Predictive Biomarkers of Sensitivity to the Aurora and Angiogenic Kinase Inhibitor ENMD-2076 in Preclinical Breast Cancer Models

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Abstract

Purpose: The Aurora kinases are a family of conserved serine-threonine kinases with key roles in mitotic cell division. As with other promising anticancer targets, patient selection strategies to identify a responsive subtype will likely be required for successful clinical development of Aurora kinase inhibitors. The purpose of this study was to evaluate the antitumor activity of the Aurora and angiogenic kinase inhibitor ENMD-2076 against preclinical models of breast cancer with identification of candidate predictive biomarkers.

Experimental Design: Twenty-nine breast cancer cell lines were exposed to ENMD-2076 and the effects on proliferation, apoptosis, and cell-cycle distribution were evaluated. In vitro activity was confirmed in MDA-MB-468 and MDA-MB-231 triple-negative breast cancer xenografts. Systematic gene expression analysis was used to identify up- and downregulated pathways in the sensitive and resistant cell lines, including within the triple-negative breast cancer subset.

Results: ENMD-2076 showed antiproliferative activity against breast cancer cell lines, with more robust activity against cell lines lacking estrogen receptor expression and those without increased HER2 expression. Within the triple-negative breast cancer subset, cell lines with a p53 mutation and increased p53 expression were more sensitive to the cytotoxic and proapoptotic effects of ENMD-2076 exposure than cell lines with decreased p53 expression.

Conclusions: ENMD-2076 exhibited robust anticancer activity against models of triple-negative breast cancer and the candidate predictive biomarkers identified in this study may be useful in selecting patients for Aurora kinase inhibitors in the future.

Introduction

Breast cancer can be biologically segregated into discrete molecular subtypes using human gene expression profiling which include luminal, HER2-amplified, and basal-like cancers (1, 2). The basal-like subgroup includes tumors that are described as triple negative as they lack expression of the estrogen receptor (ER), progesterone receptor (PR), and do not display HER2 amplification (3). Triple-negative breast cancer (TNBC) is associated with a shortened disease-free and overall survival at all stages of diagnosis when compared with other breast cancer subtypes and carries an increased risk of developing distant metastases (4–6). TNBC remains an area of unmet need in terms of effective anti-cancer agents with innovative patient selection strategies (7).

The Aurora kinases are a family of serine-threonine kinases integral to mitotic cell division and have recently emerged as novel anti-cancer targets (8–10). Overexpression of Aurora Kinase A (AURKA) has been detected in many human cancers, including breast cancer, which correlates with decreased survival in women with early stage disease (11). The Aurora kinases represent promising targets for the treatment of TNBC due to their higher growth fraction and an increased sensitivity to microtubule-targeting antimitotic agents (12).

ENMD-2076 (EntreMed, Inc.) is a novel, orally bioavailable, small molecule inhibitor of Aurora kinases as well as multiple kinases involved in angiogenesis, cell-cycle progression, and cellular proliferation (13). The antiproliferative activity of ENMD-2076 is more selective for AURKA (half-maximal inhibitory concentration [IC50] 4 nmol/L) as compared with Aurora Kinase B (AURKB; IC50 350 nmol/L); however, inhibition of both AURKA and AURKB has been shown in multiple myeloma and
Translational Relevance

Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype that carries a high risk of developing distant metastasis. A major barrier to the successful treatment of metastatic TNBC is the lack of effective targeted anticancer agents. We show that the novel Aurora and Angiogenic kinase inhibitor ENMD-2076 has activity against preclinical models of breast cancer with more robust activity against TNBC. In this study, differences in the p53 pathway were associated with alternate cellular fates following exposure to ENMD-2076, including apoptosis and senescence. This study supports further clinical investigation of ENMD-2076 in patients with metastatic TNBC with an emphasis on the continued development of p53-based predictive biomarkers.

Materials and Methods

Cell lines and culture

The human breast cancer cell lines HCC1143, MDA-MB-436, HCC1806, HCC1937, MDA-MB-157, Hs578T, HCC38, A549, BT474, TC7, T47D, HCC70, andZR-75-30 were obtained from the American Type Culture Collection. CAL-120, HIDQ-P1, and CAL-85-1 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. MDA-MB-134-VI, MDA-MB-468, MCF7, MDA-MB-231, BT20, T47D, HCC70, and ZR-75-1 were obtained from the University of Colorado Cancer Center Tissue Culture Core laboratory. Cell lines were authenticated by DNA profiling at the University of Colorado Cancer Center DNA Sequencing and Analysis Core that compared this data to the ATCC profile. Cells were cultured in Dulbecco’s modified eagle media (DMEM, Cellgro Mediatech) supplemented with 10% FBS (Invitrogen), 1% nonessential amino acids (Cellgro Mediatech), and 1% penicillin/streptomycin. Cells were maintained in an incubator at 37°C containing 5% CO2. Routine screening for Mycoplasma(MycoAlert; Cambrex BioScience) was conducted.

Drugs

ENMD-2076 [2-(phenylvinyl-4-[4-methylpiperazin-1-yl])-6-(5-methy-2H-pyrazol-3-yl-amino)-pyrimidine] was provided by EntreMed, Inc. and prepared as a 10 mmol/L stock solution in dimethyl sulfoxide for in vitro studies and at 25 mg/mL in water for the in vivo studies. The free base of ENMD-2076 was used for in vitro experiments and the tartrate salt was used for oral gavage administration in vivo.

Evaluation of cytotoxicity

To evaluate cytotoxic effects, the sulforhodamine B (SRB) method was used as previously described (19, 20). Briefly, cells were plated in 96-well flat-bottomed plates with lids during logarithmic growth, incubated overnight, and then exposed to increasing concentrations of ENMD-2076 from 0 to 20 μmol/L for 24 to 96 hours. Cells were fixed and stained with 0.4% SRB (MP Biomedicals). The SRB staining intensity was measured on a plate reader (Biotek Synergy 2) at an absorbance wavelength of 565 nm. Cell proliferation curves were derived from the raw absorbance data and IC50 values calculated from a minimum of 3 experiments.

In the HCC1937, MDA-MB-134-VI, HCC70, and MDA-MB-468 cell lines, the bromodeoxyuridine (BrdUrd) cell proliferation ELISA assay was also conducted. Cell suspensions containing 5,000 viable cells in logarithmic growth phase were seeded into 96-well black-walled plates and allowed to adhere overnight. Cell lines were exposed to increasing concentrations of ENMD-2076 from 0 to 5 μmol/L for 96 hours at 37°C. The ELISA procedure and analysis were conducted according to the manufacturer’s protocol (Roche Cell Proliferation ELISA, BrdUrd, Roche Diagnostics).

Clonogenic colony formation assay

MDA-MB-468, HCC1806, SK-BR-3, and BT474 cells were seeded at 10,000 cells/well in a 6-well plate for 24 hours and subsequently treated with ENMD-2076 (0.5 or 1.0 μmol/L) for 96 hours. Following treatment, the wells were washed with 1X PBS and incubated in drug free DMEM with 10% FBS for 7 days to allow for regrowth. At the end of the regrowth period, the media was removed, and cells were fixed with methanol (100% v/v) for 30 minutes, and then stained for 1 hour with crystal violet (0.05% crystal violet in 50% methanol). After staining, excess dye was gently washed 3 times with Milli-Q water, plates were then dried at room temperature and photographed.

References

Fluorescence in situ hybridization for AURKA

Dual-color FISH assays were done on prepared slides of select cell lines using 75 ng of Spectrum Green-labeled AURKA (University of Colorado Cancer Center Cytogenetics Lab) and 1 µL of a diluted Spectrum Orange-labeled CEP20 (Abbott Molecular) as previously described (19). A total of 20 metaphase spreads and 50 interphase nuclei were analyzed per specimen.

Flow cytometric analysis of cell-cycle distribution

Cells were seeded in 6-well plates (2 x 10^5 per well) and allowed to adhere for 24 hours before being treated with ENMD-2076 (1.25 µmol/L) for 6, 24, 48, or 96 hours. Cells were then washed with PBS, resuspended in Krisha-stain and allowed to incubate at 4°C for 24 hours. Cells were analyzed for cell cycle by flow cytometry at the University of Colorado Cancer Center Flow Cytometry Core Facility.

Caspase 3/7 activity

Cells were plated at 5,000 cells per well in 96-well, white-walled plates. After 24 hours, cells were exposed to ENMD-2076 (0–5 µmol/L) for 6, 24, 48, or 72 hours. Apoptosis was then determined by measurement of caspase-3 and 7 activity using a luminometric Caspase-Glo-3/7 assay (Promega) according to the manufacturer's protocol and read using a 96-well plate reader. Apoptosis was expressed as a fold increase over untreated control cells.

Phospho-receptor tyrosine kinase array

HCC1937 and HCC70 cells were plated in 60 mm tissue culture dishes (5.0 x 10^5 cells/dish) and allowed to adhere overnight. Cells were then exposed to either media alone or media with 1 µmol/L ENMD-2076 for 24 hours. Floating and adherent cells were harvested and lysed. Samples were diluted to a total of 150 µg of protein in 1.5 ml and probed with the human phospho-receptor tyrosine kinase (RTK) array (R&D Systems) according to the manufacturer’s protocol. Densitometry was conducted using the ChemiDoc Imaging system and Quantity One software package (Bio-Rad).

Senescence

Cells were seeded in 24-well plates and allowed to adhere overnight at 37°C. Cells were then treated with vehicle or ENMD-2076 at 1 µmol/L for time points ranging from 4 to 17 days, and subsequently fixed and stained for senescence associated β-galactosidase activity using the Senescence β-galactosidase Staining Kit (Cell Signaling Technology). The staining solution was then aspirated and wells were overlaid with 70% glycerol. Images were acquired using a Nikon inverted microscope at x4 magnification. Four independent fields were imaged for each treatment group and percentage of β-galactosidase-positive cells relative to nonstaining cells was calculated from manual counts. The assay was conducted in triplicate for the 11-day time point.

In vivo xenograft studies

Five- to 6-week-old female athymic nude (nu/nu) mice were ordered (Harlan Sprague Dawley) and housed in groups of up to 5. Mice were allowed to acclimate for 1 week before handling, provided with sterilized food and water ad libitum and kept on a 12-hour light/dark cycle. Breast cancer cell lines MDA-MB-468 and MDA-MB-231 were harvested during logarithmic growth and resuspended in a 1:1 mixture of serum-free DMEM and Matrigel (BD Biosciences). Bilateral mammary fat pad injections were conducted using 2.5 x 10^6 cells/injection in a volume of 100 µL. Mice were monitored daily for toxicity and weighed twice weekly. Tumor measurements were conducted daily using calipers with the Study Director software package (Studylog Systems) and tumor volume was calculated using the following formula: volume = (length x width^2) x 0.52. When tumors reached a mean volume of 70 mm^3 (MDA-MB-468) or 300 mm^3 (MDA-MB-231), mice were randomized into vehicle and ENMD-2076 groups with 10 tumors/group. ENMD-2076 (100 mg/kg) or vehicle control was administered via oral gavage with continuous once daily dosing. At the end of treatment (16 days for MDA-MB-231 and 40 days for MDA-MB-468), mice were euthanized with CO2 and tumor samples were collected for gross anatomic and immunohistochemical analyses. Formalin-fixed, paraffin-embedded xenograft tumor samples were analyzed by immunohistochemistry of the MDA-MB-468 and MDA-MB-231 xenograft tumors using an antiphospho-histone H3 (pHH3) antibody (Cell Signaling Technology) as a marker of Aurora kinase inhibition (21). In a subset of animals, MDA-MB-231 xenograft tumors were harvested following 4 days of treatment with vehicle control, ENMD-2076 (100 mg/kg), or ENMD-2076 (200 mg/kg) for Western blot analysis. All xenograft studies were conducted in accordance with the NIH guidelines for the care and use of laboratory animals in a facility accredited by the American Association for Accreditation of Laboratory Animal Care with approval by the University of Colorado Institutional Animal Care and Use review board before initiation of experiments.

Gene expression profiles of breast cancer cell lines

Baseline gene expression profiles for 29 breast cancer cell lines were downloaded from the Cancer Cell Line Encyclopedia project (NCBI Gene Expression Omnibus Accession Number: GSE36133). These publicly available data were profiled using Affymetrix HG U133 Plus 2.0 gene arrays. Raw data were obtained from the website, and gene expression profiles were extracted and normalized by the Robust Multiarray Average algorithm using Affymetrix Power Tools. Probe sets representing the same gene were collapsed based on maximum values resulting in 21,009 genes for further data analysis.

Gene set enrichment analysis

Gene set analysis was conducted using the Gene set enrichment analysis (GSEA) software Version 2.0.6 obtained from the Broad Institute (22). Gene set permutations were
conducted 1,000 times for each analysis. The nominal \( P \) value and normalized enrichment score was used to sort the pathways enriched in each phenotype. We used the 313 pathways defined by the BioCarta database as the gene set in this study. One hundred and ninety gene sets passed the gene set size filter criteria (\( \min = 10 \) and \( \max = 500 \)).

**Immunoblotting analysis**  
Cells were seeded into 6-well plates and allowed to attach for 24 hours before exposure to ENMD-2076. Cells were harvested with trypsin/EDTA and then lysed in cell lysis buffer (Cell Signaling Technology). Fifty micrograms 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 for 1 hour in blocking buffer, before overnight incubation at 4°C with one of the following primary antibodies: PARP, p53, survivin, BAX, p21 (Cell Signaling Technology), p73β (AB-2, Calbiochem), and β-actin (Sigma Aldrich). Following primary antibody incubation, membranes were washed 4 times for 10 minutes in TBS-Tween (0.1%) and then incubated with the appropriate secondary anti-rabbit or anti-mouse IgG1 horseradish peroxidase–conjugated antibody at 1:50,000 (Jackson Immunoresearch) for 1 hour at room temperature. After 4 additional washes, blots were developed using the Odyssey Infrared Imaging System (LI-COR Biosciences). Immunoblot experiments were conducted in triplicate for each antibody. To enrich for phospho-Aurora kinases, HCC1806 cells were arrested at \( G_2 \)-M phase by incubation with 0.5 \( \mu \)g/mL nocodazole (Sigma Aldrich) for 18 hours. Cells were then exposed to either media alone or media with the indicated concentration of ENMD-2076 for 2 hours, harvested, and immunoblotted for p-AURKA, B and C using a pan-p-Aurora Kinase antibody (Cell Signaling Technology) according to the protocol described above. MDA-MB-231 xenograft tumors were harvested following 4 days of treatment as described above. Protein was isolated and Western blot experiments were conducted as above for p53, cleaved PARP, survivin, pHH3 (Cell Signaling Technology), and β-actin (Sigma Aldrich).

**Statistical analysis**  
Treatment groups were compared by ANOVA parametric analysis of the means using a commercially available statistical program (Prism 4.0, Graph Pad). The comparisons between sensitivity and correlations of the ER and HER2 status were conducted using Fisher’s exact test with \( p = 0.05 \) as the cut-off for statistical significance.

**Results**  
**In vitro antiproliferative activity of ENMD-2076 against 29 molecularly characterized breast cancer cell lines**  
A diverse panel of 29 breast cancer cell lines representative of the clinically defined breast cancer subtypes was screened for in vitro sensitivity to ENMD-2076. Fig. 1A depicts the in vitro response of ENMD-2076 treatment in breast cancer cell lines where \( IC_{50} \) values ranged from 0.25 to 16.1 \( \mu \)mol/L. We defined ENMD-2076-sensitive and resistant cell lines as \( IC_{50} \leq 1 \) \( \mu \)mol/L and \( IC_{50} \geq 3 \) \( \mu \)mol/L, respectively. Using this sensitivity cut-off, 8 and 11 cell lines from the breast cancer panel were classified as sensitive and resistant to ENMD-2076, respectively. Interestingly, the cell lines that were most sensitive to ENMD-2076 were enriched for TNBC, whereas the more resistant lines were enriched for HER2 and luminal subtypes (Fisher’s exact test, \( P = 0.0045 \); Fig. 1B).

Although ER, PR, and HER2 remain the dominant molecular classifiers of breast cancers, there are other well-characterized genomic changes that may affect response to targeted therapies, including TP53, BRCA1, PTEN, and PIK3CA mutations. Although TP53 mutations have been estimated to occur in approximately 50% of ER+ breast cancers, we found that TP53 mutations were uniformly present in the TNBC cell lines (23). AURKA gene amplification by FISH was detected in a subset of HER2-amplified cell lines, but did not result in increased AURKA gene expression (Supplementary Fig. S1, Fig. 1A). There was no correlation between the presence of a TP53 mutation and sensitivity to ENMD-2076 exposure; however, within the TNBC subgroup there was a correlation between increased TP53 expression and sensitivity to ENMD-2076 (Pearson’s Correlation Coefficient \( r = -0.49, P = 0.0537 \)).

**The effect of ENMD-2076 treatment on colony formation**  
To assess the delayed effects of ENMD-2076 exposure on colony formation, clonogenic assays were conducted in a subset of sensitive and resistant cell lines. ENMD-2076 treatment for 96 hours resulted in absent colony formation in the sensitive MDA-MB-468 cell line. In the HCC1806, SKBR-3, and BT474 cell lines, there was a decrease in colony formation as compared with no drug control; however, cells maintained the ability to form colonies (Fig. 2A). Interestingly, we observed an increase in cellular and nuclear size consistent with a senescent phenotype in the resistant cells remaining following exposure to ENMD-2076 (Fig. 2B).

**Treatment with ENMD-2076 induces a G2 cell-cycle arrest and induction of apoptosis in breast cancer cell lines**  
\( G_2 \) cell-cycle arrest is classically observed following AURKA inhibition and has been shown in multiple myeloma cell lines exposed to ENMD-2076 at micromolar concentrations (14, 24–26). A \( G_2 \) cell-cycle arrest was seen following exposure to ENMD-2076 at 1.25 \( \mu \)mol/L with the maximal effect observed at 72 hours (Fig. 3A and B). Polyploidy was not observed in the \( G_2 \) cell-cycle arrested population. Caspase-3 and 7 are critical enzymes in the apoptotic response, and their increase correlates with induction of apoptosis. We investigated ENMD-2076 concentrations of 0 to 5 \( \mu \)mol/L and found a dose response, with peak effects at 48 hours (Fig. 3C). A statistically significant increase in caspase-3/7 activity was observed in the MDA-MB-468 and HCC1187 sensitive cell lines at 48 hours.
Exposure to ENMD-2076 following cell-cycle synchronization with nocodazole resulted in inhibition of pAURKA, B, and C at concentration of ENMD-2076 of 0.5 and 2.5 μmol/L in the HCC1806 TNBC cell line (Fig. 3E).

RTK array
To further investigate the molecular mechanisms of ENMD-2076 inhibition of TNBC growth, we used phospho-RTK arrays that allowed us to assess the activity of numerous RTKs on a single platform. As shown in Supplementary Fig. S2, treatment with ENMD-2076 resulted in inhibition of pVEGFR1 and FGFR1/3 in the HCC70 sensitive cell line and VEGFR1 in the HCC1937 resistant cell line. As expected, pVEGFR2 was not expressed in these cell lines in vitro at a concentration sufficient for quantification, therefore, modulation of this target was not observed in this assay (Supplementary Fig. S2).

ENMD-2076 treatment leads to senescence in a subset of breast cancer cell lines
We next investigated the effects of ENMD-2076 in 2 sensitive and 2 resistant cell lines on DNA synthesis as compared with protein synthesis using the BrdUrd assay in addition to the SRB assay. In the sensitive HCC70 and MDA-MB-468 cell lines, the proliferation curves were similar using both assays (Fig. 4A). However, in the resistant...
HCC1937 and MDA-MB-134-VI cell lines, a 50% decrease in DNA synthesis was observed at submicromolar concentrations with preserved protein synthesis, consistent with a permanent cell-cycle arrest or senescence (27). As shown in Fig. 4B and C, we observed a statistically significant increase in senescence-associated β-galactosidase activity and increased cell size in the ENMD-2076 treated resistant cell line MDA-MB-134-VI compared with vehicle controls.

**ENMD-2076 shows antitumor activity against TNBC xenograft models**

To confirm our findings that TNBC cell lines are sensitive to the antiproliferative effects of ENMD-2076 in an in vivo environment, we evaluated the antitumor activity of ENMD-2076 against 2 TNBC orthotopic xenografts. As depicted in Fig. 5A, MDA-MB-468 xenograft tumors in mice treated with ENMD-2076 exhibited static growth for 40 days of treatment when compared with vehicle controls. In the MDA-MB-231 xenograft model, treatment with ENMD-2076 (100 mg/kg) resulted in tumor growth inhibition that is comparable to published results using this model (18). The dose of ENMD-2076 used to treat these mice was well tolerated and animals exhibited no signs of toxicity as manifested by illness or weight loss. As depicted in Fig. 5B, there was a trend toward an increase in pH3 staining cells in the MDA-MB-231 ENMD-2076-treated group compared with the vehicle control group; however, this was not statistically significant (104.7 ± 36.2 positive cells/mm² in treated and 79.9 ± 34.5 in control). In the MDA-MB-468 xenograft model, there were very few viable tumor cells in the excised treated tumors; however, the same pattern was exhibited (111.4 ± 78.6 positive cells/mm² in treated and 56.4 ± 6.7 positive cells/mm² in control). Following 4 days of treatment with ENMD-2076 at 100 or 200 mg/kg, no change was observed in pH3 by Western blot analysis (Fig. 5C). An increase in cleaved PARP was observed at 200 mg/kg (Fig. 5C), indicating induction of apoptosis in vivo. A decrease in p53 was observed following treatment that was more pronounced in the lower dosing group (Fig. 5C).

**Global analysis of gene expression profiles**

Baseline whole-genome transcriptome profiles of the 8 sensitive (IC₅₀ values < 1 μmol/L: HCC1187, MDA-MB-468, MCF7, HCC70, Hs578T, BT549, MDA-MB-157, and CAL-120) and 11 resistant (IC₅₀ > 3 μmol/L: HCC1806, CAMA-1, SK-BR-3, AU565, HCC1937, BT474, BT483, UACC812, MDA-MB-134-VI, ZR-75-30, and HCC1419) cell lines were obtained by microarray analysis. By comparing the gene expression profiles between the ENMD-2076-sensitive and resistant cell lines, a clear pattern of differentially expressed genes was observed. Using significance analysis of microarrays (SAM; ref. 28), 580 genes were differentially expressed between the ENMD-2076-sensitive and resistant cell lines, a clear pattern of differentially expressed genes was observed. Using significance analysis of microarrays (SAM; ref. 28), 580 genes were differentially expressed between the sensitive and resistant cell lines at least 2-fold (false discovery rate <0.03). 237 and 343 genes were identified as upregulated and downregulated in the sensitive cell lines as compared with the resistant cell lines, respectively. Supplementary Fig. S3 depicts the heatmap of the 580 differentially expressed genes between these phenotypes (28).
differentially expressed genes and pathways in TNBC

correlation of ENMD-2076 sensitivity with... 

ENMD-2076, which is consistent with the HER2 phenotype. 

representative images for the MDA-MB-468 cell line treated with vehicle or ENMD-2076 1.25 µmol/L. C, fold change in caspase-3 and 7 activity after treatment with ENMD-2076 at the indicated time points and concentrations; experiment was conducted on 1 sensitive cell line (HCC1187, MDA-MB-468) and 1 resistant cell line (HCC1937). D, fold change in caspase-3 and 7 activity after treatment with ENMD-2076 at the indicated concentrations for 48 hours; experiment was conducted on 3 sensitive (HCC1187, MDA-MB-468, and HCC70) cell lines and 2 resistant (HCC1806 and HCC1937) cell lines. P < 0.001; **P < 0.05. E, immunoblot illustrates the inhibition of p-Aurora A, B, and C after cell-cycle synchronization using nocodazole followed by exposure to ENMD-2076 for 2 hours in the HCC1806 cell line.

Gene set enrichment analysis confirms HER2 pathway enriched in ENMD-2076 resistant cell lines

GSEA was conducted on the baseline gene expression profiles of the 8 sensitive and 11 resistant cell lines. As expected by the subtypes of the cell lines, we found the ERBB2 signaling pathway (P = 0.027) as the top pathway enriched in the ENMD-2076-resistant phenotype (Supplementary Fig. S4). These results indicate that upregulation of the HER2 signaling pathway may contribute to resistance to ENMD-2076, which is consistent with the HER2 phenotype of the resistant lines (Fig. 1A).

Correlation of ENMD-2076 sensitivity with differentially expressed genes and pathways in TNBC

Although the TNBC subset was more sensitive as compared with the HER2-amplified and ER+ cell lines (Fig. 1B), there were 2 TNBC cell lines that were resistant to the antiproliferative and proapoptotic activity of ENMD-2076 exposure in vitro. To identify genes and pathways that correlated with ENMD-2076 sensitivity and resistance within the TNBC subtype, we compared the gene expression profiles of the 2 most sensitive TNBC cell lines, HCC1187 (IC50 = 0.25 µmol/L) and MDA-MB-468 (IC50 = 0.46 µmol/L) and the 2 most resistant TNBC lines HCC1806 (IC50 = 3.12 µmol/L) and HCC1937 (IC50 = 5.83 µmol/L). From the SAM analysis, 1,906 genes were differentially expressed between TNBC sensitive and resistant lines at least 2-fold (false discovery rate < 0.01). Among them, 942 and 964 genes were overexpressed and underexpressed in TNBC sensitive lines as compared with the resistant cell lines, respectively (Supplementary Fig. S5A). Using the same criteria as previously described, pathway analysis...
revealed the G2–M cell-cycle checkpoint as the top pathway (P = 0.03) enriched in the ENMD-2076 TNBC sensitive lines (Supplementary Fig. S5B). The other top 10 pathways enriched in the sensitive group included the p53 signaling pathway (P = 0.14; Supplementary Fig. S5C).

**Sensitivity to ENMD-2076 is associated with p53 mutation accompanied by p53 cellular accumulation in TNBC**

As illustrated in Fig. 1A, p53 mutations were present in all of our TNBC cell lines. Given the observed upregulation of p53 signaling in the sensitive TNBC cell lines as compared with resistant cell lines (Supplementary Fig. S5C), we conducted immunoblotting studies to evaluate for p53 cellular accumulation. As depicted in Fig. 6A and B, we observed accumulation of p53 which was accompanied by increased p53 gene expression in the sensitive TNBC cell lines as compared with the resistant TNBC cell lines. We analyzed expression levels of p53 target genes as measured by micro-array, which uncovered differential changes in transcripts in the sensitive versus resistant cell lines (Fig. 6B). In addition, the differentially expressed genes identified in the 2 most sensitive versus 2 most resistant TNBC lines were enriched in p53 target genes (hypergeometric test P = 0.02; Fig. 6B; ref. 29).

The cellular accumulation of p53 is commonly observed in cells with mutated p53, at least in part due to impaired p53-induced expression of MDM2 and decreased cellular clearance of the mutant p53 proteins (30). Previous studies have shown that the p53 family member p73 can mediate apoptosis in response to Aurora kinase inhibition in p53 mutant cell lines (31, 32). Thus, we investigated expression of p73β following exposure to ENMD-2076 and as depicted in Fig. 6C, found increased p73β expression in response to...
ENMD-2076 exposure in the p53 mutant sensitive TNBC cell lines. This increase was accompanied by upregulation of the proapoptotic protein Bax, as well as increased cleaved PARP, indicating that p73 may play a role in ENMD-2076-induced apoptotic cell death in these p53 mutated TNBC cell lines. In addition, a transient increase in survivin, a key negative regulator of p21, was observed and corresponds temporally to a decline in p21.

Discussion

While the number of anticancer agents with activity in advanced TNBC continues to increase, there is still a great need for the development of novel agents with tolerable side-effect profiles that can be coupled with accurate patient selection strategies to palliate cancer-related symptoms and prolong survival in individuals with this disease. ENMD-2076 is a novel, orally bioavailable small-molecule inhibitor of angiogenic, mitotic, and cell-cycle regulating kinases including AURKA/B and VEGFR2/KDR with rationale for activity against TNBCs due to their high proliferative index and dependence on angiogenesis. The aim of this study was to evaluate the anticancer activity of ENMD-2076 against TNBC cell lines as compared with luminal and HER2-amplified cell lines and use baseline gene expression profiling with GSEA to determine pathways predictive of sensitivity and resistance to ENMD-2076 with a final goal of developing biomarkers predictive of sensitivity to this agent in TNBC which can be applied to human clinical trials.

Our studies showed that ENMD-2076 exhibits anticancer activity against cell line-based in vitro and in vivo models of human breast cancer, with more robust antiproliferative activity in TNBC cell lines as compared with HER2-amplified and luminal cell lines. In vitro exposure to ENMD-2076 resulted in a G2–M cell-cycle arrest which was independent of sensitivity to the antiproliferative effects of ENMD-2076. While we observed in vitro a decrease in pAURKA and B in response to ENMD-2076 exposure at submicromolar concentrations, the observed G2–M arrest is most phenotypically consistent with dominant AURKA inhibition, as opposed to the failure of cytokinesis and polyploidy seen

Figure 5. ENMD-2076 causes tumor growth inhibition in vivo. A, effect of ENMD-2076 treatment (100 mg/kg) in 2 cell line-derived orthotopic xenografts. Tumor inhibition in vivo was statistically significant in both models (MDA-MB-468, **, P < 0.01; MDA-MB-231, *, P < 0.05). B, immunohistochemical analysis of phospho-histone H3 in MDA-MB-231 tumors from mice treated with ENMD-2076 or vehicle. C, Western blot analysis showing p53, cleaved PARP, survivin, pHH3, and actin in MDA-MB-231 tumors from mice treated with ENMD-2076 100 mg/kg, 200 mg/kg, or vehicle for 4 days.
primarily with AURKB inhibitors (33). We observed induction of caspase-independent apoptosis in cell lines sensitive to the antiproliferative activity of ENMD-2076 as assessed by protein-based proliferation assays, as has been previously reported (13). In addition, we observed a decline in DNA replication and senescence as an endpoint in cell lines resistant to the cytotoxic effects of ENMD-2076 treatment as measured by protein-based proliferation assays.

In vivo exposure to ENMD-2076 in MDA-MB-231 and MDA-MB-468 TNBC xenograft models resulted in significant antitumor activity. Our data also reveal that in the MDA-MB-468 TNBC xenograft model, where treatment was initiated when tumor volumes reached approximately 70 mm³, exposure to ENMD-2076 resulted in static growth for 40 days. Furthermore, when these tumors were excised, only rare viable tumor cells were found. In the MDA-MB-231 model, exposure to ENMD-2076 100 mg/kg once daily with continuous dosing, a significant decrease in tumor growth was observed. These results are similar to those reported by Fletcher and colleagues in a similar MDA-MB-231 xenograft model in which tumor growth inhibition was observed at 75 mg/kg, whereas static growth followed by modest tumor regression was observed in animals treated with a higher dose of 302 mg/kg (18). These data, along with similar dosing experiments in xenograft models of colorectal cancer reported by Tentler and colleagues, support a positive dose response correlation for ENMD-2076 treatment in vivo (15).

In addition, the antitumor activity reported here for ENMD-2076 toward models of TNBC is consistent with reports for other Aurora kinase inhibitors including MK-5108, AZD1152, PF-03814735, and ZM447439 (24, 34–36).

Figure 6. Comparison of p53 pathway between sensitive and resistant cell lines, and molecular effects of ENMD-2076 treatment. A, immunoblot of baseline p53 expression in 2 resistant (HCC1937 and HCC1806) and 3 sensitive (HCC70, MDA-MB-468, and HCC1187) cell lines. B, p53 target genes were enriched in the differentially expressed list of the 2 most sensitive versus 2 most resistant TNBC cell lines (hypergeometric test P = 0.02). The p53 target genes were obtained from Riley and colleagues (29). C, immunoblot showing molecular effects of ENMD-2076 on cell-cycle and apoptotic effectors in 1 sensitive (HCC1187) cell line and 1 resistant (HCC1937) cell line over a period of 0 to 72 hours. D, immunoblot showing effects of ENMD-2076 1 µmol/L on p53 and survivin in 2 sensitive (MDA-MB-468 and HCC1187) and 2 resistant (HCC1937 and HCC1806) cell lines over a period of 0 to 72 hours.
expression of the HER2 pathway recapitulates our data in monolayer culture systems, whereby HER2-amplified cell lines were more resistant to the *in vitro* effects of ENMD-2076 as compared with TNBC cell lines.

While our TNBC cell lines were more sensitive to the antiproliferative effects of ENMD-2076 treatment, there were resistant TNBC cell lines in our panel. To identify potential pathways mediating resistance or sensitivity to ENMD-2076 in TNBC, we used GSEA to identify differentially expressed pathways in cell lines sensitive and resistant to the antiproliferative effects of ENMD-2076. We identified multiple pathways that were enriched in the sensitive TNBC cell lines that included p53 as a core gene. Despite the uniform presence of p53 mutation in our TNBC cell lines, we found increased cellular accumulation of p53 in the sensitive cell lines as compared with absent p53 protein expression in the resistant cell lines. This difference in protein expression was accompanied by differential p53 gene expression that was increased in the sensitive cell lines as compared with the resistant cell lines. Furthermore, there were significant differences in the expression of p53 network genes in the sensitive versus resistant TNBC cell lines indicating a functional difference with regards to p53 between the 2 groups.

The p53 gene network is a complex system affecting cellular outcomes, including cell cycle arrest, senescence, autophagy, and apoptosis in response to stress (37). Further supporting the functional difference in p53 activity between the sensitive and resistant cell lines, we observed a decline in DNA replication associated with morphologic and biologic changes associated with senescence following prolonged ENMD-2076 exposure in the setting of mutant p53 accompanied by absent p53 protein expression. One hypothesis to explain these results is that genes involved in growth arrest contain high-affinity p53 binding sites in their promoters, whereas genes involved in apoptosis contain low-affinity p53 binding sites. In this quantitative model, higher levels of mutant p53 may be required for an apoptotic response (38). The induction of senescence by Aurora kinase inhibitors has previously been reported in preclinical models; however, to our knowledge this is the first report showing an association with altered p53 function (27). Therapy-induced senescence has been observed in response to other anticancer agents and radiation; however, it remains unclear as to the clinical impact of senescence as a terminal outcome (39). Markers of senescence should be evaluated in posttreatment clinical samples from patients treated with ENMD-2076 on future clinical trials to further understand the impact of senescence on tumor progression or response to therapy.

There is an emerging body of literature describing heterogeneity within p53 mutants with varying apoptotic and cell-cycle arrest capabilities (40, 41). Changes in biologic outcome have been linked to changes in the ability of different p53 mutants to transactivate and directly regulate p53 target genes (40). It has been proposed that tumor cells may select for mutant p53 based on lack of certain tumor-suppressive functions of wild-type p53; however, p53 mutants may have other retained or even exaggerated aspects of wild-type p53 function (42). Our data show an increase in mutant p53 mRNA and protein expression to be associated with a proapoptotic response to ENMD-2076 treatment in TNBC. We hypothesize that mutant p53 in our sensitive cell lines mediates the apoptotic response through direct transactivation of target genes integral to the apoptotic pathway or through interaction with p73.

The antiangiogenic activity of ENMD-2076 is unlikely to play a major role in the *in vitro* antitumor effects observed with this agent due to the lack of endothelial support cells in monolayer culture models and the paucity of VEGFR2 receptors on human breast cancer cells. However, we did observe a decrease in pVEGFR1, pFGFR1, and pFGFR3 following *in vitro* exposure to ENMD-2076. The potent antiangiogenic activity of ENMD-2076 treatment as showed in other xenograft models, however, is likely a key contributor to the *in vivo* activity observed (15, 18). In comparison to other breast cancer subtypes, TNBCs commonly overexpress VEGF and are more vascular which may be related to the *in vivo* activity observed in this study (16, 17). Antiangiogenic agents such as sunitinib have been evaluated in TNBC with modest single agent activity (43), and there is evidence that other antiangiogenic agents such as bevacizumab may potentiate the activity of cytotoxic chemotherapy (44, 45). To our knowledge, ENMD-2076 is unique in combining angiogenic and Aurora kinase inhibition.

ENMD-2076 has completed a phase I dose escalation study in patients with advanced solid tumors in which a patient with metastatic TNBC sustained clinically meaningful stabilization of disease lasting for 41 weeks (46). The drug had an acceptable safety profile, with predictable, mechanism-based side effects including hypertension and mild neutropenia and is currently being evaluated in early phase clinical trials in hematologic malignancies (46). Our data indicate that a comprehensive assessment of the p53 pathway in TNBC including p53 mutation, p53 gene expression, p53 protein expression, and expression of downstream effectors may be predictive of response to ENMD-2076 treatment. The results of data presented in this manuscript support the planned investigator-initiated multi-institutional Phase II clinical trial of ENMD-2076 in patients with advanced TNBC with an emphasis on the continued development of biomarkers predictive of response in this breast cancer subset.

**Disclosure of Potential Conflicts of Interest**

G.C. Fletcher is employed by EntreMed as a scientist. No potential conflicts of interest were disclosed by the other authors.

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