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THE SPINDLE ASSEMBLY CHECKPOINT AS A DRUG TARGET – NOVEL SMALL-MOLECULE INHIBITORS OF AURORA KINASES

by

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“Logic will get you from A to B.
Imagination will take you everywhere.”

-Albert Einstein

ABSTRACT

Cell division (mitosis) is a fundamental process in the life cycle of a cell. Equal distribution of chromosomes between the daughter cells is essential for the viability and well-being of an organism: loss of fidelity of cell division is a contributing factor in human cancer and also gives rise to miscarriages and genetic birth defects. For maintaining the proper chromosome number, a cell must carefully monitor cell division in order to detect and correct mistakes before they are translated into chromosomal imbalance. For this purpose an evolutionarily conserved mechanism termed the spindle assembly checkpoint (SAC) has evolved. The SAC comprises a complex network of proteins that relay and amplify mitosis-regulating signals created by assemblages called kinetochores (KTs). Importantly, minor defects in SAC signaling can cause loss or gain of individual chromosomes (aneuploidy) which promotes tumorigenesis while complete failure of SAC results in cell death. The latter event has raised interest in discovery of low molecular weight (LMW) compounds targeting the SAC that could be developed into new anti-cancer therapeutics.

In this study, we performed a cell-based, phenotypic high-throughput screen (HTS) to identify novel LMW compounds that inhibit SAC function and result in loss of cancer cell viability. Altogether, we screened 65 000 compounds and identified eight that forced the cells prematurely out of mitosis. The flavonoids fisetin and eupatorin, as well as the synthetic compounds termed SACi2 and SACi4, were characterized in more detail utilizing versatile cell-based and biochemical assays. To identify the molecular targets of these SAC-suppressing compounds, we investigated the conditions in which SAC activity became abrogated. Eupatorin, SACi2 and SACi4 preferentially abolished the tension-sensitive arm of the SAC, whereas fisetin lowered also the SAC activity evoked by lack of attachments between microtubules (MTs) and KTs. Consistent with the abrogation of SAC in response to low tension, our data indicate that all four compounds inhibited the activity of Aurora B kinase. This essential mitotic protein is required for correction of erratic MT-KT attachments, normal SAC signaling and execution of cytokinesis. Furthermore, eupatorin, SACi2 and SACi4 also inhibited Aurora A kinase that controls the centrosome maturation and separation and formation of the mitotic spindle apparatus. In line with the established profound mitotic roles of Aurora kinases, these small compounds perturbed SAC function, caused spindle abnormalities, such as multi- and monopolarity and fragmentation of centrosomes, and resulted in polyploidy due to defects in cytokinesis. Moreover, the compounds dramatically reduced viability of cancer cells.

Taken together, using a cell-based HTS we were able to identify new LMW compounds targeting the SAC. We demonstrated for the first time a novel function for flavonoids as cellular inhibitors of Aurora kinases. Collectively, our data support the concept that loss of mitotic fidelity due to a non-functional SAC can reduce the viability of cancer cells, a phenomenon that may possess therapeutic value and fuel development of new anti-cancer drugs.

TIIVISTELMÄ

Solujakautuminen (mitoosi) on eliön kasvun ja kehityksen perusta. Eliön elinkyvyn kannalta on oleellista, että mitoosissa kromosomit jakautuvat tasan muodostuviin tytärsoluihin. Sukusoluissa tapahtuvat solujaon virheet aiheuttavat keskenmenoja ja synnynnäisiä kehityshäiriöitä kun taas somaattisten solujen jakovirheet edesauttavat syövän syntymistä. Solujakoa säätelee mitoottinen tarkastuspiste (engl. the spindle assembly checkpoint), joka pyrkii tunnistamaan ja korjaamaan mahdollisesti tapahtuneet virheet ennen kuin genomin tasapaino häiriintyy. Tarkastuspiste koostuu monimutkaisesta, proteiinien muodostamasta signaaliverkostosta, jossa kromosomien kinetokoreista lähteviä viestejä välitetään ja monistetaan oikeaoppisen mitoosin varmistamiseksi. Tarkastuspisteen pienet häiriöt voivat johtaa yksittäisten kromosomien lukumäärämuutoksiin tytärsoluissa (aneuploidia), jonka tiedetään edistävän syövän muodostumista. Säätelyn täydellinen pettäminen sen sijaan johtaa solukuolemaan, mikä on herättänyt kiinnostusta tunnistaa säätelyjärjestelmää salpaavia pienmolekyylejä lääkekehityksen tarpeisiin.

Tässä väitöskirjatyössä hyödynsimme solupohjaista tehoseulontaa tunnistaaksemme uusia tarkastuspistettä salpaavia ja syöpäsolujen kuolemaa edistäviä pienmolekyylejä. 65000 seulotusta yhdisteestä tunnistimme kahdeksan pienmolekyyliä, jotka estivät tarkastuspisteen normaalin toiminnan ja pakottivat solut ennenaikaisesti pois mitoosista. Flavonoideihin kuuluvien fisetiinin ja eupatoriinin sekä synteettisten yhdisteiden SACi2 ja SACi4 ominaisuuksia tarkasteltiin monipuolisilla solupohjaisilla ja biokemiallisilla tutkimusmenetelmillä. Molekyylitason vaikutuskohteiden tunnistamiseksi selvitimme olosuhteet, joissa tarkastuspiste heikentyi pienmolekyylien läsnä ollessa. Eupatoriini, SACi2 ja SACi4 estivät ensisijaisesti kinetokorin fyysisen jännitteen muutoksille herkän tarkastuspisteen osan toiminnan. Fisetiini esti myös osaa, joka aktivoituu mikrotubulusten ja kinetokorien välisten kytkentöjen puuttuessa. Tulostemme mukaan kaikki neljä pienmolekyyliä estivät Aurora-B-kinaasin toimintaa. Aurora-B on solujaossa avainasemassa oleva proteiini, jota tarvitaan virheellisten mikrotubulus-kinetokori-kytkentöjen korjaamisessa, mitoottisen tarkastuspisteen toiminnassa kinetokorin jännitteen puuttuessa sekä tytärsolujen erilleen kuroutumisessa. Eupatoriini, SACi2 ja SACi4 estivät myös Aurora-A-kinaasin aktiivisuutta, jota tarvitaan sentrosomien kypsymisessä ja erkaantumisessa sekä tumasukkulan muodostumisessa. Tunnistetut pienmolekyylit estivät Aurora-kinaasien mitoottisia tehtäviä aiheuttaen tumasukkulavaurioita ja sentrosomien pirstoutumista sekä polyploidisten solujen muodostumista kuroutumisvaiheen virheiden seurauksena. Lisäksi pienmolekyylit alensivat syöpäsolujen elinkykyä.

Tässä väitöskirjatyössä onnistuimme tunnistamaan uusia solujaon säätelyjärjestelmää salpaavia pienmolekyylejä. Lisäksi osoitimme ensimmäistä kertaa, että flavonoidit voivat estää Aurora-kinaasien toimintaa ja aiheuttaa solujakovirheitä. Tuloksemme tukevat nykykäsitystä, jonka mukaan mitoosin säätelyjärjestelmän pettäminen voi vähentää syöpäsolujen elinkykyä, havainto joka voi toimia uusien syöpälääkkeiden kehitystyön pohjana.

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ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Adenomatous polyposis coli
APC/C	Anaphase promoting complex/cyclosome
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3 related
Bub	Budding uninhibited by benzimidazole
BuBR1	Bub1-related protein 1
CaMK	Calsium/calmodulin-dependent protein kinase
CCAN	Constitutive centromere-associated network
Cdc	Cell division cycle
Cdh1	CDC20 homolog 1
Cdk	Cyclin-dependent kinase
Cenp	Centromere protein
Chk	Checkpoint kinase
CIN	Chromosomal instability
CLASP	CLIP-associating protein
CLIP	Cytoplasmic linker protein
CML	Chronic myelogenous leukemia
CPC	Chromosomal passenger complex
CYP	Cytochrome P450
EB1	End-binding 1
EGFR	Endothelial growth factor receptor
ERK	Extracellular regulated kinase
FACS	Fluorescence-activated cell sorting
FGFR1	Fibroblast growth factor receptor 1
FLT3	Fms-related tyrosine kinase 3
FTI	Farnesyl transferase inhibitor
GAP	GTPase-activating protein
GSK-3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
γ-TuRC	Gamma-tubulin ring complex
HDAC	Histone deacetylase
Hec1	Highly enhanced in cancer
HTS	High throughput screen
IC_{50}	Half maximal inhibitory concentration
IF	Immunofluorescence
INCENP	Inner centromere protein

InsR	Insulin receptor
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
Kif	Kinesin family member
KMN	KNL1, Mis12, Ndc80
KSP	Kinesin spindle protein
KT	Kinetochore
Mad	Mitotic arrest deficient
MAP	MT-associated protein
MAPK	Mitogen-activated protein kinase
MCAK	Mitotic centromere-associated kinesin
MCC	Mitotic checkpoint complex
Mklp	Mitotic kinesin like protein
MM	multiple myeloma
Mps1	Monopolar spindle 1-like 1
MT	Microtubule
MTOC	MT organizing center
Ndc80	Non disjunction of chromosomes 80
NEB	Nuclear envelope breakdown
Nek	(never in mitosis gene a)-related kinase
NuMA	Nuclear mitotic apparatus protein
PARP	Poly (ADP-ribose) polymerase
PBD	Polo-box domain
PCM	Pericentriolar material
PDGFR	Platelet derived growth factor receptor
PKA	Protein kinase A
Plk1	Polo-like kinase 1
PP	Protein phosphatase
Rb	Retinoblastoma protein
RNAi	RNA interference
RZZ	Rod, Zw10, Zwilch
SAC	Spindle assembly checkpoint
SACi	Spindle assembly checkpoint inhibitor
siRNA	Small interfering RNA
Spc	Spindle pole body component homolog
STAT	Signal transducer and activator of transcription
Topo	Topoisomerase
Trk-A	Tyrosine kinase receptor A
UV	Ultraviolet
VEGFR2	Vascular endothelial growth factor receptor 2
WB	Western blot

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by Roman numerals. The original communications have been reproduced with the permission of the copyright holders. Unpublished data is also included.

I **Anna-Leena Salmela***, Jeroen Pouwels*, Asta Varis, Anu Kukkonen, Pauliina Toivonen, Pasi Halonen, Merja Perälä, Olli Kallioniemi, Gary J. Gorbsky and Marko J. Kallio. (2009) Dietary flavonoid fisetin induces a forced exit from mitosis by targeting the mitotic spindle checkpoint. *Carcinogenesis* 30(6): 1032–1040.

II **Anna-Leena Salmela***, Jeroen Pouwels*, Anu Kukkonen-Macchi, Sinikka Waris, Pauliina Toivonen, Kimmo Jaakkola, Jenni Mäki-Jouppila, Lila Kallio and Marko J. Kallio. (2012) The flavonoid eupatorin inactivates the mitotic checkpoint leading to polyploidy and apoptosis. *Exp Cell Res* 318(5): 578-592.

III **Anna-Leena Salmela**, Jeroen Pouwels, Jenni Mäki-Jouppila, Pekka Kohonen, Lila Kallio and Marko Kallio. (2012) Novel Pyrimidine-2,4-Diamine derivative suppresses the cell viability and spindle assembly checkpoint activity by targeting Aurora kinases. *Under revisions.*

IV **Anna-Leena Salmela** and Marko Kallio. (2012) Pyrimidine-2,4-Diamine derivative sensitizes cancer cells to replication stress by inhibiting Chk1 and attenuating p21. *Manuscript.*

*Equal contribution

1. INTRODUCTION

Cancer is a term to describe a group of diseases which are characterized by unrestrained proliferation of abnormal cells which can often leave the place of origin to invade and grow in different tissues in the body. Globally, cancer is the most prevalent cause of mortality with 7.6 million deaths in 2008. In Finland, cancer is the second most common cause of mortality and caused nearly 11 000 deaths in 2009. Currently used cancer therapies rely on surgery, radiation therapy and chemotherapy by drugs that target for example MT function. Although effective, the treatments are often accompanied with severe side-effects and development of resistance and therefore, novel drugs with better cancer cell selectivity are needed. One intriguing therapeutic opportunity is based on the existence of multiple checkpoints that cells have evolved to ensure controlled progression through the cell cycle. A hallmark of cancer is uncontrolled growth of a cell population, which results from unrestrained proliferation and decreased cell death via apoptosis. Malfunction of the mitosis controlling checkpoint termed the SAC causes chromosome missegregation and leads to loss or gain of chromosomes (aneuploidy), a known contributing factor in tumorigenesis. On the other hand, complete inhibition of the SAC impedes the viability of even aggressive cancer cells. Therefore, SAC inhibitors are likely to have therapeutic value, a notion which has encouraged HT drug discovery for novel small compounds targeting essential proteins involved in SAC signaling.

First inhibitors of mitotic progression targeted the MTs. This family of therapeutics has been for a long time the only mitosis-targeting group of drugs used in clinics and their history as cancer drugs stems back to 1960s. In recent years, a plethora of LMW inhibitors of other mitotic proteins including but not limited to kinases and kinesins have been identified. These inhibitors perturb normal mitotic progression either by causing an M phase arrest or premature forced exit from mitosis depending on the cellular functions of their target proteins. Currently, the most advanced inhibitors have proceeded into late clinical phases. These experimental compounds include inhibitors of Aurora kinases which target the essential functions of these proteins in various mitotic processes causing mitotic defects and cell death. According to current view, these experimental Aurora kinase inhibitors are one potential source for next-generation anti-cancer drugs.

In this study, a HTS was performed in order to find novel LMW compounds that inhibit SAC function and lead to loss of cancer cell viability. The data describes the cellular phenotypes caused by the four most potent small compounds we identified and provide evidence for their molecular targets and mechanisms of action in cells.

2. REVIEW OF THE LITERATURE

2.1 Cell cycle

The cell is the basic building block of all organisms. Cells constantly die and new cells are rapidly formed in a body to ensure growth during development of an organism. In adult organism, the homeostasis is maintained and only cells in tissues undergoing rapid renewal continue dividing constantly. New cells originate from existing cells which have duplicated their contents and split into two daughter cells. All these co-ordinated events of growth and division form a cell cycle that in eukaryotes consists of four phases: G1, S, G2 and M phase (mitosis). A typical fast-dividing mammalian cell passes through the cell cycle in approximately 24 h, mitosis being the shortest phase. S phase is the time for DNA synthesis. In mitosis, the chromosomes and cytoplasmic components are divided equally between the two forming daughter cells. At G1 and G2 (gap 1 and 2), the cell prepares for DNA synthesis and cell division, respectively. Cell cycle phases involve active gene transcription and protein translation except in mitosis when the protein synthesis is minimised. The cell cycle is driven by different cyclin-dependent kinases (CDKs) and their activators termed cyclins, each CDK-cyclin complex functioning at certain phase of the cell cycle. Cell cycle progression has to be tightly regulated so that the next phase is initiated only after successful completion of the previous phase. This is accomplished by regulation of the cyclin levels and Cdk activities. In addition, various cell cycle checkpoints exist to control transitions from one cell cycle phase to another or as in case of the SAC (discussed in chapter 2.5) work to control progression within a particular cell cycle phase. Uncontrolled cell proliferation is one hallmark of cancer.

Cells at G1, S, and G2 are called interphase cells. Typically, G1 phase duration is 8 h, whereas G2 and S phase are shorter lasting approximately 4 h and 6 h, respectively. M phase cells comprise only a small fraction of a cell population, mitosis lasting approximately 1 h. M phase of somatic cells includes division of the nucleus and division of the cytoplasm, respectively. Mitosis is divided into five steps: prophase, prometaphase, metaphase, anaphase (A and B) and telophase (Fig. 1). At prophase, chromatin starts to condense and becomes tightly packed into chromosomes, and centrosomes separate to opposite sides of the cell. Prophase ends with nuclear envelope breakdown (NEB) after which the construction of the spindle apparatus continues. In prometaphase, cells establish attachments between the chromosomes and MTs of the spindle, and start to align the chromosomes to the cell equator. When the process of chromosome alignment is completed, the cell is at metaphase. Anaphase initiates when cohesion between the sister chromatids is lost and MT-mediated segregation of sister chromatids occurs. At anaphase A, the sister chromatids move apart which is followed by anaphase B where also the parting of the two spindle poles contributes to separation of chromatids. At telophase, chromosomes decondense and membranes reform around the new daughter nuclei. Concomitantly, actin-based ring forms and the cytoplasm becomes divided into two daughter cells completing the cell division.

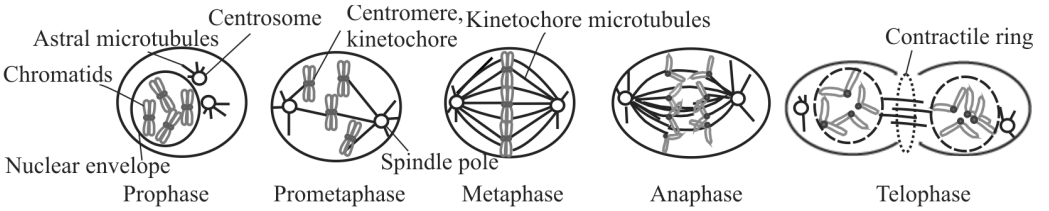

Figure 1. Mitotic phases and central structures in a mitotic cell.

2.2 Aurora B and the CPC

Since this thesis focuses on Aurora B inhibitors, the localization, function and regulation of the kinase are shortly introduced (reviewed by Carmena, Ruchaud, & Earnshaw, 2009; Ruchaud, Carmena, & Earnshaw, 2007b; van der Waal et al., 2012). More details on Aurora B can be found in the chapters below. Aurora B belongs to the Aurora kinase family of serine/threonine kinases with various essential functions throughout mitosis. The kinase is crucial for chromosome condensation, removal of arm cohesion, resolving erratic KT-MT interactions to ensure bi-orientation, SAC function and cytokinesis. The kinase functions in a cell as a part of the chromosomal passenger complex, the CPC, composed of inner centromere protein (INCENP), Survivin, Aurora B and Borealin. While Aurora B is the enzymatic core of the complex, the other complex members are crucial for the activation and localization of the kinase in mitosis. According to its name, the CPC changes its localization during mitosis. The complex localizes at chromosomal arms at prophase and moves to inner centromeres at prometaphase and metaphase. At anaphase, the CPC localizes to the spindle midzone from which it moves to the midbody at telophase. The CPC may be recruited to the chromosomal arms by binding of INCENP to heterochromatin protein-1α (HP-1α). Targeting to the inner centromere requires phosphorylation of Histone H2A and Histone H3 by Bub1 and Haspin, respectively, which creates a receptor for Borealin and Survivin. Also INCENP is needed for localizing the CPC at inner centromeres. At anaphase, Aurora B and INCENP may be responsible for the correct localization of the complex. Activation of Aurora B depends on INCENP: the kinase is a substrate for Aurora B which after phosphorylating INCENP undergoes autophosphorylation and full activation. Survivin may also have a role in activation of Aurora B. Furthermore, regulators of Aurora B may include Borealin, Bub1, Chk1, Haspin, and TD-60 which among other mechanisms may activate the kinase by promoting clustering of the kinase. BubR1 has been implicated in inhibition of Aurora B. PP1 counteracts Aurora B activity by removing the phosphorylations from the substrates. Dynamic localization of Aurora B is essential for the mitotic phase specific targeting of the diverse substrates which mediate the functions of the kinase. The well-established substrates include Histone H3 Ser10 and Cenp-A Ser7 that are generally used as markers of Aurora B activity levels. Aurora B is considered as an oncogene and is overexpressed in variety of human cancers. Targeting Aurora B kinase activity results in chromosome misalignment and increased ploidy which is followed by cancer cell death. Therefore, Aurora B inhibition is a promising therapeutic approach and several experimental drugs are undergoing clinical investigations (Kollareddy et al., 2012).

2.3 Regulation of mitosis

Post-translational protein modifications such as phosphorylation, dephosphorylation, acetylation, farnesylation and ubiquitilation are involved in the regulation of mitosis. At early phases of mitosis, phosphorylation of Cdk1 substrates is essential, while late mitosis is regulated by their dephosphorylation (Sullivan & Morgan, 2007) . Compared to mitotic kinases the roles of M phase phosphatases are only starting to be revealed. Mitosis is driven by Cdk1-cyclin B1 until metaphase after which the ubiquitin ligase activity of anaphase promoting complex/cyclosome (APC/C) is needed for mitotic progression and proteasome-mediated degradation of those mitotic proteins that halt entry into anaphase (Sullivan & Morgan, 2007). The regulation of these two processes is interrelated as Cdk1 activity is important for APC/C activation, which in turn leads to cyclin degradation, and thereby inactivation of Cdk1 (Kraft et al., 2003). Similarly to other cell cycle phases, mitotic steps are considered irreversible: they occur in a well-defined order and progression into the next step is allowed only when the conditions are appropriate and requirements of the previous step are satisfied.

2.3.1 Early mitosis

In early mitosis, interphase chromatin condenses into compact chromosomes, centrosomes separate, nuclear envelope breaks down and spindle starts to form. Sister chromatid cohesion is first removed from chromosome arms and ultimately from centromere region. These steps of mitosis require activity of various kinases implicated in the SAC, such as Bub1, BubR1 and Aurora B as well as other kinases including Cdk1, Plk1 and Aurora kinase A.

Cdk1 is a primary regulator of mitotic entry and progression of mitosis. Cdk1/cyclin B-mediated phosphorylation of numerous proteins is needed for various essential processes in early mitosis such as centrosome separation, chromosome condensation and NEB (Malumbres & Barbacid, 2005). In mitosis, the kinase first forms a complex with cyclin A and later with cyclin B which reflects the order of degradation of these cyclins (Sullivan & Morgan, 2007). Inactivating kinases Wee and Myt1 as well as activating kinases and Cdc25 phosphatases regulate the activity of Cdk1 complex and prevent mitotic entry when DNA is damaged (Ferrari, 2006). In human, three Cdc25 isoforms possibly having redundant function in G2/M transition (Boutros, Lobjois, & Ducommun, 2007). Moreover, mitotic kinases Aurora A (Seki et al., 2008) and Plk1 (Lobjois et al., 2009) can indirectly regulate Cdk1 activity to promote mitotic entry. Plk1 phosphorylates and relocalizes Cdc25B from the cytoplasm to the nucleus which results in activation of the Cdk1 complex (Lobjois et al., 2009). Furthermore, Plk1 activates the transcription factor FoxM1 which leads to high expression of mitotic regulators (Lindqvist et al., 2005). Premitotic Aurora A activity is required for centrosomal localization of Cdk1 complex (Hirota et al., 2003), phosphorylation of Cdc25B (Dutertre et al., 2004) and activation of Plk1 (Macurek et al., 2008; Seki et al., 2008).

At prophase, condensation of interphase chromatin into tightly packed chromosomes is controlled by the condensin complex (Schmiesing et al., 2000). Activation and loading of the complex on the chromatin may involve activity of Aurora B and Cdk1 (St-

Pierre et al., 2009; Takemoto et al., 2004). Histone H3 and a linker histone H1 undergo phosphorylation which may also regulate condensation in a poorly characterized manner (Freedman & Heald, 2010; Happel & Doenecke, 2009; Vader & Lens, 2008). Eg5 motor protein has MT crosslinking and sliding activity which generates one of the driving forces to push the centrosomes apart before NEB (Blangy et al., 1995). It is evident that the complex process involves contribution of multiple proteins (Tanenbaum & Medema, 2010) including Plk1, Cdk1, Nek2 and Aurora A which have been implicated in localization and activation Eg5 but have also other mechanisms to regulate centrosome separation (Barr & Gergely, 2007; E. Smith et al., 2011). Cdk1 activity towards lamins and other substrates in nuclear pore complex and nuclear membrane is essential for NEB at the transition from prophase to prometahase.

Sister chromatids of duplicated chromosomes are held tightly together by a multi-subunit cohesin complex (J. M. Peters, Tedeschi, & Schmitz, 2008). The removal of cohesion is sequential: the cohesin subunit, SA2, and the cohesion complex dissociates from arms at prophase and is preserved at centromeres until anaphase (Waizenegger et al., 2000). Plk1 and Aurora B are important for removal of arm cohesion. Plk1 has been implicated in phosphorylation and dissociation of cohesin (Hauf et al., 2005). Protection of centromeric cohesion requires Sgo1 (Salic, Waters, & Mitchison, 2004) whose centromeric recruitment is promoted by Bub1-mediated phosphorylation of H2A (Kawashima et al., 2010). Aurora B phosphorylates Sgo1, which appears to ensure the correct localization of Sgo1 at centromeres and thus, enables a loss of arm cohesion (Dai, Sullivan, & Higgins, 2006). The mechanisms that protect centromeric cohesion from prophase dissociation are not completely understood. According to current knowledge, Sgo1 interacts with a phosphatase PP2A, whose activity prevents cohesin phosphorylation (Kitajima et al., 2006; Z. Tang et al., 2006; Xu et al., 2009). Centromeric localization of PP2A requires Bub1, another Shugoshin protein Sgo2 (Kitajima et al., 2006), and Aurora B which is in turn, essential for phosphorylation and localization of both Sgo2 and PP2A (Tanno et al., 2010). Finally, Haspin (Dai et al., 2006) has been identified as a positive regulator of centromeric cohesion.

2.3.2 Late mitosis

Ubiquitilation activity of APC/C requires co-activators, Cdc20 and Cdh1, bound to the APC/C at anaphase and late anaphase, telophase and G1, respectively (Fang, Yu, & Kirschner, 1999; Morgan, 1999). The co-activators are thought to determine also substrate-specificity (Pesin & Orr-Weaver, 2008), although binding of both APC/C and co-activators to substrates may be needed (Eytan et al., 2006). APC/C is kept inactive until the metaphase-anaphase transition by the SAC which prevents Cdc20 from activating APC/C (Musacchio & Salmon, 2007), discussed in more details in 2.5.1). Protease activity of separase, an enzyme that cleaves the centromeric cohesin, is inhibited by a protein called securin. This inhibitor is intact before activation of APC/C. Moreover, Cdk1 is involved in preventing cleavage of the centromeric cohesion: the kinase phosphorylates securin which prevents ubiquitilation and degradation of the protein (Holt, Krutchinsky, & Morgan, 2008) and may also inhibit separase directly (Gorr, Boos, & Stemmann,

2005; Stemmann et al., 2001). PP1 and PP2A are thought to be responsible for dephosphorylation of separase and other Cdk1 substrates at late metaphase or anaphase and telophase, respectively (Sullivan &Morgan, 2007; Wurzenberger & Gerlich, 2011). When chromosomes have established correct attachments to MTs at metaphase, the SAC becomes satisfied and APC/C activation results in securin degradation, separase activation, cohesin cleavage and exit from M phase. Since APC/C is responsible for degradation of both securin and cyclin B1, activation of the complex couples anaphase onset to mitotic exit.

Separation of sister chromatids at anaphase is followed by telophase and cytokinesis during which the cytoplasm is divided by an actomyosin-based contractile ring. The structure forms at the position of the spindle equator and ingresses to form a cleavage furrow (Eggert, Mitchison, & Field, 2006). Plk1 and Aurora B, as well as a small GTPase RhoA, are essential for cytokinesis, though the process involves various other as important proteins (Eggert et al., 2006). Activated RhoA drives actin polymerization and contractile ring formation and ingression (Carmena, 2008). RhoA activation and localization is regulated by a centralspindlin complex of MKLP1 and Rho GTPase activating protein (RhoGAP) HsCYK-4, as well as Ect2, which is a guanine exchange factor (RhoGEF) of RhoA. Among its multiple targets within cytokinesis (Carmena & Earnshaw, 2003), Aurora B phosphorylates both centralspindlin proteins, which is required for final stages of cytokinesis (Carmena, 2008). Plk1 phosphorylates HsCYK-4 which may explain how the kinase controls localization and activation of Ect2 and RhoA (Brennan et al., 2007; Burkard et al., 2007; Burkard et al., 2009; Wolfe et al., 2009).

2.4 Mitotic structures

2.4.1 Centrosome

Centrosomes in animal cells function as major MT-organizing centers (MTOC) and become spindle poles of the bipolar spindle in mitosis (reviewed by (Barr & Gergely, 2007; Fukasawa, 2007; Nigg & Raff, 2009; Schatten, 2008). In interphase cells, the centrosomes organize interphase MTs which regulates cell motility, shape and polarity. The centrosome is comprised of a centriole pair surrounded by pericentriolar material (PCM). The centrosome undergoes a centrosome cycle consisting of duplication, maturation, separation and disengagement steps (Barr & Gergely, 2007). After cell division, each daughter cell at G1 has one pair of centrioles. Before duplication, centrioles undergo disengagement. At duplication, new centrioles form perpendicular to the old ones in a process which is tightly co-ordinated with DNA replication to ensure duplication only once per cell cycle. At G2, PCM components accumulate at maturing centrosomes. An important constituent of PCM is a gamma-tubulin ring complex (γ-TuRC) which is crucial for MT nucleation. Plk1 recruits pericentrin among other factors needed for γ-tubulin localization to centrosomes (Haren, Stearns, & Luders, 2009). At early mitosis, centrosomes separate and move to opposite sides of the cell. When nuclear envelope has dissolved, MTs start to nucleate at centrosomes and form a bipolar spindle.

A correct number and function of centrosomes is essential for ensuring that the cell forms a bipolar spindle which is crucial for mitotic fidelity. Numerical and functional changes in centrosomes are brought about by altered function of proteins regulating duplication, maturation and separation of the centrosomes, such Cdks, Plk1, Aurora A, Nek2A and Eg5. Many of these regulatory proteins are oncogenes or tumor-suppressors and consequently, centrosomal abnormality is a hallmark of cancer cells (Chan, 2011). Cdk2 is a central regulator of centrosome duplication (Matsumoto, Hayashi, & Nishida, 1999; Meraldi et al., 1999) and also a key driver of S-phase entry (Bettencourt-Dias & Glover, 2007). Therefore, Cdk2-cyclin E co-ordinates centrosome duplication with DNA synthesis and prevents hyperamplification of centrosomes (Fukasawa, 2007; Fukasawa, 2008). Activity of Cdk2 is under the control of p53-p21 pathway. When a cell is exposed to different stress conditions, p53 upregulates the levels of the Cdk2 inhibitor, p21, and entry into S phase and centrosome duplication are prevented (Fukasawa, 2007; Fukasawa, 2008). Consistently, centrosome amplification and multipolarity is observed in cells lacking functional p53 although centrosome dysregulation may require additional mutations, such as cyclin E overexpression, in human cells (Kawamura et al., 2004). Furthermore, p53 may control centrosome duplication in a transcription-independent manner, possibly by regulating as yet unknown centrosomal proteins (Tarapore &Fukasawa, 2002).

Centrosome amplification may be detrimental to the accuracy of cell division but whether it is a cause or consequence of tumorigenesis is not thoroughly understood (Acilan &Saunders, 2008). It has been reported that multipolarity caused by centrosome amplification results in multipolar anaphase and CIN (Godinho, Kwon, & Pellman, 2009). Although multipolarity and extra centrosomes are common features of cancer cells that are chromosomally instable, multipolar divisions are rare and seem to reduce cellular viability due to massive chromosome missegregation (Ganem, Godinho, & Pellman, 2009). Interestingly, it has been demonstrated that cancer cells with more than two centrosomes mostly undergo centrosome clustering which enables bipolar segregation of chromosomes and viable progeny (Ganem et al., 2009; Silkworth et al., 2009). However, merotely and lagging chromosomes are frequently seen in these cells. According to the proposed model, transient multipolar structures existing before resolution into bipolar spindles predispose to a high number of merotelic attachments. and some of the errors persist resulting in lagging chromosomes at anaphase (Ganem et al., 2009). Collectively, the studies demonstrated how extra centrosomes and multipolarity may cause CIN observed in cancer cells by promoting merotely and segregation errors.

2.4.2 Mitotic spindle

Mitotic spindle consists of MTs, centrosomes and chromosomes, and starts to assemble around the time of NEB. Bipolar spindle has characteristic shape in which two overlapping MT arrays emanate from two centrosomes and a fraction of MTs is bound to chromosomes (Kline-Smith & Walczak, 2004). Astral MTs regulate positioning of the spindle while interpolar MTs stabilize the structure and create pushing force that separates the spindle poles at anaphase (Honore, Pasquier, & Braguer, 2005). MTs are filamentous polymers consisting of α/β-tubulin heterodimers assembled into protofilaments which are laterally

associated into a lattice (Cheeseman & Desai, 2008). A MT consists of 13 protofilaments (Jordan & Wilson, 2004). One end has β-tubulin and the other α-tubulin exposed which results in polarity (Jordan and Wilson 2004) utilized by motor proteins that transport cargo along the MTs (Huszar et al., 2009; Sarli & Giannis, 2006). GTP is bound to both α-tubulin and β-tubulin but only GTP at the β-tubulin is hydrolyzed after addition of the heterodimer to the polymer. The relative rate of GTP hydrolysis and tubulin addition to the polymer is crucial for determining the growth and shrinkage of the polymer. When the rate of tubulin addition is high, MTs in GTP-bound form continue growing, whereas high rate of hydrolysis favours shrinkage of less stable, GDP-bound MTs (Stanton et al., 2011). Therefore, GTP hydrolysis enables dynamic function of MTs.

MTs are mainly nucleated at centrosomes (Khodjakov et al., 2000). However, it has been recognized for about ten years that also centrosome-independent, chromosome-driven nucleation mechanisms exist (Khodjakov et al., 2000) and MT-dependent nucleation was recently reported (H. Zhu, Fang, & Fang, 2009). Although the details of mitotic spindle formation are not understood, it is known that dynamic nature of MTs is necessary for reorganization of interface MTs into mitotic spindle (Desai & Mitchison, 1997). Defined as dynamic instability, MT plus ends can rapidly and stochastically change from slow growth phase to rapid disassembly (catastrophe) and vice versa (rescue) (T. Mitchison & Kirschner, 1984). MTs attach to chromosomes via macromolecular protein complex called a KT, which assembles on chromosomes at early mitosis (discussed in chapter 2.4.3). Chromosome attachment has been described in a classical search-and-capture model (Kirschner & Mitchison, 1986) in which centrosome-nucleated MTs randomly probe the cytoplasm and when bound to KTs are stabilized and bundle to form K-fibers. However, this mechanism alone would be inefficient and the model has also other limitations (O'Connell & Khodjakov, 2007). According to current view, MT nucleation in the vicinity of chromosomes increases the probability of capture and enables the formation of K-fibers also at KTs (Maiato et al., 2004) complementing the classical model. Collaboration of different proteins at MT plus ends and KTs is required for maintaining the architecture of MT-KT interface and generating robust attachments that can be maintained during MT polymerization and depolymerization.

A controlled function of a mitotic spindle is essential for alignment of chromosomes at the cell equator and segregation of sister chromatids at anaphase. Chromosome separation has been described in two models. Flux model involves a constant addition of tubulin dimers at plus end and removal at minus end which creates poleward flow of MT polymer (T. J. Mitchison, 1989). Alternative model states that depolymerisation of MT plus ends embedded at KTs, (Pac-Man activity) generates a driving force for the poleward movement along stationary MTs (Gorbsky, Sammak, & Borisy, 1987; Inoue & Salmon, 1995; T. J. Mitchison & Salmon, 1992)**.** It is thought that Pac-Man is the primary mechanism for chromosome segregation in somatic cells (Khodjakov &Kapoor, 2005; Kline-Smith & Walczak, 2004). Whether flux has a role in anaphase movement is under debate (Ganem & Compton, 2006). Spindle assembly and directed movement of chromosomes require MT-associated proteins (MAPs), which regulate spindle dynamics and are either stabilizers of MTs, such as CLASP1 and EB1 or destabilizers, such as

MCAK (Kline-Smith & Walczak, 2004). In addition, motor proteins at KTs are directly and indirectly involved in chromosome movement and segregation: the proteins glide along the MTs, provide attachment between dynamic MT ends and KTs and regulate spindle dynamics (Brunet & Vernos, 2001).

2.4.3 Centromere-KT Complex

KT (reviewed by Cheeseman & Desai, 2008; Musacchio & Salmon, 2007) is a proteinous assemblage built on the centromeric region and forms the linkage of a chromosome to MTs in early phases of mitosis. In eukaryotes, the main composition and structure of KTs are conserved. To date, more than 100 proteins have been identified in centromere-KT complex in human (Fukagawa, 2004a). The KT starts to assemble at G2 from the centromeric DNA outwards and is matured by early prometaphase. The macromolecular, layered structure consists of an inner centromere, inner and outer KT layer and fibrous corona and each layer has its characteristic protein constituents and consequently, distinct functions (Fig. 2) (Maiato et al., 2004).

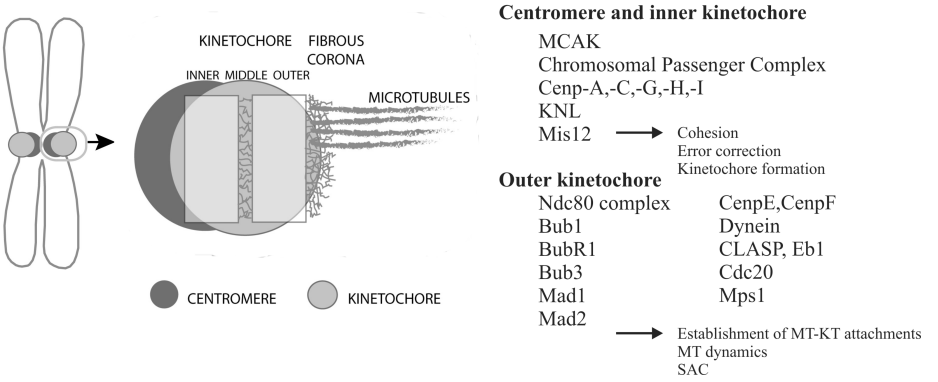

Figure 2. Animal KT organization, some of the constituents and main functions of each layer (partially adapted from Maiato et al 2004).

Centromere (reviewed by Amor et al., 2004; Cleveland, Mao, & Sullivan, 2003; Fukagawa, 2004b; Torras-Llort, Moreno-Moreno, & Azorin, 2009) has a unique structure in which histone H3 and histone variant Cenp-A irregularly alternate in nucleosomes. Pericentromeric heterochromatin on both sides of centromere is important for cohesion. Centromeric DNA is comprised of highly repetitive sequences that form regular arrays (α-satellite I regions) and more diverse arrays (α-satellite II regions). Cenp-A containing nucleosomes are associated with α-satellite I regions and form the outer centromeric region, whereas α-satellite II regions are found in the inner centromere. In humans, the sites for centromere formation are not defined by primary nucleotide sequence. Rather, establishment and maintenance of centromeres are epigenetically determined by a special chromatin with Cenp-A containing nucleosomes (Torras-Llort et al., 2009). Mechanisms of Cenp-A loading are not well characterized but experimental data supports loading in mitosis and at early G1 and requirement for several recruiting factors and modifiers of the chromatin (Torras-Llort et al., 2009).

The formation of KT structure is ordered and hierarchical. Thus, loading of each protein depends on the certain protein(s) recruited at earlier steps (Johnson et al., 2004; Vigneron et al., 2004). Most likely, the assembly is not a linear pathway but rather, the association of multiple proteins into a mature KT involves a network of interactions. The complex structure is composed of constitutive centromeric proteins, Cenp-A and CCAN (constitutive centromere-associated network) of Cenp-C and 13 interacting proteins (Cheeseman & Desai, 2008), as well as proteins that reside at KTs at G2 and in mitosis. In addition to Cenp-A, CCAN and KMN composed of KNL1, the Mis12 complex and the Ndc80 complex ($Ndc80^{Hec-1}$, Nuf2, Spc24, Spc25) are important for the KT assembly (Cheeseman & Desai, 2008). Each mitotic phase has a unique KT composition indicating that KTs are highly dynamic although the mechanisms are not well defined. For example, many of the SAC proteins respond to changes in MT-KT attachment or tension. Furthermore, anaphase onset and anaphase-telophase transition modulate the composition of the KT outer layer (Cheeseman & Desai, 2008).

KTs have three main functions. First, KTs mediate an establishment of attachments between MTs and chromosomes. Second, KTs enable chromosome movements by integrating MT dynamics and motor protein function. Third, they are the sites where SAC signals are generated. Initial contacts between MTs and chromosomes form after NEB at early prometaphase. According to the traditional model for chromosome alignment, chromosome congression is a consequence of biorientation (Rieder & Salmon, 1994). One of the two sister KTs initially forms lateral attachments to MTs. The chromosome moves rapidly via dynein-mediated mechanism to the pole where more MTs form end-on attachments to the KTs and a MT bundle, K-fiber, is formed. The mono-oriented chromosome stays at the pole until the MTs emanating from opposite spindle pole attach to the unattached sister KTs. The bioriented chromosome then congresses to the spindle equator and the attachments are completed. The model was challenged by Kapoor and colleagues whose data indicated that biorientation is not required for congression (Kapoor et al., 2006). They showed that a motor protein, Cenp-E, helps mono-oriented chromosomes to slide on the K-fiber of an already bioriented chromosome to the spindle equator. Furthermore, this was proposed to be the main mechanism of biorientation. Aurora A is thought to promote the process by phosphorylating Cenp-E at poles. According to the model (Kim et al., 2010), phosphorylation causes dissociation of PP1 phosphatase and modifies motor properties of Cenp-E to enable congression. Because of gradient of Aurora A activity, Cenp-E is dephosphorylated and PP1 is again bound at metaphase. PP1 dephosphorylates the substrates of Aurora B, Ndc80 and KNL-1 which results in stable attachments (Kim et al., 2010).

Chromosome alignment and accurate segregation require that correct attachments are stable but allow MT dynamics at MT plus ends. KMN complex has a crucial role in MT-KT interface. The complex possesses two low-affinity MT-binding domains: one in the globular domain of Ndc80 subunit of the Ndc80 complex and one in KNL-1 which appear to act co-operatively (Cheeseman et al., 2006). Furthermore, the third member of the KMN complex, Mis12 complex, increases the affinity although does

not directly bind to MTs (Cheeseman et al., 2006). Aurora B can phosphorylate all the KMN members which decreases the binding affinity to MTs (Welburn et al., 2010). This combinatorial phosphorylation provides an elegant regulation mechanism of MT-KT interactions compared to on-off switch-like regulation. When the tension is high and therefore, the distance of Aurora B from its substrates is high, KNL-1 recruits PP1 phosphatase to kinetochores which counteracts the Aurora B activity stabilizing the attachments (Liu et al 2010). When the tension is low, Aurora B inhibits the recruitment of PP1 indicating a positive feedback mechanism (D. Liu et al., 2010). In addition to the KMN core, the MT-KT interface involves various additional players. There is evidence that recently identified Ska1 complex is important in enabling dynamic MT-KT attachments (Gaitanos et al., 2009; Welburn et al., 2009). Recent data indicates that the complex is recruited by KMN complex and the process is inhibited by Aurora B (Chan et al 2012). Furthermore, other proteins such as motor proteins Cenp-E (Putkey et al., 2002) and dynein (Varma et al., 2008) as well as Cenp-F (Feng, Huang, & Yen, 2006) and proteins of Bub family (Lampson & Kapoor, 2005; Logarinho & Bousbaa, 2008; Meraldi & Sorger, 2005) modulate the establishment or stability of MT-KT interaction. Finally, various MT plus end tracking proteins that localize at both structures such as APC, EB1, CLASP1 and CLIP170 are important for the attachments (Akhmanova & Hoogenraad, 2005).

To prevent errors in chromosome segregation, each KT has to establish a bipolar, also termed as amphitelic, attachment. Erratic connections of both sister KTs to MTs from one pole (syntelic), or one sister to both poles (merotelic) are common at early prometaphase (Fig. 3) but are usually corrected before cells exit from mitosis (Cimini, 2008; Gregan et al., 2011).

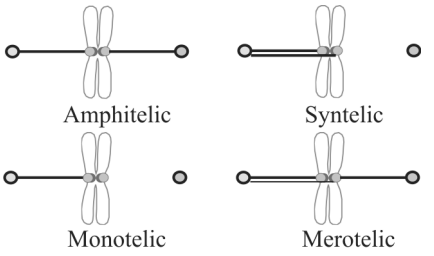

Figure 3. Types of MT-KT attachments. The figure shows a normal, amphitelic attachment and three erranous attachments.

It is evident that cells need efficient error correction mechanisms to prevent chromosome missegregation. The process involves three functions: detection of an error, detachment of faulty attachments and generation of correct ones. According to current understanding, a mitotic kinase, Aurora B, has a critical role in error correction and accordingly, chemical inhibition of Aurora B activity stabilizes both syntelic and merotelic attachments (Cimini et al., 2006; Hauf et al., 2003). Centromeric localization of Aurora B requires Bub1 and Haspin that phosphorylate histone H2A and histone H3, respectively (T. U. Tanaka, 2010). Mps1 kinase activity is also essential for error correction process (Jelluma, Brenkman, van den Broek et al., 2008) but its position

relative to Aurora B in the pathway is debatable (T. U. Tanaka, 2010). Phosphorylation of Aurora B substrates at outer KT modulates their binding to MTs. Ndc80 complex is required for MT attachment (DeLuca et al., 2005) and has lower binding affinity when phosphorylated by Aurora B (Cheeseman et al., 2006; DeLuca et al., 2006). The detailed mechanism of how incorrectly bound MTs are selectively destabilized is not completely defined and different models have been proposed (Kelly & Funabiki, 2009). When the attachments are correct, tension is generated stretching the centromere. The level of tension appears to determine the physical distance between Aurora B and its substrates. A model (T. U. Tanaka, 2010) states that the absence of tension brings Aurora B close to its substrates and their phosphorylation results in detachment of faulty attachments. The kinase activity appears to be constant independently of the type of attachment which supports the distance-based phosphorylation model (D. Liu et al., 2009). Upon proper binding and tension, Aurora B cannot reach its substrates and attachments are stabilized. PP1 phosphatase is implicated in dephosphorylation of Aurora B substrates and thereby stabilization of correct attachments (D. Liu et al., 2010). PP1 was recently shown to be recruited by KNL1 and Cenp-E (Kim et al., 2010; D. Liu et al., 2010). Interestingly, in both proteins the binding site overlapped Aurora B phosphorylation site and phosphorylation prevented PP1 binding indicating a switch-like regulation.

Incorrectly attached MTs are removed by a MT-depolymerizing kinesin, MCAK, which localizes at inner centromeres in Aurora B-dependent manner (Lan et al., 2004) and whose depolymerizing activity is negatively regulated by Aurora B (Andrews et al., 2004; Lan et al., 2004). PP1 phosphatase which is concentrated at KTs and pericentromeric chromatin may dephosphorylate and activate centromeric MCAK when the proteins become close to each other in the presence of syntelic and merotelic attachments (Gorbsky, 2004). KTs with syntelic attachments are not under tension and activate the SAC and Aurora B-mediated error correction. Merotelic attachments are not detected by the SAC (Cimini et al., 2001; Cimini et al., 2002) but tension is suggested to be reduced to reach the threshold of Aurora B activation and correction of the error (Cimini, 2007). Recently, it was shown that Aurora B phosphorylates not only Ndc80 complex but also two other members of the KMN complex in a combinatorial manner. These phosphorylations are regulated by attachment and tension status and may enable fine-tuned rather than on-off regulation of MT-KT binding affinity in error correction (Welburn et al., 2010).

2.5 Spindle assembly checkpoint (SAC)

2.5.1 Basic components and regulation of the SAC

The SAC is an evolutionary conserved safety mechanism that ensures faithful chromosome segregation in mitosis (reviewed by Musacchio &Salmon, 2007; Suijkerbuijk & Kops, 2008; Zich & Hardwick, 2010). The SAC function (Fig. 4) delays the onset of anaphase until all KTs of the chromosomes have established amphitelic attachments to the MTs. The target of inhibitory function of the SAC proteins is Cdc20, a co-activator of APC/C, whose activity initiates the onset of anaphase (discussed in 2.3.2). Only after formation of correct

attachments, the SAC becomes satisfied, the inhibitory complex dissociates and APC/C gets activated. Ubiquitylation of various APC/C substrates such as securin and cyclin B results in their degradation which causes separase activation and dramatic decrease in Cdk1 activity. Separase then cleaves cohesin enabling segregation of sister chromatids. The SAC prevents precocious exit from mitosis under conditions which would likely cause errors in chromosome segregation. In mammalian cells, complete inactivation of the SAC is lethal, while partial inactivation may result in unequal chromosome distribution and aneuploidy due to a premature anaphase (Kops, Foltz, & Cleveland, 2004).

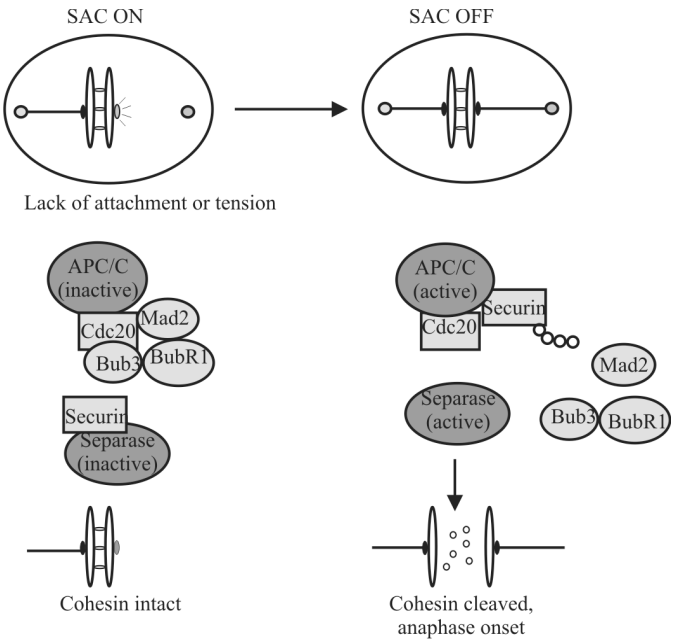

Figure 4. Basic principle of the SAC function (partially adapted from Bharadwaj and Yu 2012, Fukasawa 2007).

The SAC components were originally identified in yeast in the screens searching for yeast mutants incabable of retaining viability upon spindle poison exposure (Hoyt, Totis, & Roberts, 1991; R. Li & Murray, 1991). The protein products were named the mitotic arrest defective (Mad) and budding uninhibited by benzimidazoles (Bub) proteins and their homologues have been subsequently found in various organisms. In human, the core proteins within the SAC are Mad1, Mad2, Bub1, BubR1 and Bub3 which accumulate at KTs before formation of correct, bipolar attachments. BubR1, Bub3 and Mad2 bind to Cdc20 and form a mitotic checkpoint complex, MCC, (Sudakin, Chan, & Yen, 2001) which can function as an inhibitory complex preventing premature anaphase. MCC was shown to bind to APC/C at the site partially overlapping the binding site of Cdc20 (Herzog et al., 2009). The inhibitory complex may prevent substrate binding and perhaps also cause changes in APC/C conformation (Herzog et al., 2009). Another model originally derived from budding yeast studies suggests that Mad3 (yeast BubR1) may decrease substrate recruitment to APC/C^{Cdc20} by acting as a pseudo-substrate (Burton & Solomon, 2007). BubR1 posesses two KEN boxes, the motifs which are

recognized by APC/C^{Cdc20} to target its substrates. The first KEN box seems to be needed for MCC formation (Burton & Solomon, 2007), while the second appears essential for prevention of substrate recruitment to APC/C^{Cdc20} (Lara-Gonzalez et al., 2011). The very recently determined structure of fission yeast MCC (Chao et al., 2012) showed that Mad3 KEN box is positioned towards the KEN box receptor of the Cdc20 providing a strong support for the model of BubR1 functioning as a pseudo-substrate inhibitor of APC/C^{Cdc20}. Additional level of inhibition is brought about by phosphorylation of Cdc20 (Chung & Chen, 2003). Cdk1 activity may favour binding of Cdc20 to Mad2 and inhibit binding to APC/C (D'Angiolella et al., 2003), whereas Bub1-mediated phosphorylation of Cdc20 may strengthen the SAC without enhancing the binding of Mad2 and BubR1 to Cdc20 (Z. Tang et al., 2004). In addition to the MCC, a factor of unknown identity called mitotic checkpoint factor 2 (MCF2) has been suggested to participate in APC/C inhibition (Eytan et al., 2008).

SAC signals are generated at KTs. However, how and when the proteins interact to form MCC and whether the complex is also assembled in a KT-independent manner is not understood in details. Recruitment of proteins at KTs is thought to increase concentration and autophosphorylation of the SAC proteins and enhance binding of co-factors resulting in kinase activation (Kang & Yu, 2009). A model of the mitotic timer states that Mad2 and BubR1 might work as a cytosolic complex and control the duration of mitosis before efficient checkpoint signal is generated (Meraldi, Draviam, & Sorger, 2004). It has been proposed that MCC may be present in interphase but is functional only in mitosis because APC/C is recruited to KTs and sensitized to inhibition (Acquaviva et al., 2004). In addition to MCC, subcomplexes of Mad2-Cdc20 and BubR1-Bub3 may exist (Fang, 2002; Nilsson et al., 2008; Z. Tang et al., 2001). Based on *in vitro* studies, MCC formation enhances inhibition (Sudakin et al., 2001) and both Mad2 and BubR1 are needed for the functional SAC in cells (Musacchio & Salmon, 2007).

Laser ablation studies indicated that a single unattached KT is able to activate the SAC (Rieder et al., 1995). Extreme sensitivity is believed to be possible because the signal is diffusible and is amplified in the cytoplasm. The model called a template model states that Mad2-Cdc20 facilitates formation of the MCC and amplifies the signal (DeAntoni, Sala, & Musacchio, 2005). Mad2 exists in open (O-Mad2) and closed (C-Mad2) conformations (DeAntoni et al., 2005). This forms a basis of the model in which KT-bound C-Mad2-Mad1 facilitates the change from O-Mad2 into C-Mad2, the structure needed to bind Cdc20. The complex of Cdc20 and C-Mad2 is then released and acts as a template for generation of C-Mad2 in cytosol. In this way the model proposes a receptor for Mad2 at KTs and a mechanism for signal amplification in cytosol. Mps1 contributes to the SAC activity by recruiting Mad and Bub proteins to unattached KTs (Lan & Cleveland, 2010) and by promoting Mad2 activation (Hewitt et al., 2010; Maciejowski et al., 2010). Furthermore, Mps1 inhibits the dissociation of the inhibitory complex (Maciejowski et al., 2010). Interaction of Cenp-E kinesin and BubR1 at the KTs is thought to enable amplification of weak signals from a few unattached KTs when Cenp-E stimulates BubR1 activation in the absence of MT-attachments (Mao, Desai, & Cleveland, 2005; Weaver et al., 2003). A recent model (Burke & Stukenberg, 2008)

proposes that specific proteins would bind either MTs or SAC proteins at KTs and would serve as a platform for concentrating SAC proteins. The model highlights the importance of KMN complex and RZZ complex in binding the SAC proteins in the absence of MT attachments. KMN may also promote the activation of Bub1 and Cdk1 that modulate the SAC response (Kang & Yu, 2009).

SAC signal is thought to be generated as a response to lack of attachment (Rieder et al., 1994) and lack of inter-KT tension (X. Li & Nicklas, 1995), a tension generated when chromosomes biorientate stretching the centrosomes. Mad1 and Mad2 accumulate to unattached KTs, while Bub1, BubR1, Aurora B and Shugoshin localize at KTs in a tension-dependent manner (Indjeian, Stern, & Murray, 2005; Musacchio & Salmon, 2007; T. U. Tanaka, 2008; J. Zhou, Yao, & Joshi, 2002). Aurora B is essential for correction of faulty attachments which do not generate tension. However, direct contribution of tension and Aurora B in SAC signaling is difficult to evaluate because unattached KTs lack tension and tension stabilizes attachments (Pinsky & Biggins, 2005). Importantly, correction of erratic attachments generates unattached KTs which activate the SAC. It is debatable whether the attachment and tension are separately monitored and can independently result in generation of the SAC signal (Nezi & Musacchio, 2009; Pinsky & Biggins, 2005). It is possible that loss of tension activates error correction which then turns on the SAC by detaching erroneous attachments. According to the model, Aurora B does not have a direct role in the SAC. Alternatively, Aurora B may be directly involved in SAC signaling (Vader et al., 2007). When the coiled-coil domain of the INCENP was perturbed in the CPC, the SAC response in taxol-arrested cells was abolished without changes in Aurora B activity or chromosome alignment indicating importance of INCENP in the SAC signaling. It was proposed that INCENP could either facilitate Aurora B in the SAC activation, possibly by improving the accessibility of an unknown substrate, or could even act independently. Finally, Aurora B kinase also contributes to the SAC activity by recruiting SAC proteins BubR1, Mad2, and Cenp-E to the KTs (Ditchfield et al., 2003).

As discussed above, inter-KT tension may be required for satisfying the SAC and onset of anaphase. Recently, two studies (Maresca & Salmon, 2010; Uchida et al., 2009) demonstrated that amphitelic attachments induce changes not only at centromeres but also at KTs. Importantly, the lack of intra-KT stretching was reflected by the phosphorylation of 3F3/2 epitope, an indicator of absence of tension (Gorbsky & Ricketts, 1993; Nicklas, Ward, & Gorbsky, 1995). Furthermore, the data pointed to the possibility that the rearrangements in KT structure may satisfy the SAC independently of the centromeric tension and suggested that intra-KT stretching may in fact be the primary tension readout and required for silencing the SAC. Several mechanisms may promote silencing of the response. Dynein removes the SAC proteins from the KTs and transports them to poles (Howell et al., 2001). Cenp-E which facilitates the formation of stable attachments (Putkey et al., 2002) inactivates BubR1 upon MT binding to KTs (Mao et al., 2005). Furthermore, p31comet is thought to prevent activation of Mad2 from open to closed conformation thereby inhibiting MCC formation (Mapelli et al., 2006). Recently, p31comet was suggested to contribute to SAC silencing by stimulating a disassembly of MCC (Teichner et al., 2011). Ubiquitilation of Cdc20 may promote dissociation of the

inhibitory complex and silencing of the SAC (Reddy et al., 2007). On the other hand, there is evidence that the modification may be involved in SAC maintenance (Nilsson et al., 2008) and therefore, it seems that consensus is lacking. Finally, physical separation of Aurora B from its substrates (Andrews et al., 2004; D. Liu et al., 2009; T. U. Tanaka, 2008) and counteracting function of PP1 phosphatase on the substrates (D. Liu et al., 2010) that mediate MT binding may stabilize the attachments and enable SAC silencing.

2.5.2 Errors of the SAC and aneuploidy in human cancer

Abnormal chromosome number resulting from gain or loss of chromosomes (aneuploidy) is a hallmark of human cancers (Jallepalli & Lengauer, 2001). Most cancer cells undergo continuous change in chromosome structure and number, termed chromosomal instability (CIN). Importantly, aneuploidy has been suggested to be a potential contributor of tumorigenesis rather than a consequence of malignant cell transformation. First described by Theodor Boveri for more than 100 years ago, the hypothesis stated that incorrect combination of chromosomes promotes tumorigenesis arising from a single progenitor cell (Hardy & Zacharias, 2005). Yet, whether aneuploidy is a consequence or cause of cancer remains debatable (Weaver & Cleveland, 2006). There is evidence that rather than initiating tumorigenesis, aneuploidy may promote tumor formation (Kops, Weaver, & Cleveland, 2005; Weaver & Cleveland, 2006).

Aneuploidy is generated due to unequal chromosome segregation in mitosis. Chromosome missegregation may result from diverse mitotic defects, such as multipolarity, abnormal centrosome number, cohesion defects and failure of the SAC function which allows the precocious anaphase (Chi & Jeang, 2007; Decordier, Cundari, & Kirsch-Volders, 2008; King, 2008). Mutations of cohesin subunit SA2 have been found in human cancers and have been implicated in aneuploidy and CIN as a result of sister chromatid cohesion (Solomon et al., 2011). Observations that a failure of the SAC generates segregation errors led to the hypothesis of SAC inefficiency as an underlying event behind CIN and a role of SAC in tumorigenesis. Indeed, SAC errors have been observed in certain cancers. Increased and decreased expression of checkpoint proteins is found in SAC-defective tumors overexpression being a more common alteration (Holland & Cleveland, 2009). The changes may result from genetic aberrations such as DNA amplification, deletion and point mutations or epigenetic changes, such as promoter methylation (Perez de Castro, de Carcer, & Malumbres, 2007). In addition, mutations in various tumor suppressors and oncogenes may alter the levels of key SAC proteins at the transcriptional and post-transcriptional level in cancer cells (Kops et al., 2005). Germline mutations in BubR1 have been identified as a cause of a rare cancer-susceptible disorder, mosaic variegated aneuploidy (Hanks et al., 2004). However, mutations in SAC genes are rare and thus, are not the main mechanism of how the SAC becomes weakened (Kops et al., 2005; Weaver & Cleveland, 2005). In addition to clinical samples and cancer cell lines, transgenic mice with SAC protein overexpression or disruption of endogenous SAC gene have been utilized to study the role of SAC errors and aneuploidy in cancer. Manipulation of SAC protein levels resulted in deregulated SAC function, an increased CIN, and often increased cancer susceptibility in normal conditions or upon addition of carcinogenic compounds

depending on the SAC protein. These observations suggested that impairment of the SAC and induction of aneuploidy may contribute to tumorigenesis (Perez de Castro et al., 2007; Suijkerbuijk & Kops, 2008; Weaver & Cleveland, 2007)**.** On the other hand, there was no correlation between aneuploidy and tumor incidence and tumors did not develop in all aneuploid animals suggesting that deregulation of SAC proteins may cause additional changes which together with aneuploidy may sensitize to malignancy (Holland & Cleveland, 2009). Many SAC proteins have crucial functions outside the SAC in mitosis as well as in other phases of the cell cycle which complicates interpretation of these data on importance of aneuploidy in tumorigenesis (Weaver & Cleveland, 2007).

Lagging chromosomes are typically observed in cancer cells undergoing anaphase. Those chromosomes are often merotelically attached and cause chromosomal imbalance in daughter cells (Cimini et al., 2001). In fact, merotely is now generally accepted to be the main cause of aneuploidy and CIN (Gregan et al., 2011). Merotely can result from increased formation of erratic attachments or decreased efficiency of correction mechanisms. Alterations in the KT and/or centromere structure or in MT-KT interactions contribute to merotely because erratic attachments are formed more frequently (Cimini, 2008). Moreover, cancer cells may lack efficient correction mechanisms due to changes in proteins such as CPC which increases the frequency of merotely (Cimini, 2008).

Although SAC depletion studies support the idea that SAC defects are important in cancer formation, there is also plenty of evidence against this hypothesis. Many studies show that SAC proteins are unaltered in CIN cancer cells. In addition, the cells often have functional SAC in normal conditions although may be unable to maintain strong arrest upon exposure to spindle drugs (Thompson, Bakhoum, & Compton, 2010). Based on sequencing data from various tumor types, SAC proteins are not generally mutated in cancer cells (Thompson et al., 2010). Examination of mitotic progression has revealed no errors at anaphase indicating that the SAC is functional. Furthermore, cancer cells often show a SAC-mediated mitotic delay which is likely induced by extra centrosomes and chromosomes (Z. Yang et al., 2008) and various other genetic changes present in cancer cells (Dalton & Yang, 2009). Accumulating evidence indicates that a prolonged SAC activation can facilitate tumorigenesis (Dalton & Yang, 2009). The mechanisms are unclear but at least some of the genetic changes delaying satisfaction of the SAC may increase segregation errors (Dalton & Yang, 2009).

Not only promoting tumorigenesis, chromosomal instability may confer resistance to chemotherapeutic drugs (Thompson et al., 2010). These reasons highlight the importance of suppression of CIN. On the other hand, while missegregation may be tumorigenic due to gain or loss of a critical gene, it may also bring about changes that are detrimental to tumor cell growth (Thompson & Compton, 2008; Weaver & Cleveland, 2007). Whether aneuploidy promotes tumorigenesis or has tumor suppressive role seems to depend at least on the cell type and on the presence of other genetic changes in the tissue (Holland & Cleveland, 2009). Anti-tumorigenic concept can be utilized in cancer treatment by elevating aneuploidy. Anti-mitotics that induce a transient mitotic arrest are efficient cancer drugs whose efficiency may at least partially depend on induction of aneuploidy

(Dalton & Yang, 2009). Other means to increase aneuploidy are based on inhibition of SAC or centrosomal clustering of multipolar cells. Those strategies would result in massive aneuploidy and induce cell death (Thompson & Compton, 2008). It has been recently proposed that aneuploidy may represent an Achilles heel of cancer cells. Aneuploid cells try to cope with protein overload and activate unfolding and degradation of the proteins. This response is thought to generate stress the amount of which correlates with the severity of aneuploidy (Y. C. Tang et al., 2011). There is evidence that these cells are vulnerable to additional stress and therefore, inhibitors of stress response pathways may enable more selective targeting of cancer cells (Y. C. Tang et al., 2011).

2.6 Mitosis as an anti-cancer drug target

2.6.1 Traditional anti-mitotic drugs

Traditional anti-mitotic cancer drugs target MTs. When used at high concentrations, MTs are either stabilized (taxanes and epothilones) or depolymerized (vinca alkaloids). At low concentrations used in cancer clinics, the microtubule polymer mass is unaffected but the drugs suppress MT dynamics (Stanton et al., 2011). Taxanes are widely used to treat cancers of breast, ovarian, prostate, Kaposi's sarcoma and non-small-cell lung cancer whereas vinca alkaloids are utilized in treatment of haematological cancers (Jordan & Wilson, 2004; Stanton et al., 2011). The drug treated cells have several possible fates: they undergo a prolonged cell division arrest due to disrupted function of the mitotic spindle which can then result in death from mitosis, abnormal division or mitotic slippage followed by cell death, cycling or terminal arrest (senescence) of the polyploid pseudo-G1 cells (Fig. 5) (Keen & Taylor, 2009; Yamada & Gorbsky, 2006b).

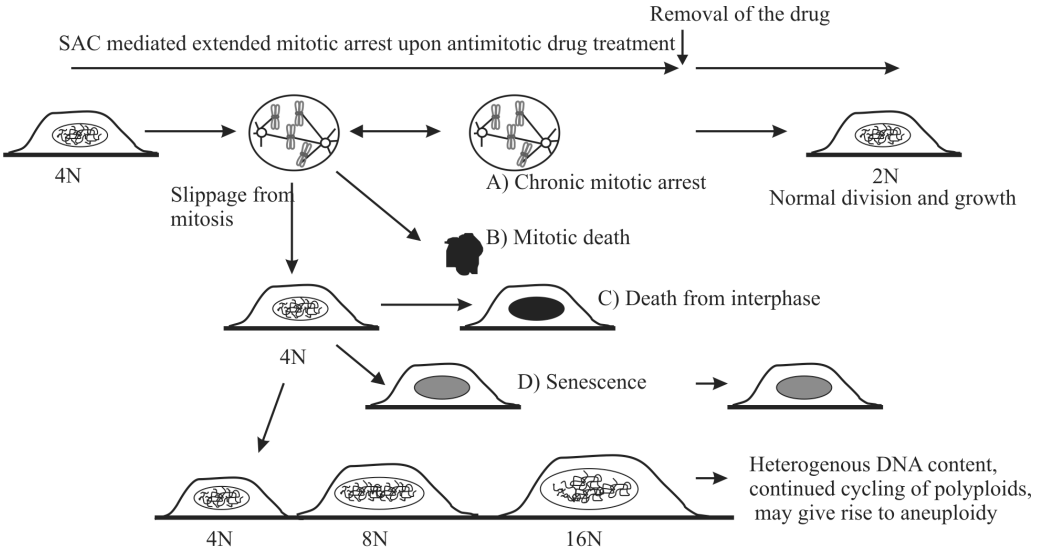

Figure 5. The possible fates of anti-mitotic drug treated cells (adapted from Yamada and Gorbsky 2006b).

Mitotic arrest is a consequence of SAC activation induced by lack of attachment or tension when MTs are destabilized or stabilized, respectively. The molecular mechanisms how anti-mitotics result in cell death are not completely understood (Gascoigne & Taylor, 2009). Whether the SAC needs to be functional for efficient apoptosis is controversial and has been extensively reviewed (Gascoigne & Taylor, 2009; Jackson et al., 2007; Schmidt & Bastians, 2007; Weaver & Cleveland, 2005; Yamada & Gorbsky, 2006a). There is a body of evidence that the SAC status is important for MT-drug sensitivity. The notion is supported by studies showing that SAC protein depletion negatively affects the sensitivity (Masuda et al., 2003; Sudo et al., 2004; W. Tao, 2005). However, even genetically identical cells have a profound variation in response to MT-drugs (Gascoigne & Taylor, 2009). Importantly, evidence is accumulating that rather than the SAC status sensitivity to apoptosis may be an important predictor of the response (Chin & Herbst, 2006; Gascoigne & Taylor, 2009; J. Shi, Orth, & Mitchison, 2008). A model of two "competing networks" (Gascoigne & Taylor, 2009) states that during mitotic arrest, the relative rates of cyclin B degradation and accumulation of apoptotic signals would determine whether a cell undergoes apoptosis or slippage from mitosis.

Mitotic slippage in the presence of spindle drugs or inhibitors of cytokinesis results in a tetraploid daughter cell. It has been proposed that cells have a so called tetraploidy checkpoint which enables the ploidy control at G1 phase. When the checkpoint gets activated, the cells arrest or undergo cell death and therefore, endoreduplication in the presence of extra chromosomes and centrosomes is prevented (Lanni & Jacks, 1998; Margolis, Lohez, & Andreassen, 2003; Vogel et al., 2004). p53 protein has been implicated in this post-mitotic checkpoint which may function as a backup mechanism for the SAC (Ganem & Pellman, 2007). It is believed that the checkpoint results in apoptosis or G1 arrest (senescence) in p53-proficient cells. In the absence of a functional p53, cells can continue cycling, endoreduplicate and give rise to aneuploid progeny (Margolis et al., 2003)**.** Although a plausible mechanism for preventing potentially dangerous proliferation of polyploid cells, the existence of a checkpoint monitoring chromosome number is debatable (Ganem & Pellman, 2007; Uetake & Sluder, 2004). It is possible that checkpoint activation is not a response for tetraploidy but instead, may result from abnormal cytoskeleton or numerical or structural changes in centrosomes (Ganem & Pellman, 2007). SAC proteins Bub1 and BubR1 have been implicated in stabilizing p53 after slippage which may contribute to removal of the cells (Suijkerbuijk & Kops, 2008)**.**

Although commonly used in clinics, traditional anti-mitotics that interfere with MT dynamics have major drawbacks. Besides being constituents of mitotic spindle, MTs have other important functions unrelated to cell division. Therefore, side-effects such as neuropathy, an impairment of peripheral nervous system function, are commonly encountered. Moreover, tubulin mutations and enhanced activity of efflux pumps among other mechanisms may lead to resistance which limits the use of these anti-mitotics.

2.6.2 Novel anti-mitotics

Due to several limitations of MT-drug based therapies, search for drugs with mitosis-specific targets and less severe side effects has become essential. The efforts for identifying novel drug candidates have generated inhibitors of various kinases and kinesins that may possibly enable efficient yet more specific targeting of cancer cells. Based on the effects on mitosis, the novel inhibitors can be categorized as inducers of mitotic arrest or forced exit (discussed in chapter 2.6.3). Several experimental compounds from both classes have proceeded into clinical trials. Potential use of these novel agents in clinics involves challenges such as understanding the pathway from target inhibition to apoptosis and finding predicitive markers for the response (Jackson et al., 2007).

2.6.2.1 Kinesin inhibitors

Kinesins are motor proteins that use ATP energy to translocate along MTs (Bergnes, Brejc, & Belmont, 2005). To date, 12 kinesins have been shown to have mitotic functions (Sarli & Giannis, 2006) ranging from spindle assembly to chromosome alignment and segregation, as well as cytokinesis (Bergnes et al., 2005). Due to essential mitotic functions and mitosis-specific expression, these kinesins are attractive druggable targets. To date, clinical trials have been performed with inhibitors of kinesin-5 (KSP/Eg5) and Cenp-E and a handful of experimental compounds are in preclinical phases (Huszar et al., 2009). KSP/Eg5 regulates centrosome separation and consequently, its inhibition resulted in a monopolarity and mitotic arrest (Blangy et al., 1995). KSP/Eg5 is overexpressed in various tumors but the significance may be questionable possibly reflecting higher mitotic index (Huszar et al., 2009). Seven inhibitors are undergoing clinical trials and four of them are in phase II (Huszar et al., 2009). One of the first in clinical trials is SB-715992, ispinesib, developed by Cytokinetics and GlaxoSmithKline. There is clinical evidence on efficacy against breast cancer (Purcell et al., 2010) and phase I results on patients with childhood solid tumors encouraged for initiation of phase II studies (Souid et al., 2010). MK-0731 from Merck (Cox & Garbaccio, 2010) was investigated in phase I trials which supported further investigations in patients with taxane-resistant solid tumors (K. Holen et al., 2011). SB-743921 from Cytokinetics, was evaluated in Phase I/II clinical trial in Hodgkin lymphoma (www.cytokinetics.com/sb_743921) and in phase I in patients with advanced solid tumors or relapsed/refractory lymphoma with promising outcome (K. D. Holen et al., 2011). AZD4877 (Theoclitou et al., 2011) developed by AstraZeneca is in phase I and II for solid tumors and hematological malignancies (http://www.astrazenecaclinicaltrials.com/). First reports did not show promising efficacy against solid tumors (Esaki et al., 2011) or AML (Kantarjian et al., 2011). ARRY-520 by Array Biopharma is evaluated in patients with multiple myeloma (MM) (http://www.arraybiopharma.com/_documents/Publication/PubAttachment494.pdf). In general, clinically effective KSP/Eg5 inhibitors have resulted in stable disease or partial response (Huszar et al., 2009). Possibilities of combining traditional anti-mitotics and KSP/Eg5 inhibitors require further investigations (Huszar et al., 2009). KSP/Eg5 inhibitors that proceeded into clinical trials are mostly non ATP-competitive targeting a unique, induced-fit pocket on KSP/Eg5. Mutations of this target site may lead to drug

resistance (Maliga & Mitchison, 2006) and ATP-competitive inhibitors (Luo et al., 2007; Parrish et al., 2007) may be tools to combat against such tumors.

Cenp-E is a plus end directed kinesin expressed in G2/M and degraded after mitosis (Yen et al., 1992). Its dysregulation may contribute to tumorigenesis and the kinesin may be a potential anti-cancer target. Cenp-E overexpression is detected in various tumor tissues (Wood et al., 2008) and its heterozygous disruption decreases cancer susceptibility at certain conditions in mice (Weaver & Cleveland, 2007). Disruption of Cenp-E function induced alignment defects, mitotic arrest or delay followed by erratic chromosome segregation, aneuploidy and apoptosis which indicated a role in stabilization of MT-KT attachments, chromosome alignment and SAC signaling (Putkey et al., 2002; Tanudji et al., 2004; Weaver et al., 2003; Yao et al., 2000). The crystal structure of the Cenp-E motor domain has been solved (Garcia-Saez et al., 2004) which enables a virtual screening for inhibitors of ATPase activity. To date, two inhibitors have been reported. Inhibitor UA62784 was poorly soluble indicating a need for chemical optimization (Henderson et al., 2009). GSK923295 is an allosteric inhibitor of Cenp-E ATPase activity and prevented detachment of the protein from MTs and stabilized ADP-Pi-Cenp-E-MT complex (Wood et al., 2010). The mechanism of GSK923295 and KSP/Eg5 inhibitor, ispinesib, is different: ispinesib weakened the binding of KSP/Eg5 to MTs and retarded the release of ADP from KSP/Eg5 (Lad et al., 2008). GSK923295 resulted in mitotic arrest and cell death in preclinical models (Wood et al., 2010) and is evaluated in phase I trials (Good, Skoufias, & Kozielski, 2011). c-Myc overexpression or amplification represents a potential biomarker of enhanced sensitivity for Cenp-E inhibition (http://www.cytokinetics.com/press_releases/release/pr_1207592692).

2.6.2.2 Farnesyl transferase inhibitors (FTIs)

Another means to inhibit Cenp-E activity is to target farnesylation of the kinesin. Although FTIs were originally developed to inhibit an oncogene, ras (Kohl et al., 1993), the inhibitors were soon recognized to elicit their effects in a more complex manner. Clinical studies demonstrated anti-tumor activity preferentially against ras-independent cancers (Sousa, Fernandes, & Ramos, 2008). Because FTIs induce prometaphase arrest it was suggested that FTIs might target mitotic proteins (Crespo et al., 2001). Cenp-E and Cenp-F function in mitosis and are known to undergo farnesylation and therefore, were plausible candidates for being effectors of FTIs. Prevention of farnesylation induced a phenotype resembling depletion of Cenp-E or Cenp-F: induction of chromosome misalignment, loss of sister KT tension and a mitotic delay (Crespo et al., 2001; Schafer-Hales et al., 2007). FTIs also abolished localization Cenp-E and Cenp-F (Hussein & Taylor, 2002; Schafer-Hales et al., 2007). Furthermore, FTIs inhibited binding of Cenp-E to MTs *in vitro* (Ashar et al., 2000; Crespo et al., 2001). On the other hand, a distinct phenotype of monopolar arrest was described suggesting that FTIs may target protein(s) required for pole separation and spindle formation (Crespo et al., 2001). Many proteins have been shown to undergo farnesylation but it is unclear which proteins mediate the FTI effects (Harousseau, 2007). Clinical trials indicate that FTIs, especially when combined with other agents, may have a therapeutic value in hematologic malignancies

(Braun & Fenaux, 2008; Harousseau, 2007) and in metastatic breast cancer (T. Li & Sparano, 2008).

2.6.2.3 Plk1 inhibitors

Plk1 is highly expressed in variety of human cancers and its expression has a prognostic value (McInnes, Mezna, & Fischer, 2005; Takai et al., 2005). Abrogation of Plk1 function caused mitotic arrest resulting in apoptosis in several cancer cell lines (Spankuch-Schmitt et al., 2002; Strebhardt & Ullrich, 2006), whereas caused only minor effects on proliferation of normal cell lines (Guan et al., 2005; X. Liu, Lei, & Erikson, 2006; Spankuch et al., 2007). Constitutive expression of Plk1 using mRNA injection transformed NIH 3T3 cells (M. R. Smith et al., 1997) indicating that excess of the kinase can contribute to the cell fate and therefore, Plk1 overexpression is more than a marker of increased proliferation. Furthermore, Plk1 depletion increased the sensitivity to taxol and herceptin (Spankuch et al., 2006; Spankuch et al., 2007). These features justify Plk1 as a drug target and inspire the development of chemical inhibitors. Here, I will review literature about inhibitors that are investigated in clinical trials.

Most Plk1 inhibitors are ATP-competitors which inhibit catalytic activity by binding to ATP-binding domain of the kinase. To date, five Plk1 inhibitors, three of which are ATP-competitors, have entered clinical trials and several inhibitors are in preclinical development (McInnes & Wyatt, 2011). BI 2536, developed by Boehringer Ingelheim, is among the best characterized anti-Plk1 compounds. The inhibitor had anti-cancer activity in xenograft murine models and against solid tumors in phase I trials (Mross et al., 2008; Steegmaier et al., 2007). However**,** further investigations were halted due to development of BI 6727, another member of the same class of experimental inhibitors (D. Rudolph et al., 2009) with improved pharmacokinetics (Medema, Lin, & Yang, 2011). The compound has proceeded in phase II on patients with advanced solid tumors (Schoffski et al., 2012). GSK461364A, an inhibitor optimized from a thiophene benzimidazole named compound 1 (Gilmartin et al., 2009; Lansing et al., 2007), was anti-proliferative in several cancer cell lines as well as resulted in tumor regression in xenograft models (Gilmartin et al., 2009) and is currently in phase I for advanced solid tumours and NHL (Lapenna & Giordano, 2009). Recommendations for phase II doses have been suggested (Olmos et al., 2011). A recently identified, oral inhibitor of Plk1, NMS-P937, is currently in phase I trials (Beria et al., 2011).

Recently, two Plk inhibitors that are unrelated to ATP, ON 0190.Na and HMN-214, entered clinical trials (Schoffski, 2009). ON 0190.Na (Gumireddy et al., 2005) induced apoptosis in a broad range of cancer cell lines including MDR-positive, treatment-resistant cells lines and was anti-tumorigenic in animal models. Plk1 activity was inhibited *in vitro* although ON 0190.Na also inhibited Abl, Flt-1 and PDGFR. In cancer cells, the phenotype resembled Plk1 depletion (Sumara et al., 2004), whereas normal cells were not affected suggesting cancer cell specificity (Gumireddy et al., 2005). ON 0190.Na has entered several phase I studies (Jimeno et al., 2009). Although the primary target is suggested to be Plk1 (Gumireddy et al., 2005), the effects may

be partly attributed to inhibition of other targets (Olmos, Swanton, & de Bono, 2008). HMN-214 is a stillbene derivative whose cellular effects, mitotic arrest, spindle abnormalities and cell death (DiMaio et al., 2009; Takagi et al., 2003; H. Tanaka et al., 2003), are suggested to involve Plk1 pathway (H. Tanaka et al., 2003) although the compound only mislocalized the protein. The compound has been reported to be the first anti-centrosome drug which inhibits MT nucleation at centrosomes (DiMaio et al., 2009). Whether Plk1 is involved in these effects was not addressed. The compound resulted in a stable disease in a phase I study in patients with advanced solid tumors (Garland et al., 2006) and is currently investigated in phase II and III (DiMaio et al., 2009). Biomarker studies are needed for validation of target inhibition, optimization of the administration and prediction of responsive patients (Olmos et al., 2008). Cyclin B1 is an example of a potential predictive biomarker in context of pancreatic cancer (Jimeno et al., 2009).

Plk1 possesses a Plk family specific phosphopeptide binding domain (polo-box domain, PBD) (Elia, Cantley, & Yaffe, 2003). The domain mediates Plk1-substrate interactions by specifically binding to phosphopeptides which contain Ser-pThr/pSer-(Pro/X) motif (Elia et al., 2003) and is essential for proper intracellular localization of Plk1 (Elia et al., 2003; K. S. Lee et al., 1998). PBD inhibitors are thought to provide specificity compared to kinase domain targeting inhibitors (Strebhardt & Ullrich, 2006). To date, three phosphopeptide ligands of PBD have been identified (Strebhardt & Ullrich, 2006). Thymoquinone and purpurogallin (PPG) are natural products capable of inhibiting PBD *in vitro* and in cells (Reindl et al., 2008; Watanabe et al., 2009). Poloxin is a synthetic thymoquinone derivative with improved specificity (Reindl et al., 2008). Consistently with phenotypes of PBD overexpression (Hanisch et al., 2006; Seong et al., 2002), the inhibitors mislocalized Plk1 and induced chromosome alignment defects resulting in SAC-mediated mitotic delay and apoptosis. It is possible that the inhibitors mislocalize Cenp-E and thus, destabilize MT-KT attachments as was shown for PPG (Watanabe et al., 2009). Although the cancer cell specificity remains to be investigated and the concentrations needed for cellular effects are rather high, these compounds validate PBD as a drug target and may be used as lead compounds.

The mechanism how Plk inhibitors induce cell death and activation of apoptotic machinery is not completely understood (Schmidt & Bastians, 2007). However, the phenotype of chemical inhibition is well-established: induction of SAC activation and mitotic arrest followed by apoptosis which resembles the effects of KSP/Eg5 inhibitors and traditional anti-mitotic drugs. Therefore, apoptosis-inducing mechanisms may also turn out to be similar (Schmidt & Bastians, 2007). Consistently, synergism with paclitaxel has been reported (Spankuch et al., 2006; Spankuch et al., 2007)**.** Factors that determine the sensitivity to these drugs are poorly understood. There is evidence that tumors with defective p53 or ras overexpression may be suitable for Plk1 inhibitor therapy (Medema et al., 2011). Furthermore, inhibition of DNA damage response increases the amount of cell death in Plk1 silenced cells (X. Liu & Erikson, 2003) suggesting that cells with defective repair system may be more vulnerable (Olmos et al., 2008).

2.6.2.4 Aurora A inhibitors

Aurora A is a mitotic kinase which plays an essential role in centrosome maturation at G2, regulates centrosome separation at prophase and assembly of bipolar spindle at prometaphase (Marumoto, Zhang, & Saya, 2005). Dysregulation of the kinase is associated with cancer-susceptibility and induction of chromosomal instability which rationales the development of Aurora A inhibitors in anti-cancer drug development. Aurora A is overexpressed or amplified in various cancers and may correlate with tumor grade (Boss, Beijnen, & Schellens, 2009; Marumoto et al., 2005). Aurora A overexpression transformed NIH3T3 cells and induced tumor formation in nude mice (H. Zhou et al., 1998). However, additional genetic changes are probably needed for tumorigenesis (Giet, Petretti, & Prigent, 2005). A plethora of different mechanisms may account for tumorigenic function of Aurora A: overexpression of the kinase induced centrosome amplification, inhibition of the SAC and cytokinesis and resulted in tetraploidy (Anand, Penrhyn-Lowe, & Venkitaraman, 2003; Jiang et al., 2003; Meraldi, Honda, & Nigg, 2002). Depletion of the kinase by RNAi induced incomplete centrosome separation, spindle defects and abnormal mitotic progression and resulted in cell death (Marumoto et al., 2005). Indeed, available chemical inhibitors possess anti-tumorigenic properties in cell lines and in animal models (Kollareddy et al., 2012)

Most Aurora kinase inhibitors target both Aurora A and B the phenotype showing Aurora B inhibition. I will only discuss specific Aurora A inhibitors here. MLN8054, developed by Millennium Pharmaceuticals, was the first of few small compounds against Aurora A activity. It is ATP-competitive and specific until 1 μM (Manfredi et al., 2007). Drug-induced effects on mitosis and proliferation have been extensively characterized in various cancer cell lines (Hoar et al., 2007; Manfredi et al., 2007) resulting in a model of inhibitor function in mitosis (Hoar et al., 2007). MLN8054 induced spindle abnormalities majority of cells having unseparated centrosomes. Interestingly, the cells were mainly bi- or multipolar. It was suggested that the monopolar spindles could eventually rearrange into bi- or multipolar ones as a result of acentrosomal spindle pole formation. In addition, multipolar spindles might undergo resolution into bipolar structures. Spindle abnormalities and alignment defects activated the SAC and induced a transient mitotic delay. Due to chromosome bridges and lagging chromosomes among other segregation defects, daughter cells were aneuploid which eventually resulted in cell death. Anti-tumor effects were confirmed in murine xenograft model showing inhibition of tumor growth, increase in mitotic index and induction of apoptosis (Manfredi et al., 2007). MLN8054 entered phase I trials for advanced solid tumors in 2005 but due to adverse effects on the central nervous system the concentrations achieved in plasma were insufficient (Dees et al., 2011). An analogue of MLN8054, MLN8237, has improved specificity and multiple phase II trials on patients with solid tumors and haematological malignancies are being initiated (Kollareddy et al., 2012). Preclinical studies suggested potential in treatment of childhood acute lymphoblastic leukemia (ALL) and solid tumors (Carol et al., 2011; Maris et al., 2010) as well as MM (Gorgun et al., 2010) and aggressive B-cell non-Hodkin's lymphoma (Qi et al., 2011).

MK-5108 developed by Vertex stabilized the disease as a single agent and caused also partial response when combined with docetaxel in patients with advanced solid tumors (Kollareddy et al., 2012). ENMD-2076 from EntreMed has proceeded in phase II in patients with ovarian cancer and holds a great promise for treatment of AML (Cheung et al., 2011; Kollareddy et al., 2012). Phase I studies in advanced solid tumors and MM are ongoing (Kollareddy et al., 2012). MP529 pyrimido[4,5-b]indole series of compounds developed by SuperGen Inc seem to be very potent and specific *in vitro* and in various cancer cell lines and reduce tumor growth in xenograft model (http://www.healthtech.com/conferences_track_overview.aspx?id=77554&c=). MP529 was reported to be in late preclinical stages in 2007 (http://kcancer.com/node/84.). Data from clinical trials have not been published.

Despite high preclinical anti-tumor activity, clinical utility of Aurora A inhibitors remains to be determined. Before the inhibitors can be used in clinics, various questions still remain (discussed in chapter 2.6.3.1) such as how to monitor the effective inhibitor dose in tumor. Mitotic index has been thought to confirm target inhibition. However, Aurora A inhibition may lead to mitotic death, or mitotic slippage followed by death or post-mitotic cell cycle arrest, suggesting that additional markers are likely required (Chakravarty et al., 2011). Aurora A phosphorylates TACC3 on Ser558 which is required for localization of the protein to spindles and centrosomes. Consistent with Aurora A inhibition, MLN8054 inhibited TACC3 phosphorylation and mislocalized the protein. The effects correlated with the status of Aurora A autophosphorylation site Thr288, a widely used marker for activity of the kinase in preclinical models. These data suggested that TACC3 localization may represent a direct pharmacodynamic marker for activity of Aurora A in tumors (LeRoy et al., 2007). The marker may have technical advantages over measuring the Thr288 phosphorylation in clinical samples (LeRoy et al., 2007). Finally, a recent study proposed that spindle morphology and chromosome alignment together reflect the response in skin and tumor biopsies (Chakravarty et al., 2011).

2.6.2.5 Hec1/NEK inhibitors

Hec1 (highly expressed in cancer) is a member of a conserved Ndc80 complex comprised of Hec1, Nuf2, Spc24, Spc25 (Bharadwaj, Qi, & Yu, 2004; Y. Chen et al., 1997; Nabetani et al., 2001). The complex localizes to the outer KT plate where it is required for the formation of proper MT-KT attachments, chromosome congression and SAC function (Cheeseman et al., 2006; DeLuca et al., 2006; McCleland et al., 2003; Meraldi, Honda, & Nigg, 2004). Hec1 is overexpressed in various human cancers (Wu et al., 2008). Furthermore, Hec1 depletion in tumor cell lines and animal models induced mitotic abnormalities and cell death (Gurzov & Izquierdo, 2006; L. Li et al., 2007; Lin et al., 2006) suggesting that inhibition of Hec1 function may have therapeutic value. Most available inhibitors target enzymes with small molecule substrates such as ATP and therefore, the knowledge on inhibition of protein-protein interactions is scarce. Furthermore, these interactions are transient and binding sites are structurally more difficult to target (J. Rudolph, 2007). However, abrogation of

protein-protein interaction was successfully used for inhibiting Hec1 (Wu et al., 2008). The authors performed a yeast two-hybrid screen to identify LMW inhibitors of Hec1-Nek2 kinase interaction shown to be important for Hec1 function in chromosome segregation (Y. Chen et al., 2002) and stabilization of MT-KT attachments (Du et al., 2008). The screen identified a small compound termed INH1 that bound to Hec1 and inhibited the interaction (Wu et al., 2008). Interestingly, INH1 also reduced Nek2 protein levels. Reminiscent to depletion of Hec1 and Nek2, INH1 resulted in mitotic delay and induced a massive apoptosis after slippage from aberrant mitosis. In addition to reduced viability of a panel of cancer cell lines, INH1 showed preclinical anti-tumor activity in mouse xenograft model. Importantly, data indicated that INH1 effects may be cancer cell specific. Hec1-Nek2 interaction seems therefore a potential target for anti-cancer drug development.

2.6.2.6 Inhibitors of mitotic exit

Slippage from mitosis before apoptosis is executed is one of the mechanisms responsible for resistance to anti-mitotic treatment (Manchado, Guillamot, & Malumbres, 2012). Therefore, delaying the mitotic exit have been envisioned to enhance the treatment efficacy. In fact, there is evidence that when cyclin B1 degradation is prevented by genetic ablation of APC/C activator Cdc20 the cancer cells die massively and even more efficiently than when treated with drugs targeting spindle function (H. C. Huang et al., 2009). Furthermore, introduction of non-degradable cyclin B1 led to similar effects (H. C. Huang et al., 2009). These results support the hypothesis that targeting mitotic exit may be a promising, novel, MT-independent approach to target mitosis for therapeutic purposes. Alternative possibilities to prevent cyclin B1 degradation and therefore, exit from mitosis, could include drugs targeting the proteasome or components of the APC/C (Manchado et al., 2012). In cancer clinics, these novel drugs could potentially be used together with conventional anti-mitotics and target wide range of tumors independently of the status of p53 or the SAC (H. C. Huang et al., 2009; Janssen & Medema, 2011; Manchado et al., 2012).

2.6.3 LMW compounds inducing a forced mitotic exit

It has been proposed that anti-mitotics require functional SAC to be effective killers. On the other hand, there is a consensus that complete SAC inactivation is lethal itself (Kops et al., 2004; Kops et al., 2005). Homozygous deletion of BubR1 or Mad2 causes embryonic lethality and silencing causes cell death in cell lines. The essential nature of the SAC has created the concept of "mitotic drivers" (Keen & Taylor, 2009) referring to drugs that override the SAC and force cells out of abnormal mitosis. "Mitotic drivers" identified to date mainly target Aurora B. The outcome of a forced mitotic exit may be cell death, senescence or endocycling (Fig. 6) (Keen & Taylor, 2009). It is believed that the use of "mitotic drivers" would improve specificity against proliferating cells and therefore, would not cause severe side-effects of tubulin-targeting drugs. Besides Aurora B, other SAC kinases such as Bub1 and BubR1 could potentially be targeted to cause forced exit and cell death. In fact, data from Gao and colleagues support

inhibition of Bub1 as a potential anti-cancer strategy in p53-proficient cells (Gao et al., 2009). The basis for possible tumor cell selectivity is not understood but preclinical data suggests that tumor cells might be more vulnerable to certain SAC inhibitors (Kaestner & Bastians, 2010). It is possible that the presence of extra chromosomes, a characteristics of cancer cells, contributes to the increased sensitivity to "mitotic drivers" by increasing the time required for the chromosome alignment (Janssen & Medema, 2011). Furthermore, cancer cells possess abnormal DNA content and therefore, might be more sensitive than normal cells to drug-induced increase in genetic imbalancies (Janssen & Medema, 2011).

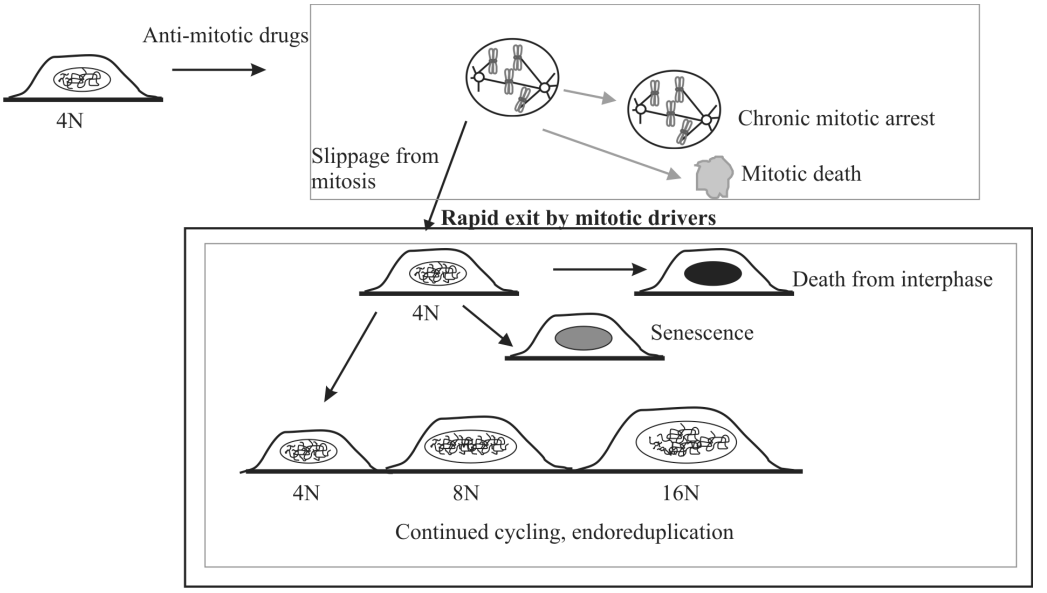

Figure 6. The mitotic driver concept and cellular fates (modified from Keen and Taylor 2009, Yamada and Gorbsky 2006b). Gray boxes: the fate of anti-mitotic drug treated cells, black box: mitotic driver concept. A mitotic driver drug rapidly overrides a drug-induced mitotic arrest.

2.6.3.1 Aurora B inhibitors

Aurora B kinase is essential for mitosis. The kinase functions together with INCENP, Borealin and Survivin as a part of the CPC in which the other members are needed for its activity and localization (Vader, Medema, & Lens, 2006). Aurora B is involved in key mitotic processes (Carmena & Earnshaw, 2003; Vader & Lens, 2008; Vagnarelli & Earnshaw, 2004) from early mitosis to cytokinesis including chromosome condensation, cohesion, resolution of incorrect attachments, SAC function and cytokinesis (Fig. 7).

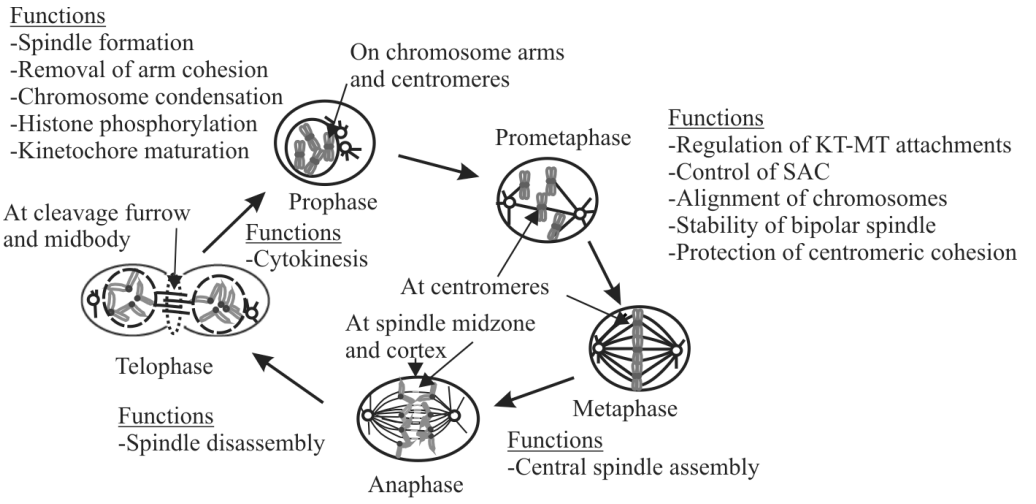

Figure 7. Localization and functions of Aurora B in mitosis (adapted from Ruchaud et al 2007).

Furthermore, Cenp-E, and the SAC proteins BubR1 and Mad2 depend on Aurora B for KT localization (Ditchfield et al., 2003). Overexpression of Aurora B has been detected in multiple tumor types of different origin (Kollareddy et al., 2008; Mountzios, Terpos, & Dimopoulos, 2008) and the expression correlates with tumor grade and prognosis in various cancers (Kurai et al., 2005; Sorrentino et al., 2005; S. Tanaka et al., 2008; Zeng et al., 2007)**.** CIN is observed in Aurora B overexpressing tumors. However, a role of the kinase in tumorigenesis is not well-established (Vader & Lens, 2008). Aurora B overexpression may have transforming potential alone (Ota et al., 2002) or together with H-Ras (Kanda et al., 2005). Cancer-associated mutations of the gene have not been determined (Perez de Castro et al., 2007) and the locus is not known to be amplified (Mountzios et al., 2008; Vader & Lens, 2008). However, expression data of different cancers has encouraged for development of chemical inhibitors for cancer therapy purposes. LMW inhibitors of Aurora B caused severe defects: SAC abrogation and a failure of chromosome alignment and cytokinesis (Ditchfield et al., 2003; Hauf et al., 2003). The catastrophic mitosis led to premature forced exit and pseudo G1-arrest or alternatively, endoreduplication both of which were followed by apoptosis (Carvajal, Tse, & Schwartz, 2006; Ditchfield et al., 2003; Hauf et al., 2003). Although not completely understood, lethality of Aurora B inhibition is thought to arise from severe polyploidization (Kaestner & Bastians, 2010).

Currently, Aurora B can be specifically targeted with two inhibitors, namely AZD1152 and compound 677, both developed by AstraZeneca (Carvajal et al., 2006). p53-deficient cells were sensitized to the compound 677 and preclinical data on combining the inhibitor with other anti-cancer agents was promising (Carvajal et al., 2006). Clinical data has not been published. AZD1152 is a pro-drug which is metabolized into active form, A-ZD1152-HQPA, in plasma. The selectivity of the drug is 1000-fold higher for Aurora B than for Aurora A (Walsby et al., 2008). The inhibitor induced apoptosis and inhibited tumor growth in colorectal and lung cancer models (Wilkinson et al., 2007). Besides in solid tumors, the drug has been investigated in preclinical models of

haematological malignancies (R. P. Evans et al., 2008; Wilkinson et al., 2007; J. Yang et al., 2007). AZD1152 was anti-proliferative and triggered cell death in several leukemic and myeloma cell lines, primary cells from MM patients as well as in murine xenograft models for the diseases. Moreover, the effects were synergistic with MT-depolymerizing agent vincristine and topoisomerase II inhibitor daunorubicin which are used as anti-leukemic drugs (J. Yang et al., 2007). In MM cell lines, AZD1152 was synergistic with dexamethasone (R. P. Evans et al., 2008). Recently, sensitization for radiotherapy response, micronucleation and mitotic catastrophe were investigated (Y. Tao et al., 2008). Shown in cell lines and colon carcinoma mouse model, sequential combination of AZD1152 and radiation resulted in synergistic effects on cell death and tumor growth delay. The drug was particularly anti-tumorigenic in p53-deficient cells proposing that combination of DNA-damaging agents and Aurora B may have therapeutic potential on p53-defective background. AZD1152 has been studied in patients with solid tumors and disease stabilization was observed (Cheung et al., 2009; Kollareddy et al., 2012). Clinical data indicated that the drug may have therapeutic in AML patients (Kollareddy et al., 2012).

Inhibitors that target both Aurora A and B appear to result in phenotype of Aurora B inhibition. VX-680 (MK-0457) (Harrington et al., 2004) and PHA-739358 (Carpinelli et al., 2007) are the most extensively characterized inhibitors of both Aurora A and B. ZM447439 (Ditchfield et al., 2003) and hesperadin (Hauf et al., 2003) are more potent against Aurora B and are commonly used to explore biological functions of the kinase. Hesperadin was discovered in cell-based assays as a compound inducing polyploidization (Hauf et al., 2003), whereas ZM447439 and VX-680 were identified to inhibit Aurora activity *in vitro* (Ditchfield et al., 2003; Harrington et al., 2004). VX-680 was investigated in phase I clinical trials in patients with CML and Philadelphia chromosome-positive ALL but the trials were discontinued due to side effects on heart function (Cheung et al., 2009). At least nine small compounds which inhibit Aurora A and B (and some also C) with similar concentrations, have proceeded into clinical trials in patients with solid or haematological malignancies (Kollareddy et al., 2012). Three of the inhibitors, R-763, PF-3814735 and CYC-116, appeared to be suitable for oral and intravenous administration (Cheung et al., 2009). One of the pan-Aurora inhibitors, danusertib (PHA-739358), (Fancelli et al., 2006) is currently in phase II trials (Cheung et al., 2011) and is expected to soon enter phase III in several solid malignancies (Kollareddy et al., 2012). Interestingly, besides Aurora B, VX-680 and PHA-739358 inhibit BCR-ABL and therefore, are considered as potential second-generation drugs for imatinib resistant patients (Gontarewicz et al., 2008; Weisberg et al., 2007). In addition, a plethora of Aurora inhibitors are in preclinical development and novel inhibitors are still being identified (Kollareddy et al., 2012; Schmidt & Bastians, 2007).

The determinants of the sensitivity to Aurora inhibition have been intensively studied but the subject is very challenging and the crucial factors are not yet well-established. Aurora kinases are highly expressed in mitosis and therefore, it is thought that highly dividing cells are most vulnerable (Keen & Taylor, 2009). There is a body of evidence that the status of p53 may influence the cell fate upon Aurora inhibition (Ditchfield et al.,

2003; Gizatullin et al., 2006; Margolis et al., 2003). Typically, p53-deficient cells with inhibited Aurora B become polyploid and undergo cell death, whereas p53-proficient cells arrest at pseudo-G1 phase (Ditchfield et al., 2003; Gizatullin et al., 2006; Harrington et al., 2004; Hauf et al., 2003). In line with the arrest, many Aurora inhibitors induce p53 accumulation (Dreier et al., 2009; Gizatullin et al., 2006; Kaestner, Stolz, & Bastians, 2009). There is evidence that p53 may not totally prevent the cells from endoreduplication and may not arrest the cells immediately after the first erratic mitosis (Dreier et al., 2009). It was shown that upon drug removal after long-term treatment, some colonies emerged independent of p53 status indicating that some cells were capable of resuming proliferation. Importantly, those cells were not resistant to the drug suggesting that other mechanisms enabled the cells to evade the killing and in case of cancer treatment those cells could possibly be targeted by repeating the exposure (Dreier et al., 2009). Altogether, predicting cell fate based on the p53 status may be too simplistic. In fact, a characterization of selective Aurora B inhibitor effects performed with an extensive panel of cell lines with different p53 status indicated that Aurora inhibition can abrogate p53-mediated arrest and induce polyploidy independent of p53 functionality (Nair et al., 2009). In conclusion, data from several laboratories (Ditchfield et al., 2003; Dreier et al., 2009; Gizatullin et al., 2006; Kaestner et al., 2009) suggest that p53 alone cannot determine the cell fate upon Aurora inhibition.

Not only p53 but also p21 and Rb, which all are essential for postmitotic checkpoint arresting cells at pseudo-G1 state, may be involved in the response to Aurora inhibitors. Supporting the role of p21 and Rb, cell lines responded differently to Aurora inhibitors depending on p21 induction and Rb phosphorylation (Gizatullin et al., 2006). It was suggested that not only p53 functionality but the integrity of p53-p21 pathway and possibly status of Rb may affect the drug response. On the other hand, there is evidence that induction of p53 or p21 does not necessarily correlate with the extent of polyploidy (Nair et al., 2009). In the same study, Rb was found to be a substrate of Aurora B adding a Cdk-independent connection between Aurora B and Rb (Nair et al., 2009). It was proposed that Aurora B-dependent phosphorylation of Rb on Ser780 is crucial for regulating the post-mitotic checkpoint after erroneous mitosis. Furthermore the data suggested that upon Aurora inhibition, Rb could actually promote polyploidy after erroneous mitosis (Nair et al., 2009). Therefore, it is possible that Rb deficient tumors might undergo less endoreduplication upon Aurora inhibition (Nair et al., 2009). Finally, Myc expression levels may represent a predictor of the response for Aurora B inhibition (D. Yang et al., 2010) because Myc-overexpressing tumors may depend on Aurora B for the malignancy (den Hollander et al., 2010).

Due to mitotic roles of Aurora kinases, it has been proposed that their inhibition may represent a means to target rapidly proliferating cancer cells and the preclinical data support the concept (Ditchfield et al., 2003; Harrington et al., 2004). Clinical data indicates that novel mitosis-targeting drugs, including Aurora inhibitors, have anti-tumor potential although weaker than traditional MT-targeting drugs (Manchado et al., 2012). Currently, it seems that the therapeutic effects of Aurora inhibition are lower than originally expected (Kollareddy et al., 2012; Komlodi-Pasztor, Sackett, & Fojo, 2012). This outcome likely

reflects the fact that doubling-times of cells in human tumors are in fact much longer than those in preclinical models that may contest the use of such models in the study of early state inhibitors affecting mitosis (Kollareddy et al., 2012; Komlodi-Pasztor et al., 2012). The importance of Aurora kinases in tumorigenesis is not well-established and it is possible that their role during cancer formation is only transient (Kollareddy et al., 2012). Moreover, the lack of means to identify most responsive patients may partially explain why the inhibitors have not yet met the expectations (Manchado et al., 2012). It is likely that induction of tetraploidy affects also normal cells and potentially might also predispose healthy cells to tumorigenesis (Janssen & Medema, 2011). Reflecting the effects on rapidly dividing haematological cells, neutropenia has been encountered as a side-effect in clinical trials (Kollareddy et al., 2012). Duration of the treatment, monitoring the efficacy, and identification of the most responsive patients are examples of the issues that require further investigations before these inhibitors can be approved for clinical use (Keen & Taylor, 2009). Furthermore, it will be important to determine whether targeting a single or several Aurora kinases simultaneously is a more potent approach. Finally, careful evaluation of drug combinations are needed (Cheung et al., 2009).

2.6.3.2 Histone deacetylase inhibitors (HDACIs)

Genetic mutations can cause inactivation of tumor suppressors or activation of proto-oncogenes which can ultimately drive tumorigenesis. However, it has been known for years that also epigenetic changes are tightly involved in cancer formation (Yoo & Jones, 2006). Epigenetic modifications include DNA methylation and acetylation, as well as methylation, ubiquitilation, and glycosylation of histones which may, depending on the type and place of modification, activate or inactivate gene expression. Consequently, changes in these modification patterns result in deregulated gene expression and genomic instability and are typical for cancer cells. Methylation and acetylation are reversible and therefore, restoring the modification patterns is an attractive cancer treatment possibility. Potential targets for such epigenetic drugs include DNA methyltransferases (DNMTs) and histone deacetylases (HDACs). Currently, there are at least eleven inhibitors of HDACs (HDACIs) undergoing clinical trials and one inhibitor, vorinostat (SAHA), has got an FDA approval for treatment of cutaneous T-cell lymphoma (Cang, Ma, & Liu, 2009). HDACIs possess anti-proliferative properties (Eot-Houllier et al., 2009) and seem to have cancer cell specificity (Gabrielli et al., 2004; Warrener et al., 2003) targeting both proliferating and non-proliferating tumor cells (Burgess et al., 2004). Numerous cellular functions, such as apoptosis, cell cycle progression, angiogenesis and differentiation are potentially affected by HDACIs (Yoo & Jones, 2006). As expected, HDACIs target gene expression but only 5 % of the genes are likely affected (Robbins et al., 2005). The inhibitors may activate transcription of tumor suppressors and apoptosis-inducing genes and repress oncogenes. HDACIs may also result in acetylation of non-histone proteins such as p53 which prevents DNA-repair. Indeed, the inhibitors may exert their effects in transcription-dependent and independent manner (Yoo & Jones, 2006).

Effects on mitotic progression and in particular, SAC function are especially interesting in context of this thesis. A plethora of HDACI-induced mitotic defects have been described

including insufficient chromosome condensation (Cimini et al., 2003), impaired KT assembly, mistargeting of the CPC (Stevens et al., 2008), spindle defects (Stevens et al., 2008) and decrease in the amount of Aurora-A kinase (J. H. Park et al., 2008). Indeed, HDACIs may induce congression defects (Warrener et al., 2003), override of the SAC (Dowling et al., 2005; Warrener et al., 2003), segregation errors and abnormal cytokinesis and polyploidy (Eot-Houllier et al., 2009). Interestingly, SBHA (Stevens et al., 2008), TSA, apicidin and sodium butyrate (Magnaghi-Jaulin et al., 2007), which are in clinical trials (Eot-Houllier et al., 2009), inactivate the SAC prematurely and induce apoptosis. Data from several laboratories suggest that non-transcriptional changes, such as disruption of centromere structure and function or prevention of histone deacetylation involved in mitotic progression, may be involved in these mitotic abnormalities (Y. Li et al., 2006; Magnaghi-Jaulin et al., 2007; Taddei et al., 2001). In addition, transcriptional changes such as reduced expression of Aurora B (LaBonte et al., 2009), Plk1, survivin and cyclinB1 (Noh et al., 2009) are likely involved.

Given the multi-targeting nature of HDAC inhibitors, specific targeting of mitosis may be difficult. However, HDACIs which can inhibit the SAC and kill G2 checkpoint-deficient cancer cells without affecting normal cells are considered promising anti-cancer agents. Development of more selective inhibitors may decrease side-effects reported in clinical trials. Finally, evidence is accumulating that HDACIs may increase efficiency of certain anti-cancer agents, such as proteasome inhibitors, CDK inhibitors and demethylating agents (Al-Janadi, Chandana, & Conley, 2008; Grant, 2008).

2.6.3.3 Mps1 inhibitors

Mps1/TKK is a kinase which localizes at KTs and whose activity peaks in mitosis (Stucke et al., 2002). Mps1 activity is essential for correction of improper attachments (Jelluma, Brenkman, van den Broek et al., 2008) and SAC function (Abrieu et al., 2001; Stucke et al., 2002). The kinase is able to activate Aurora B via phosphorylation of Borealin (Jelluma, Brenkman, van den Broek et al., 2008). On the other hand, very recent data from three laboratories indicated that Mps1 functions downstream of Aurora B proposing that Aurora B does not mediate error correction and chromosome alignment functions of Mps1 (Hewitt et al., 2010; Maciejowski et al., 2010; Santaguida et al., 2010). These discrepancies have been suggested to reflect the complexity of interactions and existence of signaling network rather than linear dependencies between the kinases (Lan & Cleveland, 2010). It is possible that chromosome alignment depends on functions of both Mps1 and Aurora B on a common substrate, Cenp-E (Lan & Cleveland, 2010), whose phosphorylation by both kinases enables congression (Espeut et al., 2008; Kim et al., 2010). Mps1 has been shown to contribute to the SAC in various ways (discussed in chapter 2.5.1) in unperturbed mitosis and upon MT drug treatment in human cells (Jelluma, Brenkman, McLeod et al., 2008; Stucke et al., 2002; Tighe, Staples, & Taylor, 2008). Outside mitosis, Mps1 stabilizes p53 upon spindle damage and thus, promotes activation of G1 checkpoint and prevents genomic instability (Y. F. Huang, Chang, & Shieh, 2009). Few Mps1 substrates have been identified in vertebrates Borealin (Jelluma, Brenkman, van den Broek et al., 2008) being the only target involved in the

SAC function. BubR1 phosphorylation requires Mps1 but whether it is a direct substrate remains to be resolved (H. Huang et al., 2008). Cancer-associated mutations in the gene have not been found (de Carcer, Perez de Castro, & Malumbres, 2007) but overexpression is common in chromosomally instable tumors (Carter et al., 2006). Given that Mps1 activity is important in essential mitotic functions and it is overexpressed in several cancers, pharmacological inhibition of Mps1 may be an attractive therapeutic strategy.

To date, eight inhibitors of Mps1 have been identified. Three of them were effective on analogue-sensitized Mps1 (Maciejowski et al., 2010; Sliedrecht et al., 2010; Tighe et al., 2008) and one, cincreasin, was effective only in yeast (Dorer et al., 2005). SP600125 (Schmidt et al., 2005) was the first inhibitor of mammalian Mps1. Although originally identified as an inhibitor of JNK (Bennett et al., 2001), the compound inactivated the SAC independent on JNK and also at concentrations below JNK inhibition which indicated that the effects were JNK-independent. Indeed, the induced defects in the SAC were shown to result from Mps1 inhibition (Schmidt et al., 2005). In line with the SAC inhibition, BubR1 was mislocalized from KTs and its phosphorylation was reduced indicating that its activity was decreased. The authors suggested that Mps1 activity is needed to recruit BubR1 but is not necessary for Mad1 localization the latter being confirmed later (Tighe et al., 2008). As shown later, Mps1 activity is required for recruitment of Mad2 (Tighe et al., 2008). SP600125 did not abolish Mad2 localization at KTs, raising the question of the target responsible for SAC inhibition (Tighe et al., 2008). In fact, the drug inhibited a wide range of kinases *in vitro* but possible mitotic functions for those kinases remain to be identified (Schmidt et al., 2005). In summary, the relevance of SP600125 as a real Mps1 inhibitor remains to be seen. The structure of Mps1 catalytic domain alone and in complex with SP600125 is available (Chu et al., 2008; W. Wang et al., 2009) for the structure-based design of related inhibitors and analogues.

Since identification of SP600125, several cellular inhibitors of Mps1 have been characterized including AZ3146 (Hewitt et al., 2010), Mps1-In-1 and -2 (Kwiatkowski et al., 2012), and reversine (Santaguida et al., 2010), a compound originally identified as an inhibitor of Aurora B (D'Alise et al., 2008). MPI-0479605 was reported to be anti-tumorigenic in mice but the growth inhibition of normal cells and side-effects seen in mice indicated that the drug affected also normal cells (Tardif et al., 2011).The most potent and selective Mps1 inhibitor to date is NMS-P715 (Colombo et al., 2010). The compound induced SAC override, aneuploidy and massive cell death in various cancer cell lines. Furthermore, the inhibitor was highly anti-proliferative in cancer cell lines and also in mouse models but had minor effects on growth of normal cells. Altogether, the data justify further investigations of Mps1 inhibitors as a potential novel means for cancer treatment.

2.7 Phenotypic screens of small compounds

HTS of small molecules is performed in pharmaceutical industry and academia to identify new hit compounds for drug development. When designing the screen of

small compounds, several aspects are considered. Nowadays done in HT format, the screens have to be automatable, cost-effective, and easy to perform. Libraries of LMW compounds of synthetic and natural origin are commercially available and utilized for screening purposes. While natural compounds increase the diversity of the library, modification steps may be difficult (Verkman, 2004). Screening concentrations are inevitably a compromise between the number and selectivity of hits discovered, toxicity effects, and other issues. Usually, concentrations used are within micromolar range (Schriemer, Kemmer, & Roberge, 2008). Similarly, the length of the treatment is a compromise between being long enough for the cellular effects to take place and short enough to reduce the likelihood of unspecific effects. The activity of the small compounds is preferentially measured with a "positive readout", such as increase in fluorescent signal intensity, because it reduces the amount of hits that are not selectively targeting the process or a protein of interest (Schriemer et al., 2008). Hits from the primary screens are typically validated in secondary screens and further characterized by various assays aiming to determine specificity and cytotoxicity, activity range, and structure-activity relationship (SAR).

In cellular target-based screens, the aim is to identify LMW compounds that have an effect on a pre-determined target as for example a certain kinase. In contrast, phenotypic screens identify compounds that affect a cellular process as for example SAC signaling or execution of cytokinesis and by definition, targets are not known beforehand. Various different approaches for target identification have been developed ranging from traditional affinity chromatography and expression cloning to modern genome-wide systems biology methods (Hart, 2005). Although not always easy, potential targets can be narrowed down by making "educated guesses" based on existing literature or silent knowledge (Perrimon et al 2007). All in all, target identification process is challenging and is a rate-limiting step in phenotypic screens (Eggert et al., 2004).

In context of mitotic research, phenotypic screens have revealed several interesting LMW compounds such as Eg5 inhibitor monastrol (Mayer et al., 1999), Aurora B inhibitor hesperadin (Hauf et al., 2003) and Mps1 inhibitor cinreasin (Dorer et al., 2005). These inhibitors affect key mitotic processes such as bipolar spindle formation, SAC activity, and chromosome alignment. Nowadays, fluorescence microscopy has turned out to be an extremely powerful technique in basic mitotic research and also in HT screens. It can be used in live cell assays to monitor fluorescently-tagged protein(s) or in experiments with fixed samples stained for DNA and immunolabeled with fluorescently tagged antibodies detecting the protein of interest. Compared to plate reader based detection, imaging increases the amount of information of cellular effects induced by the library compounds (Yarrow et al., 2003) but as a consequence, makes the data analysis and handling more demanding (Eggert & Mitchison, 2006). Due to these difficulties in data analysis and target identification in the phenotypic screens, target-based screens are more widely used (Perrimon et al., 2007). Cell-based small compound screening by imaging has been successfully utilised in identification of inhibitors of processes such as cell migration (Yarrow et al., 2005), cytokinesis (Eggert et al., 2004) and mitotic progression (Wilson et al., 2006). Comparison of the phenotypes of parallel chemical screening and genome-

wide RNAi screens is a potent approach to identify new inhibitors and speed up the target identification process although the target has to be validated by other methods (Eggert et al 2004).

2.8 Flavonoids and their medicinal effects

2.8.1 Structure and function

Secondary metabolites protect plants that produce them against different stresses such as reactive oxygen species, parasites and UV radiation. The largest group of secondary metabolites is comprised of polyphenols, including tannins, phenolic acids, stilbenes, lignans, and flavonoids among others (Link, Balaguer, & Goel, 2010). More than 9000 flavonoids have been identified in plants (Y. Wang, Chen, & Yu, 2011). Flavonoids have a common phenylbenzopyrone (C6-C3-C6) skeleton comprised of two phenolic rings connected by oxygen-containing pyran ring (Fig. 8). Based on the level of saturation and ring substituents, flavonoids are further classified into seven main groups: flavonols, flavonones, flavones, isoflavones, flavanones, flavanols, and anthocyanins (Singh & Agarwal, 2006). In plants, flavonoids are typically found as various glycoside conjugates.

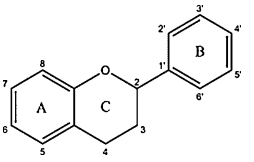

Figure 8. General structure, ring labeling and carbon atom numbering system of a flavonoid.

Medicinal effects of plants have been utilized for thousands of years in traditional medicine and are nowadays of high interest also in modern medicine and drug discovery. More than half of the cancer drugs in clinical use originate from natural sources (E. H. Liu et al., 2009) plants being among the most diverse source of drug candidates. Besides treatment of disease, plant-derived small compounds have raised interest in disease prevention. It is estimated that 1/3 of cancers are preventable, mainly by avoiding smoking and having a healthy diet (Bode & Dong, 2009). Epidemiological data suggests that diet rich in fruits and vegetables may lower the incidence of various diseases including cancer (Lamoral-Theys et al., 2010). Edible plants are a versatile source of different polyphenols which are mostly flavonoids (Chahar et al., 2011). Especially rich dietary sources of polyphenols are vegetables, fruits, cocoa, tea and berries (Egert & Rimbach, 2011; Paredes-Lopez et al., 2010). The inverse correlation of the vegetable and fruit-rich diet and lower cancer risk has fuelled animal studies on flavonoids and other polyphenols. In vast number of animal studies, flavonoids appear to inhibit cancer initiation, promotion and progression (Scalbert et al., 2005), which points to the possibility that flavonoids may have potential in cancer treatment and chemoprevention in humans. However, many epidemiological studies have failed to show any correlation between high intake of flavonoids and lower disease risk. The discrepancies between animal studies and epidemiological data have

been suggested to partially reflect the fact that animal and cell line studies involve high flavonoid concentrations not present in normal diet (Scalbert et al., 2005). Furthermore, flavonoids have a low bioavailability due to rapid conjugation reactions in the body and poor water solubility (Lamoral-Theys et al., 2010). Considering rapid metabolism of flavonoids in human body, there is a need to put efforts not only to research of pure small compounds but also to anti-cancer potential of their metabolites (Scalbert et al., 2005). This requires better knowledge on flavonoid metabolism and characterization of the metabolites present in the body. Moreover, the availability of biomarkers of polyphenol intake is limited, and flavonoid composition in diet is not fully explored which hamper the research on health effects (Scalbert et al., 2005). Relying on food consumption questionnaires also gives rise to uncertainties in determination of flavonoid intake (Manach et al., 2004).

Flavonoids may affect human health by multiple means of which anti-oxidation is among the most thoroughly studied (Nijveldt et al., 2001). Ability to scavenge free radicals and reactive oxygen species, prevent their formation and enhance detoxification has been generally believed to account for the health benefits but it has turned out that flavonoids can modulate activity of multiple cancer-related kinases (Lamoral-Theys et al., 2010). Flavonoids are able to target key enzymes involved in proliferation, apoptosis or cell cycle such as Akt, Cdks and MAP kinases and prevent angiogenesis as well as metastasis (K. W. Lee, Bode, & Dong, 2011). Furthermore, epigenetic alterations may explain some of the anti-cancer effects of flavonoids (Link et al., 2010). Flavonoids modulate the activity of cytochrome P450 enxymes (CYP) and phase II enzymes which metabolize endogenous and exogenous substances such as drugs and carcinogens. Therefore, phytochemicals can inhibit activation of carcinogens and enhance their clearance from the body (Moon, Wang, & Morris, 2006). However, whether this holds true *in vivo* is largely unknown (Androutsopoulos et al., 2008). Flavonoids can disrupt existing blood vessels in tumor (McKeage & Baguley, 2010). Interestingly, flavonoids target mainly the tumor core suggesting that when combined with conventional therapies often having limited penetrance into the tumor center the flavonoids may offer a means to efficiently kill solid tumors. Each flavonoid and polyphenols in general modulates activity of multiple proteins. This multi-targeting is considered advantageous in treating complicated diseases such as cancer and this type of therapy could be associated with less side-effects (Lamoral-Theys et al., 2010). On the other hand, multi-targeting complicates target identification.

Polyphenolic phytochemicals, mainly components of green tea and soy as well as pure curcumin or quercetin have been studied in healthy individuals, in patients with premalignant lesions and in cancer patients (Thomasset et al., 2007). An interesting example is soy which has a high content of isoflavones, such as genistein. Isoflavones belong to phytoestrogens which are plant-derived estrogen-like compounds with anti-estrogenic and estrogenic properties (Ososki & Kennelly, 2003). Phytoestrogens are growth-inhibitory and induce G2/M arrest in several cancer cell lines (Virk-Baker, Nagy, & Barnes, 2010). Epidemiological data indicate that phytoestrogens may decrease a risk for prostate and breast cancer (Virk-Baker et al., 2010) and has fuelled the investigations

on therapeutic potential of these anti-estrogenic properties. Anti-carcinogenic effects of pure genistein or soya-based diet have been studied in animal models of breast and prostate cancer as well as in some other cancers. These studies have reported a decrease in metastasis, inflammation and angiogenesis (Virk-Baker et al., 2010). Clinical studies on phytoestrogens alone and in combination with chemotherapy are ongoing for various cancers (Pavese, Farmer, & Bergan, 2010; Virk-Baker et al., 2010). There are indications that phytoestrogen-rich diet may lower the risk of cancer recurrence and improve survival in breast cancer patients but there is clearly a lot of inconsistency and therefore no recommendations on the "therapeutic" intake can be made at the moment (Andres et al., 2011; Velentzis et al., 2008). Estrogenic properties manifested upon low estrogenic conditions have led to use of phytoestrogen supplements as alternatives for the traditional hormone replacement therapy in alleviating menopausal symptoms. Yet, there are uncertainties regarding possible adverse effects of the estrogenic functions which is also supported by animal studies (Andres et al., 2011). Without better knowledge on the associated risks, the use of supplements especially in postmenopausal women should be critically evaluated (Andres et al., 2011). Another example of a polyphenol in clinical trials is quercetin, the most common flavonoid in diet (Russo et al., 2012) which may have a potential in prevention and treatment of certain cancers and cardiovascular diseases. Importantly, as a multi-target flavonoid, quercetin has been shown to be able to target all the phases of carcinogenesis namely initiation, promotion and invasion. Clinical data has been mainly obtained from studies on healthy individuals and the studies in cancer patients are being initiated. Considering that high concentrations are needed for therapeutic effects, the safety of supplements should be carefully studied. Furthermore, similarly to the phytochemicals in general, there is still much to be learned about molecular targets of quercetin.

Berries are a rich source of bioactive, anti-oxidative constituents such as flavonoids. According to a Finnish study, berries represent 80 % of the most abundant dietary polyphenol sources (Ovaskainen et al., 2008). *In vitro* studies suggest that direct scavenging of reactive oxygen species and activity of anti-oxidative enzymes present in berries may explain the anti-oxidative properties. The anti-oxidative capacity not only varies from berry type to another but is also influenced by a plethora of other factors (Stoner, Wang, & Casto, 2008). However, the functionality of those protective enzymes in human body has not been proven (Stoner et al., 2008). In fact, there is evidence that anti-oxidative potential of berries in human may be questionable (Stoner et al., 2008). Animal studies indicate that berry extracts can inhibit carcinogen-induced tumor formation in models of cancers of colon, breast, oral cavity and esophagus. Epidemiological studies on association of berry consumption and cancer risk in human is rather new area partly due to the investigation of fruits and berries under the same category (Pajari, 2010). Clinical data indicated that berry bioactives may prevent cancer formation in colon, oral cavity and esophagus supporting the data from animal studies (Stoner et al., 2008; Stoner, 2009).

Vast amount of preclinical and epidemiological data indicating health benefits of polyphenols have not only increased the interest in polyphenol-rich diet but also created

a business of dietary supplements. The origin of natural products has been widely taken as a quarantee for their safe use (Ulbricht & Chao, 2010). However, as discussed above, there are still many uncertainties and caveats in herbal medicine research that need to be addressed before their use is regarded safe. In fact, long-term supplementation may even increase the risk for cancer (Ulbricht & Chao, 2010). Potential carcinogenic effects have to be considered; for example use of phytoestrogens in estrogen-receptor positive breast cancer patients may include risks (Ulbricht & Chao, 2010). Furthermore, there are indications that flavonoids may be pro-oxidative at high concentrations. Quercetin is abundantly used as a dietary supplement but is also known to form mutagenic metabolites in pro-oxidative reactions which result in formation of mutagenic or carcinogenic adducts with DNA and proteins. On the other hand it has been stated that before DNA adduct stability *in vivo* is known, investigations on mutagenicity *in vitro* may not be used for risk prediction in human (Rietjens et al., 2005). Furthermore, flavonoids' bioavailability is low and therefore, the genotoxic concentrations observed *in vitro* may not be relevant. Inadequate quality and information of dietary supplement content and concentrations are also considered problematic (S. F. Zhou et al., 2007). Importantly, it has been shown that herbal medicines may interfere with the metabolism of conventional drugs. These herb-drug interactions result from the fact that phytochemicals, including flavonoids, modulate the levels of enzymes of cytochrome P450 (CYP) family and function as CYP inhibitors or activators depending on flavonoid structure and concentration (Hodek, Trefil, & Stiborova, 2002). These interactions may inhibit carcinogen activation but may enhance elimination of the conventional drugs or even lead to toxicity (Hodek et al., 2002).

In light of the existing data on bioavailability and high concentrations needed in animal studies, use of flavonoids as a therapeutic means most likely requires optimized delivery systems (Lamoral-Theys et al., 2010). The benefits of polyphenol-rich diet are widely accepted but recommendations of intake are still to be awaited. Especially, it is possible that intake of polyphenolic dietary supplements causes adverse effects as it may cause unknown flavonoid-flavonoid interactions and affect pharmacokinetics of traditional drugs (Egert & Rimbach, 2011). Effects of phytoestrogen supplements or soy infant formula on estrogen-responsive breast cancer patients and neonates, respectively, should be carefully considered (Stopper, Schmitt, & Kobras, 2005). Before used in clinics, long-term effects of phytochemical supplementation need to be characterized. Furthermore, reliable biomarkers are needed for efficacy evaluation. Those markers are potentially derived from proteomic or metabolomic profiling of treated patients (Scott et al., 2009).

3. AIMS OF THE STUDY

Cells possess an evolutionary conserved checkpoint termed the SAC that regulates the fidelity of chromosome segregation in order to preserve genomic stability. Weakened SAC may result in gain or loss of chromosomes (aneuploidy) which is a hallmark of cancer cells. On the other hand, while SAC errors may promote tumorigenesis, cell line studies and animal models have shown that total ablation of the mitotic checkpoint is detrimental to cell viability. Therefore, SAC abrogation may hold a therapeutic value. One important class of cancer drugs rely on inhibition of MT function in cells. However, these tubulin targeting drugs cause severe side-effects in patients due to perturbation of essential MT functions throughout the human body and not only in the dividing tumour cells. This and other reasons such as development of resistance to the anti-cancer treatments have created a need for discovery of new anti-neoplastic compounds that would possess more cancer cell selective mechanism of action. It is currently thought that inhibition of SAC function may improve the efficacy and alleviate the problems associated with MT-targeting drugs. The purpose of this study was to identify novel SAC-inhibitory hit compounds for drug development.

The specific aims of this thesis were:

1. To identify novel, low molecular weight inhibitors of the SAC using cell-based HTS.

2. To characterize the cellular phenotypes of the newly discovered compounds in details.

3. To identify the mitotic target(s) and the mechanisms of action of the most potent anti-SAC compounds.

4. MATERIALS AND METHODS

More detailed information on methods is available in the original publications (I-IV).

Cell line	Source	Used in
HeLa	human cervical adenocarcinoma	I, II, III, IV
HeLa H2B-GFP	human cervical adenocarcinoma	I, II, III
A549	human lung adenocarcinoma	I, II, III
DU145	human lung adenocarcinoma	I, II, III
PC3	human prostate adenocarcinoma	I, II, III
MCF-7	human breast adenocarcinoma	I
MCF-10A	breast epithelial	I, II, III, IV
22Rv1	human prostate adenocarcinoma	II
LnCaP	human prostate adenocarcinoma	II

Chemicals/reagents	Application	Supplier	Used in
Nocodazole	mitotic arrest	Sigma	I, II, III
Taxol	mitotic arrest	Molecular Probes	I, II, III
MG132	proteasome inhibition	Sigma	I, II, III, IV
Monastrol	monopole induction	Sigma	I, II, III
Vinblastine	mitotic arrest	Sigma	I, II, III
Mitomycin C	G2 arrest/Chk1 activ.	Sigma	IV
Thymidine	G1/S arrest	Sigma	IV
ZM447439	Aurora B inhibition	Tocris	I, II, III, IV
MLN8054	Aurora A inhibition	Selleck	II, III, IV
Fisetin	experimental drug	Sigma	I
Eupatorin	experimental drug	ExtraSynthese	II
SACi2	experimental drug	ChemDivIncorp.	III, IV
zvad-FMK	caspase inhibition	Calbiochem	III, IV
Staurosporine	apoptosis induction	Sigma	II, III
DAPI	DNA staining	Molecular Probes	I, II, III
SyberGold	DNA staining	Molecular Probes	I, II, III
Doxorubicin	Apoptosis/G2 arrest/Chk1 activ.	Sigma	IV
SB-218078	Chk1 inhibition	Calbiochem	IV
Ro-31-8220	HTS positive control	LC Laboratories	I, II, III
Crystal violet	Colony stain	Sigma	III

Antigen	Species	Supplier	Used in
Aurora A	rabbit	Abcam	I, III
Aurora B	rabbit/mouse	Abcam/BD Biosciences	I, II, III
Aurora A pThr288	rabbit	Cell Signaling Tech.	II, III
Aurora B pThr232	rabbit	Rockland	II, III
Bub1	mouse	Upstate	I, III
BubR1	mouse	Abcam	I, II, III
Cdc25C pSer216	rabbit	Cell Signaling Tech.	IV
Cdc27	mouse	Dr. P. Hieter	I
CenpF	mouse	BD Transduction Laborat.	I, III
Cenp A pSer7	rabbit	Upstate	I, II, III
Chk1 pSer296	rabbit	Cell Signaling Tech.	IV
cleaved PARP	mouse	Cell Signaling Tech.	II, III, IV
CREST	human	Antibodies Incorporated	I, II, III
Cyclin B1	mouse	BD Transduction Laborat.	I, III
GAPDH	mouse	Advanced Immunochemicals	II, III, IV
Hec1	mouse	Abcam	I, II, III
Histone H3 pSer10	rabbit	Upstate	I, III
Histone H2AX pSer139	mouse	Upstate	IV
INCENP	rabbit	Abcam/Dr. E. Nigg	II, III
NuMA	mouse	Dr. M. Kallajoki	III
Pericentrin	rabbit	Abcam	II, III
p21	rabbit	Cell Signaling Tech.	IV
Survivin	rabbit, mouse	Abcam	II, III
Tubulin alpha, DM1A	mouse	Abcam	II, III
Tubulin gamma	mouse	Abcam	II, III
Tubulin alpha YL1/2	rat	Abcam	I, III

Library	Library size	Supplier
ChemDiv	25 000	ChemDiv Incorporated
ChemBridge	30 000	ChemBridge Corporation
IBS Natural products	2 000	InterBioScreen
Spectrum Microsource	2 000	Microsource Discovery Systems
Tripos	6 000	Tripos International

Methods	Used in
Cell culture	I, II, III, IV
Immunofluorescence	I, II, III
Live cell microscopy	I, II, III, IV
Western blotting	I, II, III, IV
Flow cytometry	I, II, III
High-throughput screening	I, II, III
In vitro kinase assay	I, II, III
In vitro tubulin polymerization assay	II, III
3D organotypic cell culture	II
Colony formation assay	III
Monastrol washout	II, III
Cold Ca lysis	II

Equipment	Manufacturer	Used in
Tecan PW384 plate washer	Tecan	I, II, III
Zeiss Axiovert 200M microscope	Zeiss	I, II, III
Odyssey infrared imaging system	LI-COR Biosciences	I, II, III, IV
BD FacsArray™	BD Biosciences	I, II, III
LSR II flow cytometer	BD Biosciences	II
Incucyte live cell imager	Essen Instruments	IV
Acumen cell cytometer	TTP LabTech Ltd	I, II, III

5. RESULTS AND DISCUSSION

5.1 Phenotypic screen for inducers of a forced mitotic exit (I, II, III)

Recent preclinical and clinical data on novel experimental LMW inhibitors of cell division regulators supports the concept of mitosis as anti-cancer drug target. This promising outcome encourages for design and execution of new HTS to discover novel anti-mitotic compounds. We developed a phenotypic HTS with the aim to identify LMW inhibitors of the SAC that can induce loss of cancer cell viability. The published results of this study are presented in this thesis. Unpublished results are also included (figures are found after the original publications). The screen set-up, a modified version of the screen originally developed by our collaborator Gary Gorbsky (OMRF, OK, USA) (DeMoe et al., 2009), relied on the observations that mitotic, round-shaped HeLa cells attach loosely to the substratum, whereas flat interphase cells attach tightly. The SAC was hyperactivated with a low nocodazole concentration, which partially depolymerized MTs and accumulated the cells at M phase. After shake-off and re-plating, the mitotic cells were exposed to LMW compounds for 4 h. Subsequently, DNAase treatment and washing step removed sticky mitotics and cells that remain in mitosis in the presence of the compounds (Fig. 9). Finally, the cells were stained with a nucleic acid stain and fluorescence of the wells was measured. High fluorescence was an indicator of a large cell number and thus, suggested that the cells in these wells had exited mitosis.

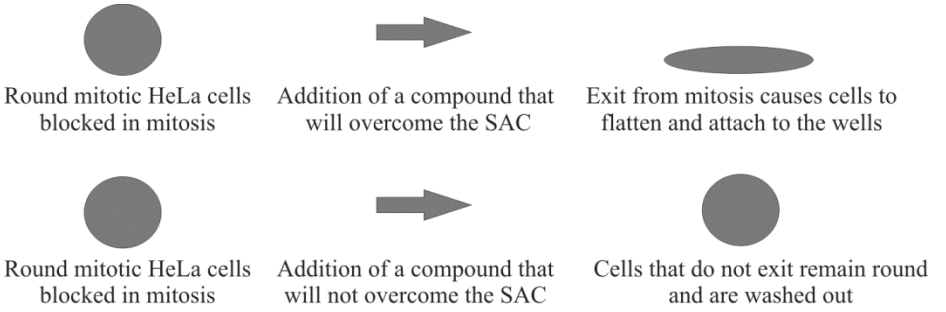

Figure 9. The principle of screen for identification of LMW inhibitors of the SAC

In the HTS of 65 000 LMW compounds from five libraries, we identified altogether eight compounds causing a forced mitotic exit, a phenotype that was validated in secondary screens (I, II, III and unpublished results). Structures and source libraries are depicted in unpublished results Fig. 1. Using the same HTS platform, our collaborator Gary Gorbsky published their results on aminothiazole derivative, named OM137, as an inhibitor of Aurora B (DeMoe et al 2009) which provides further proof-of-concept for the HTS methodology employed in this thesis work.

Cell-based screens typically measure changes in reporter genes (expression and/or localization), post-translational modifications of proteins, or viability (Aherne et al., 2002). A phenotypic approach could, in principle, also enable identification of novel SAC inhibitors targeting essential proteins within the signaling pathway. In comparison to target-based *in vitro* screens, cell-based screens possess several advantages in drug discovery in general. The effective compounds can penetrate through the cell membranes and target the substrates in their natural biochemical environment (Aherne et al., 2002). Importantly, the toxicity can be observed at the early stages of the study (Soleilhac, Nadon, & Lafanechere, 2010). These are notable advantages in drug discovery. Phenotypic screens are not restricted to one target or end-point measurement but allow broader analysis of the signaling cascade and possibly identify also novel components of the anticipated target pathway. At the time my Ph.D. project was started, phenotypic HTS, to our best knowledge, had not revealed any LMW inhibitors of the SAC. Instead, phenotypic screens had identified novel mitosis-targeting compounds, such as Eg5 inhibitor monastrol (Mayer et al., 1999), Aurora B inhibitor hesperadin (Hauf et al., 2003) and Mps1 inhibitor cinreasin (Dorer et al., 2005), all of which perturb normal mitotic progression. A few years from the start of my project, a HTS for premature mitotic exit causing compounds was described in which the end-point was mitotic index measured using MPM2-FACS analysis (Stolz et al., 2009). The group identified an indolocarbazole Gö6976 abrogating the function of Aurora A and B kinases. Recently, a screen comparing viability of taxol alone and in combination with LMW compounds identified a novel series of Aurora inhibitors (Kwiatkowski et al., 2012).

High scaffold diversity of the compounds included in the screen likely increases the probability of hit identification (Koehn & Carter, 2005; Mishra et al., 2008). We utilized high-quality libraries in which the compounds were either purely synthetic (ChemDiv, ChemBridge and Tripos libraries of 25 000, 30 000 and 6000 compounds, respectively), of natural origin (IBS Natural Compounds library, 2000 compounds), or combination of both types (Spectrum Microsource, 2000 compounds). Natural products are thought to possess extremely high chemical diversity and they have a profound history in drug discovery and development. The natural compounds of IBS library were isolated from variety of sources including many plants, micro-organisms, and marine species. In addition, the library included derivatives and analogues of natural compounds. Spectrum collection was comprised of drugs (50 %), highly divergent natural products (30%) and other bioactives (20%). Chemical properties of the compounds in libraries are important as high charge, reactivity, alkylation capacity, or toxicity among other factors will often lead to false positives (Aherne et al., 2002). Lipinski "rule of five" describes a set of physicochemical properties (molecular weight $\leq$ 500, hydrogen bond donors $\leq$ 5, hydrogen bond donors and acceptors $\leq$ 10, and partition coefficient log $P < 5$) that are statistically shared by drugs (Lipinski et al., 2001). High proportion of compounds that obey "the rule of five" can be considered to increase the quality of the library in drug discovery (Aherne et al., 2002). Despite the diversity of the libraries, our hit rate in the HTS (~0.01%) was 10-fold lower than the rate considered typical (Aherne et al., 2002). The rate is also influenced by the drug concentration which is often a compromise between high cytotoxicity and low efficacy (Schriemer et al., 2008). Even though our

HTS included a range of concentration from 0.05 to 60 μM it is possible that some of the hits were missed because of the aforementioned reasons. In cell-based screens the choice of cell line is of crucial importance. We used HeLa cells because this cell line has a robust SAC and round cellular morphology in mitosis which are optimal properties regarding the set-up of the screen. Amount of false positives is generally minimized by performing a secondary screen using the same assay set-up. Similarly, in our HTS this step was found to be of great importance. Fluorescence plate reader-based end-point measurement was certainly a fast and non-laborious method but since it cannot differentiate between cells in interphase, apoptosis and mitosis, microscopic evaluation was required to minimize the amount of false positives. Most likely, the automated HTS microscopic approach could have identified more hit compounds because the image-based screening produces data rich of biological information and enables measurement of morphological features at a single cell level. Because of the high efficacy and speed, microscopy-based HTS is becoming a method of choice in cell-based screens (Emery et al., 2011).

5.2 Inhibition of the SAC (I, II, III)

In order to study the cellular effects of the identified compounds in live cells, we utilized live cell imaging of HeLa H2B-GFP cells. We studied initially seven LMW compounds (unpublished results Fig. 1, A-H except D which was not commercially available at the time). For more detailed analyses we selected five most effective ones that caused a rapid and prominent mitotic exit. Data on one of these compounds, SACi3, (unpublished results Fig. 1, compound H) are only preliminary and further investigations would be required for drawing further conclusions on the effects of the compound. Notably, the chemical structure of small compound D was similar to JNK inhibitor SP600125 (Bennett et al., 2001), an anthrapyrazolone, which was later recognized as inhibitor of Mps1 (Schmidt et al., 2005). However, our hit compound has a double bond between two nitrogens and a hydroxyl group in position of hydroxy group of SP600125. It would have been interesting to study how D affects mitosis and which kinases are involved in the observed phenotype but the compound was not anymore available by the library provider at the time we wished to purchase more of it. The live cell analysis confirmed that the flavonoids fisetin (I, Fig. 2A) and eupatorin (II Fig. 2A), as well as SACi2 (III Fig. 1B), SACi3 (unpublished results) and SACi4 (unpublished results) consistently induced a rapid flattening of round mitotic cells indicating exit from mitosis. Concomitant decrease in cellular levels of Cenp-F and Aurora A, proteins mainly expressed in mitotic cells, confirmed that fisetin (I Fig. 2C) and SACi2 (III Fig. 1D) induced exit from M phase and not only decondensation of chromosomes. The SAC targets APC/C, a mitotic ubiquitin ligase, whose activity is required for proteasomal degradation of anaphase inhibitors (Musacchio & Salmon, 2007). To clarify whether the targets of our exit-causing small compounds reside up- or downstream of APC/C, we investigated whether the exit was dependent on proteasome activity. Indeed, all the compounds failed to cause exit from nocodazole-induced mitotic arrest in the presence of a proteasome inhibitor,

MG132 (I, II Fig. 2A-B, III Fig. 1B-C, SACi4 unpublished results), as expected when the compounds inhibit proteins that regulate the SAC activity.

Cells are thought to monitor both MT-KT attachment and the resulting inter-KT tension and activate the SAC as a response to lack of these conditions (Maresca & Salmon, 2010; Zich & Hardwick, 2010). There is a model of two arms of the SAC, one arm being responsive to the loss-of-attachment and the other to the loss-of-tension (Morrow et al., 2005). In an attempt to investigate how the identified small compounds affect these proposed two arms of the SAC, we added the compounds to the cells exposed to different chemicals hyperactivating the SAC. Low concentration of nocodazole influences the MT-attachments, however, without full depolymerization of MTs (Jordan, Thrower, & Wilson, 1992). In contrast, MT-stabilizing drug taxol and Eg5 inhibitor monastrol preserve the majority of attachments but prevent generation of inter-KT tension. We observed a rapid forced mitotic exit in all these conditions (I Fig. 2B, II Fig. 2B, III Fig. 1C, SACi3 and SACi4 unpublished results). However, all hit compounds except fisetin were unable to overcome the arrest induced by high concentration of MT depolymerizing drugs nocodazole and vinblastine (I Fig. 2B, II Fig. 2B, III Fig. 1C, SACi3 and SACi4 unpublished results), suggesting that these small compounds could not override SAC induced by lack of MT attachment. Accumulating evidence indicates that Aurora B is required for the SAC activity (Vader & Lens, 2008). However, there is an ongoing debate, whether the effect is direct or reflecting the Aurora B-mediated error correction of the faulty MT attachments during which unattached KTs are transiently generated (Maresca & Salmon, 2010; Nezi & Musacchio, 2009; Pinsky & Biggins, 2005). Aurora B inhibitors have been consistently shown to cause a rapid exit from taxol-induced mitotic arrest (Ditchfield et al., 2003; Hauf et al., 2003). In contrast, nocodazole-induced arrest was not overridden (Girdler et al., 2006) or was abolished only when the cells were arrested for long periods of time (Ditchfield et al., 2003). Considering the nocodazole concentrations used in those studies, the results are in line with our results showing that eupatorin (II Fig. 2B), SACi2 (III Fig. 1C), SACi3 and SACi4 (unpublished results) forced the cells out of mitosis only when the nocodazole concentration was low. It is possible that fisetin-induced exit from both taxol and nocodazole (I Fig. 2B) reflects Aurora B-independent mechanism. We also speculate that this could be due to differences in the effective concentrations of fisetin and established Aurora inhibitors, a notion supported by a study showing similar phenomenon when the concentration of ZM447439 was increased over 10 μM (Ditchfield et al., 2003).

In conclusion, using the cell-based HTS we identified several small compounds which all forced the chemically arrested cells out of mitosis. Inhibition of the proteasome activity completely prevented the premature mitotic exit indicating that the targets reside within the SAC. Our screens were performed on mitotic cells arrested with a low concentration of nocodazole resulting in partial depolymerization of MTs (Jordan et al., 1992). The condition can be hypothesized to enable identification of compounds targeting proteins from both arms of the SAC. Only fisetin overrode the SAC induced by lack-of-attachment suggesting that the small compounds primarily abrogated tension-sensing arm of the SAC. Flavonoids generally exert their effects on cell cycle at rather high concentrations,

typically concentrations reported in literature being in high μM range. In concordance, fisetin and eupatorin both required high concentration to elicit efficient override of the SAC as indicated by forced exit induced at 30 and 50 μM, respectively. This might reflect high metabolism of flavonoids or other properties influencing the intracellular accumulation/activities.

5.3 KT accumulation of SAC proteins (I, II, III)

SAC signals are generated at KTs from which they are thought to diffuse throughout the cell to prevent precocious exit from M phase. According to the basic principle of the SAC, the KTs that are unattached and/or in physically relaxed state recruit various proteins which keep the SAC activated. To clarify the mechanism of the forced mitotic exit and SAC abrogation, we quantified the amount of KT bound BubR1 in mitotic cells with hyperactivated SAC. The KT level of the protein was dramatically decreased by 94%, 82% and 86% in the presence of fisetin (I Fig. 4A-B), eupatorin (II Fig. 3A) and SACi2 (III Fig. 2A-B), respectively. Mad2 levels at the KTs were not determined due to the technical problems with the antibody stainings. Besides BubR1, a member of the inhibitory complex MCC, we also investigated the localization of Aurora B and Bub1 which reside upstream in the signaling pathway (Vigneron et al., 2004). We found a notable decrease in Bub1 KT intensity in the presence of the abovementioned three hit compounds (I Fig. 4A-B, eupatorin unpublished results, III Fig. 2A-B). Interestingly, all the small compounds mislocalized Aurora B (I Fig. 4A, II Fig. 3B, III Fig. 2C, unpublished results Fig. 2). Survivin and INCENP, which are subunits of the CPC together with Aurora B and Borealin (Ruchaud, Carmena, & Earnshaw, 2007a), were also mislocalized from inner centromeres to chromosome arms (II Fig. 3B, III Fig. 2C, unpublished results Fig. 2). Our results indicated that the compounds perturbed KT localization of key SAC proteins which was likely contributing to the observed phenotype in these cells. The result is in line with the earlier findings showing that Aurora B kinase is required for accumulating BubR1 at KTs upon SAC activation (Vader & Lens, 2008). Moreover, Aurora inhibitors ZM447439 and Hesperadin caused a similar reduction of BubR1 at KTs (Ditchfield et al., 2003; Hauf et al., 2003). The mislocalization of the CPC is not a hallmark of Aurora inhibition: Aurora B and Survivin were reported to be retained at centromeres in the presence of ZM447439 (Ditchfield et al., 2003). However, in our hands the inhibitor mislocalized the complex (III Fig. 2C).

5.4 Molecular targets (I, II, III)

5.4.1 Aurora kinases

Cell-based screens typically lead to challenging target identification process, which is one of the reasons for the popularity of target-based screens (Perrimon et al., 2007). The effective concentrations of hit compounds from cell-based screens are typically relatively high, meaning that the interactions with the targets are weak which hampers

the affinity-based target identification (M. J. Evans et al., 2005). We used candidate-based target identification approach because there are only a limited number of mitotic kinases whose inhibition could abrogate a chemically-induced mitotic arrest. Although Cdk1 inhibition phenotype is SAC abrogation, we demonstrated that our small compounds induced a forced mitotic exit in a proteasome-dependent manner suggesting that the exit was not a consequence of Cdk1 inhibition (Potapova et al., 2006). The observations on Aurora B mislocalization and the forced exit from taxol-induced mitotic arrest urged us to investigate whether the activity of Aurora B was affected. Aurora B creates specific phosphorylations on two proteins in nucleosome structure, namely on CenpA Ser7 (Zeitlin, Shelby, & Sullivan, 2001) and on Histone H3 Ser10 (Hsu et al., 2000). According to current knowledge, these epitopes can be used as markers of the kinase activity the latter being suggested to have also potential as a biomarker in clinical samples (Soncini et al., 2006). Immunofluorescence analysis of pHistone H3 showed that fisetin (I Fig. S2) and SACi2 (III Fig. 3B) abrogated the epitope phosphorylation, the signal being decreased by 92%, and 72%, respectively. Western blotting analysis confirmed that SACi2 inhibited Histone H3 Ser 10 phosphorylation (Fig. 3C). Furthermore, fisetin (I Fig. 4A-B), SACi2 (III Fig. 3B) and eupatorin (II Fig. 3A) dramatically inhibited CenpA phosphorylation on Ser7. All these compounds decreased the epitope phospho-signal by > 90 %. SACi4 effects on the phospho-signal were moderate (unpublished results Fig. 3).

These effects of fisetin, eupatorin, SACi2 and SACi4 were comparable to those of Aurora inhibitor ZM447439 that we used as a reference compound in our studies (I Fig. 4A-B, S2). Because well-established Aurora B inhibitors are known to abrogate phosphorylation of these substrates (Soncini et al., 2006), we concluded that the kinase was a common target for these LMW compounds. Downregulation of Aurora B autophosphorylation on Thr232 (Walter et al., 2000) supported the notion that one target of the compounds is Aurora B (II Fig. 3C, III Fig. 3B, unpublished results Fig. 4C). The antibody signals were detected not only at KTs but also at the poles. The same phenomenon was also observed by others (Posch et al., 2010) and was suggested to result from unspecific detection of Aurora A (Posch et al., 2010). However, the signal was not abolished when Aurora A was depleted using RNAi (our unpublished observation) and therefore, it is possible that the signals reflect the detection of other antigens consistent with the unspecificity we observed in Western blots (unpublished results). Alternatively, it is possible that a minor fraction of Aurora B phosphorylated on Thr232 locates to the poles and is detected with the antibody. However, this is unlikely as no earlier study has reported Aurora B pole localization. Interestingly, eupatorin did not notably affect pHistone H3 levels (unpublished results), although downregulated the two other markers of Aurora B activity.

Aurora kinase inhibitors identified to date are ATP-competitors. The ATP-binding pockets of Aurora A and B are nearly identical with only 3 out of 26 residues around the pocket differing between the kinases (Brown et al., 2004). This in mind, we were interested in investigating whether fisetin, eupatorin, SACi2 and SACi4 could modulate also Aurora A. The kinase possesses autophosphorylation site Thr288 which is a marker for activity

(Walter et al., 2000). The phosphorylation of the epitope decreased dramatically at poles in the presence of SACi2 (down by 96%, III Fig. 3B) or a reference compound, Aurora A inhibitor MLN8054 (III Fig. 3B). Eupatorin (II Fig. 3D) and SACi4 (unpublished results Fig. 4A) effects on the epitope were moderate decreasing the signal by 57% and 20 %, respectively. In addition, we performed Western blotting which confirmed the effects on Aurora A activity (II Fig. 3D, III Fig. 3C, unpublished results Fig. 4B). Fisetin did not change the phosphorylation intensity of the kinase (I Fig. 4D). In conclusion, our cell-based assays indicated that the identified LMW compounds targeted Aurora A and/or Aurora B kinase. Notably, eupatorin (unpublished results) and SACi2 (III Fig. 3B-C) did not change the levels of Aurora kinases which could have explained the reduction in autophosphorylations. Aurora A and B inhibitor VX680 and Aurora A inhibitor MLN8054 were previously shown to cause a partial loss of the kinase from centrosome (Manfredi et al., 2007; Tyler et al., 2007). Notably, our compounds did not affect kinase localization (unpublished results).

Cell-based assays can be complicated by indirect effects which arise from drug-induced effects on upstream regulators. Aurora B activators include INCENP, Borealin and TD-60, whereas Ajuba, Tpx2, and Bora are activators of Aurora A (Carmena et al., 2009). To further clarify the mechanisms how our LMW compounds inhibited Aurora activity, we performed *in vitro* kinase assays. We demonstrated that all the compounds studied in the assay, namely fisetin (I Fig. 4C and Fig. S3), eupatorin (II Fig. 3C) and SACi2 (III Fig. 3A) inhibited Aurora B. IC_{50} value of fisetin was estimated to be 2 μM for inhibition of Aurora B which indicates that it is less potent in comparison to ZM447439 with the IC_{50} value of 0.13 μM (I Fig. S3). SACi2 had an IC_{50} value of 5 μM indicating potency similar to that of fisetin. Eupatorin was significantly less potent with IC_{50} value of approximately 20 μM. Notably, *in vitro* kinase assay was a simple and quick approach to show a direct inhibition, but it was limited by the selection of recombinant kinases available in house. Bub1, Nek2 and Mps1 are among other candidates whose activity in the presence of our small compounds would deserve investigation. Our *in vitro* kinase assay on Aurora A showed IC_{50} of >30 μM for fisetin (I Fig. 4C) and 15 μM for SACi2 (III Fig. 3A) supporting the results of the cell-based activity studies. Eupatorin was not included in Aurora A *in vitro* assay. Collectively, our results indicate that fisetin inhibits preferentially Aurora B, whereas eupatorin, SACi2 and SACi4 inhibit both Aurora A and B kinases. Although eupatorin slightly modulated Aurora A activity in cells, the compound was clearly more potent against Aurora B. The data demonstrates that fisetin and SACi2 are most potent Aurora B inhibitors identified in this study. Moreover, of these compounds SACi2 is the most effective Aurora A inhibitor. We propose that eupatorin, SACi2, and SACi4 most likely inhibit Aurora A directly as supported by normal levels of Aurora A at poles, *in vitro* kinase assays (SACi2) and autophosphorylation (eupatorin, SACi2, SACi4). The potency against Aurora kinases was relatively weak suggesting that none of the compounds would be considered as a drug candidate without further chemical optimization. However, fisetin being a dietary flavonoid may contribute to human health as a constituent of diet rich in fruits, vegetables and berries whose consumption is implicated in cancer prevention in wide number of epidemiological studies (Lamoral-Theys et al., 2010). Bisanilinopyrimidine scaffold present in SACi2 has been shown to

be well suitable for combinatorial synthesis (Aliagas-Martin et al., 2009) and therefore, analogues of SACi2 with enhanced potency could possibly be developed. In fact, a class of these 2,4-diamino-5-fluoropyrimidine derivatives were recently characterized as efficient inhibitors of Aurora kinases (Aliagas-Martin et al., 2009) which is interesting because they share a similar structural scaffold with SACi2. These compounds inhibited Aurora A with submicromolar IC_{50} values.

Although inhibition of protein-protein interactions could offer an alternative, indirect means to inhibit Aurora kinase activity, the approach in general is not trivial (Arkin & Wells, 2004) and for the time being, such allosteric inhibitors remain to be identified. Our results indicated that fisetin, eupatorin, SACi2 and SACi4 were capable of directly inhibiting Aurora B and all but fisetin directly abrogated autophosphorylation of Aurora A. Not only kinase activity but also localization of Aurora B and the other CPC members, survivin and INCENP, was abolished (II Fig. 3B, III Fig. 2C, unpublished results Fig. 2). Whether the complex remains intact in compound treated cells could be studied by pull-down assays. Delocalization of the intact, active complex leads to a disappearance of CenpA phosphorylation while pHistone H3 signals are preserved as shown by Becker and colleagues (2010). It was shown that intercalation of actinomycin D in DNA was capable of inducing a displacement of the active complex from centromeres, possibly due to alteration in tertiary structure of DNA (Becker et al., 2010; van der Waal &Lens, 2010). Interestingly, many flavonoids within flavones and flavonols subgroups, including fisetin, are known to intercalate DNA (Webb &Ebeler, 2004). We cannot exclude the possibility that fisetin could change CPC function by causing intercalation-induced changes in DNA. In our studies, we noticed that the flavone eupatorin induced a phosphorylation of Histone H2AX Ser139 in mitotic cells indicating DNA damage (unpublished results). Therefore, we suggest that besides its direct effects on Aurora B activity, eupatorin may inhibit CPC indirectly. Interestingly, one of the original hits SACi3 that in our preliminary assays decreased BubR1 and pCenpA at KTs (unpublished results) was actually later recognized as anthracycline aclacinomycin A (aclarubicin) known to intercalate DNA and cause chromosomal aberrations (Steinheider, Westendorf, & Marquardt, 1987). We suggest that also SACi3 effects on Aurora B could be at least partially indirect being a consequence of DNA damage which may prevent correct localization of the kinase. Interestingly, we found no decrease in pHistone H3 signals supporting indirect effect of SACi3 on Aurora B (unpublished results). Immunostainings of pAurora B Thr232 and pHistone H2AX would be required to investigate this issue further. A natural small compound, an angucycline jadomycin B, was recently identified as a direct Aurora B inhibitor (Fu et al., 2008). We propose that SACi3 is another example of Aurora B inhibitors belonging to polyketide secondary metabolites. Since the results are only preliminary further investigations are required to confirm this hypothesis correct.

5.4.2 Other kinases (unpublished results)

During our studies, we found that fisetin induced a phosphorylation shift in one of the APC/C subunits, Cdc27 (unpublished results). We propose this dephosphorylation could result from Cdk1 inhibition since APC/C is mainly phosphorylated by Cdk1, whereas

Plk1 may have a minor role (Kraft et al., 2003). Supporting this notion, fisetin was previously shown to target Cdk1 at high 60 μM concentration (L. Y. Lu & Yu, 2009) and was moderately inhibited *in vitro* in our assays at 30 μM concentration (I Fig. 4C) used also in cell-based assays.

In order to get an insight into other targets, fisetin, eupatorin and SACi2 were subjected to a kinase panel of non-mitosis specific kinases (unpublished results Table 1). The assay was performed by Millipore and paid for by Bayer Healthcare. Because of high structural similarity of Aurora A and B kinase domains and identity of residues around ATP-binding pocket (Aliagas-Martin et al., 2009), it is not surprising that our phenotypic screens identified LMW compounds inhibiting both kinases. Interestingly, especially SACi2, but also fisetin and eupatorin inhibited Aurora kinase C (unpublished results Table 1), a third kinase of the family with essential functions in spermatogenesis (Dieterich et al., 2007). It was previously shown that overexpression of KD Aurora C on Aurora B-deficient background phenocopied the deficiency of Aurora B function including cytokinesis defects and override of the SAC in response to lack of tension (Slattery et al., 2009). WT Aurora C overexpression rescued the phenotype suggesting overlapping functions of Aurora B and -C in mitosis (Slattery et al., 2009). Furthermore, indications of the proto-oncogenic nature suggest that Aurora C is a potential anti-cancer drug target (J. Khan et al., 2011; Tsou et al., 2011). Considering that Aurora C may be involved in the SAC function, the inhibition of the kinase may contribute to the phenotype of our compounds.

The kinase panel (unpublished results Table 1) indicated wide specificity of fisetin which is supported by the ample of evidence on flavonoids as multi-targeting inhibitors (Amin et al., 2009; K. W. Lee et al., 2011; Singh & Agarwal, 2006). SACi2 similarly inhibited multiple kinases. In fact, compounds with broad specificity are likely to provide benefits over the strictly specific ones (S. C. Gupta et al., 2010). It has been suggested that multi-targeting inhibitors, including the ones targeting Aurora kinases, may alleviate the problem of drug resistance decreasing its development or efficiently targeting the resistant mutants (Kollareddy et al., 2012; McLaughlin et al., 2010). Broad specificity is also expected to increase the efficacy of the treatment because tumorigenesis involves alterations of multiple proteins (S. C. Gupta et al., 2010; Kollareddy et al., 2012). On the other hand, multi-targeting inhibitors might increase the toxicity of the treatment. Aurora inhibitors generally appear to inhibit various kinases involved in different phases of tumor formation such as growth factor receptors KDR (VEGFR2), FLT3 and FGFR1, as well as JAK2 (Kollareddy et al., 2012). Also in our studies, the Millipore *in vitro* kinase panel indicated that fisetin and SACi2 inhibited KDR and JAK2 (unpublished results Table 1). There is evidence that Aurora kinase inhibitor R763/AS703569 which inhibits Aurora kinases, FLT3, and KDR, may have enhanced anti-cancer potential due to inhibition of kinases from different cell cycle phases (McLaughlin et al., 2010).

The *in vitro* kinase panel (unpublished results Table 1) indicated that SACi2 is a very potent inhibitor of Janus-activated kinase 2 (JAK2). The JAK-STAT signaling regulates proliferation in response to interferones, cytokines and growth factors and has been implicated in regulation of the cell cycle but the mechanisms of action are poorly defined

(Reiterer & Yen, 2006). Interestingly, it has been shown that inhibition of all JAK kinases induced failure of cytokinesis, polyploidy and downregulation of Mad2 expression suggesting that JAKs have important functions within mitosis (Reiterer & Yen, 2006). The data indicated that ERK upregulation could mediate the effects of JAK inhibition. The JAK family member responsible for the mitotic defects remains to be identified. The data did not indicate an obvious mitosis-regulating role for JAK2. However, considering that off-target effects may be associated with use of kinase inhibitors, it is difficult to exclude JAK2 from being a contributor in SACi2 phenotype. Depletion of each JAK individually by RNAi or comparison of kinase inhibitor effects to each other could be two different experimental approaches to address the question.

Fisetin inhibited eight kinases with submicromolar IC_{50} values, namely EGFR, PDGFR, KDR (VEGFR2), GSK3β, CaMK II δ, InsR kinase, Trk-A and PKA in Millipore *in vitro* kinase assays (unpublished results Table 1). The first three are growth factor receptors that are among major players in angiogenesis. Because blood vessel formation is essential for tumor growth and survival, the kinases are also attractive therapeutic targets (S. C. Gupta et al., 2010). Fisetin is known to have anti-angiogenic properties (Fotsis et al., 1997) which may involve stabilization of endothelial cell MTs (Touil et al., 2009). Furthermore, a recent study indicated that the flavonoid inhibited expression of angiogenic mediators VEGF and eNOS and inhibited formation of capillary-like tubular structures by endothelial cells (Bhat et al., 2012; Touil et al., 2011) as well as angiogenesis in mice (Touil et al., 2011). We speculate it is possible that inhibition of KDR (VEGFR2), PDGFR and EGFR as well as angiopoietin receptor Tie2 (Davis et al., 1996) also mediate the reported anti-angiogenic functions of fisetin. In fact, *in silico* screening recently suggested that fisetin binds to KDR (J. Y. Lee et al., 2009). Based on partially overlapping angiogenesis-related targets of fisetin, eupatorin and SACi2 (unpublished results Table 1), it would be interesting to see whether all these compounds have anti-angiogenic properties. In fact, there is evidence that wide range of nutraceuticals can inhibit angiogenesis (S. C. Gupta et al., 2010). The effects of our small compounds could be investigated for example by measuring proliferation and survival effects, levels of pro-angiogenic factors such as VEGF, and cell migration in HUVEC cells.

The finding that fisetin inhibited glycogen synthase kinase 3 (GSK3β) is especially interesting in context of the SAC and this thesis. There is evidence that inhibition of the kinase causes spindle abnormalities, chromosome alignment defects (Tighe et al., 2007; Wakefield, Stephens, & Tavare, 2003) and missegregation of chromosomes after a mitotic delay (Tighe et al., 2007). The delay likely arose from chromosome alignment defects and spindle abnormalities potentially due to altered function of the GSK3β substrates such as MAPs or downregulation of Aurora A levels (Fumoto et al., 2008; Ong Tone et al., 2010). Interestingly, the SAC response was weaker in the absence of GSK3β activity and the amount of Bub1 and BubR1 were decreased at aligned KTs (Tighe et al., 2007). The mechanism of SAC attenuation is to be revealed but was suggested to involve APC, a plus end stabilizing protein with a function in the maintenance of chromosomal stability. It was shown that especially when pre-phosphorylated by GSK3β, APC is phosphorylated by Bub1 and BubR1 *in vitro* (Kaplan et al., 2001). The role of this

modification is poorly understood (Tighe et al., 2007). Altogether, the reported functions of GSK3β encourage us to speculate that the kinase could be involved in a mitotic phenotype observed in our studies.

5.5 Mitotic progression and cytokinesis (I, II, III)

The spindle apparatus starts to form at NEB and is followed by the establishment of MT-KT attachments. The SAC is active from prophase to metaphase-anaphase transition and prevents a cell from undergoing a premature exit from mitosis in the presence of misaligned chromosomes. After showing that our LMW compounds overrode the chemically hyperactivated checkpoint, we were curious to investigate the effects on unperturbed cell population. Fisetin (I Fig. 3A-B), eupatorin (II Fig. 4A) and SACi2 (III Fig. 4) rapidly forced the cells out of mitosis, as expected. In sharp contrast, the cells that were at G2 upon addition of a compound to the culture medium, exhibited a moderate mitotic delay by SACi2 (NEB to completion of telophase 196±20 min, III Fig. 5C) and fisetin (unpublished results) or significant prolongation of mitosis by eupatorin (489±156 min, II Fig. 4C). SACi2 completely prevented formation of metaphase plate in these cells (III Fig. 5A-B). In live cell analysis, we noticed that fisetin did not totally perturb the chromosome alignment but unaligned chromosomes persisted longer in the vicinity of the spindle poles (unpublished results). Cells were mostly multipolar in the presence of eupatorin as indicated by the chromosome configuration. We concluded that the compounds interfered with chromosome alignment and caused a prolongation of mitotic progression upon acute treatment of cycling cells.

Besides determining the mitotic duration, live cell imaging of unperturbed cells allowed us to observe the effects of the compounds on cytokinesis. Aurora B regulates the localization and function of the central spindlin complex that regulates RhoA which in turn, controls the formation of contractile ring and cleavage furrow at the position of central spindle (Vader & Lens, 2008). Considering the high number of Aurora B substrates within cytokinesis, the kinase is a crucial regulator of proper separation of the two daughter cells. Consequently, inhibitors of Aurora B cause cytokinesis defect (Ditchfield et al., 2003; Hauf et al., 2003; Lens, Voest, & Medema, 2010). Indeed, fisetin (I Fig. 3A-B), eupatorin (II Fig. 4B) and SACi2 (III Fig. 4) inhibited normal cytokinesis in vast majority of the cells recapitulating the well-established phenotype of Aurora B inhibition. The cells initiated anaphase and cleavage furrow started to form but neither process was completed. In some cells, abscission of the furrow failed. Both types of defects resulted in formation of a polyploid daughter cell. These results are in contrast to SACi4 treated cells, the majority of which were able to undergo cytokinesis and form two daughter cells (unpublished results). The reason for the different phenotype despite inhibition of the same target, Aurora B, remains unclear. It was reported that Gö6976 (Stolz et al., 2009), an Aurora inhibiting indolocarbazole, did not inhibit cytokinesis. In this light, the late M phase phenotype of SACi4 is not exceptional for an Aurora inhibitor. Whether cytokinesis is perturbed could depend on Aurora B subcomplex selectivity of different drugs as speculated previously in context of Gö6976 (Stolz et al., 2009).

5.6 Polyploidy and apoptosis (I, II, III)

SAC function is critical for the maintenance of genomic stability. A well-established phenotype of Aurora B inhibition is polyploidy resulting from defective cytokinesis (Lens et al., 2010). Therefore, we hypothesized that a continued exposure of cells to our LMW compounds that prevent cytokinesis may induce deleterious effects on the cells' ploidy and cellular viability. To analyze the DNA content of the cells, we performed a fluorescence activated cell sorting analysis (FACS) using four cancer cell lines (A549, DU145, PC3 and HeLa) and one non-tumorigenic cell line (MCF-10A) exposed to fisetin (I Fig. 5), eupatorin (II Fig. 6A) and SACi2 (III Fig. 6A). Although the sub-G1 peak in FACS profile of PI-stained cells reflects cell death, additional methods are required to confirm the mechanisms of cell elimination. To this end, we detected induction of PARP cleavage in HeLa cells using Western blotting. Fisetin (I Fig. 5), eupatorin (II Fig. 6A) and SACi2 (III Fig. 6A) resulted in increase in sub-G1 cell population in all cancer cell lines which was shown to be due to apoptosis in HeLa cells (fisetin unpublished, II Fig. S4, III Fig. 6C). Caspase inhibitor zvad-FMK prevented SACi2-induced cell death of HeLa cells (III Fig. 6D) which further supports the conclusion that the compound triggered the apoptosis pathway. HeLa cells were very sensitive to cell death induced by eupatorin (II Fig. 6A, Fig. S4) and SACi2 (III Fig. 6A and C) as shown by high increase in sub-G1 peak and cleaved PARP intensity by three days of incubation with the compounds. Fisetin resulted in a dramatic loss of viability in A549 and DU145, whereas cell death in HeLa and PC3 cells was moderate (I Fig. 5). Eupatorin and SACi2 also suppressed viability of non-tumorigenic cell line, MCF-10A, as indicated by a prominent sub-G1 peak (II Fig. 6A, III Fig. 6A). In contrast, fisetin only marginally increased sub-G1 peak in MCF-10A (I Fig. 5). Interestingly, we found that SACi2 did not result in cleavage of PARP in MCF-10A cells despite the presence of sub-G1 population indicating that the mode of cell death was different in MCF-10A and HeLa cells. In conclusion, all the identified small compounds triggered cell death in all tested cancer cell lines. Our studies suggest that the fisetin preferentially suppressed viability of cancer cell lines proposing possible cancer cell specificity.

Eupatorin (II Fig. 6A) and SACi2 (III Fig. 6A) increased the number of cells within 4N population by day one in all the cell lines examined which was consistent with the observed M phase exit and a failure of cytokinesis. Interestingly, we found that eupatorin induced a transient mitotic arrest at day one which could partially explain the increase in 4N cells (II data not shown). We cannot explain why fisetin did not increase the amount of 4N MCF-10A or DU145 cells although the flavonoid could override the hyperactivated SAC in all cell lines tested (I). However, the lack of 4N increase could reflect different effects that fisetin may have on cytokinesis in different cell lines. Fisetin effects on unperturbed cells could be studied using live cell imaging. Especially PC3 cells were able to undergo several rounds of replication after the first erroneous mitosis as indicated by the appearance of 16N cells in the continued presence of eupatorin (II Fig. 6A). Both PC3 cells and A549 cells were able to continue cycling until 16N in the continued presence of SACi2 (III Fig. 6A). Interestingly, MCF-10A cells did not reach 16N DNA content over the course of the assay when treated with any of our compounds.

This suggests that non-tumorigenic cells may have a mechanism(s) restricting the proliferation after erroneous mitosis and that the cancer cells investigated in our studies have lost this mechanism. Furthermore, a moderate cell death but significant difference in cell number upon incubation with SACi2 in comparison to DMSO (III Fig. 6A and C) pointed to the possibility that MCF-10A cells underwent a cell cycle arrest or delay in growth. In fact, SACi2 retarded the growth of MCF-10A cells later shown by live cell analysis (unpublished results) and colony formation assay (III Fig. S3). Moreover, the cells were incapable of resuming growth when the compound was washed out (III Fig. S3). We suggest that SACi2 induces cellular senescence in the MCF-10A cells which is supported by the colony formation assay showing cells with extremely large cytoplasm. There was only a slight increase in polyploidy in MCF-10A over the time which is in line with the presented hypothesis.

Phytochemicals in general are able to induce apoptosis because they can modulate a plethora of cell survival and apoptosis-regulating proteins (N. Khan, Adhami, & Mukhtar, 2008). Fisetin has been shown to induce caspase activity via activation of ERK1/2 (Ying et al., 2012), decrease anti-apoptotic protein levels due to inhibition of NF-κB (J. Li et al., 2011; Sung, Pandey, & Aggarwal, 2007), upregulate pro-apoptotic proteins of Bcl-2 family (N. Khan et al., 2008), induce p53 (J. Li et al., 2011; Lim do & Park, 2009) and inhibit PI3K/Akt pathway (N. Khan, Afaq et al., 2008). Our studies indicated that polyploidy induction is a novel mechanism how fisetin and eupatorin could induce apoptosis. SACi4 caused a rapid cell death even though the majority of cell underwent normal cytokinesis suggesting that the loss of viability was not only a consequence of cytokinesis errors and polyploidy. When comparing the different cell lines, we did not find a correlation between the status of tumor suppressor p53 (Lane, 1992) and cell fate. This result is supported by the data from several other laboratories suggesting that the cell fate is not solely determined by the p53 status, although it appears to be involved in the response (Ditchfield et al., 2003; Dreier et al., 2009; Gizatullin et al., 2006; Kaestner et al., 2009). High sensitivity of HeLa cells to flavonoids observed in our studies resembled the previously reported effects of ZM447439 (Ditchfield et al., 2003). It was again proposed that p53-independent mechanisms were involved in the sensitivity on Aurora B inhibition (Ditchfield et al., 2003). Fisetin, eupatorin and SACi2 abrogated the SAC in all the cell lines used in the cell fate assays (I, II, III) indicating that the cells were not resistant to the compounds. We propose that these differences in the cell fate may involve variation in drug response pathways or variations of the drugs' mechanisms of action. We cannot explain the finding of eupatorin-induced transient M phase arrest in HeLa and MCF-10A cells. However, as eupatorin was shown to be metabolized by CYP1 enzyme in breast cancer cell line (Androutsopoulos et al., 2008) it could be speculated to involve cell type-dependent variations in the metabolism of eupatorin in different cell lines. Fisetin is also known to be extensively metabolized in cells due to its four OH groups (Touil et al., 2011). Fisetin effects in cells could therefore also reflect effects of the metabolites in each cell line. Regarding a therapeutic potential, a drug should cause minimum effects on normal cells but kill cancer cells. Fisetin caused less apoptosis in MCF-10A than in cancer cell lines pointing to the possibility of cancer cell specificity, a hypothesis that should be further tested using a broader panel of cell lines.

5.7 Spindle and centrosomes (I, II, III)

Fisetin, eupatorin and SACi2 prolonged mitosis (fisetin unpublished results, II Fig. 4C, III Fig. 5C) which we reasoned could result from the chromosome alignment defects we observed in live cell imaging. Aurora B has a profound role in correcting erratic attachments and inhibitors of the kinase interfere with chromosome alignment (Lens et al., 2010). Another obvious cause for incomplete chromosome alignment is abnormal spindle structure or function, a phenotype of Aurora A abrogation (Asteriti et al., 2011; Barr & Gergely, 2007; De Luca et al., 2008; Schmidt & Bastians, 2007). Therefore, we wanted to investigate the spindle effects of our compounds. To study the effects on spindle formation and maintenance, we added eupatorin (II Fig. 5A) or SACi2 (III Fig. S1A-B) either before mitosis or at metaphase, respectively. We then stained the cells with α-tubulin antibody as well as antibodies against a pole marker NuMA and a marker of centrosomal region pericentrin. Upon premitotic exposure to the compound, a vast majority of eupatorin treated cells formed multipolar spindles with several extra MT asters (satellite poles) and multiple centrosomes (II Fig. 5A). SACi2 cells had mainly bipolar spindles which, however, were abnormal being accompanied with multiple satellite poles (III Fig. S1B). Only half of the cells were able to form two centrosomes upon SACi2 treatment. When added on metaphase cells, eupatorin (II Fig. 5A) and SACi2 (III Fig. S1A) abolished normal spindle structure as indicated by the appearance of satellite poles around either bipolar or multipolar main spindle, respectively. Eupatorin did not abolish centrosome structure in these cells, whereas only half of the SACi2 cells preserved two centrosomes. In conclusion, we suggest that the mitotic delay induced by SACi2 and eupatorin in the cells exposed to the compounds at G2 could result from spindle anomalies and alignment defects. The same may apply to fisetin treated G2 cells. We propose that the defects evoked a SAC-mediated mitotic delay which was only transient in time due to inhibition of Aurora B. Similar phenomenon has been reported for a prototype of Aurora B inhibitors, ZM447439 (Girdler et al., 2006), Aurora A inhibitor MLN8054 (Hoar et al., 2007), as well as dual Aurora inhibitors VX680 (Tyler et al., 2007) and Gö6976 (Stolz et al., 2009). Spindle abnormalities are typical in cells deficient of functional Aurora A and it is highly likely that the anomalies seen in the presence of eupatorin and SACi2 were due to Aurora A inhibition. A delay in mitotic progression is also a characteristic phenotype of Aurora A inhibition (Lens et al., 2010). Whether the delay in the presence of our LMW compounds depends on the SAC could be studied further using chemical inhibition of Aurora B in delayed cells or by following compound effects in cells lacking critical SAC proteins, such as BubR1.

Spindle defects caused by our compounds deserve further discussion. Eupatorin and SACi2 induced formation of acentrosomal satellite poles which we suggest to reflect Aurora A inhibition: similar phenomenon was reported earlier for specific inhibitor MLN8054 (Hoar et al., 2007) and for *N*-phenyl-4-(thiazol-5-yl)pyrimidin-2-amines which inhibit both Aurora A and B (S. Wang et al., 2010). Consistent with the function of Aurora A in the centrosome separation (Glover et al., 1995), Aurora A inhibitors were shown to induce monopolarity and when the deficiency of the kinase function was milder result in shortened spindles (Kwiatkowski et al., 2012). However, even though eupatorin, SACi2 and SACi4 inhibited both Aurora A and B kinases, cells with one

pericentrin-positive centrosome were present only upon SACi2 or SACi4 addition. We observed cells with unseparated centrosomes in the cell population exposed to SACi2 before mitosis or in mitosis (III Fig. S1A-B) which suggested that the compound was able to inhibit both formation and maintenance of bipolar spindles. Spindle effects of SACi4 were especially interesting as we found that the compound induced a rapid and prominent collapse of the bipolar metaphase spindle into monopolar configuration: 83 % of the cells had monopolar spindles by 5 h after addition of the compound to cells arrested in mitosis (unpublished results Fig. 5). The phenotype is reminiscent of Plk1 inhibition (McInnes et al., 2005; U. Peters et al., 2006; Santamaria et al., 2007). However, compounds termed 1 and 32 inhibiting both Aurora A and B induced a similar phenomenon (Kwiatkowski et al., 2012) suggesting that decrease in Aurora A activity possibly contributed to the phenotype. Moreover, in our hands, MLN8054 caused a collapse of the spindle (unpublished results Fig. 5). Interestingly, spindles did not collapse upon eupatorin addition (II Fig. 5A) and the phenomenon was rare in SACi2 treated cells (III Fig. S1A). This suggests that Aurora A inhibition does not always lead to collapse of the mitotic spindle. We hypothesize that SACi4 may inhibit also additional protein(s) that are required for maintenance of spindle bipolarity. According to the current knowledge, a collapse of the spindle may result from decreased stabilization or increased destabilization of KT-fibers caused by alterations in proteins regulating depolymerization and polymerization such as MCAK or CLASP, respectively (Tillement et al., 2009). Kif2a (Ganem & Compton, 2004) is a kinesin-like protein whose MT-depolymerizing function at poles is required for MT flux in which MTs depolymerize at minus ends, subunits move towards poles and the generated tension induces MT polymerization at plus ends (Ganem, Upton, & Compton, 2005). Depletion of Kif2a is associated with collapse of the bipolar spindle (Ganem & Compton, 2004; C. Zhu et al., 2005). It was proposed that without functional Kif2a and therefore, without flux, the tension at KTs is lost which abrogates polymerization at plus ends. Eventually, depolymerization of KT-MTs results in collapse of the spindle (Ganem & Compton, 2004). Conditions that balance the loss of Kif2a activity such as the ones that abolish MT-KT attachments, or stabilize MT plus ends rescued the phenotype (Ganem & Compton, 2004). The potential role of Kif2a in SACi4-induced phenotype could be examined using similar sets of experiments as performed by Ganem and Compton (Ganem & Compton, 2004).

SACi4 induced another unique alteration of the spindle, namely formation of excessive astral MTs (unpublished results Fig. 6). Perturbation of Aurora B function by injection of function neutralizing antibodies induced growth of prominent astral MTs (Kallio et al., 2002) pointing to the possibility that Aurora B inhibition could explain this spindle phenotype. However, considering the reported phenotypes of multiple well-characterized inhibitors of the kinase, other mechanisms are more likely involved. Prominent astral MTs were observed in the presence of MG132 excluding the possibility that these changes were a consequence of initiated forced exit. MT length is balanced by the flux in which tubulin subunits are added at plus ends of MTs and removed at minus ends. Decreased MT depolymerization at poles could result from inhibition of the proteins that are crucial for the process such as Kif2a as discussed above (Ganem & Compton, 2004; Tillement et al., 2009). It was proposed that especially non-KT MTs were eventually elongated

because MCAK depolymerizes only KT-MTs (Tillement et al., 2009). Considering that the two phenotypes observed in the SACi4 treated cells, excess of astral MTs and monopolarity, have both been associated with deficiency of Kif2a function, contribution of the protein for the SACi4 phenotype should be examined in future.

A common phenotype to all our compounds was formation of extra MT-nucleating, NuMa positive foci or satellite poles outside the main spindle. Furthermore, cells had multiple or fragmented centrosomes when incubated with eupatorin or SACi2. All the LMW compounds inhibited Aurora B, and therefore, the deficiency of the kinase function could explain the spindle phenotype. However, to our best knowledge the main phenotype of Aurora B inhibition does not involve such extremely prominent defects in poles or centrosome structure. The abnormalities appeared within a short period (3 h) of compound addition excluding the impact via defective centrosome duplication or errors from previous mitosis. We suggest that the centrosome structure was less stable in the presence of SACi2 or eupatorin which caused the formation of extra poles in these cells. Accumulating evidence indicates that Aurora A is involved in the maintenance of pole integrity (Asteriti et al., 2011; De Luca et al., 2008; S. Wang et al., 2010) and a model for the kinase function has been proposed (Asteriti et al., 2011; De Luca et al., 2008). The model emphasized that Aurora A has a crucial role in regulating the spindle forces and that the imbalance of the forces has major consequences on the pole structure. When Aurora A was inhibited, MTs appeared to be hyperstable (Asteriti et al., 2011) and the generated polewards pressure possibly caused fragmentation of the poles. The altered forces appeared to arise from excess of TOG and decreased levels of MCAK at the poles (De Luca et al., 2008). Supporting the model, conditions that reduced the polewards pressure, such as Eg5 inhibition and TOG depletion (Asteriti et al., 2011), as well as disruption of MT-KT attachments using nocodazole (De Luca et al., 2008) rescued the phenotype of Aurora A inhibition and depletion, respectively. In our assays, Eg5 inhibitor monastrol rescued the centrosome effects of SACi2 and eupatorin (II Fig. S2, III data not shown). To conclude, we speculate that extra poles induced by these compounds involve changes in MT forces which are potentially due to Aurora A inhibition.

Various flavonoids including quercetin (K. Gupta & Panda, 2002), casticin (Haidara et al., 2006), genistein (Mukherjee et al., 2010) and 5,7,3'-Trihydroxy-3,4'-dimethoxyflavone (Torres, Quintana, & Estevez, 2011) perturb MT function by binding on tubulin. Fisetin was shown to retard MT depolymerization induced by cold treatment indicating that the flavonoid stabilized MTs (Touil et al., 2009). Interestingly, the study indicated that fisetin did not affect MT assembly but increased tubulin acetylation. *In vitro* tubulin polymerization assay is a generally used method to study direct effects on tubulin polymerization. Eupatorin did not have major effects on tubulin polymerization *in vitro* with any concentration tested (II Fig. 5C) indicating that tubulin was not a direct target of eupatorin. There is obviously a need to use cell-based and biochemical assays to investigate whether a compound has indirect effects on MTs. None of the small compounds investigated in the cold calcium lysis assay notably decreased MT-KT attachment stability in the main spindle (II Fig. S3, SACi2 and SACi4 unpublished results). To investigate whether hyperstabilization of MTs presented in the Aurora A inhibition model (Asteriti et al., 2011; De Luca et al., 2008)

was involved in fragmentation of poles and centrosomes observed in our studies, it would be interesting to determine the relative speed of cold-induced MT-depolymerization. In contrast to eupatorin, high concentrations of SACi2 changed tubulin polymerization *in vitro*: samples incubated with 0.5 and 5 μM SACi2 did not differ from control reaction but 15 and 25 μM SACi2 promoted *in vitro* polymerization of tubulin suggesting that SACi2 may also stabilize MTs in cells (III Fig. S2). Therefore, SACi2 may interfere with the structure and function of the mitotic spindle and centrosomes directly due to binding on tubulin and indirectly via inhibition of Aurora A.

Eg5 inhibitor monastrol causes monopolarity due to inhibition of centrosome separation and arrests the cells with syntelic attachments (Kapoor et al., 2000). The inhibition is reversible which can be utilized to study efficiency of attachment correction processes and reformation of bipolar spindle upon monastrol wash-off and Eg5 reactivation (Kapoor et al., 2000). We were curious to find out whether eupatorin or SACi2 disturb these processes and therefore, performed a drug wash-off experiment. The monastrol-arrested cells released into medium containing eupatorin (II Fig. 5B) or SACi2 (III Fig. S1C) were unable to form normal, bipolar spindles. In the presence of eupatorin, spindles mainly remained monopolar but accompanied with satellite poles (II Fig. 5B). Majority of the eupatorin treated cells had multiple centrosomes while a few had only one centrosome. Nearly all cells released from monastrol-induced arrest into SACi2 containing medium reformed bipolar main spindle but again exhibited with satellite poles (III Fig. S1C). Majority of these cells had two centrosomes similar to control cells. These results collectively suggest that eupatorin and SACi2 interfere with the reformation of bipolar spindles. Furthermore, we propose that eupatorin interferes with centrosome separation.

Aurora B has a key role in correction of erroneous attachments which is possibly regulated by the physical distance of the kinase from its substrates (D. Liu et al., 2009). When the attachments are erroneous (syntelic and merotelic attachments), the kinase is thought to reach its substrates such as Hec1 and decrease their binding affinity to MTs (Cheeseman et al., 2006; DeLuca et al., 2006). The detached MTs are then selectively depolymerized by MCAK which is under negative control of Aurora B (Andrews et al., 2004; Lan et al., 2004). A model has been proposed in which MCAK is activated by the collapse of the outer KTs or stretching of the centromere which enables PP1 to counteract Aurora B activity in the presence of merotelic or syntelic attachments, respectively (Gorbsky, 2004). Finally, the generated unattached KTs activate the SAC response. Yet, it is under debate whether the kinase has also a direct role in the SAC signaling. According to the current knowledge, Aurora B inhibition prevents correction of MT-KT attachment errors causing persistent chromosome alignment defects. Eupatorin and SACi2 perturbed normal chromosome alignment and spindle recovery upon Eg5 reactivation which is in line with inhibition of Aurora B.

5.8 Flavonoids and the cell cycle (I, II)

Regarding the anti-cancer effects of flavonoids, modulation of cell cycle progression is considered to be one of the key mechanisms of their action (Singh & Agarwal, 2006).

Knowledge on how flavonoids modulate mitotic kinases is, however, scarce. There is plenty of evidence that flavonoids induce arrest at different phases of the cell cycle by targeting Cdks: flavonoids directly inhibit Cdk activity, downregulate cyclin expression and upregulate expression of cyclin inhibitors among other mechanisms (W. Y. Huang, Cai, & Zhang, 2010; Lamoral-Theys et al., 2010; Shanmugam, Kannaiyan, & Sethi, 2011). Fisetin has been shown to induce G1 arrest for example in prostate cancer cells (N. Khan, Afaq et al., 2008) and melanoma cells (Syed et al., 2011). These effects on interphase cells could be mediated by inhibition of Cdk2, -4 and -6 (N. Khan, Afaq et al., 2008; X. Lu et al., 2005) and induction of p53 (Y. C. Chen et al., 2002). Furthermore, the flavonoid prevented mitotic progression of colon (X. Lu et al., 2005) and prostate cancer cells (Haddad et al., 2006). Considerably less data is available on the cell cycle effects of eupatorin, a methoxylated flavone identified in our HT screen. The flavonoid has a long history in traditional medicine (Androutsopoulos et al., 2008). Eupatorin prevented proliferation of human cancer cell lines including HeLa, gastric adenocarcinoma MK-1 (Nagao et al., 2002) and breast cancer cell line MDA-MB-468 (Androutsopoulos et al., 2008). The anti-proliferative effects were mainly associated with effects on enzymes of CYP family which is important for metabolism and detoxification of carcinogens and drugs but is also involved in activation of pro-carcinogens. Interestingly, one of the studies pointed to the possibility that the flavonoid inhibited proliferation of MDA-MB-468 but not MCF-10A because CYP1 enzymes are expressed at different levels in these cell lines (Androutsopoulos et al., 2008). Eupatorin inhibited CYP1 activity *in vitro* but was also readily metabolized by the enzyme in cells. In more specifics, it was shown that eupatorin underwent a bioactivation into more active metabolites in CYP1-expressing cell line, MDA-MB-468, but not in MCF-10A which lacks the enzyme. Collectively, it was suggested that while flavonoids can inhibit CYP family enzymes, they can also be substrates of the enzyme or have both characteristics. It is therefore possible that the metabolites of flavonoids account for some of the effects seen in our studies. The results on cancer cell specificity are in contradiction to our studies and further studies with larger cell line panels would be required to clarify the effects in more details. In our studies, eupatorin was anti-carcinogenic in 3D organotypic prostate cancer cell culture model in 22RV1 (II Fig. 6B) and in LNCaP (unpublished results). It is possible that CYP1 metabolism has a role in sensitivity observed in these cells. In order to test the hypothesis, it would be interesting to investigate whether CYP1 inhibitors affect the sensitivity of the cells to eupatorin.

Our studies indicate that the flavonoid effects on mitosis might be much more versatile than previously thought. We propose that the reports showing a fisetin-induced G2/M arrest based on the FACS profiles may in fact reflect the increase of polyploidy due to a forced mitotic exit. To our best knowledge, our study on fisetin provides the first indication of flavonoids being able to inhibit SAC function and Aurora kinase activity (Salmela et al., 2009). Recently, quercetin, a structural analogue of fisetin, was found to inhibit nocodazole and taxol from arresting cells in mitosis (Samuel et al., 2010). In fact, the flavonoid was later shown to be a relatively potent inhibitor of all Aurora kinases (Boly et al., 2011) a result likely explaining how the flavonoid abrogated nocodazole and taxol function. 3-hydroxyflavone was reported to inhibit Aurora B with IC_{50} of 1 μM

(Lang et al., 2010) which indicates similar potency with fisetin. A polyphenol curcumin delocalized Aurora B and inhibited the kinase indirectly by downregulating the expression of survivin (Wolanin et al., 2006). Direct inhibition remains to be resolved. Luteolin is the most recently identified Aurora B inhibitor belonging to the flavonoids (Xie et al., 2012). Altogether, flavonoids seem to be relatively weak inhibitors of Aurora B. Keeping in mind that these phytochemicals are highly similar in structure novel Aurora inhibitory functions may continue to be identified for other flavonoids.

5.9 DNA damage and Chk1 inhibition (IV)

It is thought that inhibition of Aurora kinase activity causes cell death because the cells undergo erroneous mitosis characterized by spindle anomalies and cytokinesis defects. To our surprise, SACi2 caused apoptosis not only in cells that had progressed through mitosis as shown earlier (III) but also in cells that were arrested at G1/S with a double thymidine block indicating that mitotic defects were not alone responsible for the SACi2-induced cell death. In fact, as shown by live cell imaging (IV Fig. 1 B, Suppl. video 1) and Western blotting detection of cleaved PARP (IV Fig. 1A), SACi2 sensitized thymidine-arrested cells to undergo cell death, whereas G1/S cells treated with Aurora inhibitors ZM447439 and MLN8054 retained viability without increase in PARP cleavage upon 6 h compound treatment (IV Fig. 1A). This suggests that sensitization was independent of Aurora kinase inhibition. The results encouraged us to seek for additional target(s) of SACi2 that could be required for the maintenance of cell viability upon replication stress. Literature search indicated that the sensitization phenotype was reminiscent of Chk1 inhibition (Bolderson et al., 2004; T. Chen et al., 2012). The kinase is crucial for DNA-damage checkpoints at S and G2/M phases upon genotoxic stress, is involved in SAC function and regulates initiation of mitosis and replication during unperturbed cell cycle (Fig. 10) (Garrett & Collins, 2011). The kinase induces degradation of Cdc25A and promotes sequesteration of Cdc25B and Cdc25C thereby inhibiting Cdk activities required at these cell cycle transition points (Bucher & Britten, 2008; T. Chen et al., 2012).

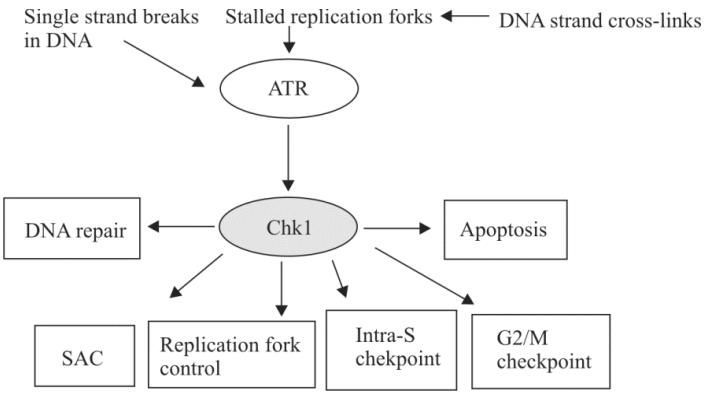

Figure 10. Functions of Chk1 (adapted from Garrett and Collins 2011).

Anti-cancer therapies are typically based on induction of DNA damage. Upon damage, cells normally activate the DNA damage response which leads to a checkpoint-mediated arrest allowing repair of the damage. It is thought that the survival of p53-deficient cells from DNA-damaging anti-cancer therapy is highly dependent on functional Chk1 which has led to a concept of Chk1 inhibition as a strategy to enhance treatment efficacy (Garrett & Collins, 2011; Massey et al., 2010). Doxorubicin, mitomycin C and excess of thymidine are genotoxic agents that activate DNA damage response and Chk1 (Blasina et al., 2008; Cho et al., 2005; Heffernan et al., 2009; Xiao et al., 2003). Doxorubicin intercalates into DNA and inhibits Topo II (Tewey et al., 1984), mitomycin C cross-links DNA (Ma, Janetka, & Piwnica-Worms, 2011) and thymidine induces replication fork stress (Bolderson et al., 2004). In the presence of these agents, SACi2 decreased Chk1 autophosphorylation at Ser296 (IV Fig. 2A-B and data not shown). As the epitope is a marker for the kinase activity (T. Chen et al., 2012; Clarke & Clarke, 2005), the observation supports our hypothesis that Chk1 is one of the targets of SACi2. The effect was relatively weak in comparison to commercial Chk1 inhibitor SB-218078 (Jackson et al., 2000) indicating that SACi2 was less potent inhibitor of Chk1. Supporting Chk1 inhibition, SACi2 also decreased the phosphorylation of Cdc25C on Ser216 in thymidine-arrested cells (IV Fig. 2C).

According to the current understanding, lethality of DNA-damaging drugs is potentiated in cells lacking Chk1 function (Bucher & Britten, 2008; T. Chen et al., 2012; Garrett & Collins, 2011; Ma et al., 2011). Similarly, Chk1 suppression seems to enhance the effects of anti-metabolites that inhibit DNA synthesis (Bolderson et al., 2004; T. Chen et al., 2012) consistent with our study which showed a synergism between SACi2 and thymidine. Thymidine is regarded as a weak inducer of cell death (Rodriguez & Meuth, 2006). However, thymidine-SACi2 co-treatment rapidly induced classical cell morphology changes associated with apoptosis which were confirmed with detection of PARP cleavage (IV Fig. 1 and 3A). Chk1 inhibitor SB-218078 also increased the cell death induced by thymidine (IV Fig. 3A) supporting the hypothesis of Chk1 as a contributor in SACi2 phenotype. Our findings resembled the ones reported previously by others: Chk1 inhibition or depletion sensitized the cells to anti-metabolites including thymidine (Rodriguez & Meuth, 2006; Sampath, Shi, & Plunkett, 2002; Z. Shi et al., 2001). These data provide further support for the notion that SACi2 sensitization may result from inhibition of Chk1. There is discrepancy whether p53-status is important in determining how the cells respond to genotoxic agents upon Chk1 inhibition (T. Chen et al., 2012). Our results suggested that p53 protects the cells from sensitization at G1/S transition because cell death was not induced when SACi2 was added to thymidine-arrested MCF10-A cells possessing a functional p53 (IV Suppl. video 2). Interestingly, SACi2 also sensitized HeLa cells to apoptosis upon release from thymidine-mediated arrest (IV Fig. 3B). Similar phenomenon was induced when Chk1-depleted cells were released from the replication block induced by ribonucleotide reductase inhibitor, hydroxyurea, or DNA-polymerase inhibitor, arabinoside-C (Cho et al., 2005). SB-218078 similarly induced cell death upon thymidine wash-off (IV Fig. 3B). More rapid induction of apoptosis by SB-218078 likely reflected different kinetics and/or stronger

inhibition of Chk1 as confirmed by much more rapid and profound attenuation of kinase autophosphorylation by SB-218078 (Fig. S1).

We showed that SACi2 increased phosphorylation of Histone H2AX on Ser139 (γ-H2AX), a marker of DNA double-strand breaks, in thymidine-arrested cells and also upon release from the arrest into SACi2-containing medium (IV Fig. 3D-E). The same phenomenon has been previously shown when Chk1 depleted cells were released from a replication block (Cho et al., 2005) and when gemcitabine or topoisomerase I inhibitor-induced replication block was abrogated by Chk1 inhibition (Ewald, Sampath, & Plunkett, 2007; Furuta et al., 2006). In those cells, the signal increased already before apoptosis induction which was an important finding because γ-H2AX is also upregulated in cells undergoing apoptosis. It was proposed that the increase reflected the role of Chk1 in preserving replication fork integrity to resume DNA replication and that inhibition of this function likely caused collapse of the replication fork and the loss of cell viability (Cho et al., 2005; Ewald et al., 2007). We suggest that the abovementioned mechanism is involved in the SACi2-induced cell sensitization. To be able to clarify the issue further, it would be important to exclude the possibility that changes in γ-H2AX were due to apoptosis-associated DNA fragmentation.

There is ample of evidence that Chk1 inhibition enhances the efficacy of genotoxic therapies that are based on the checkpoint response at G2. We demonstrated synergism between SACi2 and a DNA-crosslinking agent, mitomycin C (IV Fig. 3C, Suppl. video 3). Compared to mitomycin C-treated cells, the apoptosis-associated morphological changes occurred earlier and the cell death was more pronounced in the co-treated cell populations. These results indicated that SACi2 enhanced mitomycin C-induced cell elimination. Again, MCF-10A cells showed no notable sensitization to apoptosis (IV Suppl. video 4) which pointed to the possibility that p53 status affects the cell sensitivity to these conditions. It is believed that Chk1 inhibitors enhance the effects of DNA damaging drugs at G2 phase because they abrogate the G2 checkpoint and drive the cells into deleterious mitosis (Bucher & Britten, 2008; Ma et al., 2011). Whether SACi2-induced cell death was preceded by override of G2 checkpoint should be studied further although we could not observe cells entering mitosis in the presence of mitomycin C and SACi2 in our live cell analysis (unpublished results).

It is well-established that p53-p21 pathway inhibits Cdk activation and is essential for G1 arrest upon DNA damage. Therefore, we studied whether p21 was induced in the thymidine-arrested cells cultured in the presence of SACi2. Unexpectedly, we found that the amount of p21 was decreased in thymidine-SACi2 co-treated HeLa cells (IV Fig. 4A) as well as in unperturbed HeLa and MCF10-A cells (IV Fig. 4B) indicating that SACi2 attenuated cellular levels of p21. ZM447439 did not induce similar changes but rather upregulated p21 levels (unpublished results) which is in line with previous reports (Kaestner et al., 2009) and suggests that attenuation is independent on Aurora inhibition. Notably, p21 levels were not rescued back to the control levels upon inhibition of caspase activity which excludes the effects of cell death on the measurements (IV Fig. 4D). When proteasome activity was inhibited, p21 levels were upregulated (IV Fig. 4A)

suggesting that SACi2 enhanced the degradation of p21. Cycloheximide assays could be used to investigate whether synthesis is also affected. The function of p21 as a regulator of Cdks (Xiong et al., 1993) is well-established. It is however evident that the protein has multiple other functions such as regulation of apoptosis. There is evidence that p21 abrogation may increase the efficacy of DNA damage based anti-cancer therapy (S. H. Park et al., 2008; S. H. Park, Park, & Weiss, 2008) which has encouraged scientists to perform HTS for identification of p21 inhibitors (S. H. Park, Wang et al., 2008). Based on these data, we suggest that p21 downregulation by SACi2 might be involved in the sensitization. Our finding that proteasome inhibition partially rescued p21 levels and rescued the cells from apoptosis (Fig. 4A and C, Suppl. video 5) supports our hypothesis. A model of p21 function upon Chk1 depletion and replication stress was previously proposed (Rodriguez & Meuth, 2006). According to the model, p21 is induced earlier in thymidine-arrested Chk1-depleted cells to prevent entry into S phase and thereby the protein slows down the replication stress-induced cell death. High p21 protein levels were detected at 24 h corresponding to the time point we measured decrease in p21 which supports the notion that SACi2 downregulated p21. Importantly, the model proposed that p21 is dispensable for protecting Chk1-proficient cells from cell death but is crucial when Chk1 is depleted. We present a model integrating Chk1 and p21 in the SACi2-induced response on replication stress (Fig. 11 D).

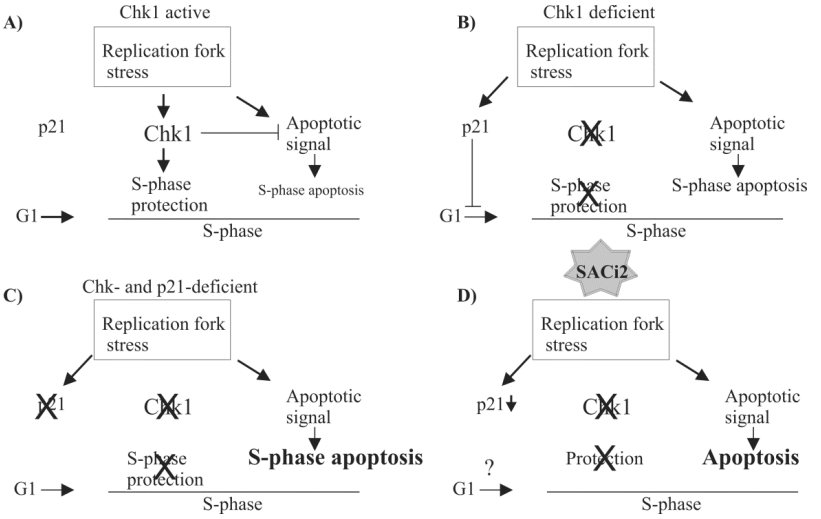

Figure 11. The function of Chk1 and p21 in protecting from cell death upon replication fork stress (A-C, figures and legend adapted from Rodriquez and Meuth 2006) and our model of the SACi2-induced sensitization to replication stress (D). (A) DNA synthesis inhibitors activate Chk1 that protects replication forks and prevents S-phase apoptosis. (B) The lack of Chk1-induced protection favors the induction of apoptosis in the S phase. p21 activation triggers an alternative protective mechanism by preventing cells from entering S phase. (C) Depletion of both Chk1 and p21 eliminates the protection of G_1 phase cells and results in apoptosis of virtually the entire cell population. D) SACi2 inhibits Chk1 and attenuates p21 which results in loss of protection and massive cell death upon replication stress.

In the model, we propose that SACi2 might cause conditions which resemble Chk1-p21 co-depletion (Rodriguez & Meuth, 2006) in which the exposure of cells to thymidine is

deleterious (Fig. 11). We propose that the SACi2-induced cell sensitization may be due to a combined effect of Chk1 inhibition and attenuation of p21. Whether the SACi2-thymidine co-treated cells underwent death from S phase should be investigated in future.

In conclusion, our results showed that SACi2 is a multifaceted compound inhibiting fundamental kinases namely Aurora A, Aurora B and Chk1 which are involved in checkpoints evoked by lack of MT-KT attachment and intra-KT tension, and induction of DNA-damage and replication stress, respectively. Inhibition of Aurora kinases and Chk1 by the same pharmacophore was not surprising considering the structural similarities in the ATP-binding pockets of the kinases (Massey et al., 2010). Moreover, inhibitors targeting Aurora kinases and Chk1 have been previously reported (Blasina et al., 2008; Gorgun et al., 2010; Ma et al., 2011; Massey et al., 2010). Furthermore, in our preliminary cell-based assays, we observed that SACi2 caused a reduction in the stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK) Thr183/Tyr185 phospho-signals in UV treated HeLa cells indicating that the compound decreased the activity of the kinase (unpublished results). Considering that JNK is activated due to genotoxic agents such as hydroxyurea and aphidicolin that inhibit replication (Damrot et al., 2009), and further, is implicated in DNA damage checkpoint at G2/M (Gutierrez et al., 2010), it is tempting to speculate that DNA damage sensitization effects of SACi2 reflect inhibition of Chk1 and JNK. More studies are needed to explore this notion further.

Recent data indicates that Chk1 is not only involved in DNA damage checkpoint but also SAC signaling and maintenance of SAC activity in taxol but not in nocodazole (Zachos et al., 2007). Chk1 seems to be essential for proper BubR1 localization and full activation of Aurora B kinase (Zachos et al., 2007). Based on these findings, we hypothesized that Aurora B activity could be modulated by SACi2 both directly and indirectly. Moreover, it could be even speculated that simultaneous inhibition of Chk1 and Aurora B might enhance the SAC override by SACi2.

6. SUMMARY AND CONCLUSIONS

SAC is an essential conserved surveillance mechanism that prevents the initiation of anaphase until functional connections between the MTs and KTs of the chromosomes have been established. Weakened SAC permits chromosome missegregation that can lead to aneuploidy, a condition known to contribute to tumorigenesis. On the other hand, severe ablation of the SAC signaling may result in loss of cell viability due to induced massive genomic imbalance. The latter event has raised interest toward SAC as a potential anti-cancer drug target. Consequently, a plethora of SAC inhibitors are currently investigated in preclinical and clinical studies for their ability to suppress proliferation of cancer cells. This thesis work was conducted in order to identify novel LMW inhibitors of the SAC signaling using a cell-based HTS. The main findings and conclusions of the work are the following:

1. The study demonstrates that a phenotypic HTS based on compound-induced effects on the cell morphology and attachment properties is a powerful method for identifying novel inhibitors of the SAC.

2. We identified four LMW compounds that override SAC-dependent mitotic arrest. The cellular effects of the compounds were characterized in more details using versatile cell-based and *in vitro* assays.

3. All four small compounds inhibited the activity of Aurora kinases, key facilitators of cell division. Abolishment of Aurora B kinase activity provides a plausible explanation for the observed abrogation of the tension-sensitive arm of the SAC.

4. Two of the discovered anti-mitotic compounds were flavonoids which for the first time were shown to be potent inhibitors of SAC signaling.

5. All four compounds suppressed cancer cell viability by inducing apoptosis. Data from the fisetin assays raises a notion that the flavonoid may possess cancer-cell specificity.

6. The phenotypes induced by the compounds were not identical which suggests that additional, yet unidentified, targets may contribute to the observed cellular effects.

7. The LMW compounds identified in our screens may have value as template structures in the development of future anti-cancer drugs.

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Anna-Leena Salmela

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