibitors like GA and 17-AAG keeps the open ADP-bound conformation of Hsp90 dimer. Thus, these compounds efficiently target and block the function Hsp90 chaperone machinery. Due to their toxicity, they are failed in clinical trials (Zuehlke et al., 2018). Unlike N-terminal ATP-pocket inhibitors, C-terminal nucleotide pocket binding inhibitors do not induce the heat shock response (Wang et al., 2020b). Direct protein-protein interface (PPI) inhibitors that block the interaction between Hsp90/Cdc37 are another class of Hsp90 inhibitors. For instance, conglobatin A leads to the

degradation of client kinases of Hsp90 (Huang et al., 2014). Moreover, conglobatin A selectively blocks the activity of K-Ras and therefore impairs cancer stemness properties (Najumudeen et al., 2016). Conglobatin A is a bacterial macrolide obtained from Streptomyces conglobatus, which bears some difficulties for its synthesis. Therefore, chemo-synthetically more accessible inhibitors that block Hsp90/Cdc37 are of interest. Here we first developed a cell lysate-based assay to identify inhibitors that block the interaction between Hsp90/Cdc37 (Figure 14).

5.2.1 Assay workflow and cell lysate preparation

In order to discover novel Hsp90/Cdc37 inhibitors we used a previously developed split *Renilla* based assay (Jiang et al., 2010). In this assay the N-terminus of *Renilla* luciferase (NRL) was fused with full length human Hsp90 (NRL-Hsp90) and the C-terminus of *Renilla* luciferase (CRL) was fused with human Cdc37 (Cdc37-CRL) (II-Figure 1A). In the reaction mixture, when Hsp90 interacted with Cdc37, the fragments of *Renilla* luciferase interacted to form fully functional *Renilla* luciferase protein that produces luminescence signal in the presence of the substrate coelenterazine (II: Figure 1A). Any compound that inhibits the interaction between Hsp90/Cdc37, is able to significantly decrease the luminescence signal. We used this approach for the identification of novel Hsp90/Cdc37 inhibitors in the final part of this thesis.

We overexpressed NRL-Hsp90 and Cdc37-CRL separately in HEK cells. Harvested cells were lysed in the buffer provided in a commercial kit (*Renilla* luciferase assay kit). The supernatant was collected after centrifugation (II: Figure 2A-C). We used 96 well plate with 50 µl reaction mixture. Thus also a 384 well plate can be used for high-throughput screening. The components of the reaction mixture were cell lysate assay buffer, a test compound, (NRL-Hsp90 and Cdc37-CRL) and substrate coelenterazine. All of these components were added sequentially as illustrated in (II: Figure 2D-F).

Next, we determined that the luminescence signal produced in the reaction is only because of interaction between Hsp90/Cdc37 not of direct interaction between NRL and CRL. We, therefore, measured the luminescence signal only in the presence of NRL and CRL. The signal produced by only NRL and CRL was approximately 5 folds lower than the signal produced by NRL-Hsp90 and Cdc37-CRL (II: Figure 3B). These results confirmed that luminescence activity in the reaction is due to the Hsp90/Cdc37 interaction.

5.2.2 Compounds can be preincubated with either NRL-Hsp90 or Cdc37-CRL

NRL-Hsp90 and Cdc37-CRL require some time to form fully mature *Renilla* luciferase. Therefore, we measured the time needed by split *Renilla* fragments to form a fully active protein. In the reaction mixture containing assay buffer, NRL-Hsp90, Cdc37-CRL and then immediately substrate were added by an injector to the well. Then, immediately the luminescence signal was read every 10 s for 700 s. We found a maximum signal at 150 s and this signal was stable for up to 400 s (II: Figure 4C). Therefore, we selected 2 min incubation time to form fully functional *Renilla* luciferase.

5.2.3 Order of components in reaction mixture

NRL-Hsp90, Cdc37-CRL, assay buffer, test compound and substrate were the components of the assay. In order to determine the order of components adding to the reaction, we first incubated test compound with NRL-Hsp90 in following order: assay buffer followed by 20 μM conglobatin A or withaferin A. Cdc37-CRL was added after 0 to 10 min. Interestingly, no difference in the signal was seen, suggesting the binding of inhibitor to Hsp90 was very fast (II: Figure 4A). Similar results were obtained if we preincubated compounds first with Cdc37. Thus, we concluded that the preincubation of inhibitors with NRL-Hsp90 or Cdc37-CRL did not affect the inhibition of Hsp90/Cdc37 complex.

5.2.4 Effect of temperature on assay

To test whether there is any effect of temperature on the reaction, we ran the reaction at various temperatures. Substrate was added to the reaction containing NRL-Hsp90 and Cdc37-CRL, then readings were measured after 10 s of shaking at 24 °C or 30 °C or 35 °C. The highest reading was detected at 24 °C after 30 s, this signal was stable for up to 180 s (II: Figure 4E). The lowest *Renilla* luciferase activity was observed at higher temperatures (II: Figure 4E). These results suggest that the maximum activity of *Renilla* luciferase may be at 20 to 25 °C. Therefore, we selected 24 °C temperature, 2 min complex formation time and a reading taken 40 s after substrate addition including 10 s plate shaking.

5.2.5 Validation of the assay with known Hsp90 and Hsp90/Cdc37 inhibitors

To ensure that our assay is suitable for screening for Hsp90/Cdc37 inhibitors, we first tested various types of known Hsp90 and Hsp90/ Cdc37 inhibitors. We started by comparing activities at 20 μM concentration to test these compounds. As expected, N-terminus ATP-pocket Hsp90 inhibitors like 17-AAG, geldanamycin and luminespib were almost inactive on the Hsp90/Cdc37 complex inhibition. But C-terminus ATP binding pocket inhibitor EGCG inhibited the Hsp90/Cdc37 complex formation. But novobiocin had no effect on this interaction (II: Figure 2A). Allosteric C-terminal Hsp90/Cdc37 interaction inhibitors like celastrol and withaferin A also showed the expected inhibitory effect. Direct PPI Hsp90/Cdc37 complex inhibitors significantly decreased the luminescence signal but platycodin D had no inhibitory effect at 20 μM (II: Figure 5A) (Li et al., 2017).

To confirm that the decrease in signal is only due to the inhibition of complex formation but not due to direct inhibition of *Renilla* luciferase enzyme, we performed the same assay using only full length *Renilla* luciferase. None of the compounds had an effect on the luminescence signal except for celastrol and to a small extent also withaferin A (II: Figure 5B). Celastrol most efficiently decreased the luminescence signal. In the absorption spectra we found that celastrol's maximum absorption was at 480 nm, which was close to the emission peak of *Renilla* luciferase with coelenterazine. This experiment suggested that celastrol absorbed the *Renilla* luciferase catalyzed luminescence signal (II: Figure S1B). Withaferin was the 2nd most potent compound against the Hsp90/Cdc37 complex and did to a small, but significant extent inhibit the luminescence signal of full length *Renilla* luciferase, while no absorption was detected at 480 nm. These data may suggest that these compounds directly bind to *Renilla* luciferase and thereby, inhibit its activity unspecifically.

Next, we determined the IC_{50} of all compounds by using our optimized assay. We found similar results as we got with 20 μM concentration. N-terminus Hsp90 inhibitors had poor IC_{50} but C-terminus nucleotide pocket inhibitors exhibited IC_{50} -values between 40 to 95 μM (II: Figure 5C,D). The C-terminus allosteric inhibitors showed very potent IC_{50} (II: Figure 5E). Hsp90/Cdc37 PPI inhibitor conglobatin A also showed good IC_{50}, 7.2 μM (II: Figure 5G). To confirm that this assay was good enough for screening compounds, we calculated the Z' value with

withaferin A or conglobatin A as positive controls. Z' is a measure of statistical effect size. It has been proposed for use of in high-throughput screening. For high-throughput screening the Z' should be more than 0.5 (Zhang et al., 1999). The Z' determined at 20 μM concentration was 0.71 for withaferin A (C-terminal allosteric inhibitor) and 0.52 for conglobatin A (II: Figure 5G,H). Thus, our assay is suitable for medium-throughput screening for the identification of Hsp90/Cdc37 inhibitors.

In conclusion, the assay has a good Z' value and therefore, is suitable for medium-throughput screening of compounds. The split *Renilla* luciferase, cell lysate-based assay can be used for the identification of two types of Hsp90/Cdc37 inhibitors. These two types are direct PPI inhibitors like conglobatin A and C-terminal allosteric inhibitors like withaferin A. Of note, preparation of a mammalian cell lysate-based assay is less time consuming because it does not require the purification of proteins, which can be difficult for some proteins.

Previously the direct PPI inhibitor, Platycodin D reduces the fraction of Hsp90-bound Cdc37 without affecting the expression levels of both Hsp90 and Cdc37 (Li et al., 2017). By contrast, platycodin D did not inhibit the Hsp90/Cdc37 complex formation in our assay. It may therefore be concluded that the described inhibition of Hsp90/Cdc37 complex formation may be because of an indirect effect of Platycodin D or it is not an inhibitor of Hsp90/Cdc37. Finally, one limitation in our assay which we have seen with celastrol is that colored compounds, which absorb the luminescence signal are not good candidate to test in the split *Renilla* luciferase assay.

5.3 Targeting Hsp90/Cdc37 by novel small molecules to disrupt K-Ras nanoclustering and signaling (III)

The Hsp90 chaperone machinery helps in folding and maturation of hundreds of client proteins. These client proteins comprise kinases (Raf, Akt, ERK) and transcription factors (HIF-1α, NF-κB) (Schopf et al., 2017; Taipale et al., 2012). Conglobatin A binds to the N-terminal of Hsp90 thus inhibiting the interaction with its co-chaperone Cdc37 (Huang et al., 2014). Our group discovered that conglobatin A selectively disrupts the K-Ras membrane organization and inhibits cancer stemness (Najumudeen et al., 2016). However, how exactly conglobatin A or inhibition of Hsp90/Cdc37 blocks activity of K-Ras and cancer stemness is unclear. Conglobatin A showed the good anti-tumor activity both in vitro and in vivo in breast cancer cells (Huang et al., 2014). Thus, because of promising results of Hsp90/Cdc37 inhibitor we identified novel Hsp90/Cdc37 inhibitors by using our well established split *Renilla* luciferase assay (Siddiqui et al., 2020).

5.3.1 Inhibition of Hsp90/Cdc37 selectively disrupts K-Ras nanoclustering and signaling

Ras proteins are organized into nanoscale, oligomeric signaling packages in the plasma membrane, called nanocluster. Ras proteins tagged with FRET-fluorophores give high FRET because of their dense packing in nanoclusters (Abankwa et al., 2007).

Conglobatin A selectively blocked the K-RasG12V nanoclustering in HEK cells (III: Figure 1A) (Najumudeen et al., 2016). To confirm that conglobatin A disrupts the interaction between Hsp90/Cdc37, we used split *Renilla* assay. Treatment of conglobatin A inhibited the interaction between Hsp90/Cdc37 (III: Table 1; Figure SI 1A-C) (Huang et al., 2015; Siddiqui et al., 2020).

Previous studies show that Glu47 and Asp54 of Hsp90 interact with Arg167 of Cdc37 and Gln133 of Hsp90 binds with Asp170 of Cdc37 (III: Figure SI ID) (Roe et al., 2004). The computational docking model of conglobatin A with the N-terminus fragment of human Hsp90 suggested that conglobatin A forms hydrogen bonds with residues Ser50, Asp54, Asp57 and Gln133 (III: Figure 1C, Figure SI 1E). The conglobatin A binding site overlaps with the Cdc37 interaction site. Additionally, conglobatin A formed hydrogen bonds to Glu47 and Ser50 with

Hsp90 and, thereby, hinders the most important interactions between Hsp90 and Cdc37.

Further, to test the effect of Hsp90 or Cdc37 on K-Ras and H-Ras nanoclustering, we knocked down both human cytoplasmic Hsp90α/Hsp90β or Cdc37 in our FRET assay. Intriguingly, knock down of both Hsp90 or Cdc37 decreased only K-RasG12V nanoclustering but no effect was seen on H-RasG12V nanoclustering (III: Figure 1D,E). Moreover, inhibition of Hsp90 by the ATP competitive inhibitor, 17-AAG also selectively decreased the K-RasG12V nanoclustering. These results suggested that conglobatin A inhibited the K-Ras activity by inhibiting the function of Hsp90 chaperone machinery. Inhibition of Hsp90 results in proteasomal degradation of client proteins (Schneider et al., 1996). To rescue this effect we pretreated HEK cells with proteasomal inhibitor bortezomib. Interestingly, the treatment of conglobatin A in the presence of bortezomib incompletely but significantly, rescued the K-RasG12V nanoclustering (III: Figure 1F). Thus, inhibition of Hsp90/Cdc37 decrease the expression of client proteins by proteasomal degradation that are directly or indirectly required for the K-Ras nanocluster formation.

Inhibition of K-Ras nanoclustering by inhibiting Hsp90 has a direct effect on MAPK signaling (Tian et al., 2007). We therefore checked the expression level of ERK and phosphorylated ERK after treatment with conglobatin A. Conglobatin A or 17-AAG decreased the expression level of ERK1/2 and pERK1/2 in K-Ras mutant MDA-MB-231 cells. Moreover, conglobatin A also decreased the expression of Akt which confirmed the effect of Hsp90/Cdc37 inhibitors on the degradation of kinases (III: Figure 1G). Furthermore, conglobatin A decreased MDA-MB-231 cell proliferation at IC_{50} 32 ± 2 μM (III: Figure 1H). To test the effect of inhibition of Hsp90/Cdc37 by conglobatin A on microtumor growth, we grew MDA-MB-231 cells on the chick chorioallantoic membrane (CAM) to form a microtumor. Inhibition of Hsp90 by both conglobatin A or 17-AAG significantly decreased the microtumor growth.

5.3.2 Inhibition of Hsp90 downregulates K-Ras nanocluster scaffold galectin-3 by depletion of Hsp90 client HIF-1α

Higher expression of galectin-3 (Gal3) increases K-RasG12V nanoclustering but not H-RasG12V nanoclustering (Shalom-Feuerstein et al., 2008). Conglobatin A or 17-AAG treatment decreased the expression level of Gal3 in K-RasG12V

overexpressing HEK and MDA-MB-231 cells (III: Figure 2A). This explains that inhibition of Hsp90 decreases the K-RasG12V nanoclustering by decreasing the expression of Gal3. Moreover, the silencing of Hsp90 or Cdc37 by using siRNA, decreased the expression of Gal3 in both HEK and MDA-MB-231 cells (III: Figure 2B). To study the direct effect of Gal3 on K-RasG12V nanoclustering in our system, we overexpressed Gal3 with the K-RasG12V FRET pair in HEK cells. As expected Gal3 significantly, increased the K-RasG12V nanoclustering. Moreover, overexpression of Gal3 partially rescued the conglobatin A decreased K-RasG12V nanoclustering (III: Figure 2C). According to Picard's laboratory Hsp90 interactors list, Gal3 is not a client of Hsp90, hence the downregulation of Gal3 on inhibition of Hsp90 may be due to the blocking of the transcriptional activity of the Hsp90 client HIF-1α. HIF-1α upregulates in the hypoxic response, and regulates several genes necessary for the survival of cancer cells in unfavorable conditions (Jun et al., 2017). The expression of Gal3 is regulated by HIF-1α. Therefore Gal3 is upregulated in the hypoxic response (Ikemori et al., 2014; Zeng et al., 2007). It is well established that $CoCl_2$ enhances the stability of HIF-1α, thus upregulates the expression of HIF-1α targeted genes (Zhang et al., 2014). Stabilization of HIF-1α by treatment of $CoCl_2$ increased the expression level of Gal3 in HEK and MDA-MB-231 cells (III: Figure 2D). Furthermore, treatment with conglobatin A or 17-AAG decreased the nuclear accumulation of HIF-1α in both HEK and MDA-MB-231 cells (III: Figure 2E). Silencing of HIF-1α or blocking of the transcriptional activity by CAY10585 in K-RasG12V overexpressing HEK and MDA-MB-231 cells decreased the expression of the Gal3 (III: Figure 2F,G). Interestingly, inhibition of HIF-1α significantly decreased the K-RasG12V nanoclustering to the same magnitude as knockdown of Gal3 or treatment with conglobatin A (III: Figure 2H). No effect of inhibition of HIF-1α or knockdown of Gal3 was observed on H-RasG12V nanoclustering (III: Figure 2I). We conclude that inhibition of Hsp90 by conglobatin A blocked the transcriptional activity of HIF-1α and thereby, decreased the expression of K-Ras nanocluster scaffold Gal3, downstream of HIF-1α.

5.3.3 Identification of novel Hsp90/Cdc37 inhibitors by using computational docking and split *Renilla* assay based screening

For the identification of novel Hsp90/Cdc37 inhibitors, we first computationally screened 120,000 compounds from the Institute for Molecular Medicine Finland

(FIMM), by using conglobatin A as a docking model. We did this work in collaboration with Dr. Tiina Salminen, Åbo Akademi University (III: Figure 3A). In silico screening from the FIMM chemical library resulted in 82 hit compounds. Compounds from in silico hits were further screened in vitro by using the split *Renilla* luciferase assay (III: Figure SI 1C, 2A). This in vitro validation identified 14 hit compounds with four different scaffolds (III: Figure SI 2B). Next, we screened compounds based on the identified scaffolds. For the 2nd round FIMM in silico screening, we also computationally screened 7 million compounds from the MolPort library. These screenings resulted in 54 hit compounds from FIMM library and 58 compounds from MolPort. Out of these FIMM (2nd round) and MolPort hits, only 41 compounds were available for the repurchasing for testing on split *Renilla* luciferase assay. In split *Renilla* assay we found that three scaffolds with 9 hit compounds significantly inhibited the Hsp90/Cdc37 interaction with an IC_{50} of less than 60 μM. Three out of nine hit compounds were large polyphenols, therefore we excluded those compounds because of their potential toxicity. The remaining 6 compounds had drug-like characteristics based on Lipinski's rule of five (III: Figure 3B, Figure SI 2C-F).

Four compounds were selected from two scaffolds (x1540, x1742 and x6506, x1625) for testing of their effect on binding to N-terminus Hsp90 ATP pocket. Interestingly, none of these compounds displaced the ATP competitive inhibitor geldanamycin at 20 μM concentration from the Hsp90 ATP pocket (III: Figure 3C, SI 3A). The most active compounds x6506 and x1540 were incapable to displaced geldanamycin from Hsp90 up to 50 μM concentration (III: Figure 3B,C). In computational docking to N-terminus Hsp90 (N-Hsp90), both x6506 and x1540 bounded to N-Hsp90 in a different manner in our docking system (III: Figure 3D,E). Compounds x6506 and x1540 interacted with the Asp54 as well as Mg^{2+} ion which is a part of the ATP-pocket. Compound x1540 additionally formed the hydrogen bond with Glu47 of Hsp90, Glu47 of Hsp90 is crucial for binding Arg167 of Cdc37. Furthermore, x1540 interacted with Ser50 and Gln133 by hydrogen bonding and by π-cation interaction with Arg46 (III: Figure 3E). Compound x6506 orients with its bulky adamantyl substituent toward a small hydrophobic pocket demarcated by Phe213 (III: Figure 3D). Other two compounds (x1742 and x1625) of the same scaffolds also showed the same binding pattern, although x1625 seems to implement a mixed orientation (III: Figure SI 3D,E). Compound x6506 seemed to be the most potent in the split *Renilla* assay with an IC_{50} of only 5 μM, while compound x1625 from the same scaffold was less potent, IC_{50} = 41 μM. This high potency of x6506 was probably due to the presence

of a large adamantly substituent closer to Phe213, which constitutes a stronger sterical hindrance for the binding of Cdc37 to Hsp90. We conclude that x6506 and x1540 may bound to N-Hsp90 other than the ATP pocket, thus inhibiting the interaction with Cdc37. To differentiate real N-terminal Hsp90 PPI inhibitors from allosteric ones, we constructed the N-terminus of Hsp90 tagged with NRL paired with the Cdc37-CRL. C-terminal allosteric inhibitors failed to inhibit the interaction between N-terminus Hsp90 and Cdc37 (data not shown).

5.3.4 Both x6506 and x1540 downregulate Hsp90 clients and Gal3 to selectively target K-Ras

We next tested x6506 and x1540 Hsp90/Cdc37 inhibitors on various cancer cell lines. KAS mutant cancers such as pancreatic and lung adenocarcinoma have a high level of HIF-1α and Gal3 (III: Figure SI 4A,B). High levels of HIF-1α and Gal3 associated with poor survival of human cancer patients (III: Figure SI 4C,D). For the selection of cell lines to test our novel inhibitors, we used ATARiS dependency data from RNAi screen in Project DRIVE (McDonald et al., 2017). On the basis of K-Ras, HIF-1α and Gal3 dependency we selected various cell line like MDA-MB-231, MIA PaCa-2 and HCC-44 (III: Figure SI 4E).

To further establish these compounds as inhibitors of Hsp90/Cdc37, we checked the expression level of Hsp90 client kinases and Gal3 upon treatment with x6506 and x1540. Treatment of both x6505 and x1540 decreased expression level of HER2, C-Raf, AKT and ERK1/2 in a dose dependent manner in SK-BR-3, MDA-MB-231, MIA PaCa-2 and HCC-44 cells.-(III: Figure 4A-D). Moreover, 17-AAG induced the heat shock response by heat shock factor-1 (HSF-1) and thereby, increased the expression of both Hsp70 and Hsp90. Interestingly, Hsp90/Cdc37 inhibitors did not induced the heat shock response (Li et al., 2009; Wang et al., 2019). We also have not seen any effect of x6506 or x1540 treatment on the expression levels of Hsp70 and Hsp90, confirming that these compounds do not induce the heat shock response (III: Figure 4A-D). K-Ras nanoclustering scaffold Gal3 and transcriptional activator of Gal3 known as HIF-1α were also decreased upon the treatment of x6506 and x1540 (III: Figure 4A-D). We next tested these compounds on K-Ras and H-Ras nanoclustering-FRET. Like conglobatin A both x6506 and x1540 selectivity inhibited the K-Ras nanoclustering (III: Figure 4E,F). Thus, both x6506 and x1540 inhibited Hsp90 client proteins in similar manner as conglobatin A.

5.3.5 x6506 and x1540 decrease cancer cell proliferation and microtumor growth CAM assay

We next studied the effect of x6506 and x1540 on cancer cell proliferation in breast, pancreatic and lung cancer cell lines. Both compounds showed higher activity in pancreatic cancer cell line MIA PaCa-2 (III: Figure 5A, Table 2). We found IC_{50} values between 23 μM to 49 μM, which was comparable to the potencies found in vitro (III: Table 1). We further examined the effect of these compounds on spheroid formation in 3D culture, we found IC_{50} between 26 μM to 67 μM (III: Figure 5B). Compound x6506 showed more potency than x1540. Moreover, inhibition of Hsp90 by x6506 or x1540 or 17-AAG significantly decreased the microtumor growth of MDA-MB-231 cells in CAM assay (III: Figure 5C).

5.3.6 Conglobatin A depletes Hsp90 client kinases B-Raf and C-Raf (unpublished data)

B-Raf and C-Raf are client kinases of the Hsp90 chaperone machinery (Neckers and Workman, 2012). Therefore, we checked the expression level of all Raf proteins upon inhibition of Hsp90/Cdc37 in various cell lines. Interestingly, inhibition of the Hsp90/Cdc37 interface by conglobatin A significantly decreased the protein expression level of B-Raf and C-Raf in a dose dependent manner (Figure 15A). The effect of conglobatin A was least observed on the expression level of A-Raf in HEK and MDA-MB-231 cells. Next to confirm this degradation of kinases was due to the inhibition of Hsp90/Cdc37 complex, we silenced both HSP90 or CDC37 in K-Ras overexpressing HEK and MDA-MB-231 cells (III Figure 2B). Interestingly, silencing of HSP90 or CDC37 degraded B-Raf and C-Raf in both cell lines. Similar to the above results knockdown of HSP90 or CDC37 showed less effect on degradation A-Raf (Figure 15B). Our concluded from our results, that both B-Raf and C-Raf depend upon the Hsp90/Cdc37 chaperone machinery for their maturation but A-Raf is less depended upon the Hsp90.

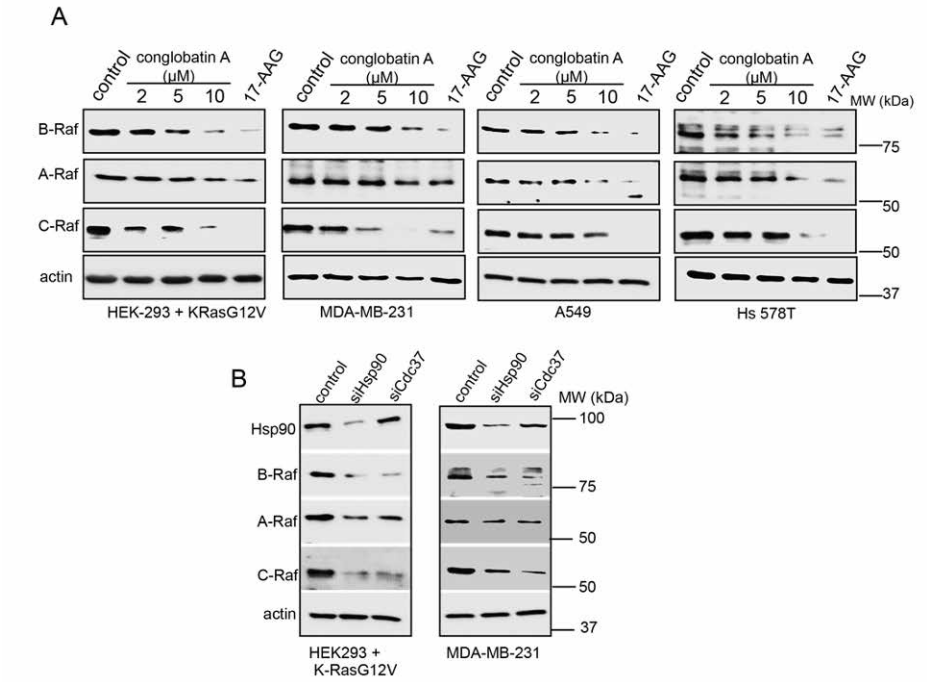


Figure 15: Conglobatin A decreases Hsp90 client Raf kinases (A) Western blots of HEK cells transfected with mGFP-K-RasG12V or of MDA-MB-231 or A549 or Hs 578T cells treated with either 0.1 % DMSO vehicle control or with indicated concentrations of conglobatin A or 2 μM 17-AAG. All drug treatments were for 24 hours (**B)** Western blots of HEK cells transfected with mGFP-K-RasG12V and of MDA-MB-231 cells 48h after knockdown using 50 nM for scramble control or 50 nM siRNA-Hsp90 or 50 nM siRNA-Cdc37.

5.3.7 Downregulation of B-Raf and C-Raf disrupt K-Ras membrane organization (unpublished data)

Inhibition of Hsp90/Cdc37 by conglobatin A selectively blocks K-Ras nanoclustering. In our previous results we also found that Hsp90/Cc37 inhibition depletes both B-Raf and C-Raf. Moreover, we previously showed that A-Raf and Gal1 stabilize H-Ras nanocluster (Blazevits et al., 2016). In analogy to H-Ras nanocluster, we assumed that Raf kinases are important for K-Ras nanocluster formation. We, therefore, analyzed the effect on B-Raf and C-Raf on K-Ras nanoclustering. Silencing of B-Raf significantly decreased the K-Ras nanoclustering but not H-Ras nanoclustering (Figure 16A,B). Next we treated cells with Raf inhibitors sorafenib or PLX-4720, which not only inhibit the kinase

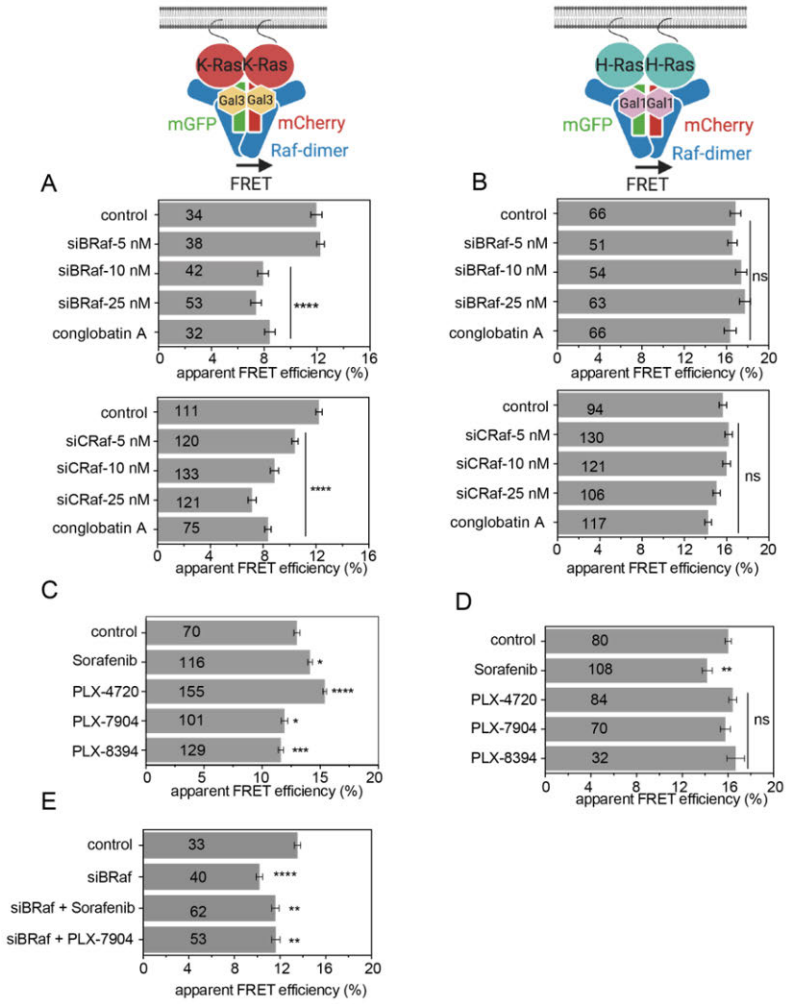


Figure 16: B-Raf or C-Raf disrupts only K-Ras nanoclustering. (A, B) Ras membrane organization studied with nanoclustering-FRET in HEK cells co-expressing mGFP or mCherry tagged K-RasG12V (A) or mGFP or mCherry tagged H-RasG12V (B). Cells were co-transfected with siRNA-B-Raf or siRNA-C-Raf for 48 h or treated with 0.1% DMSO control, 2 μM conglobatin A for 24 h. (C-D) Ras membrane organization in HEK cells co-expressing K-RasG12V-(C) or H-RasG12V-(D) FRET pair, were treated with 10 μM Raf inhibitors that induce dimerization (sorafenib or PLX-4720) or that don't induce dimerization (PLX-7904 or 8394) for 4 h. (E) K-Ras membrane organization co-expressing K-RasG12V FRET pair or 25 nM siRNA-B-Raf. Cells were treated with 10 μM sorafenib or PLX-7904 for 4 h. The numbers on the bars indicate numbers of analyzed cells. Graphs show mean values ± SEM. Statistical significance levels as compared to control are annotated as ****$p < 0.0001$, **$p < 0.001$, *$p < 0.01$; ns, not significant.

activity of B-Raf and C-Raf but induce the dimerization of Raf (hetero or homodimer) (Adnane et al., 2006; Lavoie et al., 2013). Interestingly, both sorafenib and PLX-4720 significantly increased K-Ras nanoclustering-FRET but this effect was not observed on H-Ras nanoclustering-FRET (Figure 16C,D). Furthermore, silencing of B-Raf reversed the K-Ras nanoclustering induced effect of sorafenib and PLX-4720 (Figure 16E). Another type of B-Raf and C-Raf inhibitor are PLX7904 and PLX8394, so called paradox-breaker, that have a very low potential to induce the dimerization of Raf (Tutuka et al., 2017). Consistently, PLX7904 and PLX8394 did have a small negative effect on K-Ras nanoclustering-FRET, but no effect on H-Ras nanoclustering-FRET (Figure 16C,D). Here, we conclude that both B-Raf and C-Raf are required for the K-Ras nanocluster formation. Thus, B-Raf and C-Raf play themselves a critical role in K-Ras nanocluster formation. Based on these results and those from the previous chapters, here, we propose a nanocluster model for K-Ras, where Gal3, potentially as di/oligomer, forms bridges between two or more Raf proteins to stabilize K-Ras nanocluster signaling complex.

This study provides an additional mechanism for how inhibition of the Hsp90/Cdc37 complex by conglobatin A selectively blocks the activity of K-Ras but not H-Ras. Therefore, we concluded that targeting of Hsp90/Cdc37 inhibits cancer cell stemness by selective K-Ras inhibition (Najumudeen et al., 2016; Wang et al., 2015a). Inhibition of Hsp90/Cdc37 depletes a broad range of kinases and HIF-1α. Specifically, conglobatin A treatment decrease expression of HIF-1α and thereby, decreases the expression of its target Gal3. Gal3 stabilizes the K-Ras nanocluster on the plasma membrane thus, the downregulation of Gal3 disrupts the K-Ras nanoclustering and signaling output (Shalom-Feuerstein et al., 2008). Higher expression of HIF-1α and Gal3 is found in pancreatic and lung cancer cells. Therefore, inhibition of Hsp90/Cdc37 complex formation may provide an alternative approach to treat these types of cancers.

Inhibition of Hsp90 affects multiple client proteins including established drug targets like AKT or B-Raf (Kudchadkar et al., 2012; LoPiccolo et al., 2007). This we can also see in our results that inhibition of Hsp90/Cdc37 blocks the activity of many drug targets. Moreover, high expression of Hsp90 and Cdc37 is found in many cancers. Isoforms of Hsp90 vary in cancer types which correlate with drug resistance and stages of cancer (Kim et al., 2019; Sreedhar et al., 2004). High expression of Cdc37 is found in highly proliferative cancer cells (Gray et al., 2007).

Hsp90/Cdc37 inhibition targets multiple oncogenic client proteins, we therefore identified novel Hsp90/Cdc37 inhibitors. Hsp90/Cdc37 inhibitors seem to be a promising strategy as compared to targeting Hsp90, as they may have low on-target toxicity. Moreover, Hsp90/Cdc37 inhibitors do not induce heat shock response which is seen in N terminal ATP pocket inhibitors like 17-AAG. Our molecular docking data showed that novel small molecule inhibitors bind to the N-terminus of Hsp90 close to the ATP pocket but they did not inhibit ATP binding. Similar to conglobatin A, recently discovered inhibitors DDO-5936 and 18h (Wang et al., 2020a; Wang et al., 2019) and both (x6506 and x1540) interact Glu47 and Gln133 on Hsp90, which are crucial residues for the binding of Cdc37. In the light of the anti-cancer and anti-aging potential of Hsp90 inhibitors, Hsp90/Cdc37 interface inhibitors warrant further development, given their potentially low on-target toxicity.

Hsp90 is involved in many diseases other than cancer. Diseases caused by protozoans and elimination of senescent cells are being tested using Hsp90 inhibitors. An increased number of senescent cells in various organs promote aging and age-related diseases. Thus, senolytics are drugs that kills senescent cells are explored to extend the life and healthy life span. The combined therapy of tyrosine kinase inhibitor (dasatinib) and quercetin is currently under clinical trial for aging-related diseases. We also identified quercetin as a Hsp90/Cdc37 inhibitor in our results. Our selected compounds are Hsp90/Cdc37 interface inhibitors and good candidates for further development to combat Hsp90 related diseases including cancer.

5.3.8 Elaiophylin disrupts K-Ras nanoclustering by inhibiting Hsp90/Cdc37 complex (unpublished data)

After getting promising results from conglobatin A as Hsp90/Cdc37 inhibitor, we searched for compounds that have some similarities in structure to conglobatin A. We found elaiophylin which is a related polyketide. Moreover, we also tested some biological variants of conglobatin A to study their effect in blocking K-Ras signaling and cancer stemness.

5.3.8.1 Elaiophylin inhibits Hsp90/Cdc37 complex (unpublished data)

Elaiophylin structure resembles that of conglobatin A (Figure 17 A,B). Therefore, we docked the crystal structure of Hsp90 to conglobatin A and elaiophylin in collaboration with Dr. Tiina Salminen and Dr. Vladimir Vukic, Åbo Akademi

University. Both conglobatin and elaiophylin binding sites overlap with the Cdc37 interaction site. Conglobatin A and elaiophylin seem to form a steric hindrance for binding of Cdc37 to Hsp90. Moreover, like conglobation A, elaiophylin formed hydrogen bonds to Glu47 and Ser50 to Hsp90 and thereby, hinders the most important interactions between Hsp90 and Cdc37 (Figure 17 C,D).

To confirm, elaiophylin inhibits the complex formation between Hsp90/Cdc37. We checked the effect of elaiophylin in the split *Renilla* luciferase assay. Interestingly, elaiophylin inhibited the interaction between Hsp90/Cdc37 with IC_{50} 14 μM (Figure 18A). Moreover, elaiophylin decreased the interaction between N-terminus Hsp90 and Cdc37 more potently than full length Hsp90 with IC_{50} 11.4 μM (Figure 18B). Thus, elaiophylin similarly bound with Hsp90 as conglobatin A and thereby blocked the interaction between Hsp90/Cdc37.

5.3.8.2 C-Raf and Gal3 are degraded after elaiophylin treatment (unpublished data)

Previous results confirmed that elaiophylin blocked the interaction between Hsp90 and Cdc37. Moreover, our results also concluded that Gal3 and C-Raf stabilize the K-Ras nanocluster complex on the plasma membrane. Here, we focused the effect of elaiophylin only on the K-Ras signaling. Therefore, we next tested the effect of elaiophylin on Hsp90 client kinase C-Raf and K-Ras nanocluster scaffold, Gal3. Interestingly, treatment of elaiophylin decreased the expression of C-Raf and Gal3 potently in only in (1 μM range) in MDA-MB-231 cells (Figure 18C). Degradation of Hsp90 client kinase, C-Raf again established that elaiophylin may be a novel inhibitor of Hsp90/Cdc37 complex formation.

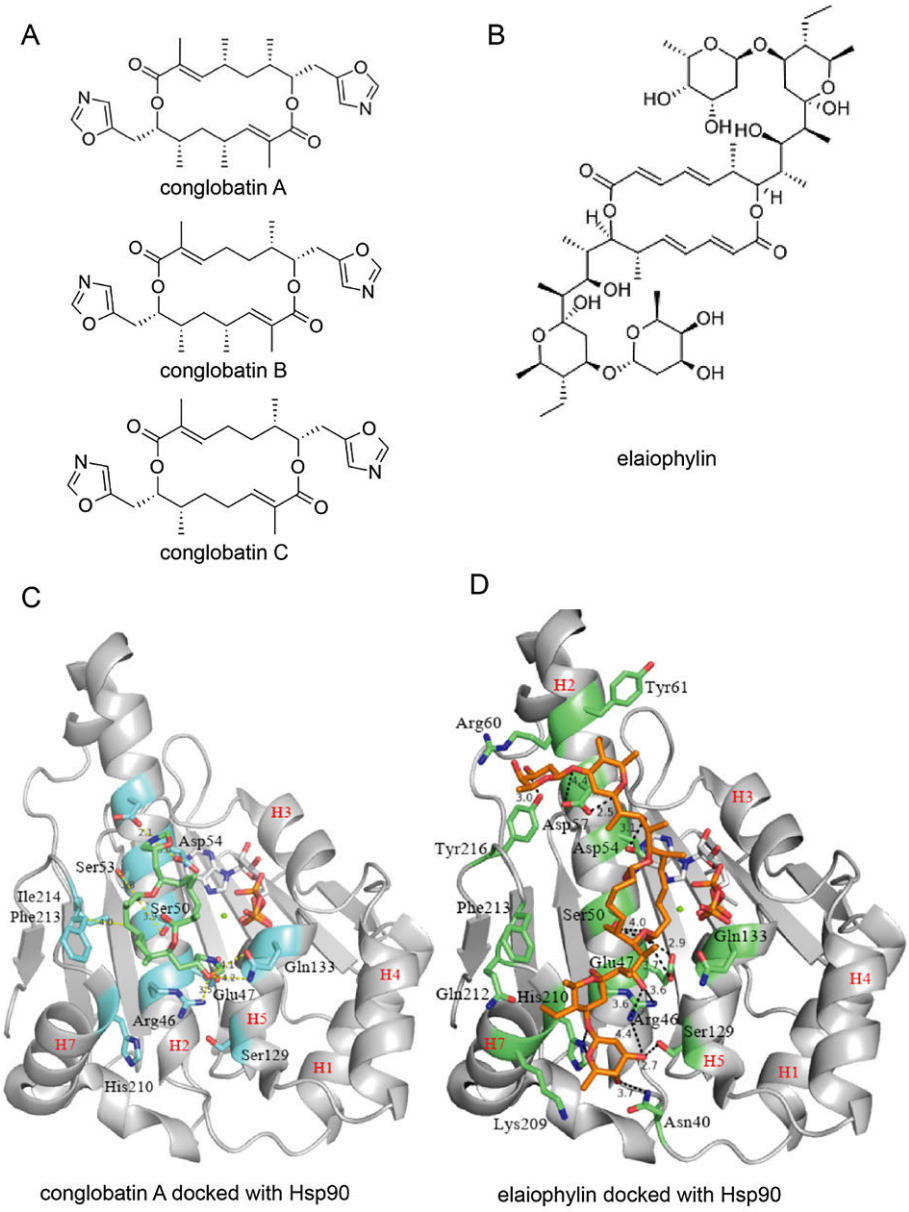


Figure 17: Elaiophylin docked to the N-terminus of Hsp90 in a similar manner as conglobatin A. (A-B) Structure of conglobatin A, B, C and elaiophylin. (C) N-terminus Hsp90 with docked conglobatin. Conglobatin (green sticks) interacts with Hsp90 (cyan sticks) through hydrogen bonding and hydrophobic interactions on one side. (D) N-terminus Hsp90 with docked elaiophylin. Elaiophylin (orange sticks) interacts with Hsp90 (green)

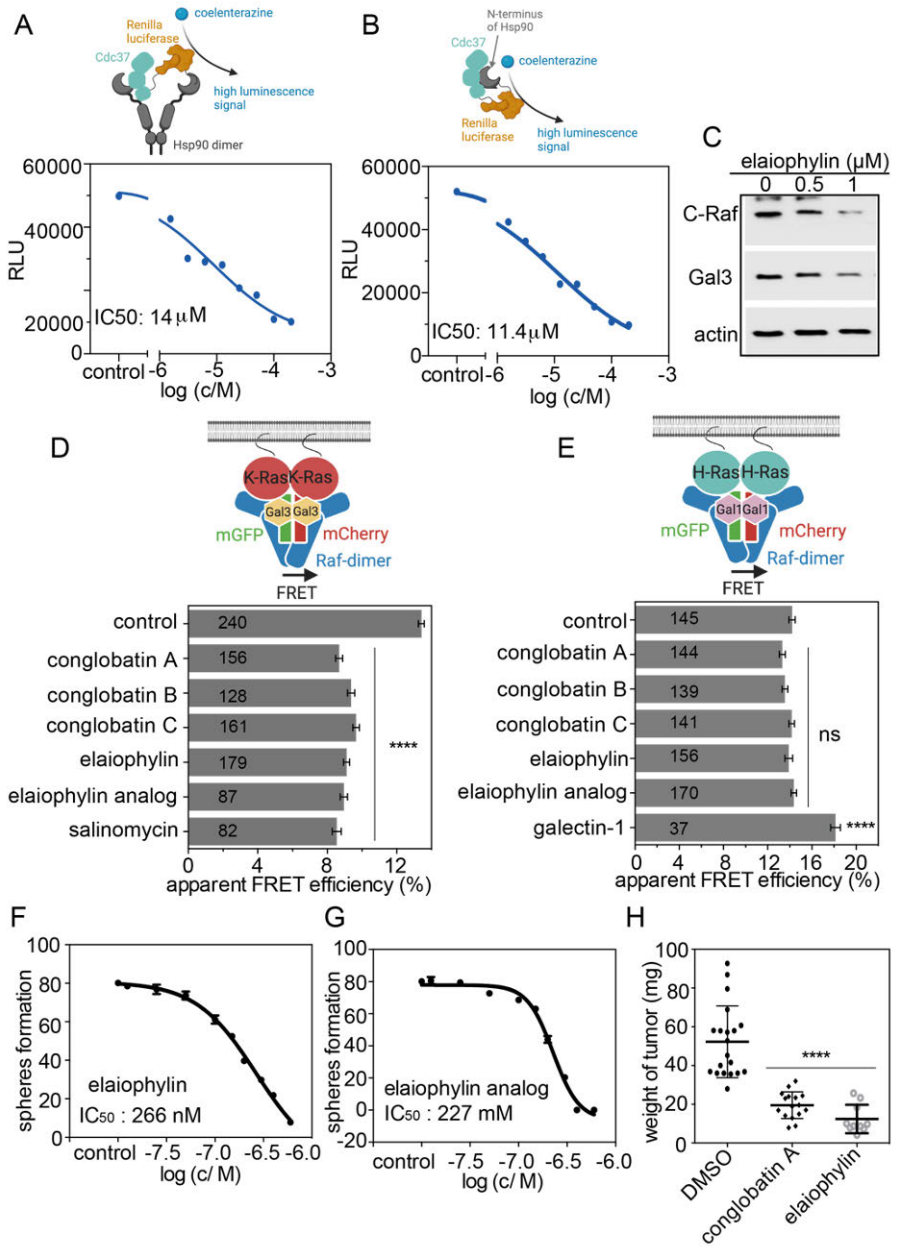


Figure 18: Elaiophylin selectively targets K-Ras via inhibition of Hsp90/Cdc37 (A) Elaiophylin was tested at increasing concentrations in a full length Hsp90/Cdc37 interaction split *Renilla* luciferase assay. (B) Elaiophylin was tested at increasing concentrations in a N-terminal Hsp90/Cdc37 interaction split *Renilla* luciferase assay. Western blots of MDA-MB-231 cells treated with either 0.1 % DMSO vehicle control or with indicated concentrations of elaiophylin for 24 h (D,E) Ras membrane

organization studied with nanoclustering-FRET in HEK cells co-expressing mGFP- or mCherry- tagged K-RasG12V (D) or mGFP- or mCherry- tagged H-RasG12V (E). Cells were treated with 0.1% DMSO control, 2 μM conglobatin A or B or C and 1 μM elaiophylin or elaiophylin analog or salinomycin for 24 h. (F,G) Dose response analysis of elaiophylin and its analog tested on MDA-MB-231 cells in spheroid formation assay for 72 h (H) Microtumor formation of MDA-MB-231 cells on chick CAM. Cells were treated with 0.1 % DMSO vehicle control, 10 μM conglobatin A or 5 μM elaiophylin for 5 days

5.3.8.3 Elaiophylin selectively disrupts the K-Ras membrane organization (unpublished data)

Downregulation of Gal3 together with C-Raf would disrupt the K-RasGa12V nanoclustering. Therefore, we tested elaiophylin and some conglobatin variants on K-RasG12V and H-RasG12V nanoclustering in HEK cells. Like conglobatin A, all conglobatin variants or elaiophylin and its analog significantly and selectively disrupted the K-Ras nanoclustering but these inhibitors did not affect H-Ras membrane organization (Figure 18 D,E). Thus, elaiophylin selectively blocked the activity of K-Ras but not H-Ras.

5.3.8.4 Elaiophylin inhibits tumorosphere and microtumor growth (unpublished data)

Our previous results demonstrated that compounds that selectively disrupt the K-RasG12V nanoclustering but not H-RasG12V nanoclustering, have the ability to inhibit the stemness like properties of cancer cell, as measured by mammosphere formation (Najumudeen et al., 2016; Posada et al., 2017). Elaiophylin and its analog significantly decreased the mammosphere formation of MDA-MB-231 cells in a dose-dependent manner with IC_{50} 266 nM and 227 nM respectively (Figure 18F,G). Both compounds were the most potent in the spheroid assay amongst those we tested so far. We next, tested elaiophylin on the inhibition of microtumor growth of MDA-MB-231 cells in CAM assay. Consistently, elaiophylin decreased microtumor growth more potently than conglobatin A. (Figure 18H).

Our results showed a binding of elaiophylin to Hsp90 similar to conglobatin A. Moreover, elaiophylin depleted the Hsp90 client kinase, C-Raf, thereby disrupted the K-Ras nanoclustering. Furthermore, similar to conglobatin A, elaiophylin also downregulated the expression level of K-Ras nanocluster scaffold, Gal3. This is another explanation of inhibition of K-Ras nanoclustering upon the treatment of

elaiophylin. Two more conglobatin derivatives, also selectively disrupted the K-Ras signaling. Elaiophylin decreased tumorosphere formation in 3D culture in low concentration. Here, we find that the working concentration of both elaiophylin and elaiophylin analog is two to five folds lower than conglobatin A in our cellular assays. Moreover, the same potency effect of elaiophylin was also seen on inhibition of microtumor growth in CAM assay. Our results suggest that elaiophylin is a novel Hsp90/Cdc37 inhibitor, which is more potent than conglobatin A.

6. FUTURE PERSPECTIVES

Oncogenic K-Ras is involved in multiple processes and signaling pathways in cancer. Thus, K-Ras is an important therapeutic target in various types of cancers. K-Ras is organized on the plasma membrane as nanoclusters to recruit its downstream effectors like Raf kinases. Thus, the plasma membrane organization is necessary to initiate downstream signaling. In order to block the activity of oncogenic K-Ras, targeting nanoclustering or plasma membrane organization represents an alternative strategy. The results of my thesis suggest that targeting the plasma membrane organization of K-Ras can enable K-Ras over H-Ras selectivity. This has the potential to target CSCs.

PDE6D is a trafficking chaperone of farnesylated proteins like K-Ras and RHEB. In this study, we designed novel inhibitors of PDE6D that provide suggestions how to solve the current problems of inhibitors, like ejection by ARL2 and selective targeting of the farnesylated K-Ras. Our current inhibitors, Deltaflexin-1 and -2 selectively target PDE6D and may already be sufficient to study the effect of PDE6D on cellular functions. For instance, inositol polyphosphate-5-phosphatase E (INPP5E) translocates to the primary cilium for ciliogenesis (Humbert et al., 2012). Mutations in INPP5E are associated with Joubert Syndrome, which is characterized by ciliopathic defects like renal cyst formation (Xu et al., 2017). INPP5E is a prenylated protein and a client of PDE6D, therefore, inhibition of INPP5E transport to the cilium may affect the functioning of the primary cilium and may in this way exert effects on stemness signaling pathways such as Hedgehog, Wnt and PDGF signaling pathways. However, INPP5E is a high affinity client of PDE6D, as compared to K-Ras, hence our relatively weak inhibitors may enable selective targeting of low affinity clients of PDE6D, such as K-Ras. Development of next-generation compounds may in general benefit from additional hydrogen bonding to PDE6D and improved flexibility against ARL2 ejection. Such improvements may also benefit future PDE6D PROTACs, which are currently based on molecules that have a Deltasonamide 1 moiety.

Compounds with potentially low toxicity important for neurodegenerative diseases, that patients live with for many years. More optimization is required in the development of our identified Hsp90/Cdc37 inhibitors to enhance selectivity, potency and characterize their overall toxicity. It would interesting to know that why Hsp90/Cdc37 inhibitors are less toxic than ATP pocket inhibitors.

Given that Hsp90/Cdc37 protects several kinases like CDK5, ERK, Akt, PKC, GSK3β and microtubule affinity regulating kinase 2 (MARK2) that promote the development of neurodegenerative diseases like Alzheimer and Parkinson disease, our inhibitors may have future applications in these diseases (Schopf et al., 2017). Aggregation of intraneuronal tangles, which are composed of tau protein is the hallmark of Alzheimer disease. Protection of the above kinases by Hsp90/Cdc37 leads to hyper phosphorylation of tau protein, which further plays a role in the development of Alzheimer disease (Dickey et al., 2007; Schopf et al., 2017). Thus, Hsp90/Cdc37 inhibitors could also be used for inhibiting the development of Alzheimer disease.

Similarly, Parkinson's disease is characterized by the decline in mitochondrial function, deregulation in multiple signaling pathways, oxidative stress and formation of Lewy bodies (Schapira and Jenner, 2011). Lewy bodies are the aggregation of misfolded and phosphorylated α-synuclein (Jinwal et al., 2011). Both Src family kinases (Lck and Fyn) and G-protein couple receptor kinases regulate the phosphorylation of α-synuclein (Luo and Benovic, 2003; Taipale et al., 2012). Thus, Hsp90/Cdc37 inhibitors, which have low cell toxicity could be used for the treatment of neurodegenerative diseases.

Finally, many viral proteins also depend on Hsp90 for the maturation of their important proteins. Phosphoprotein (P) of the rabies virus (RABV) is a nonviral protein associated with virus transcription and replication. Cdc37 assists the loading of the P protein onto the Hsp90 chaperone machinery. Thus Hsp90/Cdc37 helps to mature the non-kinase P protein, to assist in the replication of the virus. Similarly, in duck hepatitis B virus (DHBV) and human hepatitis B virus (HBV), overexpression of Cdc37 results in increased stability of reverse transcription and pgRNA packaging into nucleocapsids (RT), while overexpression of dominant-negative Cdc37 mutant Cdc37 reverses the effect (Hu et al., 2004; Hu et al., 1997). Thus Hsp90/Cdc37 inhibition may have important applications in viral disease control.

7. CONCLUSIONS

The objective of my thesis was to develop drugs that block oncogenic K-Ras signaling. Here, we developed some compounds that bind to the prenyl -pocket of PDE6D, thus inhibiting the interaction between PDE6D and farnesylated K-Ras. We added some unique features like a flexible hexyl linker and a cell penetration group to the compounds. The flexible hexyl linker provides probably the resilience against ARL2 ejection. This feature was absent in previously developed PDE6D inhibitors. PDE6D inhibitors developed by other groups have an in-vitro activity in the nanomolar but the cellular activity in micromolar. Our compounds, Deltaflexin-1 and 2 have a similar in vitro and cellular activity in the micro-molar. This provides evidence that Deltaflexins have improved cell penetration power and good flexibility against ARL2 ejection.

In the second part, we explain how conglobatin A decreases the expression of K-Ras nanocluster scaffold Gal3 by inhibiting the Hsp90/Cdc37 complex. Blocking the Hsp90/Cdc37 complex decreases the nuclear accumulation of HIF-1α, which is a transcriptional activator of Gal3. Furthermore, inhibition of Hsp90/Cdc37 depletes the kinases B-Raf and C-Raf. Knockdown of B-Raf and C-Raf disrupts K-RasGa12V nanoclustering but not H-RasGa12V. Thus, B-Raf or C-Raf are also important for K-Ras nanocluster formation on the plasma membrane. Thus, we established a molecular mechanism explains how the molecular chaperone Hsp90 indirectly regulates K-Ras signaling.

Hsp90/Cdc37 inhibitors may have a low on-target toxicity without heat shock response. Thus, we developed an in vitro split *Renilla* luciferase assay to identify Hsp90/Cdc37 inhibitors. Computational screening of millions of compounds and then in vitro validation of hundreds of compounds using the split *Renilla* luciferase assay resulted in 9 hit compounds. Two compounds, x6506 and x1540 were validated in detail as novel Hsp90/Cdc37 interface inhibitors.

Previous studies suggest that K-Ras drives the stemness activity in CSCs. Our results from both projects support a potent activity against tumorosphere growth of Deltaflexins and novel Hsp90/ Cdc37 interface inhibitors. Further development of these compounds may lead to novel therapies against K-Ras in cancer and other human diseases.

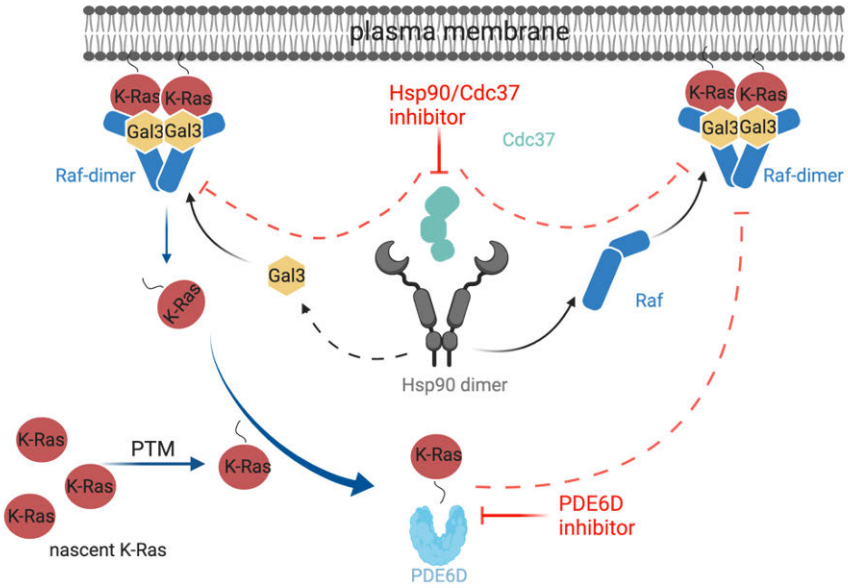


Figure 19: Inhibition of Hsp90 or PDE6D blocks the activity of K-Ras. Inhibition of PDE6D and farnesylated K-Ras interaction blocks the translocation of K-Ras through cytoplasm, thus, disrupting the K-Ras plasma membrane localization. Inhibition of Hsp90/Cdc37 interaction, depletes Hsp90 client kinases like B-Raf and C-Raf. Thus, inhibiting the K-Ras nanocluster formation. Moreover, inhibition of Hsp90/Cdc37 also downregulates Gal3 by decreasing nuclear accumulation of HIF-1α. Both, loss of Gal3 and B-/C- Raf disrupts K-Ras nanoclustering.

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