PRECLINICAL STUDY

Co-treatment with vorinostat synergistically enhances activity of Aurora kinase inhibitor against human breast cancer cells

**Warren Fiskus · Stacey L. Hembruff · Rekha Rao · Priyanka Sharma ·
Ramesh Balusu · Sreedhar Venkannagari · Jacqueline E. Smith ·
Karissa Peth · Stephen C. Peiper · Kapil N. Bhalla**

Abstract Aurora kinases (AKs) regulate multiple components of mitotic cell division in eukaryotic cells. Aurora A is frequently amplified or overexpressed in breast cancer cells leading to aberrant chromosome segregation, genomic instability, and activation of oncogenic pathways. In the present studies, we determined the effects of treatment with the pan-AK inhibitor MK-0457 and/or the pan-histone deacetylase inhibitor vorinostat against human breast cancer cells that were either ER-, PR-, and HER2- (MDA-MB-468 and MDA-MB-231) or exhibited Aurora A amplification (BT-474 and MDA-MB-231 cells). Treatment with MK-0457 depleted p-AKs levels and their activity, as well as induced G2/M accumulation, DNA endoreduplication, multipolar mitotic spindles, and apoptosis of the breast cancer cells. Similar apoptotic effects were observed with treatment with the Aurora A-specific inhibitor, MLN8237. Treatment with vorinostat induced hsp90 acetylation and inhibited its chaperone association with AKs, leading to depletion of AKs and Survivin. Exposure of the siRNA to AK A also induced apoptosis, which was augmented by co-treatment with MK-0457 and vorinostat. Co-treatment with vorinostat enhanced MK-0457-mediated inhibition of the activities of Aurora A and Aurora B, leading to synergistic in vitro activity against human breast cancer cells. Co-treatment with MK-0457 and vorinostat also caused greater tumor growth inhibition and superior survival of mice bearing MDA-MB-231 xenografts. These pre-clinical findings indicate that combined treatment with a pan-AK inhibitor or an Aurora A-specific inhibitor and vorinostat represents a novel therapeutic strategy for the treatment of Aurora A-amplified and/or triple negative breast cancers.

Keywords Aurora kinase inhibitor · Vorinostat · Hsp90 · Triple negative breast cancer · Survivin

Introduction

The Aurora kinases (AKs), Aurora A, Aurora B, and Aurora C, are a family of evolutionarily conserved serine/threonine kinases [1, 2]. AKs function to regulate mitotic spindle formation, chromosome segregation, centrosome duplication, and cytokinesis during mitosis [3, 4]. The gene encoding Aurora A is on the chromosome 20 (20q13), a region that is frequently amplified in pancreatic, colon, bladder, ovarian, and breast cancers [5–7]. Overexpression of Aurora A in cancer cells leads to aberrant chromosome segregation, genomic instability, and activation of oncogenic pathways [3, 4, 6, 8]. In normal cells, ectopic overexpression of Aurora A causes transformation, leading to aberrant chromosome segregation, genomic instability, and activation of oncogenic pathways [6, 8, 9]. Recently, Aurora A and Aurora B were identified as part of a gene expression signature that predicted poor prognosis [10]. Novel targeted agents which inhibit the activities of Aurora A and/or Aurora B have been developed and are being tested for anti-tumor efficacy [1, 2, 11, 12]. MK-0457

W. Fiskus · S. L. Hembruff · R. Rao · P. Sharma · R. Balusu ·
S. Venkannagari · J. E. Smith · K. Peth · K. N. Bhalla (✉)
The University of Kansas Cancer Center, 3901 Rainbow Blvd.
Robinson Hall 4030, Mail Stop 1027, Kansas City, KS, USA
e-mail: kbhalla@kumc.edu

S. C. Peiper
Jefferson Medical College, Philadelphia, PA, USA

(VX-680) is a small molecule pan-AK inhibitor with inhibition constants (Ki) of 0.6, 18.0, and 4.6 nmol/L, respectively [2, 13, 14]. MK-0457 inhibits the auto-phosphorylation of Aurora A and Aurora B at threonine 288 and threonine 234, respectively [14, 15]. MK-0457 treatment is also known to deplete Histone H3 Serine 10 phosphorylation, inhibit cell division, cause misalignment of the chromosomes, and induce DNA polyploidy [13–15]. In transformed cells with mitotic checkpoint errors, MK-0457 treatment blocks cell cycle progression leading to accumulation of the cells with greater than 4N DNA content, as well as causes mitotic slippage (early exit from mitosis), thereby inducing apoptosis [14, 15].

Vorinostat is a class I and II histone deacetylase (HDAC) inhibitor (HDI) [16, 17]. Treatment with vorinostat alters the expression of up to 10 % of the genes in transformed cells [18, 19]. Vorinostat treatment induces p21 and p27, up-regulates pro-apoptotic proteins, e.g., BAX, BAK, and BIM, and depletes anti-apoptotic proteins e.g., BCL-2, BCL-xL, MCL-1, XIAP, and Survivin in breast cancer cells [20–22]. Vorinostat was shown to disrupt mitosis in breast cancer cells by inducing cell-cycle G2 phase accumulation and by inducing mitotic slippage in HeLa cells [21–23]. Treatment with pan-HDI such as vorinostat has also been shown to decrease the levels of p-Ser10 Histone H3 and induce accumulation of cells in the G2 phase of the cell cycle [24]. Further, by inhibiting HDAC6, a predominantly cytosolic HDAC known to de-acetylate hsp90, treatment with vorinostat induces hsp90 hyperacetylation, which inhibits the chaperone function of hsp90 [21, 25]. This directs the hsp90 client proteins, e.g., c-RAF, AKT, ERα, and HER2/neu, in breast cancer cells to polyubiquitylation and degradation by the 26S proteasome [20–22, 25]. Recently, AKs were shown to exhibit chaperone association with hsp90, and HDI-mediated inhibition of the chaperone function of hsp90 was shown to deplete the levels of AKs [26, 27]. Treatment with vorinostat also demonstrated in vivo anti-tumor activity against breast cancer models, and has been evaluated in phase I and II clinical trials in advanced malignancies [16, 17, 28–31]. The "triple negative" breast cancer (TNBC) is an aggressive form of breast cancer characterized by the lack of hormone receptors (estrogen and progesterone receptors) and HER2/neu expression, for which specific and effective systemic treatment options need to be developed [32, 33]. In the present studies, we determined the in vitro and in vivo anti-tumor activity of the combination of vorinostat and MK-0457 against "triple negative" and/or Aurora A-amplified breast cancer cells. Because MK-0457 is no longer in clinical development, we also determined the effects of treatment with an Aurora A-specific inhibitor, MLN8237 alone, and in combination with vorinostat against TNBCs.

Methods and materials

Reagents and antibodies

MK-0457 and vorinostat were kindly provided by Merck & Co., Inc. (North Wales, PA). The Aurora A-specific inhibitor, MLN8237, was obtained from Selleck Chemicals LLC (Houston, TX). All antibodies used here were obtained as previously described [25, 27].

Cell lines and cell culture

MDA-MB-231, MDA-MB-468, and BT-474 cells were obtained from ATCC and cultured as described [21, 22]. Logarithmically growing cells were used for the studies.

Cell cycle analysis

Following the designated treatments, cell cycle analysis was performed as previously described [27].

Assessment of apoptosis by annexin-V staining

Untreated or drug-treated cells were stained with annexin-V (Pharmingen, San Diego, CA) and propidium iodide (PI), and the percentage of apoptotic cells was determined by flow cytometry [21, 25, 27]. Combination indices for each drug combination was obtained by median dose effect analysis utilizing Calcusyn (Biosoft, Ferguson, MO) [34].

Cell lysis and protein quantitation

Untreated or drug-treated cells were harvested and cell pellets were washed with 1× PBS, then lysed, and quantitated as previously described [21, 27].

Immunoprecipitation of hsp90 and immunoblot analyses

Following the designated treatments, immunoprecipitation and immunoblotting of hsp90 was performed as previously described [25]. Immunoblot analyses with specific antibodies against acetyl-K69 hsp90, Aurora A, Aurora B, or total hsp90 levels were performed on the immunoprecipitates [27].

SDS-PAGE and immunoblot analyses

Western blot analyses of p-Aurora A/B, Aurora A, Aurora B, Survivin, pSer10 Histone H3, and Histone H3 were performed on total cell lysates using specific antisera or monoclonal antibodies as described [27, 35]. The expression levels of β-actin were used as the loading control for

the Western blots. Immunoblot analyses were performed at least twice and representative blots were subjected to densitometric analysis. Densitometry was performed using ImageQuant 5.2 (GE Healthcare, Piscataway, NJ).

In vivo model of breast cancer

MDA-MB-231 cells (5 million) were injected into the mammary fat pad of female NOD/SCID mice. Tumor growth was monitored by external caliper measurement. Treatment with MK-0457 and/or vorinostat was initiated when mean tumor volume was approximately 100 mm^3 for all groups. MK-0457 was diluted in 100 % DMSO and administered by intraperitoneal injection at 25 mg/kg IP twice daily 5 days per week for 2 weeks. Vorinostat was diluted in DMSO and administered by IP injection at a dose of 25 mg/kg once a day 5 days per week for 2 weeks. General condition of the mice was monitored daily. Mice were humanely euthanized when tumor volumes exceeded 1,500 mm^3. Survival of the mice is represented by Kaplan–Meier plot as previously described [36]. A log rank sum test was used to compare the differences in the survival of mice treated with vorinostat or MK-0457 and mice treated with the combination.

Statistical analysis

Significant differences between values obtained in a population of breast cancer cells treated with different experimental conditions were determined using the Student's t test. P-values of <0.05 were assigned significance. Differences between breast cancer cells transfected with siRNA followed by treatment with MK-0457 and vorinostat were determined by one-way ANOVA with a Tukey's multiple comparison test. P-values of <0.05 were assigned significance. For the in vivo studies, differences in the mean tumor volume between single agent treatment cohorts and the combination cohort were calculated with a two-tailed, paired t test. P-values <0.05 were assigned significance.

Results

Treatment with MK-0457 induces loss of Aurora kinase activity in breast cancer cells

We first determined the gene copy number of Aurora A in breast cancer cells. MDA-MB-231 and BT-474 cells contained increased copy numbers of the Aurora A gene (gene amplification) as determined by fluorescence in situ hybridization (FISH) utilizing a probe containing the entire Aurora A gene and an additional 71-kb of the downstream sequence of the long arm of chromosome 20 (20q13.2) (Fig. 1a). MDA-MB-468 cells did not exhibit any copy number alteration in the Aurora A gene. These findings were confirmed by CGH array (data not shown). We correlated our FISH results with immunoblot analyses of Aurora A protein expression in the same number of cells for each cell line. As demonstrated in Fig. 1b, MDA-MB-231 cells and BT-474 exhibit significantly greater expression of Aurora A per cell as compared to MDA-MB-468. Treatment with MK-0457 (20–100 nmol/L) inhibited Aurora kinase activity as measured by a decrease in levels of auto-phosphorylated Aurora A and depletion of phosphorylated Serine 10 on Histone H3 (Fig. 1c–e), a marker for Aurora B inhibition, while the levels of Aurora A and total Survivin were unaffected [2, 37, 38].

MK-0457 treatment induces cell cycle arrest, endoreduplication, apoptosis, and multi-polar spindle formation in human breast cancer cell lines

We next determined the effects of MK-0457 treatment on the cell cycle status of MDA-MB-231, MDA-MB-468, and BT-474. As demonstrated in Fig. 2a, treatment with MK-0457 dose-dependently induced accumulation of cells in the G2/M phase with a concomitant decline in the G1 and S phases of the cell cycle. Following 24 h of treatment with 100 nmol/L of MK-0457, greater than 80 % of cells were in the G2/M phase of the cell cycle in MDA-MB-231 and MDA-MB-468 cells. BT-474 cells were relatively less sensitive to the cell cycle effects of MK-0457. MK-0457 treatment also dose-dependently induced apoptosis in MDA-MB-231, MDA-MB-468, and BT-474 cells, with approximately 40 % of cells showing apoptosis, following exposure to 250 nmol/L of MK-0457 (Fig. 2b). The apoptotic effect on MDA-MB-468 plateaued at 50 nmol/L MK-0457, and following exposure to 250 nmol/L of MK-0457, MB-468 cells were as sensitive to the effects of MK-0457 as MDA-MB-231 cells (Fig. 2b). MDA-MB-231 cells treated with 50 nmol/L of MK-0457 for 24 h demonstrated an increase in the number of cells with 4N DNA content, with a 2.6-fold increase in the number of polyploid cells as well as an increase in the % apoptotic (sub-G1) cells (Fig. 2c). Treatment with MK-0457 for 48 h induced endoreduplication (cell with greater than 4N DNA content). Treatment with ≥20 nmol/L MK-0457 for 48 h also induced DNA endoreduplication in MDA-MB-468 cells (Online Resource 1). These effects were notable following exposure to 100 nmol/L of MK-0457, but declined after exposure to 250 nmol/L, most likely due to an increase in the % of apoptotic cells with less than 2N DNA content (sub-G1 cells) (Online Resource 1). We also determined
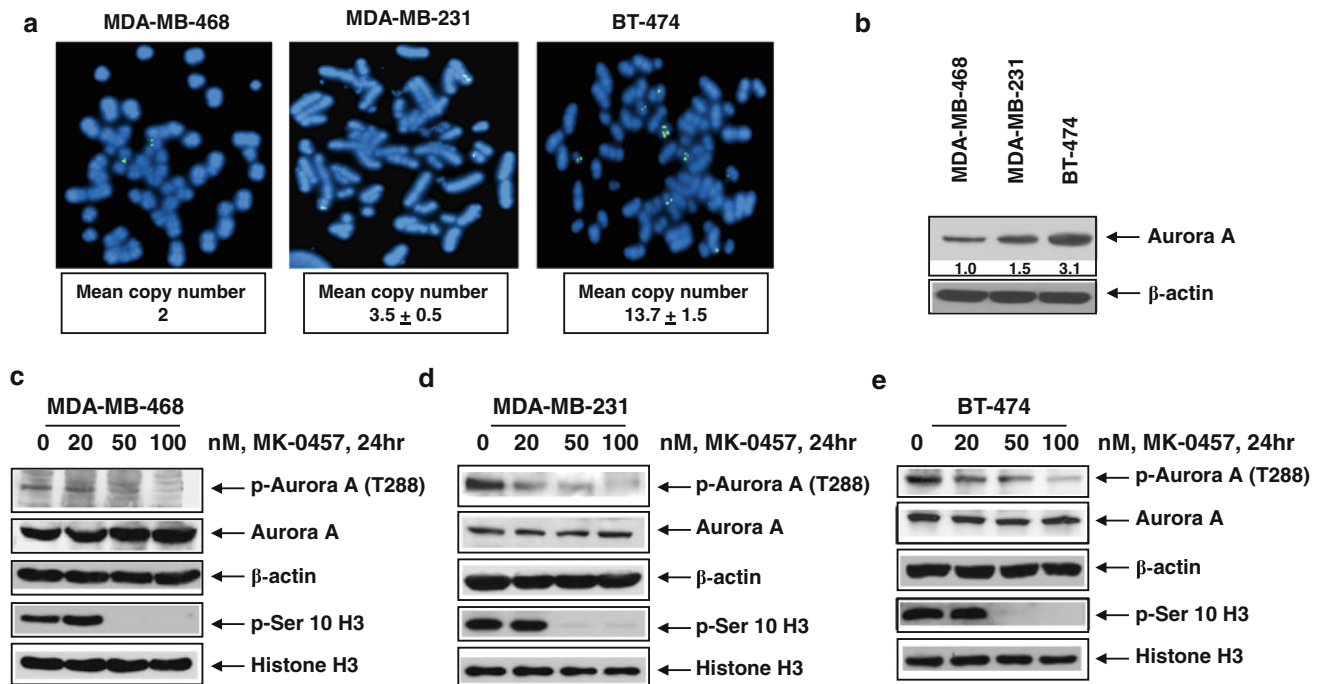
Fig. 1 Treatment with MK-0457 depletes the activity of Aurora kinases in triple negative and Aurora A-amplified breast cancer cells. **a** FISH analysis of the Aurora A locus in MDA-MB-468 (non-amplified), MDA-MB-231, and BT-474 cells. **b** Immunoblot analysis of Aurora A from 1 milli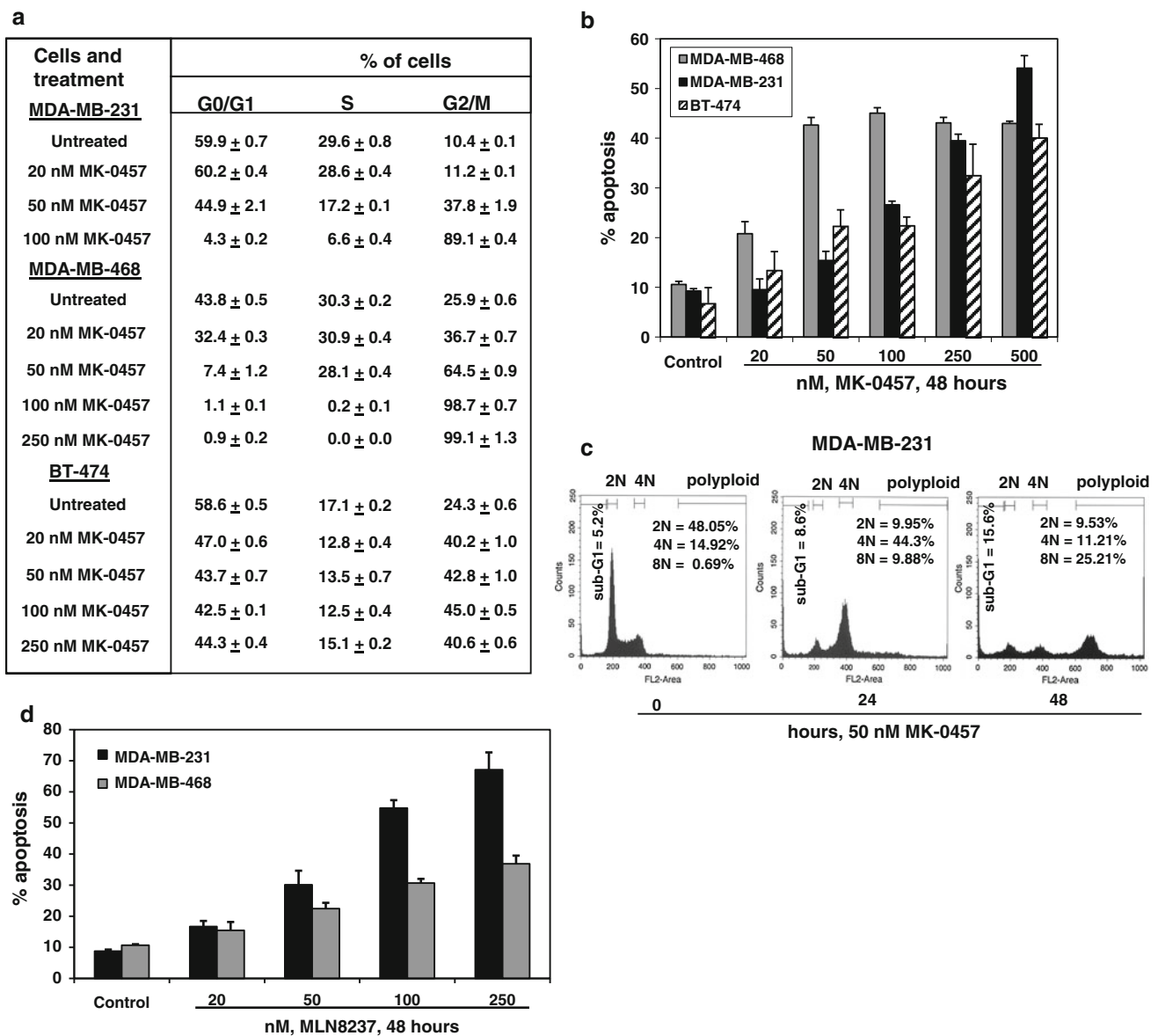on MDA-MB-468, MDA-MB-231, or BT-474 cells. Equal volume (15 μL) of cell lysate from each cell line was used for Aurora A determination. The expression levels of β-actin in the lysates served as the loading control. **c–e** Immunoblot analyses of p-Aurora A, Aurora A, Aurora B, p-Ser10 Histone H3, Histone H3, and β-actin after 24 h treatment with MK-0457

the effects of treatment with an Aurora A-specific inhibitor, MLN8237 (MLN), against MDA-MB-231 and MDA-MB-468 cells. Although treatment with MLN for 48 h induced dose-dependent apoptosis of both MDA-MB-231 and MDA-MB-468 cells, the MDA-MB-231 cells demonstrated significantly greater sensitivity compared to the MDA-MB-468 cells. In addition, treatment with 20–100 nmol/L MK-0457 or 0.5 μmol/L of vorinostat for 24 h resulted in the development of aberrant mitosis showing multiple spindle poles, as revealed by immunofluorescent staining of α-tubulin, Aurora A, and Aurora B followed by confocal immunofluorescent microscopy (Fig. 3; Online Resource 2). Aurora A localized to the mitotic spindle and the centrosomes, while Aurora B staining localized with the chromatin (Fig. 3). MK-0457 treatment had no effect on the staining intensities of Aurora A or Aurora B, whereas vorinostat treatment resulted in the loss of the signal intensity for Aurora A.

Vorinostat disrupts chaperone binding to hsp90 and depletes levels of Aurora A and Aurora B in breast cancer cells

We next determined the effects of vorinostat on Aurora A mRNA levels. Treatment with vorinostat for 16 h resulted in a 42 % decline in the levels of Aurora A mRNA in MDA-MB-231 cells (Fig. 4a). Vorinostat treatment for 24 h also markedly depleted Aurora A, Aurora B, and Survivin protein levels in a dose-dependent manner, which was associated with induction of hsp70 levels in the breast cancer cells (Fig. 4b). We next determined whether vorinostat-mediated depletion of Aurora A and Aurora B was due to inhibition of their chaperone association with hsp90. Figure 4c demonstrates that exposure to vorinostat for intervals as short as 8 h induced hsp90 hyperacetylation in MDA-MB-468 and MDA-MB-231 cells, as determined by immunoblot analyses utilizing an acetylated lysine 69 and lysine 100-specific hsp90 antibodies [25]. Hsp90 hyper-acetylation was associated with inhibition of its chaperone function and abrogation of the binding of Aurora A and Aurora B with hsp90 (Fig. 4c). Similar findings were observed in BT-474 cells treated with vorinostat (Online Resource 3a). Following exposure to vorinostat for 8 h, we also observed depletion of the levels of Aurora in total cell lysates as well as induction of hsp70 (Fig. 4c). We next determined whether vorinostat-mediated depletion of Aurora A and Aurora B was due to proteasomal degrada-tion. Figure 4d demonstrates that, in MDA-MB-231 cells, depletion of Aurora A, Aurora B, Survivin, and c-Raf by vorinostat was partially restored by co-treatment with the proteasome inhibitor bortezomib (BZ). Levels of the hsp90 client protein c-Raf served as a positive control. These data

a

Cells and treatment	% of cells		
MDA-MB-231	**G0/G1**	**S**	**G2/M**
Untreated	59.9 ± 0.7	29.6 ± 0.8	10.4 ± 0.1
20 nM MK-0457	60.2 ± 0.4	28.6 ± 0.4	11.2 ± 0.1
50 nM MK-0457	44.9 ± 2.1	17.2 ± 0.1	37.8 ± 1.9
100 nM MK-0457	4.3 ± 0.2	6.6 ± 0.4	89.1 ± 0.4
MDA-MB-468			
Untreated	43.8 ± 0.5	30.3 ± 0.2	25.9 ± 0.6
20 nM MK-0457	32.4 ± 0.3	30.9 ± 0.4	36.7 ± 0.7
50 nM MK-0457	7.4 ± 1.2	28.1 ± 0.4	64.5 ± 0.9
100 nM MK-0457	1.1 ± 0.1	0.2 ± 0.1	98.7 ± 0.7
250 nM MK-0457	0.9 ± 0.2	0.0 ± 0.0	99.1 ± 1.3
BT-474			
Untreated	58.6 ± 0.5	17.1 ± 0.2	24.3 ± 0.6
20 nM MK-0457	47.0 ± 0.6	12.8 ± 0.4	40.2 ± 1.0
50 nM MK-0457	43.7 ± 0.7	13.5 ± 0.7	42.8 ± 1.0
100 nM MK-0457	42.5 ± 0.1	12.5 ± 0.4	45.0 ± 0.5
250 nM MK-0457	44.3 ± 0.4	15.1 ± 0.2	40.6 ± 0.6

b

c MDA-MB-231

d

Fig. 2 MK-0457 induces G2/M arrest, apoptosis, and endoreduplication in human breast cancer cells. **a** Cell cycle status of MDA-MB-231, MDA-MB-468, and BT-474 cells treated with the indicated concentrations of MK-0457 for 24 h. Values represent the mean of three experiments ±SEM. **b** Percentage of apoptotic cells following 48 h treatment with MK-0457. **c** Percentage of 2, 4, and 8N cells following 24 and 48 h treatment with MK-0457. The percentage of cells with less than 2N DNA content (Sub-G1) cells was also calculated. Values represent the mean of three experiments. **d** Percentage of apoptotic cells following 48 h treatment with MLN8237. *Columns represent* the mean of three experiments; Bars represent the standard error of the mean

indicate that vorinostat-mediated depletion of Aurora A and Aurora B is at least partially due to the disruption of chaperone association of Aurora A and Aurora B with hsp90 leading to their degradation by the 26S proteasome.

Vorinostat enhances Aurora kinase inhibitor-mediated apoptosis in breast cancer cells

We next determined the effects of vorinostat and/or MK-0457 on the levels and activity of AKs. Figure 5a

demonstrates that treatment with 1.0 μmol/L of vorinostat alone attenuated the levels of Serine 10-phosphorylated Histone H3 in MDA-MB-231 and BT-474, associated with the decline in the levels of Aurora B and Aurora A (Fig. 5a; Online Resource 3b). Consistent with the decline in Aurora kinase A and B levels and activities, vorinostat attenuated total Survivin levels. Notably, co-treatment with vorinostat (1.0 μmol/L) and MK-0457 (20–50 nmol/L) caused greater depletion of pSer10 on Histone H3 than treatment with either agent alone (Fig. 5a). As compared to
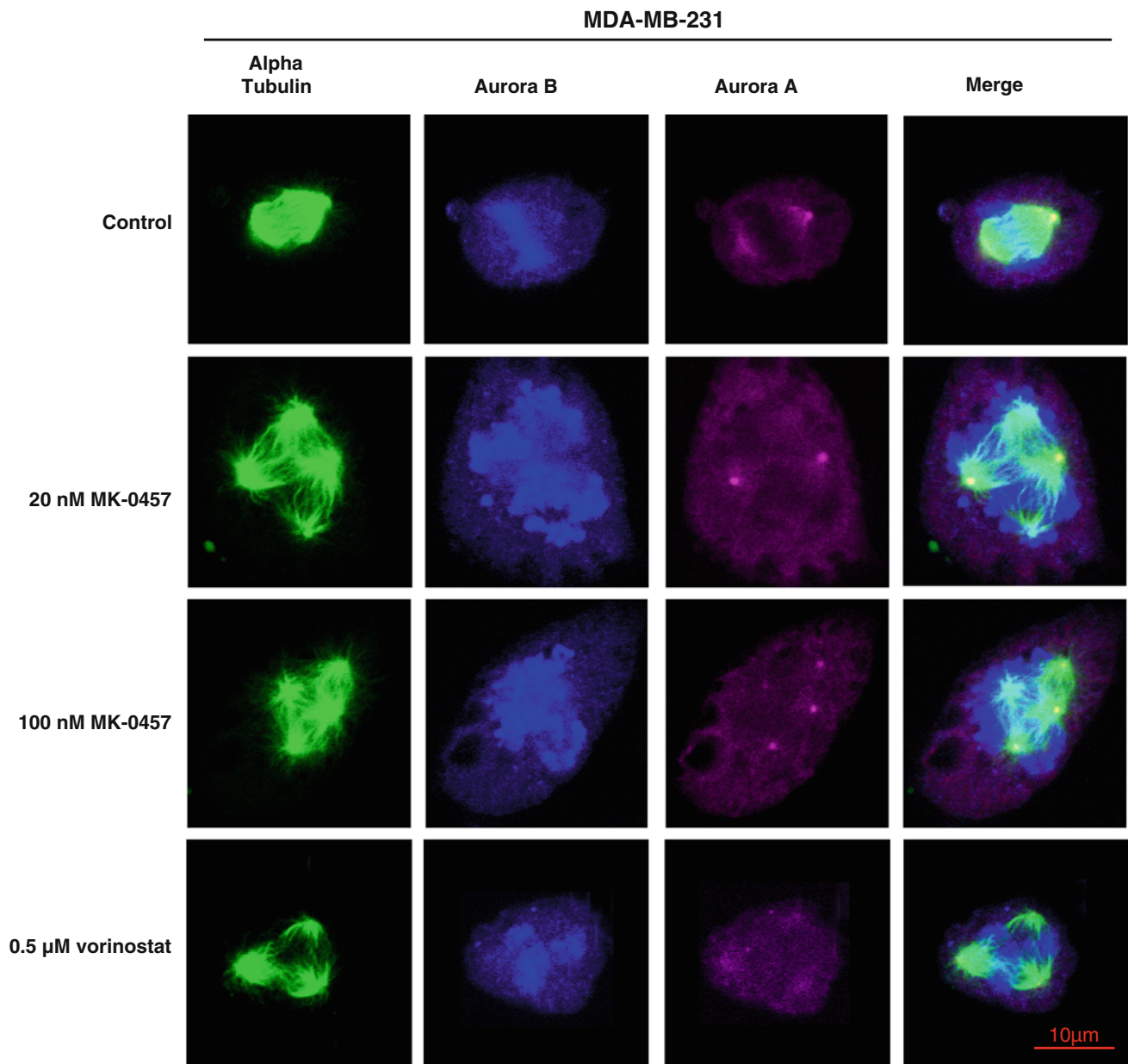
Fig. 3 MK-0457 or vorinostat treatment induces multi-polar spindle formation in breast cancer cells. Confocal immunofluorescent microscopy of α-tubulin, Aurora A, and Aurora B following 24 h treatment with each agent alone, co-treatment with vori- nostat also significantly enhanced MK-0457-induced apoptosis of MDA-MB-231 cells (Fig. 5b) and BT-474 cells (Online Resource 3b) (*P* < 0.05). Notably, combined treatment with vorinostat and MK-0457 synergistically induced apoptosis of MDA-MB-231 (Fig. 5c) and BT-474 cells (Online Resource 3c), as determined by the median dose effect analysis described by Chou and Talalay (Fig. 5c; Online Resource 3d). For MK-0457 and vorino- stat, the combination index (CI) values were less than 1.0 for all the tested doses of the two drugs. Similar synergistic

treatment with MK-0457 or vorinostat. Cells were imaged by confocal microscopy with a 63 × 1.2 NA objective. *Scale bar* indicates 10 μm

effects of the combination were also noted against MDA- MB-468 cells (data not shown). We also determined the effects of combined treatment with vorinostat and MLN8237 against MDA-MB-231 and MDA-MB-468 cells. Similar to the effects observed with MK-0457, combined treatment with vorinostat and MLN8237 synergistically induced apoptosis of MDA-MB-231 (Fig. 5d; Online Resource 4a) and MDA-MB-468 cells (Online Resource 4b–c). This was associated with greater decline in p-Aurora A and Aurora B, and greater depletion of pSer10 on His- tone H3 (Fig. 5e).
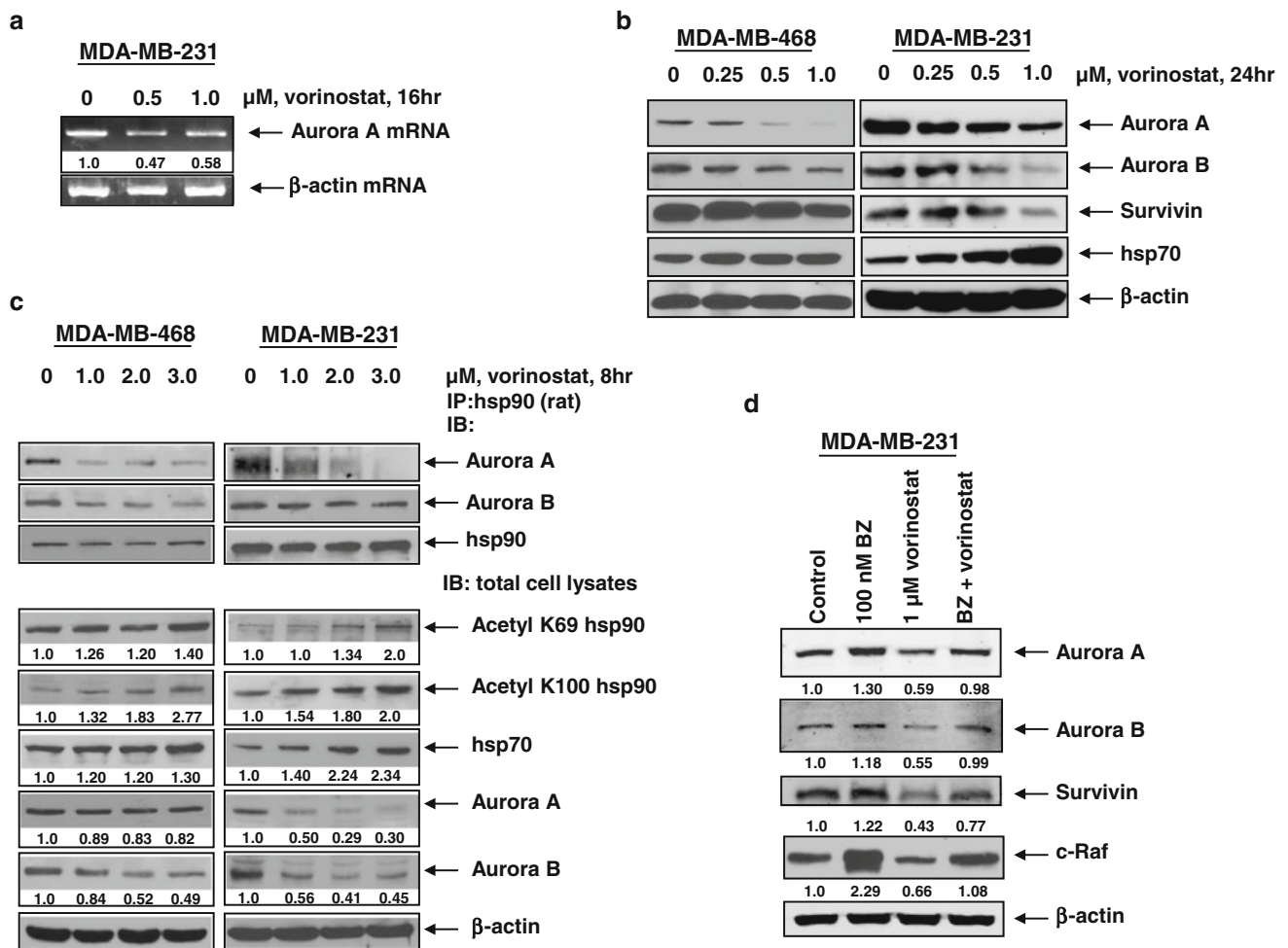
Fig. 4 Vorinostat depletes Aurora kinase levels partly through inhibition of chaperone association with hsp90 and proteasomal degradation. **a** RT-PCR for Aurora A following 16 h treatment with vorinostat. **b** Immunoblot analyses of Aurora A, Aurora B, Survivin, hsp70, and β-actin, following 24 h treatment with vorinostat. **c** Immunoblot analyses of Aurora A, Aurora B, Acetyl K69 hsp90, and hsp90 in immunoprecipitates of hsp90 following 8 h treatment with vorinostat. Immunoblot analyses were also performed on the total cell lysates. The expression levels of β-actin in the lysates served as the loading control. **d** Immunoblot analyses of Aurora A, Aurora B, Survivin, c-Raf, and β-actin following 8 h treatment with vorinostat and/or bortezomib. The *numbers underneath the bands* represent densitometric evaluation of the proteins relative to the control cells

Depletion of Aurora A by siRNA enhances MK-0457 and vorinostat-mediated cytotoxicity in MDA-MB-231 cells

We next determined whether siRNA-mediated depletion of Aurora A would have similar effects as vorinostat on MK-0457-mediated inhibition of Aurora kinase activity. Exposure to siRNA for 48 h was more effective than 72 h in depleting Aurora A levels (Fig. 6a). Notably, Aurora A knockdown by siRNA followed by treatment with vorinostat resulted in further depletion of Aurora A levels (Fig. 6b). Knockdown of Aurora A by siRNA resulted in approximately twofold increase in the sensitivity of MDA-MB-231 cells to 50 and 250 nmol/L of MK-0457 compared to control siRNA transfected cells ($P < 0.05$ by one-way ANOVA with Tukey's multiple comparison test) (Fig. 6c).

Knockdown of Aurora A also enhanced vorinostat-induced cell death compared to control siRNA transfected cells ($P < 0.05$ by one-way ANOVA with Tukey's multiple comparison test), as determined by % increase in the PI (propidium iodide) stained cells, although this effect was not as pronounced as co-treatment with MK-0457 (Fig. 6d).

Co-treatment with MK-0457 and vorinostat exerts greater in vivo anti-tumor activity against breast cancer

We next determined the effects of MK-0457 and/or vorinostat against breast cancer xenografts implanted into the mammary fat pad of female NOD/SCID mice. As shown in Fig. 7a, as compared to vehicle alone, treatment with MK-0457 delayed tumor growth over the duration of the
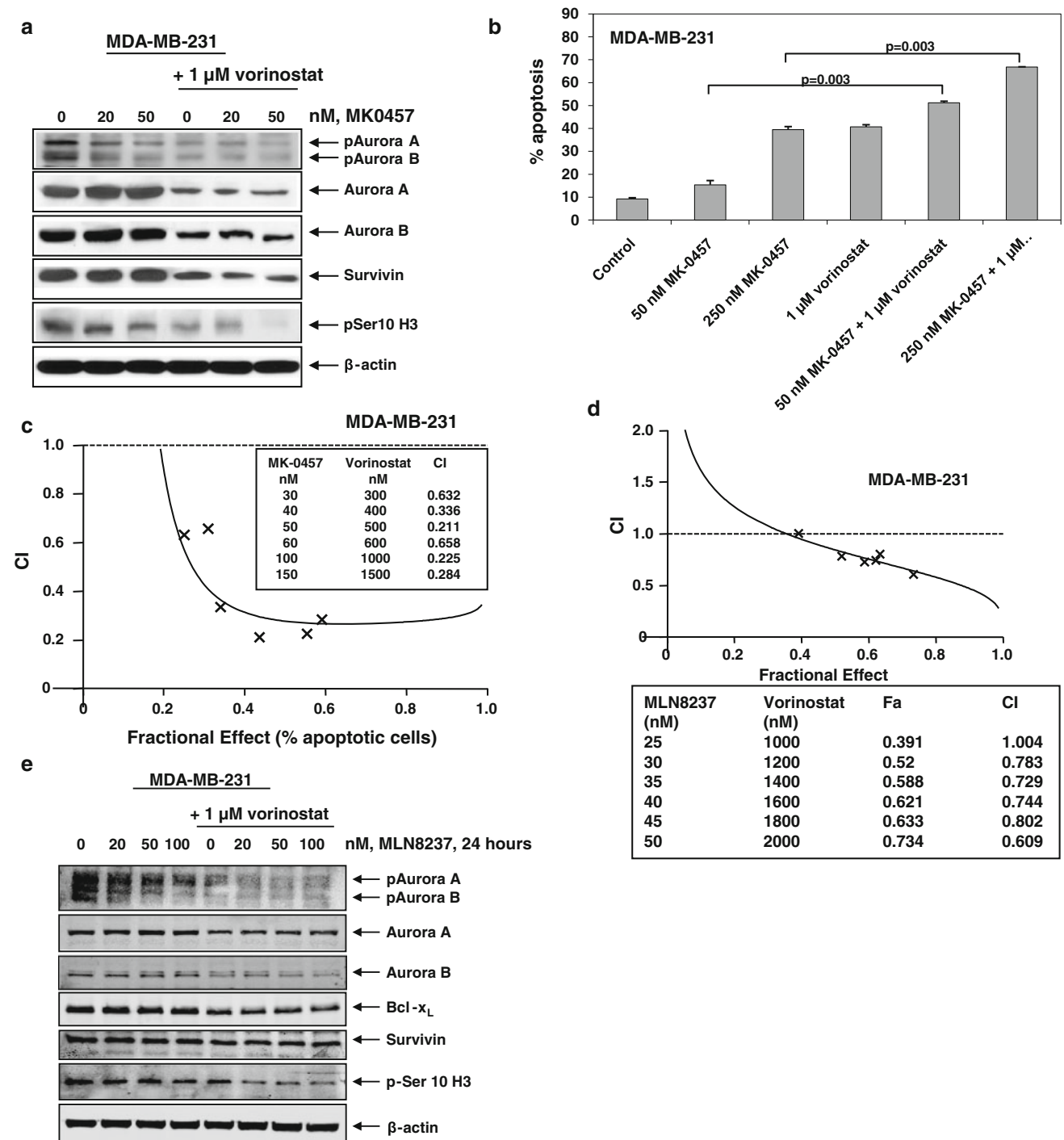
Fig. 5 Co-treatment with vorinostat and an Aurora kinase inhibitor further depletes Aurora kinase activity and induces synergistic apoptosis of Aurora A-amplified breast cancer cells. **a** Immunoblot analyses of p-Aurora A, Aurora A, Aurora B, Survivin, and p-Ser10 Histone H3 and β-actin from MDA-MB-231 cells treated with MK-0457 and/or vorinostat for 24 h. **b** Percentage of annexin-V positive apoptotic cells following 48 h treatment with MK-0457 and/or vorinostat. *Values* represent the mean of three experiments ±SEM.

c–d Isobologram analyses of MDA-MB-231 cells treated with MK-0457 or MLN8237 and vorinostat for 48 h. Combination indices (CI) were calculated utilizing the commercially available software Calcusyn. CI values less than 1.0 indicate synergism of the two agents against breast cancer cells. **e** Immunoblot analyses of p-Aurora A, Aurora A, Aurora B, Survivin, Bcl-x_L, p-Ser10 Histone H3, and β-actin from MDA-MB-231 cells treated with MLN8237 and/or vorinostat for 24 h
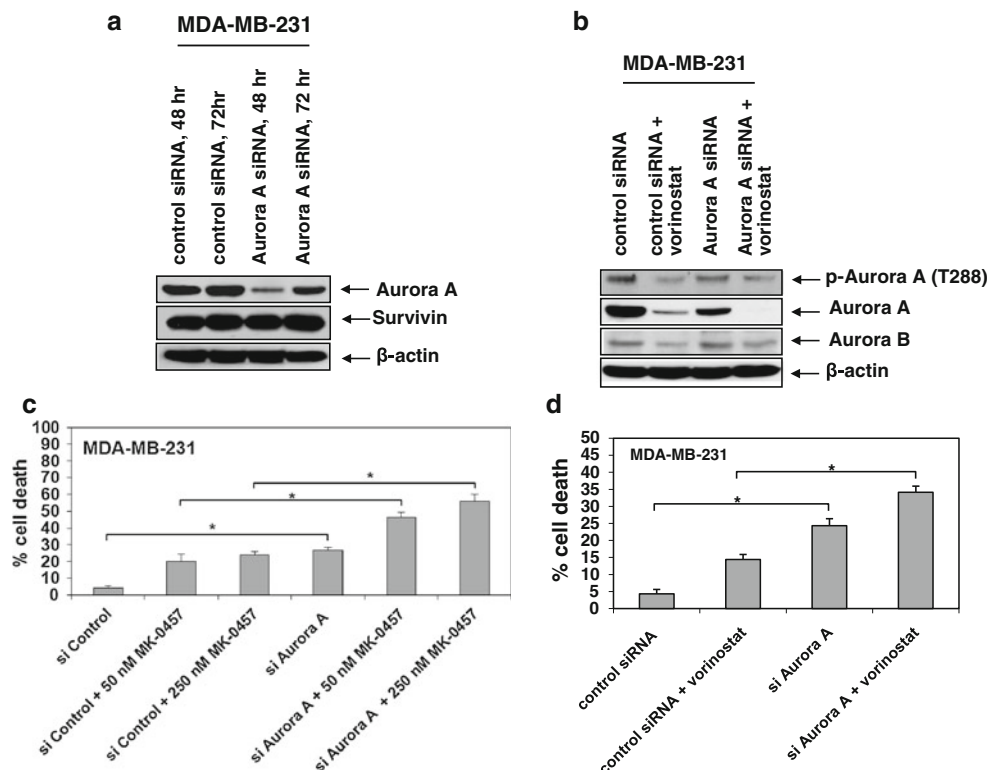
Fig. 6 Depletion of Aurora A by siRNA enhances MK-0457 or vorinostat-mediated cell death of Aurora A-amplified breast cancer cells. **a** Immunoblot analyses of Aurora A, Survivin, and β-actin in cells transfected with scrambled or Aurora A siRNA. **b** Immunoblot analyses of p-Aurora A, Aurora A, Aurora B, and β-actin in cells transfected with siRNA for 48 h and treated with vorinostat for 24 h. **c–d** Cell death of MDA-MB-231 cells transfected with Aurora A siRNA and then treated with vorinostat (1 μM) or MK-0457 (50 and 250 nM) for 48 h measured by PI staining. *Values* represent the mean of three experiments ±SEM. *indicates cell death values significantly greater ($P < 0.05$ as determined by one-way ANOVA with a Tukey's multiple comparison test) in Aurora A siRNA transfected cells compared to control siRNA transfected cells treated with MK-0457 or vorinostat

treatments. Further, co-treatment with MK-0457 and vorinostat resulted in significantly greater inhibition of tumor growth than treatment with each agent alone ($P < 0.05$ by two-tailed, paired t test). In order to determine the effect of MK-0457 and/or vorinostat on the survival of the tumor-bearing mice, mice with established tumors (~ 100 mm³) were treated for 2 weeks, during which the tumor growth was monitored. The survival data were plotted in a Kaplan–Meier plot (Fig. 7b). As shown, co-treatment with MK-0457 and vorinostat yielded significantly superior survival compared to treatment with either agent alone (MK-0457 vs combination [$P = 0.031$]; vorinostat vs combination [$P = 0.001$]). (Fig. 7b). At the dosage administered, treatment with MK-0457 and/or vorinostat did not induce any weight loss or other notable toxicity. As shown in Fig. 7c, co-treatment with MK-0457 and vorinostat caused greater attenuation in the levels of p-Aurora, Aurora A, and Survivin than treatment with either agent alone. It is noteworthy that treatment with vorinostat induced in vivo hyperacetylation of hsp90 in the tumors, as demonstrated by immunoprecipitation and immunoblot analyses with the site-specific anti-

acetylated K69 or K100 hsp90α antibodies (Fig. 7d). Immunoblot analyses on the total cell lysates from the harvested tumors confirmed the immunoprecipitation findings. It is also noteworthy that in a recent trial of vorinostat administered to patients with metastatic breast cancer, vorinostat was demonstrated to induce in vivo hyperacetylation of hsp90 and inhibition of hsp90 chaperone function, as evidenced by depletion of AKT and c-RAF levels in the tumor biopsy samples obtained from patients who had surgically accessible tumors [39, 40].

Discussion

In the present studies, we demonstrate that treatment with the pan-AK inhibitor MK-0457 causes marked accumulation of the cultured TNBC and Aurora A-amplified breast cancer cells in the G2/M phase of the cell cycle, while also inducing endoreduplication of the DNA, multi-polar mitotic spindle formation, mitotic slippage, and apoptosis. MK-0457 is known to induce endoreduplication in cells
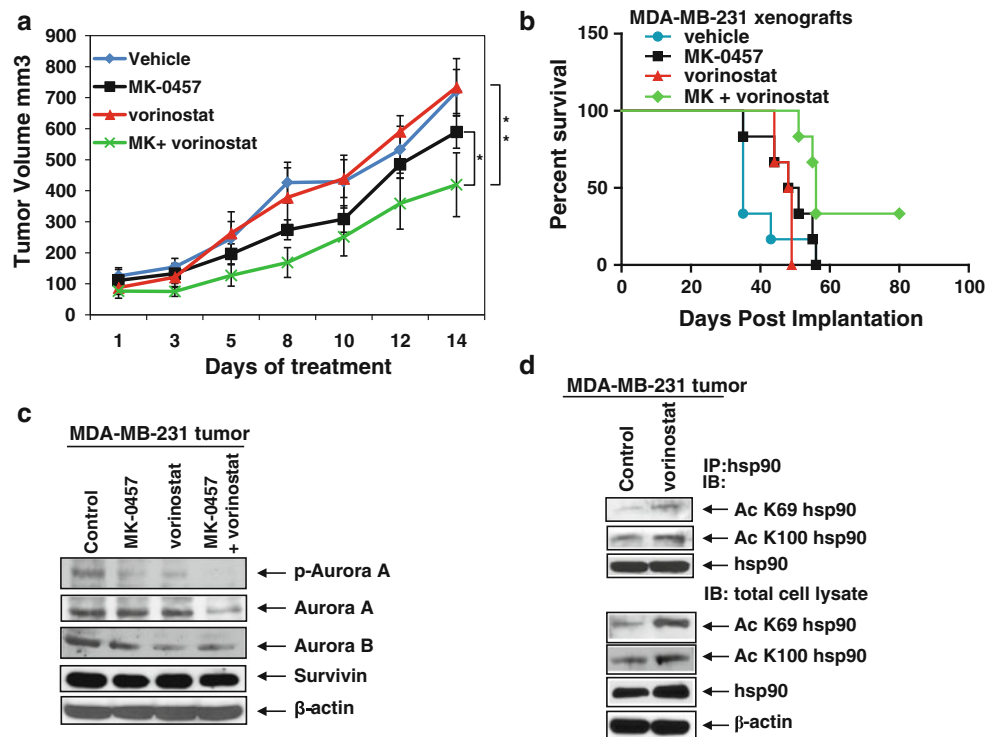
Fig. 7 Co-treatment with MK-0457 and vorinostat decreases tumor growth and improves survival in mouse models of breast cancer. **a** 5 million MDA-MB-231 cells were implanted into the mammary fat pad of NOD/SCID mice. Treatment commenced 13 days after implantation of the cells, when palpable tumors (mean volume 100 mm³) were evident in the animals (designated as day 1). Mice were treated with 25 mg/kg of vorinostat and/or 25 mg/kg of MK-0457 for 14 days. *Data* are presented as mean tumor volume (±SE) of the animals in each treatment group. *indicates tumor growth values significantly less ($P < 0.05$ by two-tailed paired t test) in combination than treatment with MK-0457 alone. **indicates tumor growth values significantly less ($P < 0.05$ by two-tailed paired t test) in combination

than treatment with vorinostat alone. **b** Kaplan–Meier plot of survival for mice treated with MK-0457 and/or vorinostat. Combined treatment with MK-0457 and vorinostat significantly prolongs survival of mice compared to treatment with either agent alone (MK-0457 vs combination [$P = 0.031$]; vorinostat vs combination [$P = 0.001$] by log rank sum test). **c** Immunoblot analyses of p-Aurora A, Aurora A, Aurora B, and Survivin on cell lysates extracted from the tumors after 14 days of treatment with 25 mg/kg of MK-0457 and/or 25 mg/kg of vorinostat. **d** Representative immunoblot of hsp90 acetylation in hsp90 immunoprecipitates or whole cell lysates extracted from the tumors after 14 days of treatment with vorinostat

with a compromised post mitotic checkpoint and allows cells to proceed through S-phase without undergoing cell division and cytokinesis [15, 21]. Cells which fail to divide accumulate greater than 8N DNA content and ultimately undergo cell death [15, 21]. It is noteworthy that in our studies the effects of MK-0457 were seen regardless of the copy numbers and gene amplification of Aurora A in the breast cancer cells. Treatment with MK-0457 inhibited p-Aurora A and p-Aurora B levels in the breast cancer cells, suggesting that it inhibits the autophosphorylation activities of Aurora A and Aurora B. MDA-MB-468 cells (without Aurora A amplification) were more sensitive than MDA-MB-231 cells (with Aurora A amplification) to the lower concentrations of MK-0457 for the induction of apoptosis. However, this difference was eliminated following exposure to higher concentration of MK-0457 (250 nmol/L). It also appears that MK-0457 had similar effects on Aurora B activity, since MK-0457 treatment attenuated p-Ser10 on Histone H3, a known substrate of

Aurora B in the breast cancer cells [1, 2]. We also demonstrated that treatment with the Aurora A-specific inhibitor MLN8237, induced dose-dependent apoptosis more in breast cancer cells with Aurora A amplification than cells without Aurora A amplification.

Clinically achievable concentrations of vorinostat induced lysine-specific (lysine 69 and lysine 100) hyperacetylation of hsp90. Vorinostat-mediated hsp90 acetylation caused inhibition of the chaperone function of hsp90 as demonstrated by induction of hsp70 levels. This was associated with inhibition of chaperone association with hsp90, proteasomal degradation, and depletion of the levels and activity of Aurora A and Aurora B. Following co-treatment with the proteasome inhibitor bortezomib, incomplete restoration of vorinostat-mediated depletion of Aurora A and Aurora B levels suggests that attenuation of the mRNA levels contributes to the overall decline in the levels of Aurora A and Aurora B by vorinostat in the breast cancer cells. In the present studies, we demonstrate for the first time

that combined treatment with vorinostat and MK-0457 or MLN8237 synergistically induces apoptosis of TNBC and Aurora A-amplified breast cancer cells. This was associated with greater decline in the levels of p-Aurora A, Aurora B, and p-Ser10 Histone H3. Targeted attenuation of Aurora A contributed to the synergistic effect of the combination, since siRNA-mediated knockdown of Aurora A induced cell death of MDA-MB-231 cells, which was further enhanced by co-treatment with vorinostat. It is noteworthy that combined treatment with Aurora A siRNA and MK-0457 treatment induced more cell death than treatment with each agent alone. These findings are consistent with our previous report showing that co-treatment with vorinostat, which also lowers the levels of HER-2, significantly enhances the activity of trastuzumab against HER2-amplified breast cancer cells [22].

Present findings also demonstrate that vorinostat induces in vivo hyperacetylation of hsp90 in the MDA-MB-231 breast cancer xenografts. This was also observed in primary breast cancer samples collected from patients receiving treatment with vorinostat [39, 40]. As compared to the treatment of the mice orthotopically implanted with MDA-MB-231 cells with each agent alone, combined treatment with vorinostat and MK-0457 caused greater in vivo depletion of p-Aurora A, Aurora B, and Survivin levels in the MDA-MB-231 tumors. Importantly, co-treatment with MK-0457 and vorinostat resulted in greater tumor growth inhibition and significant improvement in the overall survival of the tumor-bearing mice. It is likely that greater depletion of Aurora A, Aurora B, and Survivin contributes to the superior anti-tumor activity of the combination. Also, in breast cancer cells, by inhibiting hsp90 chaperone function, vorinostat has been shown to deplete pro-survival signaling proteins such as c-RAF and AKT [21, 22]. In addition, as previously reported, treatment with vorinostat also induces pro-death proteins including BIM and depletes pro-survival proteins including BCL-2, BCL-x_L, and MCL-1, which could lower the threshold for apoptosis and further sensitize breast cancer cells to apoptosis induced by depletion of the levels and activity of Aurora A and Aurora B [25]. Collectively, these observations explain why the combination of vorinostat and Aurora kinase inhibition (MK-0457 or MLN8237) exerts superior in vitro and in vivo activity against breast cancer cells. Since the superior activity of the combination extended to TNBC and/or Aurora A-amplified breast cancer cells, our preclinical findings presented here support the rationale to test co-treatment with Aurora kinase inhibitors and vorinostat as a novel therapeutic strategy in advanced TNBC and/or Aurora A-amplified breast cancers.

Ethical standards All experiments performed in this manuscript are in compliance with the current laws of the United States.

Conflict of interest All authors declare that they have no competing financial interests.

References

1. Mountzios G, Terpos E, Dimopoulos MA (2008) Aurora kinases as targets for cancer therapy. Cancer Treat Rev 34:175–182
2. Dar AA, Goff LW, Majid S, Berlin J, El-Rifai W (2010) Aurora kinases inhibitors—rising stars in cancer therapeutics? Mol Cancer Ther 9:268–278
3. Fu J, Bian M, Jiang Q, Zhang C (2007) Roles of Aurora kinases in mitosis and tumorigenesis. Mol Cancer Res 5:1–10
4. Marumoto T, Zhang D, Saya H (2005) Aurora-A—a guardian of poles. Nat Rev Cancer 5:42–50
5. Fukushige S, Waldman FM, Kimura M, Abe T, Furukawa T, Sunamura M et al (1997) Frequent gain of copy number on the long arm of chromosome 20 in human pancreatic adenocarcinoma. Genes Chromosomes Cancer 19:161–169
6. Nishida N, Nagasaka T, Kashiwagi K, Boland CR, Goel A (2007) High copy amplification of the Aurora-A gene is associated with chromosomal instability phenotype in human colorectal cancers. Cancer Biol Ther 6:525–533
7. Zhou H, Kuang J, Zhong L, Kuo WL, Gray JW, Sahin A et al (1998) Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nat Genet 20:189–193
8. Wang X, Zhou YX, Qiao W, Tominaga Y, Ouchi M, Ouchi T et al (2006) Overexpression of Aurora kinase A in mouse mammary epithelium induces genetic instability preceding mammary tumor formation. Oncogene 25:7148–7158
9. Hontz AE, Li SA, Lingle WL, Negron V, Bruzek A, Salisbury JL et al (2007) Aurora A and Aurora B overexpression and centrosome amplification in early estrogen-induced tumor foci in the Syrian hamster kidney: implications for chromosomal instability, aneuploidy and neoplasia. Cancer Res 67:2957–2963
10. Finetti P, Cervera N, Charafe-Jauffret E, Chabannon C, Charpin C, Chaffanet M et al (2008) Sixteen-kinase gene expression identifies luminal breast cancers with poor prognosis. Cancer Res 68:767–776
11. Kollareddy M, Zheleva D, Dzubak P, Brahmkshatriya PS, Lepsik M, Hajduch M (2012) Aurora kinase inhibitors: progress towards the clinic. Invest New Drugs. doi:10.1007/s10637-012-9798-6
12. Schmit TL, Ahmad N (2007) Regulation of mitosis via mitotic kinases: new opportunities for cancer management. Mol Cancer Ther 6:1920–1931
13. Harrington EA, Bebbington D, Moore J, Rasmussen RK, Ajose-Adeogun AO, Nakayama T et al (2004) VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo. Nat Med 10:262–267
14. Tyler RK, Shpiro N, Marquez R, Eyers PA (2007) VX-680 inhibits Aurora A and Aurora B kinase activity in human cells. Cell Cycle 6:2846–2854
15. Gizatullin F, Yao Y, Kung V, Harding MW, Loda M, Shapiro GI (2006) The Aurora kinase inhibitor VX-680 induces endoreduplication and apoptosis preferentially in cells with compromised p53-dependent postmitotic checkpoint function. Cancer Res 66:7668–7677
16. Richon VM (2006) Cancer biology: mechanism of antitumor action of vorinostat (suberoylanilide hydroxamic acid), a novel histone deacetylase inhibitor. Br J Cancer 95:S2–S6
17. Siegel D, Hussein M, Belani C, Robert F, Galanis E, Richon VM et al (2009) Vorinostat in solid and hematologic malignancies. J Hematol Oncol 2:31

18. Khan N, Jeffers M, Kumar S, Hackett C, Boldog F, Khramtsov N et al (2008) Determination of the class and isoform selectivity of small-molecule histone deactylase inhibitors. Biochem J 409:581–589

19. Minucci S, Pelicci PG (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer 6:38–51

20. Fuino L, Bali P, Wittmann S, Donapaty S, Guo F, Yamaguchi H et al (2003) Histone deacetylase inhibitor LAQ824 down-regulates Her-2 and sensitizes human breast cancer cells to trastuzumab, taxotere, gemcitabine and epothilone B. Mol Cancer Ther 10:971–984

21. Fiskus W, Ren Y, Mohapatra A, Bali P, Mandawat A, Rao R et al (2007) Hydroxamic acid analogue histone deacetylase inhibitors attenuate estrogen receptor-alpha levels and transcriptional activity: a result of hyperacetylation and inhibition of chaperone function of heat shock protein 90. Clin Cancer Res 13:4882–4890

22. Bali P, Pranpat M, Swaby R, Fiskus W, Yamaguchi H, Balasis M et al (2005) Activity of suberoylanilide hydroxamic acid against human breast cancer cells with amplification of Her-2. Clin Cancer Res 11:6382–6389

23. Stevens FE, Beamish H, Warrener R, Gabrielli B (2008) Histone deacetylase inhibitors induce mitotic slippage. Oncogene 27:1345–1354

24. Li Y, Kao GD, Garcia BA, Shabanowitz J, Hunt DF, Qin J et al (2006) A novel histone deacetylase pathway regulates mitosis by modulating Aurora B kinase activity. Genes Dev 20:2566–2579

25. Yang Y, Rao R, Shen J, Tang Y, Fiskus W, Nechtman J et al (2008) Role of acetylation and extracellular location of heat shock protein 90alpha in tumor cell invasion. Cancer Res 68:4833–4842

26. Park JH, Jong HS, Kim SG, Jung Y, Lee KW, Lee JH et al (2008) Inhibitors of histone deacetylases induce tumor-selective cytotoxicity through modulating Aurora-A kinase. J Mol Med 86:117–128

27. Fiskus W, Wang Y, Joshi R, Rao R, Yang Y, Chen J et al (2008) Co-treatment with vorinostat enhances activity of MK-0457 (VX-680) against acute and chronic myelogenous leukemia cells. Clin Cancer Res 14:6106–6115

28. Butler LM, Agus DB, Scher HI, Higgins B, Rose A, Cordon-Cardo C et al (2000) Suberoylanilide hydroxamic acid, an inhibitor of histone deacetylases, suppresses the growth of prostate cancer cells in vitro and in vivo. Cancer Res 60:5165–5170

29. Cohen LA, Marks PA, Rifkind RA, Amin S, Desai D, Pittman B et al (2002) Suberoylanalide hydroxamic acid (SAHA), a histone deacetylase inhibitor, suppresses the growth of carcinogen-induced mammary tumors. Anticancer Res 22:1497–1504

30. Kelly WK, O' Connor OA, Krug LM, Chiao JH, Heaney M, Curley T (2005) Phase I study of an oral histone deacetylase inhibitor in patients with advanced cancer. J Clin Oncol 23:3923–3931

31. Kirschbaum M, Frankel P, Popplewell L, Zain J, Delioukina M, Pullarkat V et al (2011) Phase II study of vorinostat for treatment of relapsed or refractory indolent non-Hodgkin's lymphoma and mantle cell lymphoma. J Clin Oncol 29:1198–1203

32. Pal SK, Childs BH, Pegram M (2011) Triple negative breast cancer: unmet medical needs. Breast Cancer Res Treat 125:627–636

33. Foulkes WD, Smith IE, Reis-Filho JS (2010) Triple negative breast cancer. New Engl J Med 363:1938–1948

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

35. Rao R, Nalluri S, Kolhe R, Yang Y, Fiskus W, Chen J et al (2010) Treatment with panobinostat induces glucose regulated protein 78 (GRP78) acetylation and endoplasmic reticulum stress in breast cancer cells. Mol Cancer Ther 9:942–952

36. Fiskus W, Wang Y, Sreekumar A, Buckley K, Shi H, Jillella A et al (2009) Combined epigenetic therapy with the histone methyltransferase EZH2 inhibitor 3-deazaneplanocin A and the histone deacetylase inhibitor panobinostat against human AML cells. Blood 114:2733–2743

37. Manfredi MG, Ecsedy JA, Meetze KA, Balani SK, Burenkova O, Chen W et al (2007) Antitumor activity of MLN8054, an orally active small-molecule inhibitor of Aurora A kinase. Proc Natl Acad Sci USA 104:4106–4111

38. Wilkinson RW, Odedra R, Heaton SP, Wedge SR, Keen NJ, Crafter C 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

39. Ramaswamy B, Bhalla K, Cohen B, Pelligrino C, Hershman D, Chuang E, et al (2009) Phase I–II study of the histone deacetylase inhibitor vorinostat plus paclitaxel and bevacizumab in patients with metastatic breast cancer: New York Cancer Consortium trial P7703. Proc Am Assoc Cancer Res. Apr 18–22, Denver CO, Philadelphia, AACR; 2009: Abstract 3571

40. Ramaswamy B, Fiskus W, Cohen B, Pellegrino C, Hershman DL, Chuang E et al (2012) Phase I–II study of vorinostat plus paclitaxel and bevacizumab in metastatic breast cancer: evidence for vorinostat-induced tubulin acetylation and Hsp90 inhibition in vivo. Breast Cancer Res Treat 132:1063–1072