Identification of Novel Small Molecules Targeting Aurora B Kinase

by

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This is to certify that I have examined this copy of a master's thesis by

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and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
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to my dearest twin sister and family

for always being beside me

ABSTRAC*T*

Cell division is a complex process which is regulated by several checkpoints and feedback mechanisms. Aurora B is a mitotic checkpoint kinase having essential roles during cell division. It is involved in proper chromosome alignment and attachment of mitotic spindle to centromeres. It localizes microtubules near kinetochores during mitosis in connection with CPC (chromosome passenger complex). That complex consists of INCENP (inner centromere protein), Borealin and Survivin proteins all of which are phosphorylation targets of Aurora B. Kinase activity and complex binding of Aurora B is increased after interacting with CPC partners, especially INCENP[(1)]. Abnormal expression patterns of Aurora B are shown to result in chromosome instability and aneuploidy followed by tumorigenesis[(2,3)]. And yet, Aurora B over-expression is observed in a large number of cancers[(4)]. Previous studies have revealed that inhibition of Aurora B induced G2/M arrest or apoptosis in cancer cells making it a novel therapeutic target for cancer treatment. There are several Aurora kinase inhibitors, some of which are under clinical trial. However all of those inhibitors target kinase domain. In this study we aimed to characterize novel potential inhibitors that target Aurora B kinase via different molecular mechanisms.

ÖZET

Hücre bölünmesi birçok kontrol noktası ve geri bildirim mekanizmasıyla düzenlenen karmaşık bir süreçtir. Aurora B hücre bölünmesinde gerekli role sahip bir mitotik kontrol kinazıdır. Kromozom dizilimi ve bölünme ekseninin sentromerlere bağlanmasında görev alır. Mitoz bölünmede kromozom passenger kompleksiyle birlikte mikrotübülleri kinetokorların yakınına getirir. Kromozom passenger kompleksi Aurora B'nin fosforilasyon hedefleri olan INCENP, Borealin ve Survivin proteinlerinden oluşur. Aurora B'nin komplekse tutunabilmesi ve kinaz aktivitesi kromozom passenger kompleksi proteinleriyle, özellikle de INCENP ile olan etkileşimiyle artar[1]. Aurora B'nin olağandışı ifadesi kromozomal instabilite ve aneuploidy ile, beraberinde de tümör oluşumuyla sonuçlanmaktadır[2,3]. Keza, birçok kanser türünde Aurora B'nin aşırı ifadesi gözlemlenmektedir[4]. Daha önceki çalışmalar göstermiştir ki Aurora B'nin inhibisyonu kanser hücrelerininde G2/M evresinde durma ya da programlı hücre ölümüne sebep olmakta ve Aurora B'yi kanser tedavisinde özgün bir tedavi hedefi haline getirmektedir. Aurora kinazlar için farklı inhibitörler bulunmaktadır ve bazı kinaz inibitörleri klinik açıdan test edilmektedir. Ancak bu inhibitörlerin tümü benzer kinaz bölgesini hedef almaktadır. Bu çalışmamızda Aurora B kinazını farklı moleküler mekanizmalar ile inhibe eden özgün potansiyel inhibtörlerin karakterizasyonu yapılmıştır.

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NOMENCLATURE

BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
C	Cytokinesis
CPC	Chromosomal Passenger Complex
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
G0	Gap Zero
G1	Gap One
G2	Gap Two
GFP	Green Fluorescent protein
HeLa	Human cervical cancer cell line
HRP	Horseradish peroxidase
kDa	Kilodalton
M	Mitosis
PBS	Phosphate buffered saline
P/S	Penicillin-Streptomycin
RT	Room Temperature
S	Synthesis

SAC	Mitotic Spindle Assembly Checkpoint
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STC	S-Trityl-L-cysteine
TBS	Tris buffered saline

Chapter 1

INTRODUCTION

Cell cycle consists of three major phases: DNA Synthesis (S phase), Mitosis (M phase) and Cytokinesis (C phase) which are filled with growth and cell division preparation stages named G1 and G2 [5]. At the boundaries of each stage, there are strictly controlled checkpoint mechanisms which provide successful completion of each event.

Cell division is the most complex and highly regulated part of cell cycle. Several regulations, degradation and synthesis of proteins take place during that period. And proper completion of the process is essential for the future of the organism since mistakes might result in abnormalities and death.

Aurora B is one of those checkpoint kinases, having major functions in mitosis and cytokinesis. It forms Chromosomal Passenger Complex (CPC) in conjunction with inner centromere protein (INCENP), Borealin and Survivin, which form the core of the CPC and are phosphorylation substrates of Aurora B. CPC is involved in correction of chromosome-microtubule attachment errors and activation of the spindle assembly checkpoint. It is required for proper localization of spindle microtubules to kinetochores before segregation of the chromosomes. INCENP regulates localization of Aurora B and increases the kinase activity of it.

Aurora B over-expression is detected in a large number of cancers, inducing chromosomal instability and aneuploidy [(2)]. Inhibition in cancer cells is shown to lead to formation of polyploidy, followed by death of the cells. There are several drugs targeting Aurora B kinase activity. However like many protein kinase inhibitors, those are not monospecific to Aurora B. Kinase inhibitors such as VX-680 usually target ATP binding sites of the kinases which share similarity among kinases decreasing specificity of those drugs. Considering that matter, our previous co-worker Bahar Degirmenci has focused on finding inhibitors targeting different mechanisms on Aurora B such as interaction with its substrates. Docking and virtual screening of compounds from ZINC database and molecular modeling using OpenEye and GOLD software revealed 1,000,000 small molecules which were screened for binding efficiency against 14 different kinases's catalytic domain and Aurora B-INCENP interaction pocket. (For Further information about the compounds and their chemical structure, see the thesis of Bahar Degirmenci[(6)]) 5 of those molecules showed Aurora B-INCENP interaction selective binding efficiency. Since the interaction is necessary for Aurora B to get completely activated, drugs were expected to have the potential to inhibit proper functioning of it. Interfering with that pocket, instead of kinase pocket of Aurora B would provide specificity of the drug. We have set series of biochemical analyses to reveal the effect of drugs on AuroraB-INCENP interaction and cell division.

In Chapter 2, a detailed literature review related to this study is given. Starting with introductory information about cell cycle, importance of Aurora B and its interaction partners on cell division are mentioned. Chapter ends with brief information about recent developments in the issue.

Chapter 3 contains materials and methods used for this project, including cell synchronization methods, immunofluorescence and immunoprecipitation experiments.

In Chapter 4, results of the study are mentioned and analyzed in detail, which is further discussed in chapter 5. The thesis ends with future insights about the topic.

Chapter 2

LITERATURE REVIEW

2.1 Overview of Cell Cycle

Cell cycle is the term used for the series of events occurring in cells ending with either cell division or resting phase (G_0). Cell division is an essential feature of any living organism which is required for reproduction, repair and maintenance of the tissues and development of the organism. Two daughter cells having the same genetic content is formed from a single mother cell. Cell cycle is separated to defined stages which are strictly regulated with checkpoint mechanisms (Figure 2.1). Interphase stage consists of G1, S and G2 which are preparation stages for cell division. In Mitosis stage, chromosome segregation and nuclear division takes place and finally the cell cycle is completed with Cytokinesis phase in which cytoplasmic division happens [(7)].

For a typical dividing human cell, the whole cell cycle takes 24-hours, with 23 hours for interphase and 1 hour for mitosis and cytokinesis phases. Depending on the cell type and conditions, interphase span might increase. Many cells in an organism remain in G0 phase until extracellular conditions are favorable and signals to divide are present to initiate the process of cell division with G1 phase[(8)]. Cell cycle starts with G1 stage in which cells grow and carry out normal metabolism. Preparation for cell division starts at that stage with duplication of organelles. After that, S phase follows in which DNA replication and chromosome duplication take place. At G2 phase, cell continues to grow and prepares for

mitosis. G2 checkpoint passes and M phase starts, which consists of 5 sub stages named prophase, prometaphase, metaphase, anaphase and telophase. To proceed through mitosis, spindle assembly checkpoint requirements should be fulfilled. Following it, cytokinesis happens with division of the cell to two daughter cells with equal number of chromosomes [7]. See Figure 2.2 for illustration of the each phase in cell cycle in HeLa S3 cells.

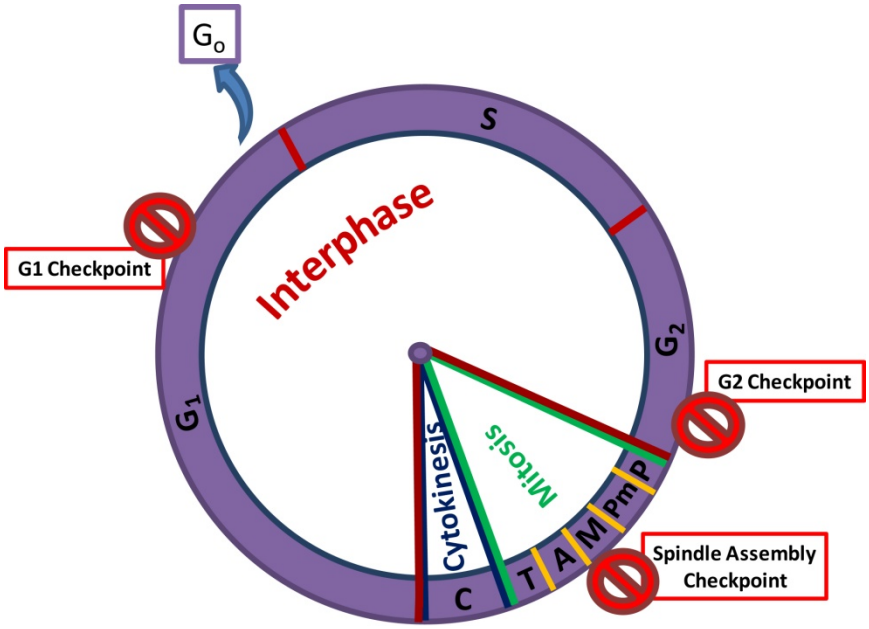

Figure 2.1 Structure of Cell Cycle with regulatory checkpoints. (Adapted from Collins and Garret, 2005[9])

2.1.1 Mitosis and Cytokinesis

The main purpose of a mitotic cell is to properly and equally distribute its genetic content and centrosomes between two daughter cells. Mitosis consists of 5 distinct phases [10]. During prophase, mitotic spindle starts to assemble and chromosome condensation starts forming sister chromatids.Nuclear envelope disassembles and following it, sister chromatid

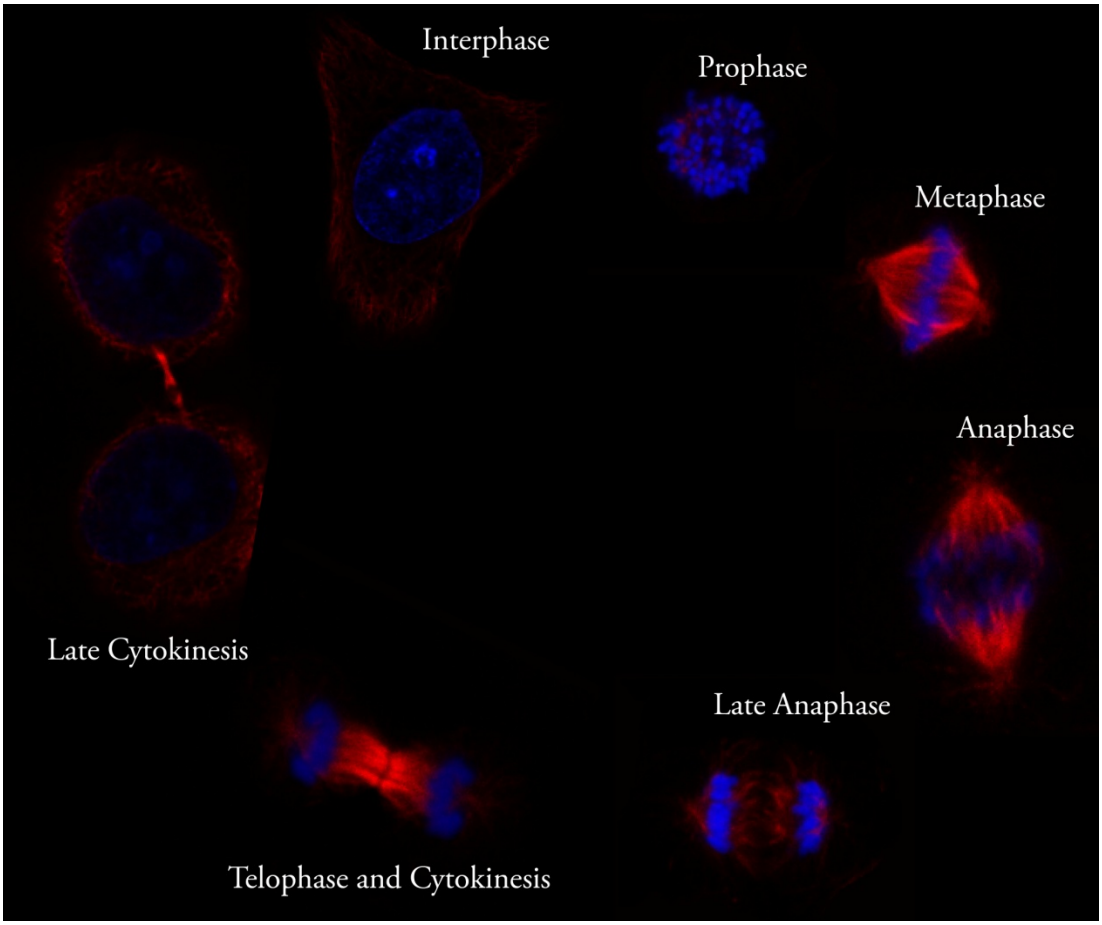

Figure 2.2 Cell Cycle Phases of a eukaryotic cell. HeLa S3 Cells were stained for DNA (blue) and α-tubulin (red). Images were taken at Nikon Eclipse 90i Confocal Microscope.

pairs attach to the mitotic spindle which consists of microtubules and acts as a backbone to hold the chromosomes. At prometaphase, microtubules emerge from the centrosomes and start searching for kinetochores of the chromosomes to attach and capture. Non-kinetochore binding microtubules interact with each other from each pole of centrosome forming the mitotic spindle[11]. At metaphase, sister chromatids align at the spindle equator of the cell forming metaphase plate. Sister chromatids within a pair are connected to opposite spindle poles which creates a tension across the centromeres. At the start of anaphase, destruction of sister-chromatid cohesion begins which causes separation and pulling of the sister chromatids to opposite poles of the spindle. At telophase, spindle is disassembled and successfully segregated chromosomes are packaged into newly being formed nuclei [7, 12]. Finally at Cytokinesis phase, cell starts to divide forming a cleavage furrow in the middle ending up with two daughter cells having equal genetic content. Nuclear envelope reforms around segregated chromosomes.

2.2 Checkpoint Mechanisms

There are several checkpoint mechanisms during progression through cell cycle to ensure that each crucial step of previous phase is passed with high fidelity. Failure in those checkpoints and completion of cell cycle can result in genomic instability ending up with either cell death or diseases including cancer [13, 14].

Three major checkpoints during cell cycle, named as the stages they are functioned in are G1, G2 and Mitotic Checkpoints (Figure 2.1). G1 checkpoint is known as restriction point and the stage of decision for entering cell cycle depending on internal and external

conditions. Depending on those conditions which are determined by several factors, cell will either enter another round of cell division or proceed through G0 phase. G1 checkpoint also provides a control of DNA integrity before the onset of DNA synthesis which will take place at S phase. G2-checkpoint aims to control genomic integrity before entry into mitosis. If there is a problem happened in DNA synthesis, cell will stop progression through cell cycle and DNA repair mechanisms will be activated[15].

Mitotic checkpoint prevents onset of anaphase until it makes sure that all the chromosomes have aligned properly and sister chromatids are connected to opposite spindle poles. When the correct alignment is succeeded, opposite pulling forces create a tension preventing destabilization of microtubules that are attached to centromeres which result in silencing of the checkpoint and initiates anaphase [16].

Progression through the checkpoints during cell cycle is controlled by cyclin–CDK (cyclin-dependent kinase) complexes and their activating subunits cyclins in a complex and highly regulated mechanism to ensure that cell cycle phases are executed in the correct order. Those CDK-cyclin complexes are degraded by ubiquitin mediated proteolysis [17]. In addition, relative abundance of those positive and negative regulators affects the rate of the cell cycle [18].

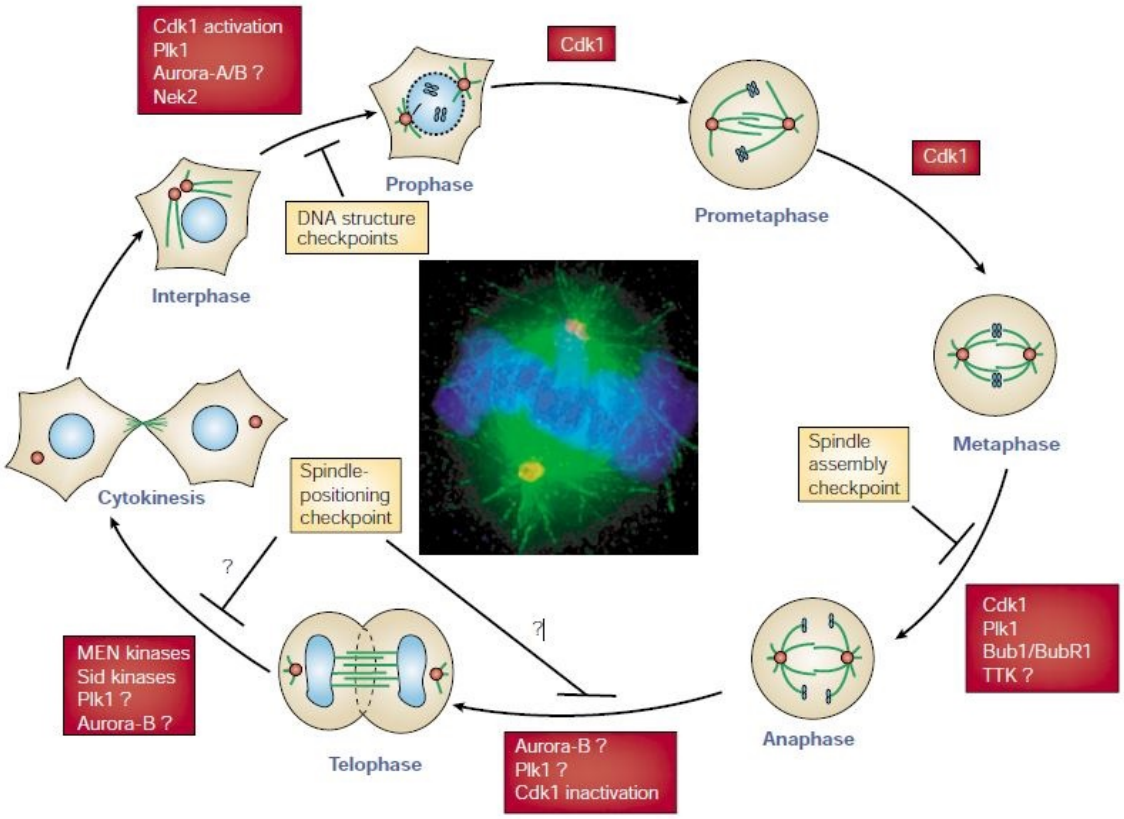

Figure 2.3 Overview of different stages of cell division and its regulatory mechanisms (Retrieved from Nigg, E, 2001 [(11)])

2.3 Aurora Kinases

The Aurora protein kinase family which consists of Aurora-A, -B and -C is an important group of enzymes that have functions in several control mechanisms and aspects of cell division in mammalian cells. Although those kinases have a high homology in their kinase domain, they are differentiated from each other in their cellular localization and functions[(19)] .

Aurora A has essential functions starting with transition from G2 to M phase. It is localized to centrosome at G1 and S phases and during G2 and Mitosis, concentrates at mitotic poles and spindle microtubules nearby. It is involved in formation of mitotic spindle, centrosome duplication and proper separation of centrosomes after formation of mitotic spindle. It is connected to microtubule formation and stabilization during mitosis [20,21].

Aurora B kinase, which is mentioned in the below sections in detail, is a component of chromosomal passenger complex, acting as a key regulator of mitosis. It has essential functions during mitosis and cytokinesis, particularly on the attachment of spindle microtubules to kinetochores, spindle assembly checkpoint and cleavage furrow formation [22,23].

Less is known about Aurora C, which is shown in a study to bind members of CPC and rescue Aurora B knockdown via ectopic expression suggesting that there might be a functional overlap between those kinases [8,24,25].

It is important to note that some studies revealed the possible intervention of Aurora A and Aurora B since they share the same substrate –CENP. Together with that, Aurora B continues to actively function at the late stages of mitosis, where both Aurora A and Aurora C are inactive. Giving credits to their essential roles during cell division, dysfunction of these kinases has shown to be associated with a failure to maintain a stable chromosome content which can lead to tumorigenesis or cell death.

Aurora B kinase is found to be over-expressed in a variety of tumor types. Several therapeutic inhibition studies has been done on those kinases, some of which shown that they can be good candidates for cancer treatments [19].

2.3.1 Aurora B and Chromosomal Passenger Complex

2.3.1.1 Aurora B Kinase

Aurora B kinase has major functions at centromeres and the central spindle for chromosome segregation and cytokinesis. Aurora B kinase activity and the formation of the Aurora B/INCENP/Survivin complex is required for its proper localization to centromeres and central spindle during mitosis and cytokinesis. And yet, over-expression of a catalytically inactive, dominant-negative mutant of Aurora B was shown to lead to impaired localization of CPC members to those regions which disturbed progression through mitosis [26].

Aurora B levels start to increase at late G2 phase and localizes diffusely in the nucleus. Eventually in prophase, Aurora-B is located on chromosome arms and starts to accumulate at centromeres between kinetochores where it stays during prometaphase and metaphase. When cells enter anaphase, Aurora B leaves the centromere and moves to the midzone. Subsequently, in telophase, it localizes to the midbody. Phosphorylation of Histone H3-S10 which is one of Aurora B phosphorylation targets increases dramatically when cells enter mitosis and stays high until cells enter anaphase [27, 28]. During cytokinesis, Aurora B is located at cleavage furrow, at the end being inactivated till the next cycle of mitosis.

Activation of Aurora B is a two-step process first requiring binding of INCENP which partially activates Aurora B and secondly phosphorylation of Aurora B near conserved C terminus of INCENP which is also known as IN-Box. Studies revealed that phosphorylation of INCENP by Aurora B is necessary to enhance kinase activity of Aurora B through inducing auto-phosphorylation of Aurora B [29,30].

Aurora B is directly involved in localization of kinesin-like proteins MKLP and ZEN4 to the central spindle at anaphase[31]. At the critical stage of progressing from telophase to cytokinesis, Aurora B phosphorylates a number of substrates including myosin II regulatory light chain and vimentin which are involved in cleavage furrow formation making this modification essential for entry and progression through cytokinesis [32].

2.3.1.2 Chromosomal Passenger Complex (CPC)

Chromosomal passenger complex (CPC) is a controller taking major functions in mitosis and cytokinesis. It maintains chromosome integrity and mitotic spindle structure, functions in correcting kinetochore-microtubule attachment errors and proper completion of cytokinesis [28, 33]. Aurora B kinase, INCENP, Survivin and Borealin forms the core of that complex[34] (See the figure 2.4). INCENP is the central targeting and regulatory subunit and acts as a scaffold of the complex; while Aurora B functions as enzymatic subunit of the complex[35].

CPC is essential for correcting non-bipolar chromosome attachments during mitosis and has a role in the mitotic spindle assembly checkpoint (SAC), Aurora B-INCENP complex is localized at centromeres/inner kinetochores during mitosis and mediates SAC activity upon spindle disruption [36].

Disruption of interaction of INCENP with Borealin and Survivin leaded to distinct CPC subcomplexes which failed to function properly, ending up with mitotic defects [37, 38]. Even though it is not certain whether survivin is necessary to activate Aurora B [39, 40]. A similar study targeting Aurora B-Survivin interaction in vitro showed that the interaction might be substantial for progression through cytokinesis [41].

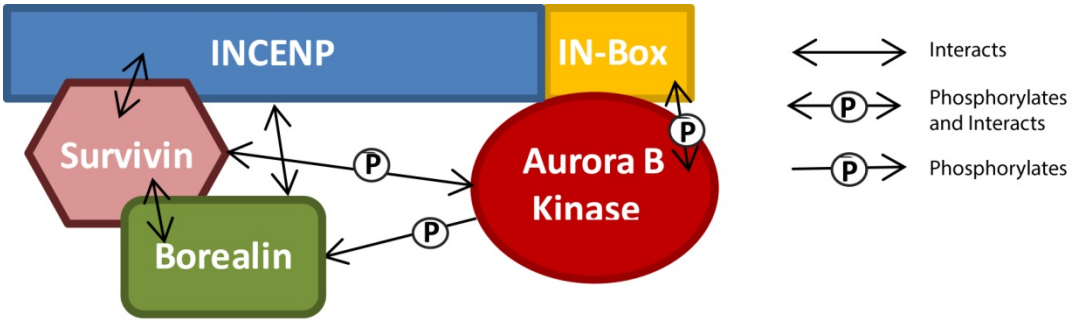

Figure 2.4 Demonstration of Chromosomal Passenger Complex members and Aurora B interactions. (Adapted from Becker, 2010[36] and Vader, 2006[42])

Blocking functioning of Borealin with RNAi interference also caused problems in spindle assembly, followed by Aurora B, INCENP and Survivin mislocalization on mitotic spindle and cleavage furrow[34]. Binding of both Borealin and Survivin is required to target CPC to centromeres which is independent of Aurora B [43].

Chapter 3

MATERIALS AND METHODS

3.1 Cell Culture

HeLa S3 PC cells were grown in DMEM (Lonza) complete medium supplemented with 10% FBS, 1%Penicillin/Streptomycin and 1%w/v L-glutamine in 5%CO2-37°C incubator. Cells were passaged each 3 days and avoided from overconfluency and overpassaging. Passaging was done via trypsinization and dilution of cells to new plates. Cells were washed once with PBS and appropriate amount of Trypsin/EDTA (Sigma/Aldrich) was added to detach the cells from the plate. After incubation at 37°C for 3-5minutes, complete DMEM was added to inactivate trypsin and proper dilution of the cells was done. For immunoflourescence experiments, cells were cultured on glass coverslips in 12-well plates. Aurora B-GFP stably expressing cell lines were grown in geneticin (400ug/ul) containing DMEM complete for selection of the cells.

For harvesting the cells, media was aspirated and cells were washed once with PBS. Following incubation with trypsin for 5 minutes, cells were resuspended in complete medium and transferred to a pre-cooled tube. Centrifugation was done at 1200rpm for 3min at +4°C to pellet the cells. Supernatant was discarded and pellet was gently resuspended in ice-cold PBS. After repeating centrifugation step and withdrawing the supernatant, cell pellets were frozen in liquid nitrogen and removed to -80°C for upcoming experiments.

3.2 Cell Synchronization for different cell cycle stages

3.2.1 G1/S and Mitosis arrest using Double Thymidine

HeLa S3 cells were grown to 40% confluency. Coverslips were placed into 12-well plates if staining procedure was to be taken place. Cells were washed once with 1X PBS and incubated for 20hours in complete DMEM containing 2mM Thymidine (Santa Cruz, 296542A) At the end of 20 hours, media was removed and cells were washed 3 times with PBS to release cells from excess thymidine. Fresh media was added and cells were incubated for 8 hours. Then, 2mM thymidine containing complete DMEM was given to the plates and incubated for 17 hours. At the end of 17 hours, cells would be at G1/S phase border. At that stage, interphase samples were collected, either via fixating coverslips for immunofluorescence or pelleting the cells. Remaining plates were washed 3 times in PBS and fresh complete DMEM was given to release the cells from excess thymidine. After incubation for 8-12 hours, cells were found to be in different states of mitosis. For a higher yield of metaphase, cells were collected at 9-9.5th hour from thymidine release.

3.2.2 Mitosis Synchronization using S-trityl Cysteine (STC)

Double Thymidine Block Method in 3.2.1 was applied till the release step after 17 hours of second thymidine incubation. Plates were washed 3 times with PBS and Complete DMEM containing 10 µM kinesin 5 inhibitor S-Trityl-L-cysteine (STC) (Sigma, 164739) was given. After incubation for 12 hours at 37°C, 5%CO_2 cells were collected with a 80-90% of mitotic phase.

3.2.3 Cytokinesis Synchronization using Nocodazole

Double Thymidine Block Method in 3.2.1 was applied till the release step after 17 hours of thymidine incubation. At the end of 17 hours, cells were washed 3 times with PBS and complete DMEM was added to release them from excess thymidine. After incubation for 7 hours, differing concentrations of Nocodazole (Calbiochem, 487928) was added to the media. After differing hours of incubation with nocodazole, cells were arrested at early stages of mitosis and could be collected depending on the purpose. At that stage, nocodazole was carefully washed out 3 times with PBS and fresh complete DMEM was added to cells to release from nocodazole and let cells proceed through mitosis. Cells entering cytokinesis after sufficient incubation time were collected.

3.3 Cell Imaging

3.3.1 Immunofluorescence

Cells in 12-well plates were washed with PBS and fixed in either 3.7%PFA for 15min or -20°C Methanol for 4 min depending on the purpose of staining. Fixatives were washed out with incubation in PBS-0.1% Triton-X (PBS-Tx) 3times for 5minutes. Blocking was done in 2%BSA in PBS-Tx for 1hour to overnight. Primary antibodies in proper dilutions were prepared in 2% BSA-PBS-Tx and added to cover slips and incubated for 3 hours at RT to overnight at +4°C. After washing 3 times for 5minutes in PBS-Tx, secondary antibody was given in proper dilutions in 2% BSA-PBS-Tx and incubated for 1 hour at RT. After washing 3 times, 5 minutes each in PBS-Tx, DAPI was added in to stain nuclei (1:1000 in 2% BSA-PBS-Tx) and incubated 10 min. Wash step was repeated. Cover slips were mounted on mounting medium (Sigma-Aldrich, M1289) and sealed with nail polish. Slides were stored at -20°C till imaging.

3.3.2 Confocal Microscopy and Quantification of Cell Cycle States

Synchronized and immunostained HeLa S3 cells were imaged with Nikon Eclipse 90i Confocal Microscope using EZ-C1 Imaging Software. For Cell Cycle Quantification Experiments, 20 shots were taken from different spots for each slide in 60x magnification. Images for the localization and quantification of Aurora B and INCENP were taken in 100x magnification. Cell cycle stages of cytokinesis optimization experiments and fluorescence intensities of Aurora B and INCENP with drug treatments were quantified using ImageJ [Wayne Rasband, NIH] software.

3.4 Preparation of Cell Lysates and Protein Analysis

3.4.1 Cell Lysis for pH3 level comparison experiments

Cells were grown and pelleted following the protocol at 3.1 PBS-0.1%Triton-X was added to cell pellets and incubated on ice for 10 minutes. After centrifugation at 7500 rpm at 4°C for 15 min, supernatants were collected and concentrations were measured with BCA assay in NanoDrop 2000 UV-Vis Spectrophotometer.

Lysates were mixed with 2X Laemmli Sample Buffer (4% (w/v) SDS, 20% Glycerol, 120mM Tris-Cl (pH 6.8), 0.02% (w/v) bromophenol blue, 100mM DTT) and boiled at 90°C for 10 minutes for denaturation. Samples were removed to -20°C for long term storage.

3.4.2 SDS-PAGE and Western Blotting

SDS-PAGE gel was prepared following the indicated amounts for separating and stacking gel. 10% separating gel contained 5ml 30% acrylamide/ 0.8% bisacrylamide, 3.75ml 4X Tris.Cl/SDS pH 8.8, 6.25ml ddH_2O, 0.05ml 10% (w/v) APS, 0.01ml TEMED and stacking gel contained 0.65ml 30% acrylamide/ 0.8% bisacrylamide, 1.25ml 4X Tris.Cl/SDS pH 8.8, 3.05 ml ddH_2O, 0.025ml 10% (w/v) APS, 0.005ml TEMED. Equal amounts of protein samples were loaded and run on the gel at 160 V for 1.5 hours.

Separated proteins were transferred to nitrocellulose membrane (Whatman Protran BA85) overnight at 40V at 4°C. Transfer was verified by staining with Ponceau dye. After 45 minutes blocking with 4% nonfat milk in TBS/0.1 % Tween20 (TBST), the membrane was incubated with primary antibodies which were prepared in 2%BSA-TBST for 3hours at RT to overnight at 4°C. Then the membrane was rinsed 3 times with TBST for 10min each. Secondary antibody incubation was done for 1.5 hours at RT with donkey anti-rabbit or anti-mouse secondary antibodies prepared in 4% nonfat milk-TBST. Then the membrane was rinsed 3 times with TBST Buffer for 10min each. For the final wash, only TBS was used to avoid interfering of the detergent with horse radish peroxidase detection system. Proteins were detected with ECL (Pierce ECL western blotting substrate 32106).

3.5 Immunoprecipitation

Aurora B-GFP expressing cells were grown to 40% confluency in 15-cm dishes to obtain a final amount of 10^6-10^7 cells at the end of the synchronization procedure which is sufficient for a proper immunoprecipitation. Double Thymidine Block + STC synchronization method was used to synchronize the cells to G1/S and M phase. Right after second thymidine release, cells at G1/S stage were trypsinized and collected. Drug2 at indicated

concentrations was given together with STC. After incubation for additional 12 hours, cells were collected and pelleted.

Cell pellet was re-suspended in 250µl ice-cold lysis buffer (20mM Tris-CL pH7.4, 150mM NaCl, 1mM $MgCl_2$, 10%Glycerol, 0.5mM EDTA, 10mM NaF, 0.5%NP-40, 1mM beta-glycerolphosphate, 1mM sodium pyrophosphate, 1mM sodium orthovanadate, 1mM DTT, EDTA free protease inhibitor (Pierce, 88266, 1Tablet for 10 ml Lysis Buffer) by pipetting and passing through 25 gauge needle. Then the tube was incubated on ice for 30 min with pipetting every 10 min. Cell lysate was centrifuged at 14000 rpm for 15 min at +4°C. The supernatant was carefully transferred to a pre-cooled eppendorf tube. Dilution buffer (20mM Tris-CL pH7.4, 150mM NaCl, 1mM $MgCl_2$, 10%Glycerol, 0.5mM EDTA, 10mM NaF) was added to the tube in 2:3 proportions to decrease the percentage of the detergent in the solution and reduce nonspecific binding. Some of the whole cell lysate was saved for western blotting analysis. GFP-Trap®_A (Chromotek, gta-20) which is a GFP binding protein coupled to a monovalent matrix was used for analysis of Aurora B-GFP fusion protein and its interaction partners. 30µl slurry GFP-Trap®_A beads per sample were washed 3 times in 600µl dilution buffer with centrifugation at 5000rpm for 3min at +4°C. Diluted lysate was mixed with the beads and rotated 3hours at +4°C. Then the beads were centrifuged at 5000rpm for 3min and supernatant was discarded saving some of it as an unbound control. For wash, beads were re-suspended in 600µl dilution buffer and centrifuged which was repeated 3 times. For elution, beads were re-suspended in 100µl 2X Laemmli Sample Buffer (4% (w/v) SDS, 20% Glycerol, 120mM Tris-Cl (pH 6.8), 0.02% (w/v) bromophenol blue, 100mM DTT) and boiled for 10 min at 95°C to dissociate immunocomplexes from them. SDS-PAGE and Western Blotting was done for analysis of protein interactions.

Table 3.1 Antibodies used for Western Blotting with their dilutions

Antibody	Host	Dilution	Company	Catalog No
Anti-Aurora B	Mouse	1:1000	Abcam	3609
Anti-INCENP	Mouse	1:250	Santa Cruz	Sc-376514
Anti-INCENP	Rabbit	1:2500	Abcam	Ab-12183
Anti-PhosphoH3 pSer10	Rabbit	1:400	Thermo Scientific	PA5-12526
Anti-PhosphoH3 pSer10	Rabbit	1:400	Santa Cruz	Sc-8656-R
Anti-Beta Actin (AC-15)	Mouse	1:7500	Thermo Scientific	MA1-91399
Anti-α-Tubulin (DM1A)	Mouse	1:1000	Cell Signaling Technology	3873S
Anti-GFP	Mouse	1:1500	Roche	11814460001
Anti-Mouse IgG-HRP linked	Horse	1:2000	Cell Signaling	#7076
Anti-Rabbit IgG-HRP linked	Goat	1:2000	Cell Signaling	#7074

Table 3.2 Antibodies used for immunoflourescence with their dilutions.

Antibody	Host	Dilution	Company	Catalog No
Anti-Aurora B	Mouse	1:200	Abcam	3609
Anti-INCENP	Rabbit	1:250	Bethyl Labs	IHC-00060
Anti-PhosphoH3 pSer10	Rabbit	1:400	Thermo Scientific	PA5-12526
Anti-PhosphoH3 pSer10	Rabbit	1:400	Santa Cruz	Sc-8656-R
α –tubulin (DM1a)	Mouse	1:1000	CST	3873S
Alexa 488 Anti-mouse IgG	Donkey	1:1000	Invitrogen	A21202
Dylight 488 Anti- rabbit IgG	Goat	1:1000	CST	35552
Dylight 594 Anti-rabbit IgG	Goat	1:1000	CST	35560
Dylight 594 Anti-mouse	Goat	1:1000	CST	35510

Chapter 4

RESULTS

In this project we aimed to identify novel candidate inhibitors targeting Aurora B which carries major functions during mitosis and has been a potential drug target for cancer treatment. There are several aurora kinase inhibitors found and being clinically tested. However their major disadvantage is that they could bind and inhibit other kinases since they target kinase pockets which have similar structures in many kinases. Our previous co-worker Bahar Degirmenci has focused on finding inhibitors having different binding sites specific to Aurora B. INCENP, being both a binding partner and activator of Aurora B has been a good candidate for that purpose.

She has docked 200,000 natural products and screened 5 million compounds through OpenEye scientific software and determined possible candidate drugs specifically binding to the Aurora B-INCENP interaction site. She screened those drugs for binding efficiency against 14 different kinases's catalytic domain and Aurora B-INCENP interaction pocket [(6)] 5 of those molecules showed Aurora B-INCENP interaction selective binding efficiency. Since the interaction is necessary for Aurora B to get completely activated, drugs were expected to inhibit proper functioning of it and to be specific to Aurora B. Her preliminary data showed promising results with drugs 1 and 2.

We have set series of experiments based on biochemistry and microscopy to analyze the effect of drugs on AuroraB-INCENP interaction and cell division.

4.1 Arresting HeLa S3 cells in cytokinesis via Nocodazole Treatment

Aurora A and Aurora B kinases have similar structures, raising the question that drug might bind to Aurora A, instead of or together with Aurora B. That might interfere with seeing the effect of the drug on Aurora B properly. As it is mentioned in the previous chapters, Aurora B differs from Aurora A with its active functions in cytokinesis phase, in which Aurora A is inactive. Following that knowledge, we aimed to focus on working with cytokinesis synchronized cells to analyze the effect of drug treatment on Aurora B and cell cycle.

The initial problem with cytokinesis synchronization was that known methods were not convincing and enriched enough to follow. Double thymidine release gives a very low efficiency of cytokinesis. Purvalanol A increases the percentage but leads to abnormal conditions starting with monopolar cytokinesis. We focused on Nocodazole, which interferes with polymerization of microtubules and commonly used to arrest cells at G2/M phase. That mitotic block is due to a reduction in microtubule dynamic turnover in a dose dependent manner which is recoverable. That feature of nocodazole can be utilized such that if the mitosis synchronized cells are released from nocodazole, microtubule polymerization will restart allowing cells to proceed through mitosis and enter cytokinesis. Our strategy was to determine parameters of nocodazole amount, incubation and release time to increase the percentage of cytokinesis synchronized cells in the population.

First phase of cytokinesis enrichment experiments was to determine the best working concentration of nocodazole. Cells were grown in 6- well plates and treated with increasing amounts of nocodazole from 0ng-1000ng in differing incubation times of 1-5 hours. After release from nocodazole, cells were observed under microscope for 30 minutes intervals. First findings showed that (data not shown), cells couldn’t overcome nocodazole over

50ng. Together with that, nocodazole incubation times lower than 3 hours were inefficient to synchronize cells to mitosis (data not shown), which is the first required condition to synchronize the cells to cytokinesis.

At the second phase, cells were grown on cover slips in 12-well plates and synchronized to interphase with double thymidine block. Cells were released from excess thymidine and incubated with 0ng, 5ng, 10ng and 25ng of nocodazole for 4 hours. After washing out nocodazole with PBS and release for 40 minutes, cells were fixed and stained with DAPI for DNA and tubulin to determine cell cycle stages properly. 12 images from different spots were taken per sample with 90i Nikon Eclipse Confocal Microscope. Different phases of cell cycle were quantified with ImageJ software (Plugins>Analyze>Cell counter). Total count of cells per sample was normalized to 585 (smallest sample size) and pie charts and graphs for average cell count with standard error values were drawn.

Results (see Figures 4.1.1 and 4.1.2) showed that in 5&10ng of nocodazole treated sets, there was a significant increase in cytokinesis stage cells from 6% to 31% comparing to 0ng control. In 25ng treated ones, increase was only to 13%.

Interestingly, in 25ng nocodazole treated ones, cells in metaphase were dramatically increased from 52% comparing to 9% in 0ng control and ~25% in 5&10ng nocodazole conditions. That data was in concordance with the deduction that, release time from 25ng nocodazole was not enough to recover from microtubule depolymerization comparing to 5&10ng nocodazole conditions.

Important finding of the second phase of the cytokinesis optimization experiments was that cells treated with nocodazole were successfully proceeding through mitosis and in higher percentage of cytokinesis stage comparing to 0ng nocodazole treated one.

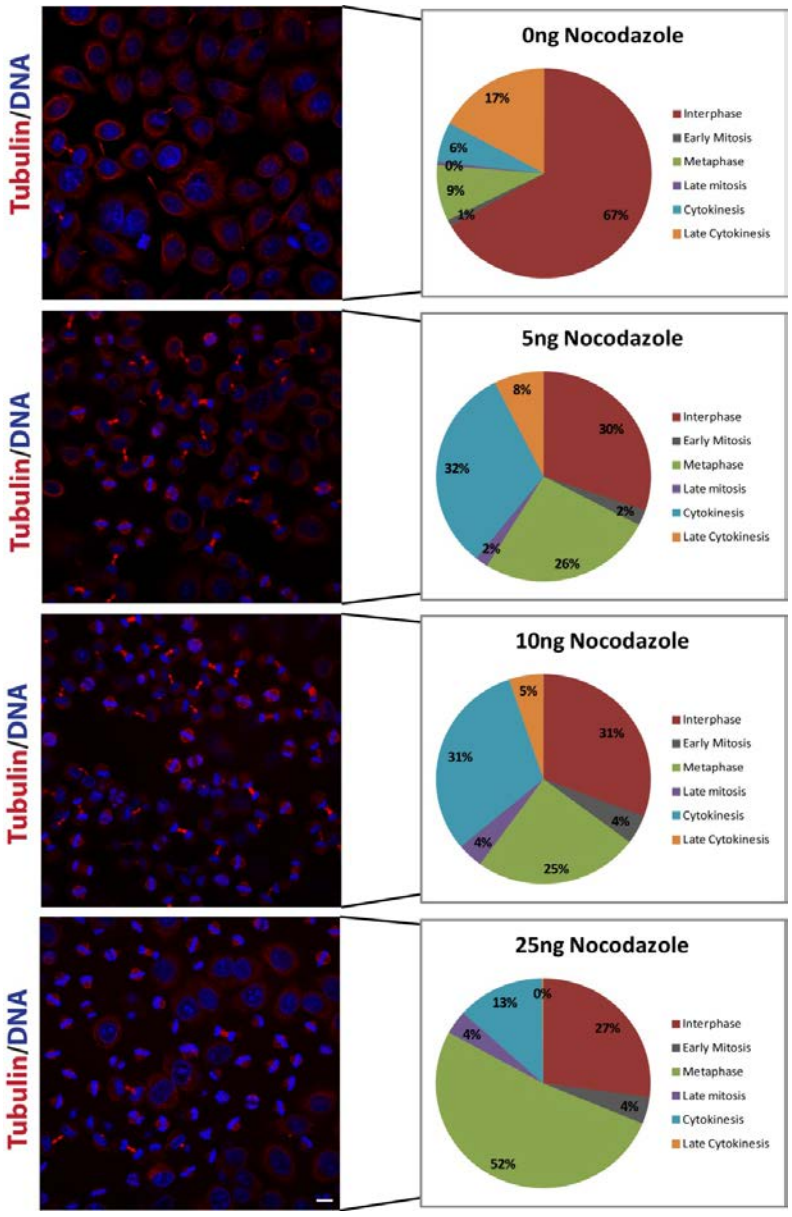

Figure 4.1.1 Cell cycle stage distributions of HeLa cells treated with different concentrations of nocodazole. Cells were stained for DNA (blue) and α-tubulin (red). Images taken at 60x with 90i Nikon Eclipse Confocal Microscope. Quantification was done with ImageJ. Scale bar, 10μm.

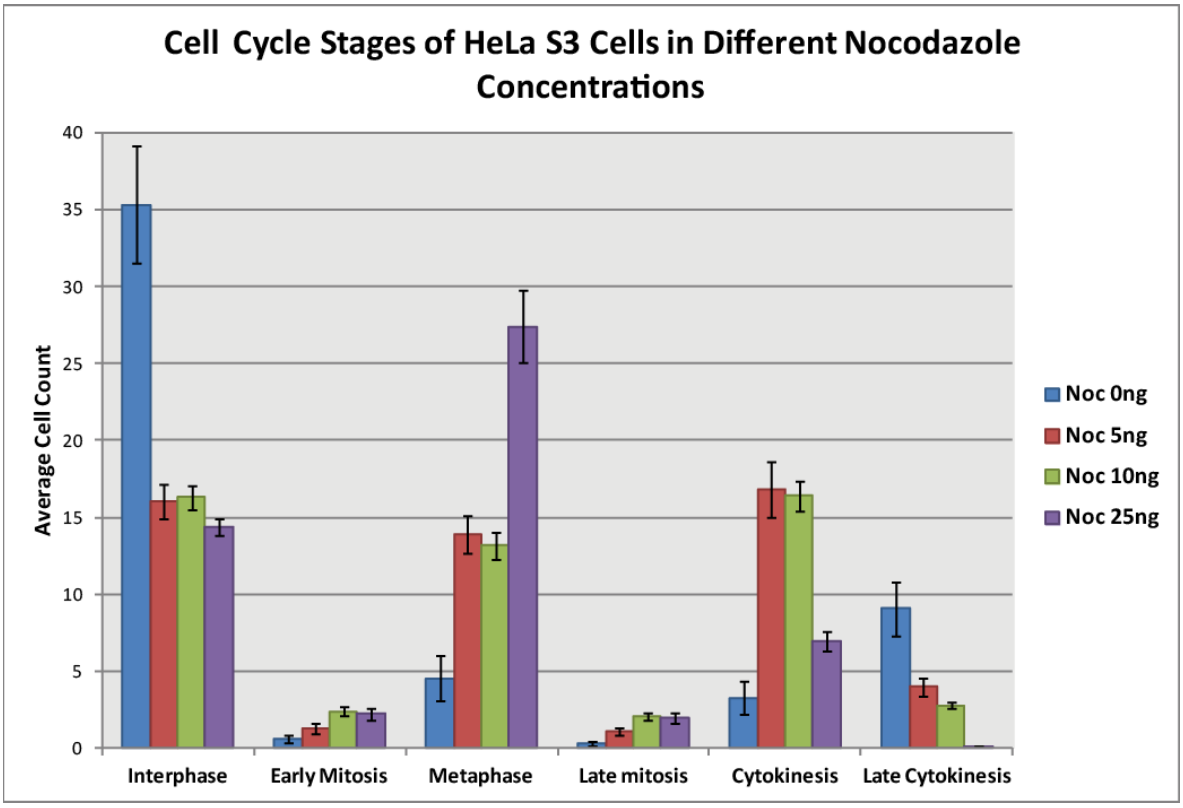

Figure 4.1.2 Comparison of Cell Cycle stages under different Nocodazole concentrations. 5-10ng of nocodazole treated plates have higher amount of cytokinesis phase cells comparing to 0ng and 25ng treated ones. Quantification of the cell cycle stages was done with ImageJ software.

If the nocodazole release time is increased enough, remaining mitotic cells in the population will have enough time to proceed through cytokinesis. However, if the release time exceeds the critical limit, cells at cytokinesis will proceed through late cytokinesis and interphase. Considering those concerns, a final phase of optimization was set. Working nocodazole amount was fixed to 10ng and remaining two parameters of nocodazole incubation and release time were targeted. Cells were grown on cover slips in 12-well plates and synchronized to interphase with double thymidine block. After 7 hours of release from second thymidine, cells were given 10ng of nocodazole and incubated for 4 to 5 hours at 37°C incubator. Nocodazole was washed out with PBS and fresh DMEM complete was given to release cells. After incubation for 40-60 minutes, cells on cover slips were collected, fixed and stained with DAPI for DNA and tubulin. 11 images from different spots were taken per sample with 90i Nikon Eclipse Confocal Microscope. Different phases of cell cycle were quantified with ImageJ software (Plugins>Analyze>Cell counter). Total count of cells per sample was normalized to 714 (median of the set) and pie charts and graphs for average cell count with standard error values were drawn.

Result at figure 4.1.3 and 4.1.4 concluded that highest enrichment of cytokinesis was with the parameters of 10ng nocodazole incubated for 5 hours and released for 60 minutes. Interesting finding was the significant difference between 50min and 60min release times with a shift of cytokinesis stage from 12% to 29% (see figure 4.1.3) Since passage from telophase to cytokinesis takes a very short span during cell cycle, it can be expected to see that dramatic increase. And yet, consistent with that idea, in 0ng nocodazole treated controls, percentage of interphase cells in the population shifted from 57% to 64% and cells in late cytokinesis decreased from 26% to 18% in 60 minutes released cells comparing to 50min released ones. That data suggests that cells which were in late cytokinesis might have entered G1 stage in 10 minutes.

In addition, cytokinesis percentage in the population was 8% higher in 5hours of nocodazole treatment, comparing to 4 hours in both 50 min and 60 min release conditions.

Increasing nocodazole incubation time to 5 hours and release time to 60 minutes significantly increased overall cytokinesis percentage in the population. However, that percentage was same with the previous phase of enrichment experiments which has parameters 10ng nocodazole incubation for 4 hours and release for 40 minutes. The main reason behind the no-change was probably washing out weakly attached mitotic cells during nocodazole release step. On the other hand, since 5hours of incubation and 60 minutes of release times relatively increased cytokinesis percentage comparing to 4 hours and 40 minutes conditions, it can be concluded that those conditions give the highest enrichment in cytokinesis.

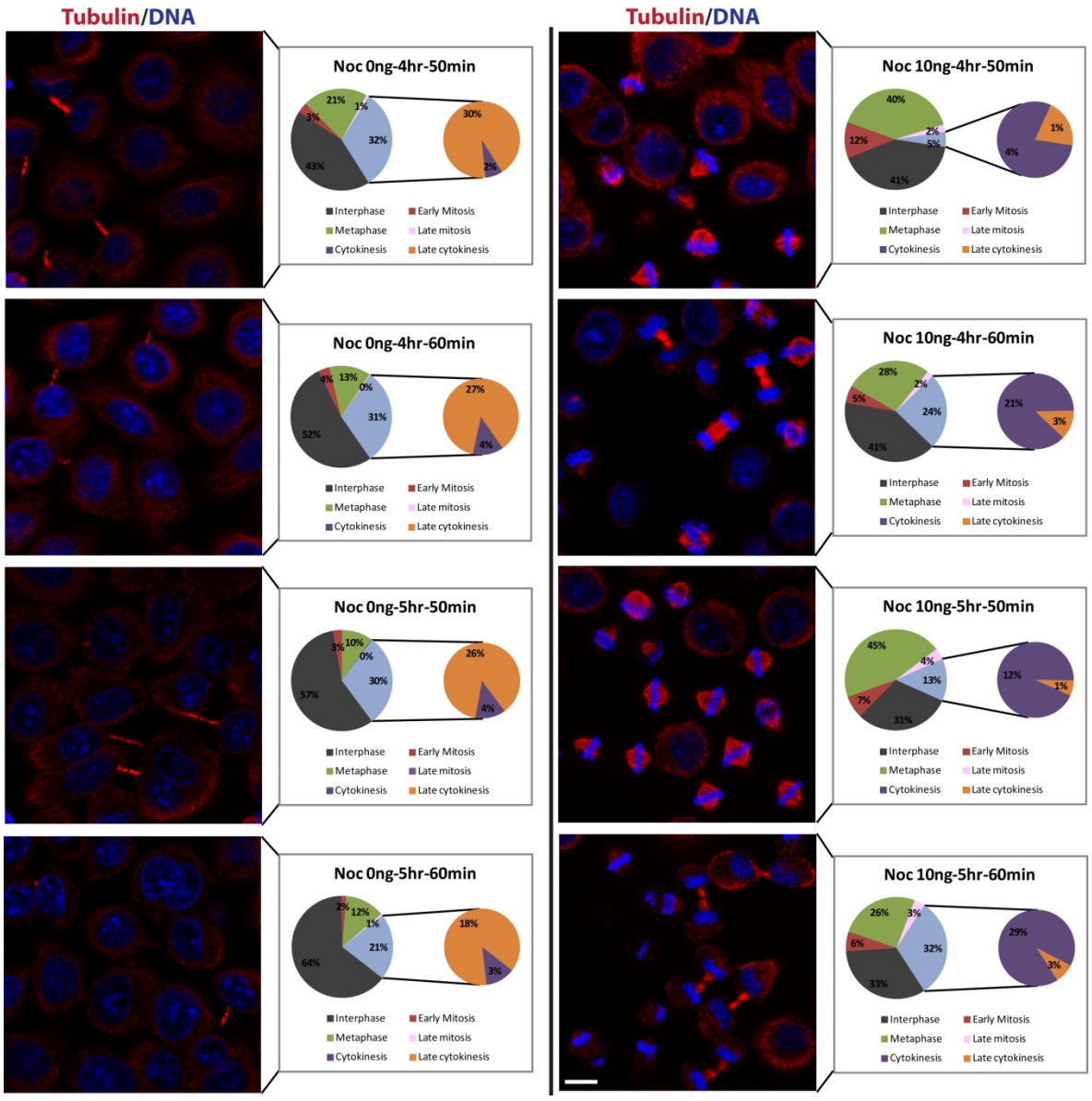

Figure 4.1.3 Cell cycle stage distributions of HeLa cells under different nocodazole treatment parameters. Cells were stained for DNA(blue) and α-tubulin(red) Images taken at 60x with 90i Nikon Eclipse Confocal Microscope. Quantification was done with ImageJ. Scale bar, 10 μm

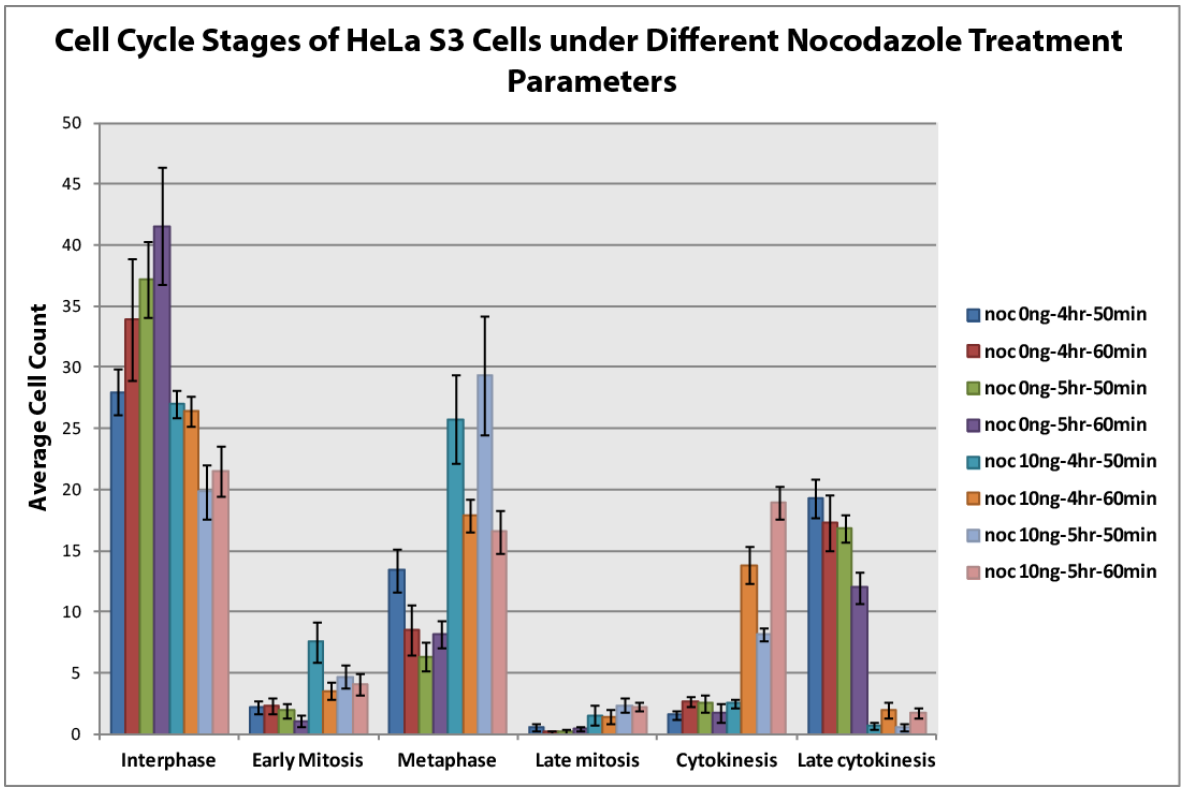

Figure 4.1.4 Comparison of Cell Cycle stages under different nocodazole treatment parameters. 60minutes of release after 5hours of 10ng nocodazole incubation gives the highest peak at cytokinesis phase of the cell populations. Quantification was done with ImageJ software (Plugins>Analyze>Cell counter) Raw data was normalized and average cell counts were calculated.

Since there is not an available marker specific to cytokinesis, the only way to show the effect of nocodazole on cytokinesis percentage of the cell population through western blotting was comparing the levels of pH3 between mitosis, interphase and nocodazole synchronized cytokinesis stage cells.

Ser-10 phosphorylated H3 is a widely used mitosis indicator. Its phosphorylation increases dramatically when cells enter mitosis and stays high until cells enter anaphase[18] making it a suitable marker for comparison of effect of nocodazole on distributions of cell cycle phases

For that purpose, cells were grown at 6-well plates and synchronized to interphase with double thymidine block and to mitosis and cytokinesis with nocodazole arrest method using the last optimized parameters. Interphase control cells were collected at the end of second thymidine incubation. After 7 hours of release from thymidine, cells were given 10ng of nocodazole and incubated for 5 hours at 37°C incubator. Then, mitosis control cells were collected via mechanical shake off from nocodazole arrested plates. For cytokinesis, remaining plates were washed with PBS and given DMEM complete for release. Following 60 minutes of incubation, cells were collected. 0ng nocodazole containing control samples were incubated for the same time unit with nocodazole synchronized cells and collected. SDS-PAGE and Western Blotting Analysis was done on the cells to compare mitosis marker Phospho-Histone H3 levels. Result at figure 4.1.5 shows that 10ng Nocodazole treated cells had lower levels of pH3 comparing to mitosis control and higher levels of pH3 comparing to 0ng nocodazole control. Consisted with the previous results from quantification experiment, this result was interpreted as cells treated with nocodazole were successfully proceeding through mitosis and in higher percentage of cytokinesis stage comparing to 0ng nocodazole treated ones.

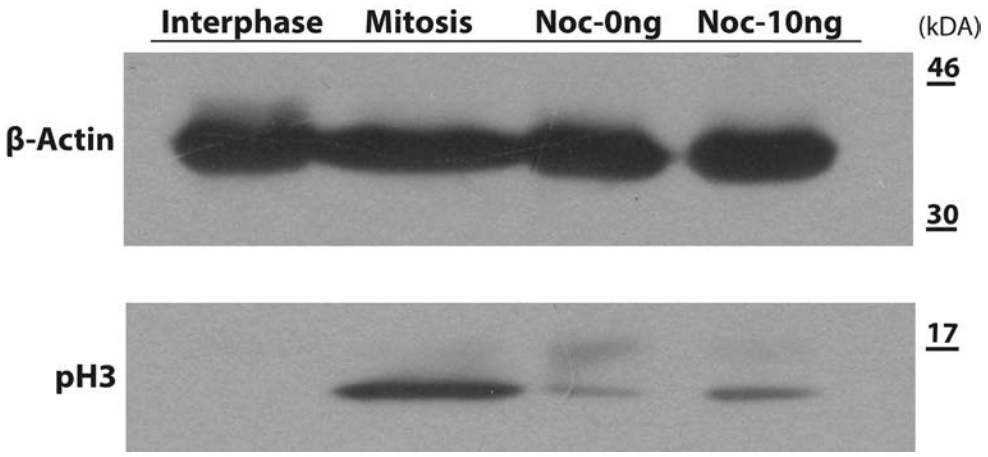

Figure 4.1.5 SDS-PAGE analysis of pH3 levels in different cell cycle stages. Level of mitosis marker phospho-Histone H3 decreases less in 10ng nocodazole treated cells comparing to 0ng nocodazole treated controls. See the results for further explanation. Beta-actin was used as a loading control.

4.2 Effects of potential inhibitors on Cell Cycle Stages of HeLa S3 cells

As it was stated in section 4.1, synchronization to cytokinesis stage was an essential requirement for the analysis of effect of drugs on the cells. Our hypothesis was that if the drugs are interfering with Aurora B kinase activity, they might have a negative effect on progress through mitosis. If the drug had such an impact, drug treated cells were expected to fail in entering cytokinesis comparing to controls.

Cells were grown on cover slips in 12-well plates and synchronized to interphase with double thymidine block. At the step of release from second thymidine, Drug1-10μM, Drug2-10μM and Drug2-20μM were given individually to the cells. After 7 hours of release from second thymidine, cells were given 10ng of nocodazole and incubated for 5 hours at 37°C CO_2 incubator. Nocodazole was washed out with PBS carefully to avoid losing weakly attached mitotic cells and fresh DMEM complete was given to release cells. After incubation for 60 minutes, cells on cover slips were collected, fixed and stained with DAPI for DNA and tubulin. 18 images from different spots were taken per sample with 90i Nikon Eclipse Confocal Microscope. Different phases of cell cycle were quantified with ImageJ software (Plugins>Analyze>Cell counter). Total count of cells per sample was normalized to 600 (median of the set) and pie charts and graphs for average cell count with standard error values were drawn.

Results at Figure 4.2.1 and 4.2.2 stated that treatment with increasing concentrations of Drug2 have dramatically decreased percentage of cytokinesis stage cells in the population from 56% to 30% at 10μM and 9% at 20μM conditions. In concordance, percentage of interphase increased from 19% to 28% at 10μM and 49% at 20μM conditions. Percentage of metaphase increased from 14% to 26% at 10μM and 23% at 20μM conditions. However,

Drug1-10μM treated cells had similar percentages of each cell cycle stage with untreated DMSO control.

Those findings indicated that drug2 might have caused an impact on proceeding through cytokinesis, keeping the cells at interphase and mitosis. The results are further discussed in discussion part.

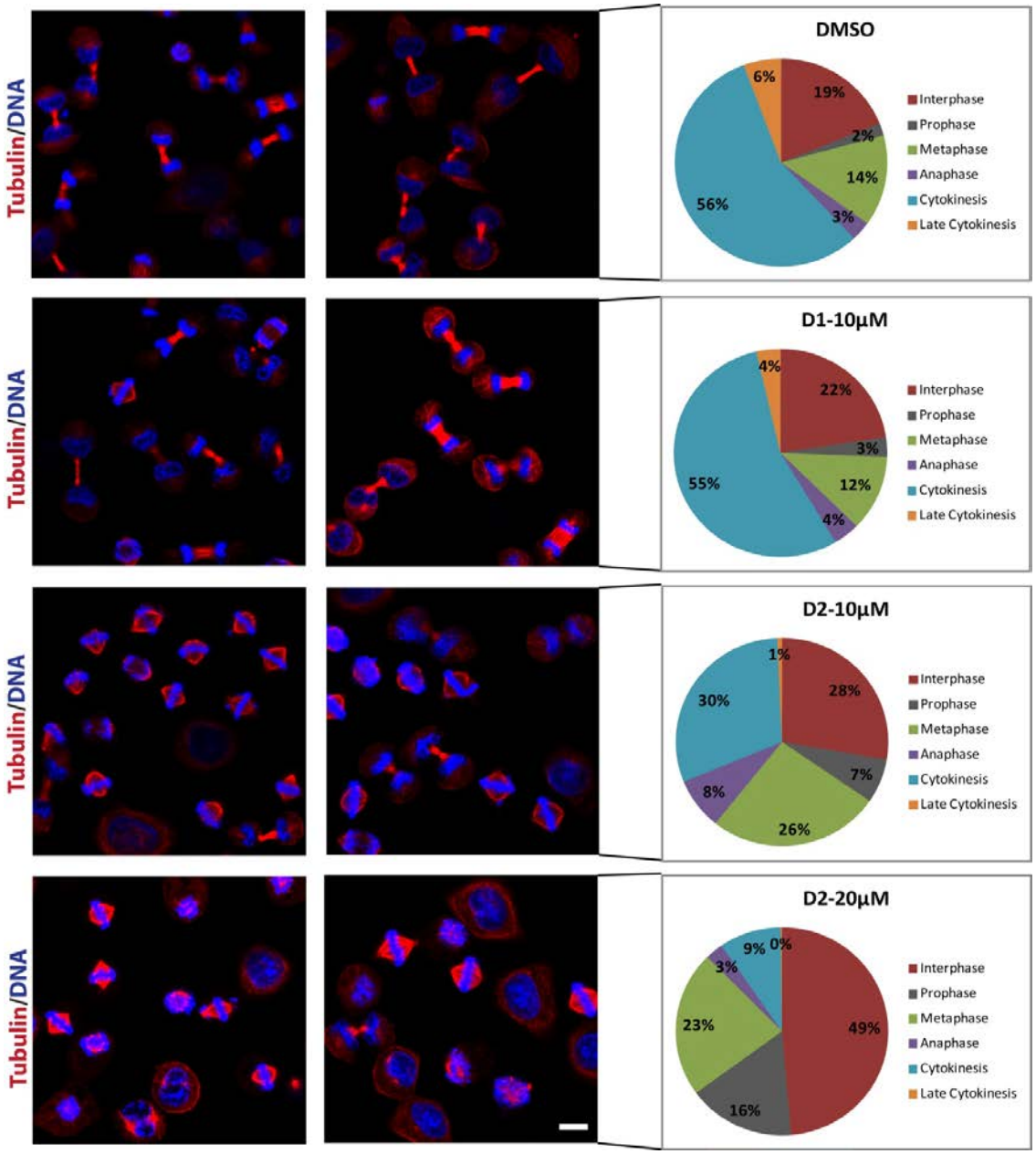

Figure 4.2.1 Cell cycle stage distributions of cytokinesis synchronized HeLa cells treated with drugs. Cells were stained for DNA(blue) and α-tubulin(red) Images taken at 60x with 90i Nikon Eclipse Confocal Microscope. Quantification was done with ImageJ. Scale bar, 10 µ

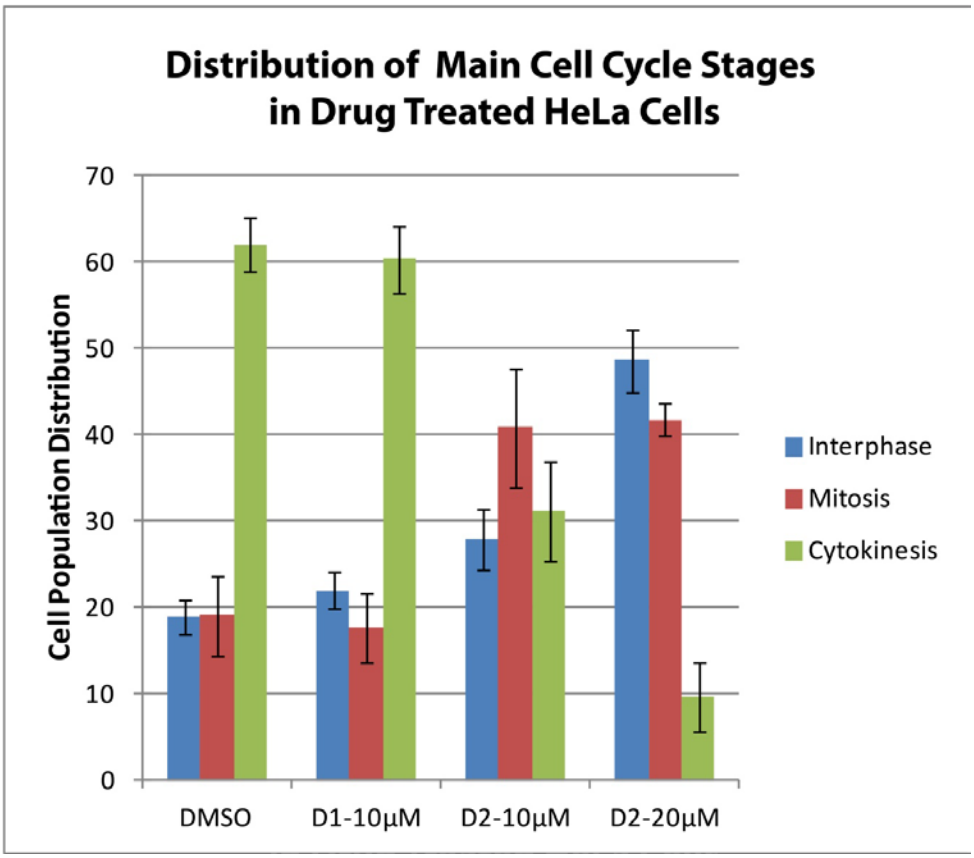

Figure 4.2.2 Distribution of three main cell cycle stages in drug treated HeLa cells. Cells were synchronized to cytokinesis with nocodazole arrest method. Drugs were given at the second thymidine release step and incubated for 13 hours. Quantification was done with ImageJ software. Raw data was normalized. Average cell counts were calculated and scaled to 100.

4.3 Effects of potential inhibitors on Histone3 Ser10 Phosphorylation in HeLaS3 cells

The effect of drugs on cell cycle was shown with quantification experiments in previous sections of this chapter. Together with that, we wanted to do an additional western blotting analysis to compare Mitosis and Cytokinesis synchronized samples were immunoblotted with mitosis marker Phospho-histone-H3. Tubulin was used as a housekeeping protein control.

Cells were grown in 6-well plates and synchronized to cytokinesis with the optimized nocodazole arrest and release method. At the step of release from second thymidine, Drug1-10μM, Drug2-10μM and Drug2-20μM were given individually to the cells. After 7 hours of release from second thymidine, cells were given 10ng of nocodazole and incubated for 5 hours at 37°C CO_2 incubator. Nocodazole was washed out with PBS carefully to avoid losing weakly attached mitotic cells and fresh DMEM complete was given to release cells. For the experiment of pH3 level analysis in mitosis, samples were collected right after release from nocodazole via mechanical shake off and pelleted. For the experiment of pH3 level analysis in cytokinesis, cells were released for 60 minutes and collected via trypsinization and pelleting. Cell Lysis, SDS-PAGE and Western Blotting procedure was done following the methods at 3.4.1 and 3.4.2.

In mitosis synchronized cells, there is no change in phospho-Histone-H3 levels in increasing concentrations of drug 2 except for 40μM condition (Figure 4.3.1). On the other hand, when we focused on cytokinesis synchronized cells, pH3 levels increase in increasing concentrations of drugs which is consistent with the quantification experiments (Figure 4.3.2).

If only the pH3 levels in mitosis synchronized cells were taken into consideration, we cannot see any difference in drug treatment. However if we couple it with pH3 level analysis of cytokinesis synchronized cells, it can be depicted that drug2 at increasing concentrations might have blocked passage through mitosis.

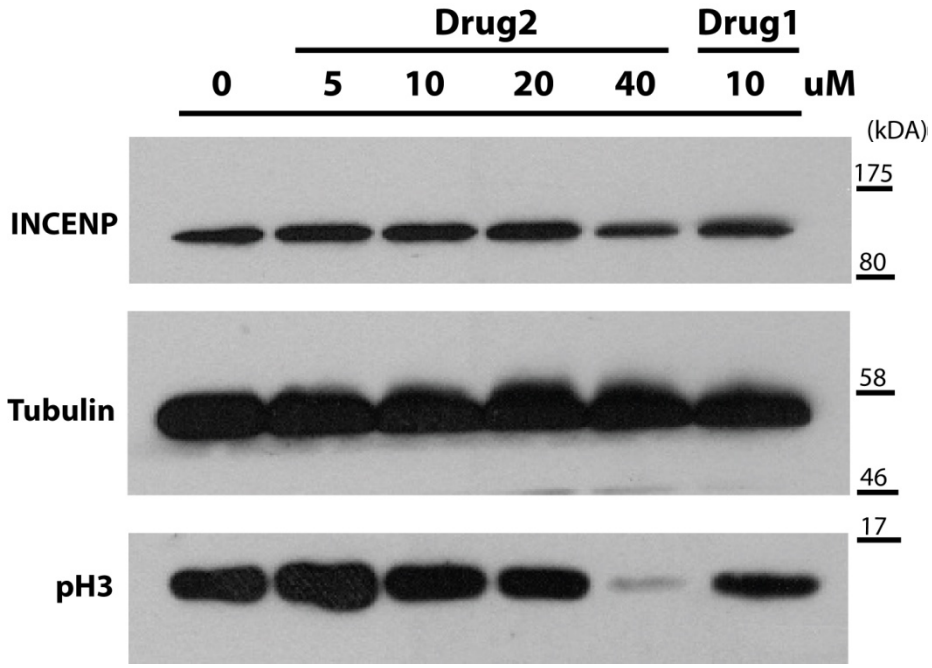

Figure 4.3.1 pH3 level analysis of Drug treated mitosis synchronized HeLa cells. Synchronization to mitosis was done with optimized nocodazole arrest method (See Materials&Methods) Drugs were given at the second thymidine release step and incubated for 13 hours.

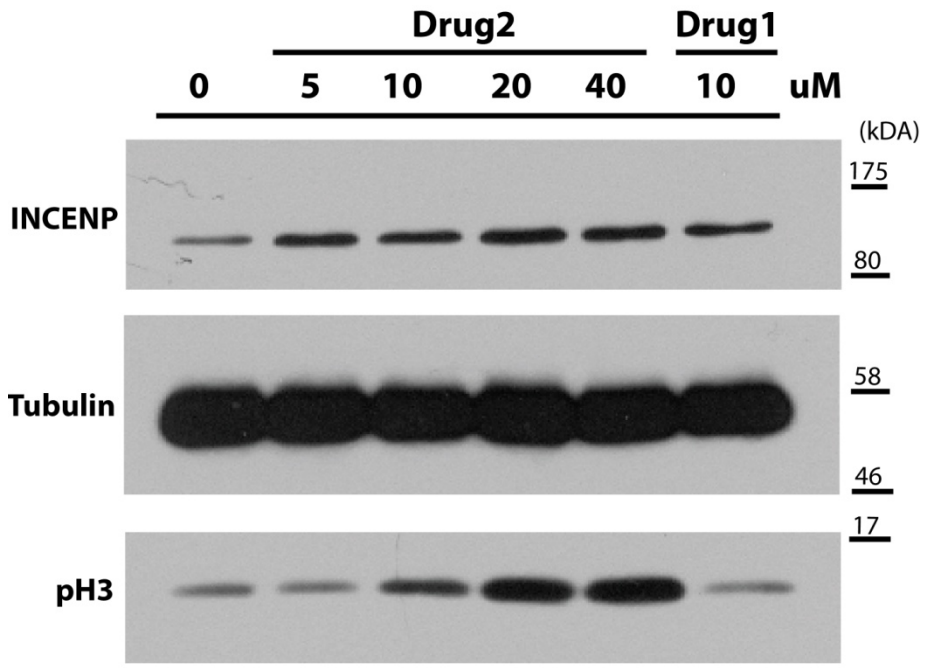

Figure 4.3.2 pH3 level analysis of Drug treated cytokinesis synchronized HeLa cells. Synchronization to cytokinesis was done with optimized nocodazole arrest method (See Materials&Methods) Drugs were given at the second thymidine release step and incubated for 13 hours.

4.4 Effects of potential inhibitors on Cellular Localization and Interactions of Aurora B and INCENP

Increasing concentrations of drug 2 caused a significant decrease in cytokinesis percentage in the population. We have questioned whether that decrease was connected to the possible intervention of the drug on Aurora B and INCENP interaction. To reveal that, we first set up a set of immunofluorescence experiment to analyze Aurora B and INCENP localization and possible phenotypic effects of the drug on the cells.

The same procedure at section 4.2 was applied to provide the same conditions of drug treatment and cytokinesis synchronization for an accurate comparison. Cells were grown on cover slips in 12-well plates and synchronized to cytokinesis. Drug2-10μM and Drug2-20μM were given individually to the cells just like in section 4.2 and incubated for 13 hours. After fixation, cells were stained with DAPI for DNA, Aurora B and INCENP. For quantification of Aurora B and INCENP intensity, 10 images of cells at metaphase from different spots were taken per sample with 90i Nikon Eclipse Confocal Microscope at 100x. Quantification of the intensity was done with ImageJ software.

Drug 2 at both 10μM and 20μM reduced Aurora B intensity on chromosomes at metaphase stage while there was no significant change in INCENP intensity(see Figure 4.4.1, Figure 4.4.2 and Figure 4.4.3), raising the deduction that the drug might have caused a failure of co-localization of Aurora B with INCENP on chromosomes properly during metaphase.

Another interesting finding was that, in both 10μM and 20μM concentrations of drug 2 an abnormal phenotype on DNA congression and localization in mitotic HeLa cells was observed (Figure 4.4.4). That abnormality can be separated to two distinct features which

were observed in many mitotic cells in both concentrations of the drugs. In some of the metaphase cells, alignment of the whole condensed chromosome was impeded leaving some portions of DNA separated from the metaphase plate. In some other cells, congressed DNA was dislocated to the poles of the cell instead of expected localization in metaphase plate which is approximately equidistant from the two poles of the cells.

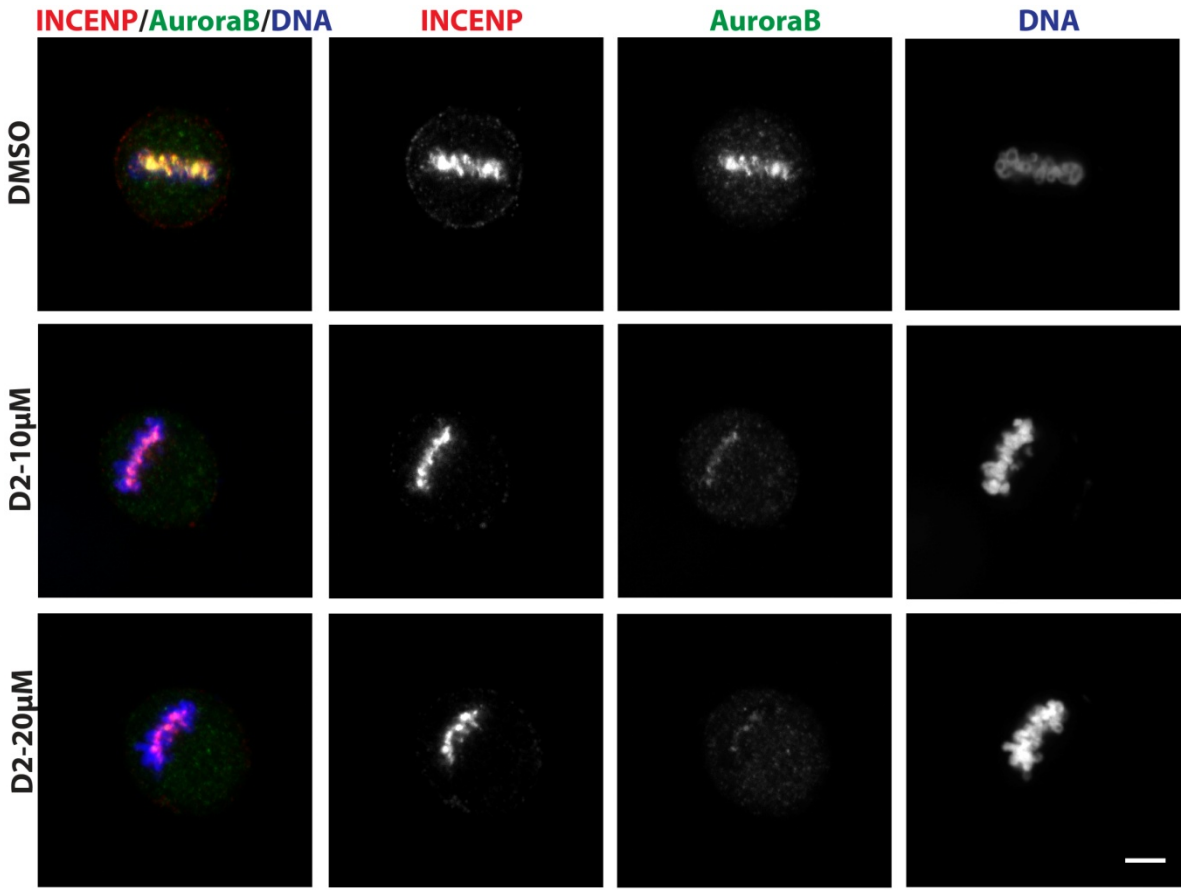

Figure 4.4.1 Drug treatment causes a decrease in Aurora B intensity in Mitotic HeLa cells. Cells were synchronized to mitosis with nocodazole arrest method (See Materials&Methods Section). Drugs were given at thymidine release step and incubated for 13 hours. Cells were stained for INCENP(red), AuroraB(green) and DNA(blue). Images taken at 100x with 90i Nikon Eclipse Confocal Microscope. Scale bar, 5µm

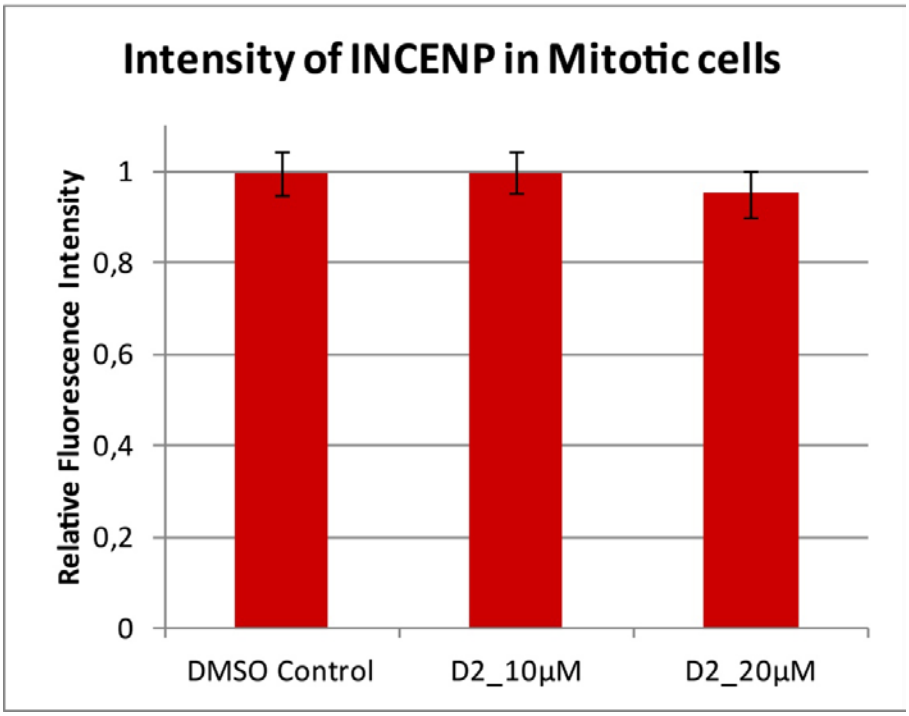

Figure 4.4.2 Quantification of INCENP intensity in mitotic cells. Comparing to DMSO control, there is not a difference in INCENP fluorescence intensity in Drug2 treated cells. Intensity quantifications were done with ImageJ Software.

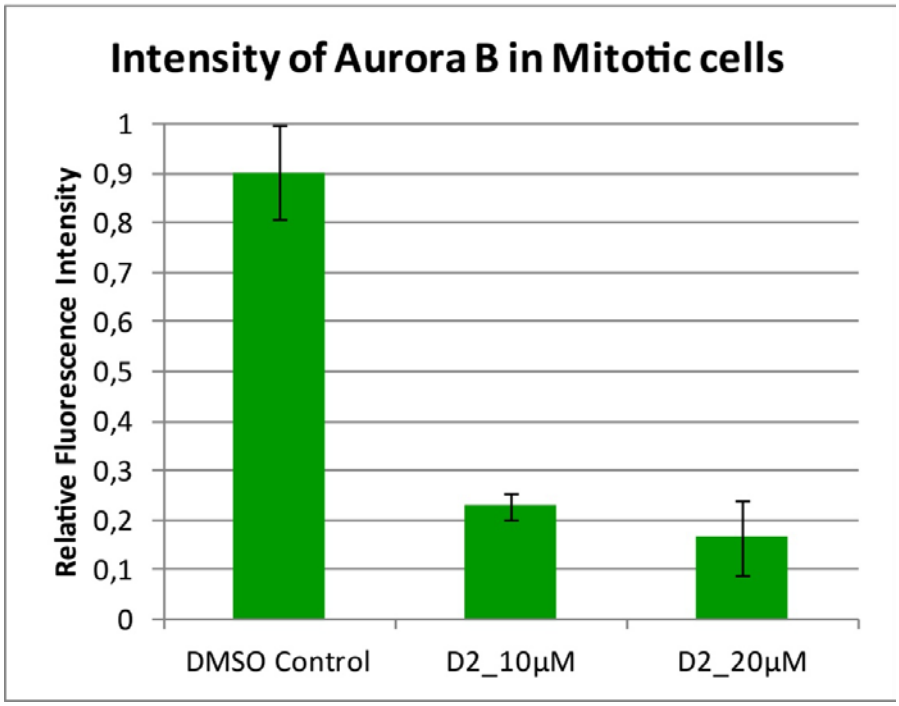

Figure 4.4.3 Quantification of Aurora B intensity in mitotic cells. There is a dramatic decrease in fluorescence intensity of Aurora B in Drug2-10µM and 20µM treated HeLa cells comparing to DMSO control. Intensity quantifications were done with ImageJ Software.

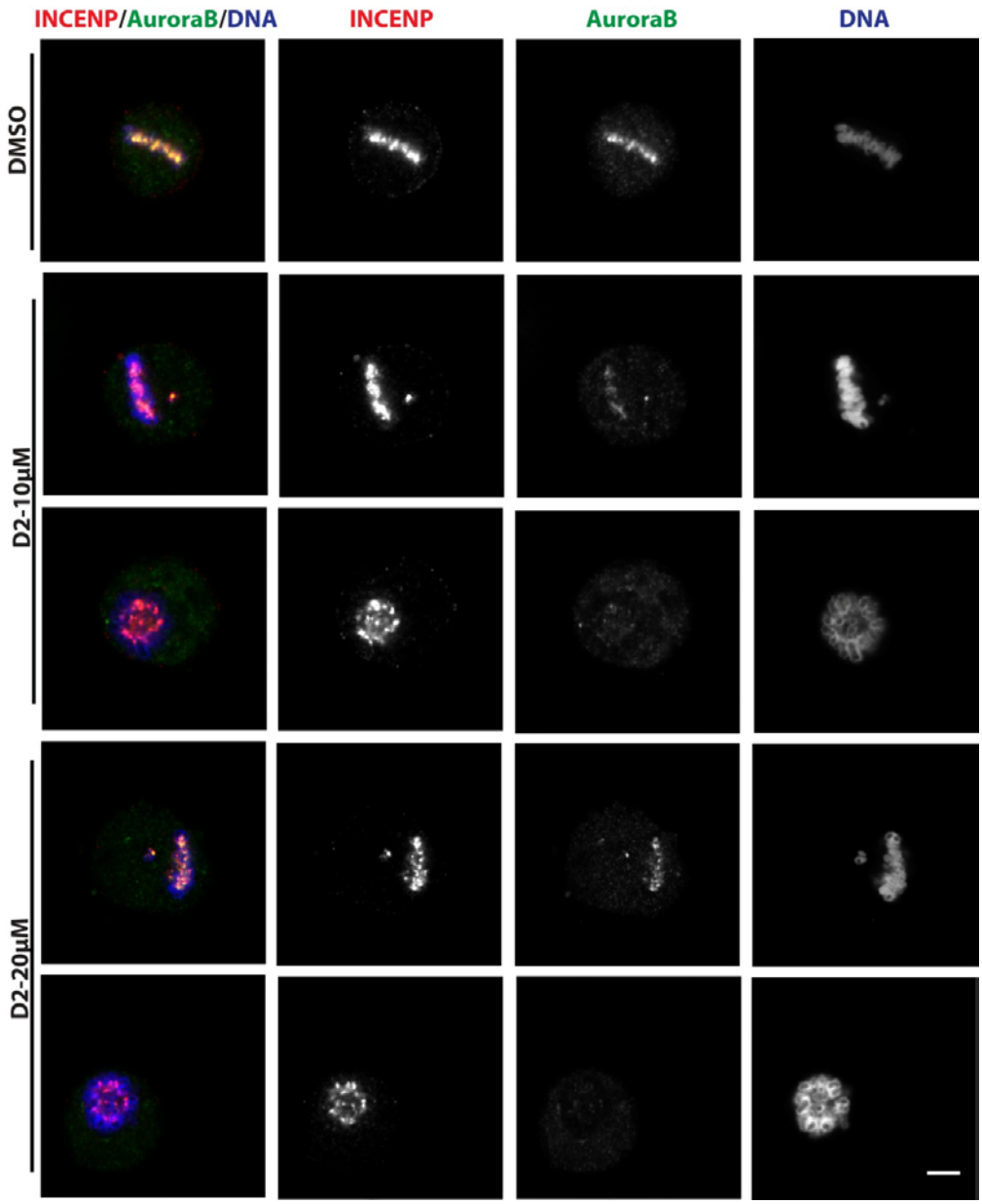

Figure 4.4.4 Drug2 treatment causes an abnormal phenotype on DNA congression and localization in mitotic HeLa cells. INCENP(red), AuroraB(green) and DNA(blue). Images taken at 100x with 90i Nikon Eclipse Confocal Microscope. Scale bar, 5μm.

4.5 Aurora B and INCENP interaction in the inhibitor treated HeLa S3 cells

Aurora B-GFP expressing stable cell lines were synchronized to Mitosis with Double Thymidine Block + STC synchronization method. Right after second thymidine release, 0 μM, 20μM and 40μM concentrations of drug2 were given together with STC. After incubation for 12 hours, cells were collected and lysed for immunoprecipitation. GFP-Trap®_A (Chromotek, gta-20) which has affinity towards GFP and GFP fusion proteins was used to trap Aurora B-GFP and its interaction partners. Non-GFP expressing HeLa S3 cells were used as a pull down control for GFP_Trap_A beads. SDS-PAGE and Western Blotting was done for analysis of protein interactions.

Drug 2 at 20μM decreased the levels of INCENP immunoprecipitated with AuroraB-GFP comparing to untreated DMSO control. (Figure 4.5)

At 40μM condition, the interaction totally vanished. However, in 40μM treated cells, pH3 levels were impeded, bringing the question that the decrease in Aurora B bound INCENP might have caused by the higher percentage of Interphase stage cells in the population. The results are further discussed in discussion section of the thesis.

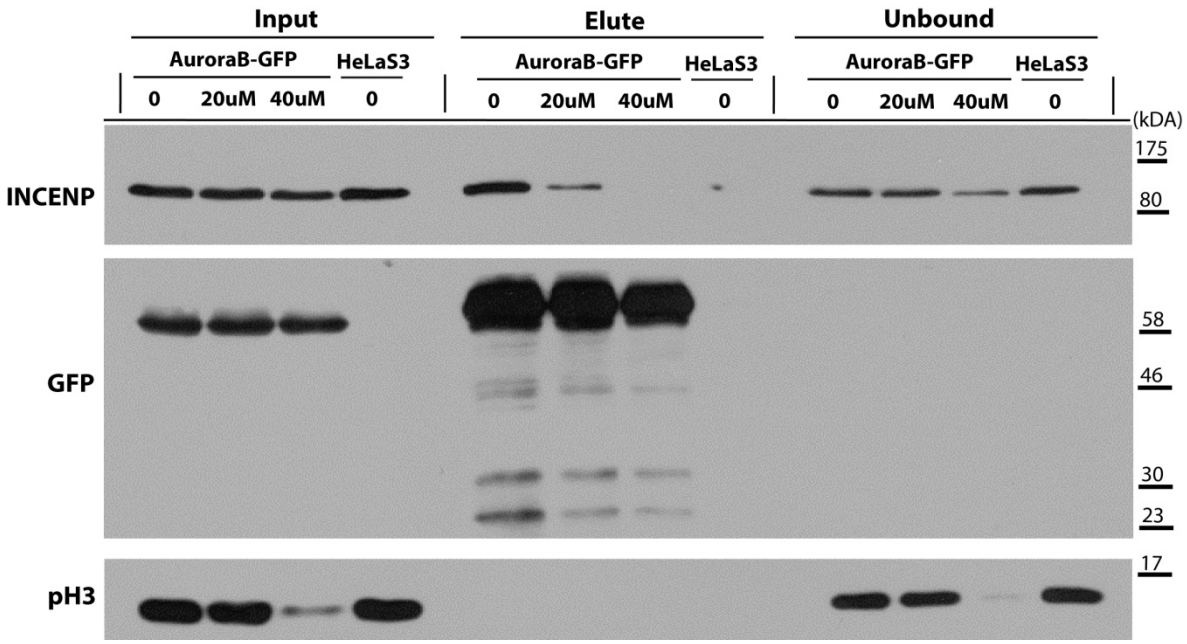

Figure 4.5 Immunoprecipitation of AuroraB-GFP with INCENP in Drug2, 0μM 20μM and 40μM treated AuroraB-GFP stably expressing cell lines. Cells were synchronized to mitosis with double thymidine release and STC treatment. GFP_Trap_A beads from chromotek were used to pulldown Aurora B-GFP fusion protein and its interaction partners. Associated proteins were detected on western blots.

DISCUSSION

The main purpose of this project was to identify and analyze novel drugs that target different parts of Aurora B kinase. The major advantage of such a drug is that it will be unique to Aurora B kinase and will not target other kinases, starting with Aurora A which has high similarity with Aurora B in structure[19]. There are several drugs targeting Aurora B and being clinically test, such as VX-680. The major problem with those drugs is that they are not specific to Aurora B and might inhibit other kinases too. And yet, those drugs failed the clinical trials due to their toxicity. Therefore targeting and finding Aurora B specific sites other than kinase pocket for those candidate drugs was essential. As Aurora B kinase would maintain the basal kinase activity, those drugs could be less toxic to the cells.

Another importance and use of finding an Aurora B specific drug is that it could be a good tool in future studies to identify Aurora-INCENP interaction dependent role of Aurora B kinase.

The major drawback of such an alternative drug design is that it is very difficult to target a protein via protein-protein interactions. And the cells need to be treated with the drug in high concentrations and for long hours. Even if drug potency can be improved it would never reach to the efficiency of drugs which target kinase pockets.

Taking all those into account, we have come up with a candidate drug targeting Aurora B INCENP interaction pocket. We have done series of biochemical analyses to identify their effect on Aurora B, its interaction with INCENP and fate of the cells.

Cytokinesis arrest optimization experiments have finalized percentage of cells at cytokinesis stage to 30% (Figure 4.1.4). With the same parameters applied, that percentage was increased to 56% in DMSO control of drug treatment experiments (Figure 4.2.2) with further The difference between those percentages might have caused by a possible loss of some of the weakly attached mitotic cells during nocodazole wash out step in the second set of nocodazole optimization experiments. Considering the results of all cytokinesis enrichment experiments, it can be concluded that nocodazole in we can conclude that the high percentage convinces about the efficiency of nocodazole optimization on enrichment of cytokinesis arrest which can be used for future purposes.

We concluded that physiological cytokinesis arrest of HeLa cells is enriched to 60% from 5% with optimized parameters of nocodazole amount, incubation and release times.

In the cell cycle distributions quantification experiment of drug treated cells, important changes comparing to untreated condition were found. Increasing concentrations of drug 2 decreased percentage of cytokinesis in the pool in inverse proportion comparing to drug 1 and DMSO control. Drug 2 $\geq$10μM might have had a negative impact on entry into cytokinesis.

In coordination with the findings of cell cycle distribution quantifications, an essential data was found in immunofluorescence experiments focused on Aurora B-INCENP interaction. In both 10μM and 20μM conditions of drug 2, fluorescence intensity quantification experiments revealed a dramatical decrease in Aurora B co-localized with INCENP on chromosomes during metaphase(Figure 4.4.3). We hypothesized that the decrease might have caused by the inhibitive effect of drug on Aurora B and INCENP interaction, since

binding and therefore activation of Aurora B to INCENP and CPC was required to proceed through mitosis.

Following that, we have set immunoprecipitation experiment to find out drugs direct effect on Aurora B and INCENP. Cells were synchronized to mitosis with STC synchronization and treated with 20μM and 40μM concentrations of drug2. INCENP immunoprecipitated with Aurora B decreased to less than half in 20μM and vanished at 40μM. The major complication of that exciting finding was that, in 40μM condition, mitosis marker phospho-Histone3 levels were lower meaning that the mitotic cells in the population were lower comparing to other conditions. Since Aurora B and INCENP interaction is initiated at the onset of mitosis, failure of INCENP immunoprecipitated with Aurora B in 40μM treated cells might be because of high percentage of interphase cells in the population. That interferes with revealing drugs inhibitory effect on Aurora B and INCENP interaction at 40μM concentration.

On the other hand, drug 2-20μM condition, which had promising results both in cell cycle quantification and immunofluorescence experiments have dramatically lowered INCENP immunoprecipitated with Aurora B in a population with adequate amount of mitotic cells as it is verified with equal amounts of pH3 levels with DMSO control. That data could be inferred as drug 2 might have bound to Aurora B-INCENP interaction pocket instead on INCENP.

Apart from intensity quantification part of the immunofluorescence experiments, we have observed interesting abnormal phenotypes on many mitotic cells in both drug 2-10μM and 20μM treatments (Figure 4.4.4). In some of the metaphase cells, alignment of the whole condensed chromosome was impeded leaving some portions of DNA separated from the

metaphase plate. In some other cells, condensed chromosome structure was different than other metaphase cells, more like in a collapsed and round form. In both phenotypes and the rest of the metaphase cells in drug treatments, chromosomes were dislocated to the poles of the cell instead of expected localization in metaphase plate which is approximately equidistant from the two poles of the cells. That phenomenon can be explained as follows. Metaphase cells were not able to connect to metaphase plate and the cells which have abnormal and round chromosomal condensation might have failed to enter metaphase, being stuck in between prophase and metaphase. Since Aurora B is directly involved in alignment of chromosomes to microtubules, those interesting phenotypes might be directly connected to drugs inhibitory effect on Aurora B and require comprehensive analysis of cells, including live cell imaging.

Recalling the primary purpose of that project, drugs specific to Aurora B kinase via targeting its interaction with INCENP were found. Several biochemical analyses were done to detect their impact on Aurora B-INCENP interaction and progression through cell division. Promising results were obtained with drug2 making it a potent candidate Aurora B kinase inhibitor, carrying the advantage of Aurora B specificity. Further in vitro analysis including kinase activity and in vitro binding assay are needed to verify direct effect of the drug on Aurora B and INCENP interaction.

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VITA

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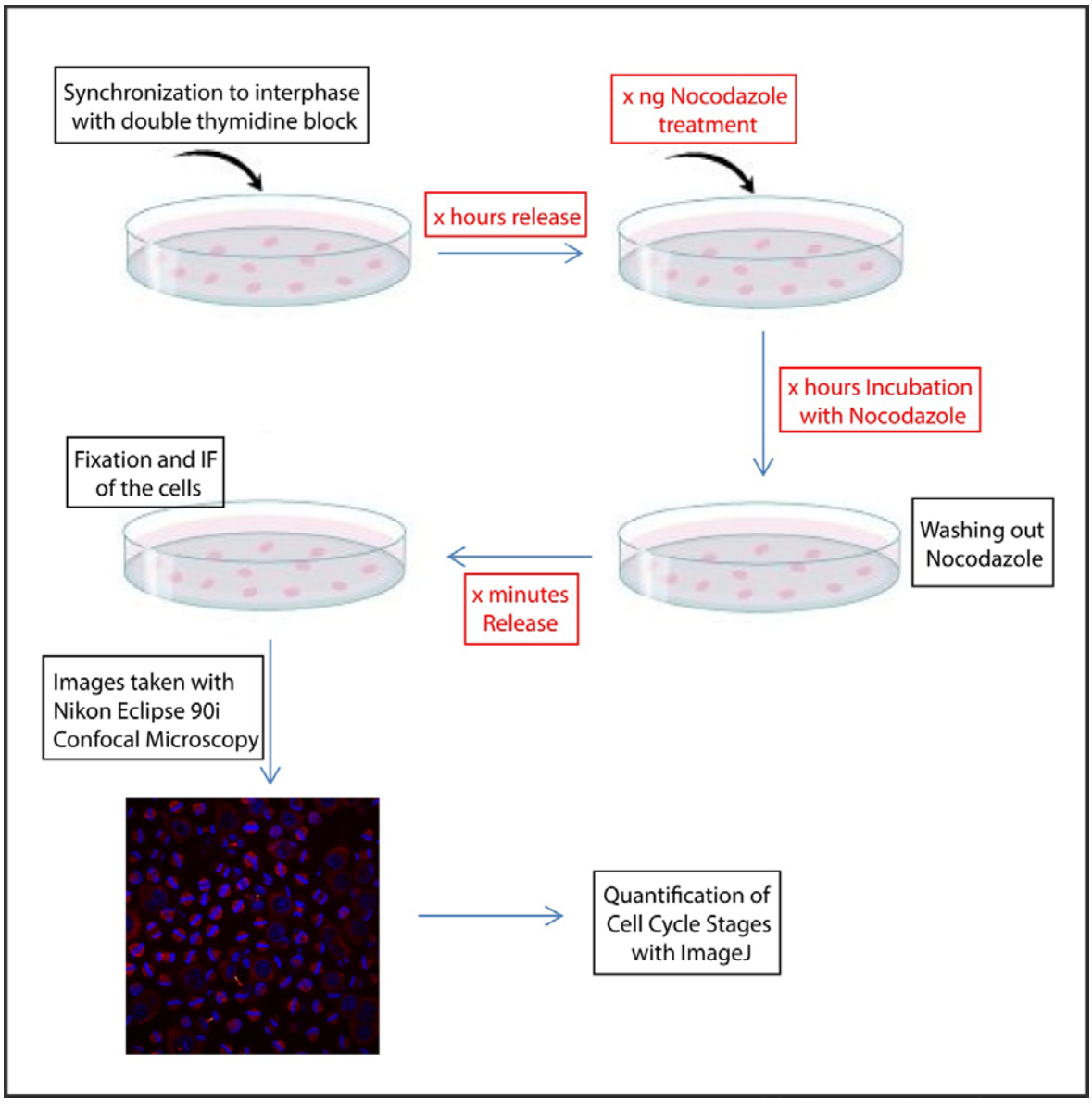
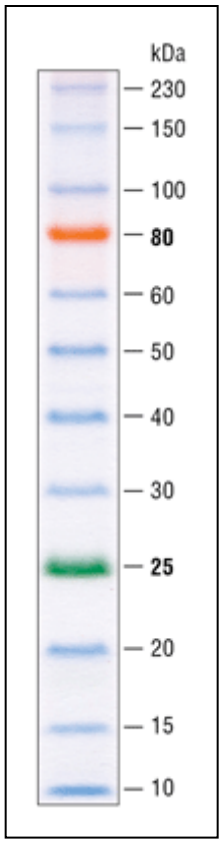
Appendix A: Workflow of Cytokinesis Synchronization Optimization Experiments



Appendix B: Protein Marker



ColorPlus Prestained Protein Ladder, Broad Range (10-230 kDa), *10-20% Tris-glycine SDS-PAGE gel*, New England Biolabs (NEB)