Combination of Eribulin and Aurora A Inhibitor MLN8237 Prevents Metastatic Colonization and Induces Cytotoxic Autophagy in Breast Cancer

Varvara K. Kozyreva¹, Anna A. Kiseleva²,³, Ryan J. Ice¹, Brandon C. Jones², Yuriy V. Loskutov¹, Fatimah Matalkah¹, Matthew B. Smolkin⁴, Kristina Marinak¹, Ryan H. Livengood⁴, Mohamad A. Salkeni¹,⁵, Sijin Wen¹,⁶, Hannah W. Hazard¹,⁶, Ginger P. Layne¹,⁷, Callee M. Walsh⁸, Pamela S. Cantrell⁸, Greg W. Kilby⁸, Sricharan Mahavadi⁹, Neal Shah¹, and Elena N. Pugacheva¹,²

Abstract

Recent findings suggest that the inhibition of Aurora A (AURKA) kinase may offer a novel treatment strategy against metastatic cancers. In the current study, we determined the effects of AURKA inhibition by the small molecule inhibitor MLN8237 both as a monotherapy and in combination with the microtubule-targeting drug eribulin on different stages of metastasis in triple-negative breast cancer (TNBC) and defined the potential mechanism of its action. MLN8237 as a single agent and in combination with eribulin affected multiple steps in the metastatic process, including migration, attachment, and proliferation in distant organs, resulting in suppression of metastatic colonization and recurrence of cancer. Eribulin application induces accumulation of active AURKA in TNBC cells, providing foundation for the combination therapy. Mechanistically, AURKA inhibition induces cytotoxic autophagy via activation of the LC3B/p62 axis and inhibition of pAKT, leading to eradication of metastases, but has no effect on growth of mammary tumor. Combination of MLN8237 with eribulin leads to a synergistic increase in apoptosis in mammary tumors, as well as cytotoxic autophagy in metastases. These preclinical data provide a new understanding of the mechanisms by which MLN8237 mediates its antimetastatic effects and advocates for its combination with eribulin in future clinical trials for metastatic breast cancer and early-stage solid tumors. Mol Cancer Ther; 15(8): 1809–22. ©2016 AACR.

Introduction

Kinases are considered to be the first-line targets for designing anticancer therapies, due to their suitability of inhibition and importance in cell signaling. Of the three members of the Aurora kinase family, Aurora kinase A (AURKA) is well known for its role in mitosis (1) and can be successfully targeted to inhibit proliferation (2). Elevated AURKA protein levels are a negative prognostic marker of breast cancer patient survival (3). Breast cancer cells have increased levels of active AURKA, which in turn could lead to an increase in mitotic slippage/escape and accumulation of aneuploid cells, resulting in more aggressive cancer (4). The potent, highly selective, and orally available AURKA inhibitor MLN8237 (alisertib, IC₅₀ = 6.7 nmol/L; ref. 5) is currently in phase I–III clinical trials to treat solid and hematologic cancers (6). Treatment with AURKA inhibitors leads to multiple defects, including mitotic deficiency, apoptosis (7), and senescence, in multiple human tumor cell lines (6, 8). MLN8237 demonstrated promising antitumor activity toward hematologic, lymphatic, and CNS malignancies (9–13). In phase I–II trials for advanced solid tumors, MLN8237 as a single agent was able to stabilize the disease in few cases (14–17) (https://clinicaltrials.gov/). The antitumor activity of MLN8237 was also tested in combination with standard-of-care agents, including paclitaxel (18). In some cases, combination therapy provided enhanced antitumor activity in comparison with either agent alone (19). Many metastatic breast cancers are resistant to taxanes and currently are treated with a nontaxane microtubule inhibitor, eribulin mesylate (20), which induces mitotic arrest and apoptosis (21, 22). Eribulin has been approved as a monotherapy for patients with metastatic breast cancer (23). The therapeutic benefit of eribulin in combination treatment with AURKA inhibitors is currently unknown. The antitumor activity of AURKA inhibition toward breast cancer.

¹West Virginia University Cancer Institute, West Virginia University School of Medicine, Morgantown, West Virginia. ²Department of Biochemistry, West Virginia University School of Medicine, Morgantown, West Virginia. ³Department of Biochemistry and Biotechnology, Kazan Federal University, Kazan, Tatarstan. ⁴Department of Pathology, West Virginia University School of Medicine, Morgantown, West Virginia. ⁵Department of Medicine, West Virginia University School of Medicine, Morgantown, West Virginia. ⁶Department of Surgery, West Virginia University School of Medicine, Morgantown, West Virginia. ⁷Department of Radiology, West Virginia University School of Medicine, Morgantown, West Virginia. ⁸Protea Biosciences, Inc., Montgomery, West Virginia. ⁹INBRE Program, West Virginia University School of Medicine, Morgantown, West Virginia. ¹⁰Department of Biostatistics, West Virginia University School of Medicine, Morgantown, West Virginia.

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

Corresponding Author: Elena N. Pugacheva, Department of Biochemistry, West Virginia University School of Medicine, 1 Medical Center Drive, Morgantown, WV 26506, Phone: 304-293-5295; Fax: 304-293-4667; E-mail: epugacheva@hsc.wvu.edu

doi: 10.1158/1535-7163.MCT-15-0688
©2016 American Association for Cancer Research.
cells in vitro has been reported (24–27). Nevertheless, little is known about the effect of MLN8237 on metastasis as a process and the treatment of established metastases. We have previously reported on the inhibition of pulmonary metastases by AURKA inhibitors (28). The mechanisms behind this phenomenon are still unknown. In our current work, we analyzed step by step the inhibitors (28). The mechanisms behind this phenomenon are not fully understood; no authentication was done by the authors. All cells were routinely screened for the absence of mycoplasma. siRNAs, cell medium, and supplements were prepared as described previously (28). Eribulin mesylate (Halaven) was kindly provided by MBR Cancer Center pharmacy.

Materials and Methods

Cell lines and reagents

BT-549 and MDA-MB-231LN cells were obtained in 2011 to 2012 from ATCC and Caliper, amplified on the basis of the manufacturer’s recommendations; from multiple frozen aliquots, one aliquot of each was thawed for this project. All cell lines come with comprehensive authentication and quality assurance testing by ATCC/Caliper via short tandem repeat profiling; no authentication was done by the authors. HCl_0001 and HCl_0002 are triple-negative breast cancer (TNBC) primary cells kindly provided by Dr. Alana Welm (Huntsman Cancer Institute, University of Utah, Salt Lake City, UT) through a cell skin kindly provided by Dr. Alana Welm (Huntsman Cancer Institute, University of Utah, Salt Lake City, UT). The mechanisms behind this phenomenon are not fully understood; no authentication was done by the authors. All cells were routinely screened for the absence of mycoplasma. siRNAs, cell medium, and supplements were prepared as described previously (28). Eribulin mesylate (Halaven) was kindly provided by MBR Cancer Center pharmacy.

Cell proliferation and nuclear morphology analysis

Cell proliferation was determined using the Countess II Automated Cell Counter. Cells (1 × 10^4) were treated with serial dilutions: 0, 25, 50, 100, and 150 nmol/L/MLN8237; 0, 1, 3, and 5 nmol/L eribulin; or combination in triplicates for 48 hours (n = 3). Viable cells were counted on the basis of trypan blue exclusion assay. Nuclear morphology was analyzed in 100 cells/treatment, n = 3 using Hoechst 33342, cleaved caspase-3, and Annexin V (BD Biosciences) staining under 100× objective, Axiovert200/Zeiss microscope. Annexin V and cleaved caspase-3–positive cells with condensed/fragmented DNA were counted as apoptotic. Cells with mitotic figures, large abnormally shaped nuclei, or multiple small nuclei were assigned in separate categories.

Western blotting

Western blotting was performed using standard procedures (28). Primary antibodies included anti-AURKA (BD Biosciences), -SQSTM1/p62, -LC3B, -Akt, -cleaved PARP, -phospho-T288 AURKA (Cell Signaling Technology), pAkt/S473 (R&D), –f-actin, –α-tubulin (Sigma), and GAPDH (Millipore). Secondary antibodies were rabbit-HRP-conjugated (Jackson ImmunoResearch), dilution 1:10,000 followed by chemiluminescence-based detection with HyGLO (Denville Scientific). Bands were quantified using the digital electrophoresis documentation and image analysis software Gene Tools (Syngene Corp.), with signal intensity normalized to β-actin, α-tubulin, or GAPDH.

3D migration/invasion

Studies were carried out in 3D Chemotaxis μ-Slides (ibidi) according to the manufacturer’s protocol. Briefly, attached cells were pretreated overnight with vehicle, 100 nmol/L MLN8237, 3 nmol/L eribulin, or combination (100 nmol/L MLN8237 + 3 nmol/L eribulin); deattached, and 3 × 10^6 cells/mL were suspended in 3 mg/mL Matrigel (BD Biosciences). Vehicle/or drugs were included in medium and Matrigel. Imaging was done using a Nikon-Sweptfield/Eclipse TE2000-E confocal microscope with 10× Plan Apo DIC objective every 15 minutes for 23 hours. Time-lapse images were combined to build the video files and analyzed using ImageJ software/NIH (Bethesda, MD). The tracks of individual cell movement were manually mapped using MTrack plugin to measure cell body and leading edge speed, distance, and directionality (30). Detailed analysis is provided in Supplementary Methods.

Immunofluorescent cell analysis

The cells were processed as described previously (31). Primary antibodies included anti-cleaved caspase-3, -LC3B, -SQSTM1/p62, phospho-mTOR/S2448, phospho-Bad/S112, phospho-Akt1/2 Thr202/Tyr204 (Cell Signaling Technology), -pAkt/S473 (R&D Systems), phospho-T288-AURKA (Bethyl Laboratories), -actin, α-tubulin (Santa Cruz Biotechnology), and phospho-histone H3 (BD Biosciences). Secondary antibodies included Alexa Fluor 488, 555, and 647 (Life Technologies). Images were captured using the confocal microscope LSM-510/Zeiss equipped with Photometrics QuantEM CCD camera (Photometrics), 63× Plan Fluor, NA 1.4 objective. Images were captured every 0.35 μm, followed by 3D reconstruction using LSM/Zeiss and ImagedNIH software. The images inside each dataset were collected with the same microscopy and image capture settings, and the raw data were used for image and statistical analysis.

FACS

FACS was performed as described previously (32). The detailed protocol is provided in Supplementary Methods.

Fluorescent IHC

The detailed fluorescent IHC (F-IHC) protocol and used antibodies are provided in Supplementary Methods. IHC images were obtained using LSM-510/Zeiss fluorescent microscope of 20× objective. Images were analyzed and intensity was calculated using ImageJ software (NIH). For quantifications, the signal from corresponding antibodies was normalized by area and number of nuclei. Metastases borders were determined by an overlay with hematoxylin and eosin (H&E), nuclear morphology, cytokeratin staining, and interpreted by a pathologist.
Anoikis assay
Attached cells were pretreated with vehicle, 3 nmol/L eribulin, 100 nmol/L MLN8237, or combination for 24 hours, then trypsinized and plated on Ultra-Low Attachment Plates (Coming) with complete medium containing drugs as indicated above for 48 hours. Drugs were reapplied every 24 hours. Anoikis-induced apoptosis was detected via staining with Annexin V-FTTC (BD Biosciences), propidium iodide (PI; Sigma) containing 10 μg/mL RNase, or anti-cleaved caspase-3 antibody (BD Biosciences) via FACS.

Cell adhesion and spreading assay
Cells were pretreated with drugs as above, trypsinized, and 1 × 10^5 cells per well were plated in vehicle/or drugs-containing medium. Multiple bright-field images were taken at 10× magnification every 20 minutes for 1 to 2 hours. The area of individual attached cells was quantified using ImageJ software, 100 cells analyzed per time point.

Xenograft models of breast cancer and treatments
Orthotopic and tail vein injections were performed as previously described (28) approved Institutional Animal Care and Use Committee (IACUC) protocol. Treatment with vehicle, 20 mg/kg MLN8237, 0.6 mg/kg eribulin, or combinations (10 mg/kg MLN8237, 0.3 mg/kg eribulin) was initiated when primary tumors reached 500 mm^3, as measured by ultrasound Vevo2100 system (VisualSonics). In tail vein models, mice were treated with similar regimens, including 20 mg/kg chloroquine and 20 mg/kg MLN8237 + 20 mg/kg chloroquine. MLN8237 was administered via oral gavage twice daily for 4 days per week for 2 to 3 weeks (28). Eribulin was administrated every other day by intraperitoneal injection for 2 to 3 weeks. Chloroquine was administrated for 5 days per week by intraperitoneal injection once daily for 2 weeks. At the end of the study, the primary tumors, lungs, and bones were collected, and embedded in paraffin. Serial sections (5 μm) through the whole lungs were made, stained with H&E, and analyzed for metastases (single cell, clustered cells) by pathologist.

Laser ablation electrospray ionization mass spectrometry analysis
Detailed protocol/analysis is provided in Supplementary Methods.

Statistical analysis
Statistical comparisons were made using two-tailed Student t test. When more than two groups were analyzed, one-way or two-way ANOVA was used. P ≤ 0.05 was considered to be significant (*) as indicated in the figure legends. All treatment groups were compared with vehicle unless mentioned otherwise. Experimental values were reported as the means with ±SEM, and calculations of statistical significance were made using GraphPad software. Additional statistical methods, including synergy analysis, are provided in Supplementary Methods.

Results
Application of eribulin leads to an increase in active AURKA
In our study, we found that the application of FDA-approved breast cancer antimetastatic agent, eribulin, as a single agent results in a significant increase in the active form of AURKA in TNBC cells and xenografts (Fig. 1A and B and Supplementary Fig. S1A and S1B). The combination of eribulin with MLN8237 eliminates this effect and decreases the amount of active AURKA.

To test whether AURKA expression could be a potential biomarker of the patient’s response to eribulin, we depleted AURKA via application of anti-AURKA siRNAs and assessed the viability of cells. The decrease in AURKA expression resulted in little to no effect on viability of MDA-MB-231 cells, which have mutant p53 and significant upregulation of endogenous AURKA. Depletion of AURKA in the patient-derived TNBC cell line HCl_002 potentiated eribulin treatment and resulted in an increase in cell death (Supplementary Fig. S1C and S1D), suggesting that combination of these drugs might significantly improve the treatment outcomes of metastatic breast cancer patients. Next, we tested the effects of MLN8237 and combination on different stages of metastatic cascade.

MLN8237 and eribulin reduce 3D motility/invasion and adhesion of breast cancer cells
We have previously reported that the activity of AURKA is critical for stabilization of actin at the leading edge of migrating tumor cells (31). Cells treated with eribulin also showed decreased motility (22). In the current work, we have tested the impact of AURKA inhibition as a single agent or in
Combination with eribulin on 3D migration/invasion using time-lapse microscopy. The treatment of TNBC cells embedded in basal membrane–like matrix (Matrigel) with MLN8237 reduces the speed, distance, and directionality of cell movement (Fig. 1C–E and Supplementary Fig. S1E–S1H; Supplementary Videos 1 and 2). Interestingly, eribulin alone decreased directionality, but not the speed of cell migration, suggesting a different mode of action. Importantly, the combination of drugs resulted in a greater decrease in cell invasion and thus may significantly decrease the initial dissemination of tumor cells from the primary site.

Adhesion is an important step in cancer cells intra/extravasation to colonize distant organs. We tested whether MLN8237 alone or in combination with eribulin impairs the ability of breast cancer cells to attach to a physiologically relevant matrix. There was a significant decrease in the ability of cancer cells to attach/spread upon treatment with MLN8237 (Fig. 1F and G), suggesting that AURKA activity is required for formation/stabilization of cell/matrix adhesions, but the combination with eribulin did not result in an additional benefit.

MLN8237, eribulin, or combination does not affect sensitivity of breast cancer cells to anoikis

Circulating tumor cells released by solid tumors must develop a resistance to anoikis and survive in the blood/lymphatic system to reach distant organs. To test whether inhibition of AURKA or application of eribulin may sensitize breast cancer cells to anoikis, attached cells were pretreated with drugs or vehicle for 24 hours, detached, and replated on low attachment plates for 48 hours in the presence of drugs or vehicle. FACS and microscopy-based observation of nuclear morphology documented no difference in sensitivity to anoikis activity of MDA-MB-231LN cells treated as indicated (Fig. 2A–C), suggesting that neither MLN8237 nor eribulin have any effect on anoikis sensitivity in TNBC cells.

Combination of eribulin and MLN8237 leads to synergistic induction of cell death in attached breast cancer cells

Treatment of breast cancer cells attached to the matrix with MLN8237 or eribulin has little to moderate effect on the
induction of cell death, with 5% to 10% of cells found to be Annexin V, cleaved PARP, or PI positive 48 hours posttreatment. Importantly, combination of these drugs led to a synergistic increase in the number of dead cells (Fig. 2D and E, FACS charts, Supplementary Fig. S2). Attached breast cancer cells were further tested for sensitivity to combination therapy via serial dilutions of both drugs (Fig. 2F). The number of live/dead cells after 48 hours of treatment in each dilution was used for assessment of drug interaction in vitro using three different statistical methods as described in the Supplementary Methods. The statistical analysis confirmed a synergistic mode of interaction between these drugs (Table 1).

**Figure 2.** MLN8237, eribulin, or combination does not affect sensitivity of breast cancer cells to anoikis. A, FACS analysis of MDA-MB-231LN cells treated with vehicle/or drugs in suspension for 48 hours, with Annexin V, PI, and Hoechst33342. Graph represents percentage of cells in each category in vehicle or drug-treated groups ± SEM. n = 3, one-way ANOVA: vehicle versus treatments. B, representative immunofluorescent images of MDA-MB-231LN cells as in A. Annexin V, PI, and DNA. Scale bar, 20 μm; white arrow, Annexin V-positive, but PI-negative cell; white square, Annexin V– and PI-negative cell. C, representative immunofluorescent images of MDA-MB-231LN cells as in A with anti-cleaved caspase-3 (cCaspase-3) antibodies, DNA. Scale bar, 20 μm; white arrow, cleaved caspase-3–positive cell; white square, caspase-negative cell. D, FACS analysis of attached cells treated with vehicle or drugs and stained as in A. All cells, including those found in suspension, were collected and analyzed. Percentage of cells ± SEM, n = 3, one-way ANOVA: vehicle versus treatments. E, representative immunofluorescent images of MDA-MB-231LN cells as in D with anti-cleaved-PARP antibodies, DNA. Scale bar, 20 μm; white arrows, apoptotic cells. F, cell proliferation/death analysis of MDA-MB-231LN cells treated with serial dilutions of MLN8237 and eribulin as indicated for 48 hours. The percentage of live/dead cells was calculated using trypan blue staining.
MLN8237 alone or in combination reduces metastases in vivo through the inhibition of proliferation/prosurvival pathways and promotion of cytotoxic autophagy

To determine the impact of MLN8237 and eribulin therapy on mammary tumors and established metastases, we utilized breast cancer orthotopic xenograft models. MDA-MB-231LN cells were injected in the mammary fat pad of NSG mice to form mammary tumors (500 mm³) and then treated with drugs as indicated. In agreement with our previous findings, MLN8237 did not suppress the growth of mammary tumors; however, it drastically reduced the number of pulmonary metastases (Fig. 3A, B, and E; refs. 28, 31). The combination therapy led to significant reduction in the size of mammary tumors and metastases (Fig. 3B). Interestingly, eribulin alone has no effect on the number of metastases but reduces their size when compared with vehicle (Fig. 3A), suggesting cytostatic rather than cytotoxic activity in lungs.

To define the potential mechanisms, we analyzed lung metastases and mammary tumors for expression of markers for proliferation (Ki67, pERK1/2), survival (pAKT/S473, p-mTOR/S2448), apoptosis (cleaved caspase-3, pBAD/S112), and autophagy (LC3B, p62/SQSTM1) via quantitative F-IHC staining and Western blot analysis. MLN8237, eribulin, and combination treatments decrease the proliferation and survival of tumor cells at the metastatic site (lungs) by 80% to 90%, as indicated by decreases in Ki67 expression and phosphorylation of AKT (Fig. 3C and E) and its downstream substrates mTOR (Fig. 3D) and BAD (Fig. 34) (Supplementary Fig. S3A and S3B). The activity of caspase-3, a prominent marker of apoptosis, was not affected in metastases, while slight decrease in phosphorylation of BAD, a proapoptotic member of the Bcl-2 family, indicates autophagy-promoting effect of BAD, potentially via release of Beclin1 from Bcl2 complex (35). Expression of p62/SQSTM1 and LC3B significantly increased in MLN8237 and combination-treated metastases, but not in metastases treated with eribulin alone, suggesting the involvement of autophagy in the clearance of metastases (Fig. 4A and C).

The effect of MLN8237 on proliferation/ki67 of mammary tumor cells was lower, but statistically significant, with minimal changes in pAKT and p-mTOR (Fig. 3D and Supplementary Fig. S3C and S3F). The increase in pERK1/2 and pBAD, which might be indicative of active cell-cycle and inhibition of apoptosis (36), was significant in MLN8237 treated tumors (Supplementary Fig. S3C and S3D). MLN8237 treatment did not induce upregulation of p62/LC3B or activation of caspase-3 in mammary tumors in agreement with the limited effect on tumor size.

Eribulin and combination therapy, however, significantly reduced the size of mammary tumors, which correlates with the decrease in Ki67, p-mTOR, pERK1/2, pBAD and increase in p62, LC3B, and cleaved caspase-3 expression (Fig. 4B and D and Supplementary Fig. S3C and S3D), which suggests involvement of both pathways (autophagy and apoptosis) in the reduction of mammary tumors. pAKT was slightly upregulated in eribulin and combination-treated mammary tumors, which correlates with the redistribution of AKT from the nucleus (observed in vehicle and MLN8237) to the cytoplasm, which was previously shown to correlate with induction of apoptosis (37). The DNA panels for the immunohistochemical staining are included in Supplementary Fig. S4. In addition to H&E staining and nuclear morphology, metastases were confirmed via cytokeratin staining (Supplementary Fig. S5).

Next, we analyzed the lysates prepared from mammary tumors and lung metastases treated with Western blotting to assess the levels of the indicated above markers. MLN8237 increased LC3B staining and conversion to the LC3B-II form in lysates of lung metastases, but not in mammary tumors (Supplementary Fig. S6A–S6C). Because of the small size of the eribulin and combination-treated mammary tumors and lung metastases, the levels of cleaved caspase-3, p62, and pAKT were not detectable.

As a complementary approach, we amplified and treated in vitro the tumor cells isolated from mammary tumors and lungs of vehicle-treated mice. Expression of most markers was similar to the levels observed in tumors and metastases by IHC, but the levels of p62 and pAKT did vary and were, on average, higher in mammary tumor cells than lung metastases (Fig. 4E). Cleaved PARP, a downstream target of active caspase-3, was elevated in eribulin and combination-treated cells, suggesting an activation of apoptosis, which is in agreement with the IHC data. Addition of chloroquine, an inhibitor of autophagy, resulted in further increases in p62 and LC3B in cells isolated from both sites, suggesting that in vitro, both types of cells had significant levels of basal autophagy potentially due to in vitro culturing conditions (Fig. 4E and F).

Similar to F-IHC data, immunofluorescent staining of p62 in MLN8237-treated cells isolated from lung metastases shows upregulation of p62 and decrease in pAKT. Addition of chloroquine leads to further increase in p62 levels, indicating the presence of active autophagic flux (Supplementary Fig. S7A and S7B). Immunofluorescent staining of p62 in cells isolated from MLN8237-treated mammary tumors shows active autophagic flux only in eribulin and combination-treated cells (Supplementary Fig. S7C and S7D). Analysis of nuclear morphology in these cells shows a significant increase in apoptotic and polynucleated cells upon treatment with eribulin or combination, whereas MLN8237 causes an increase in abnormally shaped nuclei, potentially due to an increase in aneuploidy (Supplementary Fig. S7E and S7F).

Table 1. The analysis of drug interaction in cell growth assay as in Fig. 2F using multiple statistical methods described in Supplementary Methods

<table>
<thead>
<tr>
<th>Models</th>
<th>Drug Interaction parameter</th>
<th>Estimated 95% CI</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>h &lt; 1</td>
<td>h = 1</td>
<td>h &gt; 1</td>
<td></td>
</tr>
<tr>
<td>Chou and Talalay</td>
<td>Eribulin = 1, MLN = 50</td>
<td>0.688</td>
<td>(0.573–0.825)</td>
</tr>
<tr>
<td></td>
<td>Eribulin = 3, MLN = 100</td>
<td>0.468</td>
<td>(0.334–0.654)</td>
</tr>
<tr>
<td></td>
<td>Eribulin = 5, MLN = 100</td>
<td>0.669</td>
<td>(0.519–0.816)</td>
</tr>
<tr>
<td></td>
<td>Eribulin = 5, MLN = 150</td>
<td>0.427</td>
<td>(0.269–0.677)</td>
</tr>
<tr>
<td></td>
<td>Greco α &gt; 0</td>
<td>α = 0</td>
<td>α &lt; 0</td>
</tr>
<tr>
<td></td>
<td>Machado η &lt; 1</td>
<td>η = 0</td>
<td>η &gt; 1</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; MLN, MLN8237.
MLN8237 reduces colonization potential of metastatic cells

As only MLN8237 or combination therapy was able to decrease the number and size of pulmonary metastases, the effect of MLN8237 as a single agent on seeding and colonization of new metastases by circulating tumor cells was tested. MDA-MB-231LN cells were injected intravenously into NSG male mice via tail vein and immediately treated with vehicle or MLN8237 (Fig. 5A). The total flux and average radience of injected animals was measured but did not significantly differ during the experimental time frame. Mice were euthanized and lungs were harvested at 24, 48, and 72 hours post injection/treatment. Note that based on our previously published work and in vitro results, treatment with...
Figure 4. MLN8237/eribulin combination induces cytotoxic autophagy and apoptosis. A and B, representative images of F-IHC staining of pulmonary tissue (A; metastases defined by the white outline) or mammary MDA-MB-231LN-xenograft tumor (B) with -LC3B/red, -SQSTM1/p62/green, -cleaved caspase-3/green antibodies. Scale bar, 30 μm. C and D, quantification of F-IHC as in A and B, multiple metastases (met.) or tumors; 5 to 6 animals, n = 3; percentage to control; one-way ANOVA: vehicle versus treatments. E, Western blot analysis of LC3B, p62, cleaved PARP, pAKT, AKT, and α-tubulin (loading control) with respective antibodies in MDA-MB-231LN cells isolated from vehicle-treated mice, expanded in vitro and treated with drugs for 48 hours as indicated; last two lanes contain lysates treated with MLN8237 + chloroquine (Chlo.). F, quantification of digital images as in C using Genetools software; mean gray value of corresponding band/area (% of vehicle, normalized to tubulin) ± SEM, n = 3, one-way ANOVA: vehicle versus treatments. RUI, relative intensity.
MLN8237 plus Eribulin Eradicates Metastases via Autophagy

MLN8237 did not affect the viability of tumor cells in suspension (28). In agreement with these findings, a similar number of single-cell metastases was found in the lungs of mice at 24 hours postinjection. By 48 hours, in both MLN8237 and vehicle-treated groups, some of the single-cell foci underwent one to two rounds of division forming “mixed metastases,” represented by a combination of single-cell and 2 to 4 cell clusters (Fig. 5B and C). However, by 72 hours of treatment, MLN8237 was able to reduce both the size and also total number of metastases (independent of size) in lungs (Fig. 5D). Under these conditions, the potential difference in the number of cells in circulation released by primary tumors was eliminated, suggesting that MLN8237 specifically inhibits the colonization and growth of metastases.

**MLN8237 treatment improves overall survival via eradication of metastases by cytotoxic autophagy**

To assess the impact of MLN8237 on overall survival, we injected breast cancer cells into the mammary fat pad, followed by mammary tumor growth (500 mm³) and surgical resection. Mice were then treated with MLN8237 or vehicle in adjuvant settings for 2 weeks (Fig. 5E). After treatment, mice were monitored daily and then euthanized once a critical health condition was reached based on a veterinarian evaluation. Importantly, MLN8237 treatment in an adjuvant setting was able to increase the lifespan of mice by 31%, increasing from an average survival age of 19 days posttreatment in the control group to 32 days in the MLN8237-treated group. Twelve percent of MLN8237-treated mice remained tumor free until they were euthanized at 2 years old after the end of the study (Fig. 5F), indicating that MLN8237 significantly prolongs survival when used as a postoperative standard-of-care treatment. Interestingly, only half of the MLN-treated animals had a recurrent mammary gland tumor when compared with the control group (Fig. 5G). To assess the impact of MLN8237 on the survival of animals with metastases in a gender-unbiased assay and independent from possible mammary tumor size differences or surgery quality, breast cancer cells were introduced via tail vein injection into male mice (Fig. 5H). Treatment with MLN8237 prolonged the survival of males twice more efficiently (by 62%) in the tail vein model, going from a median survival of 28 days in the control group to 46 days in the MLN8237-treated group (Fig. 5I and J). Similar to our earlier findings, the decrease in proliferation and survival markers was observed in lung metastases upon MLN8237 treatment (Supplementary Fig. S8A and S8B). To determine that decrease in metastases is driven by cytotoxic autophagy, we have performed similar metastatic colonization/growth study with addition of chloroquine, inhibitor of autophagy. Chloroquine alone did not affect metastatic growth when compared with vehicle, but its addition to MLN8237 completely blocked MLN’s potential to suppress metastatic outgrowth, confirming pivotal role of autophagy in eradication of metastases in vivo (Fig. 5K–M).

**MLN8237 is efficient against pulmonary metastases in PDX models**

To further evaluate our findings in clinically relevant models, we utilized metastatic breast cancer PDXs. PDX models are significantly better at predicting drug responses in clinic and can replicate the heterogeneity of an original tumor (38). Five metastatic PDXs (Supplementary Table S1) were used in this study: HCl_001, HCl_002, PEN27, PEN25, and PEN65. Passage one of combination even with twice less dosage of each drug, suggesting a superior performance with an increase in dosage (Fig. 6C). The mitotic index in mammary tumors was decreased in animals treated with eribulin and combination, but not with MLN8237 alone, whereas in lung metastases, only MLN8237 and combination treatment had a significant decrease in mitotic index (Fig. 6H). In addition, the number of disseminated tumor cells to the bone was significantly decreased in eribulin and combination-treated animals, potentially due to the overall decrease in tumor volume or specific action of eribulin on dormant cancer cells in this microenvironment (Fig. 6I). Notably, eribulin had no effect on the number of disseminated tumor cells to the lungs (Fig. 6J).

To test whether the limited MLN8237 efficacy in the mammary tumor is a result of poor penetration of the drug, we performed a drug distribution analysis in PDX tumors resected from mice treated with MLN8237 for 2 days using LAESI mass spectrometry imaging (Protea Biosciences). We demonstrate that based on histologic morphology, MLN8237 is present in and around the blood vessels of the tumor (Supplementary Fig. S10A–S10C). Localization was further confirmed by a correlative high content imaging of heme groups (Supplementary Fig. S10D–S10F).
Table 2. Analysis of drug interactions using Chou-Talalay method

<table>
<thead>
<tr>
<th>Drugs/nm</th>
<th>Estimated</th>
<th>95% CI</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eribulin = 1, MLN = 50</td>
<td>0.538</td>
<td>(0.334–0.741)</td>
<td>Synergy</td>
</tr>
<tr>
<td>Eribulin = 3, MLN = 100</td>
<td>0.481</td>
<td>(0.329–0.641)</td>
<td>Synergy</td>
</tr>
<tr>
<td>Eribulin = 5, MLN = 100</td>
<td>0.645</td>
<td>(0.501–0.852)</td>
<td>Synergy</td>
</tr>
<tr>
<td>Eribulin = 5, MLN = 150</td>
<td>0.341</td>
<td>(0.247–0.777)</td>
<td>Synergy</td>
</tr>
</tbody>
</table>

NOTE: Analysis Chou and Talalay Syn II < 1, Add II = 1, Ant II > 1.

Abbreviations: CI, confidence interval; MLN, MLN8237.

Discussion

This study identifies the specific stages of the metastatic process that are most efficiently inhibited by the investigational compound MLN8237 (alisertib), both as a single agent and in combination with the FDA-approved drug eribulin mesylate (Halaven) to benefit metastatic breast cancer patient care. The application of eribulin leads to significant accumulation of cells, with active AURKA justifying the introduction of combination therapy to clinic. AURKA is known to be a promigratory and proinvasive kinase (26, 31). Our findings indicate that MLN8237 alone or in combination with eribulin reduces the adhesion, speed, and directionality of individual cell invasion but did not affect the sensitivity of TNBC cells to anoikis using in vitro and in vivo models. Promising results of the antiproliferative effect of MLN8237 were previously shown in breast cancer orthotopic models (39) if applied at early stages. Our previous studies in TNBC cell lines MDA-MB-231 and BT-549, however, showed resistance of established mammary tumors to MLN8237 treatment (28, 31). The effects of MLN8237 + eribulin combination on cancer cells in the metastatic niche are currently unknown.

Our study shows that MLN8237 alone or in combination with eribulin reduces proliferation of breast cancer cells in lung metastases, suggesting a specific effect of MLN8237 on metastatic cells. Eribulin and combination therapy both reduced proliferation of the primary tumor. MLN8237 treatment also decreased the survival of cancer cells in vitro and in vivo (39) via a reduction in phosphorylation of AKT in metastases, but not primary tumors. Phosphorylated AKT was mainly found in the nucleus of tumors treated with vehicle and MLN8237 and was found to be cytoplasmic in eribulin and combination-treated tumors, suggesting the activation of apoptosis in the latter cases. The treatment of tumor cells in culture did not result in significant changes in AKT or its translocation to the nucleus, suggesting that in vitro conditions have some limitations to reproduce the in vivo phenotype.

The impact of AURKA inhibition on apoptosis is controversial so far, with some reports suggesting that AURKA inhibition induces apoptosis (9, 40, 41) and other reports considering the impact of AURKA inhibition on apoptosis to be insignificant (27). The effect of MLN8237 on breast cancer cell apoptosis in metastases was not documented. In our study, inhibition of AURKA did not induce caspase-3–dependent apoptosis in pulmonary metastases or the mammary tumor. It is possible that there is an apoptotic effect of MLN8237, which is not caspase-3 dependent. However, our results indicate that MLN8237 treatment induces cytotoxic autophagy specifically in pulmonary metastases, which may significantly contribute to the antimetastatic effect of MLN8237. It has been shown that autophagy can cause cancer cell death in a caspase-3–independent manner (42, 43). An increase in autophagy can also facilitate cancer survival (cytoprotective autophagy) and has been documented in some tumors treated with anticancer drugs (44). Although it still might be the case in the primary mammary tumor, MLN8237 clearly induces cytotoxic autophagy in metastases, as indicated by a decrease in pAKT and an increase in p62 and processed form of LC3B. Eribulin alone or in combination induces apoptosis in TNBC cells based on the presence of cleaved PARP and caspase-3 but simultaneously increases the level of autophagy markers p62 and LC3B. It was previously shown that apoptosis can suppress autophagy, and thus, accumulation of p62 and LC3B may reflect the inhibition of autophagic flux. To test for this possibility, we cotreated cells with autophagy inhibitor chloroquine along with MLN8237 or eribulin. Chloroquine treatment led to further increase in p62 and LC3B, which argues against the inhibition of autophagic flux and supports the idea of potential cooperation between both types of cell death in combination therapy. The combination therapy provided synergistic activation of apoptosis, leading to an increase in cell death, drastically reducing tumor mass at the primary site and metastases in multiple sites, including bone lesions, which are the most common and difficult to treat. Even at the half dosages used in combination, the effect was superior to either drug with twice higher concentration, suggesting that usage of this regimen may significantly reduce side effects of therapy in the clinic.

The mass spectrometry analysis of flash-frozen mammary tumors isolated from MLN8237-treated animals is indicative of blood-based MLN8237 delivery/distribution in the tumor tissue. Thus, tumors with limited blood vessel density may experience limited effects of MLN8237 when compared with lung lesions. In...
Figure 6. MLN8237 + eribulin combination reduces primary tumor, metastases, and dissemination of cancer in PDX models. A, HCI_002 cell growth analysis, 48 hours; vehicle, 100 nmol/L MLN8237, 3 nmol/L eribulin, or combination; n = 3, one-way ANOVA: vehicle versus treatments. B, analysis of the nuclear morphology, cells as in A; one-way ANOVA: vehicle versus treatments in each category: mitotic, abnormal nucleus, polynucleated (Polynucl.), apoptotic, normal. C, representative immunofluorescent images of HCI_002 cells treated as in A with anti-cleaved caspase-3 antibodies, DNA. Scale bar, 20 μm: white arrow, apoptotic DNA. D, quantification of cleaved caspase-3-positive cells using immunofluorescent images as in C; one-way ANOVA: vehicle versus treatments. E, immunofluorescent-based analysis of cells as in A with p62 antibodies; the relative intensity (RUI) of the immunofluorescent signal from 3 independent experiments; 100 cells/treatment, one-way ANOVA: vehicle versus treatments. F, tumor volume analysis of HCI_002-PDX mammary xenografts in three cohorts, n = 6 per cohort. Tumor growth was normalized to time point zero of treatment (100%) in each cohort; one-way ANOVA: vehicle versus treatments. G, statistical analysis of the data as in F using mixed model. H, mitotic index analysis of metastases/lungs or mammary tumors as in F, n = 50 cells per treatment in 3 experiments, t test. I, analysis of disseminated tumor cells in femurs of mice as in F; n = 2 per mouse; one-way ANOVA: vehicle versus treatments. J, quantification of lung metastases; serial sections of lungs from three mice per treatment, one-way ANOVA: vehicle versus treatments. MLN8237 and combination-treated mice did not have metastases; arrows and zero signs visualize the absence of signal.
agreement with our findings, limited preclinical and clinical efficacy of MLN8237 was documented in the treatment of solid tumors, where MLN8237 could stabilize the disease but was incapable of reducing the tumor size (15); meanwhile, blood malignancies in xenograft mouse models show an extreme senitivity to the MLN8237 compound (11). In both a xenograft model, which incorporates the influence of primary tumor invasion and extravasation, and tail vein injection model, which takes into account only the metastatic colonization stage of disease, we showed that the treatment with MLN8237 significantly increases survival rates. It is expected that combination treatment will improve survival to an even greater degree than MLN8237 alone. Previous, MLN8237 was shown to increase survival in neuroblastoma (45) and acute myelogenous leukemia models (9). Here, we present the first evidence of such an effect on metastatic breast cancer using PDXs. The recent findings presented at the 2014 San Antonio Breast Cancer Symposium show that breast cancer PDX models most accurately predicted the patient clinical response. Importantly, when combined with eribulin, MLN8237 demonstrates synergistic inhibition of primary tumor and metastases growth, including seeding of the metastases. Our findings indicate that MLN8237 plus eribulin can be used in adjuvant and metastatic settings to improve disease-free survival for breast cancer patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: V.K. Kozyreva, R.J. Ice, M.A. Salkeni, G.W. Kilby, E.N. Pugacheva
Development of methodology: V.K. Kozyreva, R.J. Ice

Acknowledgments
The authors thank the HSC Core Facilities, WVU Cancer Institute, and Biochemistry Department for their outstanding administrative support.

Grant Support
This work was supported by grants from the NIH/NCI, CA148671 (awarded to E.N. Pugacheva); EDeA CTR NIH/NCI, US4GM104942 pilot award (to E. N. Pugacheva), and in part by a NIH/NCRR S P 2 0 R 0 1 6 5 4 0 0 0 9 award to MBBR Cancer Center. S. Mahavadi was supported by the WV-INBRE grant 2 P 2 0 G M 1 0 3 4 3 4 - 1 3 . A.A. Kiseleva was partially supported by the funds from program of competitive growth of Kazan Federal University. The Animal Models & Imaging, Microscope Imaging, and WVU Flow Cytometry Core Facilities were supported by the MBBRC and the NIH grants P20 RR016440, GM103488/RR032138, S10 RR026378, S10-RR028066, S10-O1D01615S, and GM103434. Some of the tissue samples for PDXs were provided by the Cooperative Human Tissue Network, which is funded by the NCI.

The costs of publication of this article were deferred 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 August 18, 2015; revised May 16, 2016; accepted May 18, 2016; published OnlineFirst May 27, 2016.

Published OnlineFirst May 27, 2016; DOI: 10.1158/1535-7163.MCT-15-0688

www.aacrjournals.org
Mol Cancer Ther, 15(8) August 2016

1821

References


34. del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 1997;278:687–9.


Molecular Cancer Therapeutics

Combination of Eribulin and Aurora A Inhibitor MLN8237 Prevents Metastatic Colonization and Induces Cytotoxic Autophagy in Breast Cancer


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-15-0688

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2016/05/27/1535-7163.MCT-15-0688.DC1

Cited articles
This article cites 44 articles, 25 of which you can access for free at:
http://mct.aacrjournals.org/content/15/8/1809.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/15/8/1809.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://mct.aacrjournals.org/content/15/8/1809.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.