Doctorate Program in Molecular Oncology and Endocrinology
Doctorate School in Molecular Medicine

XXVII cycle - 2011–2014
Coordinator: Prof. Massimo Santoro

“Identification of novel small molecule inhibitors of Aurora B kinase”

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“Identification of novel small molecule inhibitors of Aurora B kinase”

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LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

1. Brendan Frett, Francesca Carlomagno, Maria Luisa Moccia, Annalisa Brescia, Giorgia Federico, Valentina De Falco, Brittany Admire, Zhongzhu Chen, Wenqing Qi, Massimo Santoro, Hong-yu Li. Fragment-based discovery of a dual pan-RET/VEGFR2 kinase inhibitor optimized for single-agent polypharmacology. (Manuscript under review;Angewandte chemie)

2. Marialuisa Moccia, Qingsong Liu, Teresa Guida, Giorgia Federico, Annalisa Brescia, Zheng Zhao, Hwan Geun Geun, Xianming Deng, Li Tan, Jinhua Wang, Marc Billaud, Nathanael S. Gray, Francesca Carlomagno, Massimo Santoro. Identification of novel small molecule inhibitors of oncogenic RET kinase. (Manuscript under review; PLOS ONE)

ABBREVIATIONS

ALL: Acute Lymphoblastic Leukemia
AML: Acute Myeloid Leukemia
AMPK: AMP-activated Protein Kinase
AMPK-r: AMPK related protein Kinase
APC/C: Anaphase Promoting Complex/ Cyclosome
APS: Amonium PerSulphate
Arf6: ADP-ribosylation factor 6
Ark1: Aurora Related Protein
Arpc1b: Actin-related protein 2/3 complex subunit 1b
ATM: Ataxia Telangiectasia Mutated
ATP: Adenosine 5'-TriPhosphate
ATR: Ataxia Telangiectasia and Rad3 Related
AXL: AXL receptor tyrosine kinase
BCR-ABL breakpoint cluster region-abelson
BMMCs: Bone Marrow Mononuclear Cells
B-NHL : B-cell Non-Hodgkin Lymphoma
BORA: Protein aurora borealis
BSA: Bovine Serum Albumin
BUB1: Budding Uninhibited by Benzimidazoles 1
BUBR1: Budding Uninhibited by Benzimidazoles Related 1
CCAN: Constituting Centromere-Associated Network
Cdc: Cell division cycle
Cdk: Cyclin dependent kinase
Cdh1: Cdc20 homolog 1
CENP: CENtromere Protein A
Cep: Centrosomal protein
Chk1/2: Checkpoint kinase 1/2
CLL: Chronic Lymphocytic Leukemia
CM: Cutaneous Melanoma
CML: Chronic Myelogenous Leukemia
CPC: Chomosomal Passenger Complex
DAPI: 4',6-DiAmidino-2-PhenylIndole
DDR2: Discoidin Domain-Containing Receptor 2
DMEM: Dulbecco's Modified Eagle Medium
DMSO: DiMethyl SulfOxide
DNA: Deoxyribonucleic Acid
DSBs: Double-Strands Breaks
Ect2: Epithelial cell transforming sequence 2 oncogene
EDTA: EthyleneDiamineTetraaceticAcid
ELISA: Enzyme-Linked Immunosorbent Assay
ESCRT: Endosomal Sorting Complex Required For Transport
FACS: Fluorescence Activated Cell Sorting
FDA: Food and Drug Administration (US)

Fbxw7: F-box/WD repeat-containing protein 7
HJURP: Holliday JUnction- Recognizing Protein
INCENP: INner CENtromere Protein
IPL1: Increase-in-Ploidy
Kif4: Kinesin family member 4A
KSP: Kinesin Spindle Protein
Mad1/2: Mitotic arrest deficient-like 1
MAPK mitogen-activated protein kinase
MCAK: Mitotic Centromere-associated Kinesin
MCC: Mitotic Checkpoint Complex
Mklp1: Mitotic Kinesin-Like Protein 1
MM: Multiple Myeloma
Mps1: Monopolar Spindle 1
MRLC: Myosin Regulatory Light Chain 2
NEDD9: Neural precursor pell Expressed, Developmentally Down-Regulated 9
NHL: Non-Hodgkin Lymphoma
NPM: Nucleophosmin/B23
OD: Optical Density
PBS: Phosphate Buffered Saline
PCM: PeriCentriolar Material
Phk: Phosphorylase kinase
Plk1: Polo-like kinase1
PP1: Protein Phosphatase 1
Prc1: Protein regulator of cytokinesis 1
RacGAP: Rac GTPase Activating Protein
Rad: Rad recombinase
Ran-GTP: RAs-related Nuclear protein-GFP
Rb: Retinoblastoma protein
RhoGAP: Rho GTPase Activating Protein
Rna: Ribonucleic Acid
RPMI medium: Medium from Roswell Park Memorial Institute
SAC: Spindle Assembly Checkpoint
SDS: Sodium Dodecyl Sulphate
SDS-PAGE: SDS polyacrylamide gel electrophoresis
TACC3: transforming, acidic coiled-coil containing protein 3
Tak1: Epithelial transforming growth factor β-activated kinase 1
TBK1: TANK-Binding Kinase 1
TD-60: Telophase Disc 60 protein
TEMED: N,N,N',N'-TEtraMethylEthyleneDiamine
Tlk1: Tousled-like kinase 1
TORC2: Transducer Of Regulated CREB Protein 2
TPX2: Targeting Protein for Xenopus kinesine like protein 2
TRKA/B: Tropomyosin Receptor Kinase A/B
γ-TuRC: γ-tubulin ring complexes
Zwi: Zwint 1 protein

ABSTRACT

Aurora kinases (A, B and C) are frequently amplified and overexpressed in a wide variety of cancers, and their up-regulation often correlates with poor prognosis. The potential role of these kinases in tumorigenesis indicates that they could be appealing target for molecular therapy. Although extensive efforts have been done to develop Aurora kinases inhibitors (AKIs), selective for A, B or both kinases, so far, none of them have yet been approved by FDA due to their severe toxicity. The aim of this study has been to identify novel and powerful AKIs, Aurora B kinase inhibitors.

In living cells, we tested the inhibition of Aurora B kinase by 7 potential novel AKIs, called MK1-7, which share a common molecular scaffold, by evaluating Serine 10 phosphorylation of Histone H3, a *bona fide* downstream target of the kinase in HeLa cells. We selected 4 compounds (MK1, 2, 6 and 7), that were able to reduce phosphorylation of Histone H3 at 10 nM dose. Then, we evaluated the effect of each inhibitor on: cell cycle progression, spindle assembly checkpoint (SAC) escape, cell proliferation and viability. Although with different efficacy, these 4 MKs perturb cell cycle progression inducing polyploidy. In addition, cells treated with the 4 MKs escape from mitosis overtaking the SAC checkpoint. These MKs blocked cell growth with an IC_{50} ranging from 0.37 to 2.27 nM (MK1 IC_{50}=1,2 nM; MK2 IC_{50}=2,27 nM; MK6 IC_{50}=0,44 nM; MK7 IC_{50}=0,37 nM). Based on MK7 most powerful efficacy, its effect on cell division and viability was more extensively investigated. HeLa cells treated with MK7 were positive for apoptotic Annexin V and propidium iodide staining and showed an increase in caspase 3 and PARP cleavages, indicating induction of apoptosis. Additionally, MK7 treatment caused alteration in mitotic spindle formation, chromosome segregation and cytokinesis. Finally, we observed that MK7 is able to inhibit growth of A2780 (human ovarian carcinoma), HL-60 (human promyelocytic leukemia), HCT116 (human colorectal carcinoma) and 8505-C (human undifferentiated thyroid carcinoma) cell lines. In conclusion, our *in vitro* data have identified in MK7 a powerful compound which could be widely used to inhibit Aurora B kinase in different cancer types.

1.0 BACKGROUND

1.1 Cell Cycle

The cell cycle is the series of events that take place in a cell leading to its division and duplication (replication) with production of two daughter cells. In cells without a nucleus (prokaryotic), the cell cycle occurs via a process called binary fission. In cells with a nucleus (eukaryotes), the cell cycle can be divided into three periods: interphase, the mitotic (M) phase, and cytokinesis. During interphase the cell grows, accumulates nutrients, duplicates its DNA and prepares itself for mitosis and cell division . During the mitosis, the cell separates sister chromatides; the cell divides during the final stage, cytokinesis. (Figure 1).

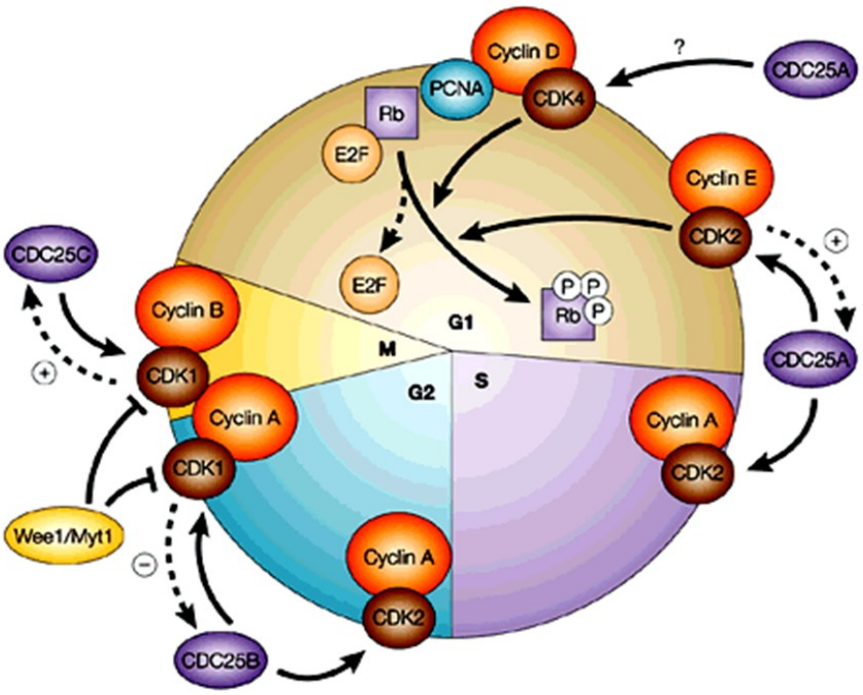


Figure 1. Stages of the eukaryotic cell cycle.
The sequential activation of the specific regulatory factors (cyclin and cyclin-dependent kinases - Cdks) of cell cycle are indicated.

1.1.1 Interphase

This is the first stage of cell cycle when the cell is engaged in metabolic activity and prepares itself for mitosis. Interphase comprises four phases (G0, G1, S and G2) and is mainly regulated by the interaction of Cyclins and cyclin-dependent kinases (Cdks) (Figure 1). G0 phase (G = gap) is a quiescent state in which the cell is unable to progress through cell cycle. When stimulated by mitogenic growth factors, Cdk4 and Cdk6 interact with D-type cyclins (cyclin D1, cyclin D2 and cyclin D3) to form Cdk-cyclin D active complexes. These complexes initiate partial phosphorylation of the retinoblastoma protein (Rb) that binds to the E2F transcription factors and inactivates its function as transcriptional repressor allowing the cell to enter the G1 phase. The G1 phase is the major period of cell growth in which the cell synthesizes RNAs, proteins and increases its size. In late G1 phase, active Cdk2-cyclin E complexes reinforce Rb phosphorylation leading to the release of E2F which participates in the expression of genes required for G1 to S phase transition and DNA synthesis. Rb hyperphosphorylation corresponds to the restriction point (G1 checkpoint), in which the cell decides either to pause or to continue cell division. Subsequent to G1, the cell enters S phase for DNA synthesis. Finally, G2 phase is the period for final DNA repair, rapid growth of organelles as well as protein synthesis before initiation of Mitotic phase (M-phase). G2 checkpoint at the end of this gap determines whether cells can enter mitosis or must extend G2 for further cell growth or DNA repair. The Cdk1-cyclin B complex is a key regulator of the G2/M transition driving cells into mitosis through phosphorylation of many substrates that contribute to chromosome condensation, nuclear envelope breakdown and spindle assembly (Enserink et al. 2010; Lapenna et al. 2009).

1.1.2 Mitosis

After successful interphase completion, somatic cells go through mitotic phase for cell division. Mitosis (nuclear division) is the most dramatic stage of the cell cycle, corresponding to the separation of daughter chromosomes and usually ending with cell division (cytokinesis). Mistakes in this stage may lead to uncontrolled cell division, aneuploidy and genetic instability that lead to cancer development. Mitosis is divided into five phases: prophase, prometaphase, metaphase, anaphase and telophase (Figure 2).

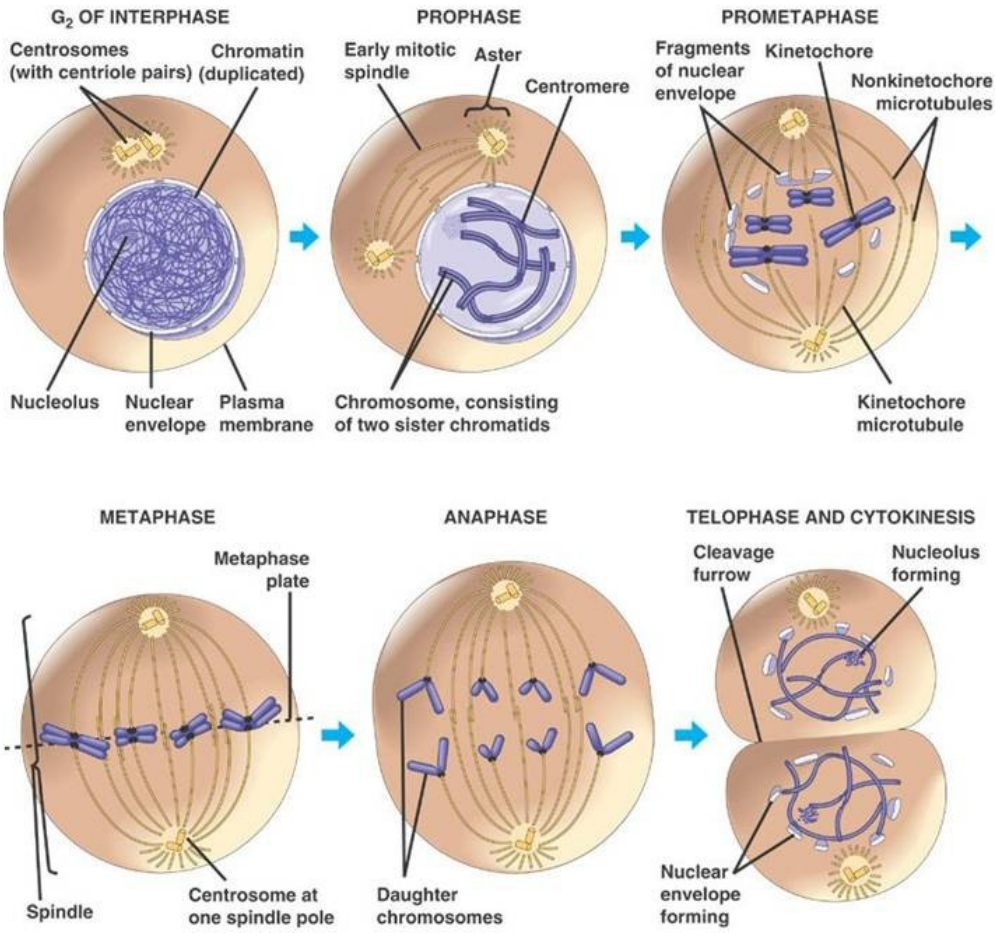


Figure 2 Stages of mitosis
In M-phase, the cell goes through 5 phases: prophase, prometaphase, metaphase, anaphase and telophase for seperation of chromosomes. Finally, the cytoplasm is divided through cytokinesis to form two daughter cells. The specific events of each phase are indicated in the picture.

At prophase, chromosome condensation begins, the duplicated centrosomes separate, and some mitotic checkpoint proteins, including Bub1 and BubR1, are recruited to kinetochores on the chromosomes (Haruki et al. 2001). At the entry of prometaphase, nuclear envelope breaks down. Microtubules emerging from the centrosomes at the spindle poles reach the chromosomes and attach to the kinetochores. During metaphase, the kinetochore-microtubules pull the sister chromatids back and forth until they align along the equatorial plate – an imaginary plane locating midway between two centrosome poles. The mitotic spindle checkpoint is activated by the signals from unattached kinetochores that delays progression to anaphase until all sister chromatid pairs are attached to the spindle microtubules and properly aligned (Musacchio and Salmon

2007). When this checkpoint is turned off, anaphase starts. Within anaphase, kinetochores provide the pulling forces which allow two sister chromatids of each chromosome to pull apart toward opposite poles. Simultaneously, the midzone is organized by a redistribution of different molecules like actin and myosin to form the cleavage furrow. Finally, at telophase, chromatids complete their movement, a new nuclear membrane is formed around each set of sister chromatids and chromosomes start to decondense into chromatin. At the end of telophase, a contractile ring called midbody is formed to initiate the process of cytokinesis. Cytokinesis is the final stage of cell division, which distributes the cytoplasm of a parental cell into two daughter cells. The actinomyosin ring constricts the plasma membrane in the equatorial region of a dividing cell to form a cleavage furrow between the cytoplasmic content of two daughter cells. The ingressing cleavage furrow compresses antiparallel microtubules of the midzone into a single large microtubule bundle that comprises the core of the midbody. A budge-like structure called the stem body is formed in the center of midbody. Midbody localizes the site of abscission where the two daughter cells will separate at the end of cytokinesis. A recent study (Hu et al. 2012) described the relocalization of midzone microtubule-interacting proteins during midbody formation. These proteins localize to three different parts of midbody: centralspindlin (Mklp1 and RacGAP1) and its partners (Cep55, Arf6 and Ect2) accumulate in the budge of midbody; Prc1 and Kif4 colocalize in the dark zone – a narrow region on the stem body unstained with tubulin; while CENP-E, Mklp1 and Aurora B were observed in the flanking zone, outside of the dark zone. Mklp1 interacting with RacGAP1 forms the centralspindlin complex that might be required to recognize the antiparallel microtubule structure of the stem body (Hutterer et al. 2009). Kif4 localizes to plus-ends microtubules to inhibit their dynamics (Hu et al. 2011). Prc1 stabilizes the microtubule overlap. Together with Mklp1, Prc1 helps to transport Polo-like kinase 1 (Plk1), a key regulator of cytokinesis, on microtubules. Aurora B kinase and Plk1 act as master regulators to ensure proper progression of abscission (Steigemann et al. 2009). Abscision, the final separation of daughter cells, requires coordination of many molecular machines, including endocytic and secretory vesicle trafficking proteins as well as ESCRT (endosomal sorting complex required for transport) proteins (Hu et al. 2012).

1.2 Structures in mitosis: mitotic spindle and centromeres/kinetochores

1.2.1 Mitotic spindle

The mitotic spindle is a bipolar, self-organizing microtubule-based machine that accurately segregates sister chromatids into the daughter cells during cell division. The major structural elements of the spindle are microtubule polymers, which are nucleated from the two centrosomes, major components of

the spindle poles. As the main microtubule-organizing center of animal cells, the centrosome participates in the regulation of cell mobility, organelle positioning, intracellular transport and mitotic spindle assembly (Lawo et al. 2012). It comprises a centriole pair surrounded by a matrix of proteins called PeriCentriolar Material (PCM) (Figure 3). Centrioles are cylindrical structures composed of nine triplets of microtubules, that are required for the formation of centrosomes (Nigg et al. 2009). In each centriole pair, there are two unequal centrioles in which the older carries appendages that are close to its distal end. During S-phase, each parental centriole (mother centriole) is duplicated by the formation of procentriole (daughter centriole) at the proximal end. Procentrioles then elongate until maximum length at the end of G2 and reach full maturation during late G2 and early M phase by the acquisition of appendages. At the end of mitosis, centrioles in each pair begin separation in a process called centriole disorientation. During centrosome maturation, the PCM increases in size and γ-tubulin ring complexes (γ-TuRC) are recruited from the cytosol, thereby promoting microtuble nucleation, the key role of centrosome. Previous studies only reported about a uniform amorphous electron-dense structure of PCM around the centrioles. Recently, by using 3D structured illumination microscopy (3D-SIM), the structure of PCM was elucidated (Figure 2). It is composed of nine centrosome components (CPAP, centrin, CEP120, CEP192, CEP152, CDK5RAP2, NEDD1, TUBG1, PCNT) organized in a toroidal manner around the proximal end of mother centriole in interphasic cells (Lawo et al. 2012). The key protein in the centrosome that nucleates assembly of microtubules is γ-tubulin (TUBG1). Complexes of γ-tubulin form ring structures that contain 13 γ-tubulin molecules and have diameters similar to those of microtubules. These γ-tubulin rings serve as nucleation sites for the assembly of microtubules and may remain bound to their minus ends. From these points, α/β-tubulin-heterodimers are attached and polymerized at their plus end, their parallel arrangement around a hollow core form microtubules.

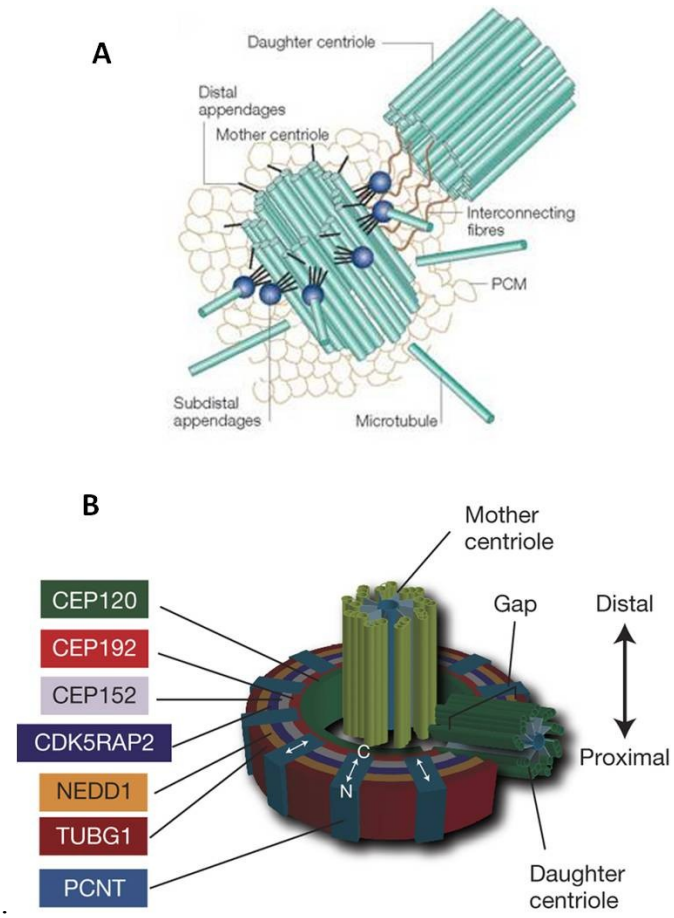


Figure 3. Structure of mitotic spindle.
Microtubules are nucleated from two centrosomes forming a mitotic spindle for chromosome separation in mitosis; (A) Each centrosome is composed of two orthogonally arranged centrioles surrounded by an amorphous mass of pericentriolar material (PCM). Each centriole comprises nine triplets of microtubles; the older of the two centrioles (mother centriole) has two sets of appendages (additional proteins) along the exterior surface. Microtubules are nucleated from the γ-tubulin ring complexes (γ-TuRC) contained in PCM. (B) Organized-structure of PCM in interphase cells. 9 PCM components are indicated (Lawo et al. 2012).

1.2.2 Centromere – Kinetochore

Centromere is the center region of chromosome. Centromeric chromatin is epigenetically characterized by the presence of specialized nucleosomes in which the canonical histone H3 is replaced by the variant CENP-A (centromeric protein –A) (Guse et al. 2011). HJURP (Holliday JUnction-Recognizing Protein) has been identified as a CENP-A chaperone, which directly interacts with newly synthesized CENP-A and guides CENP-A for its deposition into the centromeric nucleosomes (Dunleavy et al. 2009). The

deposition of CENP-A at centromeres is an important marker for the recruitment of a group of 16 proteins constituting the centromere-associated network (CCAN) (Westermann and Schleiffer 2013). Recent studies have elucidated the function of CCAN proteins as an assembly platform for the microtubule-binding interface of the kinetochore, called KMN network, consisting of KNL1, Mis12 complex and Ndc80 complex. The connection between centromeric nucleosomes and microtubules through kinetochore enables for the equal chromosome segregation at anaphase. Conventional electron microscopy studies indicated that the kinetochore has a layered structure with an electron dense inner plate that contacts centromeric chromatin, an outer plate that contacts microtubles and a "fibrous corona" middle zone that extends away from the outer plate. In recent years, advances in biochemical and proteomic approaches have identified the components of kinetochore as well as their functions. Thereby, CENP-A and CCAN subunits form a core, conserved part in the inner plate of kinetochore. CCAN proteins recruit outer kinetochore components (KMN network) that attach to the spindle microtubules. In detail, the N-terminal region of CENP-C interacts with Nnf-1 and more weakly with Nsl1, both subunits of the outer plate Mis12 complex (Screpanti et al. 2011; Przewloka et al. 2011). Through the connection with KNL1 and Ndc80 complexes, Mis12 complex enhances the binding of microtubules to kinetochores. How the microtubules are attached to kinetochore? The Ndc80 complex contains four proteins forming two dimers Ndc80/Nuf2 and Spc24/Spc25 linked by long α-helical coiled-coil rod domains. First, the Hec1/Nuf2 component of the Ndc80 complex attaches with the plus-end of microtubule; then the Spc24/Spc25 heterodimer of the Ndc80 complex interacts with the histone-fold protein Cnn1/CENP-T and Nsl1 of Mis12 complex to establish the linkage with the inner kinetochore (DeLuca and Musacchio 2012; Malvezzi et al. 2013). Meanwhile, the KNL1 complex is composed of Knl1 and Zwint1 (Zwi) proteins. The C-terminal of Knl1 protein directly interacts with Nsl 1 (Mis12 complex) and its N-terminal binds microtubules.

1.3 Control of cell cycle: checkpoints

In order to minimize mistakes in DNA synthesis as well as to ensure the proper segregation of chromosomes during cell cycle, cells have checkpoint controls at the specific phase-to-phase transitions. Normally, checkpoints consist of at least three components: a sensor for detecting the mistakes, a signal generated by the sensor via a signal transduction pathway and finally, a responsive element in the cell cycle machinery to prevent cell cycle progression until the errors are repaired (Rieder et al. 2011). There are four known checkpoint controls during the cell cycle: the DNA damage and

replication checkpoints, the spindle assembly checkpoint (SAC) and the NoCut checkpoint (Morgan 2007; Manuel et al. 2009).

1.3.1 Damage/replication checkpoints

During the development of living organisms, DNA damage might happen by the impact of various chemical and environmental agents or by errors in DNA replication. These mistakes, if not repaired properly, may lead to mutation, cancer or cell death. However, from G1 to G2/M, the cells can induce DNA repair mechanisms. The DNA damage/replication checkpoints include damage sensor proteins such as the Rad9-Rad1-Hus1 (9-1-1) complex and the Rad17–RFC complex to detect DNA damage, and the MRN complex (Mre11 Rad50 and Nbs1). In eukaryotes, the MRN/X complex plays an important role in the initial processing of double-strand DNA breaks prior to repair by homologous recombination or non-homologous end joining. The sensor complexes transduce signals to ATM, ATR, Chk1 and Chk2 kinases that finally phosphorylate p53 and Cdc25 for various responsive pathways throughout the cell cycle (Figure 4) (Jeremy and Randy 2010). The dominant checkpoint response to DNA damage through G1 depends on p53-p21 pathway and is responsible for a G1 arrest. Through both ATM and ATR, p53 is phosphorylated at serine 15, protected from MDM2-mediated ubiquitilation and proteasomal degradation, and stimulates the transcription of p21 CIP1/WAF1, the inhibitor of cyclin-dependent kinases. As a result, p21 inhibits Cdk2-cyclin E and thus stops G1/S progression. Simultaneously, in late G1, in response to genotoxic stress the ATR-activated Chk1 increases Cdc25A phosphorylation accelerating its ubiquitilation and proteolysis, thereby inhibiting Cdk2 activation. During S phase, the DNA damage/replication checkpoint is related to a Cdc25A dependent pathway. Zhou et al. (2004) distinguished 3 types of S-phase checkpoints: the replication checkpoint, the S-M checkpoint and the intra-S phase checkpoint. The replication checkpoint is initiated to delay DNA replication in response to dNTP depletion or DNA polymerase inhibition. This checkpoint firstly inactivates Cdk2-cyclin E to wait for DNA repair and then, allows cell cycle ongoing (Jares et al. 2000). The S-M checkpoint is activated when cells end S phase with incomplete replicated DNA. In this case, the checkpoint inhibits Cdk1-cyclin B and stops the cell cycle. The intra-S phase checkpoint works in response to DNA Double-Strands Breaks (DSBs) occurring at any random locus in the genome. DSBs activate ATM autophosphorylation that next phosphorylates Chk2. The combination of activated Chk2 by ATM and Chk1 by ATR increases Cdc25A phosphorylation leading to down-regulation, thereby inhibiting Cdk2-cyclin A and delaying S-phase (Zhou et al. 2004). Finally, the G2/M checkpoint prevents cells from initiating mitosis if DNA damage occurs during G2 or if cells enter in G2 with unrepaired DNA lesions. The G2/M checkpoint inhibits Cdk1-cyclin B, the

mitosis-promoting complex, by p53 pathway or through the degradation of CDC25C as above mentioned.

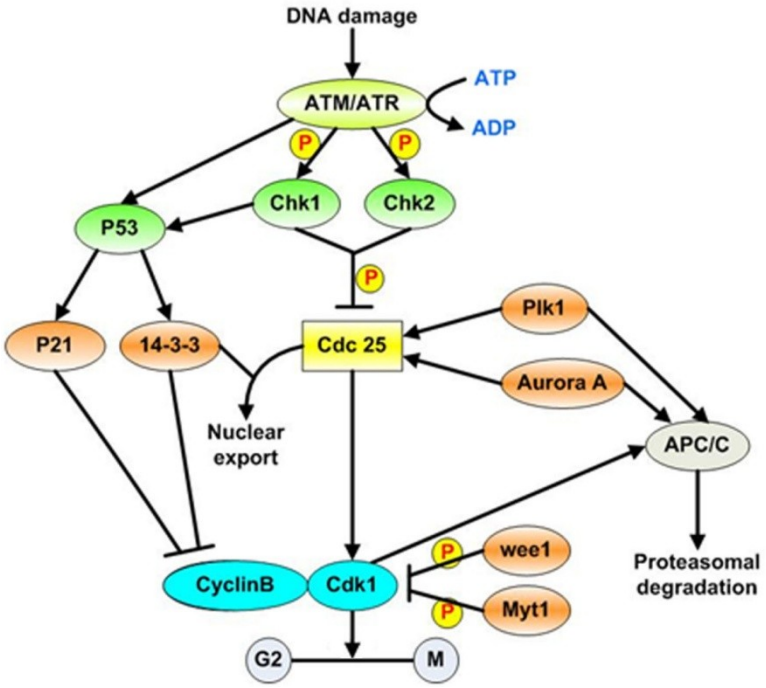


Figure 4
Activation of the G2/M checkpoint after DNA damage. (Wang et al. 2009)

1.3.2 Spindle Assembly Checkpoint

In order to minimize the mis-segregation of chromosomes and aneuploidy during cell cycle, there is a checkpoint control at the metaphase-anaphase transition called spindle assembly checkpoint (SAC) (Musacchio and Salmon 2007). The mechanism of SAC is displayed in Figure 5. SAC activation delays the anaphase onset throughout inhibitory activity on Cdc20, a key cofactor of APC/C until each chromosome is bipolarly attached to spindle microtubules (Nilsson et al. 2008). Briefly, chromosome segregation is mediated by the anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase that normally targets cyclin B for degradation by the 26S proteasome. By inhibiting the APC/C, the SAC keeps cyclin B levels high and arrests the cell in metaphase until all kinetochores are attached to spindle microtubules. Once chromosomes are properly aligned on the metaphasic plate, SAC is turned off, the inhibitory complex dissociates and APC/C gets activated initiating the

onset of anaphase. At that time, separase will cleave cohesin to resolve sister-chromatids cohesion for chromosome segregation. Separase is inactivated by securin and cyclin B. Thus, when Cdc20 binds and activates APC/C, securin and cyclin B are ubiquitylated and degraded. This in turn leads to the activation of separase for the dissociation of sister chromosomes. The core spindle checkpoint proteins are Mad1, Mad2, BubR1/Mad3, Bub1, Bub3 and Mps1 which specifically localize at the unattached kinetochores and become depleted after the proper microtubule attachment (May and Harwick, 2006). BubR1, Bub3 and Mad2, as key factors, directly bind to Cdc20 and form a mitotic checkpoint complex (MCC) which can function as an inhibitory complex preventing APC/C activity (Karess, 2005; Herzog et al. 2009). Recent studies have elucidated how the SAC proteins are recruited to unattached kinetochore. KNL1 is an important factor in the kinetochore-based SAC activation (reviewed by Foley and Kapoor, 2013). Thus KNL1 is a crucial substrate of Mps1 and its phosphorylation by Mps1 creates a docking site for the SAC kinase Bub1. Kinetochore localized Bub1 is necessary and sufficient for recruiting the SAC protein Bub3. BubR1 localizes at outer kinetochore in a Bub3-dependent manner. Additionally, BubR1 also functions as a mechanosensor monitoring CENP-E activity (Chan et al. 1999). CENP-E is a kinetochore motor protein, involved in the attachment of microtubule to kinetochore (Mao et al. 2010). Interaction of CENP-E and BubR1, at the unattached kinetochore is thought to stimulate BubR1 activation in the absence of microtubule attachments (Mao et al. 2005; Weaver et al. 2003) and conversely, inactivate BubR1 activity in the proper attachment of kinetochore-microtubules (Musacchio et al. 2007). BubR1 is considered to play an important role in the inhibition of the APC/C, together with Mad2 (Musacchio et al. 2007). The Mad2 attachment to kinetochore requires docking protein like Mad1. Mad1, a stably kinetochore bound protein, is essential for Mad2 kinetochore localization. Interaction between Mad1 and Mad2 results in the conformational modification of Mad2 enabling to bind to Cdc20. Mps1 contributes to the SAC activity by recruiting Mad and Bub proteins to unattached kinetochores (Lan & Cleveland, 2010) and by promoting Mad2 activation (Hewitt et al. 2010; Maciejowski et al. 2010). Furthermore, Mps1 prevents the dissociation of the inhibitory complex (Maciejowski et al. 2010). All these numerous actors playing together integrate kinetochore functions, SAC activation, microtubule attachments and SAC silencing to ensure accurate timely chromosome segregation (Foley and Kapoor, 2013). Aurora B activates the spindle checkpoint in response to a lack of kinetochore tension and prevents the activation of APC/C until proper bipolar spindle attachments are formed (Hauf and Watanabe, 2004).The balance of kinase and phosphatase activities has a crucial role in kinetochore functions and is not fully described. In the presence of spindle poison like Taxol, kinetochores are unable to form proper attachments to spindle microtubules. This leads to the permanent activation of the SAC and prolongation of mitotic arrest for hours. However the SAC cannot prevent a slow but continuous degradation of cyclin B that

ultimately drives the cell out of mitosis (Brito and Rieder, 2006). Mitotic checkpoint slippage leads to 4N-multinucleated cells exhibiting a micronuclei phenotype.

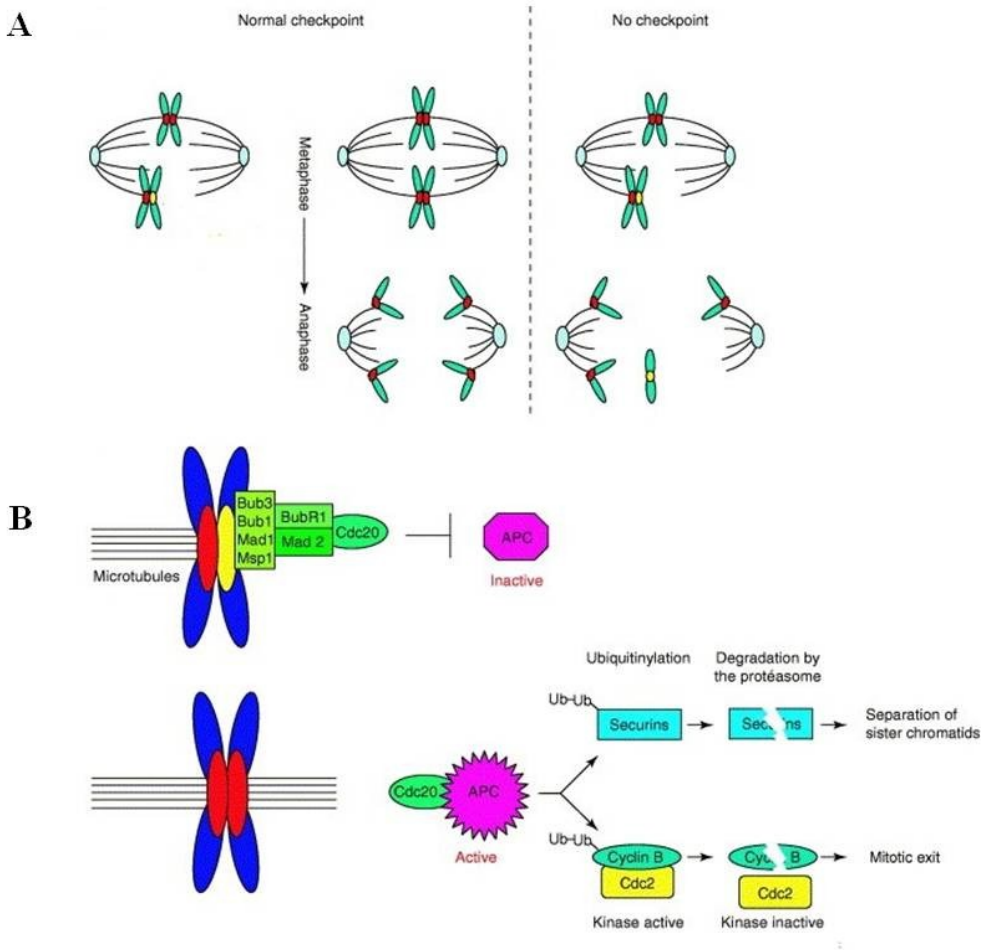


Figure 5: The spindle or metaphase checkpoint.
(a) The checkpoint monitors microtubule attachment at kinetochores. (Left) The checkpoint apparatus assembled on unattached kinetochores (yellow dots) generates an inhibitor signal that delays the metaphase–anaphase transition. In this way, the cell has more time for establishing proper kinetochore–microtubule connections (red dots) before the onset of anaphase. (Right) If the checkpoint mechanism is defective, a cell can enter anaphase even with unattached kinetochores, and this can lead to improper distribution of sister chromatids (aneuploidy). (b) Chromosome segregation triggered by the APC/C throughout the SAC turn-off. At microtubule-unattached kinetochores, SAC promotes the formation of a Cdc20 inhibitory complex, which inhibits APC/C activity. When each chromosome is bipolarly attached to spindle microtubules, SAC signaling is turned off, the inhibitory complex dissociates; Cdc20 binds to and activates APC/C promoting ubiquitylation of Securins and Cyclin B and thus, their degradations (Karess 2005).

1.3.3. NoCut Checkpoint (Abscission checkpoint control)

Cell abscission is the last step of mitosis and it does not occur until all chromatids are pulled out of the cleavage plane. In budding yeast and human cells, this event is monitored by the NoCut checkpoint, which involves the activities of the chromosome passenger Aurora B kinase and Plk1 (Carmena, 2012; Chen et al. 2012). When the NoCut checkpoint detects the presence of chromatins in the cleavage furrow, it is turned on. Active Plk1 phosphorylates a centrosomal protein of 55 kDa (Cep55) and prevents its association with the midbody (Bastos and Barr, 2010). Cep55, that efficiently bundles microtubules, binds to Mklp1 in vitro and associates with the Mklp1 (mitotic kinesin-like protein 1)-MgcRacGAP centralspindlin complex in vivo. Meanwhile, Aurora B still phosphorylates Mklp1 and prevents furrow ingression (Steigmann et al. 2009). Normally, Aurora B activity gradually decreases at cytokinesis, to be null upon abscission. If Aurora B is prematurely inactivated, the NoCut checkpoint is turned off, leading to the formation of binucleated cells (Chen et al. 2012). At the end of mitosis, once chromosomes are segregated away from the cleavage furrow, the NoCut checkpoint is turned off, Plk1 is degraded allowing the interaction of Cep55 with kinesin Mklp1 at the midbody enabling the recruitment of subsequent midbody components for the abscission initiation. In a recent study, Carmena clarified the molecular mechanism by which the CPC controls the timing of abscission through regulation of ESCRT-III (Carmena, 2012). The ESCRT (endosomal sorting complex required for transport) complexes are formed by proteins involved in membrane fission events and include six complexes (ESCRT-0, -I, -II, -III, ALIX and VSP4) in human. Different ESCRT complexes are recruited sequentially to the site of scission, ending with the recruitment of the ESCRT-III complex that brings about membrane scission. In cytokinesis, after Cep-55 is associated with kinesin Mklp1, it interacts with Tsg101 (ESCRT-I) and ALIX, which in turn recruit ESCRT-III, the complex responsible for the abscission activity. ESCRT-III can assemble in filaments around the abscission site and makes the membrane curve, eventually driving the final break between daughter cells (Carmena, 2012). This study defines a cellular mechanism that links centralspindlin to Cep55, which, in turn, controls the midbody structure and membrane fusion at the terminal stage of cytokinesis.

1.4 Aurora kinases

Aurora kinases are higly conserved serine/threonine kinases that have been recognized as regulators of mitosis, with key roles from mitotic entry to cytokinesis (Carmena et al. 2009). The founding member of the Aurora kinases is the Drosophila Aurora Kinase that was discovered in a genetic screen to identify genes involved in mitotic spindle function (Glover et al. 1995). Fungi

have only one Aurora kinase, IPL1(Increase-in-ploidy) in S. Cerevisiae (Chan et al. 1993) and Ark1 (Aurora-related-protein) in S. Pombe (Petersen et al. 2001). Ark1 is more related to the Aurora B kinases in higher organism. There are two Aurora kinases in Caenorhabditis Elegans and Xenopus Laevis called Aurora A and B (Schumacher et al. 1998), while in mammals there is a third Aurora gene called Aurora C (Bischoff et al. 1999).

1.4.1 Localization of Aurora Kinases

Aurora kinases show different cellular localization during mitosis. Aurora A localizes on duplicated centrosomes from the end of S phase (as soon as centrioles are duplicated) to the beginning of the following G1 phase, and in prophase is still there (Carmena et al. 2003). Then, from metaphase to anaphase, it associates with microtubules, close to the spindle poles. Aurora A concentrates in the midbody during cytokinesis and finally, it is degraded by the proteasome in a Cdh1-dependent manner (Castro et al. 2002).
Aurora B belongs to the Chromosome Passenger Complex (CPC). Such proteins localize to the kinetochores from prophase to metaphase and to the central spindle and the midbody in cytokinesis (Carmena et al. 2003). In particular, in prophase, Aurora B is localized at first in pericentrometic chromatins, then along the lenght of the condensing chromosomes and it gradually concentrates in the inner centromere at prometaphase. Thus, it transfers from chromosome to microtubules, localizes to the spindle midzone in anaphase. Finally, like Aurora A, it is associated with the midbody during cytokinesis (Adams et al. 2000). Aurora C is also a CPC protein with a similar localization as Aurora B; it is localized to centromeres during prophase to metaphase and is redistributed to midzone microtubules during anaphase (Sasai et al. 2004; Li et al. 2004). The different localizations of Aurora kinases are correlated with their distinct functions during mitosis (Figure 6).

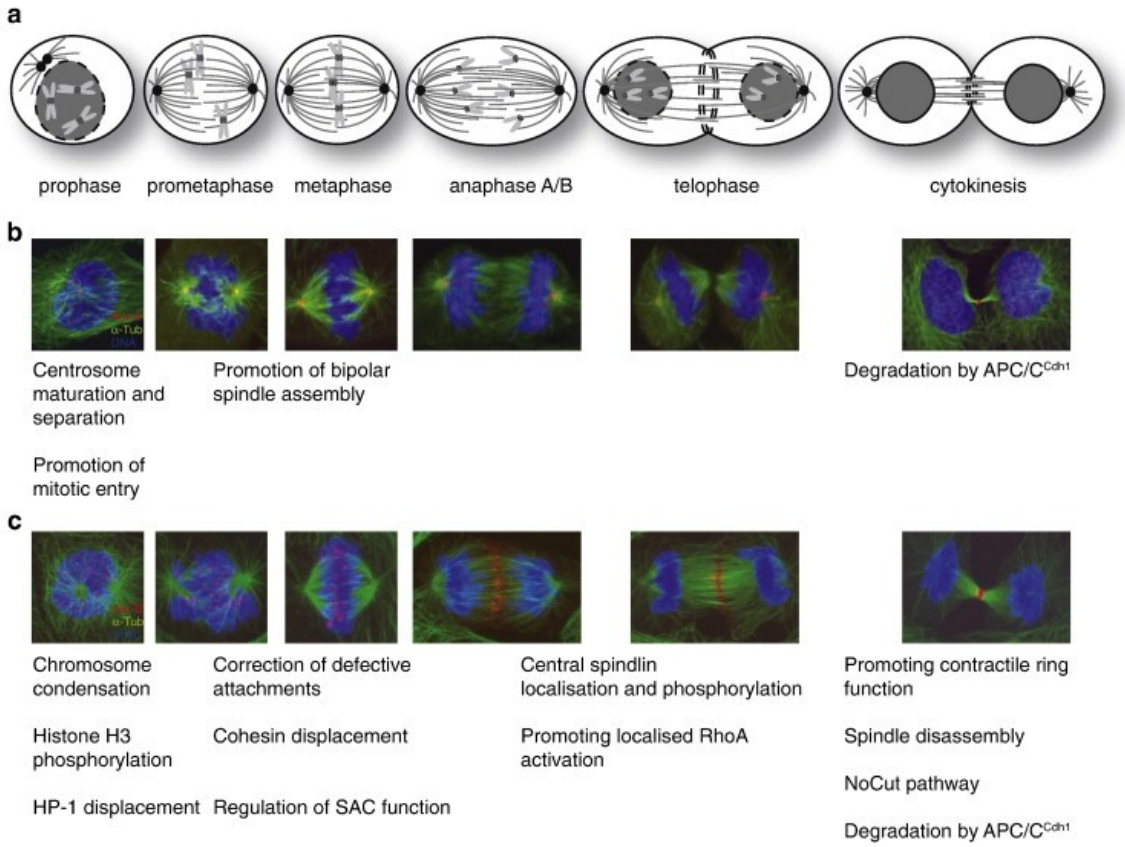


Figure 6. Localizations and functions of Aurora Kinases in mitosis
(a) Schematic representation of the different mitotic phase;
The localization pattern and functions of Aurora A (b) and B (c): DNA is stained in blue, Aurora A/B in red and α-tubulin in green: Aurora A (in red) localizes around centrosomes in prophase, on the microtubules near the spindle poles in metaphase and on polar microtubules during anaphase and telophase; Aurora B (in red) shows the typical chromosomal passenger localization. Aurora B localizes first in pericentrometic chromatins in prophase, concentrates in the inner centromere during prometaphase and metaphase, transfers to the central spindle and the cell cortex in anaphase, and finally is associated with the midbody during cytokinesis. Aurora C shares similar localization and functions of Aurora B. (Vader et al. 2008)

1.4.2 Structure and regulation of Aurora kinases

Aurora A, B and C comprise 403, 344 and 309 aminoacids, respectively. The proteins contain an N-terminal domain composed of 39-129 residues, a protein kinase domain and a C-terminal domain of 15-20 aminoacids. Like other protein kinases, the highly conserved catalytic domain consists of an activation loop, a hinge region binding ATP, an hydrophobic pocket and an allosteric site. Overall, the three Aurora kinases share high sequence identity. The kinases also share high homology between species and are evolutionarily ancient (eg: Aurora A shares 82% sequence identity between the human and rodent genes).

They also share common ancestral genes in Drosophila and Yeast. The functional similarity between Aurora A and B has been demonstrated by experiments showing that a single amino acid change in Aurora A, G198N, can convey an Aurora B kinase-like activity (Fu et al. 2009). However, the N-terminal domains of Aurora A, B and C share little sequence identity and confer unique protein–protein interaction abilities among the Aurora kinases (Carmena et al. 2003). The activity of Aurora kinases is regulated at multiple levels. Aurora A, B and C all contain a key threonine, named the T-loop residue, within their kinase domains that must be phosphorylated to allow for kinase activity (Figure 7). This occurs via autophosphorylation of the T-loop residue T288 (Aurora A), T232 (Aurora B) or T195 (Aurora C), which is driven by clustering of kinase molecole (Bischoff et al. 1998). Transcription of Aurora kinases is cell cycle-regulated. Aurora A mRNA typically peaks at G2/M with the protein expression peaking slightly later (Kimura et al. 1997). The promoter of Aurora A contains specific sequences required for transcription in the G2 phase of the cell cycle (Tanaka et al. 2002). The same is true for Aurora B: the level of this protein is cell cycle-regulated and its activity peaks just after that of Aurora A. The three kinases are differentially expressed at high levels in rapidly dividing tissues such as hematopoietic cells (A and B) and germ cells (C only) (Su et al. 2004). Conversely, Aurora kinases expression is low or absent in most adult tissues due to their lower rates of proliferation. Aurora kinases degradation is also highly regulated. All three family members contain destruction boxes (D-boxes) recognized by Anaphase promoting complex/cyclosome (APC/C), which mediates their proteasomal degradation (Figure 7). The APC/C, in conjunction with its specificity factor cdc20 homolog 1 (Cdh1), ubiquitylates Aurora A and targets it for degradation during mitotic exit (Walter et al. 2000). Moreover, in addition to its D-boxes, Aurora A contains a KEN degradation motif and an N-terminal D-Box-activating motif (Littlepage et al. 2002). Other regulators of Aurora kinases degradation have been identified, such as Cdc4/Fbxw7, checkpoint with forkhead and ring finger domain (Chfr) and Aurora A-interacting protein. Aurora B contains the same D-Box as Aurora A, but it is primarily degraded by proteasome alpha-subunit C8 in a proteasome-dependent manner (Shu et al. 2003).

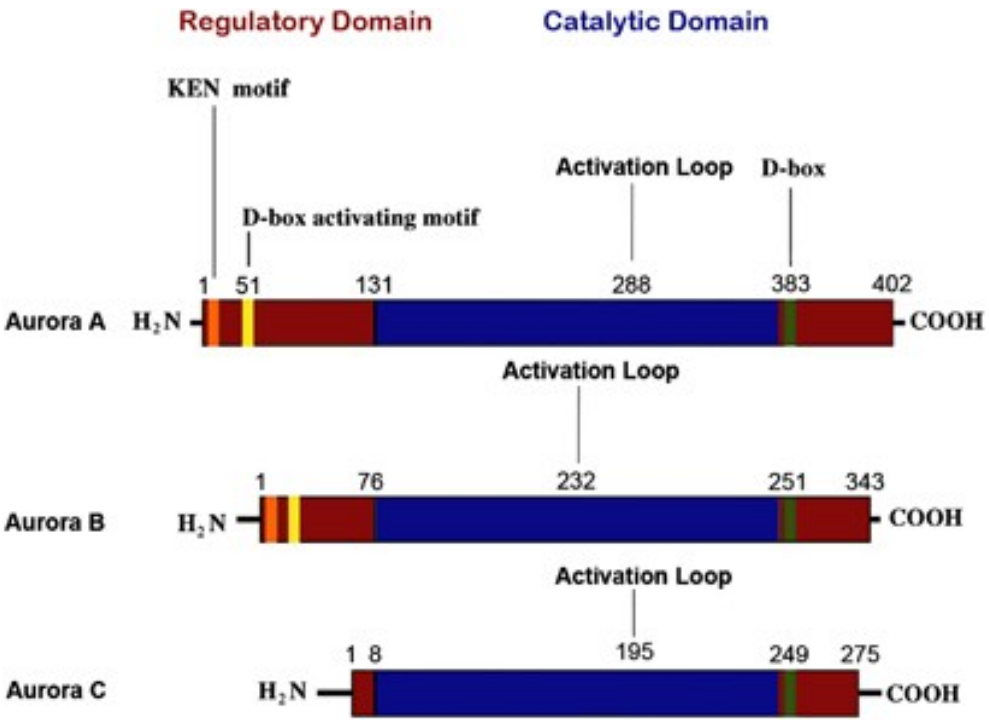


Figure 7. Domains and structure of the Aurora Kinases.
N-terminal and C-terminal portions contain D-box and KEN regulatory motifs, the central kinase domain is important for the catalytic activity. The central domain also includes key regulatory motifs, such as the activation loop residue (T-loop). (Goldenson et al. 2015)

In order to develop kinase inhibitors, the structural features of ATP-binding sites of Aurora kinases have been elucidated by X-ray crystallography. Most of the available data on the ATP binding site are from Aurora A studies, because Aurora B itself has not been crystallized in the absence of its inhibitors (Girdler et al. 2008; Sessa et al. 2005). The results showed that Aurora A has a peculiar DFG (Asp-Phe-Gly) motif in the ATP binding sites that adopts different conformations: the active DFG-in and the inactive DFG-out states (Dodson et al. 2010; Martin et al. 2012; Liu and Gray 2006). In the active DFG-in conformation, the activation loop is oriented away from the ATP site and accessibile for phosphorylation. Conversely, in the inactive-DFG state, in presence of inhibitors, the phenylalanine side chain points outwards and interacts with inhibitors, preventing the phosphorylation of the activation loop (Figure 8).

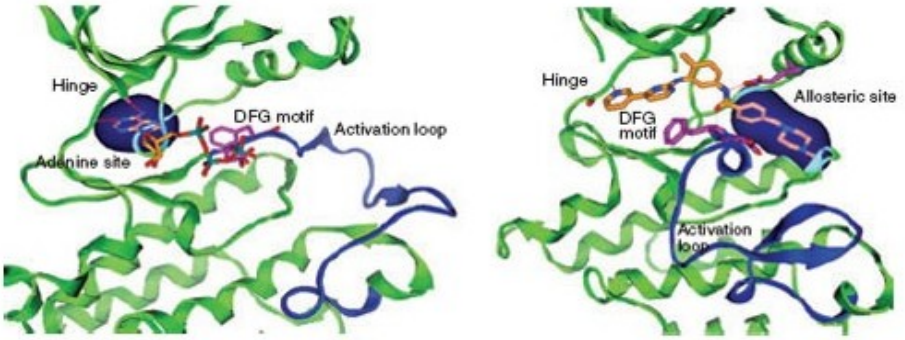


Figure 8. Conformational changes of Aurora A activation loop
The active DFG-in (left) and the inactive DFG-out states (Liu and Gray 2006)

In complex with INCENP and an aurora kinase inhibitor (VX-680), the structure of Aurora B has recently been determined (Elkins et al. 2012). The overall structure of human Aurora B resembles that of human Aurora A with the exception of the conformation of the activation loop. The catalytic domains of human Aurora A and Aurora B are 76% identical at the primary sequence level, and their ATP-binding pockets differ by just three amino acids in which Leu215, Thr217 and Arg220 of Aurora A are replaced by Arg159, Glu161 and Lys164, respectively, in Aurora B (Dodson et al. 2010). Remarkably, in complexes with the same inhibitor, both Aurora kinases showed a significant spatial difference of the DFG motif (Figure 9). In Aurora A, the DFG motif is arranged with the Phe underneath the αC helix; while in Aurora B, the αC helix is moved further out from the ATP- binding site and the Phe residue is side to the helix whereas Asp is not well-ordered in the structure (Elkins et al. 2012)

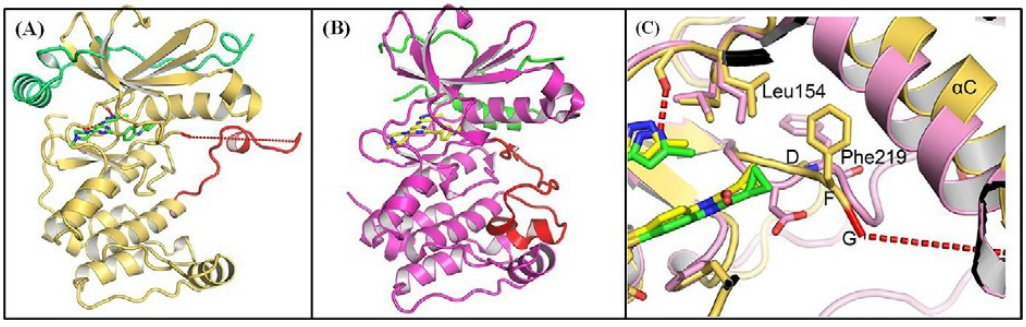


Figure 9. Comparison of human Aurora A and Aurora B structures.
(A) Structure of human Aurora B with INCENP stained green and activation loop stained red. (B) Structure of human Aurora A with TPX2 colored in green and activation loop colored in red. (C) Differences in DFG motif between Aurora B (in yellow) and Aurora A (in pink). (modified from Elkins et al. 2012).

1.4.3 Function of the Aurora kinases

Even thought the three Aurora Kinases are involved in cell division, the specific function of each kinase is different. Indeed, Aurora A but not B or C, regulates centrosome maturation and separation and bipolar spindle assembly, while Aurora B controls cytokinesis and chromosome bi-orientation as a member of the chromosome passenger complex. Aurora C coordinates meiotic spindles in spermatogenesis, while also cooperating with Aurora B to regulate mitotic chromosome dynamics.

1.4.3.1 Aurora A: a non residential centrosome kinase

During mitosis, Aurora A interacts with different substrates for its activation and regulation, corresponding to its distinct functions. The major function of Aurora A is to coordinate centrosome maturation, mitotic entry, separation of centriolar pairs, accurate bipolar spindle assembly and alignment of metaphase chromosomes. This kinase interacts with different factors.
Aurora A is activated by its phosphorylation and by the binding of activator proteins (Dodson and Bayliss 2012). Recent studies have described several activators of Aurora A. The best characterized cofactor is TPX2, a microtubule-associated protein involved in bipolar spindle assembly (Dodson and Bayliss 2012; Wittman et al. 2000). TPX2 binds to Aurora A through its conserved NH2-terminal domain, and this event induces the activation segment of the kinase to move inside its catalytic pocket. In addition, TPX2 also protects a crucial phospho-threonine (threonine 288 in human Aurora A) in the activation segment within the protein against protein phosphatase 1 (PP-1) – dependent dephosphorylation and inactivation of Aurora A (Bayliss et al. 2003) (Figure 10). In vivo, activation of Aurora A sinergistically depend on (auto) phosphorylation in its activation segment (on threonine 288) and TPX2 binding (Tsay et al. 2005). The expression, localization and activity of Aurora A are consistent with its function as a centrosomal kinase. Aurora A levels are low during G1/S phase, but increase in G2, with both function and expression peaking in early M phase (Zhou et al. 1998). With respect to localization, it is found at the centrosome in mitotic cells from late S and G2 until telophase, but also localizes to the spindle throughout mitosis (Krystuniak et al. 2006). Functionally, Aurora A regulates the progression of mitosis by phosphorylation of multiple substrates, and it promotes mitotic entry by controlling activation of Cyclin-B/Cdk-1 (Satinover et al. 2006). Aurora A also activates Polo-like kinase-1 (Plk-1) in G2 through direct phosphorylation of Plk-1 (Macurek et al. 2008). Other cofactors and substrates include Ajuba, enhancer of filamentation 1, BORA, TPX2, PLK-1, astrin, growth arrest and DNA damage-inducible 45α, transforming acidic coiled-coil containing protein 3 (TACC3) and centrosomin (Vader et al. 2008). PLK-1, which is also implicated in

centrosome maturation, is involved in targeting Aurora A to centrosomes (Lane et al. 1996). Both PLK1 and CDK11 are required for Aurora A recruitment and centrosome maturation (Terada et al. 2003). Other important functional interactions include the phosphorylation of LATS2, NDEl1 and TACC3 by Aurora A to enforce their recruitment to the centrosome and to promote centrosome maturation (Toji et al. 2004).

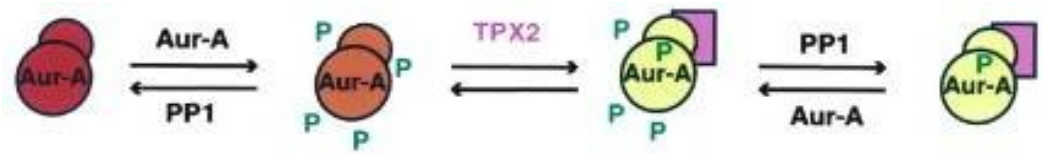


Figure 10. Regulation of Aurora A
Aurora-A ,in red, in the unphosphorylated inactive state, in orange in the phosphorylated partially active state, and in yellow in the fully active TPX2-bound phosphorylated state. TPX2 is indicated in pink. Without TPX2, the phosphatase PP1 is able to deactivate the kinase via dephosphorylation. With TPX2, most phosphorylation sites are accessible to the phosphatase, with the exception of the crucial phosphoThr288, which moves to an inaccessible position between the two kinase lobes (Bayliss et al. 2003).

1.4.3.2 Aurora B: the chromosome passenger kinase

Like Aurora A, Aurora B interacts with different substrates corresponding to its distinct roles during mitosis. Aurora B is a passenger protein, together with INCENP, Survivin and Borealin. These proteins form, together, the chromosomal passenger complex (CPC), and Aurora B is the enzymatic member of the complex. The expression of CPC members peaks in mitosis, and these proteins are interconnetted. Indeed invalidation of the expression of any CPC proteins leads to the degradation of the others (Klein et al. 2006). CPC complex has a peculiar localization; from metaphase to anaphase, it concentrates in the centromere, from where it controls kinetochore tension, and then, when the SAC is turned off at anaphase onset, it transfers to the midzone. Finally, it concentrates in the midbody and partecipate to the NoCut checkpoint. Association with the partner proteins ensures the correct localization of the kinase on the inner centromere (Figure 11).

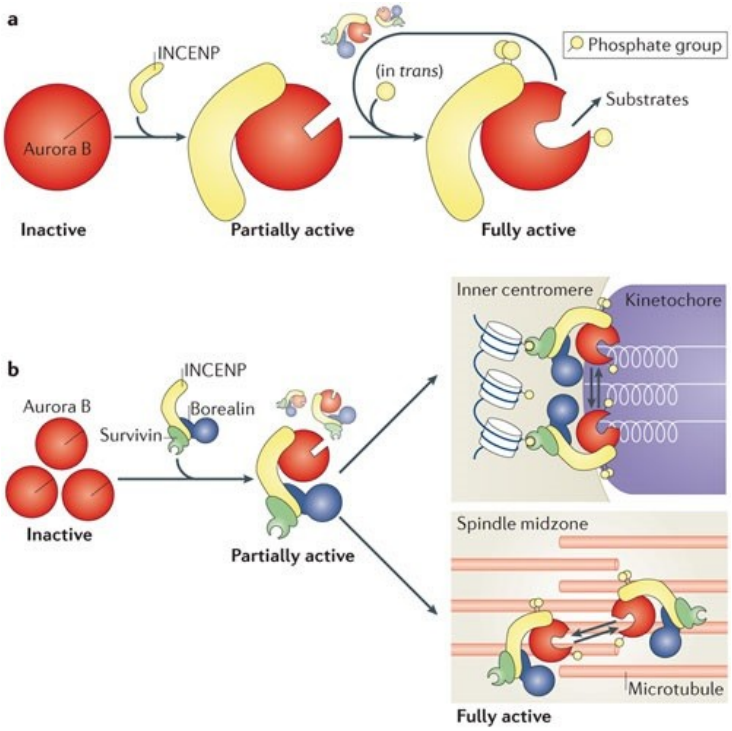


Figure 11. Aurora B and CPC complex activation
(a) Aurora B activation requires binding to inner centromere protein (INCENP) and phosphorylation . Both of these phosphorylation events are catalysed in trans.
(b) Aurora B activation is coupled to chromosomal passenger complex (CPC) localization. The localization module is composed of INCENP, survivin and borealin and targets the CPC to histones at the inner centromere and microtubules at the spindle midzone during early and late mitosis, respectively. Enrichment of the CPC at these locations facilitates autophosphorylation in trans, which leads to full Aurora B activation (Carmena et al. 2012).

Two-step mechanism promote the activation of Aurora B: first the kinase binds to INCENP through its C-terminal IN-box sequence, and is then autophosphorylated at threonine 232 (Thr 232) within its activation loop (Honda et al. 2003; Yasui et al. 2004). In the second step, Aurora B phosphorylates INCENP at the TSS sequence; in this way it is fully activated. Survivin and Borealin, together with INCENP, also partecipate in the right localization of CPC to centromeres, through the identification of two specific marks present on chromatin: haspin- dependent phosphorylation of histone H3 on Thr3 (H3-Thr3ph) and Bub1-dependent phosphorylation of histone H2A on Thr120 (H2AThr120ph) (Xu et al. 2009). In details, the Bir domain of Survivin recognizes phospho-histone H3 (Thr3), allowing its recruitment on chromatin (Figure 12). Meanwhile, histone H2A recruitis Shugoshin, another CPC member. The concomitance of the two phosphorylations on centromeres triggers the specific recruitment of the CPC (Figure 12) (Campbell and Desai 2013; Kelly et al. 2010). Simultaneously, Mps1 activity enhances H2A-T120ph and is also critical for Shugoshin recruitment to centromeres, thereby

promoting Aurora B centromeric localization (van de Walls et al. 2012). As shown in Figure 12 , when fully activated, Aurora B exercised a retro control on Haspin (Wang et al.2011).

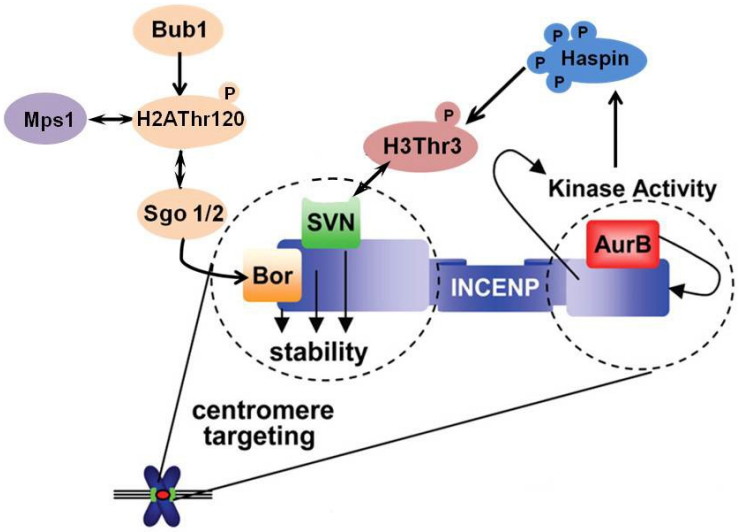


Figure 12. CPC on centromere.
Phosphorylations of histone H3 on Thr3 (H3Thr3P) and histone H2A on Thr120 (H2AThr120P) allows the recruitment of the CPC at the centromere. Survivin (SVN) interacts directly with chromatin whereas the stabilization is provided by the interaction of Borealin (Bor) with Shugoshin (Sgo1/2). Within the CPC, Aurora B is fully activated and exercises a retro control on Haspin (Xu et al. 2009).

Besides, there are two other kinases directly involved in the activation of Aurora B: the Checkpoint kinase 1 (Chk1) and Tousled-like kinase 1 (Tlk1). Chk1 kinase is a major component of DNA damage and DNA replication checkpoints. It was recently found to phosphorylate Aurora B on Ser331 during unperturbed prometaphase and during spindle disruption by Taxol. This phosphorylation is required for optimal phosphorylation of INCENP on its TSS sequence, a motif involved in Aurora B full activation (Petsalaki et al. 2011).
Aurora B is involved in chromosome condensation and cohesion, microtubule kinetochore attachment, mitotic spindle checkpoint regulation, chromosome segregation and cytokinesis.

Chromosome condensation:
Condensation of chromosome into compact structures is essential for the accurate segregation of chromosomes and is driven by Condensin complexes (Condensin I and II in human cells) (Takemotor et al. 2007; Hirano 2006). Depletion of Aurora B impairs the localization of Condensin I on chromosomes and consequently, chromosome condensation (Lipp et al. 2007) indicating a direct role of Aurora B in this process. Moreover, Aurora B is responsible for phosphorylation of histone H3 on Ser10 and Ser28 during late

G2/ prophase step of mitosis (Baek 2011). This phosphorylation is crucial for chromosome condensation during mitosis (Wei et al. 1999; Cerutti and Mollan 2009). Nevertheless, other data demonstrated that histone H3 phosphorylation is only required for the initiation rather than the maintenance of condensed chromatin state (Van Hoosser et al. 1998).

Chromosome cohesion:

Aurora B also controls chromosome cohesion. DNA is replicated during S phase and then condenses in G2 phase. The two sister chromatids require a cohesion system to keep them together. This is accomplished by a ring-like Cohesin complex that consists of Smc1, Smc3, Scc/Rad21 and Scc3/SA (Nasmyth and Haering, 2009). The cohesin complex is released from chromatin in two steps: along chromatid arms in prophase and finally, at centromere in anaphase (Remeseido and Losada, 2013). Aurora B is reported to promote the chromosome recruitment of separase for the complete removal of cohesin, at anaphase onset (Yuan et al. 2009).

Bi-oriented microtuble-kinetochore attachment

In order to ensure the proper chromosome segregation, microtubules must attach sister kinetochores to opposite sides of the mitotic spindle (bi-orientation). Errors such as syntelic attachments (both kinetochores bind microtubules from the same pole); merotelic attachments (one kinetochore attaches to both mitotic spindle poles) or monotelic attachments (single attachment of one kinetochore in two sister chromatids) exert low tension on kinetochore-to-pole connection (Figure 13). Aurora B triggers the phosphorylation of kinetochore substrates in a tension dependent manner and facilitates the bi-orientation of kinetochore-spindle pole connection. Thus, Aurora B resolves syntelic attachment by the phosphorylation of an important kinetochore-microtubule-capture factor (Dam1-ring complex), which facilitates the turnover of kinetochore-microtubule attachments (Keating et al. 2009). Similarly, the phosphorylation of Ndc/Hec1 in KMN by Aurora B is observed to increase in the merotelic attachment correction (DeLuca et al. 2006). Another study shows the enrichment of MCAK (kinesin-13 microtubule depolymerase), at points of merotelic attachment, in an Aurora B-dependent manner. When MCAK is phosphorylated, depolymerizes the improperly attached microtubules (Knowlton et al. 2006).

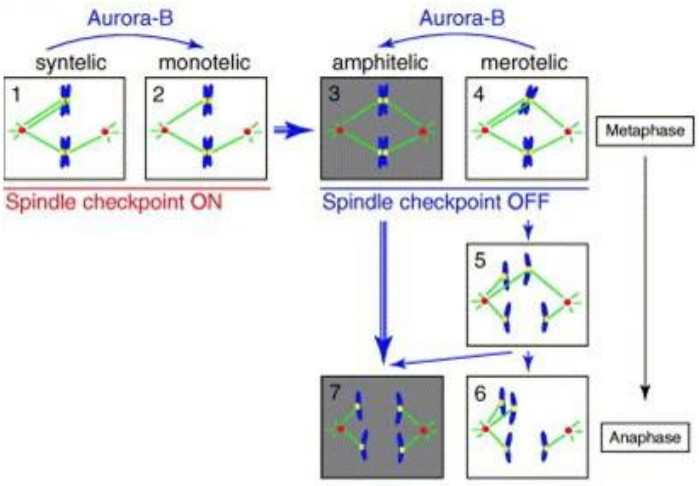


Figure 13. The model of kinetochore-microtubule attachments.
Aurora B corrects abnormal mis-attachments such as monotelic, syntelic or merotelic attachments and induces bioriented attachments (Giet et al. 2005).

Mitotic spindle checkpoint regulation

Aurora B also plays a role in spindle assembly checkpoint (SAC). The SAC guards the metaphase-anaphase transition. Aurora B activates the spindle checkpoint in response to a lack of kinetochore tension and prevents the activation of APC/C until proper bipolar spindle attachments are formed (Hauf and Watanabe, 2004). Aurora B targets two proteins for this regulation. The first is the microtubule-depolymerising kinesin MCAK, whose activity is negatively regulated by aurora B (Sampath et al. 2004), and the second is the microtubule-destabilizing protein Stahtmin/Op18, whose activity is inhibited by Aurora-B phosphorylation in the vicinity of chromosomes (Gadea and Ruderman 2006).

Anaphase and Cytokinesis:

Upon SAC inactivation, APC/Cdc20 initiates anaphase onset by destruction of Cyclin-B and Securin. This event causes a decrease in Cyclin-B-associated Cdk1 activity and a resolution of cohesion between sister chromatids (Shirayama et al. 1999). At anaphase, Aurora B localizes to both the central spindle/midbody and to the cell cortex, to which it is transported via microtubules, and regulates cleavage furrow formation (Bringmann 2005). Aurora B, on central spindle, contributes to the rapid switch in microtubule dynamics during anaphase (Higuchi et al. 2005) and it is also required, during telophase, for disassembly of the mitotic spindle (Buvelot et al. 2003).
During cytokinesis, the cytoplasm is divided into two new daughter cells with a single nucleus. Contraction of an actinomyosin ring generates a cleavage furrow that divides the cytoplasm in two. It is fundamental that this cleavage only forms after segregation of chromosomes. In this phase an important role is exerted by RhoA GTPase, which is controlled during cytokinesis by a central

spindle-localised Complex, called centralspindlin. Correct function and localisation of centralspindlin depends on Aurora-B activity (Kaitna et al. 2000). In addition, during anaphase and telophase, Aurora B interacts with others proteins, like Vimentin, Myosin II regulatory light chain and Desmin. Together, these data clearly point to a central function of Aurora B during cytokinesis.

1.4.3.3 Aurora C

The third member of tha Aurora family, Aurora C, is the least studied. This kinase is only present in germ cells in the testis, but the protein could not be detected in adult tissues, and it is involved in male meiotic cytokinesis (Dieterich et al. 2009). Like Aurora B, Aurora C is a CPC protein, and localizes on chromosomes from prophase to metaphase and at the midbody from anaphase to telophase phase. In tumor cells, it is present at centrosome, like Aurora A (Dutertre et al. 2005). The chromosomal region in which the Aurora C gene is located is known to be deleted or translocated in certain human cancer cell lines but it is unclear whether Aurora C deletion plays a causative role in tumorigenesis (Bernard et al. 1998). In conclusion, it is clear that Aurora-C plays an important role during spermatogenesis, but conclusive proof that it controls cell cycle progression in somatic and/or tumour cells is lacking.

1.5 Aurora Kinase and Cancer

Aurora kinases play essential role during mitosis; for this reason changes in their signalling could result in mitotic errors and are associated with chromosome aneuploidy and genomic instability (Dar et al. 2010). The Aurk A gene is located into a region of chromosome 20q13, that is frequently altered in human cancers. The Aurora A F31L polymorphism is associated, for example, with an increased risk of esophageal, ovarian, non small cell lung and breast cancers (Katayama et al. 2003). The role of Aurora A in carcinogenesis has been well studied. Several reports have shown that Aurora A can drive cell transformation and tumors formation in nude mice (Zhou et al. 1998) while others data suggest that overexpression alone is not sufficient to drive oncogenesis, and for this reason, others mutations are necessary to cause malignant transformation, such as activation of Ras signalling (Tatsuka et al. 2005). Overexpression of Aurora A is correlated with centrosome amplification and formation of multipolar spindle, defects in the separation of centrosome that cause formation of monopolar spindle and consequently abortive mitosis and tetraploidy (Nigg et al. 2001). Finally, overexpression of Aurora A distrupts spindle checkpoint activation by paclitaxel and nocodazole treatment,

causing the cells to became resistant to these chemotherapeutic drugs (Anand et al. 2003). Based on these strong lines of evidence, the entusiasm of exploring anticancer therapeutic target has initially been focused on Aurora A. The role of Aurora B in tumorigenesis remains unclear. Aurk B gene is located on chromosome 17p13.1, in close proximity to p53 gene. This chromosome region has not been associated with amplification in tumors. Many reports describe that Aurora B is overexpressed in certain tumor types, such as hepatocellular carcinoma (Aihara et al. 2010; Lin et al. 2010), gastric carcinoma (Honma et al. 2013), multiform glioblastoma (Zeng et al. 2007), ephitelial ovarian cancer (Chen et al. 2009), malignant mesotelioma (Lopez Rio et al. 2006), hematological malignancies (Ikezoe et al. 2007), but is not clear wether the observed disregulation of the kinase is a mere reflection of the high proliferative index of cancerous cells, or whether it is causally related to tumorigenesis. How Aurora B overexpression facilitates tumorigenesis is an interesting question; high levels of Aurora B are cause of tetraploidy and subsequent genomic instability, while in other view, a recent study proposed a new pathway in using Aurora B to prevent tumor growth (Sharma et al. 2013). In this study, Aurora B is confirmed as a target downstream of V600E B-Raf in MAP kinase signaling cascade relating to cell growth. V600E B-Raf is a popular target in melanoma cancer treatment but limited by drug resistance. Therefore, the inhibition of Aurora B kinase that is described to reduce the oncogenic growth of melanoma cells is a good alternative (Bonet et al. 2012; Sharma et al. 2013). The involvement of Aurora C in carcinogenesis has been the last explored. A few studies showed that Aurora C is overexpressed in colorectal, breast and prostate cancer (Sasai et al. 2004). Furthermore, the phenotype obtained upon Aurora C overexpression (polyploid cells containing abnormal centrosome numbers) is aggravated in the absence of a functional p53 (Dutertre et al. 2005). These data suggest that Aurora C might also be a target for cancer treatment, especially in the presence of mutated p53. All these data suggest that the involvement of Aurora kinases in cancer development makes them to be considered as potential targets in anticancer chemotherapy. Even though much effort has been made in decreasing cancer mortality, cancer is still one of the major causes of death over the world. Up to date, anti-cancer therapy mainly relies on surgery, radiation therapy and chemotherapy, which can be used alone or in combination. Both surgery and radiotherapy are only local treatments and cannot control the metastatic tumors; whereas, chemotherapy is a systemic therapy in cancer treatment. Most chemotherapeutic anti-cancer drugs used in the clinic today target the cell cycle in order to inhibit the over-proliferation state of tumor cells and then inducing apoptosis (Lee and Schmitt, 2003). Classical anti-cancer drugs interfere with DNA synthesis, DNA damage, or inhibit the function of the mitotic spindle. Among them, microtubule-binding drugs are the most exceptionally successful chemotherapeutic compounds currently used in the clinic. Traditional antimitotic chemotherapeutics, including taxanes and various Vinca alkaloids, are currently used to treat patients with breast, ovarian and non-small cell lung

cancers (Lister-Sharp et al. 2000; O'Shaughnessy et al. 2013; Socinski et al. 2013; Ueda et al. 2013). Both types of drugs create unattached kinetochores in mitosis by altering microtubule dynamics and cause long-term mitotic arrest. However, the drawback of these drugs is that they also inhibit the function of microtubules in normal cells. Lowering the blood counts and inducing peripheral neuropathies are the most common unwanted side effects. Therefore, current attempts not only try to improve novel anti-microtubule drugs, but also to develop drugs towards novel mitotic targets such as mitotic kinesins, Aurora kinases and mitotic kinases (e.g. Cdk and Plk inhibitors).

1.5.1 Aurora kinase inhibitors

Aurora kinases have focused attention as potential targets in the development of anticancer drugs. Since the kinase domains of Aurora kinases are highly homologous, it is difficult to obtain aurora kinase inhibitors that are specific for one kinase. Consequently, most inhibitors target multiple Aurora kinase members. A series of pan-Aurora kinase inhibitors have been developed and have entered clinical trials, as indicated in Table 1. Among pan Aurora inhibitors, Tozasertib (VX-680) was the first inhibitor to be tested in clinical trials for the treatment of chronic myelogenous leukemia (CML). It targets the three Aurora kinases in vitro, with IC50 of 0.7, 18 and 4.6 nM for Aurora A, B and C respectively (Ikezoe et al. 2007). Preclinical tests reveal that it inhibits the tumor growth in prostate (Lee et al. 2006), thyroid (Arlot- Bonnemains et al. 2008), ovarian (Lin et al. 2006) and oral squamous (Pan et al. 2008) cancer cells line, with IC50 values ranging from 15 to 130 nM. The inhibition of Aurora B kinase induces the accumulation of cells arrested in a pseudo- G1 state with a 4N DNA content or the accumulation of cells with >4N DNA content (the latter population representing cells that exit mitosis and subsequently proceed through S phase in the absence of cell division). Continous proliferation in the presence of aberrant mitosis and failed cytokinesis presumably result in cell death (Gizatullin et al. 2006). VX-680 was reported to induce apoptotic cell death in Aurora A- high primary leukemic blast, but not in Aurora A- low acute myeloid leukemia (AML) or in normal bone marrow mononuclear cells (BMMCs); this suggest its potentially therapeutic use for some leukemia patients (Huang et al. 2008). More interestingly, VX-680 showed in vitro activity against Bcr-Abl bearing the Imatinib resistant mutations (Giles et al. 2007; Young et al. 2006). Unfortunately, development of VX-680 was terminated due to its severe toxicity. The other pan Aurora kinases inhibitors and their clinical trials are listed in Table 1.

1.5.1.1Aurora A inhibitors

Studies validating Aurora A as potential therapeutic target have been mainly carried out using gene silencing approaches such as siRNA and antisense oligonucleotides. These reports showed for the first time that Aurora A-specific inhibition by an antisense oligonucleotide resulted in cell cycle arrest in the G2/M phase and in the induction of apoptosis. MLN8237 is a recently discovered ATP-competitive selective Aurora A inhibitor (40-fold selective for Aurora A compared to Aurora B, IC50 4nM) (Kollareddy et al. 2012). Treatment of cells with this compound causes defects in bipolar assembly resulting in chromosomal segregation abnormalities. In addition cells arrest in mitosis due to activation of the mitotic checkpoint. MLN8237 has proven to be effective in preclinical AML studies, and have also been tested in CML in both preclinical and clinical studies. Others Aurora A inhibitors are elencated in table 1.

1.5.1.2 Aurora B inhibitors

Aurora B has been investigated as a potential target in solid tumors, and in leukemia. It has been shown that Aurora B is overexpressed in leukemia cells of ALL, AML and CLL patients (Walsby et al. 2008).
Treatment with Aurora B kinase inhibitors induces the accumulation of cells arrested in pseudo-G1 state with >4N DNA content or the accumulation of cells with >4N DNA content; the latter population representing cells that exit mitosis and subsequently proceed through S phase in the absence of cell division (Gizatullin et al. 2006). Continued proliferation in the presence of aberrant mitosis and failed cytokinesis presumably give rise to cells with higher DNA content due to an increase of the cell diameter, resulting in apoptosis (Gautschi et al. 2008; Keen et al. 2004).
AZD1152 is a selective Aurora B inhibitor (IC50 0.36 nM). Data on results from the first phase I trial in advanced solid tumors were recently reported; these studies evaluate the effect of the treatment on colorectal, melanoma, prostate and pancreatic cancer, and recently also in leukemic malignancies (Schellens et al. 2006) Preclinical studies performed on AML cells lines and primary AML samples show a reduction of cell proliferation and increase cell death (Yang et al. 2007). Others Aurora B inhibitors are elencated in table 1.
Although Aurora A was initially considered to be the more appealing therapeutic target, the validation of Aurora B as a potential drug target revealed its equally critical role in carcinogenesis since it was demonstrated that pharmacological agents aiming to target either Aurora A or both aurora kinases produced a biological response equivalent to Aurora B inhibition alone (Mountzios et al. 2008).

Target	Molecule	Preclinical Activity	Clinical Development
Aurora A	MLN8237/ MLN-8054 MK-5108	Leukemia and Solid tumors Solid tumors	Phase II and Phase III Phase I
Aurora B	AZD1152 PHA-739358	Leukemia and Solid tumors	Phase II
Aurora A and Aurora B	VX-680 AT9283 MSC1992371A PF-03814735 AS703569	Leukemia and solid tumors Leukemia and solid tumors Leukemia and solid tumors	Terminated due to toxicity Phase II Phase I

Table 1. Aurora kinases inhibitors in clinic

1.5.1.3 Combinated therapy with Aurora kinases inhibitors

Combination of Aurora kinases inhibitors and conventional therapies (citotoxic anticancer agents or radiotherapy) are currently under study. For instance, MLN8237 enhanced vincristine or docetaxel chemosensitivity in aggressive B-cell non-Hodgkin lymphoma (B-NHL) (Mahadevan et al. 2012). Similarly, pan-aurora inhibitor SNS-314 also enhanced antitumor activity of microtubule-targeting agents in a colon carcinoma model (Vander-Porten et al. 2009). Treatment with AZD115 was found to be synergistic with a variety of chemotherapeutic agents, including irinotecan, docetaxel, vinorelbine, gemcitabine, oxaliplatin, and 5-fluorouracil (Nail et al. 2004), and with vincristine and topoisomerase inhibitors, in leukemia cell lines (Yang et al., 2007). Moreover, this compound also potentiates the radiation response in p53-deficient cancer cells, suggesting synergy with radiotherapy (Tao et al. 2007).
This suggests that Aurora kinases inhibitors, like many other molecular targeted agents, may optimize their efficacy in combination with classic chemoterapeutic agents or other molecular therapies, as well as overcoming the drug resistance of kinases inhibitors.

2.0 AIM OF THE STUDY

The process of cell division is instrumental for the development and progression of tumours and targeting cell division has classically been a successful antitumour therapy. So far, most antimitotic compounds (such as the vinca alkaloids and taxanes) target the mitotic spindle and trigger mitotic arrest, but recent efforts have yielded promising compounds targeting regulatory proteins during mitosis (Vader and Lens 2008). The Aurora Kinase family of mitotic regulators, have received much attention as potential targets for novel anti-cancer therapeutics. In the recent years several small molecule ATP-competitive inhibitors have been described that target the Aurora kinase. Among a large number of agents evaluated preclinically, the pan-Aurora-Agents AMG900 and AT9283, the Aurora-A and -B targeting agents AZD1152, ZM447439, and ENMD2076, the Aurora-A-specific agent MLN8237 (alisertib), and the Aurora-B/C inhibitor GSK1070916A have progressed towards clinical trials (Niconova et al. 2013; Dees et al. 2012; Hardwicke et al. 2009). The most advanced compound, MLN8237 (alisertib), is being evaluated in phase II trials for multiple types of cancer and phase III trials for the treatment of peripheral T-cell lymphoma (Dees et al. 2011).
These data are encouraging, suggesting that Aurora kinase inhibitors may have real potential as anti-cancer drugs. If inhibiting a single Aurora kinase mediates the anti-tumor activity, it may be beneficial to develop selective inhibitors of that particular Aurora kinase in order to minimise potential side effects. Thus, it would be important to identify more effective anti-Aurora B inhibitors.
Aim of our study has been to characterize potential Aurora B kinase targeting agents in order to identify novel inhibitor of this kinase. For this purpose we:

- tested ability of seven kinase inhibitors (MK1, 2, 3, 4, 5, 6 and 7) to inhibit the histone H3 phosphorylation (downstream target of Aurora B kinase) in HeLa cells, and we selected four of them (MK1, 2, 6 and 7) on the basis of their efficacy;
- evaluated effect of Aurora B kinase inhibition by MK compounds on cell cycle distribution and DNA content;
- evaluated how Aurora B inhibition induced by MK 1, 2, 6 and 7 compounds affects taxol- induced SAC activation.
- studied the effects of the four compounds on proliferation and viability of HeLa cells;
- evaluated the mechanism of cell death following the treatment with MK7, which is the most potent of the 4 compounds;
- analyzed alteration induced by MK7 treatment in mitotic spindle formation, chromosome segregation and cytokinesis in HeLa cells;
- tested the ability of MK7 to inhibit growth and viability of a panel of tumoral cell lines.

3.0 MATERIALS AND METHODS

3.1 Compounds

Compounds were synthesized in the Li laboratory. For *in vitro* experiments, compounds were dissolved in dimethyl sulfoxide (DMSO) at 10 mM concentration and stored at -80°C.

Selectivity profiling

DiscoveRx 442 kinome-wide selectivity profiling was conducted by DiscoveRx Bioscience with KinomeScanTM Technology.

3.2 Cell Culture

Cell cultures.

HeLa, A2780, HL-60 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO), 2 mM L-glutamine and 100 units/ml penicillin-streptomycin (GIBCO).
8505-C cells were grown in DMEM with 10% fetal bovine serum, 2 mM L-glutamine and 100 units/ml penicillin-streptomycin (GIBCO). HCT-116 cells were grown in McCoy's supplemented with 10% fetal bovine serum (GIBCO), 2 mM L-glutamine and 100 units/ml penicillin-streptomycin (GIBCO).

Nocodazole and Taxole treatment.

10^5 cells were seeded in 60 mm dishes and incubated overnight for cell adherence. Next day, nocodazole (100 ng/mL) (Sigma) or Taxol (Sigma) were added in complete medium and the culture kept for 16 hours. Mitotic cells were harvested upon mitotic shake off, washed three times in PBS and then replated in fresh media with or without the drug.

Growth Curves.

HeLa, HCT116 and 8505-C cells (10,000/dish) or A2780 and HL-60 cells (3,000/dish) were seeded in 60-mm dishes. The day after, different concentrations of MK1, MK2, MK6 and MK7 or vehicle, were added to the medium and refreshed every 1-2 days. Cells were counted every day (HL-60 and A2780) or every 2 days (HeLa, HCT-116 and 850-5C).

3.3 Protein Analysis

Protein lysates were prepared according to standard procedures. Briefly, cells were lysed in a buffer containing 50 mM N-2- hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.5), 1% (vol/vol) Triton X-100, 150 mM NaCl, 5 mM EGTA, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 2 mM phenylmethylsulphonyl fluoride (PMSF) and 1 □g/ml aprotinin and clarified by centrifugation at 10,000 xg for 15 min.
Protein concentration was estimated with a modified Bradford assay (Bio-Rad, Munich, Germany) and lysates were subjected to Western blot analysis. Immune complexes were detected with the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfort, UK).

Antibodies

Anti-phospho-H3 (#9701), that recognizes H3 proteins when phosphorylated on Ser10, was from Cell Signaling. Anti-H3 (FL-136), Anti-Cyclin B1 (sc-594) and Anti-Geminin (sc-8450) were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal Anti-□ tubulin (Clone DM 1A) was from Sigma. Anti-Cleaved Caspase-3 Asp175 (#9661) that detects endogenous level of the large fragment (17/19 KDa) of activated caspase-3, was from Cell Signaling. Anti-PARP (#9542), that recognize cleaved PARP by Caspase cleavage, was from Cell Signaling.
Secondary antibodies coupled to horseradish peroxidase were from Santa Cruz Biotechnology.

3.4 Cytofluorimetric Analysis

Cell cycle analysis.

Synchronized cells or unsynchronized cells were either incubated in the presence of MK compounds or vehicle. At the indicated intervals, cells were harvested by tripsinization and fixed in 80% ethanol over night at -20° C. After washing with PBS, cells were treated with RNAse A (100 U/ml) and stained with propidium iodide (25 µg/ml) (Sigma) for 30 min. DNA content was measured using the BD FACS Calibur (BD Biosciences, US). A total of at least 10^4 events were acquired for each experiment. Data were analyzed with the Cell Quest software (BD Biosciences, US).

Cell apoptosis assay.

Treated cells were harvested and rinsed with cold PBS once. After centrifugation for 10 min, cells were resuspended in 500 ul of 1X Annexin V

binding buffer (#130-092-052; Miltenyi Biotec) and then added 10 ul of Annexin V-FITC and 5 ul of propidium iodide. After incubation for 15 min at room temperature in the dark, the samples were analyzed by BD FACS Calibur (BD Biosciences, US). A total of at least 10^4 events were acquired for each experiment. Data were analyzed with the Cell Quest software (BD Biosciences, US).

3.5 Viability Assay

For the evaluation of the cytoxic effects of the MK compounds, cells were seeded in 96-well plates. After 24 h, the medium was replaced with medium containing increasing concentrations of compounds. Three days later, the cells were fixed with 50% trichloroacetic acid and stained with 0.4% sulforhodamine B in 1% acetic acid. The bound dye was solubilized in 10 mmol/L unbuffered Tris solution, and the absorbance was determined at 540 nm in a microplate reader.

3.6 Immunofluorescence Staining

For indirect immunofluorescence, cells were fixed in 4% paraformaldehyde and permeabilized with Triton 0.2% X-100 (5 minutes on ice), and then incubated with anti-□tubulin antibody (Sigma) for 1h at room temperature. Coverslips were washed and incubated with an Alexa-488 goat anti-rabbit antibody (Invitrogen) for 30 minutes at room temperature. After 5 minutes of Hoechst counterstaining, coverslips were mounted in Glycerol/PBS (1:1) and observed with a Zeiss LSM 510 META confocal microscope (Carl Zeiss). At least 300 cells were counted in triplicate.

3.7 Statistical Analysis

Unpaired Student's *t* test using Instat software program (Graphpad Software Inc) was performed to compare cell growth. All *P* values were two-sided, and differences were considered statistically significant at $P < .02$. IC_{50} doses were calculated through a curve fitting analysis from last day values using the PRISM software program (Graphpad Software Inc). To compare tumour growth we used an unpaired t-student test (InStat program, GraphPad software). *P* values were statistically significant at $P < .02$.

4.0 RESULTS

4.1 Evaluation of MK compounds potency against Aurora B activity in HeLa cells

In order to identify new Aurora B kinase chemical inhibitors, we tested seven compounds, MK1, MK2, MK3, MK4, MK5, MK6 and MK7, obtained by combinatorial chemistry optimization of a common molecular scaffold, provided by Dr Hong-Yu Li (University of Arizona, Tucson, USA). In an in vitro kinase assay, these compounds proved to be efficient Aurora B inhibitors with an IC_{50} <10 nM and selective for Aurora A (IC_{50} >100 nM). However, the Aurora B assay is not sensitive enough to differentiate compounds with an IC_{50} less than 10 nM.

To provide evidence that these compounds acted as Aurora B inhibitors and determine which compounds were most potent, we initially evaluated their effect on phosphorylation of Histone H3, a *bona fide* Aurora B target in HeLa cells. Histone H3 is a direct downstream substrate of Aurora B kinase, and its phosphorylation on serine residue 10 (pS10-H3) is crucial for cell entry into mitosis. To determine whether MK compounds inhibit pS10-H3, cells were synchronized in M phase (prophase) with nocodazole (100 ng/ml for 16 hours). Thus, by promoting mitotic arrest nocodazole enriches for cells with high Aurora B activity and maximal phosphorylation of Histone H3. Upon mitotic shake off, cells in M phase were reseeded in nocodazole containing media in the presence or not of different concentrations (10, 50 and 250 nM) of anti-Aurora B compounds for 3 hours. We determined phosphorylation status of H3 by Western Blotting with Phospho-specific H3 antibody, able to recognize H3 only when phosphorylated on Serine 10. As shown in Figure 14, compared to untreated control cells, in MK1, MK2, MK3, MK4, MK6, and MK7 treated cells there was a dose dependent reduction of H3 phosphorylation on S10. In particular, all six compounds were able to significantly decrease H3 phosphorylation already at 10 nM dose, while MK5 did not display any significant activity. H3 protein levels were not affected by the treatment, demonstrating that the decreased Phospho-H3 (Ser10) signal was due to the inhibition of phosphorylation and not to the degradation of total H3 protein. We selected MK1, MK2, MK6 and MK7 for further studies for their stronger activity at 50 nM dose.

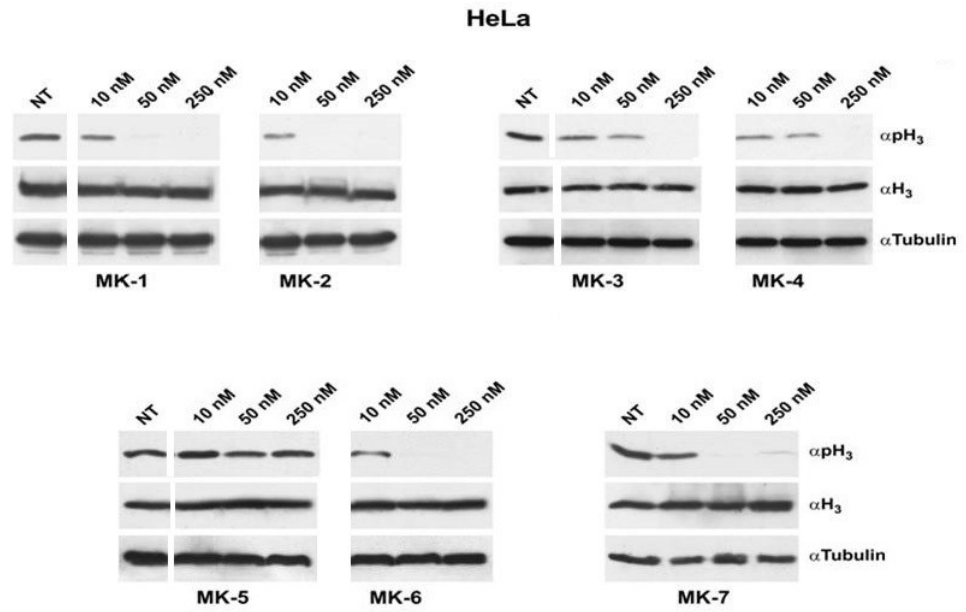


Figure 14. MK compounds potently inhibit Aurora B activity in HeLa cell lines.
HeLa cells were incubated with nocodazole (100 ng/ml) for 16 hours to arrest them in M phase. DMSO (NT) or the indicated compounds were added to cell media at different concentrations as indicated. Cells were harvested and protein extracts were subjected to Western blotting using an antibody against phosphorylated serine 10 of histone H3 as a marker of Aurora B kinase activity. H3 and alpha tubulin antibodies were used as a loading control. MK1, MK2, MK6 and MK7 displayed the highest activity.

4.2 Effect of Aurora B kinase inhibition by MK compounds on cell cycle distribution and DNA content

Aurora B is necessary for correct progression through M phase, mitotic spindle attachment to chromosomes and cell division, thereby playing a key role in the maintenance of normal ploidy. Therefore, to investigate the effect of MK inhibitors on cell cycle, HeLa cells distribution in cell cycle phases, based on their DNA content, was analysed by flow cytometry upon cell staining with propidium iodide (PI). The cells were synchronized in M phase with Nocodazole (100 ng/ml for 16 hours) which prevents polymerization of microtubules. Synchronized cells were released in the presence of 10, 50 and 250 nM of MK compounds or vehicle (DMSO) for 24 hours and then subjected to flow cytometry. A graphic presentation of cell cycle phase distribution following treatment with MK compounds is shown in Figure 15; Figure 16

shows the percentage of the cells in each cell cycle phase. Treatment with MK induced a profound change in cell cycle progression. After release of nocodazole block, control cells progressed normally throught G1 and S phase, showing a cell cycle profile indistinguishable from that of unsyncronized cells (data not shown). At 10 nM dose, MK1, MK6 and MK7 compounds arrested cells and induced an increase of the G2/M cell fraction with concurrent accumulation of cells with DNA content >4N (polyploid cells). MK2 at same dose had no detectable effects. 50 nM dose of MK1, MK6 and MK7 was sufficient to induce polyploidy (8N) in the majority of cells, while for MK2, this effect occurred only at 250 nM dose.

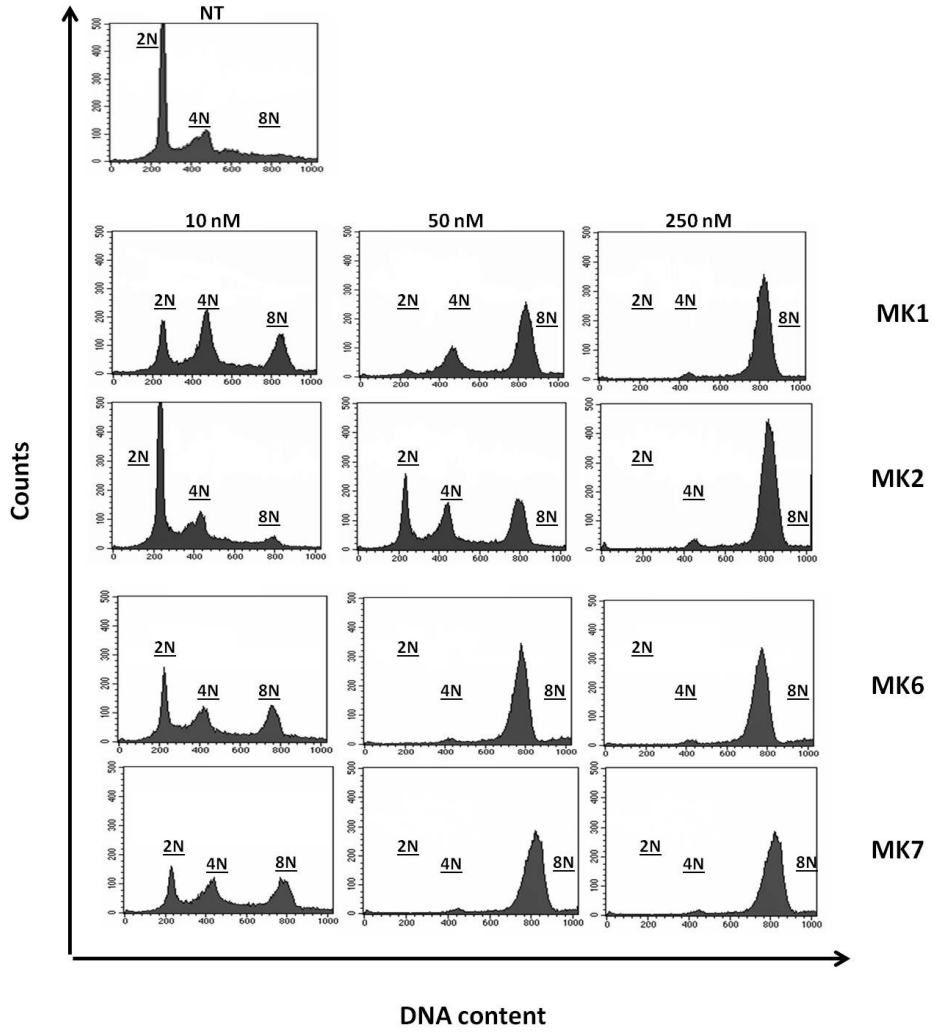


Figure 15. Effect of MK compounds on cell cycle distribution and DNA content.
HeLa cells were synchronized in M phase by nocodazole treatment (100ng/ml for 16 h). Upon release from nocodazole, cells were incubated with vehicle (NT) or MK1, MK2, MK6 and MK7 at the indicated concentrations for 24 h. Cells were then harvested, DNA was stained with propidium iodide and samples were analyzed by flow cytometry. N: aploid DNA content. MK1, MK6 and MK7 exerted the most potent effects on induction of polyploidy (8N)

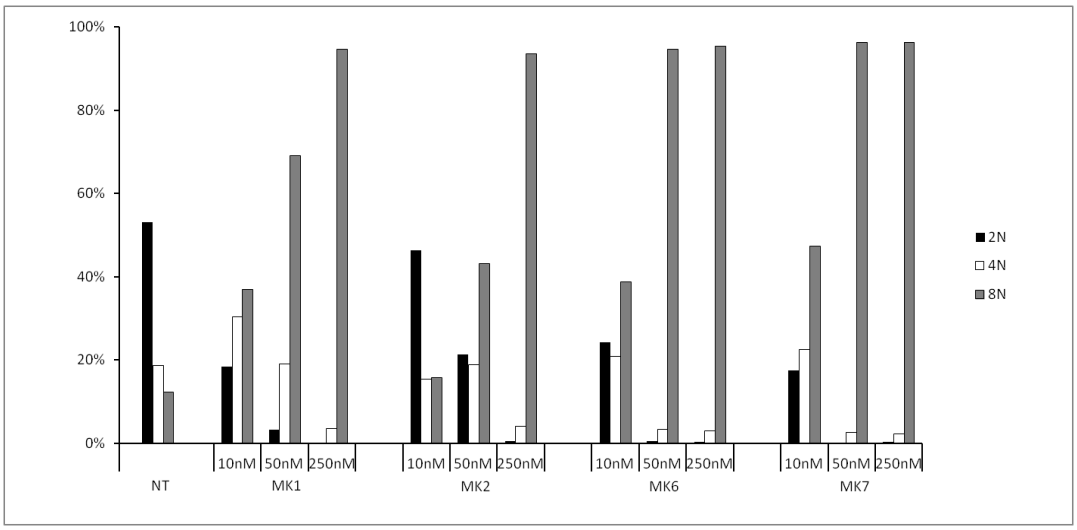


Figure 16. Percentage of cells in the different phases of cell cycle upon treatment with MK compounds.
HeLa cells were synchronized in M phase by nocodazole treatment (100ng/ml for 16 h). Upon release from nocodazole, cells were treated subsequently with vehicle (NT) or MK1, MK2, MK6 and MK7 at indicated concentrations for 24 h. Cells were then harvested, DNA was stained with propidium iodide and samples were analyzed by flow cytometry. This figure depicts quantitative analysis of the experiment reported in Figure 15. The histograms depict the cell cycle phase distribution: 2N: diploid DNA; 4N: tetraploid DNA content; 8N: octaploid DNA content (corresponding to polyploid cells).

4.3 Effect of Aurora B kinase inhibition by MK compounds on the spindle assembly checkpoint (SAC)

The proper segregation of replicated chromatids during mitosis requires that the two kinetochores on each sister chromatid are attached to the opposing spindle poles. This process is strictly controlled and anaphase onset is delayed until all chromosomes are bi-oriented and under proper tension (spindle assembly checkpoint: SAC). Upon SAC inactivation, APC/Cdc20 complex initiates anaphase by promoting degradation of Cyclin B and Securin, causing a decrease in Cyclin B-associated Cdk1 activity and resolution of cohesion between sister chromatids. In early G1, APC/Cdh1 complex promotes geminin degradation in order to allow G1 entry and DNA replication origin licensing by recruitment of MCM2/7 complex. The SAC response activated by either unattached kinetochores or by lack of tension on kinetochores has been studied by using microtubule-destabilizing (Nocodazole) or microtubule- stabilizing (Taxol) drugs, respectively. Whereas both treatments cause a SAC-dependent mitotic arrest, Aurora B is required for the arrest in response to Taxol, but less essential for the arrest induced by Nocodazole (Biggins et al. 2001; Carvalho et al. 2003).

We asked therefore whether Aurora B inhibition induced by MK compounds, was able to suppress Taxol-induced SAC activation. Hela cells were treated with Taxol (100 ng/ml) for 16h and then replated in media containing Taxol plus MKs at different concentrations (10, 50 and 250 nM) for 5 hours. In order to monitor SAC escape with consequent mitosis exit and entry in G1, Cyclin B and Geminin protein levels were evaluated by Western blotting. As shown in Figure 17, MK compounds were able to induce Cyclin B and Geminin degradation in a dose dependent manner suggesting an overcome of Taxol-induced SAC and an inappropriate mitosis exit. In particular, at 50 nM MK1 and MK2 induced Cyclin B degradation and at 250 nM Geminin degradation, while MK6 and MK7 induced Cyclin B degradation already at 10 nM and Geminin degradation at 50 nM (Figure 17).

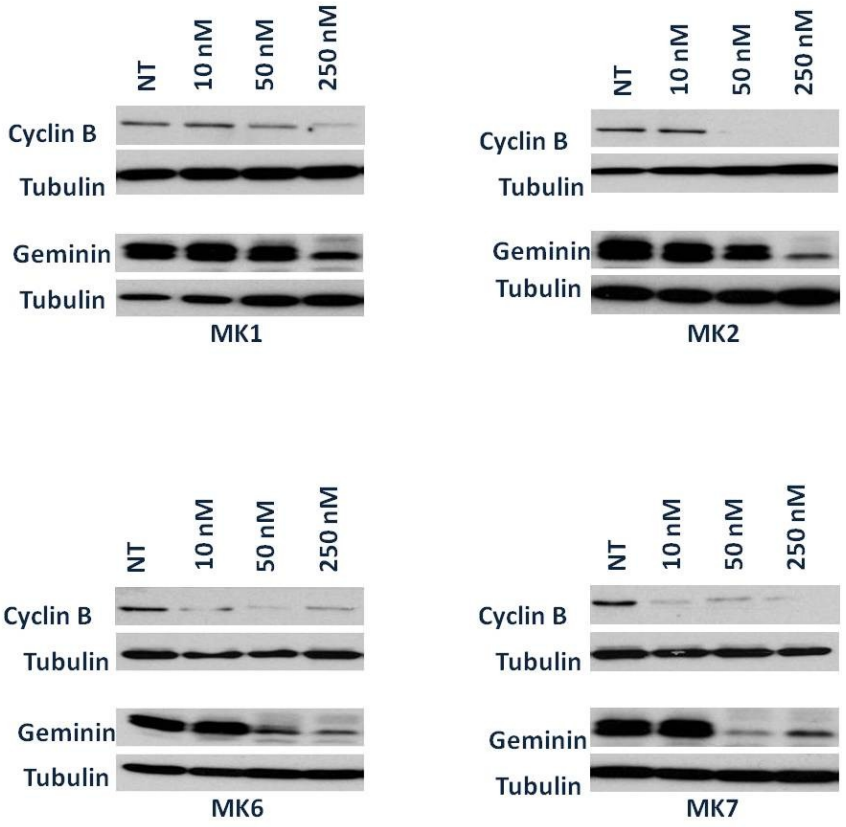
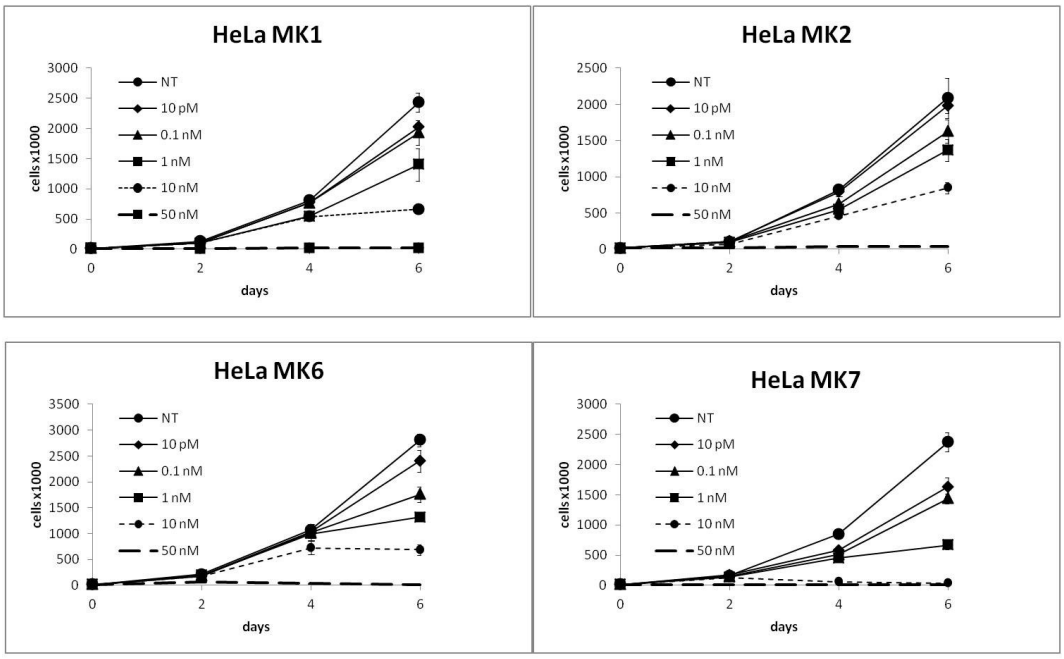


Figure 17. Effect of MK compounds on Taxol-dependent activation of the spindle-assembly checkpoint (SAC).
HeLa cells were treated with Taxol (100 ng/ml) for 16 hr to arrest them in M phase and then with the indicated concentrations of MK compounds for 5 hr. Cells were harvested and protein extracts were subjected to Western blotting with the indicated antibodies to assess M phase exit (Cyclin B degradation) and subsequent G1 phase entry (Geminin degradation). Tubulin staining was used as a loading control.

4.4 Effect of MK compounds on cell proliferation and viability

Since Aurora B plays a key role in cell cycle progression, its inhibition could result in defects in cell proliferation. We performed cell growth curves of HeLa cells treated with MK compounds. Cells were treated with MK1, MK2, MK6 or MK7 at different concentrations (10 pM, 0.1-1-10-50 nM) for six days and counted every two days, using the vehicle alone as control. As shown in Figure 18, all the four compounds were able to reduce proliferation of HeLa cells. In particular, MK6 and MK7 were the most effective compounds, inhibiting proliferation with an IC_{50} of 0.44 and 0.37 nM respectively, while MK2 was the least effective one with an IC_{50} of 2.3 nM. MK1 displayed an intermediate efficacy, with an IC_{50} of 1.2 nM. The lower IC50 dose displayed by MK compounds in growth curves with respect to biochemical Aurora B kinase inhibition (see Figure 14) raised the possibility that cell treatment caused a cytotoxic rather than a cytostatic effect.



IC50 dose

	MK1	MK2	MK6	MK7
HeLa	1.2nM	2.27nM	0.44nM	0.37nM

Figure 18 Effect of MK compounds on cell proliferation.
Top) HeLa cells were incubated with DMSO (NT) or with the indicated concentrations of MK compounds in 10% fetal bovine serum. Cells were counted at the indicated time points. Data are the mean ± SD of two experiments performed in triplicate. Bottom) Growth inhibition IC50 of MK compounds for HeLa cells.

We explored this possibility by measuring cell viability via Sulforhodamine B assay. Based on the high efficacy of MK7, we selected this drug for such an experiment and thereafter. HeLa cells were seeded in 96-well culture plates and allowed to grow for 24 hr followed by MK7 treatment for 3 days. As shown in Figure 19, 10 nM MK7 showed a potent cytotoxic effect, reducing viable cells to 60% of the control.

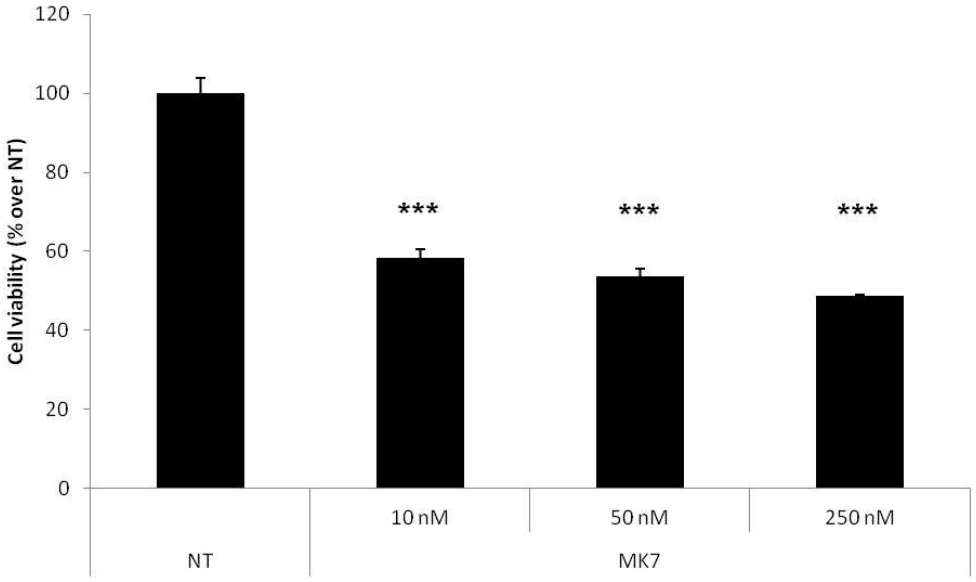


Figure 19 Effect of MK7 on cell viability.
HeLa cells were subjected to Sulforhodamine assay to detect viable cells upon treatment with vehicle (NT) or the indicated concentrations of MK7. Viable cells were plotted as percentage of control. Experiment was performed in triplicate and SD are indicated; *** $p < 0.001$ compared to NT.

4.5 Evaluation of cell death induced by MK7

Since MK7 treatment resulted in a decrease of cell viability, we explored the mechanism of cell death upon MK7 treatment. Apoptosis, or programmed cell death, is an essential mechanism used by multicellular organisms to negatively select cells that are deemed deleterious to the host. Typical apoptosis is distinguished from necrosis as only the former involves the activation of specific pathways that result in characteristic morphological features including DNA fragmentation, chromatin condensation and formation of apoptotic bodies. Many stimuli can trigger various pathways to apoptosis, but all these pathways converge to a common process involving the activation of a cascade of caspases that specifically cleave protein substrates at aspartic acid residues. One of the distinct early cellular changes during apoptosis is phosphatidylserine translocation from the internal to the external portion of the cellular membrane. Annexin V-FITC is a fluorescent probe which detects early apoptosis by binding to externalized phosphatidylserine in the presence of calcium and can be detected by flow cytometry. Staining of cellular DNA with propidium iodide (PI) in the absence of cellular permeabilization allows detection of cells whose membrane has been totally compromised as it occurs in late apoptotic stages or in necrosis. Thus, live cells show no staining with either Annexin V-FITC or PI. Cells which are early apoptotic will stain only with the Annexin V-FITC. Late apoptotic cells are stained by both Annexin V-FITC and PI. Finally, necrotic cells stain with PI only. HeLa cells were treated with MK7 (0.01, 0.1, 1.0 and 10 nM) for three and six days, followed by staining with Annexin V-FITC/PI and analysis by flow citometry. As shown in Figure 20, percentages of cells in late (upper right panel) apoptosis increased parallel to increasing concentration and duration of exposure to MK7.
At 10 nM concentration, late apoptotic cells were 31% after three days of treatment, and 57% after six days, compared to 4% and 1.9% in the untreated control cells. Interestingly, after 6 days of treatment late apoptotic cells already increased by more than 2 fold at a dose as low as 0.01 nM and more than 4 fold at 0.1 nM. After 3 and 6 days of treatment necrosis was also detected but only at 10 nM (Figure 20). Apoptosis following MK7 treatment was also confirmed by Western blotting to detect Caspase-3 cleavage by Caspase-9 and PARP (Poly ADP-Ribose Polymerase) cleavage by Caspase-3. As shown in Figure 21, both apoptotic markers were increased in a dose dependent manner following MK7 treatment, compared with the untreated control. These data support the notion that treatment with MK7 resulted in the accumulation of polyploid cells that subsequently undergo apoptosis-mediated cell death.

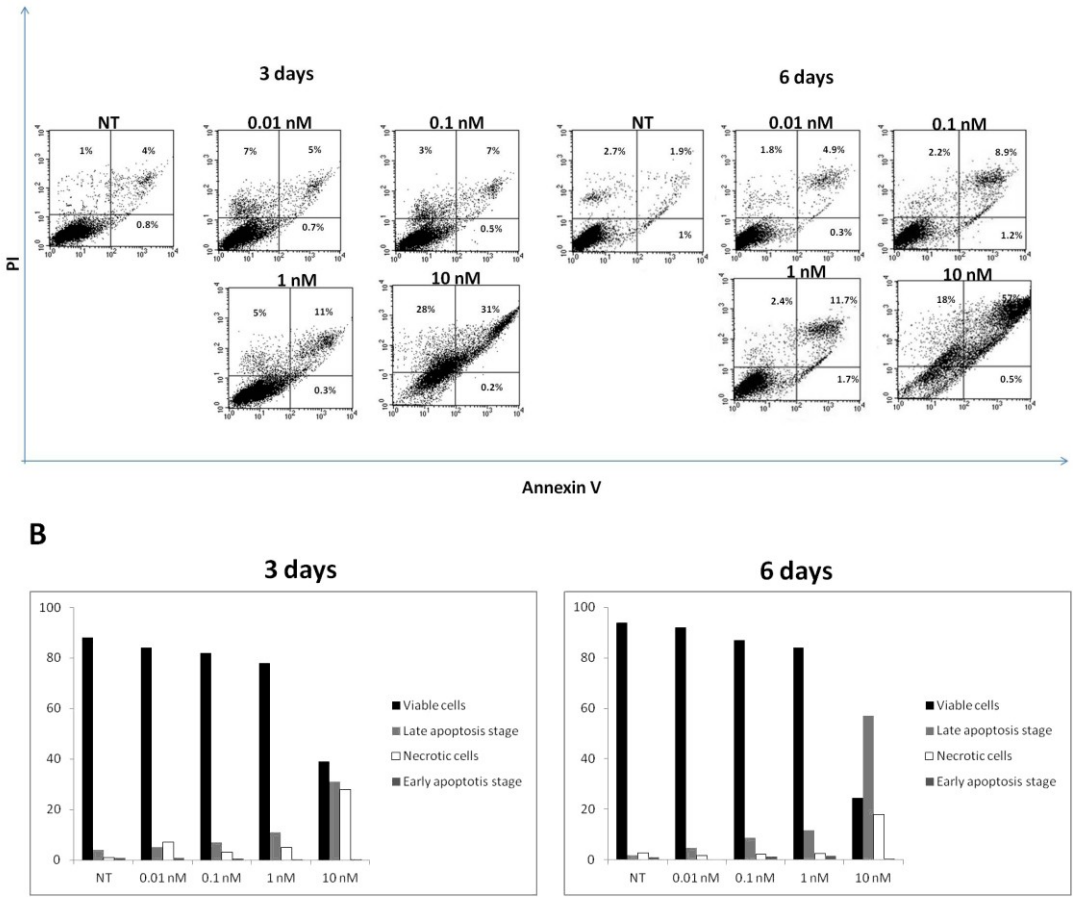


Figure 20. Cell death induced by MK7 in HeLa cells.
A) HeLa cells were treated with vehicle (control) or MK7 at the indicated concentrations for 3 or 6 days. Cell death was detected by flow cytometric analysis based on propidium iodide (y-axis; necrosis), annexin V staining (x-axis, early apotosis) or both (late apoptosis). B) Percentages of cells displaying different types of cell death are shown in the bar graphs.

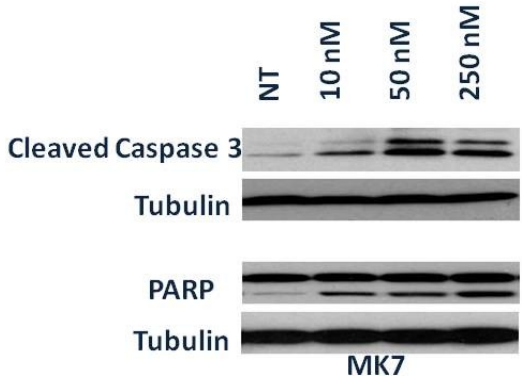


Figure 21. Western blotting analysis of PARP and Caspase 3 Cleavage after MK7 treatment
HeLa cells were treated with MK7 at indicated concentrations for 72 hr. Cells were harvested and protein lysates were subjected to Western Blotting with the indicated antibodies to detect apoptosis. Tubulin staining was performed as a loading control.

4.6 Effect of MK7 on mitotic spindle and on cell division

To analyze effects of MK7 treatment on mitotic spindle formation, chromosome segregation and cytokinesis, Taxol-synchronized HeLa cells were released in MK7 (10 and 50 nM) containing medium for 2 hours, fixed in paraformaldeyde and incubated with anti-tubulin antibody to detect mitotic spindles and with Hoechst to analyze chromatin structure by fluorescence microscopy. Cells treated with 10 nM dose exhibited only a few kinetochore-attached microtubles and were unable to organize the ordered mitotic spindle that was seen in control cells. As a consequence, chromosomes could not be fully separated and cells prematurely escaped mitosis as shown by chromatin decondensation before chromosome segregation. Furthermore, chromosomes appeared to be either splayed out throught the cell, or, rather than aligning at the spindle equator, lined up along the fibers of the spindle (Figure 22).

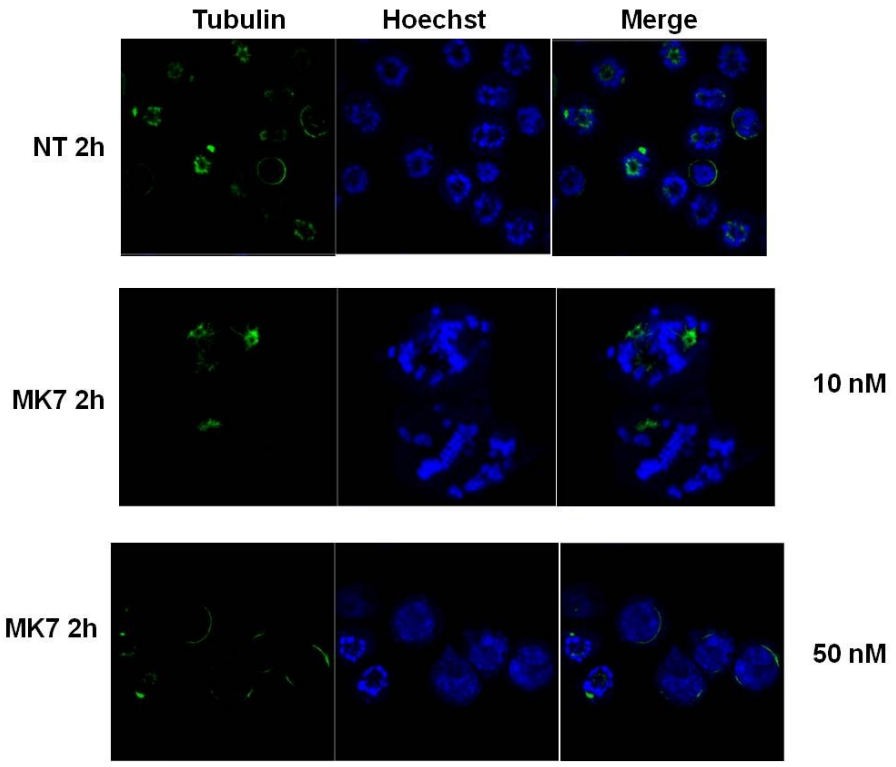
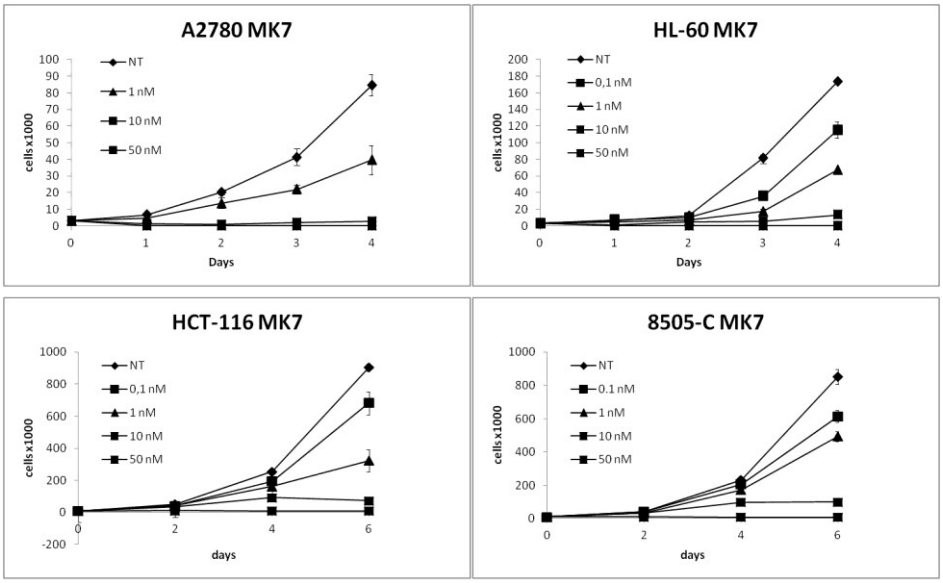


Figure 22. **Immunofluorescence staining of mitotic spindle in MK7 treated cells.** Exponentially growing HeLa cells were synchronized with Taxol in M phase (100 ng/ml), then released from M phase arrest and treated with 10 or 50 nM MK7 or vehicle (NT). After 2 hr, cells were fixed and stained by immunofluorescence with anti-□-tubulin (green) to visualize polymerized microtubules and Hoechst (cyan) to visualize chromatin at different time points.

4.7 Effect of MK7 on proliferation of a panel of human tumor cell lines

We evaluated the effects of MK7 on viability of a panel of different neoplastic cell lines. To this aim, we selected four human cell lines, derived from different tumor types: A2780 (human ovarian carcinoma), HL-60 (human promyelocytic leukemia), HCT116 (human colorectal carcinoma) and 8505-C (human undifferentiated thyroid carcinoma). Initially, we performed cell growth curves. HCT-116 and HL-60 cells were treated with MK7 at different concentrations (0.1, 1, 10 and 50 nM) for six and four days, respectively, using the vehicle alone as control. Instead, 8505-C and A2780 cells, were treated by 1, 10 and 50 nM dose of MK7, because preliminary experiments showed that 0.1 nM of the drug was poorly effective (data not shown). As shown in Figure 23, all the cell lines were sensitive to MK7, with an IC50 in the same range as that calculated for HeLa cells. In particular, HCT-116 and HL-60 showed an IC50 of 0.475 nM and 0.490 nM, respectively. 8505-C and A2780 cell growth was inhibited with IC50 values of 1.451 nM and 1.571 nM, respectively.



IC50 Dose

	MK7
A2780	1.571nM (0.817-3nM)
HL-60	0.490nM (0.290-8.26nM)
HCT-116	0.475nM (0.301-0.75nM)
8505-C	1.451nM (1.082-1.945nM)

Figure 23 Effect of MK7 on cancer cell proliferation.
Top) Cells were incubated with DMSO (NT) or with the indicated concentration of MK7 in 10% fetal bovine serum. Cells were counted at the indicated time points. Data are the mean ±SD of two experiments performed in triplicate. Bottom) Cell growth inhibition IC50 of MK7. In brakets, 95% confidence intervals are indicated.

Then, we measured cell viability upon MK7 treatment via Sulforhodamine B assay in A2780, HCT116, 8505-C and HL-60. Cells were seeded in 96-well culture plates and allowed to grow for 24 hours, followed by MK7 treatment for three days at concentrations of 10, 50 and 250 nM. As shown in Figure 24, of the four cancer cell lines analysed, the A2780 cell line was the most sensitive to MK7 treatment. In details, 3-days incubation of A2780 cells with MK7 at 10 nM reduced viable cells to 20% of the control. In the same experimental conditions, treatment of HCT-116, 8505-C and HL-60 cells reduced cell number by approximately 20, 30 or 40%, respectively.

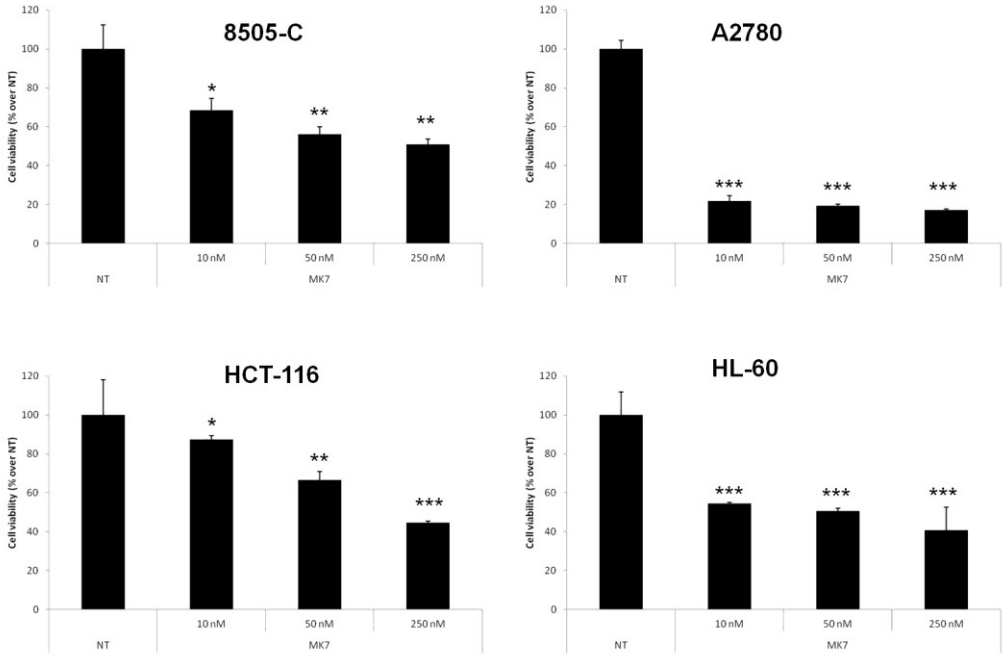


Figure 24. Effects of MK7 on cell viability.
Cells were subjected to Sulforodamine assay to detect viable cells upon treatment with vehicle (NT) or the indicated concentration of MK7. Viable cells were plotted as percentage of control. Experiment was performed in triplicate and SD are indicated; *** p< 0.001; ** p< 0.05; * p<0.3.

4.8 Kinase selectivity of MK7

In order to evaluate the specificity of MK7 compound we performed a kinome scan selecting 96 kinases distributed throughout the AGC, CAMK, GMGC, STE, TK, TKL, lipid and atypical kinase families (scanEDGE, Discoverex) at a concentration of 300 nM. The scan mapped 11 interactions with a binding score < 35% of the control and only 2, including Aurora B, with a binding score < 1% of the control (Aurora B and CSF1R), indicating high selectivity (Figure 25).

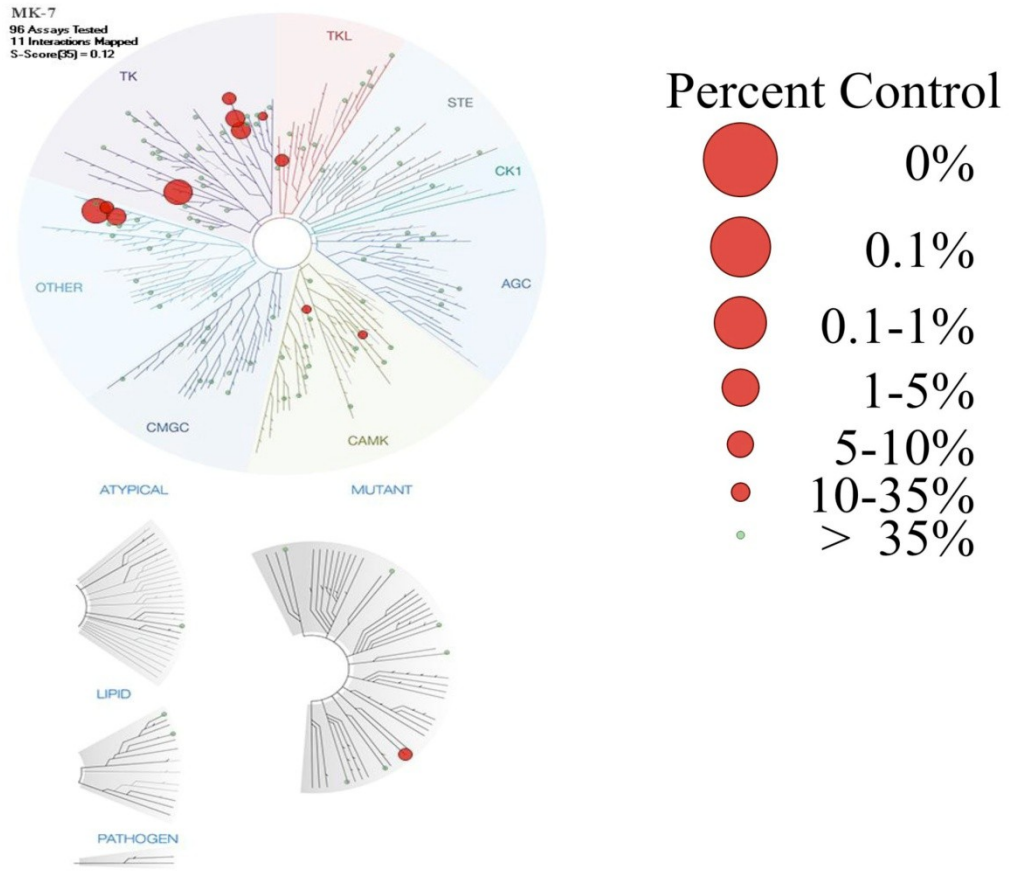


Figure 25. Kinome wide selectivity profiling of MK7.
Data were generated with DiscoveRx Treespot Version 4. Red dots indicate % of binding at 300 nM concentration of drug compared to DMSO control. S-score (35) indicated the selectivity when threshold was set at ≥ 65% binding. The size of the red circles is proportional to the strength of the binding, e.g. large circles imply high affinity.

5.0 DISCUSSION

The process of cell division is instrumental to the development and progression of tumours. Targeting the structural components of the mitotic machinery is a consolidated strategy in the treatment of cancer. Microtubule-binding agents, such as taxanes or vinca alkaloids, are strongly active against a variety of human cancer types, but MDR (multiple drug resistance), that limits the efficacy of many current anticancer therapies, as well as general and specific side effects (e.g. peripheral neuropathy) remain persistent problems. Consequently, there is a need for novel antimitotic drugs that target non-microtubule proteins, such as the mitotic kinases, that might display less severe side effects. The Aurora kinases have been frequently found to be overexpressed in many types of human tumors, and, as key mitotic regulators, have shown to be promising clinically relevant targets In the recent years, several small molecule ATP-competitive inhibitors have been describe, including VX680 (pan Aurora kinases inhibitor), AZD1152 (Aurora B inhibitor) and MLN8237 (Aurora A inhibitor). More than 30 small molecule Aurora kinase inhibitors are currently in different stages of preclinical and clinical development, however none have yet been approved by the U.S. Food and Drug Administration for clinical use.

Here, we describe preclinical analysis of seven compounds, MK1, MK2, MK3, MK4, MK5, MK6 and MK7, obtained by a common molecular scaffold, in order to identify a novel powerful Aurora B kinase inhibitor. In an in vitro kinase assay, these compounds proved to be efficient Aurora B inhibitors with an IC_{50} <10 nM and selective for Aurora A (IC_{50} >100 nM). In this study we show that four MK compounds (MK1, 2, 6 and 7) are able to decrease the phosphorylation level of Histone H3 at Ser 10 at nanomolar concentration in HeLa cells. These 4 compounds perturb the normal progression of cell cycle, cause an overcoming of taxol-induced SAC and promoting an inappropriate mitotic exit. In addition they efficiently block proliferation. In particular, one of them, MK7, is the most effective with an IC50 dose comprised between 0.37 and 2 nM. In details, MK7 treatment induces apoptosis and causes alterations in mitotic spindle formation, chromosome segregation and cytokinesis. Starting from these data and based on the high efficacy of MK7, we test the effects of this molecule on viability of a panel of different neoplastic cell lines demonstrating its efficacy in blocking proliferation of variuos types of cancer cells.

It is important to note some limitations of the studies described in this thesis. Firstly, we evaluated the effectiveness of MK7 on tumoral cell lines, but we didn't describe the effect on normal cells. We might expect that MK7 does affect normal cell viability as well, being an important component of the mitotic machinery. Nevertheless, due to their high mitotic index and aneuploidy, cancer cells are more dependent on the correct functioning of proteins regulating chromosome partition and cell division compared to normal

cells, a phenomenon known as non-oncogene addiction. This consideration indicates a greater sensitivity of cancer cells to Aurora kinase inhibitors with respect to normal cells. In addition, in order to increase efficacy and selectivity of action on cancer cells, combination regiments of Aurora kinase inhibitors and conventional therapies (citotoxic anticancer agents or radiotherapy) might represent a breakthrough and are currently under study. For instance, MLN8237 enhanced vincristine or docetaxel chemosensitivity in aggressive B-cell non-Hodgkin lymphoma (B-NHL) (Mahadevan et al. 2012). Similarly, pan-aurora inhibitor SNS-314 also enhanced antitumor activity of microtubule-targeting agents in a colon carcinoma model (Vander-Porten et al. 2009). Treatment with AZD115 was found to be synergistic with a variety of chemotherapeutic agents, including irinotecan, docetaxel, vinorelbine, gemcitabine, oxaliplatin, and 5-fluorouracil (Nail et al. 2004), and with vincristine and topoisomerase inhibitors, in leukemia cell lines (Yang et al., 2007). Moreover, this compound also potentiates the radiation response in p53-deficient cancer cells, suggesting synergy with radiotherapy (Tao et al. 2007). In summary, these data indicate that Aurora kinases inhibitors, like many other molecular targeted agents, may optimize their efficacy and specific action on cancer cells in combination with classic chemoterapeutic agents or other molecular therapies.

Another important limitation of our study is the lack of *in vivo* data; with this respect, an important point is the choice of formulation. Oral administration certainly improves drug compliance for patients. Many of the existing agents have been developed as intravenous drugs and only the Aurora A inhibitor, MLN8054, is available in oral formulas. Nevertheless, it should be noted that, although oral administration seems to be much more convenient for the patients, it is generally correlated with a greater variation in pharmacodynamic parameters and particularly with an unpredictable bioavailability. Moreover, since neutropenia has been, thus far, the major dose-limiting toxicity in this class of compounds, it appears that intermittent parenteral formulation may constitute the optimal choice. Chemical properties of MK7 compound allows us to predict its possible use via an oral formulation (data not shown). Therefore, further experiments are needed to test the pharmacokinetics and pharmacodynamics properties of MK7 and whether it would be effective as a drug given per os and which might be the best administration regiment, also in light of assessing its possible toxic effect and off target activity.

In spite of these limitations, our findings suggest that MK7 represent a promising selective inhibitor of Aurora B kinase.

6.0 CONCLUSION

Here, we describe MK7, a promising powerful compound which could be widely used to inhibit Aurora B kinase in several tumor cell lines.
At first we tested seven compounds, MK1-7 that shared a common molecular scaffold. In an in vitro kinase assay, these compounds proved to be efficient Aurora B inhibitors with an IC50 <10 nM and selective for Aurora A (IC50 >100 nM).
Briefly, we performed a series of experiments in HeLa cells to determine which compound is most potent. Our data demonstrate that MK7, in Hela cells, is able to: reduce phosphorylation of Histone H3 at 10 nM dose and perturb cell cycle progression inducing polyploidy already at 50 nM. Treatment with MK7 also promote escape from mitosis overtaking the SAC checkpoint and block cell growth with an IC_{50} of 0.37nM. Our data demonstrate also that MK7 has effect on cell division and viability, and promote cell death by apoptosis. Additionally, MK7 treatment caused alteration in mitotic spindle formation, chromosome segregation and cytokinesis. Finally, MK7 is an efficient Aurora B kinase inhibitor in several cancer cell lines. Further studies will assess the efficacy of MK7 compound in *in vivo* settings.
In conclusion we reckon that MK7 is a potent novel Aurora B kinase inhibitor which deserves important attention for its potential use in patients, upon its testing in preclinical models to assess efficacy, toxicity and best administration modality.

7.0 ACKNOWLEDGEMENTS

This study was carried out at the Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università di Napoli "Federico II" and Istituto di Endocrinologia ed Oncologia Sperimentale "G. Salvatore" of the Consiglio Nazionale delle Ricerche, Napoli.
At the end of my PhD programme, I would like to acknowledge the continuous help and encouragement that I received during this period.
I am thankful to Professor Massimo Santoro, coordinator of the International Doctorate Program and my co-supervisor, whose advices and insights were invaluable to me.
Words of thanks would fail to express my indebtness to my supervisor, Professor Francesca Carlomagno, who welcomed me in her group and allowed me to carry on my research work; her continuous support, insights and suggestions have constantly stimulated my interest for research.
Thanks to all the Lab 11 friends, my adventure mates. All of them have been invaluable to my activity and have contributed to my good spirit during the past three years. In particular, I want to thank Lisa and Giorgia, my great and sincere friends, for their smiles and their good spirit able to light my being exhausted.
I would also like to thank the colleagues of the other labs; they have been wonderful friends. In particular, I thank Dr. Gregory Alexander Raciti, Dr. Paola Mirra and Dr. Francesco Morra for their precious advice and help.
I want to thank Dr Hong Yu Li for providing us with the compounds.
I would like to thank my family, my sweet mother and my brother, Luigi, for always believing in me, for their continuous love and their support. A special thought is for my father, who, with his smile and love, was always beside me during this adventure.
Finally, I am thankful to Marco because, despite having crossed my path recently, he never fails to make me smile every day.

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