Published in final edited form as:

ACS Chem Biol. 2010 November 19; 5(11): 1015–1020. doi:10.1021/cb1001685.

Binucleine 2, an isoform-specific inhibitor of *Drosophila* Aurora B kinase, provides insights into the mechanism of cytokinesis

Yegor Smurnyy, Angela V. Toms, Gilles R. Hickson, Michael J. Eck, and Ulrike S. Eggert*
Dana-Farber Cancer Institute and Dept. of Biological Chemistry and Molecular Pharmacology,
Harvard Medical School, Boston, MA

Abstract

Aurora kinases are key regulators of cell division and important targets for cancer therapy. We report that Binucleine 2 is a highly isoform-specific inhibitor of *Drosophila* Aurora B kinase and we identify a single residue within the kinase active site that confers specificity for Aurora B. Using Binucleine 2, we show that Aurora B kinase activity is not required during contractile ring ingression, providing insight into the mechanism of cytokinesis.

Aurora B kinase is a main regulator of cell division (1). It functions in the chromosomal passenger complex, which includes at least three other proteins: the inner centromere protein (INCENP), survivin and borealin/DASRA (2). This Aurora B containing complex has multiple functions throughout mitosis and cytokinesis. During cytokinesis, Aurora B activity has been implicated in the formation of the microtubule midzone and cytokinesis completion (2). Using Binucleine 2, a small molecule inhibitor of Aurora B kinase, we show that kinase activity is not required during ingression of the cleavage furrow.

Three Aurora kinases (A, B and C) are expressed in mammals and two (A and B) in invertebrates (3). Aurora A is associated with centrosomes and is responsible for various aspects of mitotic progression (4). Less is known about Aurora C, which appears to be mainly expressed in testes (3). Aurora kinases are overexpressed in many cancers, making them potential targets for cancer chemotherapy (5), with many compounds currently in clinical trials (6). Most known Aurora inhibitors are ATP-competitive active site inhibitors and show little selectivity between the different Aurora kinases *in vitro*. Some isoform-specific Aurora inhibitors have been reported (7-9), which derive their selectivity from interactions with hydrophobic pockets adjacent to the hinge region of the ATP binding pocket, a key region responsible for determining activity and specificity (10). Here, we report a unique example of an ATP-competitive inhibitor that interacts mostly with hinge residues and exhibits a >300-fold isoform selectivity. We find that the major determinant of specificity is hinge residue Ile 132.

We discovered Binucleine 2 (Fig. 1a) in a phenotypic screen for small molecule inhibitors of cytokinesis (11). *Drosophila* Kc167 cells treated with Binucleine 2 exhibited mitotic and cytokinesis defects, as did cells where Aurora B kinase was depleted by RNAi. Based on comparisons between these phenotypes, we had predicted that the Aurora kinase B pathway was the cellular target of Binucleine 2 (11). To test this hypothesis, we purified a complex of *Drosophila* Aurora B kinase and an INCENP fragment (Supporting Fig. 1), which is needed for optimal kinase activity (12). Confirming our original prediction, we showed that

*To whom correspondence should be addressed: ulrike_eggert@dfci.harvard.edu.

Supporting Information Supporting information is available for free via the internet.

Binucleine 2 inhibits the kinase (Fig. 1b-c) and demonstrated ATP-competitive inhibition, with $K_m^{ATP} = 130 \pm 34 \mu M$ and $K_i^{B2} = 0.36 \pm 0.10 \mu M$ (95% confidence interval, Supporting Fig. 2). This result illustrates that phenotypic comparisons can be a useful approach for successful target identification.

Given that most Aurora kinase inhibitors inhibit all isoforms, we next evaluated Binucleine 2's effect on purified *Drosophila* Aurora A kinase and were surprised to find that it is highly isoform-specific (Fig. 1d), with no significant inhibition of Aurora A up to 100 μM . Similarly, Binucleine 2 did not inhibit the closely related human or *Xenopus laevis* (13) Aurora B kinases (Supporting Fig. 3). Kinase active sites are usually well conserved, both within and across species, and many ATP-competitive kinase inhibitors are notoriously promiscuous. To get some clues about possible reasons for Binucleine 2's selectivity, we inspected sequence alignments (Fig. 2a) from different Aurora kinases, focusing on residues around the "gatekeeper" residue in the hinge region of the ATP binding pocket (14). We noticed that *Drosophila* Aurora B kinase had two changes in this highly conserved region: an Ile at the position two residues C-terminal to the gatekeeper where other Aurora kinases have an aromatic residue such as Phe or Tyr, and a Ser four residues C-terminal to the gatekeeper (Fig. 2a). We hypothesized that these residues might be responsible for Binucleine 2's specificity. We "humanized" the *Drosophila* kinase by mutating Ile 132 to Tyr and Ser 134 to Pro and found that the mutant has similar enzyme kinetic properties as the wild type enzyme (Supporting Fig. 4), but it is no longer inhibited by Binucleine 2 (Fig. 1c and Supporting Fig. 4). Although we were unable to express the single Ile132Tyr mutant, we were able to purify the single Ser134Pro mutant and found that it is still inhibited by Binucleine 2 (Supporting Fig. 5), suggesting that Ile 132 is the key determinant of Binucleine 2 activity.

To explore how Ile 132 and Binucleine 2 might interact so specifically, we turned to docking experiments. The structure of *Drosophila* Aurora B kinase has not been solved, so we prepared a homology model based on the closely related *Xenopus* Aurora B structure (12). We then carried out computational docking studies using the program GLIDE, to determine potential binding conformations for Binucleine 2 (Fig. 2b). A lowest energy model (Fig. 2b) revealed a predicted hydrogen bond between N2 of the pyrazole and the backbone amide of Ala 133 and hydrophobic interactions between the aromatic substituents on Binucleine 2 and the side chain of Ile 132, which appear to be key for Binucleine 2's specificity. Other Aurora kinases have a tyrosine at this position (Fig. 2a), which is too bulky to allow a similar binding conformation.

To further test our mutational and docking-based hypothesis that hydrophobic interactions between Ile 132 and the aromatic substituents on Binucleine 2 are the primary determinants of specificity, we synthesized and tested a series of derivatives, where we systematically varied the substitution patterns (Fig. 1b). The phenyl rings of the most active compounds are either 3- or 3,4-substituted, for example, the 3,4-di-Cl derivative has an IC_{50} of 2 μM , much lower than 3,5-di-Cl (15 μM) or 2,4-di-Cl (30 μM). Substitution at the meta position is more important than at the para, as illustrated by 3-Br ($IC_{50} = 0.9 \mu M$) compared to 4-Br ($IC_{50} = 20 \mu M$). These data support the binding conformation predicted by the docking studies. It appears that the meta substituent fits nicely into a hydrophobic pocket lined by the Ile sidechain (Fig. 2b).

Although Binucleine 2 is relatively small for a highly specific kinase inhibitor, it can form both hydrogen bonds and hydrophobic interactions with the hinge region of the ATP binding pocket. Mutations in this region (and specifically at the residue corresponding to Ile132) in the human Aurora B kinase as well as in other kinases such as the clinically important Bcr-Abl have been shown to confer resistance to small molecule inhibitors (15,16), but have not

been used to gain binding specificity. Unlike many hinge-binding kinase inhibitors that rely mostly on hydrogen bonds (10), Binucleine 2 selectivity benefits from specific hydrophobic interactions with a hinge-region residue near the gatekeeper residue (Ile 132). Although the importance of the gatekeeper residue in determining inhibitor specificity is widely appreciated, the potential role of this hinge residue appears to have been largely ignored. We suggest that it might be more broadly exploited in the design of selective kinase inhibitors.

Our biochemical data strongly suggest that Aurora B kinase is a major cellular target of Binucleine 2, but they do not give us any information about other potential targets. We therefore performed rescue experiments with cells expressing Binucleine 2-resistant Aurora B kinase. We created *Drosophila* Kc cells lines that stably expressed enzymatically active mutant *Drosophila* Aurora B kinase (Ile132Ser and Tyr134Pro) fused to GFP. The mutant kinase localized normally (Fig. 3), suggesting that it can integrate into the chromosomal passenger complex. The majority of cells expressing the mutant kinase (10/12 dividing cells) were no longer affected by Binucleine 2, *i.e.* cells looked normal with no cell division defects (Fig. 3). Also, the Binucleine 2 derivatives we synthesized (Fig. 1b) exhibit a strong correlation between their *in vitro* kinase inhibition activities and their cellular effects, providing additional evidence that Aurora B kinase is indeed the primary target of Binucleine 2.

The principal goal in the discovery of small molecule probes such as Binucleine 2 is to use the compounds to study the biology of the probe's cellular target. Because they can be added with high temporal control, small molecules have been used very successfully to investigate other mitosis/cytokinesis regulators, for example Plk1 (17). Studies with other Aurora kinase inhibitors have also resulted in new insights into the mechanisms of cytokinesis (18,19), and especially into the regulation of mitosis (17), but they have been limited by the potential of off-target effects due to lack of isoform specificity. Since we now have a highly Aurora B specific tool in hand, we used it to study the role of Aurora B kinase in cytokinesis using live imaging in *Drosophila* cells. *Drosophila* cells are commonly used models to study cytokinesis because the regulation of cytokinesis is highly conserved across species. For example, both Aurora and Polo kinases were originally discovered in *Drosophila* (20,21), but have since been shown to be key regulators of cell division in human cells. In addition to providing insight into the mechanism of cytokinesis, Binucleine 2 will also be a useful tool to study the role of the chromosomal passenger complex during development in this important model organism, which was not possible previously because other Aurora inhibitors are not active in *Drosophila*.

To test the effects of Aurora B kinase inhibition on cells at different stages of cell division, we added Binucleine 2 to cells expressing GFP-tagged Aurora B or Anillin, a contractile ring marker (Fig. 4 and Supporting Figs. 6-8). Binucleine 2 addition to cells that had not yet assembled a contractile ring showed that Aurora B kinase activity is absolutely required for ring assembly, confirming previous data (18,22). Binucleine 2 produced an effect in metaphase and early anaphase cells within 2 min (Fig. 4), suggesting that it can easily enter mitotic cells. Binucleine 2 addition to cells that had already assembled a ring, surprisingly, had no significant effect on ring ingression (Supporting Fig. 6), suggesting that kinase activity is not required for this process. This result is unexpected because the kinase and its complex partners localize to the contractile ring and interzonal microtubules and are maintained there throughout ingression. Since the Aurora B kinase complex consists of several proteins, it is likely that they have additional functions such as binding to effector proteins or serving as scaffolds in addition to supporting and modulating the kinase's activity (23). It is possible that such a role predominates during ring ingression.

In this letter, we report a series of experiments that have more general implications for small molecule probe development. We show that systematic comparisons between small molecule and RNAi phenotypes can be used to identify small molecule targets. We also show that hydrophobic interactions between a small molecule and a residue in a kinase's hinge region can lead to highly specific binding. Finally, taking advantage of a small molecule that can be added at specific stages of the cell cycle, our study challenges the idea that Aurora B kinase's catalytic activity is its only function. Since Binucleine 2 has no effect on ring ingression, even though the kinase is maintained at specific cytokinetic structures, we propose that this kinase has additional functions during cytokinesis. In summary, this study demonstrates that it is possible to obtain species-specific kinase inhibitors even when the ATP binding pocket is highly conserved, which has implications in the design of fungicides or insecticides.

Methods

Kinase activity assays and kinetic data processing

For protein expression and purification, see Supporting Methods. For the ^{32}P incorporation assay 250 ng of kinase, 20 μg of myelin basic protein (Sigma), 5 μCi of ^{32}P -ATP (Perkin-Elmer), cold ATP and a drug, if necessary, were diluted into kinase reaction buffer: 20 mM Tris pH 7.5, 1 mM MgCl_2 , 25 mM KCl, 1 mM DTT, 40 $\mu\text{g}/\text{mL}$ BSA. After 10 minutes at room temperature, reactions were spotted onto P81 paper circles (Whatman), circles were washed 4 times with 0.75% phosphoric acid and once with acetone, and the amount of incorporated ^{32}P was measured using a scintillation counter.

The pyruvate kinase – lactate dehydrogenase coupled assay was performed as follows: first, 2x reaction buffer was prepared: 100 mM HEPES pH 7.5, 20 mM MgCl_2 , 2 mM DTT, 3 mg/mL BSA, 4% pyruvate kinase/lactate dehydrogenase from rabbit muscle (Sigma), 2mM phosphoenolpyruvate and water. Finally, 250 ng/reaction of kinase, 600 μM substrate peptide and 1 mM NADH were added. 50 μL of the 2x mixture was dispensed into each well of a 96 well plate, followed by 25 μL of 4x drug stocks in 100 mM HEPES pH 7.5, and, at the very last moment, 25 μL of 4x ATP in 100 mM HEPES pH 7.5. The reaction was monitored by measuring the decrease in OD_{340} due to conversion of NADH into NAD^+ . The substrate peptide is comprised of amino acids 1-20 of histone H3, Aurora B's natural substrate: ARTKQTARKSTGGKAPRKQL.

Kinetic data were interpreted in terms of the classic competitive inhibition Michaelis-

Menten model: $v = \frac{V_{\max} * S}{S + K_m * (1 + I/K_i)}$, where S is substrate (ATP) concentration, I is inhibitor concentration, $V_{\max}$ is maximally attainable reaction speed, K_m is Michaelis-Menten constant and K_i is inhibition constant.

Origin 8.0 (Originlab) and Excel (Microsoft) packages were used to perform non-linear fit and plot the data.

Homology modeling

All homology modeling and ligand docking calculations were performed at the Structural Biology Grid (SBGrid) facility at the Harvard Medical School.

The crystal structure of *Xenopus laevis* Aurora B-C-terminal INCENP binary complex, (PDB ID 2BFY) was used as a starting point. Residues Phe172 and Pro174 were mutated *in silico* to Ile and Ser, respectively using Prime homology modeling software (Schrodinger Inc). The resulting structure has the Ile-Ala-Ser motif that is present in the WT *Drosophila*

Aurora B kinase and was used as a receptor in further steps. All of other residues in the active site remain the same as in the crystal structure.

Ligand docking

Structures of Binucleine 2 and analogs were docked to the receptor using the Glide software (Schrodinger Inc). First, 3D models of ligands were prepared using the LigPrep tool from the Glide package. Then, the $20 \times 20 \times 20$ Å docking grid was generated using the OPLS2001 force field with default (1.0) scaling of van der Waals atomic radii. The grid was centered on the center of masses of the Ile172 and Ser174 (X.l. numbering) of the receptor. Finally, molecules were docked using the extra-precision (XP) method, as implemented in the Glide package. Resulting structures were ranked by Glide docking score that measures feasibility of the found ligand pose and the structure with the minimal score was further analyzed. No additional constraints (explicitly defined hydrogen bonds, fixed interatomic distances, and the like) were used during docking.

Cell culture

Drosophila Kc₁₆₇ cells were grown at 25°C in Schneider's medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and penicillin/streptomycin (Cellgro) in T25 and T75 flasks (BD Biosciences).

Antibody staining and microscopy

Cells were grown on glass coverslips, with or without Binucleine 2, fixed and permeabilized in 100 mM Pipes/KOH (pH 6.8), 10 mM EGTA, 1 mM MgCl₂, 3.7% formaldehyde, and 0.2% TritonX-100 for 15 min and washed in PBS. DNA was stained with 5 µg/ml Hoechst 33342 in TBST (TBS with 1% TritonX-100) for 15 min. Cells were then washed twice with AbDil (TBST with 2% BSA) and incubated with DM1α primary monoclonal mouse anti-tubulin antibody (Sigma), 1:500 dilution, 1 hour, room temperature, washed three times with TBST and incubated with anti-mouse IgG Alexa Fluor 568 conjugated secondary antibody, 1:500 dilution, 1 hour at room temperature. Finally, coverslips were washed three times with AbDil and mounted on glass using Prolong Gold antifade reagent (Invitrogen). Cells were imaged using a Nikon TE2000U Inverted Microscope and PerkinElmer Ultraview Spinning Disk Confocal (100x DIC objective) at the Nikon Imaging Center at Harvard Medical School.

Live imaging

Two *Drosophila* S2 cell lines expressing GFP-Aurora B and mCherry-Tubulin/GFPAnillin were received as a gift from Gilles Hickson (Université de Montréal). Since expression of GFP constructs is controlled by metallothionein promoter, 0.25 mM CuSO₄ was added to cells 24 hours before imaging. One hour before imaging 50-70% confluent cells were transferred to glass coverslips. Cells were imaged using a Nikon TE2000U Inverted Microscope and PerkinElmer Ultraview Spinning Disk Confocal (100x DIC objective) at the Nikon Imaging Center at Harvard Medical School. Images were acquired once in 4 minutes, with 3-5 planes of z-stack with 1 µm step. In drug-treated cells, 40 µM (final concentration) Binucleine 2 was added after the first frame.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the staff at the Nikon Imaging Center at Harvard Medical School for their assistance and Nathanael Gray, Ryoma Ohi and Piotr Sliz for helpful discussions. We also thank Ryoma Ohi for the gift of *Xenopus* Aurora B kinase and we are grateful to Gilles Hickson for the gift of GFP-Anillin and GFP-Aurora cell lines. A.V.T. and M.J.E. were supported by NIH grant R01 CA080942. Y.S. and U.E. were supported by NIH grant R01 GM082834 and the Dana-Farber Cancer Institute.

References

1. Eggert US, Mitchison TJ, Field CM. ANIMAL CYTOKINESIS: From Parts List to Mechanisms. *Annu Rev Biochem.* 2006; 75:543–566. [PubMed: 16756502]
2. Carmena M, Ruchaud S, Earnshaw WC. Making the Auroras glow: regulation of Aurora A and B kinase function by interacting proteins. *Curr Opin Cell Biol.* 2009; 21:796–805. [PubMed: 19836940]
3. Carmena M, Earnshaw WC. The cellular geography of aurora kinases. *Nat Rev Mol Cell Biol.* 2003; 4:842–854. [PubMed: 14625535]
4. Marumoto T, Zhang D, Saya H. Aurora-A - a guardian of poles. *Nat Rev Cancer.* 2005; 5:42–50. [PubMed: 15630414]
5. Andrews PD. Aurora kinases: shining lights on the therapeutic horizon? *Oncogene.* 2005; 24:5005–5015. [PubMed: 16049526]
6. Harrington EA, Bebbington D, Moore J, Rasmussen RK, Ajose-Adeogun AO, Nakayama T, Graham JA, Demur C, Hercend T, Diu-Hercend A, Su M, Golec JM, Miller KM. VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo. *Nat Med.* 2004; 10:262–267. [PubMed: 14981513]
7. Manfredi MG, Ecsedy JA, Meetze KA, Balani SK, Burenkova O, Chen W, Galvin KM, Hoar KM, Huck JJ, LeRoy PJ, Ray ET, Sells TB, Stringer B, Stroud SG, Vos TJ, Weatherhead GS, Wysong DR, Zhang M, Bolen JB, Claiborne CF. Antitumor activity of MLN8054, an orally active small-molecule inhibitor of Aurora A kinase. *Proc Natl Acad Sci U S A.* 2007; 104:4106–4111. [PubMed: 17360485]
8. Wilkinson RW, Odedra R, Heaton SP, Wedge SR, Keen NJ, Crafter C, Foster JR, Brady MC, Bigley A, Brown E, Byth KF, Barrass NC, Mundt KE, Foote KM, Heron NM, Jung FH, Mortlock AA, Boyle FT, Green S. AZD1152, a selective inhibitor of Aurora B kinase, inhibits human tumor xenograft growth by inducing apoptosis. *Clin Cancer Res.* 2007; 13:3682–3688. [PubMed: 17575233]
9. Pollard JR, Mortimore M. Discovery and development of aurora kinase inhibitors as anticancer agents. *J Med Chem.* 2009; 52:2629–2651. [PubMed: 19320489]
10. Zhang J, Yang PL, Gray NS. Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer.* 2009; 9:28–39. [PubMed: 19104514]
11. Eggert US, Kiger AA, Richter C, Perlman ZE, Perrimon N, Mitchison TJ, Field CM. Parallel chemical genetic and genome-wide RNAi screens identify cytokinesis inhibitors and targets. *PLoS Biol.* 2004; 2:e379. [PubMed: 15547975]
12. Sessa F, Mapelli M, Ciferri C, Tarricone C, Arces LB, Schneider TR, Stukenberg PT, Musacchio A. Mechanism of Aurora B activation by INCENP and inhibition by hesperadin. *Mol Cell.* 2005; 18:379–391. [PubMed: 15866179]
13. Ohi R, Sapra T, Howard J, Mitchison TJ. Differentiation of cytoplasmic and meiotic spindle assembly MCAK functions by Aurora B dependent phosphorylation. *Mol Biol Cell.* 2004; 15:2895–2906. [PubMed: 15064354]
14. Ghose AK, Herbertz T, Pippin DA, Salvino JM, Mallamo JP. Knowledge based prediction of ligand binding modes and rational inhibitor design for kinase drug discovery. *J Med Chem.* 2008; 51:5149–5171. [PubMed: 18710211]
15. Girdler F, Sessa F, Patercoli S, Villa F, Musacchio A, Taylor S. Molecular basis of drug resistance in aurora kinases. *Chem Biol.* 2008; 15:552–562. [PubMed: 18559266]
16. Branford S, Rudzki Z, Walsh S, Grigg A, Arthur C, Taylor K, Herrmann R, Lynch KP, Hughes TP. High frequency of point mutations clustered within the adenosine triphosphate-binding region of

- BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. *Blood*. 2002; 99:3472–3475. [PubMed: 11964322]
17. Taylor S, Peters JM. Polo and Aurora kinases: lessons derived from chemical biology. *Curr Opin Cell Biol*. 2008; 20:77–84. [PubMed: 18249108]
18. Guse A, Mishima M, Glotzer M. Phosphorylation of ZEN-4/MKLP1 by aurora B regulates completion of cytokinesis. *Curr Biol*. 2005; 15:778–786. [PubMed: 15854913]
19. Steigemann P, Wurzenberger C, Schmitz MH, Held M, Guizetti J, Maar S, Gerlich DW. Aurora B-mediated abscission checkpoint protects against tetraploidization. *Cell*. 2009; 136:473–484. [PubMed: 19203582]
20. Sunkel CE, Glover DM. polo, a mitotic mutant of *Drosophila* displaying abnormal spindle poles. *J Cell Sci*. 1988; 89(Pt 1):25–38. [PubMed: 3417791]
21. Glover DM, Leibowitz MH, McLean DA, Parry H. Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles. *Cell*. 1995; 81:95–105. [PubMed: 7720077]
22. Fuller BG, Lampson MA, Foley EA, Rosasco-Nitcher S, Le KV, Tobelmann P, Brautigan DL, Stukenberg PT, Kapoor TM. Midzone activation of aurora B in anaphase produces an intracellular phosphorylation gradient. *Nature*. 2008; 453:1132–1136. [PubMed: 18463638]
23. Xu Z, Ogawa H, Vagnarelli P, Bergmann JH, Hudson DF, Ruchaud S, Fukagawa T, Earnshaw WC, Samejima K. INCENP-aurora B interactions modulate kinase activity and chromosome passenger complex localization. *J Cell Biol*. 2009; 187:637–653. [PubMed: 19951914]

Figure 1. Initial biochemical characterization of Binucleine 2, a novel isoform-selective Aurora kinase inhibitor

(a) Chemical structure of Binucleine 2, with numbering of the positions in the phenyl ring. (b) IC₅₀ values of Binucleine 2 analogs (see Supporting Methods for details of their synthesis). Data points are color-coded according to the substituent. Compounds with the same substitution pattern are located on the same line. In vitro values correlate well with results of cellular assays: Binucleine 2, the 3-halogen derivatives and the 3,4-di-Cl compound all have ED₅₀ values in the range of 5–10 μM, while the unsubstituted analog, 4-halogenated, 2,4-di-Cl, and 3,5-di-Cl compounds are inactive up to 100 μM. (c) Dose-response curves for Binucleine 2 at [ATP] = 100 μM. The black curve shows the enzymatic activity of full-length wild-type *Drosophila* Aurora B, co-expressed with a residue 654–755 truncation of INCENP. The red curve shows the same binary complex, with two mutated residues: Ile132Tyr, Ser134Pro, as in the human homolog. Data points were fitted with sigmoidal dose-response curves. Error bars represent standard errors. (d) Enzymatic activity of *Drosophila* Aurora A in presence of either Binucleine 2 (black curve) or Staurosporine, a non-selective kinase inhibitor, (blue curve) at [ATP] = 100 μM. Error bars represent standard errors.

Figure 3. Aurora B is the main target of Binucleine 2 in cells

Rescue experiments with a Binucleine 2-resistant Aurora B mutant. Ectopic wild-type or mutant Aurora-GFP (red), tubulin (green) and DNA (blue) have been visualized in fixed *Drosophila* Kc₁₆₇ cells. Two examples of representative cells are shown for each condition.

Figure 4. Aurora B kinase activity is not required for contractile ring ingression

(a) Quantification of live imaging experiments with WT Aurora B-GFP cell lines, time in minutes is plotted on the horizontal axis. In small molecule-treated cells, Binucleine 2 was added at the second minute of imaging, with a final concentration of 40 μ M. Groups of normally dividing cells are color-coded in green, abnormally dividing cells in red. (b) Representative still images from live imaging experiments. See Supporting Fig. 6 for quantitation of the rates of ring ingression.