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8. Targeting mitotic kinases for anti-cancer treatment

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1. Background

Cancer cells proliferate unlimitedly. Therefore, targeting the proliferative capacity of cancer is one of the smartest ways to treat cancer. In this vein, understanding the basis of mitosis is essential for the advanced treatment of cancer, let alone its importance in basic biology. Indeed, recent progress in anti-cancer strategy has come from the development of a group of inhibitors targeting mitosis.

Opposed to meiosis, mitosis aims to preserve its genome during proliferation. In mitosis, genetic information of the parental cell is passed to two daughter cells through equal segregation of the replicated genome. Thus, the genetic integrity in proliferating cells is guaranteed through accurate chromosome separation in mitosis.

Mitosis is an ordered process orchestrated by a series of mitotic kinases and associated proteins. Sets of checkpoint mechanisms ensure the accurate segregation of the replicated genomes. Prior to mitosis, G1 restriction point,

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G1/S checkpoint, G2 checkpoint ensures that any type of DNA damage is repaired before entry into mitosis. p53 plays the pivotal role in interphase checkpoints; hence numerous anti-cancer strategies have focused to activate the p53 pathway.

In mitosis, failure in the bipolar attachment of microtubule spindles to even one chromosome would result in loss or gain of chromosomes in daughter cells, resulting in massive alteration of genetic information. Therefore, spindle assembly checkpoint has evolved in eukaryotes to ensure that not a single chromosome is left behind in mitosis. Errors in this process can be detrimental; deformity in development, abortion, Down's syndrome, neurodegenerative disorders, and cancer.

Principal strategy of anticancer agents was the stabilization or destabilization of microtubule polymerization. This effectively controlled mitotic cells, the cancer cells, and not the quiescent normal cells. These include taxanes and vinca alkaloids. Recently, mitotic kinases emerged as the target for anti-cancer therapy.

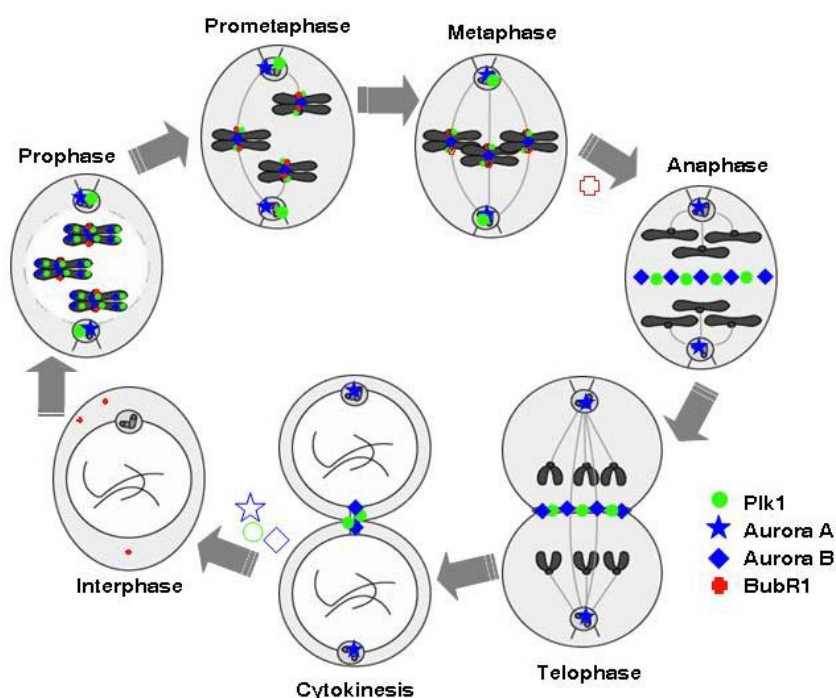


Figure 1. Dynamic localization of mitotic kinases. At the prophase, Plk1 recruits Aurora A and B kinases to the centrosomes and chromosome arms respectively. BubR1 is recruited to the outer kinetochores after nuclear envelope break-down (NEBD). During prometaphase, Plk1 and Aurora B converge to the kinetochores; Aurora B to the inner centromeres and Plk1 to the outer kinetochores. At the metaphase when all the chromosomes are attached to the bipolar spindles and under proper tension, BubR1 is degraded and anaphase begins. At the anaphase, Plk1 and Aurora B are relocated to the midzone and regulate cytokinesis. With completion of cytokinesis, Plk1 and both Aurora kinases banish.

Table 1. Inhibitors for Aurora kinases and Plk1 in clinical trials.

Aurora kinase inhibitors		
Company	Name	Remarks
Astex Therapeutics	AT9283	Phase I-II trials, Aurora A/B inhibitor
AstraZeneca	AZD1152	Phase I-II trials, Aurora B/C inhibitor
	ZM447439	Preclinical development, Aurora B inhibitor
Cyclacel	CYC116	Phase I trials, pan Aurora inhibitor
GlaxoSmithKline	GSK1070916	Phase I trials, selective Aurora B/C inhibitor
Millenium	MLN8054	Phase I trials, Aurora A inhibitors
	MLN8237	Phase I-II trials, Aurora A inhibitors
Pfizer	PHA-739358	Phase II trials, pan Aurora inhibitor
	PHA-680632	Aurora A inhibitor
	PF-03814735	Phase I trials, Aurora A/B inhibitor
Sunesis Pharmaceuticals	SNS-314	Phase I trials, pan Aurora inhibitor
Vertex / Merck	VX-680 (MK-0457)	Phase I-II trials, Aurora A/B inhibitor
Plk1 inhibitors		
Company	Name	Remarks
Boehringer Ingelheim	BI 2536	Phase I-II trials
	BI 6727	Phase I-II trials
GlaxoSmithKline	GSK461364	Phase I trials
Nippon Shinyaku	HMN-214	Phase I trials
Onconova	ON01910Na	Phase I-II trials

As illustrated in Figure 1, mitosis is orchestrated by several mitotic kinases at distinct cellular locations. Thus, malfunction in any of these key kinases can activate the spindle assembly checkpoint and ultimately end up in apoptosis in an as-yet-undefined mechanism. Among these mitotic kinases, Aurora kinases and Polo-like kinases have proven to be effective targets for cancer therapy; specific inhibitors are on clinical trials (Table 1).

In this chapter, we will review on how mitosis is regulated by key kinases, such as Aurora kinases, Plk, and BubR1, and discuss on the potential of the recently developed anti-cancer drugs. The future of anti-cancer strategy based on the understanding of mitosis and its novel mechanisms will also be discussed.

2. Aurora kinases

Aurora kinases are Serine/Threonine kinases that are associated with microtubule apparatus and chromosome in mitosis. While cyclin-dependent kinase-1 (CDK1) and Polo-like kinase-1 (Plk1) trigger cell division and work as global controllers of mitosis, Aurora kinases directly regulate mitotic events largely at three critical points; at the mitotic entry building up centrosomes for the bipolar spindle formation, at the prometa- to metaphase orchestrating amphitelic attachment of spindles to kinetochores, and during cytokinesis delaying abscission until chromosome segregation is complete. In yeasts, a single Aurora kinase exists, Ipl1 [increased in ploidy] in the budding

yeast and Ark1 [Aurora related kinase] in fission yeast. In metazoans Aurora A and B were discovered; Aurora A is prominently localized at the centrosomes whereas Aurora B is localized at the inner kinetochores early in the mitosis and relocalized to the midbody during cytokinesis (Figure 1). Yeast Ipl1 is localized at the chromosome to midbody and forms a complex with INCENP-Survivin (Sli15-Bir1) similar to mammalian Aurora B. Reminiscent of their localization, Aurora A plays a crucial function in the centrosome maturation and bipolar spindle formation whereas Aurora B is involved in the chromosome condensation, microtubule-kinetochore attachment, and cytokinesis. Vertebrates have a third Aurora kinase C; the expression of Aurora C is restricted to the testis and shows similar localization and function as Aurora B.

2.1. Structure

Aurora kinases have a variable N-terminal domain, a highly conserved protein kinase domain and a regulatory C-terminal domain. Aurora A and Aurora B kinases share over 60% amino acid identity and structural similarities. The substrate binding surface of Aurora A and Aurora B catalytic domain is almost identical except for a few amino acid residues. The difference is responsible for the binding of a unique partner for each kinase; Aurora A to Tpx2 and Aurora B to INCENP. A single amino acid substitution, Gly-198 of Aurora A with the Aurora B equivalent Asn residue disrupts Tpx2 association and confers INCENP binding for the mutant Aurora A [1]. The exchange of binding partner leads to the localization of Aurora A at the inner centromeres and midzone. More importantly, Aurora A G198N compensates the loss of Aurora B. These indicate that the basis for the divergent function of Aurora A and B is their interaction partners. Aurora A-Tpx2 complex and Aurora B-INCENP complex have distinct structure and different substrate specificities [2,3] Autophosphorylation of Thr-288 in Aurora A stimulated by Tpx2 and Thr-232 in Aurora B by INCENP are required for the full activation of kinase activities as well. The variable N-terminus mediates many protein-protein interactions for Aurora A and Aurora B kinases. C-terminal D box and N-terminal A box are responsible for APC/Cdh1 dependent degradation during mitotic exit [4] for both kinases.

2.2. Function of Aurora A

Aurora A is prominently localized at the centrosomes and along the microtubules during mitosis. At the centrosome, Aurora A is involved in the centrosome separation and centrosome maturation. Aurora A also regulates

centrosome-dependent and independent spindle assembly. In the absence of Aurora A, mitotic entry is significantly delayed, implicating Aurora A in cell cycle progression as well. Also important is that Aurora A locus is frequently amplified and the expression elevated in cancer cells including breast and colon. We will discuss the role of Aurora A in the regulation of centrosome and bipolar spindle formation, mitotic entry, and its oncogenic potential.

2.2.1. Centrosomal function

The first discovery of Aurora A kinase was made in *Drosophila*, in search for mutations that affect centrosome cycle [5]. In the *aur* mutant embryos, two centrosomes were paired and acted as a single pole to nucleate monopolar spindles. Those embryos had circular chromosome arrangements around a large centrosomal structure, resembling aurora in the polar region. Centrosomes and the pericentriolar materials (PCM) function as a main microtubule nucleation center in animal cell division. A single centrosome comprises of two centrioles perpendicularly arranged. In G1-S phase, centrosomes duplicate in concert with DNA replication, giving rise to two centriole pairs surrounded by PCM. In late G2-prophase, the two paired centrosomes undergo maturation, expanding PCM to nucleate sufficient mitotic microtubules. Centrosome separation occurs before or after nuclear envelope break down (NEBD) depending on the cell types. After NEBD, the centrosomes nucleate microtubule asters to form bipolar spindles around metaphase chromosomes. In all organisms examined, Aurora A inhibition resulted in defects in centrosome maturation, failing to build up enough PCM components such as γ -tubulin. In *C. elegans*, Aurora A depleted *air-1*(RNAi) embryos and somatic cells failed to accumulate γ -tubulin and additional PCM components at the centrosome [6]. In flies, *aur* mutant sensory organ precursor cells failed to recruit γ -tubulin, Centrosomin [7], and Minispindles (XMAP215 homolog) . In HeLa cells, RNAi for aurora A impaired gamma-tubulin recruitment and induced split centrioles [8]. The failure to recruit enough PCM components may perturb in centrosome separation and bipolar spindle formation.

Apart from the role in centrosome maturation, Aurora A plays a direct function in mitotic spindle formation. In animal cells, centrosome-dependent and chromosome-dependent spindle assembly pathways exist and Aurora A is an important player in both. In centrosome-dependent spindle assembly, a link between Aurora A and the spindle formation is found in TACC, a highly transforming acidic-coiled-coil-containing protein. TACC is a substrate of Aurora A whose centrosomal targeting relies on Aurora A. TACC forms a complex with TOG/XMAP215 protein which directly binds to microtubules.

Aurora A mediated recruitment of TACC-TOG complex to centrosomes stabilizes microtubule minus ends by counteracting microtubule-destabilizing kinesins. Involving Aurora A's function in centrosome maturation and bipolar spindle formation several upstream and downstream factors were identified in various model systems. Although the contribution and exact function of those factors are not fully defined, Barr and Gergely [9] put together those factors in a model where, the kinase CDK11 is responsible for the localization of Plk1, which in turn recruits Aurora A to the centrosome. Centrosomal Aurora A phosphorylates factors like PAK1, Tpx2 and Ajuba, all of which facilitate Aurora A autophosphorylation. The active Aurora A kinase recruits downstream factors like TACC and Ndel1. Ndel1 is known to restore many defects caused by Aurora A depletion.

In the chromosome-dependent spindle assembly, microtubules assemble around chromosomes which can be readily shown in oocytes of many animal species. This pathway is known to require the activity of Ran-GTP. In fact, microtubule nucleation takes place even in the absence of centrosome or chromatin when the activity of Ran-GTP is present in xenopus egg extracts. The high concentration of Ran-GTP around the chromosomes promotes release of spindle assembly proteins from Importins at the nuclear envelope. The released factors form a complex called EXTAK, which contains Eg5, XMAP215, Tpx2, Aurora A, and HURP as well as γ -TURC. Aurora A coated beads in the xenopus egg extracts could form EXTAK complex and promote microtubule assembly in the absence of centrosome or chromatin. Aurora A recruits and phosphorylates Tpx2, which leads to the autophosphorylation of Aurora A. The active Aurora kinase recruits other components of EXTAK complex where, γ -TURC nucleates; XMAP215 stabilizes; HURP bundles; and Eg5 and Tpx2 crosslink microtubules. Thus Aurora A is at the center of mitotic microtubule organization.

2.2.2. Mitotic entry

Aurora A depletion causes delay in mitotic entry. Mitotic entry and exit are controlled by the key mitotic kinase Cdk1, and Aurora A is known to regulate it in two ways. First, the earliest activation of Cdk1-cyclin B occurs at the centrosome and Aurora A is essential for the centrosomal recruitment and activation. In human cell lines, Aurora A depletion impaired the recruitment and initial activation of Cdk1-cyclin B at the centrosome. As Cdk1 inhibition impairs Aurora A activation, there seems to be a positive feedback loop between Aurora A and Cdk1. When Aurora A is depleted, delay in mitotic entry was also observed in *C. elegans*. In the first mitotic division of *C. elegans* embryos, male pronuclei-associated centrosomes are

crucial for the timely mitotic entry and Aurora A is the major player in this process. Secondly, Aurora A regulates mitotic entry via regulating CPEB (CYtoplasmic PPolyadenylation EElement BBinding PProtein). The polyadenylation-induced translation of cyclin B was initially shown to be critical to the meiotic progression in xenopus oocytes. Similar translational control was found in xenopus embryos for mitotic progression where phosphorylation of CPEB by Aurora A regulates polyadenylation and cell cycle [10]. Embryonic CPEB is localized at the centrosome with other factors involved in the polyadenylation, indicating that CPEB phosphorylation by Aurora A is associated with centrosome. As oocytes do not have centrosome and centrosome disruption does not cause as significant mitotic delay as Aurora A depletion, the role of Aurora A in mitotic entry is exerted in both centrosome dependent and independent ways.

2.2.3. Aurora A and cancer

In 1997, human Aurora A was identified as a 'breast tumor activated kinase (BTAK)' mapped to the locus 20q11-13 that is frequently amplified in breast cancer [11]. The gene amplification as well as over expression of Aurora A is found in many other epithelial cancers [12]. The over-expression of Aurora A kinase transformed Rat1a cells and NIH3T3 cells to form colonies in vitro and tumor mass in nude mice [13,14]. These studies assigned Aurora A as an oncogene. However, Aurora A failed to transform primary mouse embryonic fibroblasts [15] and failed to induce tumorigenesis when expressed as a transgene in mice [16]. These results indicate that Aurora A is not a *bona fide* oncogene, and other genetic alterations are necessary for the transformation.

Nevertheless, Aurora A surely contributes to the tumorigenesis. First, over-expression of Aurora A kinase induces polyploidy and abnormal centrosome number. This is likely to cause genetic instability and may contribute to tumorigenesis, although not sufficient for transformation. Secondly, Aurora A phosphorylates p53 and inhibits its transactivation and also induces its degradation. The inactivation of p53 would abrogate the G1 checkpoint, and abnormal cells may enter cell cycle. Thirdly, reports suggest that Aurora A might regulate Ras signal and influence cell growth and enhance Ras-mediated transformation [17,18].

The association between Aurora A and cancer prompted the development of Aurora A targeted small molecules, which showed promising drug response in cancer cells and animal models. When aurora A is depleted or inhibited, abnormal spindle formation takes place. The cells -both normal and transformed- undergo abnormal cell division after delay in mitotic entry and

anaphase onset. The resulting cells end up with polyploidy. The long mitotic delay and the abnormal chromosome trigger apoptosis in most cells. Recently, it was found that the abnormal chromosome segregation leads to cell death depending on the time spent in mitosis trying to fix the problems. If cells have normal checkpoint function, apoptotic pathway is the preferred pathway after Aurora A inhibition because the absence of aurora A does not override checkpoints. This is why the aurora A specific inhibitor is a promising but potentially dangerous cancer therapeutic target. Primary mechanism how Aurora A inhibition induces cell death is through generation of abnormal cell division. If cells escape the death pathway, Aurora A inhibition could generate more genetically unstable, potentially cancer-prone cells in the treatment. Development of anti-cancer drugs targeting Aurora A specifically will be discussed later.

2.3. Function of Aurora B

Aurora B forms the chromosomal passenger complex (CPC) and regulates chromosome cohesion, chromosome-microtubule attachment, and cytokinesis. To coordinate these processes, the CPC moves dynamically from chromosomes to midbody during mitosis.

2.3.1. Chromosome passenger complex

Aurora B is the enzymatic subunit of CPC; whose nonenzymatic subunits INCENP, Survivin, and Borealin are conserved from yeast to mammals. The nonenzymatic components are phosphorylated by Aurora B and regulate the localization and kinase activity of Aurora B. INCENP binds Aurora B through the C terminal IN box, which is required for the full activation of the Aurora B kinase. INCENP also functions as a scaffold to accommodate Survivin and Borealin at the N-terminus. Survivin is primarily responsible for the CPC targeting to centromeres and central spindles. Borealin is also required for targeting of CPC by bringing INCENP and Survivin together. In fact, Survivin-INCENP fusion proteins can target functional CPC to centromeres and central spindle in the absence of Borealin. Depletion of any CPC component disturbs proper localization and function of the complex and manifests similar mitotic defects, indicating that the CPC functions as a single structural unit.

2.3.2. Chromosome condensation

Aurora B is localized at chromosome arms during prophase and concentrated on the centromere at prometaphase. The chromosome

association continues to the onset of anaphase, then Aurora B moves to the central spindle. While associated with chromosome, Aurora B is known to regulate the mitotic chromosome structure. First, Aurora B phosphorylates Histone H3 at Ser10, which is a hallmark of mitosis and linked to the chromosome condensation. In tetrahymena, nonphosphorylated S10A H3 causes elongation of mitotic micronuclei. Histone H3 phosphorylation is thought to cause dissociation of HP1 from heterochromatin, which allows chromosome to access to the condensin complexes for chromosome condensation and cohesion during mitosis. Second, Aurora B phosphorylates condensin I subunits to allow its chromosome association. Condensin I is required for the chromosome compaction and also for the displacement of cohesins from chromosome arms for chromatid separation. Third, the maximal chromosome compaction is achieved during anaphase and Aurora B inhibition causes decompaction in an unknown mechanism. Therefore Aurora B regulates the chromosome structure throughout mitosis.

2.3.3. Microtubule-kinetochore attachment

Accurate chromosome segregation is the climax of mitosis. The segregation is mediated by bi-oriented microtubule spindles attaching to the sister kinetochores and pulling them to the opposite ends. This amphitelic microtubule-kinetochore (MT-KT) attachment is critical to ensure accurate chromosome segregation. Molecular machineries in monitoring the false attachments have been evolved to sense unattached kinetochores or the lack of tension. How these physical states are biochemically translated has been a major issue in cell biology. Now Aurora B is thought to be the key translator, which destabilizes the MT-KT attachment by phosphorylating kinetochore substrates in the absence of proper tension. Microtubule attachment is a dynamic process that many weak interactions, both enforcing and destabilizing, determine stable attachment. A protein called Ndc80 destabilizes the MT-KT interaction, which requires phosphorylation by Aurora B. Thus phosphorylation by Aurora B could re-enforce MT-KT dynamics to destabilization and generate unattached kinetochores, which will resume microtubule attachment until proper tension is generated. The phosphorylation by Aurora B could be limited by regulation of kinase activity and/or spatial separation between Aurora B at the inner centromeres and its substrates at the outer kinetochores. Liu had shown that the spatial segregation indeed regulated Aurora B mediated phosphorylation [19], and proper tension led to dephosphorylation of kinetochore substrates.

Alternatively, and not exclusively, regulation of kinase activity *per se* plays a role. Aurora B kinase activity is known to require microtubules and

microtubule-associated factor TD-60 [20]. Microtubule attached to the inner centromere in the absence of tension activates Aurora B kinase activity. Nucleosomal structure also affects Aurora B kinase activity by allowing close association of CPCs and activation of Aurora B kinase activity by other CPC components in trans [21]. Contribution from the different pathway is not yet clear, but clearly Aurora B is required for the bipolar MT-KT attachment.

Aurora B, the crucial kinase for amphitelic MT-KT attachment, also plays a role for spindle assembly checkpoint (SAC) that prevents the activation of APC/Cdc20 until all the chromosomes are properly aligned at the metaphase plate and bipolar spindle attachment is established. After the inhibition of Aurora B with small molecule inhibitors, ZM447439 or Hesperadin, abnormal cell division takes place indicative of SAC inactivation and premature anaphase onset [22,23]. Consistent with these findings, microtubule stabilizing agent Taxol or Eg5 inhibitor Monastrol do not arrest cells when Aurora B is inhibited or other CPC component is depleted [24,25]. Interestingly, however, in the presence of ZM447439 or Hesperadin, cells arrest after microtubule destruction with Nocodazole, suggesting that SAC activation could take place regardless of Aurora B. In this case, concomitant depletion of Bub1 overrides SAC and cells do not arrest with Nocodazole [26]. Thus Morrow and Taylor suggested that SAC activation occur via Bub1 monitoring unattached kinetochores and Aurora B monitoring bipolar attachment. Although how SAC activation is achieved by Aurora B is not fully defined, involvement of Ndc80 [27] has been suggested.

2.3.4. Cytokinesis

When Aurora B function is disrupted, multinucleated cells are formed, suggesting for cytokinesis failure [28,29]. Cytokinesis is the last stage of cell division where actin, myosin, and other cytoskeletal components are redistributed to the contractile ring to create cleavage furrow and induce division of plasma membrane in animal cells. At the anaphase onset, Aurora B is relocated from chromosomes to central spindles and regulates cleavage furrow formation. The signals for cleavage furrow formation come from both microtubule asters and central spindles at the midzone [30,31] and Aurora B is implicated in both [31]. The stabilization of midzone requires microtubule bundling activity of microtubule associated proteins (MAPs) and centralspindlin complex composed of Mklp1 and RacGAP. The localization of centralspindlin is dependent on Aurora B and either the depletion of Aurora B or Mklp1 show similar cytokinesis defects ranging from the complete lack of furrow formation [32] to asymmetric furrow formation depending on cell types. As RacGAP/CYK-4 is a putative GTPase activating

protein for RhoA GTPase that directs contractile ring assembly, Aurora B might indirectly affect the contractile ring formation as well.

While Aurora B plays a role in the initiation of furrow formation, Aurora B also delays completion of cytokinesis until all the chromosome segregation is complete [33-35]. This “NoCut” checkpoint was dissected in the budding yeast that midspindle defects or chromosome bridges delay abscission, which is dependent on Ipl1. Similar “Abscission checkpoint” was also identified in mammalian cells, as chromosome bridges located at the cleavage furrow delayed abscission in Aurora B dependent manner. The kinase activity of Aurora B is required for the delay, and Aurora B-mediated phosphorylation of Mklp1 is implicated. Thus Aurora B regulates the initiation and completion of cytokinesis.

2.4. Inhibitors of Aurora kinases in clinical trials for anti-cancer treatment

The Aurora kinase inhibitors such as ZM447439, MK-0457 (VX-680), and PHA-739358 are pan-Aurora inhibitors, which inhibit Aurora A, B, and sometimes C activities *in vitro* [22]. Although ZM447439 is still on the preclinical development, MK-0457 (VX-680) and PHA-739358 are actively being evaluated in the clinical fields.

MK-0457 (VX-680) is a pyrimidine derivative with affinity for aurora A, B, and C at a value of nanomolar concentrations. It inhibits the growth of tumor xenograft, and is effective in chronic myeloid leukemia cells and other solid cancer cells.[36,37]. In the phase I clinical trial, MK-0457 was observed to stabilize the refractory solid tumors in 3 patients [38]. Three patients with T315I phenotype-refractory CML or Philadelphia-positive ALL have shown clinical responses to doses of MK-04547 that are not related with adverse events [39]. Phase II clinical trials are currently underway in the patients with advanced NSCLC (NCT00290550, www.clinicaltrial.gov), and in the patients with CML and Philadelphia-positive ALL (NCT00405054).

PHA-739358 is a pan-Aurora kinase inhibitor with anti-cancer activity on variable tumor xenograft models [40,41]. The results of phase I trial were presented in 2006, and PHA-739358 is currently being investigated in a phase II clinical trial in CML patients relapsed after imatinib mesylate or c-ABL therapy, including patients with T315I mutation (NCT00335868). Other phase II trials are currently being investigated in adult patients with Multiple Myeloma who have a history of at least two previous lines of treatment for the disease (NCT00872300), and in patients with metastatic hormone refractory prostate cancer (NCT00766324).

AT9283 is another multi-targeted kinase inhibitor recently developed with potent activity against Aurora A and B kinases in the clinical

development [42,43]. Side effects and maximum tolerated dose of AT9283 are being examined in phase I trials when treating patients with advanced or metastatic solid tumors or non-Hodgkin's lymphoma (NCT00443976), ALL, AML, CML, high-risk myelodysplastic syndromes, or myelofibrosis with myeloid metaplasia (NCT00522990).

MLN8054 is the first orally administered Aurora kinase inhibitor, and the first one to inhibit Aurora A specifically, and much less so to Aurora B. Treatment of MLN8054 leads to spindle defects, resulting in the activation of spindle assembly checkpoint and inhibition of proliferation in various human cancer cells [44]. Growth of human tumor xenografts in nude mice was inhibited after oral administration at well-tolerated doses, and the tumor growth inhibition was sustained even after the discontinuation of the treatment. In xenografts, MLN8054 induced mitotic arrest and apoptosis. The preliminary results of a phase I study showed that MLN8054 was absorbed rapidly with a reasonably long half-life. It inhibits Aurora B at higher doses. However, dose-limiting toxicities limited dose escalation before mechanism-based toxicity was seen [45].

MLN8237 is a second-generation, selective Aurora A kinase inhibitor designed for greater potency and fewer benzodiazepine-like effects observed in first-generation agent, MLN8054. In the phase I trial, MLN8237 was tolerable with adequate doses and exhibited favorable clinical antitumor activity when treated for the advanced solid tumors [46]. The results supported the continuation for phase II development, and MLN8237 is currently in phase II clinical trials. MLN8237 is administered for the treatment of patients with platinum-refractory or platinum-resistant epithelial ovarian, fallopian tube, or primary peritoneal carcinomas (NCT00853307). MLN8237 is also effective on hematological malignancies, as well as on solid tumors. The phase I study in patients with advanced hematological malignancies who have limited standard treatment options is active (NCT00697346). In addition, phase I/II trial is currently recruiting subjects for treating pediatric patients with relapsed, refractory solid tumors or acute lymphoblastic leukemia (NCT00739427). Phase II studies for patients with relapsed or refractory non-Hodgkin's lymphoma (NCT00807495), AML or myelodysplastic syndrome (MDS) (NCT00830518) are underway.

AZD1152 is a dihydrogen phosphate pro-drug of a pyrazoloquinazoline Aurora kinase inhibitor. It showed a potent and selective inhibition of Aurora B in tumor xenografts [47,48]. AZD1152 was reported to be effective in inhibiting growth of acute leukemia cells when used in combination with vincristine and daunorubicin both *in vitro* and *in vivo* [49]. Phase I trial on patients with colon cancer, melanoma, or various other solid tumors revealed that AZD1152 made significant disease stabilization with tolerable toxicity

[50]. Phase I studies to assess the effect of AZD1152 on the rate of complete remission in patients with relapsed AML (NCT00530699) and in patients with advanced solid tumors (NCT00338182, NCT00497679, NCT00497731) are currently on going.

Various Aurora kinase inhibitors, apart from the inhibitors described above, are currently being evaluated in clinical setting. Phase I trial is studying escalated dose and the side effects of CYC116, a pan-Aurora kinase inhibitor, in treating patients with advanced solid tumors (NCT00530465, NCT00560716). PF-03814735, a pan-Aurora kinase inhibitor, which is administered orally as single agent in patients with advanced solid tumors with preliminary results (NCT00424632) [51] and SNS-314, a selective Aurora A kinase inhibitor, in advanced solid tumors with preliminary results (NCT00519662) [52] are on phase I clinical trials. Moreover, there are many more Aurora inhibitors in preclinical development: CHR-3520, CTK-110, ENMD-981693, JNJ-7706621, PHA-680632, MP-529, MP-235, GSK1070916 and so on [53,54].

3. Polo-like kinase

3.1. Entry and exit from mitosis with Plk1

The master regulator of mitosis is Cdk1, bound and activated by cyclin B. Mitotic entry and exit is controlled by Cdk1. Following Cdk1, Polo-like kinases (Plks) play critical roles in the progression of mitosis; mitotic entry, centrosome maturation, spindle assembly, chromosome alignment, APC/C regulation, and cytokinesis. Four Plk family members, Plk1-4 are found in vertebrates. Of the four Plks, Plk1 is believed to carry out most of the functions attributed to Cdc5, Plo1, and Polo of budding yeast, fission yeast, and *Drosophila*, respectively. Plk1 exerts its multifaceted functions in mitosis through localizing to all of the important places like chromosomes, centrosomes, central spindle at the right time. As Erich Nigg describes, Cdk1 is the conductor in mitosis and Plk1 is the first violin [55].

Plks have serine/threonine kinase domain at the N-terminus, D-box degradation signals for APC/C in the middle, and the unique PBD (Polo box domain) in the C-terminus. Two polo box domains, PB1 and PB2, are thought to confer substrate selectivity and regulate subcellular localization of the kinase. The PBD domain serves as an inhibitor of the kinase when substrate binding is absent. When the docking proteins are phosphorylated by priming kinases, often Cdk1, they bind to the PBD domain in Plk1, relieving the PBD's autoinhibition of the kinase, and being further phosphorylated by Plk1. This way, substrate specificity is elegantly coordinated in a cell division

cycle-dependent manner, following CDK1-cyclin B [55,56]. It is also shown that Plk1 can activate Cdk1 at G2/M transition indirectly [55], and also can phosphorylate cyclin B and promote accumulation of this mitotic cyclin B in the nucleus [57,58]. Conceivably, this synergistic and sequential interplay between Cdk1 and Plk1 is thought to regulate timely mitotic entry.

Without Plk1, mitosis cannot be continued; depletion of Plk1 from *Xenopus* oocyte extracts impaired mitotic entry, whereas depletion of Plk1 resulted in delay in anaphase progression in human cells, flies [59], and in zebrafish embryogenesis (our unpublished data). The mitotic arrest by Plk1 abrogation is thought to result from defective spindle assembly, impaired centrosome maturation, and impaired MT-KT attachments. All of these defects provoke the spindle assembly checkpoint (SAC) activation, since SAC ensures the bipolar spindle attachments and tension at kinetochores. The outcome is the delay in mitosis followed by frequent apoptosis, through the mechanism not yet understood satisfactorily. These results indicate that Plk1 is essential for progression in mitosis, and the primary role of Plk1 may be in microtubule organization, microtubule spindle formation, and MT-KT attachments.

It was believed that Plk1 on its own is not involved in SAC, evidenced by the fact that abrogating Plk1 resulted in the activation of SAC. Nonetheless, Plk1 can regulate SAC through phosphorylating BubR1 in a tension-sensitive manner [60]. With these notions, the interaction and interplay between SAC, Plk1, microtubule spindles are not as simple as it first seemed. A good reason why revealing the mechanism orchestrating mitosis is crucial, even for the sake of developing anti-cancer drugs targeting mitosis.

Multifaceted roles of Plk1 in mitotic entry and progression have hampered the finding of its role in cytokinesis; when Plk1 function is compromised, the cells first arrest in mitosis. With the development of chemicals inhibiting Plk1 function, it became clear that Plk1 localizes to central spindle in telophase and even in the place where abscission takes place and plays essential roles in cytokinesis [59,61,62]. The advantage of the chemical biology is that treating the cells with the drugs can be controlled in a timely- controlled manner. Cells were treated with specific inhibitors to Plks after they committed chromosome segregation. With chemical biology, unveiling the function of Plk1 in the last step of cell division, cytokinesis, has just begun.

3.2. Is Plk1 an oncogene?

Like Aurora A, Plk1 over expression is observed in many tumors of diverse origin, including breast, ovary, colon, stomach, pancreas, lung, head and neck, skin, esophagus, and brain [63-66]. Its over expression correlates

with poor outcome [64,67,68]. In immortalized rodent cells, over expression of Plk1 induced transformation, growth in soft agar assays, and formed tumor in nude mice, suggesting for an oncogenic potential [69]. However, depletion of Plk1 with siRNA or small molecular inhibitors caused genomic instability accompanied by sudden death as well [70-74], suggesting that both depletion and over expression of Plk1 can attack genome integrity. Live-imaging in zebrafish embryogenesis revealed that both depletion of Plk1 and over expression of it cause spindle defects and centrosome abnormality in mitosis, resulting in arrest in mitosis (our unpublished results). These results altogether point to the hypothesis that the adequate level of Plk1 is essential for progression in mitosis and genome integrity. In this regard, Plk1 is not an oncogene. Rather, it behaves as a balance for regulated mitosis and abnormal tumorigenesis. Taken together, it is conceivable to think that targeting Plk1 with chemically designed inhibitors can be cancer cell-selective. Indeed, varieties of small molecular compounds have been developed so far.

3.3. Plk1 inhibitors for anti-cancer treatment

First generation Plk1 inhibitors such as LY294002 and other related compounds are in clinical trials. Most recently, BI 2536 has been developed, which showed increased sensitivity towards Plks. *In vitro*, BI 2536 inhibits Plk1 and induces mitotic arrest with low concentration; IC₅₀ value below 1 nM, and inhibits the growth of cancer cell lines in the IC₅₀ value ranged between 2 to 30 nM [61,62]. BI 2536 binds to the catalytic domain of Plk1 with high potency. Results of phase I trials have reported that BI 2536 was well tolerated and showed a favorable pharmacokinetic profile in advanced solid tumors [75]. Phase II trial has been conducted to evaluate the efficacy, safety and pharmacokinetics of BI 2536 in the treatment of unresectable advanced pancreatic cancer as first line or second line therapy (NCT00710710), in the treatment of advanced or metastatic NSCLC of stage IIIB or IV in patients who relapsed after or failed first-line therapy (NCT00376623), in the treatment of prostate cancer (NCT00706498), in second line treatment in sensitive-relapse SCLC patients (NCT00412880).

ON01910Na is a small-molecule Plk1 inhibitor that is not an ATP-mimetic but competes for the substrate-binding site of Plk1 [76]. Recent phase I study with an accelerated titration dose-escalation design of ON01910Na showed objective response with moderate toxicities, especially in refractory ovarian cancer patients [77]. Another phase I trial was opened for evaluation of ON01910Na when given together with gemcitabine for treating patients with advanced or metastatic solid tumors (NCT00822939).

Plk1 inhibitors in preclinical or clinical development include GSK461364, BI 6727, and HMN-214. GSK461364 is a benzimidazolylthiophene which inhibits Plk1 in an ATP-competitive way [78]. GSK461364 leads to G2 phase arrest at high concentrations and M phase arrest at low concentrations. BI 6727, the second generation dihydropteridinone derivative, shows highly potent and selective anti-tumor activity in cancer models including taxane-resistant colorectal cancer [79]. Both GSK461364 and BI 6727 are in early clinical trials. Phase I clinical trial is being conducted to determine the maximum dose and adverse effects of GSK461364 in adult patients with solid tumors and Non-Hodgkins lymphoma (NCT00536835). Preliminary results for escalating dose in the subjects with solid tumors are reported [80]. The preliminary results of phase I trial with BI 6727 also showed anti-cancer effects with tolerable dose [81]. Phase II study is underway to evaluate whether BI 6727 monotherapy or in combination with pemetrexed is effective in the treatment of advanced or metastatic NSCLC in patients with recurrent or refractory first-line platinum based therapy (NCT00824408). Phase I/IIa is also on the way to investigate for monotherapy and in combination with low dose cytarabine in patients with relapsed or refractory AML that are not eligible for intensive treatment. In the phase IIa part, the combination of BI 6727 at MTD with cytarabine and cytarabine monotherapy is under investigation to explore the efficacy of the combination schedule in previously untreated AML patients who are not eligible for intensive treatment (NCT00804856). HMN-214, an oral stilbene derivative, affects indirect disruption of Plk1 and leads to G2/M phase arrest causing anti-tumor activity *in vitro* and *in vivo* mouse model [82]. The phase I trial demonstrated the tolerable dose for the patients with advanced solid tumors [83].

Besides these Plk1 inhibitors in clinical development, Plk1 inhibitors, such as ZK-thiazolidinone and DAP-81, are under the preclinical development [84,85].

4. BubR1 kinase

4.1. The key spindle assembly checkpoint component, BubR1

The climax of mitosis is at the metaphase to anaphase transition where the chromosome segregates in a pole-ward direction. This event is critical in ensuring the genetic instability. At every kinetochores of chromosomes, amphitelic attachments of microtubule spindles are checked by the spindle assembly checkpoint (SAC). SAC inhibits APC/C E3 ligase, the multisubunit E3 ligase that is responsible for the destruction of Securin and cyclin B.

Securin is the inhibitor for Separase that cleaves Cohesins. Cyclin B is the mitotic cyclin that binds to Cdk1 and conducts mitosis. Therefore, chromosome segregation and anaphase onset, are governed by APC/C, and SAC inhibits the APC/C until the chromosomes are ready to segregate [86].

Until now, the modifications and control on the 13 subunits of APC/C, the key feature in understanding the regulation of APC/C, have not been fully elucidated yet. However, the fact that APC/C activation requires WD40 domain-containing coactivators Cdc20 in mitosis or Cdh1 in mitotic exit is firmly established [87]. Therefore, the inhibition or the control of APC/C activity in mitosis can be controlled in this layer; titrating out the coactivator Cdc20 away from APC/C. Indeed, Mad2 (first identified from the *mad2* mitotic arrest deficient mutant in yeast) binds to Cdc20 in vitro and in vivo, sequestering it from APC/C thereby inhibiting Securin and cyclin B destruction [87].

Identified later, but not less important inhibitor of APC/C is the kinase BubR1. Unlike Mad2, BubR1 is a kinase not found in yeast. The N-terminus of BubR1 resembles Mad3 of yeast, but it has the kinase domain like Bub1 at the C-terminus. Therefore, it would be fair to say that the vertebrates have evolved another kinase BubR1 to modulate the crucial APC/C activity in mitosis. In vitro experiments suggest that BubR1 is a more potent inhibitor for APC/C-Cdc20 than Mad2 [88]. At first, it seemed that the way BubR1 inhibits APC/C is similar to Mad2 by sequestering Cdc20 away from APC/C. However, accumulating evidences indicate otherwise.

Studies revealed that the way SAC functions is through forming a complex of composed of Mad2, Bub3, BubR1 and Cdc20, named MCC (Mitotic Checkpoint Complex), to inhibit APC/C [89]. This way, the coactivator Cdc20 is sequestered away from APC/C and the anaphase is delayed (Figure 2a). However, this model harbors some problems. BubR1 is capable of binding to both Cdc20 and APC/C. Furthermore, BubR1 was bound to APC/C before and after anaphase onset [90]. More detailed biochemical studies showed that Mad2 only loads Cdc20 to BubR1 to kinetochores then leaves the complex. Cdc20 is ubiquitinated and degraded until SAC is satisfied. From this model, the MCC model in SAC activation has been refined; BubR1 is bound to APC/C at kinetochores, Mad2 loads Cdc20 to BubR1 and APC/C then leaves the complex. Until bipolar spindle attachment is achieved, Cdc20 is degraded. When the kinetochores are all attached with bipolar spindles, BubR1-APC/C-Cdc20 complex changes their conformation and APC/C gets activated with Cdc20 now bound [91]. In this model, as well as the MCC model, BubR1 is the key player in the inhibition of APC/C until SAC is satisfied (Figure 2b).

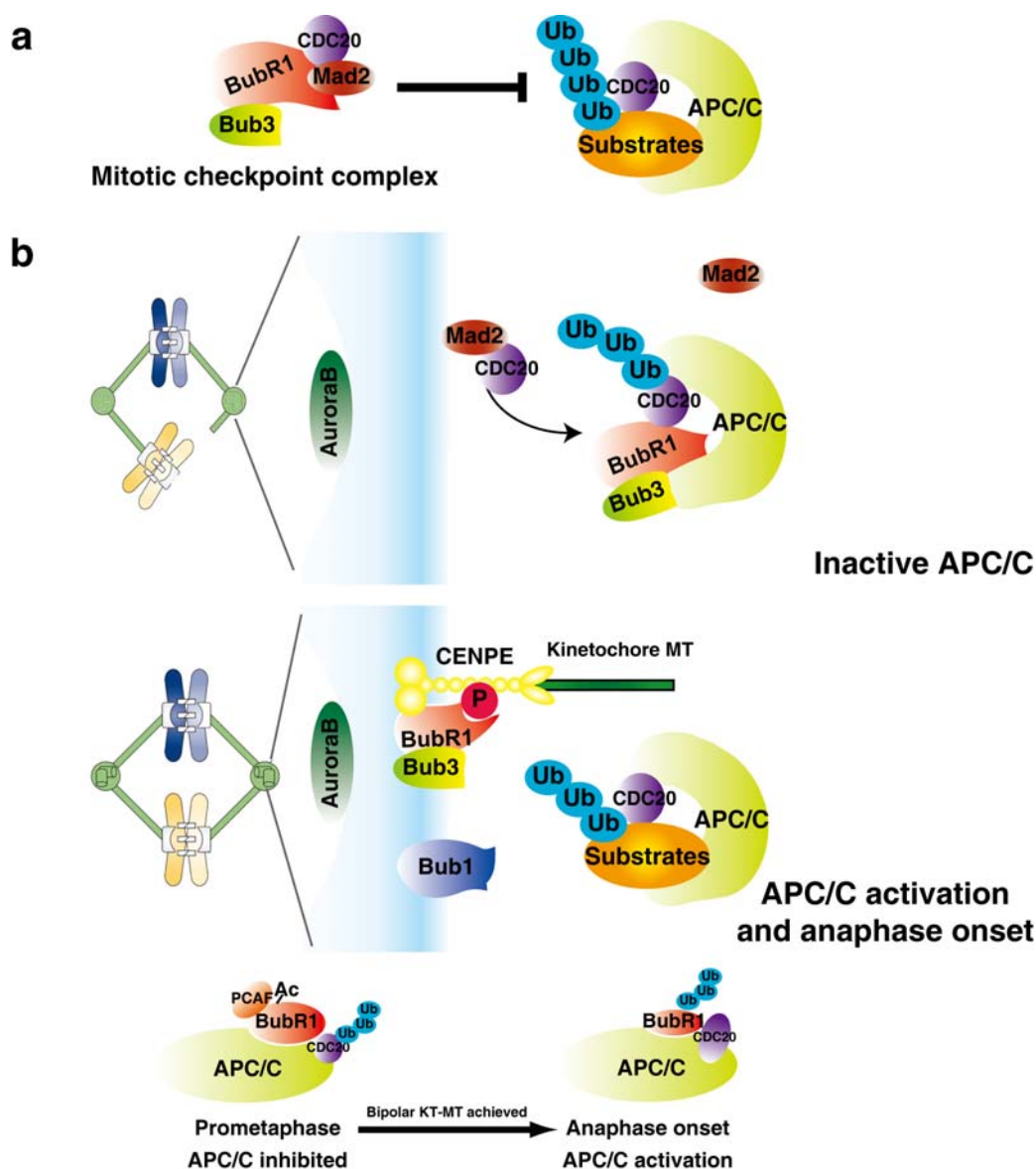


Figure 2. Proposed models of spindle assembly checkpoint. (a) In the MCC model, BubR1, Mad2, Bub3 and Cdc20 form a complex and this complex sequesters Cdc20 coactivator from APC/C, therefore APC/C E3 ligase activity is inhibited. (b) Alternatively, recent biochemical and cell biological studies suggested that Mad2 only loads Cdc20 onto BubR1, which is complexed with APC/C, and leaves the complex. Cdc20 is ubiquitinated and degraded when SAC is active and in SAC off condition, Cdc20 ubiquitination is prohibited and it serves as a coactivator for APC/C. Activated APC/C E3 ligase activity results in the anaphase onset. In addition, BubR1 acetylation/deacetylation provides another layer of modulating APC/C activity.

Then how is bipolar spindle attachments and tension sensed to SAC? The key molecule to this question seems to be BubR1. BubR1 kinase activity is dispensable for inhibiting APC/C E3 ligase activity *in vitro* [88]. However, a microtubule motor protein CENP-E is a binding partner of BubR1 and

functions like cyclins for Cdks. This way, microtubule spindle binding to kinetochores controls the activity of BubR1 kinase [92-96]. In this delicate signaling of bipolar spindle attachments to kinetochores, activation of BubR1, and inhibition of APC/C, and Plk1 [60,97] play essential roles by phosphorylating BubR1 kinase. It is noteworthy that BubR1 in prometaphase or upon spindle disruption shows a characteristic slower migrating form of phosphorylation in SDS-PAGE.

4.2. Signals that activate BubR1

All of APC/C substrates have short stretches of destruction motifs called D box or KEN boxes that are recognized by APC/C and coactivators [90]. APC/C inhibitor BubR1 also has D box and two KEN boxes. However, how BubR1 makes use of these motifs to bind to APC/C and Cdc20 and not being degraded by APC/C has not been understood. Recently, it was found that BubR1 is acetylated by PCAF in prometaphase when it is active. Acetylated BubR1 is prohibited from being a substrate of APC/C. When the SAC is satisfied, BubR1 is deacetylated and becomes a substrate of APC/C. Degradation of BubR1 further activates APC/C, initiating the anaphase onset, thus BubR1 acetylation/deacetylation is a molecular switch for BubR1 from being an inhibitor to a substrate of APC/C. [98] (Figure 2b). This study revealed that in addition to BubR1 phosphorylation, acetylation provides another layer of signaling in SAC control.

4.3. Potentials of BubR1 as a molecular target for cancer therapy

Notably, BubR1 function is crucial for cells in mitosis. In interphase, BubR1 is mainly at cytoplasm. As cells enter mitosis, Bub3 brings BubR1 to the kinetochores [99]. Localization of BubR1 at outer kinetochores is crucial for SAC function for it bound with CENP-E monitors the bipolar spindle attachment at kinetochores. Only in proliferating cells, the localization and signaling of BubR1 becomes crucial.

Conventional anti-cancer therapy has been the DNA damaging drugs or alkylating drugs to kill proliferating cancer cells. It had been noticed that upon the treatment of DNA damaging drugs, cancer cells suddenly die in mitosis. This so called mitotic catastrophe is specific to cancer cells because normal cells with intact G2 checkpoint would arrest the cells in G2 after genotoxic insult. Thus, cancer cells with abrogated interphase checkpoints, e.g. by mutation of p53, would be the ones targeted for mitotic catastrophe after treatment with DNA damaging drugs. Then what is the key molecule receiving and sending the death signals after DNA damage in mitosis?

Studies suggest that BubR1 is phosphorylated and activated in response to adriamycin [100]. Indeed, BubR1 has been suggested to induce mitotic catastrophe in HeLa cells [101]. With these notions, it is conceivable that BubR1 can be an efficient target for cancer therapy. That acetylation of BubR1 regulates SAC activity and modulation of APC/C renders another possibility that specific inhibition of BubR1 deacetylation can be adopted for targeting cancer, since HDAC inhibitors are actively being investigated for anti-cancer therapy in clinical trials [102]. So far, our understanding of BubR1 kinase substrates are poor, and targeting the kinase activity of this important protein in mitosis is yet to be explored.

5. What's next?

We are now living in the interesting times to face the most rapid development of bench to bedside research on mitotic kinase inhibitors for anti-cancer strategy. Hundreds of preclinical results indicate that mitotic kinases are effective targets for the treatment of variety of tumors. However, as we have seen from the cases of Aurora A and Plk1 where both depletion and over expression of them can be dangerous for genomic integrity, inhibition of these kinases do not straightforwardly kill cancer. In fact, we learn more on the mechanisms of how these kinases work in cell division using the specific inhibitors. Thus, clinical trials to validate the efficacy and safety of the mitotic kinase inhibitors should be accompanied with the basic side of science. Right now, we are still waiting for the further phase III clinical trials.

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