Published OnlineFirst May 18, 2017; DOI: 10.1158/1535-7163.MCT-17-0159

Cancer Biology and Signal Transduction

Molecular Cancer Therapeutics

Inhibition of Aurora A and Aurora B Is Required for the Sensitivity of HPV-Driven Cervical Cancers to Aurora Kinase Inhibitors

David Martin[1,2], Sora Fallaha[3], Martina Proctor[1], Alexander Stevenson[1], Lewis Perrin[4], Nigel McMillan[3], and Brian Gabrielli[1]

Abstract

The activity and efficacy of Aurora inhibitors have been reported in a wide range of cancer types. The most prominent Aurora inhibitor is alisertib, an investigational Aurora inhibitor that has been the subject of more than 30 clinical trials. Alisertib has inhibitory activity against both Aurora A and B, although it is considered to be primarily an Aurora A inhibitor *in vivo*. Here, we show that alisertib inhibits both Aurora A and B *in vivo* in preclinical models of HPV-driven cervical cancer, and that it is the inhibition of Aurora A and B that provides the selectivity and efficacy of this drug *in vivo* in this disease setting. We also present formal evidence that alisertib requires progression through mitosis for its efficacy, and that it is unlikely to combine with drugs that promote a G_2 DNA damage checkpoint response. This work demonstrates that inhibition of Aurora A and B is required for effective control of HPV-driven cancers by Aurora kinase inhibitors. *Mol Cancer Ther; 16(9); 1934–41. ©2017 AACR.*

Introduction

Aurora kinases are critical regulators of entry into and exit from mitosis. Aurora A is essential for centrosome maturation and separation. Aurora B regulates mitotic exit by signaling correct microtubule attachments to the kinetochore and is a component of the chromosomal passenger complex which controls correct partitioning of the replicated genome at anaphase and cytokinesis (1). Aurora kinase inhibitors have been investigated in a wide range of cancers, although as yet there is no clinically approved use for this class of drugs (2). Many of the drugs developed have activity toward all of the Aurora kinases, although Aurora A and B appear to be the primary kinases overexpressed and involved in cancer development (3). These inhibitors disrupt mitosis, and the mitotic catastrophe resulting from Aurora inhibitor treatment is thought to be a primary target for this class of drugs (4). Recently, it has been reported in melanoma that a primary outcome of Aurora kinase inhibition was senescence, although this appeared to be cell line dependent (5–8).

In previous work we have reported that Aurora A and B were identified as synthetic lethal hits with HPV E7 expression in cervical cancer, and that the investigational Aurora A inhibitor alisertib displayed selectivity for the HPV-dependent cervical cancers *in vitro* and in xenograft models (9). Alisertib has been reported to be primarily an Aurora A inhibitor, although it inhibits Aurora B at higher doses *in vitro* (2, 10, 11). It was unknown as to which of these activities was responsible for its cytotoxicity. Recently, there have been conflicting reports as to whether alisertib treatment had any effect on Aurora B activity *in vivo* (12, 13). Here, we have investigated the *in vivo* targets of alisertib, and whether the effect of Aurora A and/or B inhibition alone provides the cytotoxicity we have observed in HPV-driven cervical cancers with Aurora kinase inhibitors.

Material and Methods

Cell culture and siRNA transfection

HPV-positive cervical cancer cell lines HeLa, CaSki, HPV-negative C33A (cervix), and SCC25 (tongue SCC) were cultured as described previously (9). HPV16 E6/E7 transformed Ect1/E6E7 and VK2/E6E7 cell lines are of ectocervical and vaginal tissue origins, respectively, and CaSki cells were obtained from the ATCC. Ect1/E6E7 and VK2/E6E7 cell lines were maintained in Defined Keratinocyte Serum Free Media (Defined K-SFM; Gibco, Life Technologies) supplemented with 0.1ng/mL human recombinant epidermal growth factor and 0.4 mmol/L calcium chloride at 37°C and 5% CO_2 at 37°C and 5% CO_2. All cell lines were tested and free of *mycoplasma*, and HeLa, CaSki and C33A were authenticated with STR fingerprinting at the time of use. Inhibitors alisertib, AMG900, MK5108, and AZD2811were purchased from Selleckchem and ZM447439 from Enzo Life Sciences. Etoposide, Nocodazole, and MG132 were from Sigma.

Aurora A and Aurora B siRNA were depleted using SmartPools of Dharmacon ON-TARGET plus siRNAs (Thermo Scientific). SiRNAs were transfected into HeLa cells by reverse transfection as described previously (14). Dose response assays were performed in 384-well plate format using resazurin cell viability assay as a readout (9).

[1]Mater Research Institute, The University of Queensland, Brisbane, Queensland, Australia. [2]The University of Queensland Diamantina Institute, Translational Research Institute, Brisbane, Queensland, Australia. [3]Menzies Health Institute Queensland and School of Medical Science, Griffith University, Southport, Australia. [4]Mater Health Services, South Brisbane, Queensland, Australia.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

D. Martin and S. Fallaha contributed equally to this article.

Corresponding Author: Brian Gabrielli, Mater Research Institute, The University of Queensland, Translational Research Institute, Brisbane, QLD 4102, Australia. E-mail: brianG@uq.edu.au

doi: 10.1158/1535-7163.MCT-17-0159

Published OnlineFirst May 18, 2017; DOI: 10.1158/1535-7163.MCT-17-0159

Antibodies

Ki-67, Clone MIB-1 (Dako), Aurora B (BD) and α-tubulin (Abcam), and pHistone H3 Ser10, cleaved PARP1, pMEK Thr286, Aurora A, pAurora A Thr288 were purchased from Cell Signaling Technologies.

Immunofluorescence and immunoblotting

Cells were seeded on poly-L-lysine coated glass coverslips, then after treatments fixed with −20°C methanol. Cells were immunostained as described previously (9). For immunoblotting, cells were harvested, lysed, and immunoblotted as described previously (9). IC_{50} values for Aurora A and B kinase activity, and apoptosis (cPARP) were calculated using Prism Graphpad to fit the data using least-squares analysis.

Time lapse microscopy

Cells were seeded onto a clear 24-well tissue culture plate at least 24 hours before commencement of the experiment. Immediately after the addition of inhibitors, the plate was imaged every 30 minutes for 72 hours using the Olympus CellR Live Cell Imager with an incubator chamber maintained at 37°C and 5% CO_2. Images were made in time lapse stacks using ImageJ software analyzed manually for a minimum of 70 cells per treatment group as described previously (9).

Xenograft experiments

Female nude mice (6 weeks old) were purchased from ARC. All animal experimentations were performed according to the guidelines of the Australian and New Zealand Council for the Care and Use of Animals in Research and were approved by the Griffith University Animal Ethics number MSC/09/14/AEC and the University of Queensland Animal Ethics number UQDI/492/15/CCQ. Mice were injected with CaSki or HeLa cells, and treated with Alisertib (Selleckchem) or vehicle control. Tumors were established by subcutaneous injection, and when they reached approximately 100 mm^3, were treated as described previously (9). Tumors size was measured three times/week using calipers. Mice were sacrificed at 7 to 8 weeks post terminating the treatment or when tumor size measured >1 cm^3.

Immunohistochemical and immunofluorescence staining

Tumors were harvested and fixed in 4% paraformaldehyde, paraffin embedded, and immunohistochemical stains performed as described previously (15). For immunofluorescence, fluorophore conjugated secondary antibodies and 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) were used and imaged on a Zeiss Z1 fluorescence microscope.

Results

We have previously reported the identification of Aurora A and Aurora B as synthetic lethal targeting with oncogenic HPV-E7 expression and validated this *in vitro* and *in vivo* using the Aurora A inhibitor MLN8237/alisertib (9). We were interested to determine whether it was the Aurora A and/or Aurora B inhibition that was important for the cytotoxicity of this drug in the setting of HPV-driven cervical cancer.

One of the features of alisertib was the modest delay in mitosis and failed cytokinesis found in all cell types, although the delay was increased 3- to 5-fold in the HPV-driven tumor lines (9). Time lapse analysis of HeLa cells treated with either siRNAs to effectively deplete Aurora A, Aurora B, both Auroras (Fig. 1A), or with small molecule inhibitors alisertib or the potent pan-Aurora inhibitor AMG900 at 1 μmol/L (readily achievable serum levels *in vivo*; refs. 16 and 17), revealed that Aurora A depletion produced an extended delay in mitosis of up to 10 hours, while Aurora B depletion delayed cells in mitosis on average 3-fold over controls, and combined depletion produced a similar delay (Fig. 1B; Supplementary Fig. S1A and B). This delay mirrored the effect of the two Aurora inhibitors. Despite the long delay in mitosis, Aurora A depletion produced only a small increase in failed mitosis but 20% apoptosis. Aurora B and Aurora A+B depletion produced >50% failed mitosis with cells exiting without undergoing cytokinesis, but limited apoptosis. Both Aurora inhibitors had similar effects, with all cells failing cytokinesis (Fig. 1C). This was supported by immunofluorescence staining of cells treated in the same manner as the time lapse experiments (Fig. 1D; Supplementary Fig. S2). The similarity of the effects of alisertib on the delay in mitosis and outcomes, with the pan-Aurora inhibitor and the combined Aurora A+B depletion suggested that alisertib was behaving as a pan-Aurora inhibitor when used at 1 μmol/L concentration.

To assess the ability of alisertib to inhibit Aurora A and Aurora B kinase activities in cells, and how Aurora inhibition correlates with cytotoxicity, asynchronously growing and mitotically arrested HeLa cells were treated with increasing doses of alisertib and AMG900. Their ability to inhibit the auto-phosphorylation of Aurora A Thr288 (pAURKA) was a marker of Aurora A inhibition, and phosphorylation of histone H3 Ser10 (pH3) as a marker of Aurora B inhibition was assessed by immunoblotting. Mitotically arrested cells were maintained in mitosis by inhibition of the proteasome with MG132 to block mitotic exit, and treated with the inhibitors for 4 hours. Asynchronously growing cells were treated for 24 hours with the drugs then analyzed. Alisertib had an IC_{50} of 29 nmol/L for Aurora A inhibition, but produced a bell-shaped dose response for Aurora B inhibition detected by pH3 (Fig. 2A; Supplementary Table S1). The increased pH3 levels are likely to be a consequence of Aurora A inhibition delaying cells in mitosis, as increased pH3 and pMEK T286, and CDK1/Cyclin B mitotic substrate and marker of mitosis accumulated in similarly treated asynchronously growing cells (Supplementary Fig. S3A). This matches the mitotic arrest observed with Aurora A depletion (Fig. 1). The reduction in pH3 at higher doses indicates that Aurora B is inhibited at higher doses of alisertib, producing an IC_{50} of 350 nmol/L. Interestingly, the IC_{50} for PARP cleavage as a marker of apoptosis is 110 nmol/L, corresponding to the concentration where the pH3 bell curve peaks (Fig. 2A). This peak corresponds to the concentration at which Aurora B inhibition is impacting on the accumulation of pH3 associated with the Aurora A inhibition-induced mitotic arrest and suggests that inhibition of Aurora B is important for the apoptosis induced by Alisertib. AMG900 is an equipotent Aurora A and B inhibitor, with an IC_{50} of 8–14 nmol/L for both Aurora A and B inhibition, and for the PARP cleavage (Fig. 2B; Supplementary Table S1). Treatment of asynchronously growing cultures with alisertib and AMG900 both resulted in a 4-5 fold increase in the percentage of mitotic cells at concentrations inhibiting both Aurora A and B (Supplementary Fig. S3), supporting the time lapse data (Fig. 1B). A similar dose response experiment with the Aurora B inhibitor ZM447439 demonstrated it to be more selective for Aurora B (IC_{50}s 165 and 670 nmol/L for Aurora B and A respectively), similar to the selectivity previously reported (18), but even at

Published OnlineFirst May 18, 2017; DOI: 10.1158/1535-7163.MCT-17-0159

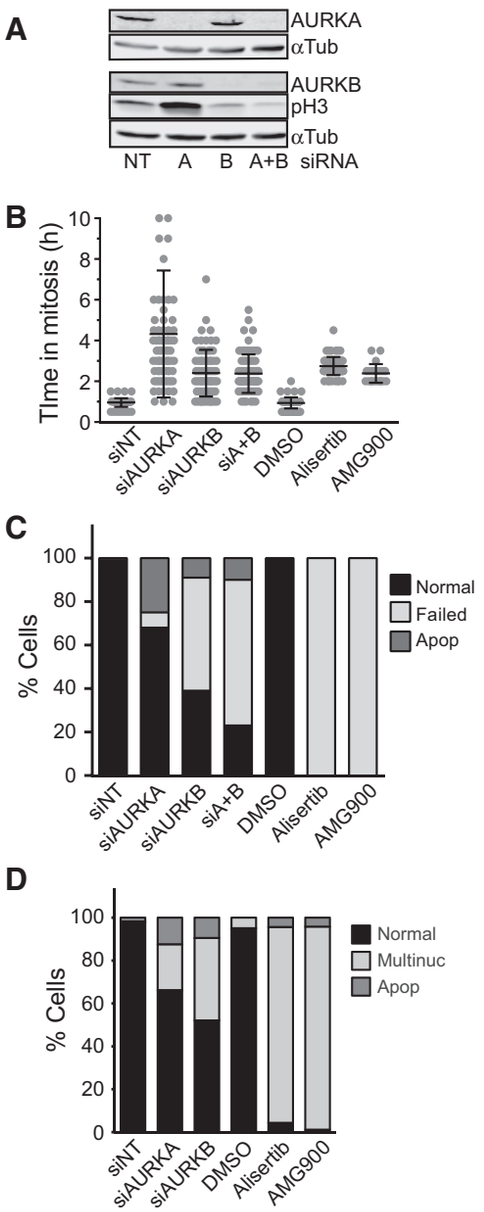

Figure 1.

Alisertib behaves as an Aurora A/B inhibitor. **A,** Immunoblot of Aurora A and B (AURKA/B) in HeLa cell transfected with either nontargeting siRNA (NT), Aurora A (A), Aurora B (B) siRNA, or both together (A + B) for 24 hours. Lysates were immunoblotted for Aurora A, B, and pH3 Ser10, a marker of Aurora B activity. α-Tubulin was the loading control. **B,** HeLa cell transfected as in A, or treated with 1 μmol/L alisertib or AMG900 were followed by time lapse microscopy for 72 hours. The time in the first mitosis was assessed for 100 cells in each case. Image acquisition was commenced 24 hours after siRNA transfection and 1 hour after drug addition. These data are representative of at least two experiments. **C,** The outcome of the first mitosis from the experiment shown in B. Normal represents the production of two daughter cells, failed represents failed cytokinesis with only a single daughter cell, and apoptosis (Apop). This is representative of at least two experiments. **D,** HeLa cells treated as in B were fixed 24 hours after drug treatment, 48 hours after siRNA transfection. The fixed cells were stained for DNA and microtubules, and the proportion of normal, multinuclear, and apoptotic cells was assessed in >200 cells for each condition.

3 μmol/L did not cause significant levels of PARP cleavage (Supplementary Table S1; Supplementary Fig. S4).

HPV-driven cervical cancers are highly sensitive to dual inhibitors of Aurora A and B

The requirement for both Aurora A and B inhibition to promote cytotoxicity of HPV-driven cervical cancer cell lines was demonstrated by the sensitivity of HeLa and CaSki cells to alisertib and AMG900, whereas C33A and SCC25 lines were relatively insensitive to these drugs. When these cell lines showed sensitivity, the drugs promoted only a cytostatic effect indicated by the 40% viable populations with 10 μmol/L drugs (Supplementary Table S2; Supplementary Fig. S5). Two other HPV-immortalized lines VK2 and Ect1 were likewise very sensitive to both alisertib and AMG900. A selective Aurora A inhibitor MK5108 was more potent in the HPV-driven lines, although high doses of the drug (10 μmol/L) were cytotoxic to both HPV-dependent and -independent lines tested. Interestingly, the selective Aurora B AZD2811 appeared to show selectivity for the HPV-driven lines and the effect was to promote cytotoxicity, with all the HPV-driven cell lines having <10% viability at 10 μmol/L drug, with HeLa as the surprising exception, although this was still less than the non-HPV lines (Supplementary Table S2; Supplementary Fig. S5A). The relative lack of effect of the Aurora A/B inhibitors on the non-HPV lines was not due to any lack of sensitivity to alisertib and AMG900, as even low micromolar concentrations of these drugs drove a similar level of mitotic failure in the C33A as in HeLa cells (Supplementary Fig. S5B).

HPV-driven cervical cancers are sensitive to combined inhibition of Aurora A and B

These data indicate that the dual inhibition of Aurora A and B was critical in the cytotoxicity of alisertib in HPV-driven cervical cancers, although alisertib at low submicromolar concentrations is primarily an Aurora A inhibitor. To assess the effects of alisertib

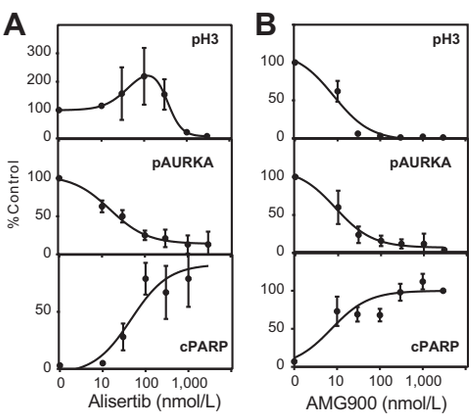

Figure 2.

Alisertib inhibits Aurora A and B at submicromolar concentrations. **A** and **B,** HeLa cells were blocked in mitosis with nocodazole and then MG132 to prevent exit, then treated with alisertib or AMG900 in a dose response for 4 hours. Lysates were prepared from each drug treatment and immunoblotted for pH3 as marker of Aurora B activity, and pAurora A Thr288 (pAURKA) as a marker of Aurora A activity. Cleaved PARP (cPARP) was assessed from asynchronously growing HeLa cells treated with the same drug concentration for 72 hours. The band densities were measured from three experiments and are presented and mean and SD of the percentage of the untreated control.

Published OnlineFirst May 18, 2017; DOI: 10.1158/1535-7163.MCT-17-0159

and other Aurora A and B inhibitors on HPV-dependent and -independent cervical cancer, cells were treated with inhibitors and followed by time lapse microscopy as described previously (9). Alisertib (0.3 μmol/L; approximately IC_{50} for Aurora B and IC_{90} for Aurora A) produced a long delay in the first mitosis after treatment, similar to treatment with the selective Aurora A inhibitor MK5018, whereas 1 μmol/L alisertib produced a shorter delay, similar to the delay observed using 1 μmol/L AMG900 (Fig. 3A; Supplementary Fig. S1, S6). Alisertib-treated cells (0.3 μmol/L) entered mitosis and attempted cytokinesis forming a cleavage furrow, but the daughter cells failed to separate, and the nuclei were retained in one cytoplasm. This dose of alisertib behaved similarly to the Aurora A inhibitor and Aurora A siRNA treatment (Fig. 3B). By comparison, cells treated with either the Aurora B inhibitor, 1 μmol/L alisertib, 0.3 or 1 μmol/L AMG900 entered and exited mitosis with no evidence of cytokinesis. Note that 1 μmol/L alisertib, AMG900, the Aurora B inhibitor and siRNA, and the combined Aurora A and B inhibitors and siRNAs all resulted in a similar length of mitotic delay and failed mitosis without evidence of cytokinesis (Fig. 3; Supplementary Fig. S6). Although the delay in mitosis was less in CaSki cells, the same trend was observed for these drugs in this HPV-driven tumor, whereas there was no difference in the effect of low and high dose alisertib, the mitotic delay being less in either HeLa or CaSki (Fig. 3A). The outcome of treatment with all siRNAs, drugs, and combinations was apoptosis (Fig. 3B). Thus, at higher doses, alisertib behaves as an Aurora A and B inhibitor in the HPV-driven tumor lines.

Alisertib requires progression through mitosis for its cytotoxic effect

Although alisertib is an Aurora inhibitor, an unanswered question is whether alisertib-induced killing is dependent on transit through mitosis. To address this, HeLa cells were treated with the TopoII inhibitor etoposide to induce a G_2 phase cell-cycle checkpoint arrest, and then treated with alisertib and

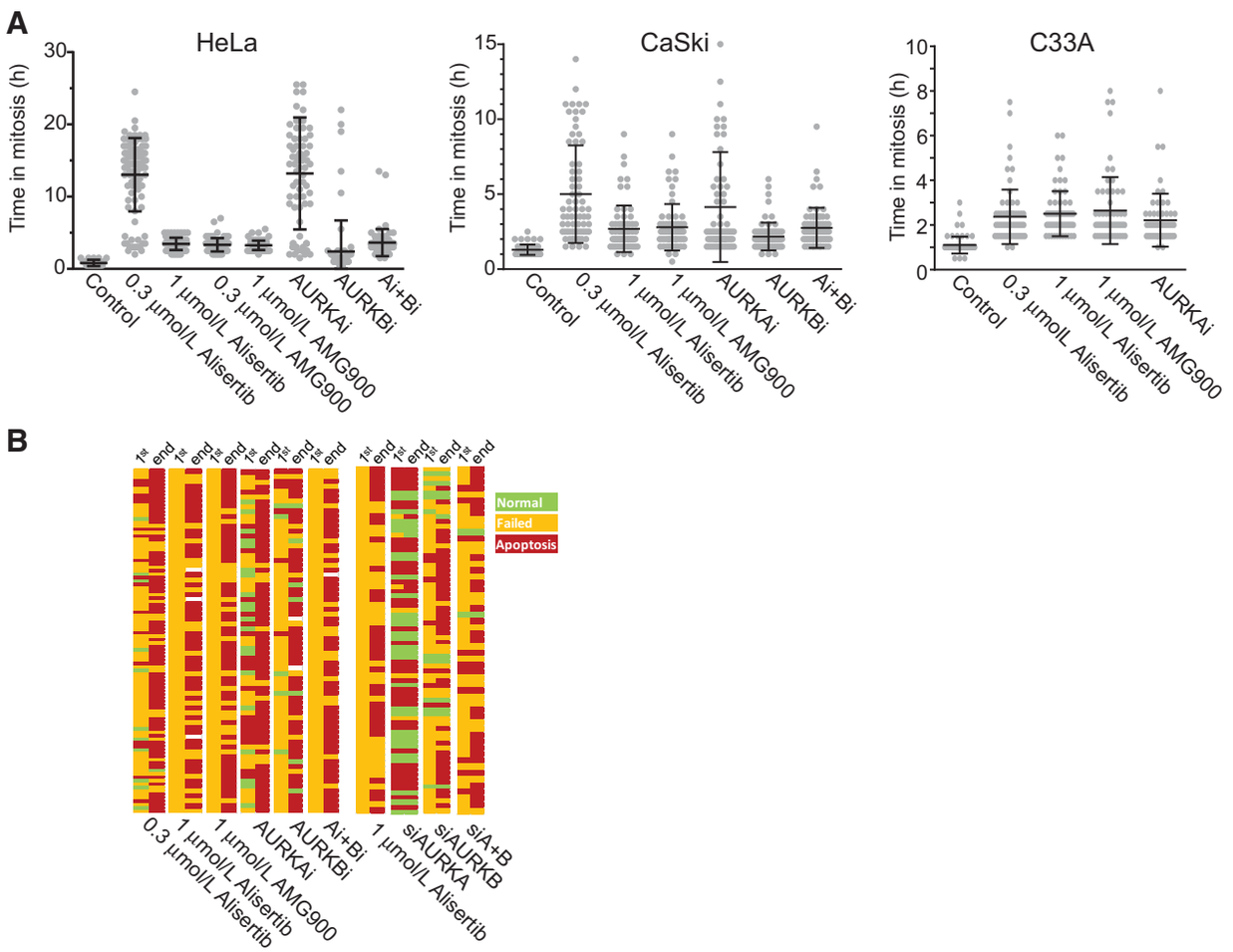

Figure 3.

Differential inhibition of Aurora A and B by alisertib in HPV-driven cervical cancers. **A,** HPV-driven cervical cancer line HeLa and CaSki, and HPV-negative C33A cells were treated with the indicated concentrations of Aurora inhibitors, and then cells were followed by time lapse microscopy. The cells were assessed for the duration of the first mitosis after drug treatment. **B,** The outcomes of mitosis of individual HeLa cells as observed from the time lapse experiment shown above and siRNA time lapse experiments similar to those shown in Fig. 1. The outcome of the first mitosis (1st), either normal mitosis (normal), failed cytokinesis (failed) or apoptosis, and the outcomes at the movie end (final) are shown for >80 cells per treatment.

Published OnlineFirst May 18, 2017; DOI: 10.1158/1535-7163.MCT-17-0159

followed for 2 days. Etoposide treatment produced an initial G_2 phase arrest as expected; then cells exited the checkpoint arrest into mitosis where they arrest again. A consequence of the mitotic checkpoint arrest is cells that have failed to separate their sister chromatids also sustained high levels of DNA damage. This is evidenced by the high level of pMEK1 Thr286, a marker of Cyclin B/CDK1 activity and mitosis (19), and γH2AX as a marker of DNA damage (Fig. 4). The treatment also promotes a high level of apoptosis indicated by the strongly elevated levels of activated, cleaved caspase-3, and the product of its activity, cleaved PARP. Alisertib treatment also promotes a mitotic delay and apoptosis, although to a lesser extent than etoposide. Cotreatment completely blocked entry into mitosis, a consequence of inhibition of PLK1 activation required for exit from the etoposide checkpoint arrest (20, 21). It also effectively reduced the levels of DNA damage and apoptosis initiated by either treatment (Fig. 4). This demonstrates that progression through mitosis is necessary for alisertib-induced cell death.

Alisertib is an Aurora A and B inhibitor *in vivo*

HeLa and CaSki xenografts were treated *in vivo* for 10 days with alisertib and were excised 6 hours after the final treatment and immunostained for the presence of pH3 Ser10 to assess Aurora B inhibition *in vivo*. In both cases, there was no effect on the level of pH3 Ser10 staining (Supplementary Fig. S7; only HeLa data are shown). However, HeLa cells treated *in vitro* for 24 hours with 1 μmol/L alisertib retained pH3-stained mitotic cells, albeit at low levels and these were exclusively later stages of a mitotic arrest, demonstrated by the hypercondensed DNA (Supplementary Fig. S8). Few early prophase mitotic cells were stained for pH3 in any of the alisertib-treated populations, although these were readily observed in both the control and cells treated with 1 μmol/L MK5018. This demonstrated that the presence of pH3 may not be an accurate indicator of Aurora B inhibition. Aurora B inhibition does block pH3, but it is the inhibition of the chromosomal passenger complex function that has the major effect on mitotic progression and the mitotic failure (1). The effect of alisertib and the selective Aurora A and B inhibitors on mitotic progression, particularly mitotic exit

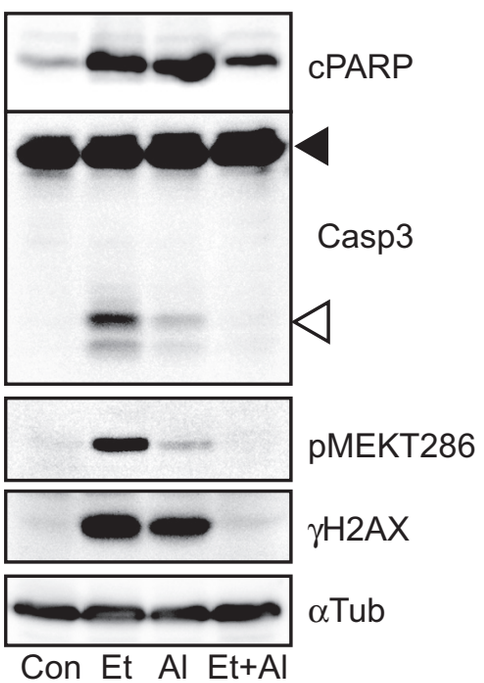

Figure 4.
Alisertib cytotoxicity is dependent on progression through mitosis. HeLa cells either untreated (Con) or treated with 1 μmol/L etoposide (Et), 1 μmol/L alisertib (Al), or etoposide for 24 hours then alisertib for a further 48 hours were immunoblotted for cleaved PARP (cPARP) and caspase 3 (the cleaved and activated caspase 3 is indicated by the arrowhead) as markers of apoptosis, pMEKT286 as a marker of mitosis, and γH2AX as a marker of DNA damage. Tubulin was used a loading control.

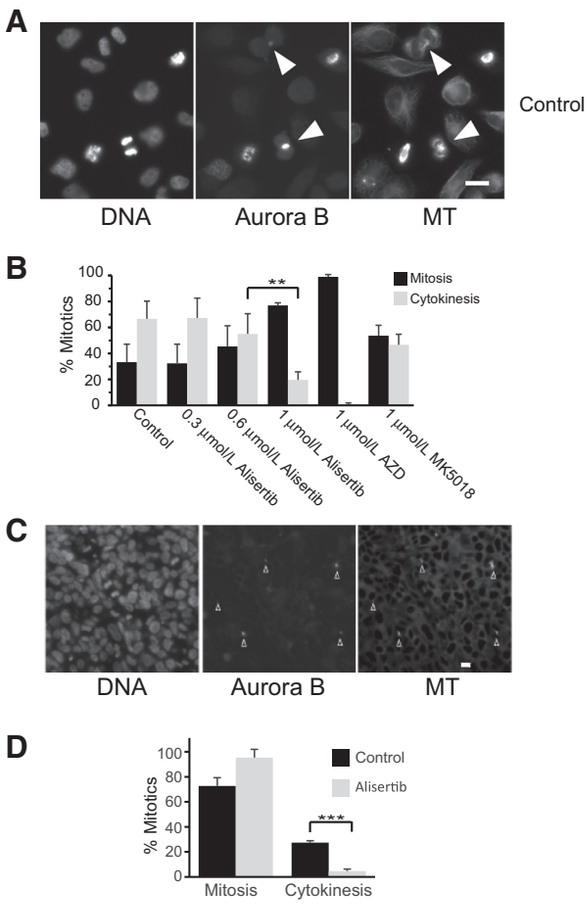

Figure 5.
Alisertib has Aurora A and B inhibitory activity at doses used *in vivo*. **A,** HeLa cells were stained for DNA, Aurora B, and α-tubulin to show microtubules (MT). Midbodies are indicated by the filled arrowheads. **B,** The proportion of cells in mitosis or undergoing cytokinesis indicated by the presence of an Aurora B stained microtubule midbody were quantified in HeLa cells treated for 24 hours with the indicated concentration of drugs. The data are from 100 cells in mitosis/cytokinesis for each condition and represent the mean and SD from three replicate experiments. **, $P < 0.01$. **C,** HeLa xenograft tumors harvested 24 hours after the final alisertib treatment were stained for DNA, Aurora B and α-tubulin to show the microtubules (MT). Midbodies positive for Aurora B and microtubule staining are indicated by the open arrowheads. **D,** Quantitation of proportion of cells in mitosis/cytokinesis as in B. The data are from >100 cells in mitosis/cytokinesis from three separate tumor for each condition.

Published OnlineFirst May 18, 2017; DOI: 10.1158/1535-7163.MCT-17-0159

and the establishment of a midbody, marking effective cytokinesis, was assessed. Cells were fixed and stained for microtubules and Aurora B to identify midbodies (Fig. 5A; Supplementary Fig. S9). These were readily detected in controls and in cells treated with low doses of alisertib (<1 μmol/L), but there was a nonsignificant decrease in the proportion of midbodies detected with 0.6 μmol/L alisertib which was highly significant at 1 μmol/L (Fig. 5B). There was a complete absence of midbodies with 1 μmol/L Aurora B inhibitor AZD2811, corresponding to lack of cytokinesis observed in the time lapse experiments (Fig. 3), whereas 1 μmol/L Aurora A inhibitor MK5018 resulted in aberrant mitosis but a high proportion of midbodies. This is in agreement with the observed cytokinesis in the time lapse data (Fig. 5B; Fig. 3).

HeLa xenografts harvested 6 hours after the final treatment with alisertib had readily detected mitotic figures in both control and drug treated samples (Fig. 5C; Supplementary Fig. S10). Midbodies were readily observed in control tumors, but few were found in the alisertib-treated tumors. There were also increased pleomorphic nuclei and multinuclear cells, similar to the cells that failed cytokinesis observed in both the time lapse and *in vitro* immunofluorescence studies (Fig. 5D). The lack of normal cytokinesis indicates that alisertib is acting as both an Aurora A and B inhibitor *in vivo*, as inhibition of Aurora A alone did not significantly reduce the occurrence of midbodies or cytokinesis (Figs. 3B and 5B).

Alisertib treatment can produce long-term disruption to mitosis *in vivo*, but not senescence in HPV cervical cancers

It was reported that alisertib treatment produced a senescent phenotype in melanoma xenografts (8). When HeLa and CaSki xenografts were treated with alisertib, then followed for up to 70 days post treatment, there was a significant inhibition of tumor growth overall, with complete inhibition of growth or even reduction in tumor size in the first 10 days after treatment (Fig. 6A). Both control and alisertib treated tumors stained strongly Ki67 positive, indicating continued proliferative potential (Fig. 6B), and mitotic figures were readily detected in both the control and alisertib-treated tumors. Senescence results in loss of Ki67 expression and mitosis (8, 22). There was a small increase in the proportion of abnormal mitotic figures found in HeLa xenografts even 70 days after treatment was completed (Fig. 6C). The lower proliferative rate of CaSki xenografts resulted in a comparatively lower number of mitotic figures, although these also showed a similar trend to increased abnormal mitotic figures.

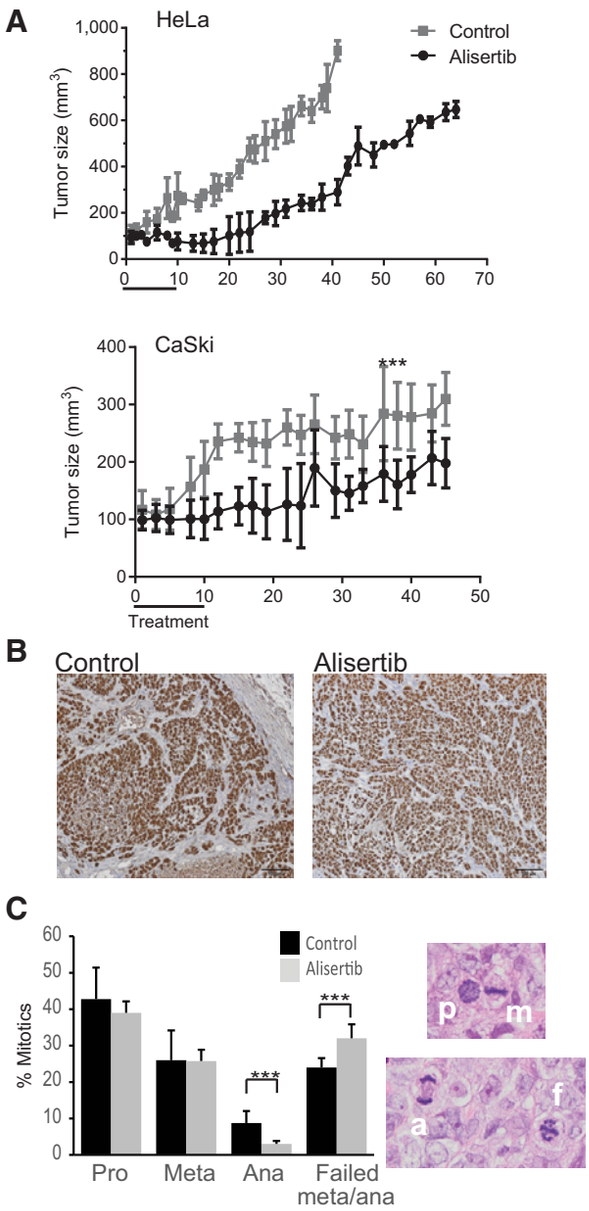

Figure 6.

A, Xenograft growth curves for HeLa and CaSki HPV-tumors either without or with 30 mg/kg alisertib treatment for the first 10 days after tumors reached >100 mm^3. The data are from 7 mice per treatment. **B,** Immunohistochemical staining for Ki67 of control or alisertib treated tumors harvested at the end of the experiment. **C,** Mitotic figures representing prophase (p), metaphase (m), anaphase (a), and failed mitosis (f) from H&E-stained tumor sections were quantified for the percentage of each. The data represent >200 mitotic figures for each tumor, from at least three individual tumors.

Discussion

Alisertib has been known to inhibit both Aurora A and B kinase activities *in vitro* with a distinct preference for Aurora A (2, 10–13). There was little evidence for Aurora B inhibition *in vivo* or it contributing to the cytotoxicity of the drug. Here, we have shown that in HPV-driven cervical cancer models, there is clear evidence for both Aurora A and B inhibition achieved using the standard 30 mg/kg dosing regime, although the effect on Aurora B activity is most readily detected by its effect on cytokinesis rather than pH3 levels. In tissue culture experiments, the effect on mitotic exit and particularly cytokinesis, detected by the loss of Aurora B stained midbodies, was observed at lower drug doses than those required to significantly influence pH3 levels. Additionally, there is a confounding effect of low doses of alisertib increasing pH3 levels by preferentially inhibiting Aurora A resulting in a mitotic arrest. The increased level of pH3 being a direct consequence of the increased proportion of cells in mitosis, also seen with the selective Aurora A inhibitor and siRNA depletion. The effect of high doses of alisertib was to ablate pH3 staining in cells in earlier stages of mitosis, but pH3 staining was detected in cells undergoing an extended mitotic arrest. *In vivo*, a similar absence of

Published OnlineFirst May 18, 2017; DOI: 10.1158/1535-7163.MCT-17-0159

cytokinesis was observed in alisertib-treated tumors, indicating that the drug at 30 mg/kg significantly inhibited both Aurora A and B kinases.

The effects of dual Aurora A and B inhibition in the setting of HPV-driven cancers were increased selectivity over either selective Aurora kinase inhibitor examined here, MK5018and AZD2811. The selective Aurora A inhibitor promoted the mitotic arrest, and coaddition of the Aurora B inhibitor reduced but did not completely abolish this arrest. This was also observed with equipotent Aurora A/B inhibitor AMG900, and combination of specific Aurora siRNAs, indicating that the failure to abolish the arrest was not a consequence of inadequate Aurora B inhibition. It is unclear as to why the prolonged mitotic arrest imposed by selective Aurora A inhibition did not produce a stronger cytotoxic effect. Aurora A kinase directly regulates the mitotic stability of the pro-apoptotic BH3-only BIM protein (23), and several other apoptotic components are regulated in mitosis with the result that the balance of pro- and antiapoptotic signals increasingly favors pro-apoptotic signaling the longer the mitotic arrest is maintained (24, 25). There was a surprising selectivity of the Aurora B inhibitor AZD2811 for the HPV-driven cancer cell line. This cannot be due to any mitotic delay, but must be associated with the failed cytokinesis that is a consequence of Aurora B inhibition (1). It is possible that the failed cytokinesis triggers a caspase-2-dependent mechanism to eliminate these aneuploid cells (26). The relative lack of cytotoxicity of the first-generation Aurora B inhibitor ZM447439 for HPV-driven cell lines (9) is surprising as it displays significant Aurora A inhibition at low micromolar concentrations. However, it has other known targets such as LCK, SRC, and MEK1 (27), and inhibition of these may reduce the selectivity of the drug compared with more highly selective Aurora inhibitors. Interestingly, both Aurora A and B were identified as individual synthetic lethal hits in the original siRNA screen (9), indicating that disrupting progression through and out of mitosis is individually lethal to HPV-driven cancers. Thus, the combined inhibition of both kinases appears to provide increased selectivity and potency over the individual inhibitors. This is well demonstrated by the potency and selectivity of AMG900.

The selectivity of Aurora inhibitors for HPV-driven cancers may be in part a consequence of the effect of the HPV E6/E7 oncogenes. We have previously demonstrated that overexpression of E7 is sufficient to sensitize toward alisertib (9), and defective p53 function, an outcome of E6 expression, promotes mitotic slippage and failed cytokinesis with Aurora inhibitors (10). Although the exact targets of E7 that promote selectivity are not known, the reported ability of alisertib to promote senescence in melanomas (8) suggests that it may be RB protein as HPV E7 expression effectively bypasses senescence (22). RB is rarely defective in melanomas (28), whereas all HPV-driven cervical cancers are RB defective by virtue of E7 expression. This would also explain the lack of senescence in the HPV-driven xenograft models.

In summary, we have demonstrated that inhibition of Aurora kinases provides cytotoxic insult in HPV-driven cervical cancer cells, and that the combined inhibition of Aurora A and B enhances the potency and selectivity of this effect. The relatively good clinical profile of Aurora inhibitors (4) suggests that this class of inhibitors, particularly dual Aurora A/B inhibitors, may have efficacy in the treatment of HPV-driven cervical cancer. It is unlikely that these inhibitors would be effective in combination with chemotherapies or radiation that produces DNA damage as Aurora A inhibition, through its downstream inhibition of PLK1 will reduce the efficacy of both agents by the ATM/ATR-dependent block of progression into mitosis (20, 21) reducing the efficacy of the Aurora A inhibitors. Aurora A inhibition maintains the cell-cycle arrest and would reduce the efficacy of chemoradiation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: D. Martin, N. McMillan, B. Gabrielli
Development of methodology: D. Martin, S. Fallaha, A. Stevenson, B. Gabrielli
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Martin, S. Fallaha, M. Proctor, A. Stevenson, L. Perrin, B. Gabrielli
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Martin, S. Fallaha, A. Stevenson, N. McMillan, B. Gabrielli
Writing, review, and/or revision of the manuscript: D. Martin, M. Proctor, A. Stevenson, L. Perrin, N. McMillan, B. Gabrielli
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Martin, M. Proctor, B. Gabrielli
Study supervision: A. Stevenson, N. McMillan, B. Gabrielli

Acknowledgments

The authors thank Dr Cameron Snell and Ms. Claire Davies for their assistance in the preparation of this work.

Grant Support

This work was supported by grants from the Cancer Council Queensland and the National Health and Medical Research Council of Australia. BG is supported by funding from Smiling for Smiddy.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 20, 2017; revised April 20, 2017; accepted May 9, 2017; published OnlineFirst May 18, 2017.

References

1. Carmena M, Wheelock M, Funabiki H, Earnshaw WC. The chromosomal passenger complex (CPC): from easy rider to the godfather of mitosis. Nat Rev Mol Cell Biol 2012;13:789–803.
2. de Groot CO, Hsia JE, Anzola JV, Motamedi A, Yoon M, Wong YL, et al. A cell biologist's field guide to aurora kinase inhibitors. Front Oncol 2015;5:285.
3. Vader G, Lens SM. The Aurora kinase family in cell division and cancer. Biochim Biophys Acta 2008;1786:60–72.
4. Bavetsias V, Linardopoulos S. Aurora kinase inhibitors: current status and outlook. Front Oncol 2015;5:278.
5. Min YH, Kim W, Kim JE. The Aurora kinase A inhibitor TC-A2317 disrupts mitotic progression and inhibits cancer cell proliferation. Oncotarget 2016;7:84718–35.
6. Sadaie M, Dillon C, Narita M, Young AR, Cairney CJ, Godwin LS, et al. Cell-based screen for altered nuclear phenotypes reveals senescence progression in polyploid cells after Aurora kinase B inhibition. Mol Biol Cell 2015;26:2971–85.
7. Wang LX, Wang JD, Chen JJ, Long B, Liu LL, Tu XX, et al. Aurora A kinase inhibitor AKI603 induces cellular senescence in chronic myeloid leukemia cells harboring T315I mutation. Sci Rep 2016;6:35533.

8. Liu Y, Hawkins OE, Su Y, Vilgelm AE, Sobolik T, Thu YM, et al. Targeting aurora kinases limits tumour growth through DNA damage-mediated senescence and blockade of NF-kappaB impairs this drug-induced senescence. EMBO Mol Med 2013;5:149–66.
9. Gabrielli B, Bokhari F, Ranall MV, Oo ZY, Stevenson AJ, Wang W, et al. Aurora A is critical for survival in HPV-transformed cervical cancer. Mol Cancer Ther 2015;14:2753–61.
10. Marxer M, Ma HT, Man WY, Poon RY. p53 deficiency enhances mitotic arrest and slippage induced by pharmacological inhibition of Aurora kinases. Oncogene 2014;33:3550–60.
11. Asteriti IA, Di Cesare E, De Mattia F, Hilsenstein V, Neumann B, Cundari E, et al. The Aurora-A inhibitor MLN8237 affects multiple mitotic processes and induces dose-dependent mitotic abnormalities and aneuploidy. Oncotarget 2014;5:6229–42.
12. Kurokawa C, Geekiyanage H, Allen C, Iankov I, Schroeder M, Carlson B, et al. Alisertib demonstrates significant antitumor activity in bevacizumab resistant, patient derived orthotopic models of glioblastoma. J Neuro-Oncol 2016;131:41–8.
13. Nair JS, Schwartz GK. MLN-8237: a dual inhibitor of aurora A and B in soft tissue sarcomas. Oncotarget 2016;7:12893–903.
14. Oakes V, Wang W, Harrington B, Lee WJ, Beamish H, Chia KM, et al. Cyclin A/Cdk2 regulates Cdh1 and claspin during late S/G2 phase of the cell cycle. Cell Cycle 2014;13:3302–11.
15. Fox C, Lambie D, Wilmott JS, Pinder A, Pavey S, Le Cao KA, et al. Multiparameter analysis of naevi and primary melanomas identifies a subset of naevi with elevated markers of transformation. Pigment Cell Melanoma Res 2016;29:444–52.
16. Cervantes A, Elez E, Roda D, Ecsedy J, Macarulla T, Venkatakrishnan K, et al. Phase I pharmacokinetic/pharmacodynamic study of MLN8237, an investigational, oral, selective aurora a kinase inhibitor, in patients with advanced solid tumors. Clin Cancer Res 2012;18:4764–74.
17. Juan G, Bush TL, Ma C, Manoukian R, Chung G, Hawkins JM, et al. AMG 900, a potent inhibitor of aurora kinases causes pharmacodynamic changes in p-Histone H3 immunoreactivity in human tumor xenografts and proliferating mouse tissues. J Translat Med 2014;12:307.
18. Yang H, Burke T, Dempsey J, Diaz B, Collins E, Toth J, et al. Mitotic requirement for aurora A kinase is bypassed in the absence of aurora B kinase. FEBS Lett 2005;579:3385–91.
19. De Boer L, Oakes V, Beamish H, Giles N, Stevens F, Somodevilla-Torres M, et al. Cyclin A/cdk2 coordinates centrosomal and nuclear mitotic events. Oncogene 2008;27:4261–8.
20. van Vugt MA, Bras A, Medema RH. Polo-like kinase-1 controls recovery from a G2 DNA damage-induced arrest in mammalian cells. Mol Cell 2004;15:799–811.
21. Spoerri L, Brooks K, Chia K, Grossman G, Ellis JJ, Dahmer-Heath M, et al. A novel ATM-dependent checkpoint defect distinct from loss of function mutation promotes genomic instability in melanoma. Pigment Cell Melanoma Res 2016;8:12466.
22. Lee WJ, Skalamera D, Dahmer-Heath M, Shakhbazov K, Ranall MV, Fox C, et al. Genome-wide overexpression screen identifies genes able to bypass p16-mediated senescence in melanoma. SLAS Discov 2016; 22:298–308.
23. Moustafa-Kamal M, Gamache I, Lu Y, Li S, Teodoro JG. BimEL is phosphorylated at mitosis by Aurora A and targeted for degradation by betaTrCP1. Cell Death Differ 2013;20:1393–403.
24. Colin DJ, Hain KO, Allan LA, Clarke PR. Cellular responses to a prolonged delay in mitosis are determined by a DNA damage response controlled by Bcl-2 family proteins. Open Biol 2015;5:140156.
25. Pedley R, Gilmore AP. Mitosis and mitochondrial priming for apoptosis. Biol Chem 2016;397:595–605.
26. Dawar S, Lim Y, Puccini J, White M, Thomas P, Bouchier-Hayes L, et al. Caspase-2-mediated cell death is required for deleting aneuploid cells. Oncogene 2017;36:2704–14.
27. Ditchfield C, Johnson VL, Tighe A, Ellston R, Haworth C, Johnson T, et al. Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. J Cell Biol 2003; 161:267–80.
28. Castellano M, Pollock PM, Walters MK, Sparrow LE, Down LM, Gabrielli BG, et al. CDKN2A/p16 is inactivated in most melanoma cell lines. Cancer Res 1997;57:4868–75.

Published OnlineFirst May 18, 2017; DOI: 10.1158/1535-7163.MCT-17-0159

Molecular Cancer Therapeutics

Inhibition of Aurora A and Aurora B Is Required for the Sensitivity of HPV-Driven Cervical Cancers to Aurora Kinase Inhibitors

David Martin, Sora Fallaha, Martina Proctor, et al.

Mol Cancer Ther 2017;16:1934-1941. Published OnlineFirst May 18, 2017.