Functional Effects of AKT3 on Aurora Kinase Inhibitor-induced Aneuploidy*

Kohji Noguchi†, Keita Hongama, Shiori Hariki, Yuma Nonomiya, Kazuhiro Katayama, and Yoshikazu Sugimoto
From the Division of Chemotherapy, Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan

Edited by Eric R. Fearon

The suppression of mitotic Aurora kinases (AURKs) by AURK inhibitors frequently causes cytokinetic failure, leading to polyploidy or aneuploidy, indicating the critical role of AURK-mediated phosphorylation during cytokinesis. We demonstrate the deregulated expression of AKT3 in Aurora kinase inhibitor (AURKi)-resistant cells, which we established from human colorectal cancer HCT 116 cells. The AKT family, which includes AKT1, -2, and -3, plays multiple roles in antiapoptotic functions and drug resistance and is involved in cell growth and survival pathways. We found that an AKT inhibitor, AZD5363, showed synergistic effect with an AURKi, VX-680, on two AURK-expressing AURKi-resistant cell lines, and AKT3 knockdown sensitized cells to VX-680. Consistent with these activities, AKT3 expression suppressed AURKi-induced apoptosis and conferred resistance to AURKi. Thus, AKT3 expression affects cell sensitivity to AURKi. Moreover, we found that AKT3 expression suppressed AURKi-induced aneuploidy, and inversely AKT3 knockdown enhanced it. In addition, partial co-localization of AKT3 with AURKB was observed during anaphase. Overall, this study suggests that AKT3 could repress the antiproliferative effects of AURKs, with a novel activity particularly suppressing the aneuploidy induction.

The dysregulation of cell division causes chromosomal abnormalities in daughter cells, including chromosomal instability and aneuploidy, which are commonly observed in many types of cancer (1). Various mitotic protein kinases, including cyclin-dependent kinases, Aurora kinases (AURKA, -B, and -C), and Polo-like kinases (PLK1–4), are frequently up-regulated in human cancers (2). The mitotic protein kinases are thus considered potential targets for anticancer drugs. The AURKs, especially AURKA and AURKB, are important in both normal and cancerous cell division (3). AURKA increases in late G2/M phase, is mainly located at the spindle poles, and functions in centrosome duplication and mitotic spindle formation (4). Its overexpression is associated with chromosomal instability and rodent fibroblast transformation (5). By contrast, AURKB is located at the centromere until metaphase and then moves to the midzone and midbody until the end of cytokinesis (3). Small molecule AURK inhibitors (AURKis) have been developed, and these cause polyploidy, arising from the failure of cytokinesis (6). VX-680 (Tozasertib, developed by Vertex Pharmaceuticals and Merck), the first clinically tested AURKi, inhibits all three AURKs (7), whereas AZD1152-HQPA (Barasertib, developed by AstraZeneca) and MLN8237 (Alisertib, developed by Millennium Pharmaceuticals and Takeda Pharmaceutical Co.) are highly selective inhibitors of AURKB (8) and AURKA (9), respectively. Their antiproliferative effects are thought to be mediated by mitotic catastrophe, including impaired chromosome segregation, abrogation of the mitotic checkpoint, and cytokinesis failure, causing polyploidy and cell death (10). Molecular predictors of the responses to AURKis would greatly facilitate favorable therapeutic outcomes (11). Previous studies have suggested that the deregulation of TP53, MYC, or drug efflux transporters, known to be involved in resistance to various anticancer drugs, also affects chemosensitivity to AURKis (11). However, the resistance mechanism specific to the antimitotic activity of AURKis is unclear.

In this study, we established AURKi-resistant cells from the human colorectal cancer cell line HCT 116 with VX-680 selection. AKT3 was elevated in several VX-680-resistant clones. AKT was originally discovered as an oncogene in the murine leukemia virus AKT8 (12), and AKT3 is one of the three isoforms (AKT1–3) of the AKT family (13). Phosphoinositide 3-kinase (PI3K)-induced phosphatidylinositol (3–5) trisphosphate activates AKT through its N terminus pleckstrin homology domain, and the PI3K-AKT axis is a frequently deregulated signaling pathway in human cancers (13, 14). AKT has antiapoptotic activities and is also involved in resistance to various cytotoxic, antihormonal, and targeted drugs by phosphorylating several apoptosis-associated molecules, including mouse double minute 2 homolog (MDM2), glycogen synthase kinase 3β (GSK3β), Forkhead box subgroup O transcription factor (FOXO), BCL-2-associated death promoter (BAD), caspase-9, p27, and tuberous sclerosis complex 2 (15). Interestingly, previous studies have demonstrated a pharmacological synergy between AKT inhibitors and antimitotic microtubule inhibitors and suggested further study to elucidate the mechanism of
the synergy between AKT inhibitors and anti-microtubule drugs for cancer chemotherapy (16–19). Because AKT3 was elevated in our AURKi-resistant clones, and AKT is activated in G2/M phase and promotes G2/M progression (20, 21), we inferred that AKT signaling affects the antimitotic effects of AURKis. Our data indicate that AKT3 is associated with an AURKi-resistant phenotype and suppresses both apoptosis and aneuploidy.

Results

Characterization of AURKi-resistant Clones—Using two independent protocols (Fig. 1A), we established VX-680-resistant (VX-resistant) clones, VX1-1, VX1-2, VX0-1, VX0-2, and VX0-4. Cell growth inhibition assays showed that these clones have cross-resistance to other AURKis, AZD1152-HQPA and MLN8237 (Fig. 1, B and C). Treatment with these AURKis caused cytokinesis failure, causing polyploidy in HCT 116 cells (Fig. 2, A and B) as described (7). Therefore, we looked at ploidy of the resistant clones by analyzing chromosome numbers in mitotic VX-resistant clones. The VX-resistant clones were aneuploid cells with about 70–80 chromosomes (Fig. 2C), suggesting that they were the cells that survived after VX-680-induced cytokinetic failure.

To examine the drug resistance, we determined the expression of the drug efflux pump ABC transporters by Western blot analysis. An apparent increase of P-glycoprotein (P-GP)/
ABCB1 expression was detected only in the VX0-2 clone, but little expression was seen in other clones; BCRP/ABCG2 was not detected in any of the VX-resistant clones (Fig. 3A). In a cell growth inhibition assay, the VX-resistant clones showed weak resistance to vincristine, an antimitotic microtubule inhibitor and a typical substrate for P-GP/ABCB1, and little resistance to a topoisomerase I inhibitor, SN-38, a typical substrate for BCRP/ABCG2. P-PG/ABCB1-overexpressing HCT 116/MDR cells and BCRP/ABCG2-overexpressing HCT 116/BCRP cells showed strong resistance to these typical substrate drugs, respectively (Fig. 3B and Table 1). Consistent with the literature, P-GP/ABCB1 and BCRP/ABCG2 conferred very strong resistance to VX-680 and AZD1152-HQPA (22) but did not confer resistance to MLN8237 (Fig. 3C, right graph). By contrast, all VX-resistant clones showed cross-resistance to the three AURKis (Fig. 3D, top graphs).

We next tested the effect of MS-209 (dofequidar), a P-GP inhibitor (23, 24), in cell growth inhibition assays. Although MS-209 completely eliminated resistance to VX-680 in HCT 116/MDR cells and AURKi resistance in VX-resistant clones was partly reduced, resistance to the three AURKis was still observed in all VX-resistant clones in the presence of MS-209 (Fig. 3D and Table 3). Therefore, we speculated that P-GP contributed to the resistance to VX-680 and AZD1152-HQPA in the five VX-resistant clones, but an undetermined factor(s) may have an impact on the cross-resistant phenotype of the VX-resistant clones.

FIGURE 2. Aneuploidy induced by AURKi in HCT 116 cells. A, HCT 116 cells were treated with the indicated AURKi at 100 nM for 2 days (2ON). The 70% ethanol-fixed cells were stained with propidium iodide (PI), and the DNA contents were analyzed with flow cytometry. B, drug-treated cells were also stained with DAPI to visualize nuclear DNA and analyzed with a microscope equipped with a fluorescence digital CCD camera. Representative results are shown. Bar, 40 μm. C, chromosome numbers in VX-680-resistant clones. Chromosomes were counted in >50 metaphase cells and photographed. A histogram is shown below. Every VX-680-resistant clone shows aneuploidy. Parental HCT 116 cells had about 45–46 chromosomes, but every VX-680-resistant clone had about 70 chromosomes. *, peak of chromosome number.
**FIGURE 3.** Drug efflux pumps in VX-680-resistant cells. 

**A.** Western blot analysis of P-GP and BCRP in VX-resistant cells. P-GP/ABCB1-transfected HCT 116/MDR cells and BCRP/ABCG2-transfected HCT 116/BCRP cells were used as controls. Little expression of P-GP or BCRP was observed, except in the VX0-2 clone cells.

**B and C.** Cell growth inhibition was examined with WST assays after cells were treated for 4 days. Open symbols, parental HCT 116 cells; filled triangles and gray squares, P-GP- and BCRP-expressing cells HCT116/MDR and HCT 116/BCRP, respectively. Results are presented as means ± S.D. (error bars) from three independent experiments.

**D.** Effect of the P-GP inhibitor, MS-209. Chemical structure of MS-209 is shown in the left panel. Cell growth inhibition was examined with WST assays after the cells were treated for 4 days. Results are presented as means ± S.D. from three independent experiments. Top graphs, AURKi-treated cells; bottom graphs, cells cotreated with AURKi and MS-209 (5 μM). MS-209 (5 μM) completely abolished the vincristine resistance of HCT 116/MDR cells (data not shown).
AURKB phosphorylates mitotic histone H3 at Ser-10 (25). Thus, we examined the inhibitory effect of VX-680 on histone H3 phosphorylation at Ser-10 (P-H3S10) in mitotic VX-resistant clones by immunofluorescence confocal microscopic analysis (Fig. 4, A and B, and Table 3). VX-680 treatment severely inhibited the P-H3S10 signal in parental HCT 116 cells but not in HCT 116/MDR cells (Fig. 4, left panels), suggesting that P-GP-mediated efflux of VX-680 from cells protected AURKB activity. However, VX-680 treatment reduced P-H3S10 in all VX-resistant clones except VX1–2 (Fig. 4, A and B), indicating that AURKB was inhibited by VX-680 in VX0-1, 0-2, 0-4, and 1-1 clones. Because P-H3S10 was not suppressed by VX-680 in the P-GP-negative VX1-2 clone, AURKB activity seemed to be unaffected in the VX1-2 clone in the presence of VX-680. We analyzed the genomic DNA sequence of AURKB in the VX1-2 clone and found a point mutation causing an amino acid substitution at position 250, H250Y (Fig. 4B). The same mutation H250Y of AURKB was reported to cause marginal resistance to VX-680 by hyperactivating the catalytic activity of the kinase (26). Thus, we speculated that the AURKi resistance of VX1-2 would be mediated by the H250Y mutation in AURKB.

AURKIs exert antiproliferative activities through inducing both cell death and polyploidy (7, 27). Therefore, we next investigated the expression of apoptosis-related molecules by Western blot analysis in cells treated with AURKIs (Fig. 4C). The expression of AURKA, AURKB, and proapoptotic BAX was quite similar in the VX-resistant cells and parental cells. Although P21 and TP53 were induced by AZD1152-HQPA in clones VX0-4, 1-1, and 1-2, their expressions were reduced by VX-680 and MLN8237 in all VX-resistant clones. Importantly, cleaved caspase-9, an initiator of the mitochondria-related intrinsic apoptosis pathway (28), induced by the three AURKIs (clearly induced by 100 nm AURKi), was suppressed in all VX-resistant clones. This indicates that the caspase-9-initiated apoptosis induced by AURKIs was suppressed in the VX-resistant clones.

Deregulation of AKT3 Suppresses AURKi-mediated Apoptosis and Aneuploidy—Our Western blot analysis showed that AKT3 was overexpressed in four of five resistant clones, but the other AKT family members, AKT1 and AKT2, were not (Fig. 5A). AKT phosphorylation (Ser-473) was increased in the VX-resistant cells, and GLUT1 and GSK-3β phosphorylation at Ser-9, which are linked to AKT signaling, were increased (Fig. 5A), suggesting activation of the AKT pathway. GLUT1 is reportedly involved in cytokinesis (29), and our data suggest a
possible role for AKT3 in the AURKi-resistant phenotype. Although growth inhibition assays showed little resistance to the AKT inhibitor AZD5363 in these VX-resistant clones (Fig. 5, B and C), the addition of AZD5363 after treatment with VX-680 sensitized the VX-resistant cells, especially VX0-1 and VX0-4, to VX-680 (Fig. 5D). Sensitivity of parental HCT 116 to VX-680 appeared to be unaffected by the AKT inhibitor, but calculated combination index values were <1 in VX-resistant clones, especially in VX0-1 and VX0-4 clones (Fig. 5D). Therefore, there may be a selective synergistic effect of the AKT inhibitor on VX-680 resistance (30).

To examine the effect of AKT3 on AURKi sensitivity, AKT3 knockdown experiments were performed in VX0-1 and VX0-4 clones (Fig. 5E and F). AKT3 knockdown efficiency was transient and incomplete in VX0-1 cells, but significant sensitization to VX-680 and MLN8237 was observed (Fig. 5E). The
transfection efficiency of siRNA transfection in VX0-4 cells was poor; thus, we performed AKT3 knockdown by shRNA plasmid transfection and found significant sensitization to VX-680 and MLN8237 in the VX0-4 clone (Fig. 5). These data indicate that the AKT3 pathway has the ability to contribute to chemoresistance against AURKis.

To examine the effects of AKT3 signaling on AURKi resistance, we established cell lines stably expressing the myristoylated active mutant AKT3 (myr-AKT3) (DA-18, -14, and -36) (Fig. 6). Exogenous myr-AKT3-HA expression was high in DA-18, but low in DA-36, and similar to endogenous AKT3 in DA-14 (Fig. 6A). The expression of total AKT1/2/3 (pan-AKT1/2/3) was unchanged in these transfectants, but AKT phosphorylation (Ser-473) was elevated, suggesting activation of AKT signaling. Every transfected clone showed resistance to the three AURKis and also slight resistance to the PLK inhibitor BI 6727 (Fig. 6B). The AURKi-induced cleavage of caspase-9 was attenuated in the VX-resistant clones (Fig. 4C), and we consistently observed that high expression of AKT3 (in DA-18 and -14) strongly suppressed the caspase-9 cleavage induced by AURKis (Fig. 6C). These data suggest that AURKi-induced apoptosis and caspase-9 activation are suppressed by AKT3 in HCT 116 cells, although the inductions of TP53 and P21 are not.

We also tested the impact of AKT3 on the pharmacological effects of AURKis in HeLa cells. Active AKT3, either myr-AKT3 (31) or AKT3 (E17K) (32), was transiently expressed in HeLa cells, and apoptosis and aneuploidy caused by AZD1152-HQPA were analyzed with flow cytometry (Fig. 7, A and B). The results showed that the expression of active AKT3 and GLUT1 suppressed both the apoptosis and aneuploidy caused by AZD1152-HQPA in HeLa cells (Fig. 5B). AKT3 also suppressed the apoptosis induced by two other AURKis (data not shown). Cell growth inhibition assays showed that IC\textsubscript{50} values against VX-680 were increased by AKT3 expression, suggesting that AKT3 conferred resistance to VX-680 in HeLa cells (Fig. 7C). Because the aneuploidy-suppressive effect of AKT3 was a novel activity, we further examined anti-aneuploidy activity of AKT3 by analyzing nuclear size in aneuploidy/polyploidy cells by confocal microscopy. In addition to HCT 116 cells, we also tested the effect of AKT3 on MCF7 and OVCAR3 cell
lines, because these cells do not express endogenous AKT3 (supplemental Fig. S1). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and AKT3-transfected cells were recognized by staining with an anti-hemagglutinin (HA) antibody (Fig. 7D). Consistent with FACS analysis (Fig. 2A), nuclear sizes of AZD1152-HQPA-treated cells were bigger than those of nocodazole-treated cells arrested at G2 phase (Fig. 7D and supplemental Fig. S2), suggesting that the nuclear size of polyploidy cells was bigger than that of G2 phase cells. The sizes of nuclei were measured in captured images with the ImageJ software, and the data were summarized as a box plot with a bee swarm dot plot overlay (Fig. 7E). AZD1152-HQPA increased the median nuclear size with a range of sizes in the control cells by the induction of aneuploidy, whereas active AKT3 expression repressed this increase of nuclear size after ADZ1152-HQPA treatment. Anti-aneuploidy activity of AKT3 was also observed in myr-AKT3-stably expressing HCT 116 cells (supplemental Fig. S3).

Ovarian cancer OVCAR5 cells express AKT3 and showed relative resistance to VX-680 compared with OVCAR3 cells, which do not express AKT3 (supplemental Fig. S1). We next tested the effect of AKT3 knockdown on AURKi-induced aneuploidy (Fig. 8). As in Fig. 7, we analyzed AURKi-induced nuclear size change in AKT3 siRNA-transfected cells (Fig. 8B). The results showed that the ratio of cells with aneuploid large nuclei was increased in AKT3-reduced cells after VX-680 treatment but not after nocodazole treatment (Fig. 8, C and D). Collectively, our data indicate that AKT3 can repress the induction of aneuploidy by AURKi.

Localization of AKT3 and AURKB during Anaphase—Some GLUT1 localizes to the midbody and is involved in the pro-
AKT3 Represses Aneuploidy

**FIGURE 7. AKT3 suppressed aneuploidy induction.** A, induction of apoptosis and aneuploidy was repressed by AKT3 in HeLa cells. HeLa cells were transiently transfected with plasmid expressing either wild-type (WT), myristoylated (myr), or mutant (E17K) AKT3 or GLUT1, and the transfected cells were divided equally on the following day. The cells were then treated with the AURKB inhibitor AZD1152-HQPA (20 nM) for 2 days. DNA contents of the control (black lines) and drug-treated cells (red lines) were analyzed with flow cytometry and quantified with the CellQuest™ software. The M1 fraction corresponds to sub-G1, apoptotic cells, and the M2 fraction corresponds to the surviving cells with normal DNA content. The M3 fraction represents high-DNA content aneuploid cells. Representative histograms are shown. B, quantitative results for each fraction are shown in the bar graph. Results are presented as means ± S.D. from three independent experiments. Statistical analysis was performed with Student’s t test. *, p < 0.01; **, p < 0.05. C, transient expression of AKT3 conferred resistance to VX-680 in HeLa cells. Transient expression of myr-AKT3-HA was shown by Western blotting analysis at 2 days posttransfection in the left panels. Next, myr-AKT3-expressing HeLa cells were seeded in triplicate wells and treated with VX-680 for 4 days. Cell growth inhibition was examined by a WST-8 assay. Two independent transfection studies (#1 and #2) were performed, and the results of the WST-8 assay are presented as means ± S.D. (error bars) from triplicated wells in each sample. D, induction of aneuploidy was repressed by AKT3. Active AKT3 mutant, either myr-AKT3 or AKT3 (E17K), was transiently expressed either in HCT 116, MCF7, or OVCAR3 cells. The cells were then treated with AZD1152-HQPA for 2 days. Control cells were not treated with the drug, and nocodazole (100 nM for 24 h)-treated HCT 116 cell nuclei were also shown as reference for G2-arrested cells. After fixation, nuclei were stained with DAPI (blue signal) and AKT3-expressing cells were detected with anti-HA staining (red signal). Confocal microscopic analysis was performed, and representative images are shown. E, nuclear areas in the control and AKT3-expressing cells (>120 cells/sample) as in D were measured with the ImageJ software. Data are summarized as a box plot with a bee swarm dot plot overlay, generated with the BoxplotR software. Results of two independent experiments (1 and 2) in HCT 116 cells are shown, and representative results of two independent experiments in MCF7 and OVCAR3 cells are shown. Center lines show the medians; box limits indicate the 25th and 75th percentiles, determined with the R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; outliers are represented by dots. Results are summarized in supplementary Table S3. Statistical analysis was performed with the Mann-Whitney U test.

Consistent with this, we found that the location of GLUT1 partly overlapped with that of AURKB in the midbody in AKT3-expressing MDA MB-231 cells (supplemental Fig. S1). We further investigated the subcellular localization of AKT1 (Fig. 9, A and D), AKT2 (Fig. 9, B and E), and AKT3 (Fig. 9, C and F) during mitosis in the VX0-1 clone and MDA-MB 231 cells using confocal microscopy (Fig. 9). Most endogenous AKT1, -2, and -3 (red signal) were not on the chromosomes (blue signal) during mitosis. However, during anaphase, we observed partial colocalization of AKT3 (red signal) and AURKB (green signal), especially around the central region of the cell, as detected by merged yellow signals (Fig. 9, C and F). In a three-dimensional reconstructed movie, rotated at right angles, yellow signals indicated overlapping of AKT3 and AURKB during anaphase in MDA-MB 231 cells (supplemental Movie S1). A signal intensity profile showed that AKT3 (red signal) was partly coincident with AURKB (green signal) in the central region during anaphase (Fig. 9G). AKT1 and AKT2 were also detected during mitosis, and the locations of AKT3s and AURKB also, in part, seemed to overlap during anaphase (Fig. 9, A, B, D, and E). These results suggest that AKT3 and AURKB partly colocalize in the central midzone during anaphase. Collectively, these observations suggest that AKT3 has a role in mitosis and potential to repress AURKi-induced cytokinetic dysregulation.
**Discussion**

AURKA and AURKB are important regulators of mitotic processes, and their deregulated expression has been demonstrated in various cancers (10). Therefore, AURKs are considered good pharmacological targets for anticancer chemotherapeutics, and various AURKis have been developed for therapeutic use (27). During the investigation of AURKi-resistant cells, we identified AKT3 with an ability to suppress AURKi-induced aneuploidy. Our data suggest that AKT3 has a potential to contribute to proper cytokinesis progression.

Previous studies have investigated the AURKi-resistant phenotypes of human cells and have shown that a point mutation in AURK reduces its sensitivity to its inhibitor (26). Our experiments show that the mitotic P-H3S10, a typical target of AURKB (25), was inhibited by the pan-AURKI VX-680 in four of the five VX-resistant cells, whereas P-H3S10 in VX1-2 clone, which harbors the AURKB mutation H250Y, was not inhibited.

Therefore, we concluded that AURKB was inhibited by VX-680 in four of the five resistant clones. Another study showed that P-GP/ABCB1 and BCRP/ABCG2 confer resistance to AZD1152-HQPA (22). Because our VX-resistant cells showed cross-resistance to P-GP-insensitive MLN8237, and the P-GP inhibitor MS-209 did not abolish the resistance of the VX-resistant cells, we presume that P-GP and BCRP contribute only slightly to the AURKi-resistant phenotype of our VX-resistant cells. Because apparent P-GP expression was detected only in the VX0-2 clone, elevations of P-GP and AKT3 were not observed in the VX1-1 clone, and apparent pharmacological synergy between the AKT inhibitor and VX-680 was seen in only two VX-resistant VX0-1 and 0-4 clones (supplemental Table S1), there is a possibility that multiple different resistance mechanisms might be involved in each VX-resistant clone.

In addition, our VX-resistant clones are near tetraploidy cells, and TP53 accumulation after AURKI treatment was
reduced in the clones (Fig. 4C), suggesting that the VX-resistant cells should proliferate after VX-680-induced cytokinetic failure. Tetraploidy after cytokinesis failure is reported to activate the hippo pathway and stabilize TP53 in postmitotic G1 arrest, and conversely growth factor signaling, such as IGF1, is sufficient to overcome such G1 arrest (33). IGF1 activates the PI3K-AKT pathway, and numerous studies have shown that the activation of the PI3K-AKT pathway is associated with a poor prognosis and the chemotherapeutic resistance of cancer cells (13, 14). Although our preliminary microarray gene expression analysis did not indicate an activation of the hippo pathway,3 we found overexpression of AKT3 in the VX-resistant clones (Fig. 5A). We confirmed that AKT3 suppresses AURKi-induced

3 K. Noguchi, K. Hongama, S. Hariki, Y. Nonomiya, K. Katayama, and Y. Sugimoto, unpublished results.
caspase-9 activation and confers resistance to AURKi, whereas the AURKi-induced accumulation of TP53 and P21 was not abolished by AKT3. A previous study reported that reactive oxygen species (ROS) and p38 MAPK activate P21 in AURKi-induced aneuploid cells (34), and the increased ROS production in aneuploid cells is attributed to high glucose metabolism (35). Intriguingly, the glucose metabolic pathway seems to be altered in our AURKi-resistant clones, because the expressions of the glucose transporter GLUT1 and some glycolysis-regulating enzyme genes were up-regulated in the VX-resistant clones (data not shown). Therefore, the AURKi-resistant clones have potentially high glucose metabolism and ROS production. Because the AURKi-induced accumulation of TP53 and P21 was reduced in our AURKi-resistant clones, the ROS-associated downstream mechanism(s) may be altered. A future study should investigate these phenomena to identify as yet undetermined resistance mechanisms to AURKi.

The AKT pathway is involved in cell cycle progression, and its activity increases during mitosis and promotes the G2/M transition (20, 21). Recent studies also showed complicated positive- and negative-regulatory networks among AURKA, AURKB, PLK, and AKT during mitosis (36–40), whereas AKT3 expression conferred slight resistance to the PLK inhibitor in our setting (Fig. 6B). AKT could activate PLK1 (39), and other studies reported that PLK1 could activate PI3K-AKT signaling by suppressing PTEN (phosphatase and tensin homolog) (41, 42). This possible cross-talk between AKT3 and PLK1 signalings might be involved in AKT3-induced cross-resistance to PLK inhibitor.

Moreover, in this study, we demonstrated that AKT3 suppresses AURKi-induced aneuploidy in colorectal cancer HCT 116 and other cells. Although the specific role of AKT3 in colorectal cancer has not been established, previous studies have suggested AKT3 as a possible mitotic regulator (43, 44). Because the inhibition of AURKB causes cytokinetic failure and aneuploidy, and a possible role of AKT in microtubule stabilization has been suggested (45), we suspected that AKT3 may have non-apoptotic activity associated with the cytokinetic machinery, which might counteract aneuploidy induction by AURKis and modulate the chemosensitivity of cancer cells to AURKi. Undoubtedly, AURKis disturb AURKB-regulated various central spindle-associated factors, such as KIF23, Rac-GAP1, KIF2A, and KIF4A (46–48), and thus AKT3 might affect various functions of these molecules. Further study is required to uncover the entire mechanism and pathway of AKT3-mediated prevention against aneuploidy by AURKis.

In addition, the expression of AKT3 is observed in several types of cancer and some patients (Cancer Genome Atlas database), so that AURKi should be administered to these patients with care, and the deregulated expression of cytokinetic molecules might affect the therapeutic effects of various antimitotic drugs for cancer.

**Experimental Procedures**

**Cells and Reagents**—HCT 116, HeLa, HEK293, MDA-MB 231, MCF7, OVCAR3, and OVCAR5 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium or RPMI1640 medium (Sigma-Aldrich) supplemented with 7% fetal bovine serum and kanamycin (50 µg/ml) at 37°C in 5% CO2. VX-680 (Tozasertib), AZD1152-HQPA (Barasertib), MLN8237 (Alisertib), and AZD5363 were purchased from Selleck Chemicals (Houston, TX). Vincristine and nocodazole were purchased from Sigma-Aldrich, and SN-38 was provided by Yakult Honsha Co., Ltd. (Tokyo, Japan). To establish VX-680-resistant cell lines, the HCT 116 cell line was initially treated with VX-680, starting from 40 nM and gradually increasing to 200 nM within 6 months. Resistant cells were cloned from two independent origins (VX0-1, VX0-2, VX0-4 and VX1-1, and VX1-2). P-GP/ABCG2-expressing HCT 116/MDR cells were established by transduction of P-GP-expressing HaMDR retrovirus (49). BCRP/ABCG2-expressing HCT 116/BCRP cells were previously established (50). Stable AKT3-HA-expressing HCT 116 cells were established by selecting cells transfected with an AKT3-expressing plasmid with G418 (800 µg/ml). AKT3-HA expression in the cloned cells was confirmed with Western blotting.

**Cell Growth Inhibition Assay**—Cell growth inhibition assays were conducted with a Coulter counter (Beckman Coulter, Brea, CA) after treatment for 4 days. Cell viability was also evaluated with a 4-[3-(2-methoxy-4-nitrophenyl)-2-[4-nitrophenoxy]-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt (WST-8)-based assay (Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan). The IC50 (drug dose inhibiting cell growth by 50%) was determined from growth inhibition curves, and the degree of resistance (-fold) was calculated by dividing the IC50 of the test cells by that of the parent cells, as described previously (51).

**Plasmids**—Human wild-type AKT3 cDNA (NM_005465.4) was cloned with conventional PCR and inserted into the CMV promoter-driven plasmid pD3HA, as described previously (52). The SRC myristoylation signal sequence peptide (MGSSK-SKP2; designated “myr”) was fused to the N terminus of AKT3 to construct active AKT3 by conventional PCR (31). AKT3 was expressed with an HA tag at the C terminus. PCR primers used in this study are listed in [*supplemental Table S1*](#).

**Genomic DNA Sequence for AURKB**—Genomic DNA from VX-resistant clones was prepared using the DNeasy blood and tissue kit (Qiagen, Valencia, CA), and DNA fragments corresponding to the AURKB open reading frame (genome assembly data: GRCh38) were amplified by PCR and directly sequenced. PCR primers used in this study are listed in [*supplemental Table S2*](#).

**AKT3 Knockdown Experiments**—For AKT3 knockdown by siRNA transfection, control and four AKT3 siRNAs (AllStars negative control siRNA and Flexi Tubes Hs_AKT3_12, _8, _7, and _17 purchased from Qiagen) were used. The shRNA plasmid for AKT3 (sc-38911-SH) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). In the siRNA transfection experiments, Lipofectamine® 2000 transfection reagent (Invitrogen) was used for transfection of VX0–1 cells, and HiPerfect transfection reagent (Qiagen) was used for transfection of OVCAR5 cells, in accordance with the manufacturers’ instructions. shRNA plasmid was transfected into VX0-4 cells using FuGENE® HD transfection reagent (Promega, Madison, WI), and cells were selected by puromycin (Sigma-Aldrich).
**Western Blotting**—For Western blotting, cells were lysed in whole cell lysis buffer (20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5% SDS, 0.5% sodium deoxycholate, 5 mM EDTA, 0.5 mM AEBSF, 10 μM pepstatin, 10 μM leupeptin, 10 μM E-64d, 0.1 μM calyculin A, 1 μM MG132) and sonicated on ice to prepare whole cell lysates. After SDS-PAGE, proteins were transferred from the gel to an Immobilon-P membrane (EMD Millipore, Billerica, MA), and the membrane was blocked and incubated with primary antibodies. Western blotting signals were developed with the SuperSignal® West Dura Extended Duration Substrate (Pierce) and recorded with an ImageQuant LAS4000 mini image analyzer (GE Healthcare Japan Corp., Tokyo, Japan). We used anti-FLAG M2 (Sigma-Aldrich), anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH, 6C5, Millipore Corp., Chemicon, Billerica, MA), anti-TP53 (DO-1, Abcam, Cambridge, UK), anti-Aurora A (A520B, EMD Millipore Japan). We used anti-FLAG M2 (Sigma), 50 μg/ml RNase A (Sigma), 50 μg/ml propidium iodide (Sigma) in PBS, and subjected to FACS (BD LSR II flow cytometer; BD Biosciences). Quantitative data were analyzed with CellQuest software (BD Biosciences). Statistical Analysis—In vitro experiments were repeated independently at least twice, and similar results were observed. Quantitative results are presented as means ± S.D. (n ≥ 3). A two-tailed Student’s t test was used to evaluate the significance of differences between two groups of data with similar variances. A value of p < 0.05 was considered statistically significant. A box plot and bee swarm dot plot were generated with BoxplotR software (55). The Mann-Whitney U test was used to evaluate the statistical significance of the box plot results, with IBM Statistics SPSS version 22.0 (IBM Corp.).

**Author Contributions**—K. N. conceived the idea of this study. K. N., K. H., and S. H. conducted most of the experiments. Y. N. contributed to the plasmid construction. K. N., K. K., and Y. S. analyzed the results and wrote the paper.

**References**

1. Holland, A. J., and Cleveland, D. W. (2009) Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. *Nat. Rev. Mol. Cell Biol.* 10, 478–487.

2. Nigg, E. A. (2001) Mitotic kinases as regulators of cell division and its checkpoints. *Nat. Rev. Mol. Cell Biol.* 2, 21–32.

3. Dominguez-Brauer, C., Thu, K. L., Mason, J. M., Blaser, H., Bray, M. R., and Mak, T. W. (2015) Targeting mitosis in cancer: emerging strategies. *Mol. Cell* 60, 524–536.

4. Nikonova, A. S., Astsaturov, I., Serebriiskii, I. G., Dunbrack, R. L., Jr, and Golemis, E. A. (2013) Aurora A kinase (AURKA) in normal and pathological cell division. *Cell Mol. Life Sci.* 70, 661–687.

5. Zhou, H., Kung, J., Zhong, L., Kuo, W. L., Gray, J. W., Sahin, A., Brinkley, B. R., and Sen, S. (1998) Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. *Nat. Genet.* 20, 189–193.

6. Hauf, S., Cole, R. W., LaTerra, S., Zimmer, C., Schnapp, G., Walter, R., Heckel, A., van Meel, J., Rieder, C. L., and Peters, J. M. (2003) The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *J. Cell Biol.* 161, 281–294.

**Immunofluorescence Confocal Microscopy**—Mitotic chromosomes were analyzed as described previously (53). Briefly, colcemid-arrested mitotic cells were suspended in hypotonic buffer (0.075 M KCl), fixed, washed with methanol/acetic acid (3:1), and stained with DAPI. Mitotic chromosomes were visualized with a conventional fluorescence microscope equipped with a fluorescence digital CCD camera (Keyence, BZ-9000, Japan).

Cells were seeded on ScientificTM NuncTM Lab-TekTM II CC2TM chamber slides (5–10 × 10⁴ cells/0.5 ml/well; Thermo Fisher Scientific) and cultured overnight. Cells were then transfected with plasmids using FuGENE® HD transfection reagent (Promega) and fixed the next day with ice-cold methanol or 3.7% formalin, phosphate-buffered saline (PBS) for 10 min at room temperature. Cells were permeabilized with NEB100 buffer for 10 min at room temperature, washed with PBS, and blocked with 3% bovine serum albumin (BSA) in TBST (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20) for 30 min at room temperature, as described previously (53). The slides were incubated overnight at 4 °C with primary antibodies diluted with 3% BSA, TBST, and then with Alexa Fluor 488-conjugated goat anti-mouse or Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen, Molecular Probes), diluted 1:2000 in 3% BSA, TBST for 2 h at room temperature. For primary antibodies, we used anti-AKT1 (C73H10), anti-AKT2 (D6G4), anti-AKT3 (E1Z3W) rabbit antibodies diluted at 1:3000. Anti-AURKB mouse monoclonal antibody (ab3609, Abcam) was used at 0.2 μg/ml in 3% BSA, TBST. Reaction specificities of anti-AKT antibodies are shown in *supplemental Fig. S4*. AKT3-HA was detected by anti-HA antibody clone 3F10 or 12CA5 (diluted at 10 or 500 ng/ml, respectively). Anti-phosphohistone H3 (Ser-10) antibody (clone RR002) was purchased from Merck Millipore (Darmstadt, Germany) and incubated with cells for 2 h at room temperature (dilution at 0.5 μg/ml). Cells were washed three times with PBS and mounted with Prolong® Gold Antifade Reagent with DAPI (Invitrogen). Acquisition of images (640 × 640 pixels) was performed using the FX1000-D IX81 confocal microscope equipped with an objective lens, UPLFLN ×40, numerical aperture 1.3 oil (Olympus Corp., Tokyo, Japan) with identical parameter settings. The EGFP signal intensity and region integration profile was analyzed with the associated viewer software (Olympus). Confocal two-dimensional TIFF images were merged with the Adobe® Photoshop CS4 Extended software (Adobe Systems Inc., San Jose, CA), and confocal three-dimensional movies were made with the accompanying FV10-ASW software, according to the instruction manual (Olympus Corp.).

Flow Cytometric Analysis—Cells were harvested after drug treatment, washed with PBS, fixed with 70% ethanol, stained with propidium iodide (0.1% Triton X-100, 200 μg/ml RNase A (Sigma), 50 μg/ml propidium iodide (Sigma) in PBS), and subjected to FACS (BD LSR II flow cytometer; BD Biosciences). Quantitative data were analyzed with CellQuest software (BD Biosciences). Statistical Analysis—In vitro experiments were repeated independently at least twice, and similar results were observed. Quantitative results are presented as means ± S.D. (n ≥ 3). A two-tailed Student’s t test was used to evaluate the significance of differences between two groups of data with similar variances. A value of p < 0.05 was considered statistically significant. A box plot and bee swarm dot plot were generated with BoxplotR software (55). The Mann-Whitney U test was used to evaluate the statistical significance of the box plot results, with IBM Statistics SPSS version 22.0 (IBM Corp.).
7. Harrington, E. A., Bebbington, D., Moore, J., Rasmussen, R. K., Ajose-Adeogun, A. O., Nakayama, T., Graham, J. A., Demur, C., Hercend, T., Diu-Hercend, A., Su, M., Golec, J. M., and Miller, K. M. (2004) VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo. Nat. Med. 10, 262–267

8. Wilkinson, R. W., Odedra, R., Heaton, S. P., Wedge, S. R., Keen, N. I., Crafter, C., Foster, J. R., Brady, M. C., Bigley, A., Brown, E., Byth, K. F., Barras, N. C., Mundt, K. E., Foote, K. M., Heron, N. M., et al. (2007) AZD1152, a selective inhibitor of Aurora B kinase, inhibits human tumor xenograft growth by inducing apoptosis. Clin. Cancer Res. 13, 3682–3688

9. Görgün, G., Calabrese, E., Hideshima, T., Ecsedy, J., Perrone, G., Mani, M., Ikeda, H., Bianchi, G., Hu, Y., Ciristea, D., Santo, L., Tai, Y. T., Nahar, S., Zheng, M., Bandi, M., et al. (2010) A novel Aurora-A kinase inhibitor MLN8237 induces cytotoxicity and cell-cycle arrest in multiple myeloma. Blood 115, 5202–5213

10. Goldenson, B., and Crispino, J. D. (2015) The aurora kinases in cell cycle and leukemia. Oncogene 34, 537–545

11. Hiltn, J. F., and Shapiro, G. I. (2014) Aurora kinase inhibition as an anti-cancer strategy. J. Clin. Oncol. 32, 57–59

12. Bellacosa, A., Testa, J. R., Staal, S. P., and Tsichlis, P. N. (1991) A retroviral oncogene, akt, encoding a serine/threonine kinase containing an SH2-like region. Science 254, 274–277

13. Manning, B. D., and Cantley, L. C. (2007) AKT/PKB signaling: navigating downstream. Cell 129, 1261–1274

14. Fresno Vara, J. A., Casado, E., de Castro, J., Cejas, P., Belda-Iniesta, C., and González-Barón, M. (2004) PI3K/Akt signalling pathway and cancer. Cancer Treat. Rev. 30, 193–204

15. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Cellular survival: a standard chemotherapeutic agents or molecular targeted drugs downstream of the mammalian midbody proteome reveals conserved cytokinesis mechanisms. Science 305, 61–66

16. Chou, T. C., and Talalay, P. (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul. 22, 27–55

17. Mende, I., Malstrom, S., Tschliss, P. N., Vogt, P. K., and Aoki, M. (2001) Oncogenic transformation induced by membrane-targeted Akt2 and Akt3. Oncogene 20, 4419–4423

18. Davies, M. A., Stemke-Hale, K., Tellez, C., Calderone, T. L., Deng, W., Prieto, V. G., Lazar, A. J., Gershenson, J. E., and Mills, G. B. (2008) A novel AKT3 mutation in melanoma tumours and cell lines. Br. J. Cancer 99, 1265–1268

19. Ganem, N. J., Cornils, H., Chiu, S. Y. O’, Rourke, K. P., Arnaud, J., Yimlamai, D., Théry, M., Camargo, F. D., and Pellman, D. (2014) Cytokinesis failure triggers hippo tumor suppressor pathway activation. Cell 158, 833–848

20. Siegel, J. J., and Amon, A. (2012) New insights into the troubles of aneuploidy. Annu. Rev. Cell Dev. Biol. 28, 189–214

21. Seki, A., Coppinger, J. A., Jang, C. Y., Yates, J. R., and Fang, G. (2008) Bora and the kinase Aurora A cooperatively activate the kinase Plk1 and control mitotic entry. Science 320, 1655–1658

22. Macurek, L., Lindqvist, A., Lim, D., Lampson, M. A., Klompmaker, R., Freire, R., Clouin, C., Taylor, S. S., Fuchs, M., and Medema, R. H. (2008) Polo-like kinase-1 is activated by Aurora A to promote checkpoint recovery. Nature 455, 119–123

23. Carmena, M., Pinson, X., Platani, M., Salloum, Z., Xu, Z., Clark, A., Maciasca, F., Ogawa, H., Eggert, U., Glover, D. M., Archambault, V., and Earnshaw, W. C. (2012) The chromosomal passenger complex activates Polo kinase at centromeres. PLoS Biol. 10, e1001250

24. Kasahara, K., Goto, H., Itoh, Y., Watanabe, N., Elowe, S., Nigg, E. A., and Inagaki, M. (2013) PI 3-kinase-dependent phosphorylation of Plk1 Ser99 promotes association with 14–3–3σ and is required for meta-phase-anaphase transition. Nat. Commun. 4, 1882

25. Chen, L., Li, Z., Ahmad, N., and Liu, X. (2015) Plk1 phosphorylation of IRS2 prevents premature mitotic exit via AKT inactivation. Biochemistry 54, 2473–2480

26. Choi, B. H., Pagano, M., and Dai, W. (2014) Plk1 protein phosphorylates phosphatase and tensin homolog (PTEN) and regulates its mitotic activity during the cell cycle. J. Biol. Chem. 289, 14066–14074

27. Moffat, J., Grueneberg, D. A., Yang, X., Kim, S. Y., Klopfer, A. M., Hinkle, G., Piqani, B., Eisenhaure, T. M., Luo, B., Grenier, J. K., Carpenter, A. E., Foo, S. Y., Stewart, S. A., Stockwell, B. R., Hacohen, N., et al. (2006) A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. Cell 124, 1283–1298
Rines, D. R., Gomez-Ferreria, M. A., Zhou, Y., DeJesus, P., Grob, S., Batalov, S., Labow, M., Huesken, D., Mickanin, C., Hall, J., Reinhardt, M., Natt, F., Lange, J., Sharp, D. J., Chanda, S. K., and Caldwell, J. S. (2008) Whole genome functional analysis identifies novel components required for mitotic spindle integrity in human cells. *Genome Biol.* 9, R44

Onishi, K., Higuchi, M., Asakura, T., Masuyama, N., and Gotoh, Y. (2007) The PI3K-Akt pathway promotes microtubule stabilization in migrating fibroblasts. *Genes Cells* 12, 535–546

Carmena, M., Wheelock, M., Funabiki, H., and Earnshaw, W. C. (2012) The chromosomal passenger complex (CPC): from easy rider to the godfather of mitosis. *Nat. Rev. Mol. Cell Biol.* 13, 789–803

Nunes Bastos, R., Gandhi, S. R., Baron, R. D., Gruneberg, U., Nigg, E. A., and Barr, F. A. (2013) Aurora B suppresses microtubule dynamics and limits central spindle size by locally activating KIF4A. *J. Cell Biol.* 202, 605–621

Uehara, R., Tsukada, Y., Kamasaki, T., Poser, I., Yoda, K., Gerlich, D. W., and Goshima, G. (2013) Aurora B and Kif2A control microtubule length for assembly of a functional central spindle during anaphase. *J. Cell Biol.* 202, 623–636

Mitsubashi, J., Tsukahara, S., Suzuki, R., Oh-hara, Y., Nishi, S., Hosoyama, H., Katayama, K., Noguchi, K., Minowa, S., Shibata, H., Ito, Y., Hatake, K., Aiba, K., Takahashi, S., and Sugimoto, Y. (2007) Retroviral integration site analysis and the fate of transduced clones in an MDR1 gene therapy protocol targeting metastatic breast cancer. *Hum. Gene Ther.* 18, 895–906

Katayama, K., Shibata, K., Mitsuhashi, J., Noguchi, K., and Sugimoto, Y. (2009) Pharmacological interplay between breast cancer resistance protein and gefitinib in epidermal growth factor receptor signaling. *Anticancer Res.* 29, 1059–1065

Kawahara, H., Noguchi, K., Katayama, K., Mitsuhashi, J., and Sugimoto, Y. (2010) Pharmacological interaction with sunitinib is abolished by a germ-line mutation (1291T>C) of BCRP/ABCG2 gene. *Cancer Sci.* 101, 1493–1500

Noguchi, K., Fukazawa, H., Murakami, Y., and Uehara, Y. (2004) Nucleolar Nek11 is a novel target of Nek2A in G1/S-arrested cells. *J. Biol. Chem.* 279, 605–621

Noguchi, K., Naito, M., Oshimura, M., Mashima, T., Fujita, N., Yonehara, S., and Tsuruo, T. (1996) Chromosome 22 complements apoptosis in Fas- and TNF-resistant mutant UK110 cells. *Oncogene* 13, 39–46

Noguchi, K., Vassilev, A., Ghosh, S., Yates, J. L., and DePamphilis, M. L. (2006) The BAH domain facilitates the ability of human Orc1 protein to activate replication origins in vivo. *EMBO J.* 25, 5372–5382

Spitzer, M., Wildenhain, J., Rappsilber, J., and Tyers, M. (2014) BoxPlotR: a web tool for generation of box plots. *Nat. Methods* 11, 121–122