AMG 900, a small molecule inhibitor of aurora kinases, potentiates the activity of microtubule-targeting agents in human metastatic breast cancer models

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Abstract

Breast cancer is the most prevalent malignancy affecting women and ranks second in cancer deaths, where death occurs primarily from metastatic disease. Triple-negative breast cancer (TNBC) is a more aggressive and metastatic subtype of breast cancer that is initially responsive to treatment of microtubule-targeting agents (MTAs) such as taxanes. Recently, we reported the characterization of AMG 900, an orally bioavailable, potent and highly selective pan-aurora kinase inhibitor that is active in multidrug resistant cell lines. In this report, we investigate the activity of AMG 900 alone and in combination with two distinct classes of MTAs (taxanes and epothilones), in multidrug resistant TNBC cell lines and xenografts. In TNBC cells, AMG 900 inhibited phosphorylation of histone H3 on Ser10, a proximal substrate of aurora-B, and induced polyploidy and apoptosis. Furthermore, AMG 900 potentiated the antiproliferative effects of paclitaxel and ixabepilone at low nanomolar concentrations. In mice, AMG 900 significantly inhibited the growth of MDA-MB-231 (F11) (parental), MDA-MB-231 (F11) PTX-r (paclitaxel resistant variant), and DU4475 xenografts. The combination of AMG 900 with docetaxel enhanced tumor inhibition in MDA-MB-231 (F11) xenografts compared with either monotherapy. Notably, combining AMG 900 with ixabepilone resulted in regressions of MDA-MB-231 (F11) PTX-r xenografts, in which >50% of the tumors failed to regrow 75 days after the cessation of drug treatment. These findings suggest that AMG 900, alone and in combination with MTAs, may be an effective intervention strategy for the treatment of metastatic breast cancer (MBC), and provide potential therapeutic options for patients with multidrug resistant tumors.
Introduction

The step-wise process of somatic cell division ensures faithful segregation of duplicated chromosomes into two equal daughter cells. Deregulation of the cell cycle is a hallmark of cancer, characterized by uncontrolled proliferation and defects in chromosome segregation. The human kinome contains a number of enzymes that specifically regulate mitotic progression and spindle assembly checkpoint (SAC) function, including two members of the aurora family of serine-threonine kinases (aurora-A and -B). Both play unique and essential roles in the G2-M phase of the cell cycle and are aberrantly expressed in many human cancers, including breast cancer (1-3).

Breast cancer is a heterogeneous disease that can be classified into subtypes with different prognosis and treatment strategies. Global gene expression profiling has defined five distinct subtypes that include luminal A, luminal B, ERBB2-enriched, basal-like, and claudin-low (4, 5). The latter two non-luminal subtypes that lack expression of estrogen and progesterone hormone receptors (ER and PR) and ERBB2 are referred to as triple-negative breast cancer. The TNBC subtype is characterized by its more aggressive and metastatic nature, high degree of genomic instability, elevated proliferation, and frequent inactivation of p53 (6, 7). Metastatic breast cancer commonly spreads to the bones, lungs, liver, and the central nervous system and remains incurable in most patients. Transcriptome-based analysis of primary breast cancers has shown that increased expression of AURKA and AURKB correlates with elevated proliferation, ER negativity, and primarily (but not exclusively) poorly differentiated non-luminal tumors (8, 9). Recently, a protein expression based biomarker algorithm analysis of cell-cycle status showed that aggressive breast cancer subtypes (ERBB2-enriched and triple-negative) were associated with significantly elevated levels of aurora-A, p-histone H3 Ser10, Mcm2, Ki67, Geminin, and Plk1 (10). Amplification of the AURKA gene locus has been observed in a subset of human cancers that includes breast tumors (2). In HeLa cells, ectopic expression of aurora-A at levels similar to cancer-associated gene amplification induces resistance to paclitaxel (11). Taken together, the critical role that aurora kinases play in mitosis and their overexpression in MBC make them attractive therapeutic drug targets.
Microtubule-targeting agents such as taxanes are among the most active drugs for the treatment of MBC. However, treatment frequently fails due to de novo or acquired resistance to taxanes. The underlying causes of cancer resistance are multifactorial and complicated by tumor heterogeneity (12, 13). One of the intrinsic properties of TNBC cells is their enhanced genomic instability, which can accelerate the generation of resistant subpopulations. One well-characterized mechanism of resistance is the overexpression of the MDR1 gene, which encodes P-glycoprotein (P-gp), a drug-efflux pump capable of efficiently extruding taxanes from cells. Another mechanism that can render tumor cells resistant to taxanes is β-tubulin modifications caused by mutation or changes in isotype expression (12-14). Epothilones, similar to taxanes, activate the SAC and inhibit cell proliferation by stabilizing microtubules. Ixabepilone, an epothilone B analog, has lower susceptibility to P-gp mediated drug efflux and has demonstrated durable clinical activity in MBC tumors resistant to taxanes (15, 16). Combining two antimitotic agents with distinct modes of action, SAC activation (microtubule stabilizer) versus SAC silencing (aurora-A/B inhibition), may provide an approach to block avenues of resistance and limit a cancer cell’s ability to evade death (3, 17).

Recently, we reported the characterization of AMG 900, a novel potent and highly selective pan-aurora kinase inhibitor with activity in tumor cell lines that are resistant to taxanes and 3 other aurora kinase inhibitors. AMG 900 was broadly active in multiple tumor xenografts, including 3 multidrug-resistant models (18). In this report, we explore the activity of AMG 900 alone and in combination with two different classes of MTAs in multidrug resistant TNBC cell lines and xenografts. In vitro, AMG 900 induced polyploidy and apoptosis, and inhibited the growth of P-gp expressing TNBC cells at low nanomolar concentrations. In combination, AMG 900 enhanced the antiproliferative effects of MTAs in TNBC cells in vitro and in established tumor xenografts. Notably, AMG 900 plus ixabepilone resulted in durable tumor regressions in MDA-MB-231 (F11) PTX-r xenografts compared with either monotherapy. Our data provide preclinical evidence that AMG 900, alone and in combination with MTAs, has the potential to treat patients with metastatic breast cancer.
Materials and Methods

Small molecule inhibitors. AMG 900 N-(4-((3-(2-amino-4-pyrimidinyl)-2-pyridinyl)oxy)phenyl)-4-(4-methyl-2-thienyl)-1-phthalazinamine) was synthesized at Amgen (Fig. 1) (WO2007087276). Paclitaxel (Sigma), docetaxel (Sanofi-Aventis), and ixabepilone (Bristol-Myers Squibb) were procured from commercial sources and molecular structures have previously been reported (19).

Cell lines. Human cancer cell lines were obtained from the American Type Culture Collection (ATCC) unless otherwise specified. Cells were authenticated and certified by ATCC. ATCC ensures each cell line is negative for Mycoplasma, bacteria, and fungi contamination; confirms species identity; and conducts DNA profiling and cytogenetic analysis to authenticate each cell line. Cell lines were cultured in media supplemented with 10% FBS using conditions specified by ATCC. MDA-MB-231 (F11) human breast cancer cells were a gift of Toshiyuki Yoneda (University of Texas, San Antonio, TX) and were derived through in vivo passage of MDA-MB-231 parental cells (ATCC, HTB-26) for selection of bone-tropic cells growing in the hind limbs of mice after intra-cardiac injection (20). MDA-MB-231 (F11) PTX-r (paclitaxel resistant) cells were established at Amgen by growing the cells in the presence of increasing concentrations of paclitaxel over a period of 6 months. MDA-MB-231 (F11) PTX-r cells were maintained in complete media supplemented with paclitaxel at 50 nmol/L.

Animals. All experimental procedures were conducted in accordance with Amgen’s Institutional Animal Care and Use Committee and U.S. Department of Agriculture regulations. Four to six-week-old female athymic nude mice (Harlan Sprague Dawley) were housed five per sterilized filter-capped cages and maintained under aseptic and pathogen-free conditions. The animal holding room provided 12 hours of alternating light and dark cycles and met the standards of the Association for Assessment and Accreditation of Laboratory Animal Care specifications. Food, water, and nutritional supplements were offered ad libitum. All drugs were administered based on the individual body weight of each mouse. AMG 900 was formulated as a suspension in 2% HPMC, 1% Tween-80, at pH 2.2. Taxanes were formulated as previously described (21). Ixabepilone (2 mg/mL stock in supplied diluent) was diluted with Lactated Ringer’s solution to 0.5mg/mL prior to dosing.

Tumor xenograft pharmacodynamic assay (p-histone H3). Mice with established MDA-MB-231 (F11) human xenograft tumors were administered a single oral dose of vehicle or AMG 900 at 3.75, 7.5 or 15 mg/kg.
(n = 3 animals per group). At 3 hours after treatment, tumor tissue was collected and processed as described in Supplementary Material and Methods. Deparaffinized sections were heated in citrate buffer to retrieve antigenicity and stained with an anti-phospho-histone H3 on Ser\textsuperscript{10} antibody (Millipore) followed by detection with an anti-rabbit IgG-alexa-568 antibody (Invitrogen) and DAPI. Imaging analysis was performed using a TE2000-PFS inverted microscope imaging system (Nikon) equipped with MetaMorph software. The number of p-histone H3 positive cells (2 image fields per tumor) was determined using a threshold based count algorithm. Blood was collected from individual mice to determine the concentration of AMG 900 in plasma.

**Tumor xenograft efficacy studies.** Mice were injected subcutaneously with 5 x 10\textsuperscript{6} MDA-MB-231 (F11) human breast tumor cells in 50% Matrigel (BD Biosciences). When tumors were established (approximately 200 mm\textsuperscript{3}), mice were randomized into experimental groups (n = 10-12 per group) and treated orally twice daily (b.i.d.) with AMG 900 at 3.75, 7.5 or 15 mg/kg intermittently for 2 consecutive days per week for 3 weeks. For the combination studies, mice were administered either docetaxel at 10 mg/kg intraperitoneally (i.p.) weekly or ixabepilone at 5 mg/kg intravenously (i.v.) weekly, one day before AMG 900 treatment at 7.5 mg/kg. Mice were provided nutritional supplements [Bacon Softies (BioServ), Transgel (Charles River), and Nutri-Cal (EVSCO)] on a daily basis during the treatment cycle. Tumor volumes and body weights were recorded twice per week using a digital caliper and analytical scale, respectively. Tumor volumes were calculated as previously described (21). Tumor data were represented by mean tumor volume ± SEM. Tumors were collected and processed for routine histology (see Supplementary Materials and Methods).

**Statistical analysis.** For the pharmacodynamic assays, the effects of AMG 900 on p-histone H3 were compared using Factorial ANOVA followed by Dunnett’s post hoc test for multiple comparisons as appropriate. For the single agent efficacy studies, the effects of AMG 900 or docetaxel on tumor growth was assessed by repeated measures analysis of variance (RMANOVA) followed by Dunnett’s test for multiple comparisons. For the combination efficacy studies, the effects of AMG 900 in combination with docetaxel or ixabepilone on tumor growth were assessed by separate RMANOVA between the combination group and each of the relevant single agents. In all statistical analysis, differences were considered significant at a $P$ value of <0.05.

**Microarray analysis.** Total RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen) and processed following the protocols described in the Agilent Two-Color Microarray-Based Gene Expression Analysis
Protocol v5.5. Cy3- or Cy5-labeled cRNA were generated using the Agilent Low RNA Input Linear Amplification Labeling Kit (Agilent Technologies) starting with 200 ng of total RNA. Labeled cRNA was purified using magnetic beads (Beckman Coulter Genomics) for competitive hybridization to the Agilent Human Whole Genome 4x44K array (AMADID 014850). Each labeled experimental sample was hybridized against its corresponding control sample in fluor-reversed pairs. Arrays were washed on the Little Dipper Processor for Agilent Arrays (SciGene) and scanned on the Agilent High-Resolution Microarray Scanner. Data was extracted from images using the Agilent Feature Extraction version 10.5, and imported into Rosetta Resolver 7.2 for analysis. Raw microarray files have been imported into the GEO database (accession number GSE47435).

Details regarding Western blot analysis, cell imaging, cell-cycle assays, cell count and colony formation assays, and histology are described in the Supplementary Materials and Methods.

Results

Inhibition of aurora-B by AMG 900 induces polyploidy and cell death in human breast cancer cell lines.

The effect of AMG 900 on aurora–B activity was evaluated by immunofluorescence-based detection of phosphorylation of histone H3 on Ser\textsuperscript{10} in MDA-MB-231 and DU4475 TNBC cell lines. As shown in Figure 2A, cells in mitosis treated with DMSO alone showed strong positive staining with anti-p-histone H3 antibody (top panel), whereas mitotic cells treated with AMG 900 suppressed aurora-B activity measured by the absence of phosphorylation (bottom panel). To further characterize the cellular effects of AMG 900 on the same breast cancer cells, flow cytometry was used to simultaneously measure DNA content, DNA synthesis (BrdUrd), and apoptosis (cleaved caspase-3). As shown in Figure 2B, treatment with AMG 900 induced an accumulation of cells with >4N DNA content by 48 hours. BrdUrd incorporation decreased in the 2N and 4N DNA cell fractions and increased in the >4N DNA cell fraction, indicating AMG 900 induced endoreduplication (Fig. 2C and 2D). Induction of polyploidy by AMG 900 was associated with apoptotic cell death measured by the increased number of cells staining positive for cleaved caspase-3 (and negative for BrdUrd). Cell death was also
detectable by an increase in SubG1 DNA content. Next, we evaluated the nuclear morphology and centrosome features of cells treated with AMG 900 for 48 hours. Microscopy of MDA-MB-231 cells treated with AMG 900 exhibited enlarged irregular-shaped nuclei with numerous centrosomes detected by anti-pericentrin antibody (Supplementary Fig.1). The mode of action of AMG 900 on TNBC cells is consistent with inhibition of aurora-B; in that AMG 900 silences the SAC, thus leading to polyploidy and cell death (3).

**Cell cycle effects and activity of AMG 900, paclitaxel, and ixabepilone in P-gp-expressing breast cancer cell lines.**

We previously reported that AMG 900 was active in P-gp expressing cancer cell lines resistant to taxanes and 3 well-characterized aurora kinase inhibitors (18). To further investigate these findings, we utilized the highly metastatic MDA-MB-231 (F11) cells, *in vivo* selected to be bone-tropic in mice, and the MDA-MB-231 (F11) PTX-r cells, a variant subline resistant to paclitaxel and docetaxel. Microarray analysis was used to evaluate the gene expression profile of 47 ATP-Binding Cassette (ABC) transporter family members in both MDA-MB-231 (F11) PTX-r and corresponding (F11) parental cells. We determined that both *ABCB1* (MDR1, P-gp) and *ABCB4* (MDR3) genes were differentially expressed (>20-fold increase) in the MDA-MB-231 (F11) PTX-r compared with (F11) parental cells (Supplementary Fig. 2). The ABCB4 gene is located on chromosome 7q21.1, proximal to the ABCB1 gene locus, suggesting that the increase in mRNA expression of both genes may be the result of an amplification on 7q21.1 (22, 23). Indeed, we determined by aCGH analysis the MDA-MB-231 (F11) PTX-r cells showed co-amplification of ABCB1 and ABCB4 gene loci on the long arm of chromosome 7 (data not shown). Next, Western analysis was used to directly compare the level of P-gp protein expressed on MDA-MB-231 (F11) PTX-r and (F11) parental cells, along with MDA-MB-231 and DU4475 cells. Human uterine sarcoma MES-SA DX5 variant cells and corresponding MES-SA parental cells served as P-gp positive and negative controls, respectively (24). Consistent with our microarray results, P-gp expression was elevated in (F11) PTX-r cells compared with the other two MDA-MB-231 cell lines (Fig. 3A). Interestingly, the βIII-tubulin overexpressing DU4475 cells also showed elevated expression of P-gp protein, which may represent a dual mechanism of resistance to taxanes (25, 26). In preparation for future combination studies, we evaluated the cell cycle effects of AMG 900, paclitaxel, and ixabepilone in the same set of breast cancer cell lines to determine single agent potency. As shown in Figure 3B, AMG 900 induced polyploidy in all four
cell lines with ≥4N DNA EC₅₀ values of 1-2 nmol/L and an associated steep slope factor (>4). The cell cycle effects observed with paclitaxel and ixabepilone showed distinct phenotypes at low and high concentrations. At lower concentrations of drug, the primary effect was cell death, as measured by an increase in the SubG₁ DNA content. At higher concentrations of drug, the fraction of cells with 4N DNA content increased, indicating a classic mitotic arrest phenotype (Supplementary Fig. 3A and 3B). Although the cell cycle profiles were largely similar for each cell line treated with ixabepilone or paclitaxel, the P-gp expressing DU4475 and MDA-MB-231 (F¹¹) PTX-r cells were more sensitive to ixabepilone. Consistent with previous reports, the cell death response observed at lower concentrations of MTAs was likely driven by a transient mitotic arrest and not a sustained mitotic arrest (27).

**AMG 900 in combination with MTAs potentiates inhibition of cell growth in MDA-MB-231 (F¹¹) parental and (F¹¹) PTX-r breast cancer cell lines.**

MTAs are the foundation of many combination therapy regimens used to treat solid and hematologic cancers. A number of reports have shown that aurora kinase inhibitors can act synergistically with MTAs, such as taxanes or vinca alkaloids, to inhibit the growth of cancer cell lines in vitro (2, 3). To explore the potential of AMG 900 in combination with MTAs, we treated MDA-MB-231 (F¹¹) cells sequentially with either paclitaxel plus AMG 900 in the (F¹¹) parental cells or ixabepilone plus AMG 900 in the (F¹¹) PTX-r cells. Based on this treatment paradigm, we anticipated transient SAC activation with the MTAs followed by SAC inactivation with AMG 900. As shown in the dosing scheme (Fig. 4A), cells were first treated with either paclitaxel or ixabepilone for 24 hours followed by AMG 900 for 48 hours. After each drug treatment, cells were washed and cultured for a total of 7 days. As controls, cells were treated with DMSO or either agent alone (paclitaxel at 3, 4, or 5 nmol/L; ixabepilone at 10, 13, or 16 nmol/L; AMG 900 at 1.5, 2.5, or 3.5 nmol/L). MDA-MB-231 (F¹¹) parental or (F¹¹) PTX-r cells treated sequentially with either paclitaxel or ixabepilone followed by AMG 900 displayed enhanced inhibition of cell growth, as measured by the decrease in cell number and colony formation (Fig. 4B and 4C). The enhanced cell growth inhibition with this combination approach was observed over a narrow concentration range compared with either agent alone, which was anticipated given the cytotoxic nature of both classes of antimitotic agents. In a previous study, we determined the sequential treatment of paclitaxel followed by AMG 900 resulted in only an additive interaction using parental MDA-MB-231 cells (data
not shown). These results show the antiproliferative effects of MTAs are potentiated by AMG 900 and underscore the potential of combining ixabepilone with AMG 900 to treat tumors resistant to paclitaxel and docetaxel.

**AMG 900 inhibits the phosphorylation of histone H3 and suppresses the growth of human breast cancer xenografts.** To confirm that AMG 900 inhibits aurora-B activity *in vivo*, mice bearing established MDA-MB-231 (F11) parental and (F11) PTX-r tumors were administered a single oral dose of vehicle or AMG 900 at 3.75, 7.5, or 15 mg/kg. As shown in Figures 5A and 5B, administration of AMG 900 for 3 hours resulted in significant inhibition of p-histone H3 in the parental [3.75 (78%), 7.5 (91%), or 15 mg/kg (98%)] and in the taxane-resistant tumors [3.75 (70%), 7.5 (87%), or 15 mg/kg (88%)] compared with vehicle-control group (*P* < 0.0001). The drug concentrations measured in plasma reflected the degree of p-histone H3 inhibition in tumors (Fig 5B).

Next, we tested if inhibition of aurora-B activity, measured by the degree of p-histone H3 inhibition, correlated with suppression of tumor growth *in vivo*. Mice bearing established MDA-MB-231 (F11), (F11) PTX-r, and DU4475 tumors were orally administered vehicle or AMG 900 at 3.75, 7.5, or 15 mg/kg b.i.d. for 3 weekly cycles of treatment consisting of 2 consecutive days per week. As shown in Figure 6A, intermittent administration of AMG 900 resulted in dose-dependent inhibition of the MDA-MB-231 (F11) tumor growth compared with vehicle-control group [3.75 (57%), 7.5 (63%), or 15 mg/kg (84%), *P* ≤ 0.0234]. Weekly administration of docetaxel at 30 mg/kg resulted in tumor regressions in MDA-MB-231 (F11) xenografts (*P* < 0.0001). The effect of AMG 900 was further evaluated in the above breast cancer models which were largely insensitive to docetaxel when dosed at the maximum tolerated dose (MTD, 30 mg/kg). In Figure 6B, treatment of AMG 900 resulted in significant tumor growth inhibition of MDA-MB-231 (F11) PTX-r at 15 mg/kg (71%), *P* = 0.0002, while in mice bearing DU4475 xenografts, treatment with AMG 900 significantly inhibited tumor growth at 7.5 mg/kg (64%) and 15 mg/kg (73%), *P* ≤ 0.0249 (Fig. 6C), compared with vehicle-control group. The main adverse effect after treatment with AMG 900 was moderate body weight loss observed at the highest dosage only (average of <11%, data not shown).
**AMG 900 in combination with MTAs enhanced tumor growth inhibition in human breast cancer xenografts.** Because MTAs are the standard-of-care in patients with metastatic breast cancer, we further assessed the antitumor effects of AMG 900 in combination with either docetaxel or ixabepilone using MDA-MB-231 (F11) or (F11) PTX-r xenografts, respectively. Based on historical dosage and scheduling (20), docetaxel was administered i.p. at 15 mg/kg which resulted in 80% tumor growth inhibition in MDA-MB-231 (F11) xenografts compared with vehicle-control group (data not shown). In contrast, ixabepilone was administered i.v. (based on published reports, 15,16) at 5 and 10 mg/kg which inhibited tumor growth by 46% (P = 0.0048) and 77% (P < 0.0001) in (F11) PTX-r xenografts respectively, compared with vehicle-control group (data not shown). In the combination studies, docetaxel, ixabepilone, and AMG 900 were administered at doses below their respective MTDs to avoid unacceptable body weight loss. Mice were first administered either docetaxel at 10 mg/kg i.p. or ixabepilone at 5 mg/kg i.v. on day 1 followed by AMG 900 dosed orally at 7.5 mg/kg b.i.d. on days 2 and 3 for three weekly cycles of treatment. As shown in Figure 7A, treatment with docetaxel plus AMG 900 resulted in significant inhibition of tumor growth (96%, P < 0.0006) in MDA-MB-231 (F11) xenografts compared with either monotherapy. Most notably, ixabepilone in combination with AMG 900 showed tumor regressions in MDA-MB-231 (F11) PTX-r xenografts (P < 0.0001) (Fig. 7B). Similar results were obtained when combining AMG 900 with ixabepilone in the DU4475 model (data not shown). An overall loss in body weight (average of <11%) was observed after 3 cycles of ixabepilone plus AMG 900 treatment (Fig. 7A, 7B). On day 42, histologic assessment was performed on the MDA-MB-231 (F11) PTX-r xenografts (5 of 12 mice) from the vehicle-control or ixabepilone plus AMG 900 groups after 3 cycles of treatment. Tumors from the vehicle-control group showed uniform cell size with many mitotic figures (>10/40Xfield) (Fig. 7C arrows) and few bi- or multi-nucleated cells. In contrast, there appeared to be an increase in the number of multinucleated cells in tumors treated with ixabepilone plus AMG 900 (Fig. 7C arrowheads). After treatment ceased, the remaining mice (7 of 12) in the combination-treated group were monitored for tumor regrowth. Four out of seven tumors failed to show rapid tumor regrowth after 75 days off treatment in this group (Fig. 7D). Together, these data provide evidence that AMG 900 inhibits the activity of aurora-B and suppresses the growth of MDA-MB-231 (F11) and (F11)-PTX-r xenografts alone and in combination with MTAs. Importantly, our
data indicate that AMG 900 has the potential to treat patients with metastatic breast cancer that have become resistant to standard-of-care antimitotic drugs.

**Discussion**

In this report, we describe the activity of AMG 900, a selective pan-aurora kinase inhibitor that shows promising activity alone and in combination with MTAs against TNBC cell lines and xenograft models. Notably, we provide evidence that AMG 900 possesses superior activity to taxanes in TNBC tumors with multiple modes of resistance. Furthermore, the combination of ixabepilone with AMG 900 leads to durable tumor regressions and limits regrowth of multidrug resistant TNBC xenografts.

In contrast to taxanes, AMG 900 was active in TNBC cell lines expressing high levels of P-gp and \( \beta \text{III-tubulin} \), suggesting AMG 900 has low susceptibility to P-gp mediated drug efflux and functions independently of altered \( \beta \)-tubulin isotype expression. Our data show that SAC silencing mediated through inhibition of aurora-B activity by AMG 900 at low nanomolar concentrations, leads to polyploidy and apoptosis in multidrug resistant MDA-MB-231 (F\(^{11}\)) PTX-r and DU4475 cell lines. Other factors may also contribute to the antiproliferative effects of AMG 900, including cellular senescence and cell death by mitotic catastrophe, giant-cell necrosis, and multipolar cell division (unpublished observations by Marc Payton, 28-31). In breast cancers, p53 is mutated in approximately 25% of cases, with a higher frequency in TNBC (32). The level of polyploidy induced by AMG 900 treatment was higher in mutant p53 MDA-MB-231 cells compared to wild-type p53 DU4475 cells; this was likely due to activation of the post-mitotic p53-dependent G\(_1\)-checkpoint. Impeding endoreduplication by activating this checkpoint may favor senescence, whereas bypassing this checkpoint (via p53 mutation/deletion) may initiate a more durable p53-independent cell death response (33). Loss of cell-cycle checkpoint control and elevated proliferation associated with TNBC may represent a vulnerability to drugs that induce mitotic stress, including MTAs and aurora kinases (34). MTAs and AMG 900 both act directly on cells during mitosis, but they inhibit mitotic progression and induce stress through distinct modes of action. MTAs disrupt microtubule dynamics, resulting in SAC activation, whereas AMG 900 inhibits the activity of both aurora-A and -B, leading to SAC silencing. We hypothesized that by first treating with a MTA, the fraction of cells in mitosis would increase due to SAC activation, rendering the cells more vulnerable to the activity of
AMG 900 through SAC silencing. The combination of MTAs with AMG 900 enhanced inhibition of cell growth and colony formation in both the MDA-MB-231 (F11) parental and (F11)-PTX-r cells, suggesting the combined effect of SAC activation follow by SAC silencing may increase mitotic stress and cell lethality. We did not examine AMG 900 in combination with vinca alkaloid class of MTAs because P-gp expressing cancer cells were insensitive to vinblastine; suggesting a low potential for enhancing the activity of AMG 900 (data not shown, 35).

We extended our findings in vivo, demonstrating that MTAs combined with AMG 900 enhanced antitumor activity in both taxane-sensitive and -resistant TNBC xenografts. Notably, we found that sequential treatment with ixabepilone followed by AMG 900 induced durable tumor regressions in the MDA-MB-231 (F11) PTX-r xenografts. Ixabepilone plus AMG 900 induced multinucleation, suggesting the tumor xenograft cells survived after the MTA induced mitotic arrest, becoming multinucleated rather than dying directly from SAC activation alone. There may be other antitumor mechanisms by which ixabepilone and AMG 900 act cooperatively. A recent study showed that ixabepilone was more effective than paclitaxel at blocking tumor angiogenesis in vivo, possibly through inhibiting the proliferation of tumor xenograft-associated mouse endothelial cells overexpressing P-gp (36). These findings may explain why we observed a more durable in vivo antitumor effect with the ixabepilone and AMG 900 combination. We should note that we did not confirm the antitumor activity of MTAs combined with AMG 900 induced apoptosis, largely because the dynamic and transient nature of apoptosis in vivo made it difficult to select an appropriate time interval to measure programmed cell death in tumor xenograft tissues. It is important to recognize the potential challenge of combining AMG 900 with other MTAs in the clinic due to the likelihood of overlapping toxicities in proliferating tissues (e.g. bone marrow and gastrointestinal mucosa). The negative impact on normal tissue homeostasis could limit the utility of this combination, although prophylactic administration of granulocyte colony-stimulating factor (G-CSF) may help decrease the duration of neutropenia (18). One area for further investigation will be to evaluate the activity of AMG 900 alone and in combination with MTAs in either patient-derived cancer xenografts or a genetically engineered mouse model of human MBC (37, 38). These alternative preclinical models may more closely mirror human disease (e.g. tumor heterogeneity, multifactorial nature of MDR) and allow for a more fateful assessment of drug efficacy.
In summary, AMG 900 is effective at inhibiting the growth of TNBC cell lines and xenografts. AMG 900 demonstrates antitumor activity that is superior to taxanes in multidrug resistant xenografts, including cells that overexpress P-gp and βIII-tubulin. The combination of ixabepilone with AMG 900 leads to durable tumor regressions and limits the regrowth of multidrug resistant xenografts. These results suggest that combining MTAs such as ixabepilone with AMG 900 holds promise in the treatment of patients with metastatic breast cancer. AMG 900 is presently in phase 1 clinical evaluation in patients with advanced cancers.

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References


Figures

Figure 1. The chemical structure of AMG 900.

Figure 2. Inhibition of aurora-B activity by AMG 900 leads to polyploidy and apoptosis in the human breast cancer cell lines. A, representative merged images of DU4475 and MDA-MB-231 TNBC cells treated with DMSO alone or AMG 900 at 50 nmol/L for 6 hours. Cells were immunostained with anti-p-histone H3 Ser10 (red) and anti-pericentrin (green) antibodies and DNA was counterstained with DAPI (blue). B, DNA content profiles of cells treated with DMSO alone or AMG 900 for 48 hours [2N (G1), 4N, 8N cell populations denoted on x-axis]. C, representative scatter plots showing DNA content and cleaved caspase-3 (red, SubG1 and anti-caspase-3-FITC) and DNA synthesis (blue, anti-BrdUrd-alexa-647). D, concentration-response relationships were plotted based on BrdUrd, cleaved-caspase-3, SubG1, and ≥4N DNA content cell populations as a percentage (in hundreds) of the DMSO control (POC).

Figure 3. AMG 900 exhibits uniform potency in human breast cancer cell lines expressing P-gp. A, cell lysates were prepared from four TNBC cell lines (MDA-MB-231, DU4475, MDA-MB-231 (F11), and (F11) PTX-r), and two human uterine sarcoma cell lines (MES-SA parental and DX5 variant). Western blot analysis was performed using anti-P-gp antibody and anti-β-actin antibody as a protein loading control. B, flow cytometry based cell-cycle analysis was used to determine degree of polyploidy in the same cell lines. Cells were treated with DMSO alone or AMG 900 (concentration range, 0.098 to 50 nmol/L) for 24 hours. Concentration-response curves were determined based on the accumulation of cells with ≥4N DNA content. Percentage of control (POC) was defined based on ≥4N DNA content cell population for DMSO control (baseline) and maximum polyploidy response for each cell line (EC50 values, error bars ± SD, in duplicate).

Figure 4. AMG 900 enhances the antiproliferative effects of MTAs in MDA-MB-231 (F11) and (F11) PTX-r cell lines. A, MDA-MB-231 (F11) parental and (F11) PTX-r cells were sequentially treated with either paclitaxel or ixabepilone and AMG 900 at the indicated concentrations [MTAs (24 hours) followed by AMG 900 (48 hours)]. As controls, cells were treated with DMSO or each agent alone at the same concentrations. Following
drug treatment, cells were washed and cultured in complete media until day 7.  B and C, cells were collected and enumerated using an automated cell counter (in duplicate).  Mean total cell count is represented as a 3 dimensional column graph, column color denoted by DMSO alone (grey), AMG 900 alone (blue), or MTA alone (dark purple), and combinations (green, red, light blue).  In separate wells, cells were stained with crystal violet dye and imaged.

**Figure 5. AMG 900 inhibits phosphorylation of histone H3 in MDA-MB-231 (F11) and (F11) PTX-r tumor xenografts.** A and B, mice bearing established tumors were orally administered a single dose of vehicle alone or AMG 900 at 3.75, 7.5, or 15 mg/kg.  Tumors were collected at 3 hours after treatment (n=3 per dose) and the level of p-histone H3 was determined by immunofluorescence-based microscopy.  Representative images of sectioned tumors are stained with anti-p-histone H3 (Ser10) antibody (red) and DAPI (blue).  B, column graphs representing the pharmacodynamic-pharmacokinetic profile of AMG 900 in mice.  The number of p-histone H3 positive cells per tumor image field (6 image fields per column, mean ± SD) and concentration of AMG 900 in mouse plasma (●, mean ± SD).  The asterisk (*) indicates statistically significant inhibition of p-histone H3 compared with vehicle-control group determined by Factorial ANOVA followed by Dunnett’s post hoc test for multiple comparisons (*P < 0.0001).

**Figure 6. AMG 900 inhibits the growth of MDA-MB-231 (F11), (F11) PTX-r, and DU4475 tumor xenografts.** Mice bearing established A, MDA-MB-231 (F11), B, (F11) PTX-r, or C, DU4475 tumors were orally administered vehicle (■) or AMG 900 b.i.d. intermittently at 3.75 (■), 7.5 (○), or 15 mg/kg (△) for 3 weekly cycles of treatment consisting of 2 consecutive days per week.  As a control, mice were administered docetaxel i.p. at 30 mg/kg (◇) once per week for 3 weeks.  Tumor volumes (cubic mm) are represented as mean ± SE (n=10).  The asterisk (*) indicates statistically significant tumor growth inhibition compared with vehicle-control group determined by repeated measures analysis of variance (RMANOVA) followed by Dunnett’s test for multiple comparisons (*P ≤ 0.0249).
Figure 7. AMG 900 enhances the antitumor effects of MTAs in both MDA-MB-231 (F11) and (F11) PTX-r tumor xenografts. Mice bearing established A, MDA-MB-231 (F11) or B, (F11) PTX-r tumor xenografts were administered docetaxel at 10 mg/kg i.p. (◇) or ixabepilone at 5 mg/kg i.v. (□) on day 1 once per week, then followed by vehicle (◼), AMG 900 at 7.5 mg/kg b.i.d. (◆) by oral administration on days 2 and 3 per week for 3 cycles of treatment or AMG 900 in combination with docetaxel (♦) or ixabepilone (▼). Body weights were recorded twice per week. Tumor volumes (cubic mm) are represented as mean ± SE (n=12). The asterisk (*) indicates statistically significant tumor growth inhibition compared with either monotherapy determined by separate RMANOVA between the combination group and each of the relevant single agent (*P ≤ 0.0006). C, representative images of hematoxylin-stained MDA-MB-231(F11) PTX-r tumors from vehicle and AMG 900 plus ixabepilone treatment groups on day 42 post-treatment [mitotic figures (arrows) and multinucleated cells (arrowheads)]. D, mice (7 of 12) were monitored for tumor regrowth in the AMG 900 plus ixabepilone treatment group for 75 days after treatment ceased.
Figure 1
Figure 2

A. DU4475

- Control
- DMSO
- AMG 900 50 nmol/L

MDA-MB-231

- Control
- DMSO
- AMG 900 50 nmol/L

B. DU4475

- Control
- AMG 900 (nmol/L)

MDA-MB-231

- Control
- AMG 900 (nmol/L)

C. DU4475

- Control
- DMSO
- AMG 900 10 nmol/L

MDA-MB-231

- Control
- DMSO
- AMG 900 10 nmol/L

D. DU4475

- BrdUrd
- Caspase-3
- SubG1
- ≥4N DNA content

MDA-MB-231

- BrdUrd
- Caspase-3
- SubG1
- ≥4N DNA content
**Figure 3**

A

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<thead>
<tr>
<th>MDA-MB-231</th>
<th>MDA-MB-231 (F11)</th>
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<tr>
<td>Parental</td>
<td>Parental</td>
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<tr>
<td>DU4475</td>
<td>Parental</td>
<td>DX5</td>
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</table>

Anti-P-gp

Anti-β-actin

B

**MDA-MB-231**

- EC<sub>50</sub> 1.1 nmol/L
- EC<sub>50</sub> 2.2 nmol/L

**DU4475**

- EC<sub>50</sub> 1.5 nmol/L
- EC<sub>50</sub> 1.4 nmol/L
Figure 4

A

Single agent

- Paclitaxel
- Ixabepilone

Combination

- Paclitaxel
- Ixabepilone

B

MDA-MB-231 (F11)

C

MDA-MB-231 (F11) PTX-r

Mean Cell Count

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Figure 5

A

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</thead>
</table>

MDA-MB-231 (F11)

PTX-r

B

MDA-MB-231 (F11)

PTX-r

Mean p-histone H3 + cells per image field

Plasma Concentration (ng/mL)

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Figure 6

A. MDA-MB-231 (F11)

B. MDA-MB-231 (F11) PTX-r

C. DU4475

Graphs showing tumor volume over days post tumor implant for different treatments.
Figure 7

**A** MDA-MB-231 (F^{11})

- Vehicle
- Docetaxel 10 mg/kg
- AMG 900 7.5 mg/kg
- COMBINATION

**B** MDA-MB-231 (F^{11}) PTX-r

- Vehicle
- Ixabepilone 5 mg/kg
- AMG 900 7.5 mg/kg
- COMBINATION

**C** Vehicle Control

- Tumor Volume (mm\(^3\))
- Days post tumor implant

**D** MDA-MB-231 (F^{11}) PTX-r

- Tumor Volume (mm\(^3\))
- Days post tumor implant

**C** Vehicle Control

- % Initial Bodyweight
- Days post tumor implant

**D** MDA-MB-231 (F^{11}) PTX-r

- % Initial Bodyweight
- Days post tumor implant

**C** Vehicle Control

- AMG 900 + Ixabepilone

- Tumor Volume (mm\(^3\))
- Days post tumor implant
Molecular Cancer Therapeutics

AMG 900, a small molecule inhibitor of aurora kinases, potentiates the activity of microtubule-targeting agents in human metastatic breast cancer models


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