p53 Family Members Regulate Phenotypic Response to Aurora Kinase A Inhibition in Triple-Negative Breast Cancer

John J. Tentler¹, Anastasia A. Ionkina¹, Aik Choon Tan¹, Timothy P. Newton¹, Todd M. Pitts¹, Magdalena J. Glogowska¹, Peter Kabos¹, Carol A. Sartorius², Kelly D. Sullivan³, Joaquin M. Espinosa³, S. Gail Eckhardt¹, and Jennifer R. Diamond¹

Abstract

Triple-negative breast cancer (TNBC) is an aggressive disease with a poor prognosis. Advances in the treatment of TNBC have been hampered by the lack of novel effective targeted therapies. The primary goal of this study was to evaluate the efficacy of targeting Aurora kinase A (AurA), a key regulator of mitosis, in TNBC models. A secondary objective was to determine the role of the p53 family of transcriptional regulators, commonly mutated in TNBC, in determining the phenotypic response to the AurA inhibitor alisertib (MLN8237). Alisertib exhibited potent antiproliferative and proapoptotic activity in a subset of TNBC models. The induction of apoptosis in response to alisertib exposure was dependent on p53 and p73 activity. In the absence of functional p53 or p73, there was a shift in the phenotypic response following alisertib exposure from apoptosis to cellular senescence. In addition, senescence was observed in patient-derived tumor xenografts with acquired resistance to alisertib treatment. AurA inhibitors are a promising class of novel therapeutics in TNBC. The role of p53 and p73 in mediating the phenotypic response to antimitotic agents in TNBC may be harnessed to develop an effective biomarker selection strategy in this difficult to target disease. Mol Cancer Ther; 14(5); 1–13. ©2015 AACR.

Introduction

Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype defined by the lack of estrogen and progesterone receptor expression and lack of HER2 amplification (1). Patients with TNBC have a higher risk of cancer recurrence and death related to breast cancer as compared with other breast cancer subtypes (1, 2). Systemic therapy is currently limited to cytotoxic chemotherapy and effective targeted anticancer therapy remains to be proven (3). TNBC is a biologically heterogeneous disease and the development of active targeted therapies in conjunction with effective biomarker selection strategies is an area of unmet clinical need (3, 4).

Aurora kinase A (AurA) is a serine/threonine kinase integral to mitotic cell division (5, 6). AurA is essential in chromosome alignment, the formation of a bipolar mitotic spindle, and cytokinesis during mitosis (7, 8). It may function as an oncogene through the promotion of genetic instability (9) and overexpression of AurA has been linked to inferior outcomes in patients with early-stage breast cancer (10). Aurora kinase inhibitors (AKI) are a promising class of drugs for the treatment of TNBC as they are mitotic inhibitors and TNBC as a subtype has a high mitotic index (11).

TP53 is the most commonly mutated gene in TNBC with an incidence of approximately 85% (12). Although the majority of mutations are missense mutations in the DNA-binding domain, more complex mutations (i.e., frameshift and nonsense mutations) occur at a higher frequency in TNBC as compared with luminal breast cancers (13). Mutations in p53 may abrogate its tumor suppressor function resulting in impairment of cell-cycle arrest, DNA repair, and apoptosis (14). AurA overexpression may lead to increased p53 degradation via phosphorylation of p53 at Ser315, leading to increased ubiquitination by MDM2 (15). Furthermore, silencing of AurA results in stabilization of p53 and a characteristic G₂–M cell-cycle arrest (15). The role of p53 in mediating sensitivity to AKIs in TNBC is critical due to its high mutation rate in TNBC and the potential for p53 to affect terminal cellular outcome following drug exposure.

Alisertib (MLN8237) is an orally bioavailable, second-generation selective inhibitor of Aurora kinases which binds to AurA and prevents its phosphorylation and activation (16). We have previously shown that p53-mutated TNBC cell lines with increased p53 protein and mRNA expression had increased sensitivity in vitro to the antiproliferative effects of the multitarget AurA and angiogenic kinase inhibitor, ENMD-2076 (17). The purpose of this study was to evaluate the antiproliferative activity of alisertib against preclinical TNBC models and investigate the...
role of p53 and the p53 family member, p73, in mediating response to selective AurA inhibition.

Materials and Methods

Cell culture and reagents

Human TNBC cell lines were obtained and cultured as previously described (17). In addition, SW527 and HCC1395 were obtained from ATCC. CAL-51 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). Cells were passaged for less than 6 months.

MLN8237 was prepared in DMSO for in vitro experimentation and suspended in a 1:1 mixture of 10% hydroxypropyl β-cyclo-dextran (HPBCD) and 2% NaHCO3 for in vivo use. Nutlin-3 (Sigma-Aldrich) was prepared as a 10 mmol/L stock solution in DMSO.

Cell viability experiments

The sulforhodamine B (SRB) proliferation assay was performed as previously described to evaluate the cytotoxic effect of MLN8237 on TNBC cell lines at least in triplicate (18, 19). The CyQuant measurement of cellular DNA content via fluorescent dye was performed using the CyQuant NF Cell Proliferation Assay Kit and Protocol (Invitrogen). In brief, cells were harvested during the logarithmic growth phase and plated in 96-well flat-bottomed plates with lids. Cells were allowed to adhere overnight and then exposed to increasing doses of MLN8237 from 0 μmol/L to 0.1 μmol/L for 96 hours. For the SRB assay, the incubated cells were fixed, stained with 0.4% SRB (MP Biomedicals), and intensity read using a plate reader (Biotek Synergy 2) at an absorbance wavelength of 565 nm. For the CyQuant assay, cellular growth media were removed followed by incubation with the CyQuant dye for 30 minute at 37°C. Next, the fluorescence intensity of each plate was measured using a plate reader (Biotek Synergy 2) with excitation at approximately 485 nm and emission detection at approximately 530 nm.

CAL-51 p53 and p73 shRNA knockdown models

The CAL-51 cell line was transduced with several clones of shRNA GFP-tagged constructs targeting p53 or p73 and grown in puromycin (2.5%) supplemented media for at least 21 days. qRT-PCR was used to confirm adequate KD using TaqMan microRNA Assay kit (Applied Biosystems).

Analysis of apoptosis

The CAL-51, CAL-51 scramble control (SCR), and p53/p73 knockdown (KD) clones were seeded in 6-well plates (3 × 104 per well) and allowed to adhere for 24 hours at 37°C. Next, they were exposed to MLN8237 (50 or 100 nmol/L) for 24 to 96 hours at 37°C and prepared using the Annexin V/Dead Cell Apoptosis Kit (Invitrogen). The CAL-51 cells were stained with propidium iodide (PI) and FITC, whereas the CAL-51 SCR and KD cell lines were stained with PI and allophycocyanin conjugate (APC), due to the GFP tag present in their plasmids. Unstained, FITC and APC controls were used as standards. Samples were analyzed using the CyAn ADP Analyzer at the UCCC Flow Cytometry Core Facility (FCCF) for viable, early apoptotic, late apoptotic, and dead cells. Apoptosis was also assessed using a luminometric Caspase-Glo-3/7 assay (Promega).

Flow-cytometric analysis of cell-cycle distribution

Cells were seeded in 6-well plates (2 × 105 per well) and allowed to adhere for 24 hours at 37°C before being treated with MLN8237 (20 or 80 nmol/L) for 2 to 10 days. Analysis was performed as previously described (17).

Senescence

Cells were seeded in 24-well plates, allowed to adhere overnight at 37°C, and treated with vehicle or MLN8237 (50 or 100 nmol/L) for 5 to 15 days. Cells were fixed and stained for senescence-associated β-galactosidase (SA-β-gal) using the Senescence β-galactosidase Staining Kit (Cell Signaling Technology). Images were acquired using a Nikon inverted microscope at x20 magnification. Images from three different representative fields of view were taken at each time point. The number of cells in each field was counted and an average with SD calculated. The average cellular size was obtained by selecting a representative total of 10 cells from each of the three fields of view followed by calculating the average with SD.

For analysis of senescence on xenograft tumor samples, staining for SA-β-gal activity was performed as previously described by Debacq-Chainiaux and colleagues (20). Hematoxylin and eosin (H&E) staining was performed on matched slides by the UCCC Pathology Core Facility and reviewed by a pathologist to confirm the presence of tumor cells.

Nutlin combination studies

CAL-51 cells were plated in 96-well plates, allowed to adhere overnight then exposed to increasing doses of MLN8237 (0–200 nmol/L), Nutlin-3 (0–20.0 μmol/L), or the combination for 96 hours. Proliferation was assessed using the SRB method as described above and synergy was determined using the Chou-Talalay method using the Calcsyn software program (Biosoft; ref. 21). For each combination, the combination index (CI) was calculated with synergy indicated by a CI < 1, additivity by a CI = 1, and antagonism by a CI > 1.

Immunoblotting analysis

Cells were seeded into 6-well plates and allowed to attach for 24 hours before exposure to MLN8237. Cells were harvested with trypsin/EDTA and then lysed in Cell Lysis Buffer (Cell Signaling Technology). Forty μg of total protein was loaded onto a 4% to 20% gradient gel, electrophoresed, and then transferred to nitrocellulose using the i-Blot system (Invitrogen). Membranes were blocked in blocking buffer and incubated overnight at 4°C with primary antibodies: PARP, p53, BAX, p21 (Cell Signaling Technology), p16 (Santa Cruz Biotechnology), and β-actin (Sigma-Aldrich). Following primary antibody incubation, membranes were washed in TBS-Tween (0.1%) and incubated with secondary antibody (anti-rabbit or anti-mouse IgG1 horseradish peroxidase-linked antibody at 1:15,000 (Jackson ImmunoResearch) for 1 hour at room temperature (RT). Blots were developed using the Odyssey Infrared Imaging System (LI-COR Biosciences). Experiments were performed in triplicate.

Immunofluorescence

CAL-51 SCR and p53/p73 KD cells were seeded onto glass chamber slides and allowed to incubate overnight at 37°C then treated with either vehicle or MLN8237 (50 or 100 nmol/L) for 4 days then fixed with paraformaldehyde. Chamber slides were...
blocked with 1% BSA in PBS for 30 minutes at 37°C and incubated with the primary antibody for 1 hour at RT [p53 mouse mAb (Cell Signaling Technology), p16 (C-20 Santa Cruz Biotecology), or BAX (ab10813 Abcam)]. Slides were washed in PBS and incubated with secondary antibodies [AlexaFluor 555 (Invitrogen) or AlexaFluor 488 (Life Technologies)] and washed with PBS. Counterstaining with 300 nmol/L DAPI in PBS for 5 minutes at RT was performed. Slides were mounted with Fluoromount-G mounting media (SouthernBiotech), cover slipped, and sealed with clear nail polish. Slides were imaged using the Olympus FV-1000 confocal microscope at ×60 magnification.

**In vivo** patient tumor-derived and TNBC cell line xenograft studies

Five- to 6-week-old female athymic nude (nu/nu) mice (Harlan Sprague Dawley) were housed in groups of five, allowed to acclimate for one week before handling, provided with sterilized food and water ad libitum and kept on a 12-hour light/dark cycle. Patient tumor tissue was obtained from consented patients under an Institutional Review Board-approved protocol and implanted and expanded initially in NOD/SCID/IL2rg(-/-) mice (22). After expansion, tumor tissue was obtained, minced into 5 mm sections and implanted subcutaneously into nu/nu mice using a 12-gauge trocar as previously described (23, 24). CAL-51 cells were harvested during logarithmic growth and resuspended in a 1:1 mixture of serum-free DMEM and Matrigel (BD Bioso- ciences). The cells were injected bilaterally into the flank using 2.5 × 10^6 cells/injection in a volume of 100 µL. Mice were monitored daily for toxicity and weighed twice weekly. Tumor measurements were performed daily using calipers with the Study Director software package (Studylog Systems) and tumor volume was calculated using the formula: volume = (length × width^2) × 0.52. When tumors reached a mean volume of 150 mm³, mice were randomized into vehicle and MLN8237 groups with 10 tumors/group. MLN8237 (30 mg/kg) or vehicle control was administered via oral gavage with continuous once daily dosing. At the end of treatment, mice were euthanized with CO2 and tumor samples were collected for gross anatomic and histologic examination. Slides were stained with 300 nmol/L DAPI in PBS for 5 minutes at RT and examined under a fluorescence microscope at 10 magnification.

**Statistical analysis**

Treatment groups were compared by ANOVA parametric analysis of the means using a commercially available statistical program (Prism 4.0, GraphPad). The comparisons between sensitivity of treatment and vehicle groups in vivo were compared by unpaired parametric t test with Welch's corrections using a commercially available statistical program (Prism 4.0, GraphPad). Specific tests applied are included in the figure legends.

**Results**

**Antiproliferative activity of MLN8237 against 18 TNBC cell lines in vitro**

A panel of 18 TNBC cell lines representing the basal-like 1 and 2 (BL1/2), immunomodulatory (IM), mesenchymal (M), and mesenchymal stem-like (MSL) subtypes was screened for in vitro sensitivity to MLN8237 (4). Figure 1A depicts the in vitro response of MLN8237 treatment in breast cancer cell lines where IC50 values ranged from 23 nmol/L to > 5 µmol/L (Supplementary Table S1). We did not observe statistically significant differences in sensitivity to MLN8237 by TNBC subtype (mean IC50 IM 0.23 µmol/L, BL1/2 0.69 µmol/L, M/MSL 0.93 µmol/L, P = 0.197). Within the MLN8237-sensitive TNBC cell lines (IC50 < 0.1 µmol/L), the IM, BL1, BL2, and mesenchymal (M) TNBC subtypes were represented (Fig. 1A). BL1 TNBC cell lines exhibited both sensitivity (MDA-MB-468 IC50 0.036 µmol/L) and resistance (HCC1957 IC50 > 5 µmol/L) to MLN8237 exposure (Fig. 1A). Although in the Lehnmann and colleagues landmark study (4), the BL1 and BL2 subtypes exhibited increased expression of genes associated with cell-cycle pathways and proliferation (including AURKA and AURKB), we did not find that these subtypes were predictive of in vitro sensitivity to MLN8237.

On the basis of the previously described interactions between p53 and AurA (15, 25), we investigated p53 mutation status and mRNA expression of TP53 and AURKA in our TNBC breast cancer panel (Fig. 1A). Microarray gene expression data were obtained from the Cancer Cell Line Encyclopedia (GSE36133). There was no correlation between TP53 mutation, AURKA expression, or TP53 expression and sensitivity to MLN8237 exposure; however, there was a trend toward increased sensitivity to MLN8237 in cell lines with increased TP53 expression (Pearson correlation coefficient r = −0.49, P = 0.066). An inverse correlation between AURKA expression and p53 expression was observed possibly explained by the ability of AURKA to phosphorylate p53 at Ser315 and accelerate its degradation (15).

**Knockdown (KD) of p53 or p73 leads to resistance to MLN8237**

AurA expression in malignant cells is associated with an abrogation of the DNA damage-induced apoptotic response, at least in part due to functional inactivation of p53 (15, 25). In addition, AurA overexpression leads to increased phosphorylation of the p53 family member p73. This results in cytoplasmic sequestration of p73 with its chaperone protein, mortalin, thus diminishing its transactivation function (26). On the basis of the regulation of both p53 and p73 by AurA and reports in the literature supporting a role for both p53 family members in determining cellular fate in response to AKI, we investigated the role of both in mediating sensitivity to MLN8237 in TNBC (26, 27). We hypothesized that sensitivity to MLN8237 in p53 WT TNBC is mediated through both p53 and p73 and selected the p53 WT CAL-51 cell line for shRNA KD of p53 and p73. Expression of p53 was decreased by 82% (P < 0.01) and 63.5% (P < 0.05) in the p53-10 and p53-12 clones, respectively, as compared with the SCR control.
Effect of p53 and p73 KD on MLN8237-induced apoptosis and cell-cycle arrest

Inhibition of AurA leads to a transient G2–M cell-cycle arrest followed by aberrant cell division and aneuploidy (28). Previous studies have shown that AurA inhibition induces a modest proapoptotic response, but can also lead to senescence as a terminal outcome (29–31). To understand the mechanism of the antiproliferative effects of MLN8237 in TNBC that are affected by p53 or p73, we investigated alterations in cellular fate, including apoptosis, cell-cycle arrest, and senescence. Beginning with the CAL-51 p53 WT cell line, we observed an increase in apoptosis which peaked at 96 hours as measured by Annexin V binding flow cytometry (Fig. 2A). Annexin V is a specific binder of phosphatidylserine which appears on a cell’s surface as a marker of apoptosis (32). On the basis of a peak in apoptosis at 96 hours in the CAL-51 cell line, this timepoint was selected for analysis.

Effect of increasing doses of MLN8237 on cellular proliferation in the CAL-51 SCR and p53, p73 shRNA knockdown clones as measured by the CyQuant assay. E, effect of MLN8237 at the top dose of 0.1 µmol/L on cellular proliferation in the CAL-51 SCR, p53-10, p53-12, p73-26, and p73-55 shRNA knockdown clones as measured by SRB assay. F, effect of increasing doses of MLN8237 on cellular proliferation in the CAL-51 SCR and p53, p73 shRNA knockdown clones as measured by the CyQuant assay.

Figure 1. Antiproliferative effects of MLN8237 against TNBC cell lines in vitro. A, absolute IC50 values of a panel of 18 TNBC cell lines. Below, TNBC subtype, TP53 mutation status and heatmap of expression levels of TP53, TP73, AURKA, and AURKB gene expression levels are depicted (TNBC subtypes as described in ref. 8. U, unknown; IF, in-frame deletion; MS, missense; FS, frameshift; NS, nonsense). B, relative expression levels of p53 RNA as measured by qRT-PCR in CAL-51 scrambled control (SCR) and p53 shRNA clones 10 and 12 (p53-10, p53-12). C, relative expression levels of p73 RNA as measured by qRT-PCR in CAL-51 SCR and p73 shRNA clones 26 and 55 (p73-26, p73-55). D, effect of increasing doses of MLN8237 on cellular proliferation in the CAL-51 SCR and p53, p73 shRNA knockdown clones as measured by the SRB assay. E, effect of the top dose of 0.1 µmol/L on cellular proliferation in the CAL-51 SCR, p53-10, p53-12, p73-26, and p73-55 shRNA knockdown clones as measured by SRB assay. F, effect of increasing doses of MLN8237 on cellular proliferation in the CAL-51 SCR and p53, p73 shRNA knockdown clones as measured by the CyQuant assay. ***P < 0.001, **P < 0.01, *P < 0.05, as compared with SCR control.

Expression of p73 was decreased by 71% (P < 0.05) and 77% (P < 0.05) in the p73-26 and p73-55 clones, respectively, as compared with the SCR control (Fig. 1C).

In the p53-10, p53-12, p73-26, and p73-55 clones, exposure to increasing doses of MLN8237 resulted in an increase in the mean IC50 to 1.5, 0.66, > 1.5, and 1.49 µmol/L, respectively, as compared with the mean IC50 assessed using the SRB proliferation assay (Fig. 1D and Supplementary Table S2). There was a statistically significant increase in residual cellular proliferation following exposure to MLN8237 0.1 µmol/L for 96 hours in the p53-10, p53-12, and p73-26 clones, compared with the SCR control (P < 0.05, Fig. 1E). These findings were confirmed using the CyQuant proliferation assay (Fig. 1F). In summary, KD of p53 or p73 resulted in increased resistance to the antiproliferative effects of MLN8237 in vitro.

Downloaded from mct.aacrjournals.org on June 20, 2017. © 2015 American Association for Cancer Research.
further experimentation using the p53 and p73 KD clones. At 96 hours, there was statistically significant increase in apoptosis following exposure to MLN8237 in the CAL-51 scramble control as compared with the CAL-51 p53-10 and p73-55 KD clones (\(P < 0.001\) and \( < 0.01\), respectively; Fig. 2B and C). In the p53 and p73 KD cell lines, we observed a decrease in the degree of apoptosis following exposure to MLN8237; however, there was not a complete abrogation of the proapoptotic response. These findings were confirmed using a caspase-3/7 activity assay (Fig. 2D).

Next, we investigated the effect of MLN8237 exposure on cell-cycle distribution using flow cytometry in the CAL-51 scramble, p53-10 KD and p73-55 KD clones. We observed that in the CAL-51 scramble cell line, treatment with MLN8237 (20 or 80 nmol/L) for 48 hours led to a dose-dependent G2-M cell-cycle arrest (Fig. 3A and 3B). This result is consistent with existing literature reporting a G2-M arrest following exposure to AKI in vitro (31, 17). There was not a difference in the G2-M population following exposure to MLN8237 in the p73-55 KD cell line (Fig. 3A and 3B). Before initiating treatment with MLN8237, we observed a marked increase in the G2-M cell population in the p53-10 KD cells (Fig. 3A). We hypothesize that this may be a result of increased genomic instability related to the functional loss of p53. Given the increased G2-M population at baseline, we did not observe further G2-M arrest with MLN8237 treatment for
48 hours (Fig. 3A). There was, however, a small increase in aneuploidy following treatment with MLN8237 at the higher dose level (80 nmol/L; Fig. 3A).

On the basis of the above results and reports describing delayed effects of AurA inhibition on cellular fate (29), we investigated the effect of MLN8237 exposure on cell-cycle distribution following a 10-day exposure. There were not sufficient numbers of CAL-51 SCR control cells viable after prolonged exposure to MLN8237 for cell-cycle analysis due to the potent antiproliferative effects. In both the p53-10 KD and the p73-55 KD cell lines, however, we observed a continued dose-dependent increase in aneuploidy (Fig. 3A). In summary, we observed a dose-dependent G2–M arrest following exposure to MLN8237 for 48 hours in the CAL-51 WT and SCR control which was diminished in the p73-55 KD and increased aneuploidy in the p53-10 and p73-55 KD clones following a 10-day exposure. An increase in aneuploidy in the p53-10 KD cell line may be related to the loss of p53-dependent elimination of tetraploid cells (33).

Resistance to Aurora kinase inhibition in vitro is associated with induction of senescence

We have previously demonstrated that AKI leads to induction of senescence as a terminal cellular fate in a subset of TNBC cell lines less sensitive to the antiapoptotic effects (17). The role of p53 and p73 in mediating induction of senescence is not clearly defined in the literature, with evidence pointing both toward a role for p53 in mediating senescence and in its paradoxical suppression of senescence (34, 35). Senescence is classically described as an irreversible terminal growth arrest that can be identified by changes in cellular morphology (large, flattened cells with increased vacuoles) and the presence of SA-β-gal activity (36). To explore the role of p53 and p73 in mediating induction of senescence TNBC, we exposed the same CAL-51 scramble, p53-10 KD and the p73-55 KD cell lines to MLN8237 (100 nmol/L) for 5, 10, or 15 days and evaluated cells for morphologic changes and the presence of SA-β-gal activity. As shown in Fig. 4A, Supplementary Figs. S1 and S2 and Supplementary Table S3, treatment with MLN8237

Figure 3.
A, quantification of cell-cycle parameters using propidium iodide staining and flow cytometry in the CAL-51 SCR, p53-10, and p73-55 knockdown clones following exposure to MLN8237 at the indicated doses for 48 hours and 10 days. A statistically significant G2–M arrest was observed for CAL-51 SCR after 48 hours of 80 nmol/L MLN8237 exposure. **P < 0.01 indicates a statistically significant increase in G2–M as compared with the corresponding ND control. ***P < 0.001; ****P < 0.01; **P < 0.05 indicate a statistically significant increase in aneuploidy as compared with the corresponding ND control. B, representative flow-cytometric analysis of cell-cycle parameters in CAL-51 SCR and p73-55 cell lines. Peaks denoting G1, G2–M and aneuploid are shown.
resulted in increased SA-β-gal activity and cellular size at all time points tested in the p53-10 and p73-55 KD cell lines as compared with vehicle control. Of note, in the CAL-51 scramble cell line, exposure to MLN8237 100 nmol/L for 5 to 15 days resulted in nearly complete cell death (Fig. 4A). These findings support the induction of senescence as a mechanism for resistance to the antiapoptotic effects of MLN8237 in cells lacking functional p53 or p73.

Nutlin is synergistic with MLN8237 in the CAL-51 p53 wild-type cell line

Our results above support a functional role for p53 in mediating a proapoptotic response to MLN8237 exposure in vitro. On the basis of this, we hypothesized that increasing intracellular concentrations of p53 would lead to increased sensitivity to MLN8237 in p53 WT cells. Nutlin-3 is a small-molecule inhibitor of p53 degradation. It exerts its effects through inhibition of the interaction between p53 and MDM2, thus decreasing MDM2-mediated ubiquitin-dependent degradation of p53 (37). Figure 4B illustrates the antiproliferative effect of increasing concentrations of nutlin-3, MLN8237, or various combinations of both drugs. Synergistic antiproliferative activity was observed for the combination at a variety of dose levels using the Chou and Talalay method (Supplementary Table S4; ref. 21). An increase in apoptosis was observed with the combination at the highest dose level of MLN8237 (Fig. 4C). These findings are consistent with a recent report from Vilgelm and colleagues in melanoma (38).

Loss of p53 leads to alterations in known p53-downstream mediators of AKI-induced cellular fate

Immunofluorescence and immunoblotting studies were performed to investigate the mechanism of the observed shift from apoptosis to senescence following MLN8237 exposure in cells...
lacking functional p53 or p73 by shRNA KD. In the CAL-51 SCR, exposure to MLN8237 led to a more robust increase in nuclear p53, cytoplasmic BAX, and nuclear BAX colocalized with p53 as compared with the p53 and p73 KD cell lines (Fig. 5A–E and Supplementary Fig. S3A). Nuclear Bax/p53 complexes are associated with induction of apoptosis in response to DNA damage (39). We hypothesize that induction of BAX is mediated via the mitotic arrest imposed by MLN8237 treatment in a p53-independent manner.

p16 is an established functional biomarker of senescence likely due to its integral role in preventing cell-cycle progression (40). A dose-dependent increase in p16 staining was observed following exposure to MLN8237 in the p53 and p73 KD cell lines, but not in the CAL-51 SCR consistent with the observed increase in SA-β-gal activity and phenotypic changes indicative of cellular senescence (Figs. 4A, 5C and D and Supplementary Fig. S3B). A significant difference in p21 or p73 expression was not observed following exposure to MLN8237 in the CAL-51 SCR or p53 KD cell lines (Supplementary Fig. S3C and S3D). As expected based on the p73 KD, expression of p73 was not observed in CAL-51 p73-55 following exposure to MLN8237 (Supplementary Fig. S3D).

Senescence is associated with acquired resistance to AUR inhibition in vivo

To confirm the in vitro antiproliferative activity of MLN8237 in a more clinically relevant system, treatment studies were performed using a CAL-51 xenograft and 2 TNBC PDX models. As depicted in Fig. 6A and B, MLN8237 treatment resulted in statistically significant TGI \( (P < 0.0007) \) at day 35.
which was associated with a decrease in Ki-67 staining (Fig. 6C). This was accompanied by an increase in cleaved caspase-3 staining (Supplementary Fig. S4A). Similar antitumor activity accompanied by an increase in cleaved caspase-3 was observed in PDTX models [CU_TNBC_003, p53 WT, and CU_TNBC_002, p53 mutant (R249S); Fig. 6D–G and Supplementary Fig. S4B and S4C]. CU_TNBC_003 exhibited slower growth kinetics for the vehicle control (Fig. 6E); therefore, treatment for 65 days was required to show a statistically significant TGI ($P < 0.0001$; Fig. 6D). CU_TNBC_003 and CU_TNBC_002 were both obtained from primary breast tumors either following neoadjuvant chemotherapy or before treatment, respectively.

Following 4 days of MLN8237 treatment (30 mg/kg daily), there was no significant difference in p53 expression as compared with vehicle control (Fig. 6H). We observed p53 expression in the vehicle control tumors, consistent with the presence of p53 cellular accumulation observed in cells with mutated p53 (41). There was not a statistically significant change in pHH3 following dosing with MLN8237 for 4 days (Fig. 6H). This is likely due to its selective AurA inhibition at this dose. We observed a decrease in p16 following prolonged dosing in the responding treated tumors (Fig. 6H), suggesting that induction of senescence as a terminal outcome is not responsible for the observed continued TGI. Of note, the expression of p16 in the vehicle-treated control tumor likely represents a population of tumor
cells exhibiting baseline senescence related to hypoxia due to rapid tumor growth (42).

To investigate acquired resistance to prolonged AKI, we continued treatment of the responding CU_TNBC_002 tumors until we observed progression in a subset of tumors (day 115, Fig. 7A). As shown in Fig. 7B, we selected a tumor exhibiting continued TGI (right flank) and a tumor from the same animal exhibiting rapid growth following approximately 90 days of growth inhibition (left flank) for further experimentation. These tumors were excised and evaluated for phenotypic changes consistent with senescence and the presence of SA-β-gal activity. Comparison was also made with a vehicle control tumor harvested earlier in the experiment (day 4, approximately 300 mm³). As depicted in Fig. 7C, we observed larger cells with increased vacuoles consistent with senescent cells in the progressing tumor not observed in the sensitive tumor. A decrease in Ki-67 staining and an increase in cleaved caspase-3 were observed in the sensitive right-sided tumor, as compared with the vehicle control and the resistant left-sided tumor. In addition, apoptotic cells were observed on H&E corresponding to areas of cleaved caspase-3 staining in the sensitive right-sided tumor (arrows). Representative images of SA-β-gal activity and H&E staining were taken at x20 magnification. Representative images of Ki-67, cleaved caspase-3, and corresponding H&E staining were taken at x10 magnification. Scale, 10 μm.

Discussion

TNBC is a highly aggressive breast cancer subtype with limited systemic treatment options. AKIs represent a promising class of targeted agents for the treatment of TNBC based
on promising preclinical and early clinical trial data (17, 43, 44). The purpose of this study was to determine the antitumor activity of MLN8237, a selective AurA inhibitor, in TNBC cell lines, and PDTX models. Another significant objective was to investigate the role of p53 and its family member p73 in mediating terminal cellular fates in response to AKI in TNBC.

Our studies showed that MLN8237 has antiproliferative activity against TNBC cell line-based and PDTX models. The sensitivity of TNBC cell lines to MLN8237 in vitro was not dependent on TNBC subtype and both p53 mutated and WT cell lines exhibited sensitivity. We did observe a trend whereby increased p53 mRNA expression was associated with sensitivity to MLN8237, consistent with our previously published data for ENMD-2076 which also target Aurora (17). MLN8237 exposure resulted in induction of apoptosis in the CAL-51 p53 WT cell line, which was partially abrogated by shRNA KD of p53 or p73. Our observations are consistent with data reported by Nair and colleagues demonstrating apoptosis following exposure to AKI in the HCT116 p53 WT colon cancer cell line (45). In their study, the apoptotic response was abrogated by knockdown of p53 where an increase in endoreduplication and polyplody was observed following AKI exposure (45). Although our results are consistent with their findings, they did not investigate whether the polyplody they observed was associated with senescence (46).

We observed the classic AKI-induced G2–M cell-cycle arrest following MLN8237 exposure and an increase in aneuploid cells with p53 shRNA KD. Our in vitro data indicate that sensitivity to MLN8237 in the CAL51 p53 WT cell line can be reversed with shRNA KD of either p53 or p73 and that this shift in sensitivity is accompanied by induction of senescence. To our knowledge, these data are unique in supporting a role for p53 and its family member p73 in mediated sensitivity to AKI in TNBC. These data are consistent with our prior report of multigene AKI leading to senescence in TNBC models resistant in vitro to the antiproliferative activity (17). Furthermore, this study confirms these findings in vivo using a clinically relevant PDTX TNBC model where we observed induction of cellular senescence in tumors as they developed acquired resistance to MLN8237 in vivo.

The p53 tumor suppressor gene is often referred to as “the guardian of the genome,” regulating the expression of a broad panel of genes responsible for DNA repair, cell-cycle arrest, apoptosis, and senescence in response to DNA damage (47). TAp73 is a p53 family member with structural and functional similarity to p53 (48). A functional loss of p53 due to deletion or mutation is found in up to 80% of TNBC; however, p53 mutations are quite heterogeneous with differing degrees of preservation of p53 function (12, 49, 50). In this study, we demonstrate that loss of WT p53 leads to resistance to AKI in TNBC; however, it does not phenocopy the p53-mutated models with regards to response to AKI and terminal cellular outcome. Although loss of WT p53 by shRNA knockdown impairs induction of apoptosis in response to AKI in TNBC, the effect of individual p53 mutations on mediating sensitivity to AKI is unknown. To answer this question, mutant p53 knockdown models are needed. Possible mechanisms for the observed sensitivity of multiple p53-mutated cell lines to MLN8237 include partially retained p53 function for individual p53 mutations, potential gain-of-function with regards to induction of apoptosis, and overlapping apoptotic capabilities of p73 in response to AKI in TNBC. We have previously reported upregulation of p73 in response to AKI in p53-mutated TNBC; however, a global analysis of baseline p73 expression in our panel of TNBC cell lines did not correlate significantly with sensitivity to MLN8237 (17). Clearly, this is an area in need of future research.

Regulation of senescence by p53 is somewhat controversial in the literature. Evidence exists in support of p53-dependent senescence and its role in impairing a favorable drug response to doxorubicin in preclinical breast cancer models (34). There are other reports, however, supporting a role for p53 in suppressing p21-mediated cellular senescence (35, 40). Our data are consistent with these latter reports in that silencing p53 activity through shRNA knockdown leads to a shift in terminal cellular outcome from apoptosis to senescence in response to AKI. Although Jackson and colleagues showed that MMTV-Wnt1 mammary tumors with WT p53 or a heterozygous p53 R172H missense mutation exhibited a senescent-like phenotype following inhibition of DNA/RNA synthesis by doxorubicin, the mechanism of cellular fate may be different with AKI (34). Our data are consistent with other studies reporting induction of apoptosis in response to AKI in p53-mutated cancer cell lines (17, 31, 43). The role of mutant p53 in the induction or suppression of senescence in response to AKI remains unknown. Mutant p53 knockdown models would be useful in answering these questions as the cell line subject to p53 knockdown in this study was p53 WT.

Cellular senescence is a physiologic process leading to permanent growth arrest in response to certain types of cellular stress (40, 51). Senescence is characterized by changes in cellular morphology (i.e., enlarged and flattened cells with increased vacuoles), high levels of p21 and p16, markers of the DNA damage response, and the senescence-associated secretory phenotype (SASP). In this study, we observed that the induction of senescence in TNBC cell lines was associated with resistance to the antiproliferative activity of MLN8237 in vitro. Furthermore, using a TNBC PDTX model, we observed induction of senescence at the time of acquired resistance to MLN8237. This is consistent with reports associating senescence with impaired response to chemotherapy, cancer recurrence following treatment, and malignant transformation of preneoplastic cells (34). Our findings may be explained by cytokines and growth factors secreted by senescent cells (SASP) which are necessary for maintenance of the senescent state, but when secreted into the tumor microenvironment can stimulate tumor growth. The effect of induction of senescence on global tumor progression deserves further investigation using preclinical in vivo modeling, but also using correlative tissue samples obtained from patients enrolled on clinical trials receiving potentially pro-senescent therapies.

TNBC represents a challenging breast cancer subtype clinically due to its aggressive disease course and limited systemic treatment options. We believe that our data support the further clinical investigation of AKIs, including MLN8237, in TNBC. Our observation of senescence associated with acquired resistance to AKI in p53-mutated TNBC may support the rational combination of AKIs with inhibitors of the mTOR pathway based on their ability to decelerate cellular senescence (52). This study also supports further investigation of the p53 landscape in TNBC and the role of individual p53 mutations in mediating response to AKI, including terminal cellular fate.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.J. Tentler, A. Ionkina, T.P. Newton, M.J. Glogowska, P. Kabos, C.A. Santorius, J.R. Diamond
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Ionkina, A.-C. Tan, T.P. Newton, T.M. Pitts, J.M. Espinosa, S.G. Eckhardt, J.R. Diamond
Writing, review, and/or revision of the manuscript: J.J. Tentler, A. Ionkina, P. Kabos, J.M. Espinosa, S.G. Eckhardt, J.R. Diamond
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Ionkina, T.P. Newton, J.R. Diamond
Study supervision: J.J. Tentler, J.M. Espinosa, J.R. Diamond

References

Acknowledgments
The authors acknowledge Millennium Pharmaceuticals, Inc., and the National Cancer Institute, NIH for providing MLN8237.

Grant Support
This work was supported by the NIH and the National Cancer Institute (NCI) through P50CA046934-25 (University of Colorado Cancer Center Support Grant), IK2CA172691-01A1 (to J.R. Diamond), and SK12CA086913-10 (to J.R. Diamond), and Howard Hughes Medical Institute through HHMI Early Career Science Award (to J.M. Espinosa).

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

Received July 24, 2014; revised February 24, 2015; accepted February 24, 2015; published OnlineFirst March 10, 2015.


Molecular Cancer Therapeutics

p53 Family Members Regulate Phenotypic Response to Aurora Kinase A Inhibition in Triple-Negative Breast Cancer


Mol Cancer Ther  Published OnlineFirst March 10, 2015.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-14-0538-T

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2015/03/11/1535-7163.MCT-14-0538-T.DC1

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, contact the AACR Publications Department at permissions@aacr.org.