Validating Aurora B as an anti-cancer drug target

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Summary

The Aurora kinases, a family of mitotic regulators, have received much attention as potential targets for novel anti-cancer therapeutics. Several Aurora kinase inhibitors have been described including ZM447439, which prevents chromosome alignment, spindle checkpoint function and cytokinesis. Subsequently, ZM447439-treated cells exit mitosis without dividing and lose viability. Because ZM447439 inhibits both Aurora A and B, we set out to determine which phenotypes are due to inhibition of which kinase. Using molecular genetic approaches, we show that inhibition of Aurora B kinase activity phenocopies ZM447439. Furthermore, a novel ZM compound, which is 100 times more selective for Aurora B over Aurora A in vitro, induces identical phenotypes. Importantly, inhibition of Aurora B kinase activity induces a penetrant anti-proliferative phenotype, indicating that Aurora B is an attractive anti-cancer drug target. Using molecular genetic and chemical-genetic approaches, we also probe the role of Aurora A kinase activity. We show that simultaneous repression of Aurora A plus induction of a catalytic mutant induces a monopolar phenotype. Consistently, another novel ZM-related inhibitor, which is 20 times as potent against Aurora A compared with ZM447439, induces a monopolar phenotype. Expression of a drug-resistant Aurora A mutant reverts this phenotype, demonstrating that Aurora A kinase activity is required for spindle bipolarity in human cells. Because small molecule-mediated inhibition of Aurora A and Aurora B yields distinct phenotypes, our observations indicate that the Auroras may present two avenues for anti-cancer drug discovery.

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Introduction

The Aurora kinases, a family of mitotic regulators, have received much attention as potential targets for novel anti-cancer therapeutics (Andrews, 2005; Keen and Taylor, 2004; Matthews et al., 2006). This enthusiasm comes largely from early observations showing that Aurora A is overexpressed in human cancers and has oncogenic properties in vitro (Bischoff et al., 1998; Zhou et al., 1998). Since then, the Auroras have been extensively studied and much learnt about their biology (Andrews et al., 2003; Carmena and Earnshaw, 2003; Ducat and Zheng, 2004; Keen and Taylor, 2004). Aurora A, which localises to centrosomes and spindle poles, has been implicated in centrosome maturation and spindle assembly (reviewed by Marumoto et al., 2005). Aurora B, a chromosome passenger protein, which localises to centromeres in early mitosis and then the spindle midzone in anaphase, is required for histone H3 phosphorylation, chromosome biorientation, the spindle assembly checkpoint (SAC) and cytokinesis (reviewed by Andrews et al., 2003; Carmena and Earnshaw, 2003). Mammals express a third family member, Aurora C, another chromosome passenger, which may play specific roles in male meiosis (Tang et al., 2006).

In the quest for novel anti-cancer agents, several small-molecule Aurora kinase inhibitors have been developed including Hesperadin, ZM447439 and VX-680 (Ditchfield et al., 2003; Harrington et al., 2004; Hauf et al., 2003). In cells, all three suppress histone H3 phosphorylation, inhibit chromosome segregation and prevent cell division. In the presence of Hesperadin and ZM447439, kinetochores attach microtubules but biorientation fails. These drugs also override the spindle checkpoint when microtubules are stabilised with taxol, but not when microtubule polymerisation is inhibited with nocodazole. ZM447439 also has anti-proliferative effects in vitro (Ditchfield et al., 2003), and VX-680 induces apoptosis in a variety of human tumour cell lines (Harrington et al., 2004). Strikingly, VX-680 has impressive anti-tumour activity in rodent xenograft models (Harrington et al., 2004). These observations are encouraging, suggesting that Aurora kinase inhibitors may have real potential as anti-cancer drugs. However, many questions remain. Specifically, it is not clear which Aurora kinase is the relevant in vivo target for these inhibitors. Although Aurora B appears to be the most likely suspect, the inhibitors described thus far are not selective for Aurora B: in in vitro kinase assays, ZM447439 inhibits Aurora A and B with equal potency (Ditchfield et al., 2003); VX-680 inhibits Aurora A and C more potently than B (Harrington et al., 2004); and the potency of Hesperadin against Aurora A and C is unknown.

Determining which Aurora is the relevant target of these inhibitors is important for several reasons (Keen and Taylor,

2004). First, to define the roles of the respective Auroras, it is essential to know which effects are attributable to which kinase. Second, the inhibitors are potentially powerful research tools. However, their true potential will only be realised if we can be confident in the nature of their targets. Finally, from the perspective of developing clinically efficacious anti-cancer drugs, identifying the target is essential. Although the existing compounds demonstrate it is possible to inhibit Aurora kinase activity, it is not yet known whether they will have clinical efficacy and whether next generation inhibitors will be needed (Keen and Taylor, 2004). If inhibiting a single Aurora mediates the observed anti-tumour activity, it may be beneficial to develop selective inhibitors of that particular Aurora kinase in order to minimise potential side effects.

Several lines of evidence suggest that the effects induced by the existing Aurora-inhibitors are due to inhibition of Aurora B. Firstly, budding yeast strains harbouring mutations in *IPL1*, arguably an Aurora B homolog, fail to resolve chromosome malorientations or sustain the spindle checkpoint in the absence of tension (Biggins and Murray, 2001; Tanaka et al., 2002). Secondly, repression of chromosome passengers that interact with Aurora B yields similar phenotypes (Carvalho et al., 2003; Lens et al., 2003). However, the situation is complicated by observations showing that inhibition of Aurora B by RNAi, gene knockouts or antibody injection approaches yield much more dramatic phenotypes (Ditchfield et al., 2003; Kallio et al., 2002; Petersen and Hagan, 2003). Specifically, kinetochore-microtubule attachment is inhibited and the SAC fails in both nocodazole and taxol.

One explanation for these differences is that Aurora B depletion may have more extensive consequences than simply inhibiting catalytic activity (Ditchfield et al., 2003; Keen and Taylor, 2004). A solution therefore might be to express catalytically inactive kinase mutants. However, when an Aurora B kinase mutant was overexpressed following transient transfection of normal rat kidney cells, chromosomes failed to attach microtubules and the SAC failed in nocodazole (Murata-Hori and Wang, 2002), consistent with a major kinetochore defect. Again, rather than simply inhibiting catalytic activity, excessive overexpression may induce more extensive effects by disrupting complex stoichiometry (Ditchfield et al., 2003; Keen and Taylor, 2004). Indeed, transient transfection of Aurora B mutants can result in ~500-fold overexpression, resulting in mislocalisation of the endogenous and exogenous protein (Ditchfield et al., 2003).

In addition to the complexities of studying Aurora B by molecular genetics, interpreting the small-molecule data is further confused by the fact that Aurora A may have multiple functions. The initial discovery of the *aurora* mutation in *Drosophila* implicated Aurora A in spindle assembly (Glover et al., 1995). Since then, elegant experiments in several model systems have confirmed this (Barros et al., 2005; Giet et al., 2002; Giet et al., 1999; Kinoshita et al., 2005; Liu and Ruderman, 2006; Peset et al., 2005). In human cells, the situation is more complicated: not only is the exact role of Aurora A kinase activity unclear, but Aurora A has been implicated in mitotic entry, the SAC, kinetochore assembly, chromosome alignment, cell division, p53 function, BRCA1 phosphorylation, the DNA damage response and mRNA translation (reviewed by Keen and Taylor, 2004; Marumoto et al., 2005). Finally, although Aurora C appears to be meiosis specific, we cannot rule out the possibility that it is a target in the tumour cell lines studied.

To define the cellular target of ZM447439 and thus resolve some of these issues, we have developed a new model system to study Aurora kinase activity. Here, we describe a panel of tetracycline-responsive stable cell lines expressing Aurora transgenes, both wild-type and kinase-inactive mutants. Expression of exogenous proteins is three to five times higher than that of endogenous levels, which – importantly – does not disrupt Aurora localisation. Using these lines, we have analysed the effects on cell division, spindle checkpoint control and cell viability. To complement this molecular genetics approach, we also describe two novel Aurora kinase inhibitors, ZM2 and ZM3. To maintain clarity in the text, ZM447439, as originally described by us (Ditchfield et al., 2003), will therefore be referred to as ZM1.

Results

Stable cell lines expressing Aurora kinase mutants.

To determine the respective roles of Aurora A, B and C kinase activity in human cells, we generated stable cell lines expressing the three wild-type Aurora kinases, as well as transgenes harbouring point mutants designed to inhibit catalytic activity (Fig. 1). In all three cases, we mutated the invariant lysine in subdomain II, which co-ordinates ATP, to arginine (K-R) (see Table S1 in supplementary material) (Hanks and Hunter, 1995). In addition, we separately mutated the aspartic acid in the highly conserved DFG motif, subdomain VII, to asparagine (D-N). The stable cell lines were generated using FRT-Flp-mediated recombination to integrate the minigene constructs at a pre-defined genomic locus in HEK293 cells. Importantly, this eliminates 'site-of-integration effects', thereby facilitating a direct comparison of the various transgenes. Transgene expression was under tight tetracycline control, with expression becoming maximal after ~4 hours (not shown), allowing us to study the first mitosis following induction. The Aurora proteins, expressed as Myc-tagged fusion proteins to enable detection, were all expressed at equivalent levels (Fig. 1A). To determine the expression levels relative to endogenous proteins, we also generated novel antibodies against the divergent N-terminal extensions of human Aurora A, B and C (Fig. 1A). Quantitative analyses indicated that Myc-Aurora A and B were expressed at levels three to five times higher than the endogenous proteins (not shown). Note that we could not detect endogenous Aurora C in HEK293 (Fig. 1A,D), HeLa or DLD-1 cells (not shown).

Because massive overexpression of Aurora B results in its mislocalisation (Ditchfield et al., 2003), we asked whether the exogenous Aurora proteins localised correctly when overexpressed three- to fivefold. Immunofluorescence analysis indicated that following induction, wild-type Aurora A and the Aurora A mutants localised to centrosomes in interphase (not shown) and spindle poles in mitosis (Fig. 1E). In addition, wild-type Aurora B and C, plus the respective kinase mutants, localised to the centromeres in prometaphase and metaphase (Fig. 1E) and the spindle midzone in anaphase (not shown). Thus, the exogenous Aurora proteins – both the wild-type and mutant – appear to localise correctly. To confirm that the Aurora mutants were indeed catalytically deficient, Myc-tagged proteins were immunoprecipitated and assayed in vitro. Although wild-type Aurora A and B phosphorylated histone

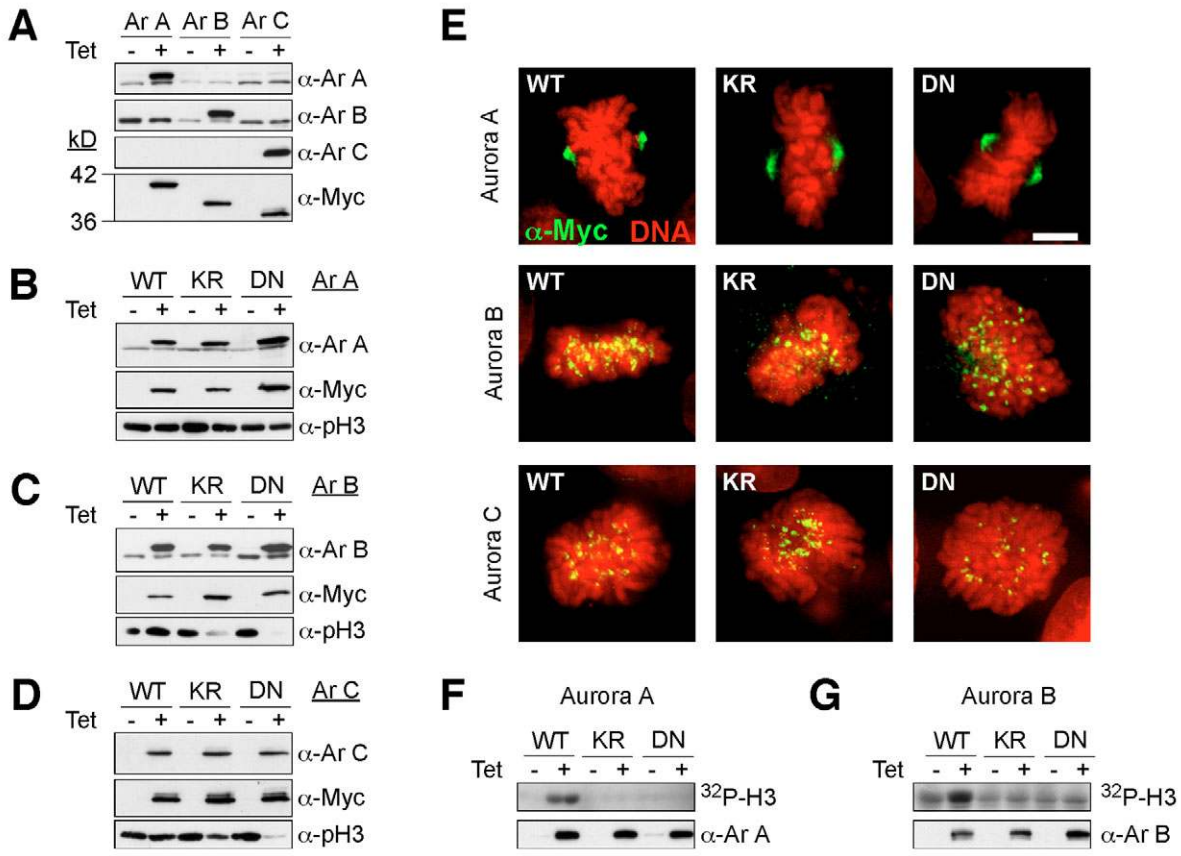


Fig. 1. Characterisation of model system. HEK293 cell lines stably transfected with Aurora transgenes were induced with tetracycline then analysed by immunoblot, immunofluorescence and immunoprecipitation kinase assays. (A) Immunoblot showing that the anti-Aurora A, B and C antibodies are monospecific for Aurora (Ar) A, B and C respectively. DN, Aurora kinase D-N mutant; KR, Aurora kinase K-R mutant; WT, wild type. (B-D) Immunoblots probed with antibodies against Aurora proteins, the Myc-epitope tag and phosphorylated Histone H3 (S10), showing tetracycline induced expression of Aurora transgenes and effects on H3 phosphorylation. (E) Immunofluorescence images showing localisation of exogenous Aurora proteins. Bar, 5 μm. (F,G) Immunoprecipitation kinase assays showing that the Aurora kinase mutants are catalytically inactive.

H3, the K-R and D-N mutants did not (Fig. 1F,G). Importantly, phosphorylation of histone H3 on Ser10 was markedly reduced following induction of the Aurora B mutants (Fig. 1C), consistent with the notion that histone H3 is a bone fide Aurora B substrate.

Suppression of Aurora B kinase activity prevents cell division.

Using this model system, we asked whether the kinase mutants exerted dominant effects on cell division, spindle checkpoint control or cell viability. First, we tested whether overexpression of the transgenes inhibited cell division. At various time points following tetracycline induction, cells were analysed by flow cytometry to measure DNA content. After 32 hours, cells expressing wild-type Aurora A, B and C exhibited normal cell-cycle profiles (Fig. 2A). Cells expressing the Aurora A K-R and D-N mutants also exhibited normal cell-cycle profiles. Indeed, as we show below (Fig. 4), these cells proliferate normally despite overexpression of the Aurora A mutants. By contrast, in the populations expressing the Aurora B kinase mutants, the cell-cycle profiles were radically different, showing a large 4N peak and cells with DNA contents >4N. Quantification showed that after 32 hours, 55% of the Aurora B D-N cells had DNA contents >4N (Fig. 2B), indicating extensive polyploidisation, a consequence of continued cell-cycle progression in the absence of cell division. Thus, although overexpression of the Aurora A transgenes had no apparent effect, suppression of Aurora B kinase activity clearly inhibited cell division.

Because we also set out to determine which Aurora kinase is the relevant target of ZM1, we directly compared expression of the Aurora kinase mutants with exposure to ZM1. Consistent with our previous report, ZM1 inhibited cell division, resulting in the accumulation of cells with DNA contents ≥4N (Fig. 2B). Note however that polyploidisation became apparent after 16 hours, several hours earlier than observed with the Aurora B mutants. One possibility is that ZM1 acts within minutes of addition (Ditchfield et al., 2003), whereas tetracycline-mediated induction of the Aurora transgenes takes 4 hours. Note also that in the presence of ZM1, the proportion of cells with DNA contents >4N dropped after 32 hours (Fig. 2B) owing to extensive cell death. Consistently, after 48 hours, cells expressing the Aurora B kinase mutant began to die (not shown, but see below). Thus, taking into account the fact that small-molecule inhibitors act rapidly whereas induction of the transgene takes several hours, these data show that suppression

of Aurora B kinase activity phenocopies the effects induced by ZM1.

Consistent with previous reports (Sasai et al., 2004; Yan et al., 2005), the Aurora C mutants also inhibited cell division (Fig. 2B). Because we could not detect endogenous Aurora C in these cells, we suspect that the Aurora C mutants compete with endogenous Aurora B as a result of their ability to bind survivin and the inner centromere protein INCENP (Li et al., 2004; Yan et al., 2005), thereby suppressing Aurora B activity. Indeed, phospho-H3 is reduced upon induction of the Aurora C mutants (Fig. 1D).

Suppression of Aurora B kinase activity compromises the spindle checkpoint.

Both Aurora A and B have been implicated in the SAC (Keen and Taylor, 2004). We asked therefore whether the Aurora kinase mutants suppressed SAC function and again we directly compared the transgene effects with those induced by ZM1. Following tetracycline induction, or exposure to ZM1, cells were exposed to nocodazole or taxol for 16 hours and the mitotic index (MI) determined by flow cytometry using MPM-2 as a mitotic marker. Consistent with our previous observations (Ditchfield et al., 2003; Morrow et al., 2005), ZM1 had only a partial effect in the presence of nocodazole, reducing the MI from 23% to 16%. However, ZM1 had a dramatic effect in the presence of taxol, reducing the MI from 28% to 5% (Fig. 3A,B). Expression of the wild-type Aurora A and B transgenes had no effect on the MI, in the presence of either taxol or nocodazole. Similarly, the Aurora A kinase mutants had no effect. Significantly however, induction of the Aurora B kinase mutants reduced the MI in the presence of taxol from 28% to 5% (Fig. 3A,B). Like ZM1 however, the effect in the presence of nocodazole was only partial, reducing the MI from 23% to 18%. Consistent with its ability to compete with Aurora B (Sasai et al., 2004), the Aurora C kinase mutants also reduced the MI in taxol. Thus, like ZM1, the Aurora B and C kinase mutants override the checkpoint in the presence of taxol.

To confirm the flow-cytometry-based observations, we used time-lapse microscopy to directly measure the amount of time cells spent in mitosis (TIM), defined as the interval between nuclear envelope breakdown and anaphase onset. In the absence of spindle toxins, ZM1 induced a brief mitotic delay, increasing the mean TIM from 26 to 69 minutes (Fig. 3C and supplementary material Table S2). However, in the presence of taxol, ZM1 reduced the average TIM from 529 to 75 minutes, consistent with checkpoint override. Notably, induction of Aurora A D-N had no effect on the TIM, either in the presence or absence of taxol (Fig. 3C,D). By contrast, expression of Aurora B D-N increased the TIM from 33 to 53 minutes in the absence of spindle toxins (Fig. 3C), and reduced the mean TIM from 355 minutes to 112 minutes in the presence of taxol, again indicating checkpoint override.

Thus, taken together, the flow cytometry measurements and the time-lapse data show that overexpression of wild-type Aurora A or the Aurora A kinase mutants had no apparent effect on the SAC. Suppressing Aurora B kinase activity does however compromise the SAC. Importantly, the SAC was more severely compromised in the presence of taxol compared with nocodazole, demonstrating that Aurora B inhibition phenocopies ZM1.

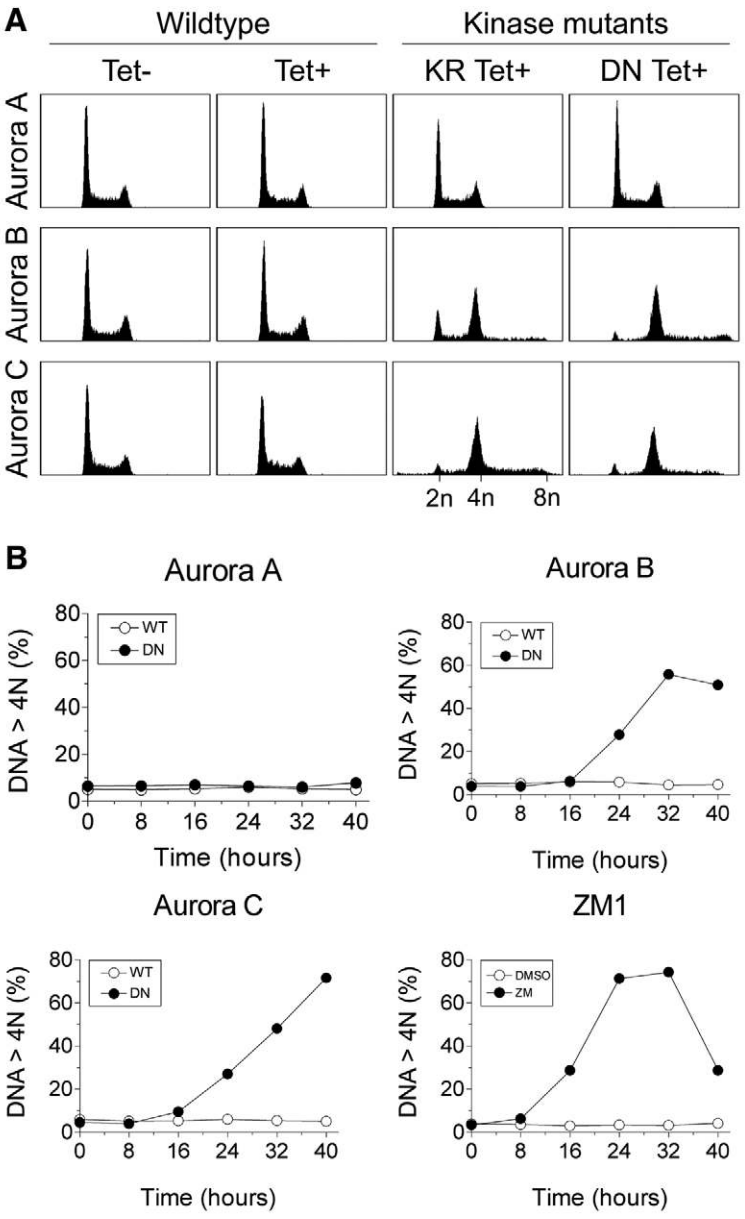


Fig. 2. Suppression of Aurora B kinase activity inhibits cell division. Aurora transgenic lines were induced with tetracycline (tet), harvested at various time points and analysed by flow cytometry to determine DNA content. (A) Histograms 32 hours post induction showing that cells expressing the Aurora B and C mutants accumulate DNA contents ≥4N. (B) Line graphs quantifying cells with DNA contents >4N over a 40-hour time course. At *t*=0 tetracycline was added to the Aurora transgenic lines indicated or, alternatively, ZM1 was added to uninduced HEK293 cells. The values shown are representative of multiple independent experiments. DN, D-N mutant; KR; K-R mutant; WT, wild type; ZM, ZM1.

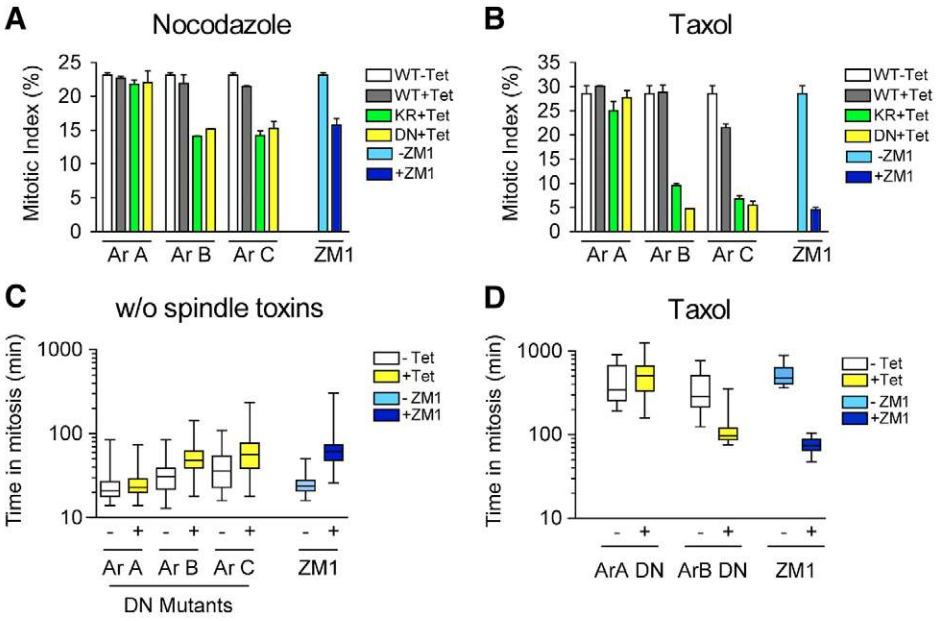


Fig. 3. Suppression of Aurora B kinase activity compromises the spindle checkpoint. Aurora transgenic lines were induced with tetracycline for 4 hours, exposed to spindle toxins then analysed by flow cytometry to determine mitotic index, or time-lapse to measure mitotic timing. In parallel, cells were exposed to 2 μM ZM1. (A,B) Bar graphs measuring mitotic index 16 hours post addition of spindle toxins nocodazole (A) or taxol (B) showing that the Aurora B and C mutants mimic the effect of ZM1. The values represent the mean ± s.e.m. derived from three independent experiments. (C,D) Box plots measuring time spent in mitosis in the absence (C) or presence (D) of taxol, showing that the Aurora B D-N mutant mimics the effect of ZM1.

Suppression of Aurora B kinase activity inhibits proliferation and viability.

The small-molecule Aurora kinase inhibitors ZM1 and VX-680 dramatically inhibit the proliferation and survival of tumour cells (Ditchfield et al., 2003; Harrington et al., 2004), properties that make them attractive as anti-cancer therapeutics. We therefore asked whether induction of the Aurora kinase mutants yielded similar effects. First, the Aurora lines were cultured in the continuous presence of tetracycline to induce transgene expression and cell proliferation was measured over an 8-day period. In parallel, cells were continuously exposed to ZM1. Although ZM1 clearly reduced proliferation, expression of wild-type Aurora A or the Aurora A kinase mutants had little effect (Fig. 4A). Interestingly, induction of wild-type Aurora B increased proliferation, such that by day 4 the cells reached confluency (Fig. 4A). Significantly however, induction of the Aurora B kinase mutants reduced proliferation, with <20% viable cells remaining by day 8. Thus, although induction of the Aurora A mutants had no apparent effect, the Aurora B kinase mutants mimic ZM1.

The above assay was performed in the continuous presence of ZM1 or tetracycline. However, in a whole-organism context, cells are typically exposed to cytotoxic drugs for a limited period. Therefore, we determined the effect of transient Aurora inhibition on long-term survival. Cells were exposed to tetracycline or ZM1 for 24 hours then harvested, washed and re-plated in fresh medium without tetracycline or ZM1. After 17 days the cells were fixed and stained with crystal violet to visualise the colonies (Fig. 4B). Consistent with our previous observations (Ditchfield et al., 2003), a pulse of ZM1 dramatically reduced colony number. By contrast, transient induction of either wild-type Aurora A, wild-type Aurora B or the Aurora A kinase mutant had no apparent effect. Transient induction of the Aurora B kinase mutant did however dramatically reduce colony number (Fig. 4B). To determine the cloning efficiency, bound crystal violet was extracted and measured. Although both ZM1 and the Aurora B kinase mutant reduced the cloning efficiency to ~10%, the Aurora A transgenes, both wild-type and D-N, had little effect (Fig. 4C). Thus, taking together the viability assay and the cloning assay, our data indicate that like exposure to ZM1, suppression of Aurora B kinase activity has a marked anti-proliferative effect. By contrast, overexpression of the Aurora A transgenes had no apparent effect.

Novel Aurora kinase inhibitors with differing selectivity and potency

In all the assays described above, suppression of Aurora B kinase activity by induction of the mutant transgenes phenocopies the effects of ZM1: cell division is inhibited (Fig. 2); the SAC is selectively compromised in response to taxol (Fig. 3); and cell proliferation is inhibited (Fig. 4). By contrast, induction of the Aurora A kinase mutants had no apparent effect in any of these assays. Thus, the simplest explanation is that the phenotypes induced by ZM1 are due to inhibition of Aurora B, not Aurora A. To test this notion further, we characterised two novel Aurora kinase inhibitors with differing selectivity and potency towards Aurora A and B.

ZM2 and ZM3 are two compounds structurally related to ZM1, which also inhibit Aurora kinase activity in vitro (see Jung and Pasquet, 2003) (Fig. 5A). In directly comparable in vitro kinase assays, ZM2 inhibits Aurora A and B with IC_{50} values of 800 nM and 7.5 nM respectively (Fig. 5B). Thus, ZM2 is ~100 times more selective against Aurora B than Aurora A. In addition, in vitro, ZM2 is five to ten times more potent against Aurora B than ZM1 (Fig. 5B). Significantly, we show below that ZM2 induces similar mitotic phenotypes to ZM1, but at much lower concentrations.

Previously, we reported that ZM1 inhibits Aurora A and B equally, with IC_{50} values of ~100 nM (Ditchfield et al., 2003). Note however that in the in vitro assays described here, which use ATP concentrations closer to physiological levels, ZM1 inhibited Aurora A and B with IC_{50} values of 1000 nM and 50

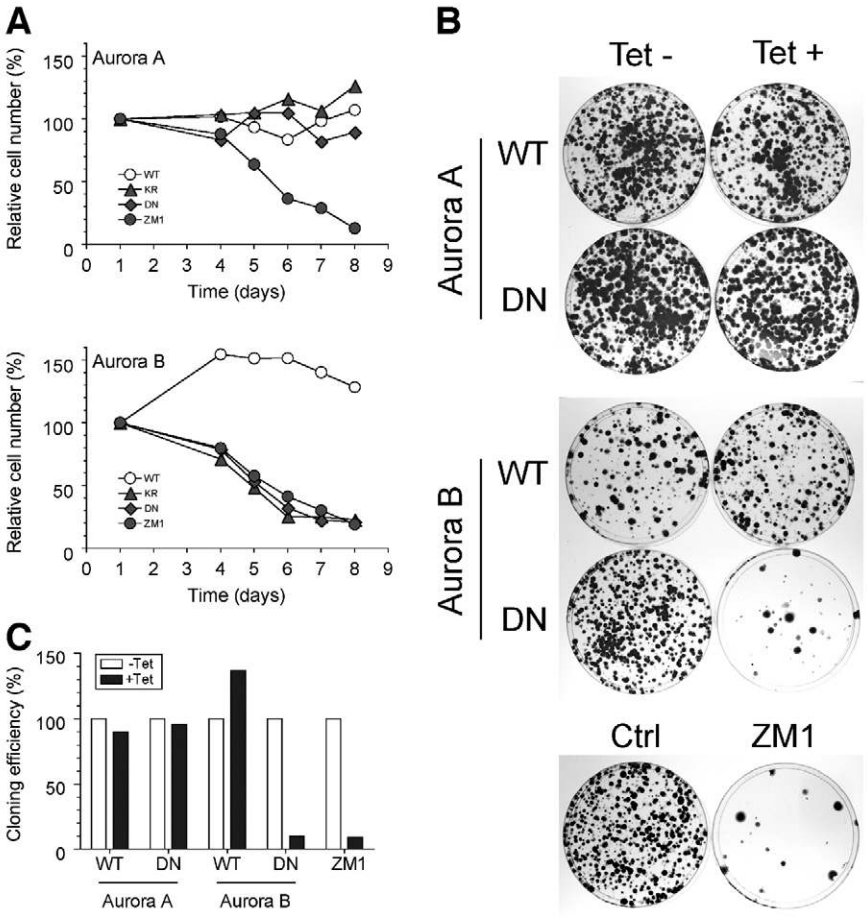
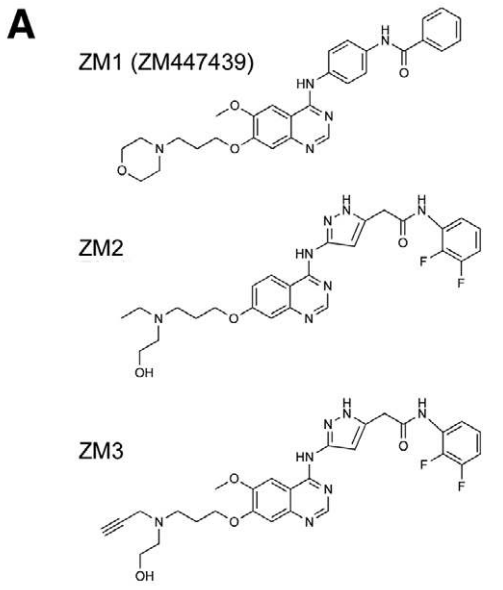


Fig. 4. Suppression of Aurora B kinase activity compromises cell proliferation and viability. (A) Line graphs plotting relative cell number over an 8-day time course in the continuous presence of tetracycline, showing that like exposure to ZM1, induction of the Aurora B kinase mutants inhibits cell proliferation. Results are from a representative experiment in which each value represents the mean of three assay wells. (B,C) Transgenic lines were induced with tetracycline for 24 hours, re-plated in the absence of tetracycline, then fixed 17 days later and stained with crystal violet to determine colony number. Images of culture plates (B) and bar graph quantifying cell number (C), both showing that the Aurora B D-N mutant mimics the effect of ZM1. The data are derived from a representative experiment in which each value represents the mean of two assay plates.



	IC_{50}(nM)			Potency vs ZM1		Selectivity
	Ar A	Ar B	Ar C	Ar A	Ar B	Ar B vs Ar A
ZM1	1000	50	250	-	-	20
ZM2	800	7.5	100	1.5	7	>100
ZM3	50	15	70	12	5	3.3

nM respectively (Fig. 5B). Thus, although ZM1 is clearly a potent Aurora B inhibitor, it is less effective against Aurora A, possibly explaining why we previously did not observe any Aurora-A-like phenotypes in cells treated with ZM1 (Ditchfield et al., 2003). By contrast however, ZM3 does appear to be a potent Aurora A inhibitor, with an IC_{50} value of 50 nM (Fig. 5B). ZM3 also inhibits Aurora B in vitro, with an IC_{50} of 15 nM (Fig. 5B). Indeed, compared with ZM1, ZM3 is more potent against both Aurora A (20-fold) and Aurora B (~3.3 fold). Consistently, ZM3 inhibits Aurora B kinase activity in cells (not shown). Significantly however, we show further below that unlike ZM1, ZM3 induces phenotypes consistent with Aurora A inhibition.

ZM2, a more selective Aurora B inhibitor, phenocopies ZM1

If the phenotypes induced by ZM1 are indeed due to inhibition of Aurora B, then a more selective and more potent Aurora B inhibitor should yield identical phenotypes, but at a lower

Fig. 5. ZM2 and ZM3: novel Aurora kinase inhibitors. (A) Chemical structures of ZM1, ZM2 and ZM3. Note that ZM1 is ZM447439 as described (Ditchfield et al., 2003). (B) Table summarising results from in vitro kinase assays to determine the effects of ZM compounds on Aurora kinase activity. Shown are the IC_{50} values; the relative potency of ZM2 and 3 with respect to ZM1; and the selectivity of each ZM compound towards Aurora B relative to Aurora A.

concentration. To test this, we analysed the cellular effects of ZM2. Consistent with it being a more potent Aurora B inhibitor, ZM2 significantly reduced phosphorylation of histone H3 at 0.1 μM, whereas 3 μM ZM1 was required for extensive inhibition (supplementary material Fig. S1A). Importantly, following release from a nocodazole block, 0.2 μM ZM2 rapidly induced mitotic exit in a manner almost identical to that observed with 2 μM ZM1 (supplementary material Fig. S1B). In addition, ZM2 selectively compromised the SAC in the presence of taxol. Specifically, when cells were exposed to 0.01-0.1 μM ZM2, their ability to maintain mitotic arrest in response to taxol was compromised yet they mounted a robust response to nocodazole (supplementary material Fig. S1C). Like ZM1, ZM2 did not prevent bipolar spindle assembly, but it did inhibit chromosome alignment, with chromosomes frequently lining up along the length of the spindle rather than at the equator (supplementary material Fig. S1D). Thus, in all the assays described here, ZM2 induces similar biological effects to ZM1 but at a much lower concentration. Because ZM2 inhibits Aurora B ~100 times more potently than Aurora A (Fig. 5B), it is highly unlikely that these effects are due to inhibition of Aurora A. Indeed, taken together with the phenotypes induced by expression of the Aurora B kinase mutants (Figs 2-4), the simplest explanation is that the biological effects of ZM1 and ZM2 are due to inhibition of Aurora B, not Aurora A.

Aurora A kinase activity is required for spindle bipolarity.

As outlined above, inhibition of Aurora B kinase activity induces phenotypes almost identical to those induced by ZM1. However, to rule out the possibility that these phenotypes are due to Aurora A inhibition, it is essential to determine the role of Aurora A kinase activity, and then ask whether or not ZM1 inhibits that process. Although Aurora A has been implicated in a number of processes, the precise role of its kinase activity in human cells remains unclear. Indeed, we were surprised that when the Aurora A kinase mutants were overexpressed three- to fivefold, we did not observe any obvious phenotypes (Figs 2-4). One possibility is that despite overexpression, the overall level of kinase activity was not suppressed below the threshold required to inhibit Aurora-A-dependent functions. Therefore, we cannot conclude that Aurora A kinase activity is not required for cell division, SAC function or proliferation, only that this methodology is not sufficient to expose the role of Aurora A kinase activity.

A potential solution would be to express the Aurora A mutants at even higher levels. However, this would risk titrating out binding partners, yielding more pleiotropic effects and therefore not providing physiologically relevant insights into the function of Aurora A kinase activity. Therefore, to expose the role of Aurora A kinase activity we used two approaches. First, we used a molecular-genetic approach to replace the endogenous Aurora A with a catalytically inactive mutant. Second, we used ZM3 in a small-molecule approach to directly inhibit the catalytic activity of endogenous Aurora A.

To inhibit Aurora A kinase activity by molecular genetics, we generated cell lines expressing Aurora A transgenes rendered insensitive to Aurora-A-specific siRNA duplexes (Fig. 6A). Following RNAi-mediated repression of Aurora A, we induced expression of wild-type or mutant Aurora A transgenes. In control populations, i.e. without repressing Aurora A, ~10-15% of the mitotic cells displayed a prometaphase appearance, with chromosomes clustered around unseparated or partially separated spindle poles (Fig. 6C). Following Aurora A RNAi, the number of prometaphase spindles increased to 35-45% and there was a marked reduction in bipolar metaphases (Fig. 6B,C). Importantly, induction of the wild-type Aurora A rescued the RNAi phenotype; metaphase spindles

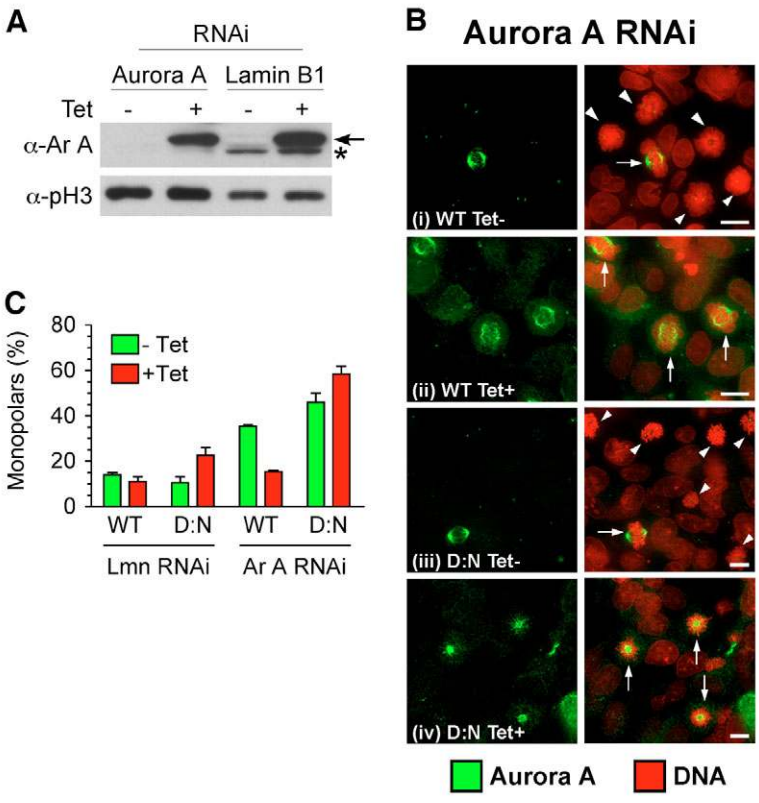


Fig. 6. Aurora A kinase activity is required for spindle bipolarity. Transgenic lines encoding RNAi-resistant Aurora A transgenes were transfected with siRNAs designed to repress Aurora A or Lamin B1, then exposed to tetracycline as indicated, to induce transgene expression. (A) Immunoblot showing simultaneous repression of endogenous Aurora A and induction of Myc-tagged Aurora A D-N. The arrow indicates the Myc-tagged exogenous Aurora A, whereas the asterisk indicates endogenous protein. (B) Immunofluorescence images showing monopolar spindles in Aurora A RNAi cells expressing the Aurora A transgenes. In panels i and iii, the horizontal arrows indicate bipolar Aurora-A-positive spindles in untransfected cells and the arrowheads indicate prometaphase-like Aurora-A-deficient cells. In panels ii and iv, the vertical arrows indicate bipolar or monopolar spindles, respectively, in cells expressing the Aurora A transgene. Bars, 10 μm. (C) Bar graph quantifying monopolar spindles showing that although the wild-type Aurora A rescues the RNAi phenotype, the Aurora A D-N kinase mutant does not. The values represent the mean ± s.e.m. derived from three independent experiments in which at least 100 mitotic cells were scored.

became readily apparent (Fig. 6Bii) and the number of prometaphases was reduced to controls levels, i.e. ~15% (Fig. 6C). Significantly however, the Aurora A D-N mutant did not rescue the RNAi phenotype, rather monopolar spindles were readily apparent (Fig. 6Biv). Indeed, quantification showed that the D-N mutant exacerbated the RNAi phenotype, increasing the number of prometaphase-like figures to ~60% (Fig. 6C) establishing that Aurora A kinase activity is required for spindle bipolarity in human cells.

ZM3 inhibits spindle bipolarity

The molecular genetics approach described above indicates that Aurora A kinase activity is required for the formation of a bipolar spindle in human cells. If this is the case, and if ZM3 can inhibit Aurora A kinase activity in cells, then ZM3 should induce a monopolar spindle phenotype. To test this, we treated asynchronous DLD-1 cells with 2 μM ZM3 for 2 hours then analysed their spindle structures. As a positive control, we treated cells with the Eg5 inhibitor monastrol (Mayer et al., 1999). Because ZM3 also inhibits Aurora B (Fig. 5), we anticipated that ZM3 would also override the SAC. Therefore, to prevent mitotic exit downstream of the SAC, we also treated the cells with the proteasome inhibitor MG132.

After a 2-hour drug exposure, bipolar spindles were readily apparent in cultures treated with MG132 alone (control) or MG132 plus ZM1 (Fig. 7A,B). By contrast, in monastrol-treated cultures, the vast majority of spindles were monopolar. Significantly, cells with monopolar spindles were readily apparent in the ZM3-treated culture (Fig. 7A). Indeed, quantification revealed that ~45% of mitotic cells were monopolar (Fig. 7B). To confirm that these were indeed monopolar spindles, we captured *z*-sections and measured interpolar distances. In controls and cells treated with ZM1, the mean interpolar distance was ~8 μm. By contrast, in monastrol-treated cells, the mean interpolar distance was less than 1 μm. The interpolar distance derived from 16 ZM3-treated cells clearly exhibited a bimodal distribution (Fig. 7C), with eight cells having well-separated poles (mean distance ~7.5 μm) and eight having poles close together (mean distance ~1.5 μm), consistent with the fact that only about half of the ZM3-treated cells were judged to be monopolar (Fig. 7B). Increasing the concentration of ZM3 increased the frequency of monopolar spindles, indicating that the effect was dose dependent (Fig. 7D). Interestingly, monopolar spindles were apparent in the ZM1-treated culture, but only at very high concentrations, ~30% at 100 μM. Importantly however, at 2 μM ZM1, a concentration where Aurora B phenotypes clearly manifest (Figs 2-4) (see Ditchfield et al., 2003), monopolar spindles were rare.

Expression of a ZM3-resistant Aurora A mutant restores spindle bipolarity.

To confirm that the ZM3-induced monopolar phenotype was due to inhibition of Aurora A rather than an off-target effect, we set out to identify a ZM3-resistant Aurora A mutant that retained catalytic activity. By systematically mutating a number of amino acids near the active site, we identified one such mutant, where the tryptophan at position 277 was converted to alanine (W277A). In vitro, Aurora A W277A is about twice as active as the wild-type enzyme but significantly, it is ~80-fold more resistant to ZM3 (IC_{50} of ~4 μM, not shown). We then generated a DLD-1 cell line expressing Aurora A W277A under tight tetracycline control (Fig. 8A). Importantly, the W277A mutant localised to spindle poles in mitosis (Fig. 8A). To test whether W277A expression reverted the monopolar phenotype, induced DLD-1 cells were exposed to 2 μM ZM3 and MG132 for 2 hours. Significantly, bipolar spindles were readily apparent in the tetracycline-induced W277A population (Fig. 8B). Indeed, quantification revealed that expression of the W277A mutant reduced the

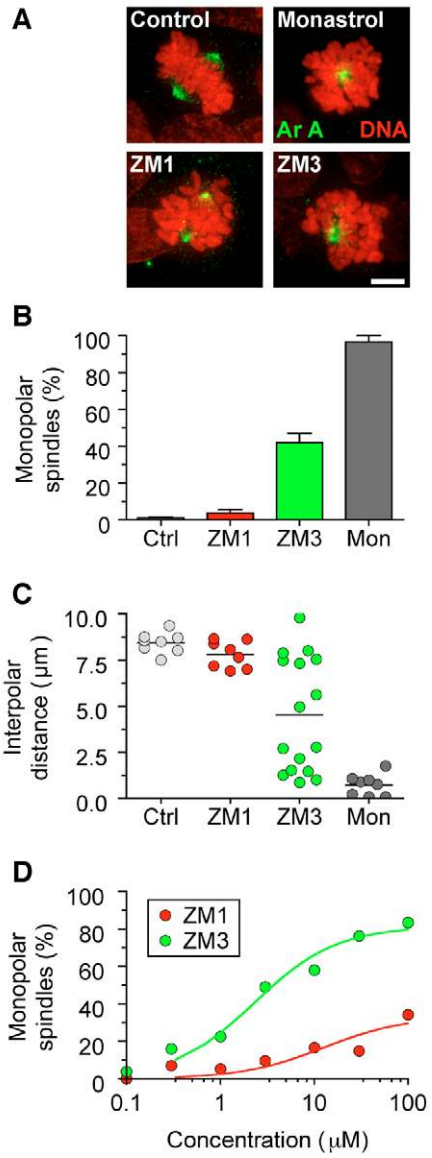


Fig. 7. ZM3 inhibits spindle bipolarity. DLD-1 cells were exposed to MG132 plus either 2 μM ZM1, 2 μM ZM3 or monastrol (Mon) for 2 hours, then analysed by immunofluorescence. (A) Images showing examples of monopolar spindles in the presence of monastrol and ZM3. Bar, 5 μm. (B) Bar graph quantifying monopolar spindles. The values represent the mean ± s.e.m. derived from three independent experiments in which at least 100 mitotic cells were scored. (C) Dot plot showing interpolar distances. (D) Line graph showing proportion of monopolar spindles over a range of ZM concentrations. The data are derived from a single representative experiment in which at least 100 mitotic cells were scored per concentration.

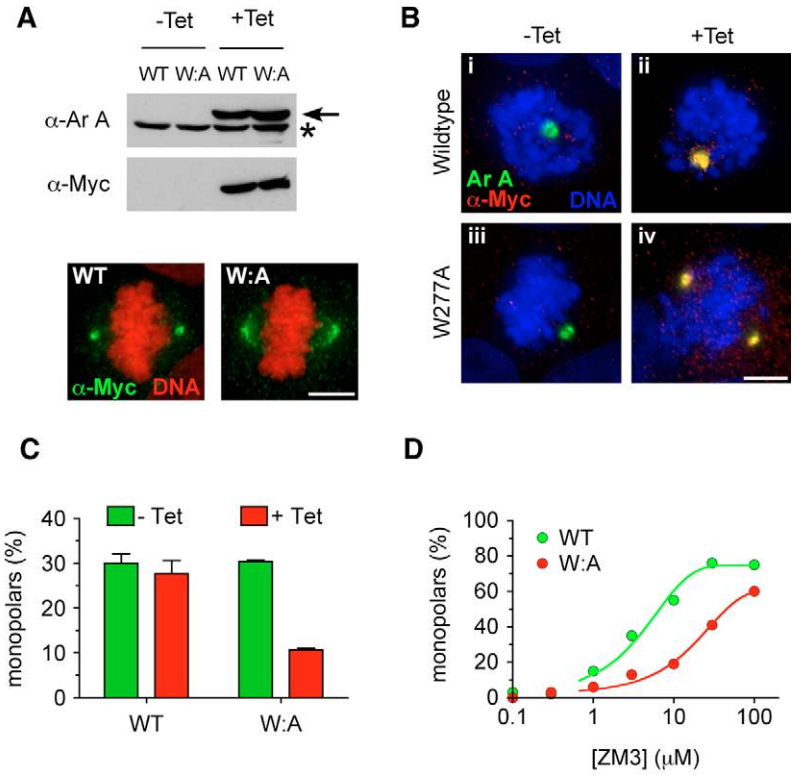


Fig. 8. Expression of a ZM3-resistant Aurora A mutant restores spindle bipolarity. (A) Immunoblots and immunofluorescence images of DLD-1 cell lines showing that the tetracycline-induced wild-type and W277A Aurora A proteins localise to the spindle poles. The arrow indicates exogenous Myc-tagged Aurora A and the asterisk indicates endogenous protein. (B) Following tetracycline (Tet) induction, cells were exposed to 2 μM ZM3 and MG132 for 2 hours then analysed by immunofluorescence. Images show examples of monopolar spindles in the absence of W277A expression. Bar, 5 μm. (C) Bar graph quantifying monopolar spindles. The values represent the mean ± s.e.m. derived from three independent experiments in which at least 100 mitotic cells were scored. (D) Line graph showing proportion of monopolar spindles over a range of ZM3 concentrations. The data is derived from a single representative experiment in which at least 100 mitotic cells were scored per concentration.

monopolar index from ~30% to ~10% (Fig. 8C). Furthermore, this effect was observed over a range of ZM3 concentrations (Fig. 8D).

Taking together the data derived from the Aurora A RNAi and D-N experiments (Fig. 6), the in vitro data showing that ZM3 is a relatively potent Aurora A inhibitor (Fig. 5), the monopolar spindle phenotype induced by ZM3 (Fig. 7), plus the observation that this phenotype can be rescued by a ZM3-resistant Aurora A mutant (Fig. 8), our data indicate not only that Aurora A kinase activity is required for bipolar spindle assembly in human cells, but that it is also possible to inhibit Aurora A kinase activity in cells with a small molecule.

Discussion

Aurora B is the target of ZM1

Small-molecule Aurora kinase inhibitors such as ZM1 have significant merit as anti-cancer drugs: by inhibiting chromosome alignment, spindle checkpoint function and cytokinesis, they prevent cell division which then results in a rapid loss of viability (Keen and Taylor, 2004). However, whether these phenotypes are due to inhibition of Aurora A, B or C is unclear. Here, we provide compelling evidence that Aurora B is the target of ZM1. First, we show that molecular genetic inhibition of Aurora B kinase activity phenocopies the action of ZM1. Specifically, suppression of Aurora B activity by expression of catalytically inactive transgenes inhibits histone H3 phosphorylation, cell division and proliferation, and compromises the spindle checkpoint following exposure to taxol (Figs 1-4). Second, we show that ZM2, which is 100 times more selective for Aurora B relative to Aurora A (Fig. 5), induces phenotypes identical to those observed following exposure to ZM1 (supplementary material Fig. S1). Third, we show that inhibition of Aurora A kinase activity induces monopolar spindles, a phenotype not observed with ZM1. And finally, with an in vitro IC_{50} value of ~1 μM, we show that ZM1 is not a potent Aurora A inhibitor.

That ZM1 is not a potent Aurora A inhibitor appears at odds with our previous report indicating that ZM1 inhibits Aurora A and B equipotently, with IC_{50} values of ~100 nM (Ditchfield et al., 2003). Note however that the in vitro kinase assays used in these two studies were designed for different purposes. The initial assays, which were optimised for high throughput screens to identify Aurora A inhibitors, used a baculovirus expression system, relatively low ATP concentrations (5-10 μM) and a biotinylated peptide as a substrate. By contrast, the assays described here, which were designed to directly compare the effects of ZM compounds on the three Auroras, used recombinant proteins purified from *E. coli*, ATP at a final concentration of 100 μM, and histones as a substrate. In the absence of a systematic comparison of the two assays, it is not clear which parameters are responsible for the differing IC_{50} values. Nevertheless, the IC_{50} values obtained here for ZM1, ~1 μM for Aurora A and 50 nM for Aurora B, appear to be more consistent with the data from cell-based assays: although ZM1 is a potent Aurora B inhibitor, it does not appear to significantly inhibit Aurora A (Ditchfield et al., 2003; Gadea and Ruderman, 2005). Taken together with our new data showing that Aurora A kinase activity is required for spindle bipolarity in human cells (Figs 6-8), we suspect that at low micromolar concentrations, ZM1 is not a significant inhibitor of Aurora A activity in cells. Consequently, these observations indicate that ZM1 is a powerful research tool for studying the downstream effects of Aurora B kinase activity.

Aurora A kinase activity is required for spindle bipolarity in human cells.

Aurora A is required for spindle assembly in several model systems, possibly by phosphorylation of targets such as Eg5 and members of the TACC family (Barros et al., 2005; Giet et al., 2002; Giet et al., 1999; Glover et al., 1995; Kinoshita et al., 2005; Liu and Ruderman, 2006; Peset et al., 2005). However, although human Aurora A has been implicated in several mitotic processes, the exact role of Aurora A kinase activity in human cells remains enigmatic. We were surprised that our initial analysis of ZM1 did not yield a monopolar spindle phenotype (Ditchfield et al., 2003). This observation is however less surprising in light of the new data presented here indicating that ZM1 is not a potent Aurora A inhibitor (Fig. 5). However, we were also surprised during the course of this study that the overexpression of Aurora A kinase mutants did not yield detectable cell-cycle effects (Figs 2-4). We suspect that this is because the endogenous, catalytically active protein is capable of providing robust Aurora A function, despite overexpression of the kinase mutants. Indeed, when we repressed Aurora A by RNAi and then induced the Aurora A D-N mutant, a striking monopolar phenotype became apparent (Fig. 6). This observation provides strong evidence that Aurora A kinase activity is required for spindle bipolarity in human cells. Further evidence for this notion comes from our analysis of a novel ZM compound, ZM3. In contrast to ZM1, ZM3 is a potent Aurora A inhibitor in vitro, and in cells ZM3 induces a monopolar spindle phenotype (Fig. 7). Significantly, this phenotype can be rescued by expression of a ZM3-resistant Aurora A mutant (Fig. 8), confirming that the phenotype is indeed due to inhibition of Aurora A, not another kinase.

Note however that in both the RNAi and ZM3 experiments (Figs 6, 7), we have no evidence to indicate that the monopolar spindle phenotype correlates with a suppression of Aurora A kinase activity in the cell. Indeed, a major limitation – not only with our studies but in the Aurora A field – is the lack of a robust, readily available cell-based marker for Aurora A kinase activity. Although antibodies that recognise the phosphorylated T-loop of Aurora A have been informative, these do not necessarily provide a robust readout of Aurora A activity. Phosphospecific antibodies that recognise downstream targets, such as TACC3 (Kinoshita et al., 2005), would be more powerful reagents. Indeed, dissecting the role of Aurora B activity has been greatly facilitated by the availability of antibodies that specifically recognise an Aurora B substrate, namely Ser10 of histone H3 (Hsu et al., 2000). There is therefore a pressing need for an Aurora A biomarker, not just to facilitate the characterisation of Aurora A function, but also to determine the efficacy of Aurora A inhibitors in animal models and patients.

Aurora kinase inhibitors as anti-cancer drugs

A number of Aurora inhibitors are in clinical trials (Matthews et al., 2006). Although the outcome of these trials remains to be seen, it is likely that second- or third-generation Aurora inhibitors will be required (Keen and Taylor, 2004). Towards which Aurora kinase should these efforts be directed? Although ZM1, -2 and -3 all inhibit Aurora C in vitro (Fig. 5), and although expression of Aurora C kinase mutants induces similar phenotypes to inhibition of Aurora B (Figs 2, 3), we suspect that Aurora C is not a valid anti-cancer target. Aurora C transcripts have been detected in human cancer cell lines (Yan et al., 2005), however, using a novel, mono-specific anti-Aurora-C antibody, we could not detect endogenous Aurora C protein in HeLa, DLD-1 or 293 cells (Fig. 1 and data not shown). Indeed, the abundance of Aurora C mRNA in testes (Kimura et al., 1999) and the detection of endogenous Aurora C protein in spermatocytes (Tang et al., 2006) suggest that significant levels of Aurora C protein may be restricted to male meiotic cells.

In contrast to Aurora C, Aurora A and Aurora B are expressed in many human cancer cells and their inhibition induces profound mitotic phenotypes (Andrews et al., 2003; Carmena and Earnshaw, 2003; Ducat and Zheng, 2004; Keen and Taylor, 2004). A number of observations suggest that Aurora B is an attractive target. Significantly, suppression of Aurora B kinase activity compromises chromosome alignment, spindle checkpoint function and cytokinesis (Ditchfield et al., 2003; Hauf et al., 2003). Consequently, following a brief mitotic delay, Aurora B-deficient cells exit mitosis without dividing and return to G1 with a 4N DNA content and they then rapidly lose proliferative potential (Fig. 4).

Another attractive feature of Aurora B as a drug target is that cells appear to be extremely sensitive to its inhibition. Induction of the Aurora B kinase mutants alone was sufficient for a highly penetrant cell-death phenotype (Fig. 4). By contrast, cells are relatively resistant to Aurora A inhibition: overexpression of the Aurora A kinase mutants had no apparent effect (Figs 2-4). Indeed, to expose the monopolar spindle phenotype using molecular genetic inhibition, we had to first repress the endogenous protein by RNAi and then overexpress the kinase mutant (Fig. 6). However, we do show that a similar phenotype can be achieved via small-molecule-mediated inhibition of Aurora A (Figs 7, 8). Thus far, we have not been able to determine the longer-term consequences of this because ZM3 also inhibits Aurora B (Fig. 5). However, it is conceivable that by preventing assembly of a bipolar spindle, a selective Aurora A inhibitor may result in activation of the SAC and prolonged mitotic arrest, which in turn may result in apoptosis. Therefore, selective Aurora A inhibitors may have potential as anti-cancer drugs in much the same way as microtubule toxins or kinesin spindle protein inhibitors (Bergnes et al., 2005). Thus, the Aurora kinases may offer two avenues for anti-cancer strategies rather than one.

Materials and Methods

Molecular biology

The human Aurora A and C open reading frames (ORFs), corresponding to GenBank accession numbers BC002499 and NM_001015878, were isolated by PCR amplification of ESTs using *Pfu* polymerase (Stratagene). PCR products were cloned and sequenced to verify their integrity. The human Aurora B ORF, corresponding to accession number NM_004217, was isolated by RT-PCR amplification of HeLa mRNA using a Superscript^{TM} one-step system (Invitrogen). Site-directed mutagenesis (QuikChange, Stratagene) was used to create the catalytic, drug-resistant and RNAi-resistant mutants (supplementary material Table S1).

Antibody generation

The N-terminal extensions of human Aurora A, B and C, encoding amino acids 2-131, 2-45 and 2-40, respectively, were PCR amplified and cloned into pGEX-4T-3 (Pharmacia). Soluble GST fusions expressed in *E. coli* were purified by affinity chromatography then used to immunise sheep (Scotland Diagnostics). Anti-Aurora B and C antibodies (SAB.1 and SAC.1 respectively) were affinity purified as described (Taylor et al., 2001). The immune sera containing anti-Aurora A antibodies (SAA.1) was sufficiently 'clean' that affinity purification was not necessary.

Cell lines

Stable, isogenic cell lines expressing Aurora transgenes under tetracycline control were generated using a FRT-Flp-based system as described (Tighe et al., 2004). Briefly, ORFs were cloned into a pcDNA5-FRT-TO vector (Invitrogen) modified to contain an N-terminal Myc-epitope tag. Resulting vectors were co-transfected into Flp-In™ TRex™-HEK293 or DLD-1 cells with pOG44, a plasmid encoding the Flp recombinase. After selection in hygromycin, colonies were pooled, expanded and transgene expression induced with 1 μg/ml tetracycline. All cell culture conditions were as described (Taylor et al., 2001). Small molecules were used at the following final concentrations: nocodazole, 0.2 μg/ml; taxol, 10 μM; monastrol, 20 μM; and MG132, 20 μM. The Aurora inhibitor ZM447439, here referred to as ZM1, was as described (Ditchfield et al., 2003). ZM2 and ZM3 (Jung and Pasquet, 2003), were dissolved in DMSO at 10 mM, stored at –20°C and used at the concentrations indicated.

Antibody techniques

Immunoblot analysis was done as described (Taylor et al., 2001) using the following antibodies: 4A6 (mouse anti-Myc, Upstate, 1:5000); SAA.1 (sheep anti-Aurora A, 1:5000); SAB.1 (sheep anti-Aurora B, 1:1000); SAC.1 (sheep anti-Aurora C, 1:500); rabbit anti-phospho (S10) histone H3 (Upstate, 1:1000). Immunofluorescence analysis was performed essentially as described (Taylor et al., 2001) using the following antibodies: 4A6 (mouse anti-Myc, Upstate, 1:750); SAA.1 (sheep anti-Aurora A, 1:5000); TAT-1 (mouse anti-tubulin, 1:1000). For IP-kinase assays, protein extracts were prepared by resuspending cells in 50 mM Tris-HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 50 mM NaF, 1 mM $NaVO_4$, 1 μM okadaic acid, 0.1% Triton X-100, 0.1% β-mercaptoethanol plus protease inhibitors, followed by centrifugation at 16,000 ***g*** for 20 minutes at 4°C. Myc-tagged proteins were isolated using 1 μg anti-Myc (4A6) antibodies per 1 ml lysate and recovered with protein-G-Sepharose beads. After four washes, beads were incubated in a reaction cocktail containing 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 100 μM EGTA, 100 μM $MgCl_2$, 0.1 mg/ml BSA, 0.1% β-mercaptoethanol, 10 μg mixed histones plus 100 μM [γ-^{32}P]ATP for 20 minutes at 30°C. Reactions were stopped by addition of SDS sample buffer, then analysed by SDS-PAGE and visualised by autoradiography.

Cell biology

DNA content and mitotic index measurements were performed using flow cytometry as described (Taylor and McKeon, 1997). Briefly, cells were fixed in 70% ethanol, stained with MPM-2 antibodies (Upstate) followed by a FITC-conjugated donkey anti-mouse antibody, then stained with propidium iodide. Flow cytometric analysis was carried out using a CyAn™ (DakoCytomation). For time-lapse analysis, stable cell lines were analysed by phase-contrast microscopy, with images collected every 2 minutes using a Zeiss Axiovert 200 as described previously (Morrow et al., 2005). *XY*-point visiting and acquisition of *Z*-sections was performed using a PZ-2000 automated stage (Applied Scientific Instrumentation). To determine mitotic timing, tetracycline was added for 4 hours and cells analysed over the subsequent 24 hours. Mitotic timing data is presented as box-and-whisker plots generated with Prism 4 (GraphPad), where the boxes show the median and interquartile ranges, whereas the whiskers show the entire range.

Viability and colony-formation assay

Cell proliferation was assessed by plating ~500 cells in each well of a 96-well plate followed 6 hours later by addition of 1 μg/ml tetracycline or 2 μM ZM1. From day 4, plates were then analysed daily using a WST1 assay according to the manufacturer's instructions (Roche). Relative cell numbers were calculated as the change in proliferation compared to control wells at each time point. To measure cloning potential, cells were treated for 24 hours with 1 μg/ml tetracycline or 2 μM ZM, then harvested, washed and ~1000 cells replated in 10 cm dishes. The media was changed every 4 days, then, on day 17, the cells were fixed in 4% formaldehyde and stained with 0.1% crystal violet. Bound crystal violet was solubilised in 10% acetic acid and the absorbance measured at 600 nm.

In vitro kinase assays

Aurora ORFs were cloned into pET28a (Novagen)-based vectors and following IPTG induction at 22°C, 6× His-tagged fusion proteins purified from *E. coli* [BL21 (DE3) pLysS, Novagen] using cobalt agarose (BD Bioscience). Purified kinases were eluted from the affinity resin with imidazole, dialysed into kinase buffer and stored at –80°C. 400 ng purified recombinant enzyme was added to a reaction cocktail containing 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.1 mM EGTA, 10 mM $MgCl_2$, 0.1% β-mercaptoethanol, 10 μg mixed histones plus 100 μM [γ-^{32}P]ATP (specific activity 100-500 cpm/pmole) then incubated at 30°C for 15 minutes. Reactions were transferred onto P81 phosphocellulose paper, washed in 0.5% phosphoric acid, dried and phosphate incorporation calculated by scintillation counting of dried papers. Under these conditions, phosphate incorporation was linear with respect to time and enzyme concentration for all three recombinant Aurora kinases.

RNA interference

siRNA duplexes (Dharmacon Research) designed to repress Aurora A were as described (Ditchfield et al., 2003). 4×10^4 cells were seeded in 24-well plates 24 hours before transfection in growth media without antibiotics. siRNA duplexes were mixed with OligofectAMINE™ (Invitrogen) in media without antibiotics and incubated for 20 minutes. siRNA-lipid complexes were then added to cells for 6 hours followed by addition of complete media containing 20% foetal calf serum. 24 hours later the cells were replated onto coverslips or in six-well plates with the addition of 1 μg/ml tetracycline to induce transgene expression. Cells were analysed 24 hours later.

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