Perspective

Discovery and Development of Aurora Kinase Inhibitors as Anticancer Agents

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Introduction

The process of cell division or mitosis is highly complex and tightly regulated. During this phase of the cell cycle two identical copies of DNA are completely separated by the microtubule spindle apparatus and packaged into two daughter cells (see Figure 1 for a description of mitosis). Inappropriate completion of mitosis leads to genetic instability and, frequently, cells that contain nondiploid DNA content (less than or greater than 2 copies of DNA). These phenotypes are hallmarks of nearly all-human cancers.[1] Targeting components of the mitotic machinery in order to block tumor progression has been an area of intense research. This effort has resulted in several marketed anticancer agents, which provide a proof of concept for the approach. Examples include the taxanes and the vinca alkaloids for which the principal target is the microtubule component of the mitotic spindle. More recently, alternative components of the mitotic machinery have been targeted in an attempt to develop novel anticancer agents. These include critical signaling kinases such as the Aurora, Plk, and the Cdk kinases and important motor proteins such as KSP1.[a,2–5]

The Aurora kinases are a family of three highly homologous serine−threonine protein kinases that play a critical role in regulating many of the processes that are pivotal to mitosis. Since their discovery in 1995[6] and the first observation of expression in human cancer tissue in 1998,[7] these kinases have been the subject of intense research in both the academic and industrial oncology communities. This effort has been very successful, and to date, more than 10 Aurora inhibitors have entered early clinical assessment. While the characterizing hallmark for these compounds is generally good selectivity against the vast majority of other kinases, many of them cross-react with a small set of kinases that are themselves implicated in tumor biology. Most notable among these are Abl and Flt-3 kinases.

The Aurora kinase inhibitors can be subdivided in to three general classes: those that have selectivity for Aurora-A over -B, those with selectivity for Aurora-B over -A, and those that are potent inhibitors of both Aurora-A and -B. So far it is not clear whether these alternative selectivity profiles will confer differences in the clinic. However, preclinical work using these compounds as tools and application of biological techniques such as siRNA depletion have provided a great deal of insight into the differential effects of inhibiting each of the Aurora kinases.

As has been the case with other targeted agents, a detailed understanding of the roles that the drug target plays in cancer biology can have a great impact on the successful outcome of clinical trials.[8] This understanding can help define the patient population most likely to respond to the test agent, help to define the end-points for the study design, and also better focus combination studies with other marketed agents. This article will summarize the roles that each of the Aurora kinases plays in mitosis and cancer biology and will discuss how different Aurora inhibition selectivity profiles could impact the cellular and in vivo behavior of the drug candidates. In addition, the wealth of structural biology information for the Aurora kinases will be reviewed. This will be used to provide a rationale for the cross-reactivity profiles of some the Aurora kinase drug

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[a] Abbreviations: Abl, Abelson murine leukemia kinase; APC, anaphase promoting complex; ATP, adenosine 5′-triphosphate; Blk, B-cell lymphocyte kinase; BrdU, 5-bromo-2-deoxyuridine; Cdc25b, cell division cycle 25 homologue B; Cdk, cyclin-dependent kinase; CPC, chromosomal passenger protein; DDR2, discoidin domain receptor2; FAK, focal adhesion kinase; FGFR1, fibroblast growth factor receptor 1; FLT3, FMS-like tyrosine kinase 3; 5-FU, 5-fluorouracil; GSK3β, glycogen synthase kinase 3β; HURP, hepatoma up-regulated protein; INCENP, inner centromere protein; JAK2, Janus kinase 2; KIT, proto-oncogene c-Kit kinase; KMN, KNL-1/Mis12 complex/Ndc80 complex; KSP1, kinesin spindle protein 1; LCK, lymphocyte-specific protein tyrosine kinase; mdm2, murine double minute 2; MCAK, mitotic centromere-associated kinesin; PDGFR, platelet-derived growth factor receptor; Plk, polo-like kinase; PP1 protein phosphatase 1; PRAK, p38-regulated/activated protein kinase; Rb, retinoblastoma protein; RECIST, response evaluation criteria in solid tumors; shRNA, short hairpin RNA; siRNA, small interfering RNA; TPX-2, microtubule-associated, homologue (*Xenopus laevis*); TrkA, tropomyosin-related kinase A; VEGFR, vascular endothelial growth factor receptor.
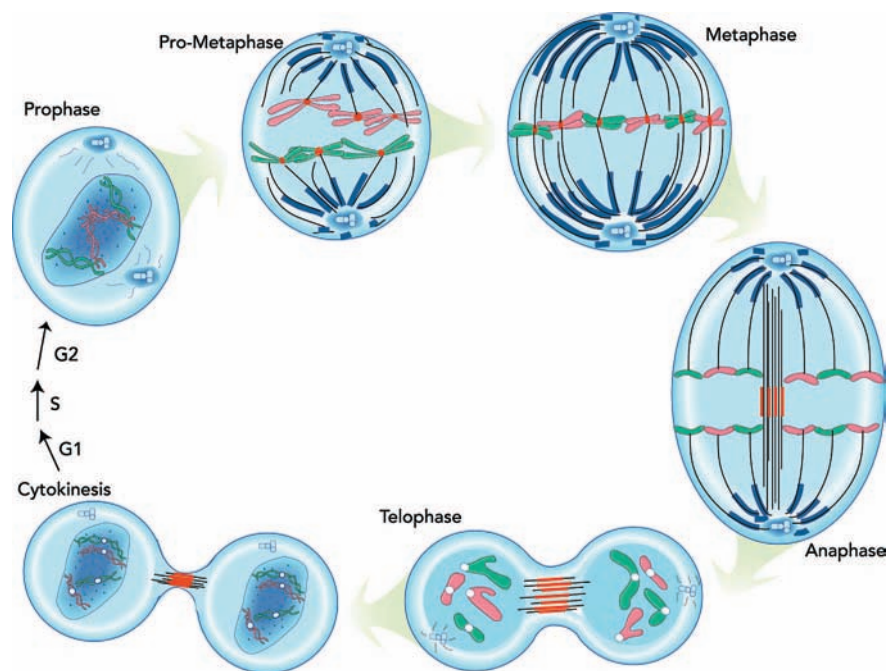
Figure 1. Schematic of mitosis with the expression of Aurora-A and -B kinases highlighted in dark blue and orange shading, respectively. The mitotic phase of the cell cycle is characterized by six distinct phases. Prophase: The loosely coiled duplicated genetic material (chromatin) within the nucleus of the cells is condensed into the ordered chromosomes (comprising two sister chromatids). The centrosomes, which were duplicated earlier in the cell cycle, begin to segregate and nucleate microtubule material that will form the spindle pole. Prometaphase: The centrosomes migrate to opposite ends of the cell, the nuclear envelope breaks down, and the microtubule network of the spindle is extended. Microtubules begin to attach to the middle of the chromosome (kinetochore), and tension within the spindle begins to exert a force on the microtubule−kinetochore, pulling it toward the centrosome. Metaphase: The spindle checkpoint machinery is activated to ensure that microtobules from opposing centrosomes successfully capture kinetochores from each of the two sister chromatids in the chromosome. Opposing tension in the spindle leads to alignment of the chromosomes at the metaphase plate in the middle of the cell. Progression beyond this phase of the cell cycle requires that all chromosomes are correctly captured and aligned. Anaphase: The two sister chromatids are separated by the anaphase promoting complex. Each chromatid is pulled toward the opposing pole, and the microtubule network begins to extend, elongating the cell. Telophase: The two identical copies of genetic material accumulate at opposite ends of the elongated cell and unfold back to form the chromatin. A new nuclear envelope is formed around each set of genetic information forming two nuclei. The extended microtubule network pinches at the middle of the cell between the two nuclei, where the metaphase plate used to be, forming a cleavage furrow. Cytokinesis: A contractile ring is formed at the cleavage furrow, which pinches off the two separate nuclei into two identical daughter cells.[9]

candidates and will review current theories for how selectivity within the Aurora kinase family has been achieved. Finally the discovery and development of the clinical candidates targeting Aurora kinases will be summarized together with a brief discussion of notable structure−activity relationships (SAR).

Aurora Kinases and Mitosis

Numerous studies using biological depletion and inhibition techniques have provided insight to the roles the Aurora kinases play in mitosis. These have been described and extensively reviewed elsewhere.[1,10–17] A brief overview of the functions of the individual isoforms, however, is merited, since it provides the basis for a discussion on the cell and in vivo profiles of Aurora inhibitors with differing selectivity profiles.

Aurora-A. Aurora-A is involved in regulating many of the early mitotic events including entry into mitosis.[18] Expression of the protein and its kinase activity rise during the G2 phase of cell cycle and peak in early mitosis (Figure 1).[19] Depletion of Aurora-A results in delayed entry into mitosis and marked disruption of the spindle with monopolar spindles a frequent observation.[20–24] At the molecular level, the roles that Aurora-A plays in many of the mitotic processes remain to be fully elucidated; however, there have been some critical discoveries that help define the profile for Aurora-A inhibition and that help identify Aurora-A specific biomarkers.

Aurora-A is able to phosphorylate Cdc25b, a direct regulator of the cyclin B1-Cdk1 complex. Activation of this complex is an essential requirement for mitotic entry and may provide a basis for the role Aurora-A plays in regulating entry into mitosis.[25] The observation of monopolar spindle formation following Aurora-A depletion can be attributed to a defect in centrosome maturation and separation and also to a defect in the organization of microtubules that form the spindle. Aurora-A regulates centrosome maturation by moderating the recruitment of proteins to the centrosome that are themselves essential for accumulating microtubule spindle components such as γ-tubulin. These critical proteins include TPX-2, Ajuba, Bora, and Lats, which can also act to regulate Aurora-A activity as part of an important feedback loop.[25]

In addition to a role in centrosome maturation, Aurora-A is also associated with the separation of centrosomes. Phosphorylation of the kinesin motor protein Eg5, a critical driver of centrosome separation, by Aurora-A has been described.[26] Eg5 acts to generate opposing forces on the overlapping microtubules that are being generated between the two centrosomes.[27,28] Finally, an understanding of the role Aurora-A plays in regulating the microtubule network that forms the spindle is beginning to emerge. In one model Aurora-A is critical to the regulation of the EXTAH multiprotein complex. This complex comprises the proteins Eg5, XMAP2154, TPX-2, Aurora-A, and HURP, which have microtubule binding, cross-linking, and kinesin motor activities. Together they act to bundle, cross-link, and stabilize the growing microtubule network. Many of the components of this complex are themselves substrates for
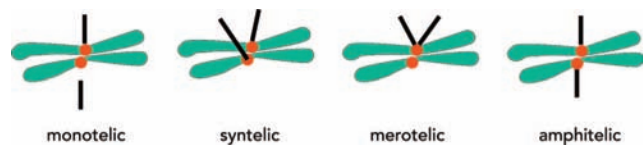
Figure 2. Kinetochore attachment to microtubules of the mitotic spindle. Monotelic attachment: One kinetochore is attached to microtubules from one spindle pole with the other kinetochore unattached. Syntelic: Both kinetochores are bound to microtubules from the same spindle pole. Merotelic: A single kinetochore is bound to microtubules from the same pole. Amphitelic: The two kinetochores are attached to microtubules from opposing poles. Only amphitelic attachment leads to appropriate segregation of the chromosomes.

Aurora-A. Disruption of any component of the complex perturbs spindle formation, leading to multi- and monopolar spindles.[25]

It is clear that Aurora-A plays an important role in many of the processes that are critical to mitosis and that major mitotic defects are observed following Aurora-A depletion. Therefore, it could be anticipated that selective Aurora-A inhibitors would have a significant antimitotic effect. The cell profile following Aurora-A inhibition would be expected to include a delay in mitotic entry followed by defects in chromosome segregation as a result of aberrant spindle formation that would lead to aneuploidy (nondiploid DNA content). Depletion studies also suggest that following these mitotic defects, cells treated with an Aurora-A inhibitor will be deleted by apoptosis.[24]

Aurora-B. Aurora-B is the catalytic component of the chromosomal passenger complex (CPC) that is critical for the correct progression through and completion of mitosis. This complex is made up of four proteins Aurora-B, survivin, INCENP, and borealin. The noncatalytic components of the complex act, in part, to regulate the localization and activation of Aurora-B.[29] Depletion of any component of the complex results in mislocalization of the other components and disruption of mitotic progression.[30] Survivin, INCENP, and borealin are known substrates for Aurora-B,[31–33] and though the impact of this phosphorylation is unknown for borealin and survivin, in the case of INCENP it appears to be part of a positive activation feedback loop.[34,35]

The CPC is initially formed along chromosome arms before concentrating at the centromeres, a region of the double chromosomal structure where sister chromatids join. It finally localizes to the spindle midzone during cytokinesis (see Figure 1). This localization of the CPC is consistent with the multiple roles it plays in mitosis. These include condensation of the chromosomes, formation of the bipolar spindle, attachment of the chromosome to the mitotic spindle, regulation of the spindle checkpoint, and completion of cytokinesis.[29,30,34]

Condensation of chromosomes is a prerequisite for segregation of duplicated DNA. Histones are known to be critical mediators of this condensation process, and Aurora-B regulates the activity of the pivotal histone, H3, by phosphorylation at Ser10 and Ser28.[36,37] Histone H3 phosphorylation at Ser10 is used as a biomarker for Aurora-B activity both in vitro and in vivo.[38] Once the chromosomes have condensed and the centrosomes segregated, a bipolar spindle is formed. The CPC is critical to promoting formation of this spindle.[39] Specifically, Aurora-B phosphorylates and negatively regulates the microtubule destabilizing protein stathmin. In addition, Aurora-B phosphorylates the depolymerizing protein MCAK; blocking this event leads to cells that are unable to support bipolar spindles.[40,41] Once the mitotic spindle has started to form, the two sister chromatids attach to microtubules from opposing spindle poles (Figure 2). This connection is made via a multiprotein complex

that resides in the center of each of the chromatids known as the kinetochore.[42,43] Failure of kinetochores to bind with fidelity to the spindle leads to aberrant segregation of the chromosomes.[42,44] To avoid inappropriate connections, the cell destabilizes incorrect attachments and stabilizes correct "amphitelic" attachment.[30,45] The CPC is a critical mediator for both of these processes.[30] At sites of inappropriate connection Aurora-B phosphorylates a multiprotein complex termed the KMN complex. This complex binds the microtubules and kinetochores together. Phosphorylation by Aurora-B disrupts its binding to the microtubule, leading to release of the kinetochore.[46,47] In contrast, at sites of correct kinetochore capture, Aurora-B phosphorylates the microtubule depolymerase MCAK, inhibits its activity, and thus stabilizes the spindle.[48]

In addition to the role Aurora-B plays in promoting kinetochore to microtubule attachments, the CPC has a very important role to play in policing the fidelity of these attachments throughout the cell. This process, known as the spindle checkpoint, acts to block further progression through mitosis until all kinetochores are correctly attached.[49] The checkpoint blocks activation of a ubiquitin ligase known as the anaphase promoting complex (APC) and also blocks activation of the protease separase. These act together to cleave the connection between two sister chromatids. The checkpoint, which is regulated by the CPC, is activated by unattached kinetochores. The checkpoint itself comprises of many proteins; most notable among them are the BubR1 and Bub1 kinases and the Mad proteins (Mad1 and Mad2). Together, these bind unattached kinetochores and directly inhibit the APC. Aurora-B has been shown to be a master regulator of the checkpoint, since it phosphorylates BubR1, activates its kinase activity, and directs its location to unattached kinetochores. BubR1 then acts as the catalyst for recruitment and activation of the other checkpoint components. Only after all kinetochores are correctly attached is the checkpoint disassembled and the APC activated.[50–52]

Toward the end of mitosis Aurora-B localizes to the spindle midzone where it is known to play a role in formation of the cleavage furrow, a critical requirement for cytokinesis. Although the molecular details for Aurora-B's role in cytokinesis and formation of the cleavage furrow are not clear, it is known that Aurora-B can phosphorylate proteins that are important for this process. These substrates include the kinesin motor protein MKPL and the intermediate filament structural proteins desmin and vimentin.[53–56] Consistent with an important role in cytokinesis, cells in which Aurora-B activity has been depleted prematurely exit mitosis without division. This leads to cells with large polyploid nuclei containing multiple copies of the DNA.[57]

A number of elegant studies have characterized the gross cellular effects of disrupting Aurora-B in cells. Given the numerous individual roles that Aurora-B plays in mitosis, this effort has been instrumental in helping predict the overall impact of small molecule Aurora-B inhibitors. Frequently these experiments have relied on expression of kinase dead protein, siRNA depletion of total protein, or microinjection of neutralizing antibodies. The most frequent observations are disruption of kinetochore—microtubule interactions, resulting in misaligned chromosomes, and multinucleated or polyploid cells. This can be attributed to abrogation of the mitotic spindle checkpoint, failure to correct inappropriate kinetochore—microtubule attachments, and a subsequent failure to complete cytokineses. Override of the spindle checkpoint has been confirmed in experiments where Aurora-B depletion or inhibition with neutralizing antibodies is able to bypass the mitotic arrest induced by the microtubule targeted agents nocodozole and

paclitaxel.[44,57–61] While these studies clearly show that depletion of Aurora-B activity leads to major defects in mitosis and the generation of polyploid cells, the fate of such abnormal cells was not generally assessed. However, the discovery and characterization of small molecule Aurora-B inhibitors have addressed this point. Mitotic defects caused by treatment of cells with Aurora-B inhibitors cause the damaged cells to be deleted by apoptosis.[59,62]

Aurora-C. Aurora-C is the least well studied of the three Aurora kinases, and details of the role that this enzyme plays in mitosis are not well defined. Aurora-C is expressed in most somatic tissues at low levels, significantly lower than that for Aurora-A or -B. The one exception is testis where Aurora-C expression is high.[15,63–65] In keeping with this expression profile, a key role in meiosis has been established with homozygous and heterozygous knockout mice. These mice show a grossly normal physiology but are subfertile with defective spermatozoa. This is associated with aberrant chromosome condensation and polyploidy.[66,67]

The localization of Aurora-C is highly dynamic. In early mitosis it is found on centromeres before relocalizing to the midzone and finally to the vicinity of the cleavage furrow. This is consistent with Aurora-C being a chromosomal passenger complex protein. In keeping with this, Aurora-C colocalizes with Aurora-B throughout mitosis and directly interacts with the other CPC components INCENP, survivin, and borealin.[68–71] Overexpression of an inactive mutant form of Aurora-C leads to a high frequency of polyploid cells with multiple nuclei.[69,72] Furthermore, expression of kinase dead Aurora-C leads to extensive apoptosis.[68] These findings are reminiscent of the Aurora-B depletion studies. Interestingly overexpression of wild type Aurora-C is able to rescue the multinuclear phenotype from Aurora-B depletion.[69,70] Taken together, these data suggest an overlapping role with Aurora-B and may indicate a degree of redundancy between the two proteins.

Aurora Kinases and Cancer

Since their discovery in the 1990s a wealth of scientific and clinical evidence has emerged that strongly links the Aurora kinases to the progression of human cancer.

The Aurora-A gene maps to the 20q13 region of the genome, which is frequently amplified in human cancers.[7] Overexpression of the Aurora-A protein is also observed in many cancers.[73] Ectopic overexpression of Aurora-A in vitro leads to the formation of cells that display many of the characteristic hallmarks of cancer such as centrosome amplification, aneuploidy, chromosomal instability, and extended telomeres.[25,74] Consistent with these observations, expression of Aurora-A, or its activating partner TPX-2, correlates with chromosomal instability in human cancer.[74,75] In addition, Aurora-A associates with, and negatively regulates, a host of critical tumor suppressors.[15] Perhaps most notable among these is p53 where Aurora-A promotes mdm2-mediated degradation of p53 and inhibition of its transcriptional activity.[17,76] Despite these oncogenic activities, Aurora-A is not a formal oncoprotein, since it does not consistently transform cells in vitro and overexpression is not generally tumorigenic in vivo.[77] The exceptions appear to be NIH3T3 and Rat1 fibroblasts where Aurora-A expression does indeed transform these cells in vitro.[7,18] Taken together, these observations suggest that Aurora-A generally requires disregulation of other oncogenes to be transforming. One potential impact of this is that selective inhibitors of Aurora-A may only show potent anticancer activity against specific cancer cells or may be best used in combination with

other agents. This appears to contrast with the previously described depletion studies in which removal of Aurora-A routinely leads to significant defects in mitosis. The disparity may be explained by the fact that these studies have only been carried out on a relatively small number of cancer cell lines. It is likely that the true impact of Aurora-A inhibition will only be revealed once selective inhibitors have been profiled against a very wide range of cancer cells.

Consistent with the hypothesis that Aurora-A inhibition may have benefit in combination with other therapies, several groups have shown that depletion of Aurora-A sensitizes cancer cells to the cytotoxic effects of chemotherapeutic agents such as the taxanes,[78,79] cisplatin,[80] and ionizing radiation.[81]

The Aurora-B gene maps to the 17p13.1 region of the genome that is altered in some human cancers.[82] Aurora-B mRNA and the protein itself are frequently overexpressed in cancer and a correlation between protein expression and disease severity has been reported in several tumor types.[73,82–85] In addition to these direct links between Aurora-B and cancer, it is interesting to note that CPC proteins, which act in concert with Aurora-B and are regulated by Aurora-B, are also commonly overexpressed or up-regulated in cancer.[86–89] Despite strong links with cancer and the critical role Aurora-B plays in regulating many mitotic processes, it is not transforming in vitro and is not generally tumorigenic in vivo.[82,90] One important exception to this is an increase in tumorigenic behavior when Aurora-B is overexpressed in a cell line expressing a mutant form of p53.[91] Results from these experiments suggest that while Aurora-B is not a formal oncogene in its own right, it can act in cooperation with other oncogenic mutations to promote tumor progression. Consistent with this concept, Aurora-B enhances Ras-mediated transformation, while depletion of Aurora-B with short hairpin RNA inhibits Ras transformation.[90] The case for Aurora-B as a key anticancer target is further strengthened by studies that show depletion of Aurora-B sensitizes cancer cells to the cytotoxic effects of therapies that include alkylating agents and ionizing radiation.[92,93]

Recent data suggest an overlapping role for Aurora-C with Aurora-B in many mitotic processes. Although Aurora-C expression has been observed in a number of human cancer cell lines, a distinct role for Aurora-C in tumorigenesis has not been defined.[63,69]

Small Molecule Inhibitors of Aurora Kinases as Chemical Probes

Depletion of Aurora kinase activity using techniques such as siRNA or expression of kinase inactive protein has helped to predict the biological profile for Aurora inhibitors. However, these techniques do not readily differentiate between inhibition of catalytic kinase activity and any scaffold function that the proteins may have. For this, selective small molecule inhibitors are required.

Dual Aurora-A and -B Inhibitors. In 2003 the first of the small molecule Aurora inhibitors was disclosed (ZM447439 **1** and hesperadin **2**; see Figure 3). Compound **1** inhibits both Aurora-A and -B (IC$_{50}$ of 110 and 130 nM, respectively) with good selectivity over other unrelated kinases (affinity for Aurora-C is not reported).[59] This selectivity profile has encouraged its wide use as a chemical probe. Compound **2** is reported as an inhibitor of Aurora-B (IC$_{50}$ of 250 nM) with significant cross-reactivity against six other kinases (no data on Aurora-A or -C are reported).[58] Both inhibitors display a similar overall phenotype: inhibition of histone H3 phosphorylation (Ser10) and endoreduplication in the absence of cell division, resulting
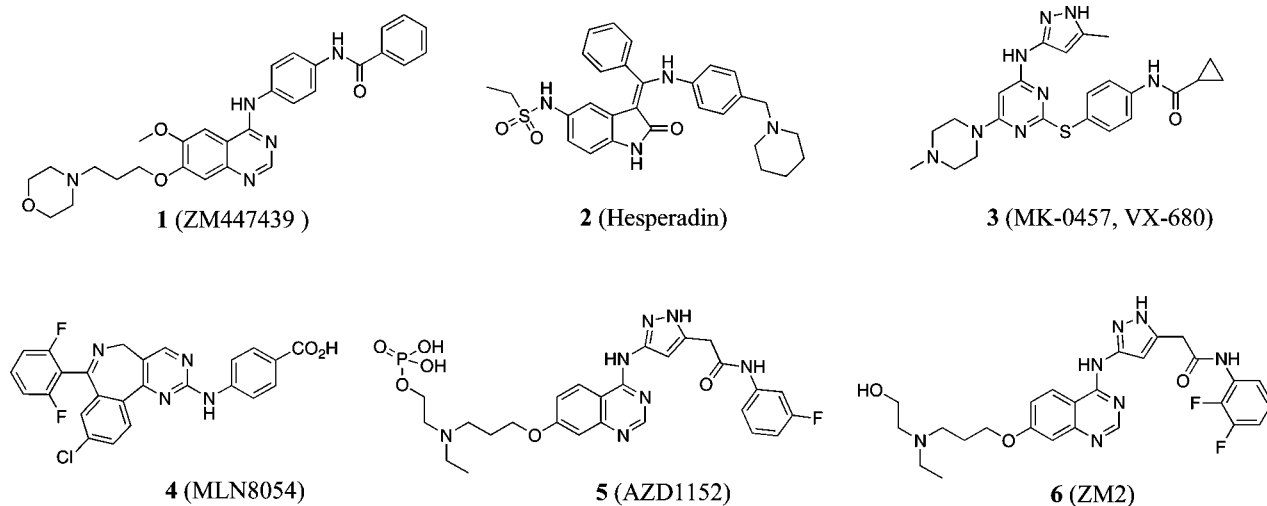
Figure 3. Small molecule inhibitors of Aurora kinases that have been used as tools to characterize the effects of inhibiting kinase activity.

in large polyploid cells. In some cases giant cells with up to 32 copies of DNA are observed. A failure to complete cytokinesis was shown in an elegant study in which cells were released from a G1 block in the presence of compound **1**.[59] Under these conditions entry and exit from mitotis proceeded with normal kinetics, though the cells failed to divide. Both inhibitors cause marked chromosomal misalignment, often with the chromosomes lining up parallel to the spindle with nonamphitelic kinetochore−microtubule connections. Despite these aberrations, separation of the two sister chromatids still occurs. Taken together, these events strongly suggest that inhibition of Aurora kinase activity leads to abrogation of the spindle checkpoint and mitotic exit in the absence of appropriate chromosomal attachment. This was confirmed when both compounds were shown to override a mitotic arrest caused by the taxane family of microtubule stabilizers. More detailed analysis showed that many of the components of the spindle checkpoint were mislocalized including BubR1, Mad2, and CENP-E. Unsurprisingly, given the major defects in mitosis that are observed, cells treated with compound **1** show decreased viability.[58,59]

The in vivo potential for Aurora kinase inhibitors was first demonstrated in 2004 with the disclosure and characterization of **3** (MK-0457 (VX-680), Figure 3).[62] Compound **3** is a potent inhibitor of all three Aurora kinases and generally has good selectivity against other kinases.[94,95] Consistent with the previous dual-Aurora-A/-B inhibitors (**1** and **2**), compound **3** rapidly induces polyploidy and blocks proliferation. Most notably cells with Aurora mediated mitotic disregulation are committed to an apoptotic cell death. This is the case for all cycling cells treated with **3** including cancer cell lines, noncancer cells, and primary tumor samples. In keeping with this cell cycle associated cell death mechanism, no loss of viability is observed in noncycling cells. In rodent models of human cancer compound **3** very effectively blocks the growth of tumors and in some cases even induces tumor regression. Immunohistochemical analysis of the compound **3** treated tumors shows clear inhibition of histone H3 phosphorylation, consistent with Aurora-B inhibition, and a marked increase in apoptosis. Efficacious doses of **3** are well tolerated, but a significant and reversible reduction in the neutrophil population is observed (>50% reduction at nadir). This is attributed to the effects of Aurora kinase inhibition on cycling noncancer cells and forms the basis for mechanism-based neutropenia that is commonly observed with Aurora kinase inhibitors in the clinic.[96] Compound **3** can induce the formation of monopolar spindles, a phenotype described fol-

lowing depletion of Aurora-A and one that is not reported for the alternative dual-Aurora-A/-B inhibitor **1**.[97] Accordingly, **3** inhibits phosphorylation of specific Aurora-A substrates in addition to the Aurora-B substrate histone H3.[97] As such, it is likely that the effects **3** has on mitosis and cell viability are driven by inhibition of both Aurora-A and -B.

Although it has been widely reported that Aurora kinase inhibitors will lead to aberrant mitosis and block proliferation in all cycling cells, a number of groups have now demonstrated that the ultimate fate of cells appears to be dependent on their genetic background. On treatment with **1** or **3**, cells that lack a functional p53 response undergo rapid and extensive endoreduplication in the absence of cytokinesis, followed by apoptosis. On the other hand, cells with a fully functional p53 response undergo less endoreduplication.[59,98] This result is attributed to the role that p53 plays in the G1 tetraploidy checkpoint.[99,100] It will be interesting to see whether such context dependency is observed in the clinic.

The emergence of mechanisms that render tumors resistant to chemotherapy is a frequent occurrence. In many cases resistance is associated with the expression of drug pumps, though mutations in the drug target itself are also observed.[101] A small number of polymorphisms in human Aurora genes have been observed though the impact of these on kinase activity or susceptibility to drugs has not been characterized.[102] A recent study has shown that by incubation of cancer cells in the presence of compound **1** for extended periods of time, it is possible to generate clones that are resistant to the compound. Detailed analysis shows that this resistance is due to a number of mutations in the Aurora-B protein. Expression of these mutant Aurora-B proteins in cells overrides the effects **1** has on histone H3 phosphorylation, cell cycle profile, and spindle checkpoint.[103] This very clearly shows that the antimitotic effects of **1** are associated with inhibition of Aurora-B and not Aurora-A. These Aurora-B mutations also block the effects of compound **3** on cell cycle profile and colony formation.[103] Again, this suggests that the anticancer properties of **3** are associated with Aurora-B inhibition and not inhibition of Aurora-A or -C. This is somewhat surprising given the antimitotic effects from Aurora-A depletion. This suggests that in this experiment there is either incomplete inhibition of Aurora-A by compounds **1** and **3**, or it suggests that inhibition of the catalytic activity of Aurora-A has only subtle effects on its own. Taking these data together with the observations that Aurora-A is generally not transforming or tumorigenic in its own right, the conclusion is that inhibiting Aurora-A may have the most effect when in combination with other

cancer therapies. Most significantly, however, this study highlights the potential for the emergence of clinical resistance to Aurora kinases inhibitors. This will be keenly monitored by those taking Aurora kinase inhibitors through the clinic.

In addition to the single agent effects of these dual Aurora kinase inhibitors, a number of groups have now shown that these compounds are able to sensitize cells to other therapeutic modalities. The efficacy of dexamethasone, doxorubicin, etoposide, vincristine, daunorubicin, and ionizing radiation has been enhanced when assessed in combination with dual Aurora-A/-B inhibitors.[23,92,104–107] While in some cases the combination effect can be attributed to the direct actions of Aurora kinase inhibition on key mitotic processes, in other cases a mechanistic basis for the observed synergy is not clear. Nevertheless these preclinical observations are likely to play a significant role in the design of clinical studies.

Selective Inhibitors of Aurora-A. The first Aurora kinase inhibitor with selectivity for Aurora-A, **4** (MLN8054, Figure 3), has recently entered clinical trials. This compound is 40-fold selective for Aurora-A over Aurora-B in enzyme assays (IC_{50} of 4 and 172 nM, respectively) and shows a greater apparent selectivity for Aurora-A over Aurora-B in cells (IC_{50} for inhibition of Aurora-A autophosphorylation of 34 nM with an IC_{50} for inhibition of histone H3 phosphorylation of 4 μM).[108] In biochemical assays, the compound is selective for Aurora-A against 226 other kinases that were tested (only seven kinases showed >50% inhibition at 1 μM). No data are available on the cross-reactivity of **4** with Aurora-C.[108]

In multiple cell lines low concentrations of compound **4** that inhibit Aurora-A but minimally inhibit Aurora-B lead to robust inhibition of proliferation (IC_{50} values typically below 1 μM). Detailed analysis revealed an accumulation of cells with 4N DNA and polyploidy along with severe defects in the alignment of chromosomes on the metaphase plate.[108] These phenotypes appear to be more consistent with Aurora-B depletion studies. In keeping with the predicted role of Aurora-A, however, cells treated with **4** have a high frequency of abnormal spindles with mono- and multipolar spindles a common observation. In addition, consistent with Aurora-A's function in centrosome maturation and segregation, cells with just one centrosome are frequently observed. Interestingly, despite the defects in centrosome segregation, cells with multiple spindle poles are still observed, though all but one of these poles lack a centrosome.[109] Unsurprisingly given all these aberrations, progression through mitosis is delayed. The average time to progress from prophase to telophase is extended from 67 min in control cells to 131 min in compound treated cells. This characteristic is consistent with biological depletion of Aurora-A. Compound **4** causes significant single agent tumor growth inhibition in mouse xenograft models. At doses of the compound that inhibit tumor growth, biomarker studies show sustained inhibition of Aurora-A but with a transient inhibition of Aurora-B at the early time points where compound exposure is highest.[108] This Aurora-B inhibition may be a contributing factor to the antitumor activity of compound **4**.

The impact of Aurora-B cross-reactivity on the anticancer properties of compound **4** was confirmed in cells expressing mutant forms of Aurora-B that are resistant to the dual Aurora-A/-B inhibitors **1** and **3**. In vitro, at concentrations of **4** predicted to completely inhibit Aurora-A and only partially inhibit Aurora-B (5 μM), the accumulation of cells with 4N DNA content and concomitant loss of the G1 population are completely blocked in cells expressing these mutant forms of Aurora-B. However, its ability to block colony formation is unaf-

fected.[103] This suggests that cross-reactivity with Aurora-B is responsible for some of the effects that compound **4** has on cells but that cytotoxicity is not associated with inhibition of Aurora-B; rather, it is most likely to be associated with Aurora-A inhibition or an off-target activity. Although many of the phenotypes observed with **4** are consistent with the anticipated roles of Aurora-A, the precise effects of a truly selective Aurora-A inhibitor remain to be reported.

Selective Inhibitors of Aurora-B. Compound **5** (AZD1152) is the first Aurora-B selective inhibitor to enter clinical trials (Figure 3). This compound is a dihydrogen phosphate prodrug that is rapidly converted in plasma to the active agent AZD1152-HQPA (**7**, Table 7). This active agent is a highly potent inhibitor of Aurora-B (K_i < 1 nM), a moderate inhibitor of Aurora-C (K_i of 17 nM), and a poor inhibitor of Aurora-A (K_i of 1.4 μM) and 20 other kinases tested (IC_{50} > 1 μM for 19/20 kinases screened; Lck was inhibited with IC_{50} of 170 nM).[110,111] The biological profile of compound **7** is similar to that seen for the dual Aurora-A/-B inhibitors such as compound **3**. This is perhaps not surprising given that the antimitotic effects of the dual-Aurora-A/-B inhibitors are blocked in cells expressing drug resistant mutant forms of Aurora-B.[103] Compound **7** induces extensive polyploidy and apoptosis in cells and blocks the phosphorylation of histone H3 at Ser 10.[110,112] Notably, treatment of cells with **7** does not affect the kinetics of mitosis, since cells are able to enter and exit mitosis at a rate that is comparable with control cells.[111] Extended studies with a close analogue, ZM2 (**6**) (Figure 3), show that this disruption in mitosis is associated with incorrect attachment of kinetochores to microtubules (**6** has a similar selectivity profile to **7**, with IC_{50} values in biochemical assays of 7.5 nM against Aurora-B and 800 nM against Aurora-A). Chromosomes fail to align on the metaphase plate and instead frequently line up parallel to the spindle.[112] The exit from mitosis in the absence of correct chromosomal attachment that is observed with these agents requires an override of the spindle checkpoint. This override of the spindle checkpoint following Aurora-B inhibition was confirmed with **6**, since it abrogates the mitotic arrest induced by treatment of cells with paclitaxel.[112] These data for compounds **6** and **7** are entirely consistent with biological studies where Aurora-B activity was depleted using techniques such as siRNA and confirm that catalytic inhibition of Aurora-B leads to inappropriate mitosis and polyploidy in vitro.

The prodrug **5** markedly inhibits tumor growth in vivo and in some cases causes significant tumor regression. A decrease in phosphorylated histone H3, an increase in cells with 4N or higher DNA content, together with an increase in large multinucleated cells and increased apoptosis are all observed in tumor tissue from compound treated animals.[110,113] A marked but transient decrease in bone marrow cells is also observed, though this had recovered within 5 days of the last dose of compound. Further analysis shows transient myelosupression with neutrophils the most affected cell population.[113] This nontumor cell effect is comparable to that seen with the dual Aurora-A/-B inhibitors.

A number of studies have used the active agent or prodrug (**7** or **5**, respectively) to explore the potential of Aurora-B inhibitors to sensitize cancer cells and tumors to chemotherapeutic agents and ionizing radiation. Compound **7** synergistically enhances the antiproliferative and cytotoxic activity of vincristine (a tubulin depolymerizing agent) and daunorubicin (a topoisomerase II inhibitor) in vitro. In vivo compound **5** potentiates the effect of both agents in mouse xenograft models of leukemia but does not increase their
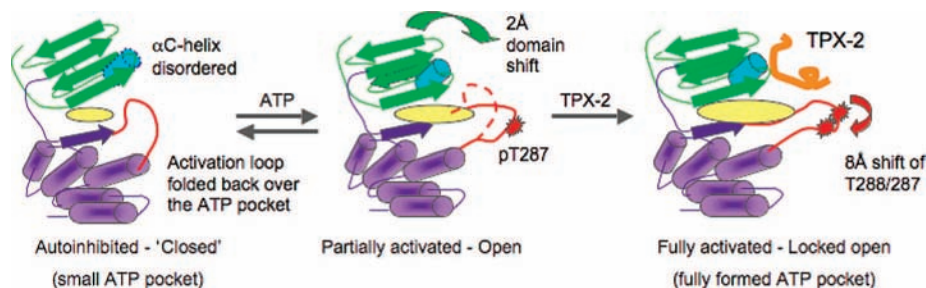
Figure 4. Cartoon representation of the conformational changes associated with Aurora-A activation. The N-terminal lobe is colored green, the C-terminal lobe colored purple, and the αC-helix colored cyan. The activation loop is shown in red, and the ATP binding pocket is represented by a yellow ellipse.

toxicity.[106] In addition, pretreatment with **7**, prior to ionizing radiation, leads to a marked enhancement of cell death and inhibition of cell growth in vitro. This effect is most pronounced in cells deficient in p53.[114]

Selective Inhibitors of Aurora-C. No selective inhibitors for Aurora-C have been reported.

Mechanism of Activation and Protein Conformational Considerations in the Design of Aurora Kinase Inhibitors

One of the most significant challenges for drug discovery efforts targeting individual protein kinases has been achieving appropriate selectivity. This arises because of the common strategy used to design ligands. Most compounds compete with the ATP substrate and as such bind in a pocket that is highly homologous across the 500 or so members of the protein kinase family.[115] Given this challenge, a detailed knowledge of the structural conformation of the targeted ATP binding site is a significant advantage when it comes to the rational design of specific ligands.

It is now accepted that many protein kinases exist in two distinct conformations: "closed", which normally corresponds to an inactive form, and "open", corresponding to the active form. The activation of protein kinases typically involves repositioning of key catalytic residues in the active site and the correct organization of regions of the protein that will form the substrate-binding pocket. In many cases phosphorylation of a critical residue on the "activation loop" is sufficient to drive these conformational changes.[117] In some cases, however, interactions with additional coactivators are required. Perhaps the best-studied example of this is activation of the CDKs by the cyclin family of proteins.[116,117]

A series of elegant biochemical and structural studies have shown that both Aurora-A and -B require phosphorylation of the activation loop along with binding to partner proteins. In the case of Aurora-A TPX-2 is required, and for Aurora-B it is INCENP.[34,35,118]

Autophosphorylation on Thr288 in the activation loop and binding to TPX-2 are required for full activation of Aurora-A.[118] Crystal structures of Aurora-A bound and unbound to TPX-2 have now been solved and have helped to define the molecular mechanism for full activation. When bound to TPX-2, the conformation of Aurora-A is very similar to that of many of the known active kinase conformations; all the important catalytic residues are correctly aligned for catalysis (Lys162 to align the phosphate for transfer, Asp271 to coordinate the Mg²⁺ ion and the catalytic base Asp256), and the activation loop is stabilized in an extended conformation that provides an open platform for ATP to bind. In addition, the phosphorylated threonine in the activation loop is buried and not solvent exposed. This acts to block dephosphorylation by the negative

regulator, the phosphatase PP1. In the absence of TPX-2 the apo-crystal structure shows much more disorder, particularly in the region of the activation loop. Despite this, clear overlap of parts of the activation loop with the ATP-binding site can be seen. The result is a small pocket that is not consistent with ATP binding. In addition the Pi-Thr288 residue undergoes an 8 Å shift, when compared with the Aurora-A structure not bound to TPX-2, and is exposed to bulk solvent.[119] Figure 4 shows a cartoon schematic for Aurora-A activation.

A cocomplex crystal structure of Aurora-A bound to the pan-Aurora inhibitor, **3**, has been published.[95] In this structure the protein adopts a closed, inactive conformation, and close inspection shows that this form is quite different from other kinase structures that have been published. For example, a small hydrophobic pocket is evident in the vicinity of the ATP binding domain that is capped by a phenylalanine residue. The closest structural homologues that can form a comparable hydrophobic pocket in the closed conformation appear to be Flt-3 and Abl kinases.[95] Given the unusual conformational nature of the inactive form of Aurora-A and the presence of an additional hydrophobic pocket, an attractive strategy is to target this form of the enzyme as a means to introduce selectivity.

Full activation of Aurora-B requires two processes: autophosphorylation at Thr248 in the activation loop (equivalent to Thr288 in Aurora-A) and binding to the CPC protein INCENP. Biochemical studies have shown that INCENP binding elevates Aurora-B activity by up to 10-fold. Interestingly during this process INCENP itself is phosphorylated at multiple positions by Aurora-B. In turn this phosphorylation leads to full activation of Aurora-B. A model for activation of Aurora-B has been proposed that is initiated with complexation of Aurora-B with INCENP; autophosphorylation of Aurora-B then takes place, followed by phosphorylation of INCENP by Aurora-B. These events then lead to a conformational change in Aurora-B and full kinase activation.[34,35] Consistent with this mechanism, crystal structures of Aurora-B bound to a truncated form of INCENP, which lacks the critical phosphorylation residues, show only a partially activated conformation for Aurora-B. Two key differences are evident when this complex is compared with the fully active Aurora-A/TPX-2 complex. First, active site residues critical for alignment of phosphates on ATP are not optimally positioned for phosphate transfer, and second, the catalytic cleft adopts a more open conformation than in the Aurora-A structure that is also not optimal for binding of ATP. Although the structures of fully activated and fully inactive Aurora-B have not been solved, it is proposed that binding phosphorylated INCENP will result in a closing of the catalytic cleft, alignment of the key catalytic residues, and generation of a fully active and open Aurora-B conformation.[35]

In the absence of a comprehensive conformational description for Aurora-B it is not possible to fully characterize the opportunities of targeting individual conformations for obtaining selectivity. However, given that compound **3** potently inhibits both Aurora-A and -B, along with other kinases that adopt a closed form, it is likely that Aurora-B adopts an inactive conformation that is similar to that for Aurora-A.[95]

No structures have been reported for Aurora-C.

Taken together, the structural studies with both Aurora-A and Aurora-B highlight multiple opportunities and tactics for designing inhibitors with good potency and selectivity. The unusual conformation of the inactive Aurora-A structure, including the presence of a small hydrophobic pocket adjacent to the ATP binding site, makes this form of the enzyme a very attractive target, particularly to gain selectivity over other kinases. In addition, the extensive rearrangements that the protein undergoes during activation highlight a high degree of flexibility. This appears to be particularly the case for the activation loop and the ATP binding pocket. This property of the Aurora kinases may be useful if inhibitors could be designed that interact with these regions of the protein to induce conformational change (in both active and inactive conformations). For example, ligands may be able to stabilize a particular protein conformation by promoting hydrophobic collapse around the compound. Alternatively the inhibitor may push against the protein causing local changes in conformation, which would not be tolerated in kinases that do not have a similar degree of flexibility.

Discovery and Development of Aurora Kinase Inhibitors

Since the discovery of the Aurora kinases and their association with cancer, there has been intense effort directed at identifying selective inhibitors as potential drugs. To date, more than 10 small molecules have entered clinical studies (Table 1). Each of these is reviewed below.

Approach Taken by Vertex and Merck to the Pan-Aurora Inhibitor, Compound 3. This is perhaps the most widely studied of the Aurora kinase inhibitors and has been assessed in multiple phase II trials as part of a collaboration between Vertex and Merck. The compound is based on an aminopyrazole hinge binding motif that was optimized for Aurora-A from a 2-phenyl substituted quinazoline lead (compound **15**, Table 2). Replacement of the phenyl group with a 4-amidothiophenyl motif (compound **16**, Table 2) provided a marked improvement in potency against Aurora-A and most notably in selectivity (as judged by cross-reactivity against Src and Gsk3β kinases). The excellent selectivity that is achieved for Aurora-A over Src and Gsk3β is likely due to the positioning of the acetamide group in the small hydrophobic pocket in Aurora-A that is not available in the other kinases.

Compound **16** showed submicromolar antiproliferative cell activity and ADME properties that were sufficient to enable its use as a tool to test the impact of Aurora inhibition on tumor growth in vivo (rat iv PK: Cl = 7 (mL/min)/kg, $T_{1/2}$ = 1 h, V_{ss} = 0.38 L/kg with F = 44%). At the maximum tolerated dose of 300 mg/kg given orally every day for 2 weeks, marked inhibition of tumor growth was observed (~50%).[129] Although these data provided an early proof of the concept for Aurora inhibition, improvements in potency and drug like properties were required. Marked improvements in both cell potency and aqueous solubility were obtained by replacing the quinazoline with 6-heterocyclic substituted pyrimidines (compounds **17–19**, Table 3). Further advances in cell potency were made by N-alkylation (compounds **20–23**, Table 3) to provide a series of compounds with excellent antiproliferative activity, the most

potent of which had an IC$_{50}$ of 2 nM (compound **23**, Table 3). From this set, compound **3** was selected as the clinical lead based on a combination of potency and ADME properties.
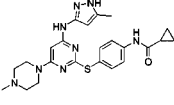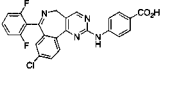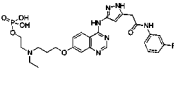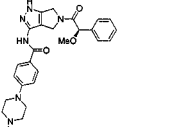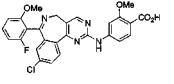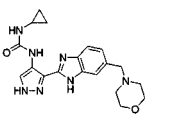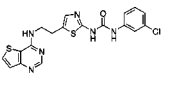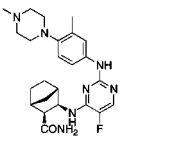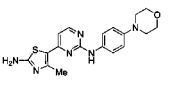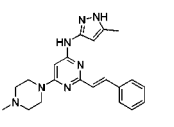
Compound **3** is a potent inhibitor of all three Aurora kinases with K_i values of 0.6 nM against Aurora-A, 18 nM against Aurora-B, and 5 nM against Aurora-C. It shows selectivity against over 190 other protein kinases, though it does cross-react with a small number of kinases that are themselves interesting cancer targets. These include Flt-3 and Abl kinases with a K_i of 30 nM against each.[94,95]

A crystal structure for **3** in complex with Aurora-A has been solved. The compound is bound to a "closed" conformation of the kinase that corresponds to an inactive structure. Important features of the structure are an extensive network of H-bonds formed between the hinge region of the kinase and the amino-pyrazole of **3**. In addition the cyclopropylamide of **3** occupies a small hydrophobic pocket that is capped by Phe275 (Figure 5). Crystal structures of activated "open" Aurora-A show that this pocket is not available in this conformation.[119] The combination of these two specific inhibitor/protein interactions and inspection of the Aurora crystal structures led to a hypothesis for a two-step binding mechanism, the first step being the formation of a complex between the compound and the open form of the enzyme, driven by the H-bond network to the hinge region, followed by hydrophobic collapse of the protein around the cyclopropyl group, leading to a high affinity complex of the compound with the inactive, closed form of the enzyme.[95] This proposed binding mechanism was supported by kinetic data, which show time dependent inhibition of active Aurora-B with **3** and a series of close analogues. The inhibition time course data are best fitted to a kinetic algorithm consistent with a two-step binding process. Most notably, the inhibition is characterized by a long residency of the compound on the enzyme (half-life for dissociation). In the case of compound **3** the half-life for dissociation is 36 min, but in some cases, such as with compound **23**, this can exceed 8 h.[130] While this proposed binding mechanism involves hydrophobic collapse of the protein onto the cyclopropyl of **3**, optimization of cell activity in this series of compounds was driven by alkyl substitution of the piperazine ring (Table 3). This group, however, is far removed from the cyclopropyl moiety and the critical hydrophobic pocket in Aurora. One explanation for this apparent disparity is that the lipophilic group on the piperazine ring may play a significant role in orienting the molecule in the active site.

Inspection of the many kinase structures that have now been solved shows that key features of the closed conformation for Aurora kinase, such as the critical small hydrophobic pocket that compound **3** occupies, are not commonly observed. Kinases that are able to form a comparable hydrophobic pocket in the closed conformation are Flt-3 and Abl, and this may provide an explanation for the ability of compound **3** to inhibit these enzymes.[95] In addition, this compound is able to inhibit a comprehensive range of clinically relevant mutant forms of the Abl enzyme. These mutations, which frequently act to stabilize an open-active protein conformation, are responsible for the emergence of resistance to the BCR-Abl kinase inhibitors such as imatinib.[94,95] This resistance arises because the binding of imatinib to Abl is dependent on interactions that are only available in the open-active conformation.[95] One of the most prevalent mutations, T315I, is also one of the most resistant forms of the enzyme.[131,132] Remarkably, compound **3** is a potent inhibitor of this T315I mutant with a K_i value of 42 nM.[95] A structure of **3** in complex with wild type Abl has been solved. Interestingly, in this case, the protein adopts an active-open conformation that is quite different from the compa-

Table 1. Aurora Kinase Inhibitors That Are Being Assessed in the Clinic

Drug candidate	Structure	Aurora profile	Notable cross reactivity	Route of Admin	Status
3		K_i (nM)[62]: Aur-A=0.6 Aur-B=18 Aur-C=4.6	K_i (nM)[62]: Abl-wt (30), Abl-T315I (42), Flt3 (30), Lck (80); K_d (nM)[94]: Abl2 (4), Jak2 (190), Plk4 (9), Ret (28), TrkA (38)	IV	Phase II
MK-5108 (VX-689)	Structure unknown	Unknown	Unknown	PO	Phase I
4		IC_{50} (nM)[108]: Aur-A=4 Aur-B=172	K_d (nM)[121]: Blk (68), PRAK2 (8)	PO	Phase I
5		IC_{50} (nM)[110]: Aur-A=1400 Aur-B<1 Aur-C=17	IC_{50} (nM)[110]: Lck (170); K_d (nM)[121]: Flt3 (8), Kit (17), PDGFRA (38), PDGFRB (41), Ret (80),	IV	Phase I
8 (PHA-739358)		IC_{50} (nM)[120]: Aur-A=13 Aur-B=79 Aur-C=61	IC_{50} (nM)[120]: Abl-wt (25), TrkA (30), Ret (31), FGFR1 (47), Lck (155), VEGFR3 (161)	IV	Phase II
9 (MLN8237)		IC_{50} (nM)[122] Aur-A=1 Aur-B=1100*	IC_{50} (nM)[122]: Lck (320)	PO	Phase I
PF-03814735	Structure unknown	Aurora-A/B[123] Data not reported	Unknown	PO	Phase I
10 (AT-9283)		IC_{50} (nM)[124]: Aur-A=3 Aur-B=3	IC_{50} (nM)[124]: Jak2 (1), Jak3 (1), Abl-T315I (4), Flt3 (10)	IV, PO	Phase I/IIa
11 (SNS-314)		IC_{50} (nM)[125]: Aur-A=9 Aur-B=31 Aur-C=3	IC_{50} (nM)[125]: TrkA (12), TrkB (5), Flt4 (14), FMS (15), DDR2 (82), Axl (84), cRAF (100)	IV	Phase I
12 (AS-703569, R763)		Aurora-A/B/C[126] Data not disclosed	Unknown	PO	Phase I
13 (CYC-116)		IC_{50} (nM)[127]: Aur-A=44 Aur-B=19 Aur-C=65	IC_{50} (nM)[127]: VEGFR2 (69), Flt3 (88)	PO	Phase I
14 (ENMD-2076)		IC_{50} (nM)[128]: Aur-A=14 Aur-B=290	IC_{50} (nM)[128]: Flt3 (3), Src (20), VEGFR2 (36), FGFR1 (93), cKIT (120), FAK (55), PDGFRa (56), Abl-wt (295), Abl-T315I (81)	PO	Phase I

(*) Data derived from cellular biomarker assay with inhibition of autophosphorylation of Aurora-B as the end-point.

Table 2. Significant Compounds toward the Clinical Lead **3**

	Ar	Aur-A K_i (nM)	Aur-B K_i (nM)	GSK3β K_i (nM)	Src K_i (nM)	Colo205[a] IC$_{50}$ (μM)
15		58	n.t.	22	81	1.40
16		4	27	1034	1258	0.48

[a] Inhibition of proliferation of Colo205 cells measured by [³H]thymidine uptake after 96 h of incubation.

**Table 3.** Optimization of Cell Potency toward the Clinical Lead **3**

	R_1	R_2	Aq. Solubility at pH$_{7.4}$ (μg/ml)	Aur-A K_i (nM)	Aur-B K_i (nM)	Colo205[a] IC$_{50}$ (μM)
17		Et	2.5	1.6	8	0.400
18		Et	5.4	1.7	14	0.135
19		Et	296	3.7	18	0.079
20		Et	63.4	1.6	20	0.024
21		Et	114.5	< 1	25	0.024
22		Et	12.8	< 1	9.5	0.075
23		Et	220.8	1.3	11	0.002
3		CyPr	6.8	0.6	18	0.019

[a] Inhibition of proliferation of Colo205 cells measured by [³H]thymidine uptake after 96 h of incubation.

rable Aurora-A cocomplex structure.[133] Taken together, the potent inhibition of wild type and mutant Abl kinases along with the structural information suggests that **3** is able to bind with high affinity to both the active and inactive conformations of Abl kinase.
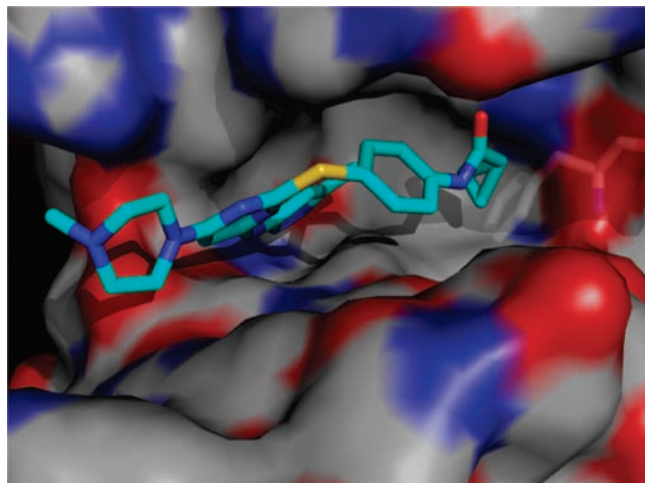
Figure 5. Cocomplex crystal structure of compound **3** bound to Aurora-A. This shows that the hydrophobic pocket, capped by Phe275 of the activation loop, is occupied by the cycylopropyl group of **3**.

Compound **3** blocks the phosphorylation of both Aurora-A and -B substrates in cells and is a potent antiproliferative agent that is active against all cycling cells (IC$_{50}$ values range from 15 to 113 nM).[62,97] Treatment of proliferating cells with **3** leads to accumulation of cells with 4N DNA and, in many cases, extensive endoreduplication in the absence of cell division. Cells that exit mitosis without completing cytokinesis are deleted by apoptosis.[62] A number of in vivo models have shown this compound to be a potent anticancer agent that leads to robust tumor growth inhibition and in some cases regression. In these studies **3** was typically dosed at 50 mg/kg ip b.i.d. everyday or by iv infusion at 1 mg/kg for 3 days/week. Predictably the major adverse effect in preclinical studies was depletion of the neutrophil cell population.[62]

Compound **3** is administered by iv infusion. It is subject to extensive hepatic metabolism, primarily by Cyp3A4, Cyp2C8, and FMO (flavin monooxygenase). Oxidation of the *N*-methylpiperazine group is the major route of metabolism, with the principle metabolites being the *N*-oxide and N-demethylated analogues.[134]

In the phase I study, **3** was dosed by continuous iv infusion for 5 days every 4 weeks in patients with refractory nonhematological malignancies. The plasma profile is consistent with the extensive metabolism seen in preclinical studies and shows a fast initial elimination phase that accounts for about 75% of compound. This is followed by a second slower terminal decay phase with a half-life of 15 h. The dose limiting toxicity is mechanism based asymptomatic neutropenia, observed at doses of 12 (mg/m^2)/h.[135] In solid tumors the compound was assessed in a phase IIa lung cancer study, dosed at 10 (mg/m^2)/h (5 day continuous iv infusion every 21 days); no data are yet available from this study. It has also been assessed in a dose escalating phase I/II study in refractory leukemias. In this study the compound was dosed as a 5-day continuous iv infusion every 14 or 21 days at doses up to 40 (mg/m^2)/h. It was well tolerated at all doses with the most significant treatment-related adverse effect being mechanism-based mucositis, which can be attributed to antimitotic effects on proliferating GI tract cells. Of the 14 compound treated patients with CML that were evaluated, 9 expressed the refractory T315I mutation and, significantly, of these, 8 showed a hematological or cytogenic response.[136,137] The white blood cell profile for one patient with accelerated disease showed that by cycle 7, blood counts had normalized on treatment with **3** at 20 (mg/m^2)/h.[138] Despite these encourag-
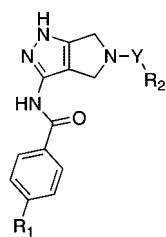
ing data, Merck and Vertex have reported that clinical studies with this compound had been stopped and that effort has been focused on an alternative Aurora kinase inhibitor MK-5108 (VX-689). The structure and selectivity profile for this compound have not been disclosed.

Approach Taken by Nerviano toward the Pan-Aurora Inhibitor, Compound 8 (PHA-739358). Researchers from Nerviano Medical Sciences identified compound **8** as a pan-Aurora inhibitor from combinatorial expansion of the 1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazole scaffold. With a ^{t}Bu substituted benzamide as the starting point, changes to the pyrrolidine ring were assessed (Table 4). Various lipophilic aryl groups were appended to the pyrrolidine ring via amide, amidomethylene, and urea linkers to provide a series of compounds with antiproliferative activity (compounds **24−27**, Table 4). These compounds suffer from poor solubility, which is improved by replacing the ^{t}Bu group with an *N*-methyl substituted piperazine (compounds **28−31** Table 4). The resulting compounds have marked antiproliferative activity with the most active analogue (**31**) inhibiting cell proliferation with an IC$_{50}$ value of 45 nM.[139] Compound **31**, known as PHA-680632, was nominated as an initial lead. Extensive profiling demonstrated encouraging kinase selectivity and, most notably, significant inhibition of tumor growth in multiple animal cancer models when dosed iv b.i.d.[140]

A cocomplex structure of **31** bound to Aurora-A shows the enzyme adopting an active open conformation.[139] This is quite different from the structure for the alternative pan-Aurora inhibitor, compound **3**, bound to Aurora-A and suggests that despite the conformational similarity between active Aurora kinase and many other kinases, it remains possible to obtain selectivity. This cocomplex structure highlighted an opportunity to increase affinity by adding further binding interactions between the compound and the glycine-rich loop, a motif common to all protein kinases that is routinely used to generate binding interactions with inhibitors. Amides of substituted phenylacetic acid were prepared, and it was shown that simple lipophilic groups at the benzylic position were optimal (Table 5).[120] Interestingly small variations in the substitution pattern at this benzylic position resulted in marked changes in isoform selectivity. For example, a fluorine substituent (compound **32**, Table 5) led to >30-fold selectivity for Aurora-A vs Aurora-B whereas a methyl substituent (compound **34**, Table 5) has only 4-fold selectivity. The reasons for this selectivity are not evident from inspection of the crystal structures, which may indicate that the selectivity is derived from subtle differences in the flexibility and dynamics of the proteins. This exploration of substituents on the benzylic position led to the identification of compound **8**, which is the clinical lead currently in phase II studies.

Despite significant structural differences between compounds **3** and **8**, there is a remarkable similarity in their biochemical and biological profiles. Compound **8** inhibits all three Aurora kinases (IC$_{50}$ values of 13, 79, and 61 nM, respectively) and has cross-reactivity against Abl kinase (IC$_{50}$ of 25 nM).[141] Furthermore, **8** potently inhibits a number of drug resistant mutants of Abl kinase including the highly refractory T315I mutation. A cocomplex structure of this compound in Abl kinase has been solved, which shows the protein adopting an active open conformation.[142]

Compound **8** inhibits cell proliferation with IC$_{50}$ values as low as 28 nM and causes accumulation of cells with 4N DNA, extensive endoreduplication in the absence of cell division, and in many cases loss of viability.[141] All these properties are consistent with data obtained from depletion of Aurora-B (see above). As with compound **3**, the extent of endoreduplication appears to be dependent on the p53 status of cells. Cells with

Table 4. SAR for the 1,4,5,6-Tetrahydropyrrolo[3,4-*c*]pyrazole Scaffold toward the Clinical Lead **8**

	R₁	Y	R₂	Aur-A IC$_{50}$ (nM)	HCT-116[a] IC$_{50}$ (μM)
24	tBu	COCH$_2$	thiophene	5	2.0
25	tBu	CO	furan	41	2.1
26	tBu	COCH$_2$	phenyl	130	3.0
27	tBu	CONH	2,6-diethylphenyl	100	2.2
28	methylpiperazinyl	COCH$_2$	thiophene	65	0.57
29	methylpiperazinyl	CO	furan	160	0.90
30	methylpiperazinyl	COCH$_2$	phenyl	140	0.22
31 (PHA-680632)	methylpiperazinyl	CONH	2,6-diethylphenyl	27	0.045

[a] Inhibition of proliferation of HCT-116 cells measured by cell counts after 72 h of incubation.

Table 5. SAR for the Substituted Phenylacetic Acid Amides toward the Clinical Lead **8**
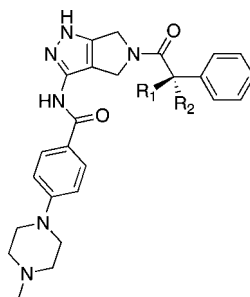
	R₁	R₂	Aur-A IC$_{50}$ (nM)	Aur-B IC$_{50}$ (nM)	HCT-116[a] IC$_{50}$ (μM)
30	H	H	130	nt	0.22
32	F	H	9	300	0.050
33	OH	H	6	48	0.097
34	Me	H	24	99	0.021
8	OMe	H	13	79	0.031
35	H	Me	452	1210	nt
36	H	OMe	354	630	nt

[a] Inhibition of proliferation of HCT-116 cells measured by cell counts after 72 h incubation. nt = not tested.

a functional p53 response arrest with 4N DNA, whereas p53 deficient cells continue through multiple rounds of cell cycle.[141]

Studies in leukemic cells show that **8** blocks Abl signaling in cells expressing both wild type Abl or multiple drug resistant

Table 6. SAR for Aniline Replacements and Quinazoline Substitutions Based on the Initial Aurora-A/-B Inhibitor **1**

	R₁	R₂	Aur-A IC₅₀ (µM)	Aur-B IC₅₀ (µM)
37			0.003	0.001
38			0.182	0.530
39			0.011	0.057
40			0.004	0.042
41			<0.001	<0.001
42			<0.001	0.007
43			-	-
44			0.450	0.002

mutations.[143] Interestingly, despite inhibition of Abl, when cells are treated with **8**, an overall profile consistent with Aurora inhibition rather than Abl inhibition is maintained. For example, there was no evidence for an Abl mediated G1 block.[141] This observation matches that seen with VE-465, an analogue of **3**, which also inhibits Abl kinase.[144] Significantly **8** blocks the growth of primary cells taken from patients suffering from varied stages of CML including newly diagnosed chronic, acute blastic, and refractory acute blastic phase CML.[143]
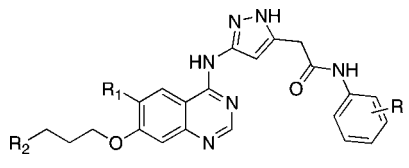
Compound **8** is administered by iv infusion. In tumor bearing mice, following 10 days of treatment with compound a bi-exponential elimination profile is observed with modest clearance (1.7 (L/h)/kg), high distribution (2.7 L/kg), and a good terminal elimination half-life (3.7 h).[141] The compound causes significant tumor growth inhibition and in some cases regression in numerous xenograft and transgenic models of cancer at a dose of 30 mg/kg b.i.d. given every day for 3–5 days.[141]
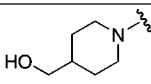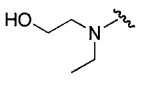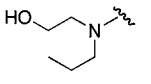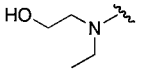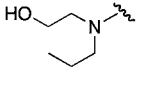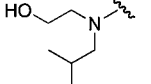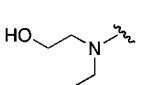
In a phase I study in advanced solid tumors, **8** was dosed as a 6 h iv infusion on days 1, 8, and 15 of a 4 week cycle at doses up to 400 mg/m². The anticipated mechanism based neutropenia was reported to be the dose limiting toxicity, which was observed at doses of 400 mg/m².[145,146] This compound is currently being assessed in a phase II study in relapsing CML. Encouraging interim data show a hematological or cytogenic response for some patients at the 330 mg/m² dose.[147]

Approach Taken by AstraZeneca toward the Aurora-B Selective Inhibitor, Compound 5. Extensive medicinal chemistry efforts based on a quinazoline scaffold core have led to a number of advanced Aurora inhibitors from AstraZeneca. Compound **1** (Figure 3) was the first to be reported and has been widely used as a chemical tool to probe the biology of the Aurora kinases (the properties of this compound are described above). The SAR for this series of compounds has been extensively reviewed elsewhere.[148]

Replacement of the central aniline ring in **1** with a variety of five- and six-membered heterocycles led to a marked improvement in affinity for both Aurora-A and -B (Table 6 compounds **37**, **39**, and **40**). Since the aromatic amide group of compounds

Table 7. Quinazoline Substitutions toward the Clinical Lead **5**

	R	R$_1$	R$_2$	Aur-A IC$_{50}$ (μM)	Aur-B IC$_{50}$ (μM)	SW620[a] IC$_{50}$ (μM)
44	3-F	MeO		0.450	0.002	0.68
45	3-F	MeO		0.19	0.001	0.27
46	3-F	MeO		0.22	0.002	0.29
47	2,3-diF	MeO		0/36	<0.001	0.045
48	2,3-diF	MeO		0.087	<0.001	0.018
49	2,3-diF	MeO		0.018	<0.001	<0.001
7 (AZD1152-HQPA)	3-F	H		1.40	<0.001	0.017

[a] Inhibition of histone-H3 phosphorylation following 24 h of incubation with SW620 tumor cells.

37, **39**, and **40** can orientate differently in the enzyme active site when attached to a five- or six-membered ring, it is suggested that the high enzyme affinity achieved indicates a degree of flexibility in the region of the protein that this group occupies (the so-called selectivity pocket).[148] Extending the phenylamide further from the thiazole enhanced the interaction of the compound with this region of the protein and provided marked improvements in potency against both Aurora-A and -B, as exemplified with compounds **41** and **42** (Table 6). These compounds are also both very potent inhibitors of cell proliferation (IC$_{50}$ < 10 nM). To enable compound **42** to be assessed in vivo, a phosphoester prodrug analogue was prepared to provide the high solubility required for administration by iv infusion (compound **43**, Table 6). Compound **43** causes marked inhibition of histone H3 phosphorylation in tumors, consistent with inhibition of Aurora-B by the active component, compound **42** (Table 6).[148] In an alternative medicinal chemistry effort, the aminothiazole group of compound **42** was replaced by an aminopyrazole. Remarkably, this change led to significant selectivity for Aurora-B over Aurora-A (compare compounds **42** and **44** in Table 6).[110] The reasons for this isoform selectivity are not clear, but subtle changes in kinase conformation brought about by the additional hydrogen bond from the pyrazole group (compared to the thiazole) to the protein backbone may be one explanation.

Subsequent modification to the substituents on the quinazoline in the pyrazole series led to improved affinity for Aurora-B and increased cell potency while maintaining selectivity over Aurora-A (Table 7).[110] Inclusion of a pendent hydroxyl group into the substituent at the 7-position of the quinazoline was necessary to provide a "handle" to attach the phosphate prodrug group. Most notably, replacing the methoxy group at the 6-position of the quinazoline with hydrogen led to a marked improvement in cell potency along with a significant decrease in potency against Aurora-A (compare compounds **45** and **7** in Table 7). Again, the reasons for this conservative change providing such a high degree of selectivity for Aurora-B over -A are not clear, though the electronic nature of the methoxy group may have an influence on the binding of the quinazoline to the hinge. The resulting compound (**7**, Table 7) has excellent affinity for Aurora-B (IC$_{50}$ <1 nM) with >1000-fold selectivity for Aurora-A (IC$_{50}$ = 1.4 μM) and is a potent inhibitor of cell proliferation (IC$_{50}$ = 17 nM). The phosphate ester prodrug analogue of this compound (**5**) exhibits potent tumor growth inhibition in vivo following iv administration and represents the first Aurora-B selective inhibitor to enter the clinic.[113]

It is interesting to note that close analogues of **7** possessing N-substituted ethanolamines on the quinazoline scaffold were found to have exceptional cell potency (Table 7, compounds **47**−**49** and **7**). The N-isobutyl-substituted compound **49** (Table 7) has an IC$_{50}$ in SW620 cells of less than 1 nM. It is intriguing to think that this dramatic cell potency may be due to a two-step binding mechanism and formation of a high affinity complex with inactive Aurora-B, in an analogous manner to that observed with the pan-Aurora inhibitor from Vertex (**3**). In keeping with this concept, it has recently been reported that

7 has an extended residency time on Aurora-B, with a dissociation half-life of 15 min.[149] In addition a cocomplex crystal structure of a 4-aminopyrimidine quinazoline analogue of **7** in Aurora-A shows the protein bound in an inactive conformation.[150]

The phosphate ester prodrug **5** has good solubility (25 mg/mL at pH 9) and is rapidly converted in plasma to the active agent. Following iv administration to rats the T_{max} for both prodrug and active agent was achieved within 2 min and the active component has good iv pharmacokinetic properties (rat iv: Cl = 14 (mL/min)/kg, $T_{1/2} \approx 5$ h).[110]

In proliferating cells **7** causes a marked disruption in mitosis, resulting in extensive polyploidy and apoptosis. Administration of the prodrug to tumor bearing mice at doses of 150 (mg/kg)/day, given as a 48 h infusion, causes inhibition of tumor growth and in some cases regression. Analysis of tumors following treatment with this prodrug shows multiple phenotypes associated with aberrant mitosis, e.g., an increase in cells with 4N or higher DNA content and apoptosis. [110,113]

Compound **5** is being assessed in a number of phase I trials in advanced solid tumors and acute myeloid leukemia. In all cases administration is by iv infusion with a number of dosing regimens being explored, e.g., 2 h infusion every week or 2 weeks, 2 h infusion for 2 consecutive days every 2 weeks, or a 48 h continuous infusion given every 2 weeks. Preliminary data from a phase I dose escalating study in solid tumors show dose limiting, mechanism based, grade 4 neutropenia in some patients at the 450 mg dose when the compound was given as a 2 h infusion every week. No other clinically significant adverse events were reported. A number of patients were reported to have shown stable disease in response to the drug.[96,151]
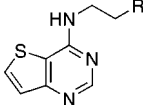
Properties of the Aurora-A Selective Inhibitor, Compound 4, Identified by Millennium. Compound **4** is the first oral selective Aurora-A inhibitor to enter the clinic. It is based on a benzazepine scaffold with a fused aminopyrimidine ring (Table 1). The in vitro biological profile is consistent with Aurora-A selective inhibition at low doses, i.e., a delay in mitotic progression, and with Aurora-B inhibition at high doses, e.g., inhibition of histone H3 phosphorylation. This compound shows rapid oral absorption to give high and sustained plasma and tumor exposure. Substantial levels were still detected in plasma and tumor tissue 24 h after dosing (30 mg/kg dose to mice gave rise to plasma levels of >1 μM and tumor levels of >2 μM 24 h after dosing).[152] It inhibits tumor growth in multiple xenograft models, and in some cases marked regression is observed (typically the compound is dosed orally at 30 mg/kg given once or twice a day). The compound is well tolerated at low doses, but at 30 mg/kg given b.i.d. a dramatic but reversible decrease in neutrophils is observed.[108] Similar effects have been observed with the pan and dual Aurora-A/-B inhibitors. Pharmacodynamic studies using histone H3 as a marker showed that at early time points, when compound exposure is very high, histone H3 phosphorylation is inhibited. This is consistent with Aurora-B inhibition. However, at later time points histone H3 phosphorylation is elevated, peaking at 8 h. This elevation is attributed to an increase in mitotic index as a result of Aurora-A inhibition.[108] These data suggest that following administration of **4** there are periods of time where both Aurora-A and -B are inhibited.
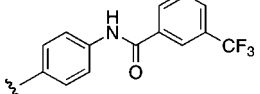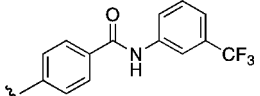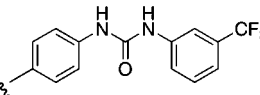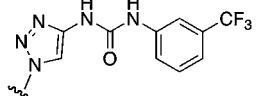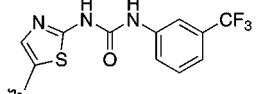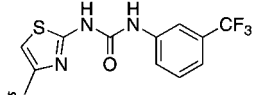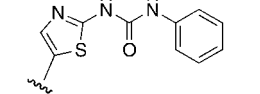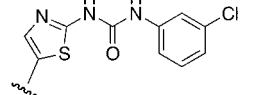
Compound **4** has been assessed in a dose escalating phase I study in advanced solid tumors. It was dosed orally up to 40 mg/d given on 7 consecutive days, repeated every 21 days. Consistent with preclinical observations, the compound was rapidly absorbed, showed dose proportional exposure, and had a long elimination half-life of 35 h. Dose limiting toxicity was found to be somnolence, which is evident at doses above 20 mg/d and dose limiting at 40 mg/d.[153] This cannot be attributed to Aurora inhibition but may instead be due to cross-reactivity with the γ-aminobutyric acid α1 benzodiazepine receptor.[96] No other adverse effects were reported. Skin punch biopsy failed to show an accumulation of cells in mitosis, which suggests Aurora-A inhibition was not complete. In an attempt to overcome the sedating affects, alternative dosing regimens, in combination with methylphenidate, are being assessed.[96]

Properties of the Aurora-A Selective Inhibitor, Compound 9 (MLN8237), Identified by Millennium. This second generation Aurora inhibitor from the Millennium group has recently entered phase I clinical trials (Table 1). It inhibits Aurora-A with an IC_{50} of 1 nM in biochemical assays and has 200-fold selectivity for Aurora-A over Aurora-B in cell assays. A broad screen of receptors and ion channels showed no significant cross-reactivity. The compound blocks the growth of multiple tumor cell lines with GI_{50} values as low as 16 nM. Growth inhibition is associated with mitotic spindle abnormalities, accumulation of cells in mitosis, polyploidy, and apoptosis. Like its predecessor (**4**), compound **9** is orally bioavailable and rapidly absorbed. Maximum in vivo efficacy, in multiple xenografts, has been achieved with oral doses of 20 mg/kg given twice a day for 21 consecutive days, although other regimens are also effective. In all cases marked tumor growth inhibition is observed, and in some cases regression is seen. As with compound **4**, biomarker studies show that at effective doses of **9** a transient inhibition of histone H3 phosphorylation is observed (consistent with Aurora-B inhibition being dominant) followed by marked elevation of histone H3 phosphorylation (consistent with Aurora-A inhibition being dominant). In a PK/PD study using elevated phospho-histone H3 as a marker for Aurora-A inhibition, it was shown that maintaining a plasma exposure in the vicinity of 1 μM leads to maximal elevation of histone H3 phosphorylation and tumor growth inhibition. Below 1 μM both the biomarker response and inhibition of tumor growth are dose dependent. Notably, oral doses of **9** that lead to profound tumor growth inhibition maintain the critical 1 μM plasma exposure for 8−12 h a day. While these data suggest that the effect on tumor growth is associated with Aurora-A inhibition, it does not rule out the possibility that partial Aurora-B inhibition is playing a role. These data are being used to guide dose selection and the dosing regimen for ongoing human phase I studies.[122,154–157] Notably in the phase I study a patient with advanced metastatic ovarian cancer showed a formal partial response (according to the RECIST criteria).[158] This represents the first clinical response to an Aurora inhibitor in a solid tumor setting.

Approach Taken by Sunesis toward the Pan-Aurora Inhibitor, Compound 11 (SNS-314). Researchers from Sunesis Pharmaceuticals identified compound **11**, which is based on a 4-aminothieno[3,2-*d*]pyrimidine scaffold. This compound was optimized from a low micromolar inhibitor of Aurora-A (compound **50**, Table 8) that was identified by in-house screening efforts.[125] Improvements to Aurora affinity were obtained by changing the nature of the linking phenylamide portion of the molecule (compounds **51**−**55**). Replacement of the amide with a urea and the phenyl group with a thiazole was optimal (compound **54**). Compound **54** is a potent inhibitor of both Aurora-A and Aurora-B (IC_{50} of 22 and 14 nM, respectively) with good antiproliferative activity in cells. Further optimization of the substituents on the terminal phenyl ring led to the clinical lead (**11**), which was selected

Table 8. SAR Leading toward the Clinical Lead **11**

Compound	R	Aur-A IC$_{50}$ (nM)	Aur-B IC$_{50}$ (nM)	HCT-116[a] EC$_{50}$ (nM)
50		3200	n.r.	n.r.
51		> 20,000	n.r.	n.r.
52		1200	n.r.	n.r.
53		250	n.r.	n.r.
54		22	14	13
55		> 20,000	n.r.	n.r.
56		52	120	45
11		9	31	6

[a] Inhibition of proliferation of HCT-116 cells measured by BrdU incorporation. n.r. = not reported.

on the basis of its tolerability under multiple dosing schedules and ease of manufacture. Cocrystal structures of compounds **54** and **11** with Aurora-A show the urea moiety of both compounds forming a hydrogen bonding network with the catalytic lysine and glutamate residues on the α-C helix of the protein. In addition the protein adopts an open-activated conformation.

Compound **11** is a pan-Aurora inhibitor with good affinity against all three isoforms (IC$_{50}$ for Aurora-A, -B, and -C of 9, 31, and 3 nM, respectively) and selectivity over the majority of kinases tested (199 out of 219 kinases had IC$_{50}$ values greater than 1 μM). In keeping with other pan-Aurora inhibitors, this compound potently blocks proliferation in a diverse panel of human cancer cell-lines (IC$_{50}$ values in the range 1.8–24 nM) and leads to accumulation of cells with >4N DNA content.[159]

In xenograft models the compound blocks tumor growth at doses of 50–170 mg/kg administered ip twice a week for 3 weeks. Apoptosis of tumor tissue along with inhibition of histone H3 phosphorylation (Ser10) in tumor, skin, and bone marrow is observed.[160] According to the clinical trials database (www.clinicaltrials.gov), **11** is currently being assessed in a dose-escalating phase I study in advanced solid tumors as an iv infusion given once a week for 3 weeks.

Properties of Compound 12 (R763, AS-703569) Identified by Rigel. In 2005 Merck Serono licensed the worldwide development rights for the pan-Aurora kinase inhibitor, compound **12**, from Rigel Pharmaceuticals. Although the data have not been disclosed, this compound is reported to be a pan-Aurora inhibitor. In addition it has been shown to have marked cross-reactivity against Flt-3 kinase. On a panel of AML

cell lines the compound displays potent antiproliferative activity with IC_{50} values as low as 0.4 nM. Interestingly the compound is 10-fold more effective in cells harboring the FLT-3-ITD mutation than in cells with wild type Flt-3. The internal tandem duplication mutation of Flt-3 leads to constitutive activation of kinase activity and is a negative prognostic marker in AML.[161] These data suggest that in this context the cross-reactivity with Flt-3 has a significant impact on the cellular outcome. In a diverse set of patient derived tumor cells, covering multiple tumor types, the compound efficiently blocks clonogenic growth (average IC_{70} of 0.34 µg/mL). Compound **12** is orally bioavailable and blocks tumor growth in multiple animal models at a dose of 10 mg/kg given orally for 4 consecutive days every 2 weeks. According to the clinical trials database, this compound is being assessed in a dose-escalating phase I study in solid tumors dosed either on days 1 and 8 of a 21-day cycle or on days 1, 2, and 3 of a 21-day cycle.[126,162]

PF-03814735 Identified by Pharmacia Italia. The structure and properties of this compound have not been disclosed. A number of patents from Pharmacia Italia claim kinase inhibitors for the treatment of cancer including compounds that inhibit Aurora. Specifically a series of potent Aurora-A inhibitors derived from a thienopyrazole scaffold functionalized with amides at the 3 and 5 positions are described.[163] An example of a generic from this series is compound **57**. An alternative series of Aurora inhibitors derived from an aminopyrimidine scaffold have also been disclosed,[164] an example of which is compound **58**. PF-03814735 is being assessed in a phase I study in advanced solid tumors. According to the clinical trials database, the compound is being dosed orally once a day for 5 or 10 days every 3 weeks.

Compound 57   Compound 58
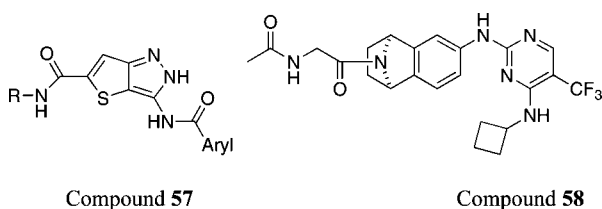
Properties of the Aurora-A/Aurora-B Inhibitor, Compound 10 (AT-9283), Identified by Astex. This compound, from Astex Therapeutics Ltd., has an aminopyrazole hinge binder on a benzimidazole scaffold. It is a potent inhibitor of Aurora-A and -B ($IC_{50} \approx 3$ nM) with a selectivity profile that is similar to the pan-Aurora inhibitor from Vertex (**3**). Specifically, potent inhibition of wild type and T315I mutant Abl is reported.[165] According to the clinical trials database, **10** is being assessed in phase I clinical studies in advanced solid tumors, non-Hodgkin's lymphoma, and hematological malignancies including AML, ALL, and CML. In all cases the compound is being dosed as a continuous iv infusion for 24 h given once a week.

Properties of the Pan-Aurora Inhibitor, Compound 13 (CYC-116), Identified by Cyclacel. The properties of **13**, which has an aminothiazole hinge binder attached to a pyrimidine core, were reported at the 2008 Annual Meeting of the American Association for Cancer Research.[127,166–169] This compound is a potent inhibitor of Aurora-A, -B, and -C (IC_{50} of 44, 19, and 69 nM, respectively). In addition to the Aurora kinases, **13** is a potent inhibitor of VEGFR2 and Flt3 kinases (IC_{50} of 69 and 3 nM, respectively). Consistent with its pan-Aurora inhibition, treatment of proliferating cells with **13** leads to polyploidy and apoptosis. Specific cellular aberrations include delayed mitotic entry, defective spindle formation, abrogation of the spindle

checkpoint, and exit from mitosis without completing cytokinesis. Interestingly, cells deficient in the retinoblastoma tumor suppressor protein (Rb) are reported to be most sensitive to the apoptotic effects of the compound. This may be associated with an override of the G1 DNA damage checkpoint that occurs in the absence of Rb.[170] Oral dosing of **13** in mice is reported to inhibit tumor growth in vivo and block phosphorylation of histone H3 in tumors. Consistent with an inhibition of VEGFR2, tumor vascularization is also decreased. In addition in vitro synergistic cytotoxicity is reported with multiple chemotherapies including cisplatin, 5-FU, and doxorubicin. According to the clinical trials database, **13** is being assessed in phase I studies in advanced solid tumors. It is dosed orally once a day on days 1−7 of a 2-week cycle.

Properties of Compound 14 (ENMD-2076) Identified by EntreMed. Based on an aminopyrazole hinge-binding motif attached to a pyrimidine core, this compound is structurally similar to the pan-Aurora inhibitor from Vertex (**3**), the difference being the substituent at the 2-position of the pyrimidine ring, which in the case of **14** is an unsubstituted styrene rather than a sulfur atom linking through to a phenylamide in **3**. This structural change provides two significant differences in the profile of **14** when compared with **3**. First, in terms of the enzyme activity properties, compound **14** is a weak inhibitor of Aurora-B with IC_{50} of 290 nM (Aurora-A IC_{50} of 14 nM) and has low affinity for wild type Abl kinase (IC_{50} of 295 nM). Furthermore, cellular inhibition of Abl kinase is very weak with $IC_{50} > 25$ µM. In addition it has a broad cross-reactivity profile and inhibits a number of kinases that **3** does not potently inhibit such as Src, VEGFR2, FAK, and PDGFRα (Table 1). This cross-reactivity profile suggests that the alkene linker positions the unsubstituted phenyl ring in such as way that **14** binds to the kinases in an open, active conformation whereas **3** is able to stabilize a closed, inactive form. In cells, at high concentration (5 µM), compound **14** leads to accumulation of cells with 4N DNA and the appearance of a small population of polyploid cells, a phenotype most commonly associated with Aurora-B inhibition. Second, **14** is orally bioavailable and has demonstrated antitumor activity in multiple models of cancer from oral doses of up to 200 mg/kg given every day. Consistent with its cross-reactivity against VEGFR2, **14** inhibits VEGFR2 activation and angiogenesis in vivo. According to the clinical trials database, this compound is being assessed in a dose-escalating phase I study in advanced refractory cancer and is being dosed orally every day throughout a 28-day cycle.[128]

Conclusions and Future Directions

The three Aurora kinases have emerged as critical regulators of mitosis. Amplification of Aurora genes in human cancers and observations that expression of Aurora genes and proteins correlate with cancer progression, poor clinical outcome, and up-regulation of chromosomal instability provide clear support for a central role of Aurora kinases in cancer progression. These observations have stimulated a great deal of research directed at identifying selective Aurora kinase inhibitors as anticancer agents. The result of this intense effort is the progression of more than 10 small molecule Aurora inhibitors into early stage clinical assessment. These compounds cover a range of selectivity profiles within the Aurora family. The clinical potential for these compounds has recently been highlighted. The Aurora-A inhibitor **9** showed a formal response in a patient with advanced solid tumor. In addition the pan-Aurora inhibitors **3** and **8** showed significant hematological or cytogenic responses in multiple patients with refractory leukemia. However, it should

be noted that the cross-reactivity of the latter two compounds against kinases that have been implicated in leukemia may contribute to their effects.

Despite their altered selectivity profiles within the Aurora kinase family, all the drug candidates show an unexpectedly similar gross phenotype: major disruptions in mitosis resulting in misaligned chromosomes, aberrant segregation of the genetic material, mitotic exit in the absence of cytokinesis, and inhibition of proliferation, leading to apoptosis. The extended residency on Aurora-B reported for both **3** and **7** is noteworthy. It will be interesting to see whether this potentially beneficial property is common to other Aurora kinase inhibitors and if it translates into a pharmacodynamic advantage in the clinic.

Preclinical studies have shown that the phenotypic response to Aurora kinase inhibition appears to be dependent on the genetic background of the cells. For example, the status of the tumor suppressor proteins p53 and Rb appears to be critical in determining the extent of endoreduplication and kinetics of cell death. How this in vitro dependence translates to the clinic remains to be established. A large number of preclinical studies have also shown that Aurora kinase inhibition, be it isoform selective or dual Aurora-A/-B, can act in synergy with other therapeutic modalities including a wide diversity of cytotoxic agents and ionizing radiation.

Crystallography studies have shown that the Aurora kinases can adopt a number of different conformations. These conformations represent distinct drug targets with alternative opportunities to derive potency and selectivity. Accordingly there are examples of advanced Aurora inhibitors that preferentially bind either an active-open or an inactive-closed conformation. Both types of compound show good selectivity against a wide variety of kinases. This suggests that unique motifs are available in both conformations or that there is a degree of flexibility in the protein that can be utilized to gain selectivity. Despite the many cocomplex structures that have been solved, in most cases a clear explanation for the observed selectivity profile within the Aurora family remains to be defined. It is possible that these differences in selectivity profiles are associated with subtle differences in the dynamic flexibility of the different Aurora proteins. One important observation is that although the closed form of Aurora is different from most other kinase structures, it has a great deal of conformational similarity with Flt-3 and Abl kinases. Unsurprisingly a number of the advanced Aurora kinase inhibitors are also potent inhibitors of these kinases. Compounds **3** and **8** are both examples of this and are potent inhibitors of wild type and drug resistant mutant forms of Abl kinase in addition to Aurora-A and -B. Despite this cross-reactivity, in vitro studies with **8** clearly show that inhibition of the Aurora kinases has a dominant effect on the cell cycle profile. It remains to be seen whether the potent cytotoxic effect these compounds have on leukemic cells and the clinical responses observed for both **3** and **8** in leukemia is due solely to inhibition of the Aurora kinases, inhibition of Abl kinase, or a combination of the two effects.

Over the past few years there have been significant advances in our understanding of the roles that Aurora kinases play in mitosis. This has been derived from elegant biomolecular studies coupled with extensive profiling of multiple Aurora kinase inhibitors. Nevertheless, a number of critical questions remain unanswered. These include how best to exploit a truly selective Aurora-A inhibitor and whether compounds with different selectivity profiles within the Aurora family will represent distinct clinical opportunities. The observations of clinical responses for **3**, **8**, and **9** are encouraging; however, a great deal of work is still required to best define how Aurora kinase

inhibitors should be used in the clinic. For example, the preclinical context dependence on p53 and Rb may mean that Aurora inhibitors are most effective in a subset of tumor types or when dosed in combination with other therapeutic modalities. Finally, any emergence of resistant mutations of the Aurora kinases must be keenly monitored, as it is already apparent that extended incubation with inhibitors in vitro can lead to the outgrowth of cells expressing Aurora-B mutations that are resistant to a number of the Aurora inhibitors.

Biographies

John R. Pollard earned his B.Sc. degree in Chemistry from the University of Southampton, U.K., in 1993. He completed Ph.D. studies in Bioorganic Chemistry, also at the University of Southampton, under the guidance of Prof. T. D. Bugg. Following this he did postdoctoral work with Prof. D. Gani at the Universities of St. Andrews and Birmingham, U.K., in Bioorganic Chemistry and Mechanistic Enzymology. In 1999 Dr. Pollard began his industrial career with Vertex Pharmaceuticals, where he currently is the Oncology Disease Area Expert for the European Research Centre and also heads the Enzyme Sciences Group. During his time at Vertex he has led a number of research and early development programs in oncology and liver disease. His major drug discovery interest is in novel mechanisms to treat cancer.

Michael Mortimore earned his B.Sc. degree in Chemistry from Durham University, U.K., and his Ph.D. in 1988 from Southampton University, U.K., working with Prof. Philip Kocienski. He then had 2 years of postdoctoral experience with Prof. Stephen F. Martin at the University of Texas at Austin. In 1991 he began his industrial career in Medicinal Chemistry with Fisons Pharamceuticals in Loughborough, U.K., which over the years turned into Astra Pharamceuticals and then AstraZeneca. In 2000 Dr. Mortimore moved to Vertex Pharmaceuticals, where he is currently a Research Fellow. His interests lie in the design and synthesis of molecules for the treatment of diseases with unmet clinical need. He has published over 50 patents and papers.

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