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PRECLINICAL STUDY

AMG 900, pan-Aurora kinase inhibitor, preferentially inhibits the proliferation of breast cancer cell lines with dysfunctional p53

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Abstract Aurora kinases play important roles in cell division and are frequently overexpressed in human cancer. AMG 900 is a novel pan-Aurora kinase inhibitor currently being tested in Phase I clinical trials. We aimed to evaluate the in vitro activity of AMG 900 in a panel of 44 human breast cancer and immortalized cell lines and identify predictors of response. AMG 900 inhibited proliferation at low nanomolar concentrations in all cell lines tested. Response was further classified based on the induction of lethality. 25 cell lines were classified as highly sensitive (lethality at 10 nM of AMG 900 >10 %), 19 cell lines as less sensitive to AMG 900 (lethality at 10 nM of AMG 900 <10 %). Traditional molecular subtypes of breast cancer did not predict for this differential response. There was a weak association between *AURKA* amplification and response to AMG 900 (response ratio = 2.53, $p = 0.09$). mRNA expression levels of *AURKA*, *AURKB*, and *AURKC* and baseline protein levels of Aurora kinases A and B did not significantly associate with response. Cell lines with *TP53* loss of function mutations (RR = 1.86, $p = 0.004$) and low baseline p21 protein levels (RR = 2.28, $p = 0.0004$) were far more likely to be classified as highly sensitive to AMG 900. AMG 900 induced p53 and p21 protein expression in cell lines with wt *TP53*. AMG 900 caused the accumulation of cells with >4 N DNA content in a majority of cell lines independently of sensitivity and p53 status. AMG 900 induced more pronounced apoptosis in highly sensitive p53-dysfunctional cell lines. We have found that AMG 900 is highly active in breast cancer cell lines and that *TP53* loss of function mutations as well as low baseline expression of p21 protein predict strongly for increased sensitivity to this compound in vitro.

Keywords AMG 900 · Aurora kinases · Pan-Aurora kinase inhibitors · *TP53* mutation · p21 · Breast cancer

Ondrej Kalous and Dylan Conklin have contributed equally to this work.

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Introduction

Aurora kinases comprise three mammalian serine/threonine kinases (Aurora A, B, C) that play a critical role in mitosis and cell division. The primary role of Aurora A relates to centrosome maturation and mitotic spindle assembly. Aurora B is essential for chromosome condensation, spindle attachment, and cytokinesis. The function of Aurora C is predominantly restricted to meiosis [1, 2]. Aurora kinases are frequently overexpressed in cancer, including breast cancer, and play an important role in oncogenesis [3, 4].

Inhibition of Aurora A leads to mitotic spindle abnormalities and accumulation of cells in mitosis, whereas inhibition of Aurora B leads to chromosome alignment defects. These effects then lead to failed cytokinesis or endoreduplication, induction of polyploidy, and eventually cell death [5–9]. In preclinical models an increase in polyploid tumor cells is specifically correlated with loss of p53 function [10–12]. Cells that lack functional p53 have an increased ability to reenter the cell cycle [13–15]. p53-dependent arrest of tetraploid cells is sometimes referred to as the "G1 tetraploidy checkpoint" and its existence remains controversial [16]. This checkpoint is hypothesized to play a role in removal of tetraploid cells either via irreversible G1 cell cycle arrest or by apoptosis [16–18].

More than a dozen small-molecule Aurora kinase inhibitors have been tested in clinical trials (reviewed in [19, 20]). They differ in their specificity and potency against the three Aurora kinase family members. To date, no consistent molecular predictors of response to Aurora kinase inhibitors have been defined [21–23]. Consequently, a subset of breast cancer patients most likely to benefit from treatment has yet to be identified.

AMG 900 is a novel, potent, orally bioavailable, and highly selective pan-Aurora kinase inhibitor currently being evaluated in Phase I clinical trials [24]. AMG 900 was shown to inhibit the enzyme activity of all three Aurora kinase family members with IC_{50} values $\leq$ 5 nmol/L [25]. AMG 900 has been previously shown to be active in multidrug-resistant tumor cell lines and to have a considerable activity in several xenograft models, including breast cancer [25]. We hypothesized that a distinct molecular subgroup of breast cancers may be more likely to respond to AMG 900. To identify this subgroup, we evaluated response to AMG 900 across a large panel of well-characterized breast cancer cell lines.

Methods

Cell lines, cell culture, and reagents

The cell line panel included 41 breast cancer and 3 immortalized breast epithelial cell lines representing the known molecular subgroups of breast cancer and was described in detail previously [26–28].

AMG 900 was obtained from Amgen Inc. (Thousand Oaks, CA) and diluted in DMSO.

TP53 mutation analysis

TP53 mutation detection in DNA extracted from the breast cell lines was performed using two different methods in parallel; the arrayed primer extension (APEX; Asper Biothech) [29] and the temporal temperature gradient gel electrophoresis (TTGE) [30]. The APEX *TP53* assay is a microarray constructed of oligo-nucleotides to capture *TP53* mutations in exon 2–9 by primer extension and fluorescent-labeled terminator nucleotides. The mutation of interest is characterized directly using APEX. The TTGE is a pre-screening method covering exon 2–11 based on a mutation sensitive denaturation of DNA using a combination of chemicals and temperature. Gene alterations detected by TTGE are uncovered as aberrant migrating bands in gel electrophoresis, and need subsequent characterization. Samples showing diverging results from the two methods were validated by sequencing, using the ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA).

Detailed information on cell lines, proliferation assay, microarray analysis, flow cytometry analysis of cell cycle and apoptosis, Western blots, and statistical analysis have been described previously [26, 27] and can be found in Online Resource Supplementary Methods.

Results

AMG 900 has potent anti-proliferative effects in breast cancer cell lines regardless of subtype

AMG 900 inhibited growth very effectively in all of the breast cancer cell lines in our panel, even at low nanomolar concentrations. The IC_{50} values for all but one cell line (UACC-812; IC_{50} = 15.2 nM) were <10 nM which is well below the clinically achievable plasma concentrations (unpublished data obtained from Amgen). This potency made it difficult to stratify the cell lines as sensitive or resistant to AMG 900 based on IC_{50} as the response metric. Alternatively, we categorized the cell lines into two groups: highly sensitive versus less sensitive. This binary classification was made using a cutoff of 10 % lethality (defined as a decrease in cell number from baseline) at 10 nM of AMG 900. Those cell lines that had greater than 10 % lethality were classified in the highly sensitive group, and, conversely, those cell lines that were below 10 % were classified as less sensitive. Based on these response criteria, 25 cell lines were classified as highly sensitive and 19 cell lines as less sensitive to AMG 900 (Table 1).

The most common clinically relevant breast cancer biomarkers including HER2 amplification status [31] and ER (estrogen receptor) status and subtypes (luminal or non-luminal subtypes) were analyzed for an association with response, but no association was found to be statistically significant (Supplementary Table S1).

Table 1 The calculated response parameters for each cell line and the response classification

Highly sensitive			Less sensitive		
Cell line	% Lethality at 10 nM	Average IC50 (nM)	Cell line	% Lethality at 10 nM	Average IC50 (nM)
HCC-1187	85.9	0.08	CAL-51	9	1.17
MDA-MB-468	62.4	0.07	MDA-MB-175	8	0.19
HCC-38	57	0.04	SUM-190	7.9	0.27
HCC-70	52.8	0.08	HCC-1806	7.6	0.02
EFM-19	49.9	0.39	EFM-192A	6.9	0.09
BT-20	46.4	0.46	184A1	0[a]	1.18
HCC-1395	46.3	0.5	184B5	0[a]	1.03
MDA-MB-157	43.8	0.45	COLO-824	0[a]	0.49
HCC-1569	40.7	0.1	DU-4475	0[a]	1.15
MDA-MB-134	39.9	0.3	HCC-1143	0[a]	0.03
UACC-893	39.2	2.12	HCC-1937	0[a]	0.06
MDA-MB-361	37.4	1.19	HCC-2218	0[a]	2.05
CAMA-1	35	0.38	KPL-1	0[a]	0.88
BT-549	25.9	0.07	MCF-10A	0[a]	0.92
ZR-75-1	24.1	0.07	MCF-7	0[a]	0.22
SUM-225	19.7	2.78	MDA-MB-231	0[a]	0.25
UACC-732	18.4	0.7	MDA-MB-453	0[a]	0.02
MDA-MB-415	16.9	2.23	T-47D	0[a]	0.46
SK-BR-3	16.2	0.12	UACC-812	0[a]	15.21
BT-474	15.3	0.11			
HCC-1954	15	0.13			
MDA-MB-435	14.8	0.04			
HCC-1419	14.3	0.08			
MDA-MB-436	11.3	1.64			
Hs578T	10.6	0.92			

Cell lines were classified as highly sensitive when the average lethality at 10 nM was >10 %. Cell lines were classified as less sensitive when the average lethality at 10 nM <10 %

[a] 0 % lethality = cell line grew from baseline at 10 nM AMG 900 (organized alphabetically)

Association analysis of Aurora kinase copy number variations (CNVs) and expression levels with response to AMG 900

We investigated the relationship between Aurora kinase levels and response to AMG 900 in our cell line panel. *AURKA* gene amplification was highly prevalent in our panel of cell lines. 13 of our cell lines have greater than 2-fold amplification ($\log_2(\text{ratio}) > 1$) of the *AURKA* gene (Table 2). Cell lines with *AURKA* amplification were more likely to be classified as highly sensitive to AMG 900 but this association failed to reach the cutoff for statistical significance (RR = 2.53 (95 % CI 0.8–8.0), $p = 0.09$) (Table 3). *AURKB* and *AURKC* CNVs were not observed in our panel.

AURKA, AURKB, and AURKC baseline mRNA expression levels by microarray did not associate with response to AMG 900 (Tables 2, 3). Similarly, Aurora A and Aurora B baseline protein levels measured by Western blot did not associate with response to AMG 900 in our panel (Table 3, Supplementary Table S2; Supplementary Figure S1).

p53 dysfunction predicts for response to AMG 900

Given the interaction of p53 and Aurora kinases at regulating cell cycle progression, we investigated the relationship between p53 dysfunction and response to AMG 900 in our cell line panel. *TP53* somatic mutations found in our genotyping analysis were cross-referenced against functional data derived from yeast transactivation assays performed on common mutations, as listed in the IARC p53 database [32] (http://www-p53.iarc.fr/) (Supplementary Table S3). Mutations whose functional status was not listed were assumed to be loss of function (LOF). We observed a higher frequency of *TP53* LOF mutants among highly sensitive cell lines (22 of 25; 88.0 %) compared to less sensitive cell lines (9 of 19; 47.3 %) (Table 4). This association was found to be statistically significant in our analysis (RR = 1.86, 95 % CI 1.1–3.1, $p = 0.004$) (Table 3).

We also investigated the role of p21 in response to AMG 900 due to the tight functional association between p21 and p53. Baseline p21 protein levels in the entire cell line panel were measured by Western blot (Table 4; Fig. 1;

Table 2 Aurora kinases and response to AMG 900

Cell line	Response to AMG900	AURKA CNV	AURKA mRNA	AURKB mRNA	AURKC mRNA
HCC-1187	Highly sensitive	No Amp	Low	High	High
MDA-MB-468	Highly sensitive	No Amp	Low	Low	High
HCC-38	Highly sensitive	No Amp	Low	High	High
HCC-70	Highly sensitive	No Amp	Low	High	Low
EFM-19	Highly sensitive	Amp	Low	Low	High
BT-20	Highly sensitive	No Amp	High	High	High
HCC-1395	Highly sensitive	Amp	High	High	Low
MDA-MB-157	Highly sensitive	Amp	Low	High	Low
HCC-1569	Highly sensitive	Amp	Low	Low	High
MDA-MB-134	Highly sensitive	No Amp	Low	High	High
UACC-893	Highly sensitive	Amp	High	High	Low
MDA-MB-361	Highly sensitive	No Amp	Low	Low	Low
CAMA-1	Highly sensitive	No Amp	Low	High	High
BT-549	Highly sensitive	No Amp	Low	High	High
ZR-75-1	Highly sensitive	No Amp	Low	Low	Low
SUM-225	Highly sensitive	No Amp	Low	Low	Low
UACC-732	Highly sensitive	Amp	High	High	High
MDA-MB-415	Highly sensitive	No Amp	Low	Low	High
SK-BR-3	Highly sensitive	Amp	High	High	High
BT-474	Highly sensitive	Amp	High	High	High
HCC-1954	Highly sensitive	Amp	High	High	Low
MDA-MB-435	Highly sensitive	No Amp	Low	High	Low
HCC-1419	Highly sensitive	Amp	High	High	High
MDA-MB-436	Highly sensitive	No Amp	Low	Low	Low
Hs578T	Highly sensitive	No Amp	Low	Low	High
CAL-51	Less sensitive	No Amp	Low	High	High
MDA-MB-175	Less sensitive	No Amp	Low	Low	High
SUM-190	Less sensitive	No Amp	Low	Low	High
HCC-1806	Less sensitive	No Amp	Low	High	High
EFM-192A	Less sensitive	Amp	High	Low	High
184A1	Less sensitive	No Amp	Low	Low	High
184B5	Less sensitive	No Amp	Low	Low	High
COLO-824	Less sensitive	No Amp	Low	High	Low
DU-4475	Less sensitive	No Amp	Low	Low	Low
HCC-1143	Less sensitive	No Amp	Low	High	Low
HCC-1937	Less sensitive	No Amp	Low	High	High
HCC-2218	Less sensitive	No Amp	Low	Low	Low
KPL-1	Less sensitive	Amp	Low	Low	Low
MCF-10A	Less sensitive	No Amp	Low	Low	High
MCF-7	Less sensitive	No Amp	High	High	Low
MDA-MB-231	Less sensitive	No Amp	High	High	High
MDA-MB-453	Less sensitive	No Amp	Low	Low	High
T-47D	Less sensitive	No Amp	Low	High	Low
UACC-812	Less sensitive	Amp	Low	Low	Low

AMG 900 response classification as described in Table 1. *AURKA* amplification (Amp) was determined by aCGH; cutoff, $\log_2(\text{ratio}) > 1$. *AURKA, AURKB, AURKC* mRNA were measured by microarray; cutoffs, high $= \log_2(\text{ratio}) > 0$, low $= \log_2(\text{ratio}) < 0$

Supplementary Table S4). High p21 protein expression correlated with classification of p53 functional status (Pearson $r = 0.58$, p value < 0.0001). Consequently, cell lines with low p21 protein levels were also far more likely to be classified as highly sensitive to AMG 900 (RR = 2.28, 95 % CI 1.4–5.8, $p = 0.0004$) (Table 3).

To verify that AMG 900 induces a p53/p21 response, a p53/p21 protein expression time course experiment was performed

Table 3 Statistical analysis of predictors of response to AMG 900

Predictor	RR (95 % CI)	*p* value
p53 (LOF vs. functional)	1.86 (1.1–3.1)	0.004
p21 baseline protein (no expression vs. expression)	2.28 (1.4–5.8)	0.0004
AURKA copy number (amplified vs. non-amplified)	2.53 (0.8–8.0)	0.085
AURKA mRNA (high vs. low)	2.0 (0.6–6.6)	0.224
AURKB mRNA (high vs. low)	1.52 (0.8–2.8)	0.153
AURKC mRNA (high vs. low)	1.0 (0.6–1.7)	0.889
Aurora A baseline protein (expression vs. no expression)	1.63 (0.6–4.1)	0.23
Aurora B baseline protein (expression vs. no expression)	1.15 (0.4–3.2)	0.78

p21, Aurora A, and Aurora B baseline protein levels were determined by Western blot, *AURKA* amplification by aCGH, and *AURKA, AURKB, AURKC* mRNA levels by microarray

in a subset of cell lines with either wild-type (wt) or mutated *TP53* and variable sensitivity to AMG 900. Significant increases in p53 and p21 expression were observed 24 and 48 h after treatment with AMG 900. These effects were more pronounced in cell lines with wild type p53 (Fig. 2).

AMG 900 effects on cell cycle and apoptosis

To investigate the mechanism of response to AMG 900, its effect on cell cycle was analyzed by flow cytometry in a subset of cell lines ($n = 18$) with variable sensitivity to AMG 900 and p53 functional status. After a 24- and 48-h treatment with 10 nM of AMG 900 we observed 4 N DNA accumulation in all tested cell lines except for UACC-812. After a 48-h treatment, polyploidy was observed in 11 of 18 cell lines (Fig. 3). Polyploidy was achieved in nearly all cell lines tested (17 of 18 cell lines) after 5 days of treatment. The onset of polyploidy was independent from the sensitivity to AMG 900 or p53 functional status (data not shown).

The effects of AMG 900 on apoptosis were analyzed by flow cytometry in a subset of cell lines ($n = 10$) with variable sensitivity to AMG 900 and p53 functional status (Fig. 4). Cells were treated with 10 nM of AMG 900 for 5 days. Highly sensitive cell lines with *TP53* LOF mutations showed a strong induction of apoptosis. The apoptotic effects were also present, albeit at lesser degree, in the highly sensitive wt *TP53* MDA-MB-134 cell line and in less sensitive cell lines with *TP53* LOF mutations. AMG 900 caused minimal or no changes in apoptosis in less sensitive wt *TP53* cell lines.

Discussion

In this study, we have evaluated the anti-proliferative activity of AMG 900, a novel pan-Aurora kinase inhibitor, on a panel of 41 cell lines representing various subtypes of breast cancer and three immortalized breast epithelial cell lines. We found that AMG 900 is highly active in breast cancer cell lines at concentrations achievable in clinical settings, with IC_{50} values below 10 nM in all but one cell line. Due to the difficulty in stratifying cell lines based on IC_{50} values alone, other outcome measures were considered for use in classifying response to AMG 900. Lethality is a less commonly used outcome measure than IC_{50} in cell proliferation assays. Percentage lethality was calculated at each concentration and can be interpreted as the percent of cell death from baseline after the 5-day treatment. We believe that % lethality may provide a more accurate quantification of cytotoxic effects as opposed to IC_{50}, which is better suited for quantification of cytostatic or growth inhibitory effects. A subset of the cell lines in our panel (25 of 44 cell lines) showed significant lethality (>10 %) at 10 nM of AMG 900 and were classified as highly sensitive. The cell lines with no significant lethality were classified as less sensitive (19 of 44 cell lines). In this study, the 10 nM cutoff point was chosen because, at this concentration, AMG 900 inhibits all three Aurora kinases and has minimal off-target effects [25]. Additionally, the 10 nM concentration is readily achievable in human plasma following treatment with AMG 900. In the current study, AMG 900 was more potent than in a previous report [25]. These differences are likely explained by the different methodologies used between the studies for calculating growth inhibition.

Breast cancers represent a genetically and phenotypically heterogeneous group of tumors. This heterogeneity is well represented in our panel of breast cancer cell lines. Despite the fact that several Aurora kinase inhibitors have already been tested in clinical trials in breast cancer, no subpopulation of patients has been identified that would benefit [21–23]. Preclinical studies with Aurora kinase inhibitors that differed in target specificity using large panels of breast cancer cell lines did not lead to a definite conclusion as far as which subtype associates with response. In vitro, response to GSK1070916 (Aurora kinase B/C inhibitor) was shown to be associated with a "claudin-low" subtype (which overlaps with our post-EMT group, Supplementary Table 1), whereas VX-680 (pan-Aurora kinase inhibitor) response was associated with cell lines that lacked HER2 amplification [21]. ENMD-2076 (Aurora A/B/angiogenic kinase inhibitor) and AS703569 (pan-Aurora kinase inhibitor) showed more activity in triple-negative breast cancer cell lines [22, 23]. In this study, we did not see a significant enrichment for any of the most commonly used clinical subgroups (HER2± , ER±, luminal/non-luminal) in our subset of cell lines classified as highly sensitive to AMG 900.

Aurora kinase inhibitors were initially developed upon the observation that Aurora kinase expression is commonly

Table 4 p53/p21 and response to AMG 900

Cell Line	Response to AMG900	p21 protein expression	p53 Status
HCC-1187	Highly sensitive	+	Likely LOF
MDA-MB-468	Highly sensitive	+	Likely LOF
HCC-38	Highly sensitive	–	Likely LOF
HCC-70	Highly Sensitive	+	Likely LOF
EFM-19	Highly sensitive	–	Likely LOF
BT-20	Highly sensitive	–	Likely LOF
HCC-1395	Highly sensitive	–	Likely LOF
MDA-MB-157	Highly sensitive	–	Likely LOF
HCC-1569	Highly sensitive	–	Likely LOF
MDA-MB-134	Highly sensitive	–	Likely functional
UACC-893	Highly sensitive	–	Likely LOF
MDA-MB-361	Highly sensitive	–	Likely LOF
CAMA-1	Highly Sensitive	–	Likely LOF
BT-549	Highly sensitive	–	Likely LOF
ZR-75-1	Highly sensitive	+	Likely functional
SUM-225	Highly sensitive	–	Likely LOF
UACC-732	Highly sensitive	+	Likely functional
MDA-MB-415	Highly sensitive	+	Likely LOF
SK-BR-3	Highly sensitive	–	Likely LOF
BT-474	Highly Sensitive	–	Likely LOF
HCC-1954	Highly sensitive	–	Likely LOF
MDA-MB-435	Highly sensitive	–	Likely LOF
HCC-1419	Highly sensitive	–	Likely LOF
MDA-MB-436	Highly sensitive	–	Likely LOF
Hs578T	Highly Sensitive	–	Likely LOF
CAL-51	Less sensitive	+	Likely functional
MDA-MB-175	Less sensitive	+	Likely functional
SUM-190	Less sensitive	–	Likely LOF
HCC-1806	Less sensitive	–	Likely LOF
EFM-192A	Less sensitive	+	Likely LOF
184A1	Less sensitive	+	Likely functional
184B5	Less sensitive	+	Likely functional
COLO-824	Less sensitive	–	Likely LOF
DU-4475	Less sensitive	+	Likely functional
HCC-1143	Less sensitive	+	Likely LOF
HCC-1937	Less sensitive	+	Likely LOF
HCC-2218	Less sensitive	+	Likely functional
KPL-1	Less sensitive	+	Likely functional
MCF-10A	Less sensitive	+	Likely functional
MCF-7	Less sensitive	+	Likely functional
MDA-MB-231	Less sensitive	+	Likely LOF
MDA-MB-453	Less sensitive	–	Likely LOF
T-47D	Less sensitive	+	Likely LOF
UACC-812	Less sensitive	+	Likely functional

AMG 900 response classification as described in Table 1. p21 baseline protein levels were measured by Western blot, the bands were quantified by densitometry (Supplementary Table S4). p53 functionality as described in Supplementary Table S3

upregulated in many cancers [3, 4]. We therefore investigated the association between the expression levels of Aurora kinases and response to AMG 900. Cell lines with *AURKA* amplification as measured by aCGH were more likely to be classified as highly sensitive to AMG 900, but this association did not reach statistical significance

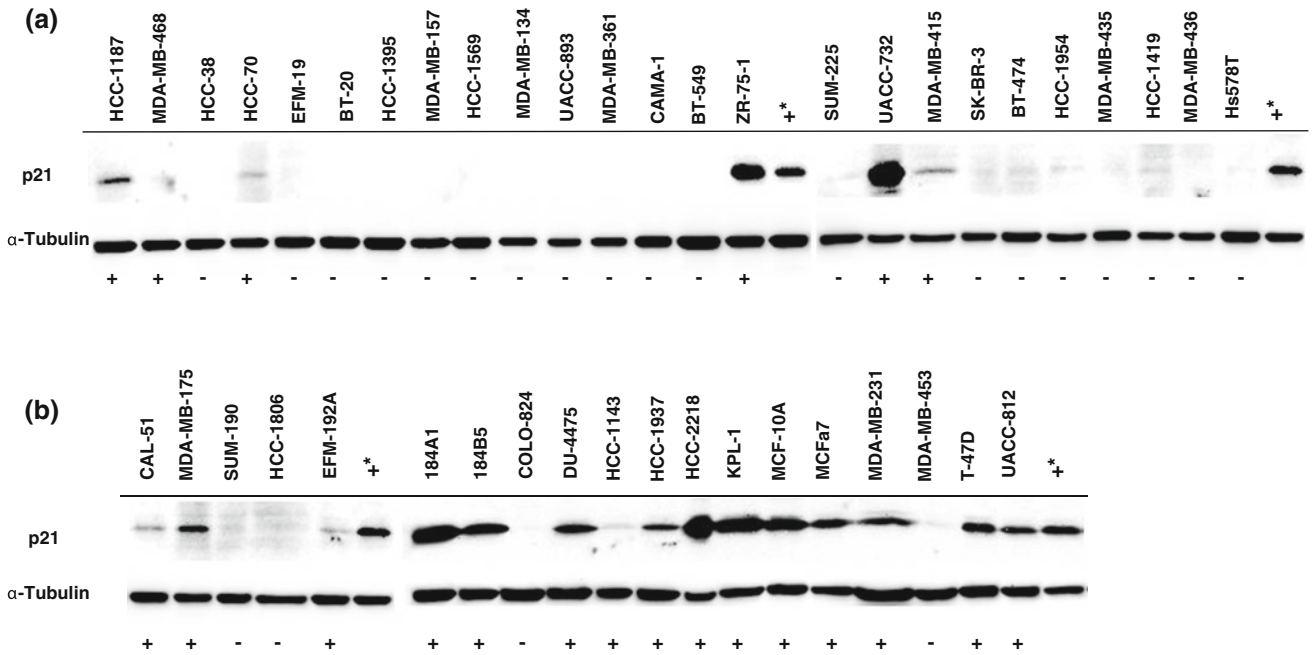

Fig. 1 p21 baseline protein levels. p21 baseline protein levels were measured by Western blots on the cell line panel as described in Supplementary Methods. Cell lines were grouped based on their sensitivity to AMG 900 as described in Table 1; **a** highly sensitive, **b** less sensitive cell lines. Highly sensitive cell lines have lower p21 protein expression compared to less sensitive cell lines. α-tubulin was used as a loading control; *plus asterisk* common control (MCF-7); densitometry data available in Supplementary Table S4

($p = 0.09$). *AURKA* baseline mRNA levels by microarrays and Aurora A baseline protein levels did not associate with response to AMG 900. No cell lines had observed copy number changes for the *AURKB* or *AURKC* genes. *AURKB* and *AURKC* baseline mRNA levels and Aurora B baseline protein levels (Aurora C baseline protein levels were not measured in this study) did not associate with response to AMG 900. These data indicate that neither gene copy number changes nor expression of Aurora kinases A/B/C are good predictive markers of response to AMG 900 in breast cancer cell lines.

TP53 mutations are found in approximately 30 % of breast cancers [33]. However, the phenotype of these mutations is variable and much uncertainty remains as to whether p53/p21 function correlates with sensitivity or resistance to chemotherapeutic agents [34–36]. Several studies have made conflicting observations regarding p53 status and response to Aurora kinase inhibitors. Some investigators have observed that Aurora kinase inhibitors have increased apoptotic or anti-proliferative activity in p53-deficient cells [37–39], while others have found that these effects were more pronounced in cell lines with wt p53 [40–42], and yet others, that the effects of Aurora kinase inhibition are independent of p53 [43–47].

Using a carefully characterized database of p53 function in our panel of breast cancer cell lines, we observed that *TP53* LOF mutations were significantly more frequent in cell lines classified as highly sensitive to AMG 900 ($p = 0.004$; in vitro sensitivity = 0.88, specificity = 0.62). In addition to p53, we found that low protein expression of p21, a downstream target of p53, associated with increased sensitivity to AMG 900 ($p = 0.0004$). We found that baseline p21 protein expression correlated with p53 functional status in our panel. However, we did not have a sample large enough to investigate the independent effects of p21 outside of p53, so it remains unclear whether low p21 expression is an independent predictor of response to AMG 900 or simply a surrogate biomarker for p53 dysfunction.

Analyses of cell cycle and apoptosis by flow cytometry were conducted to elucidate the mechanisms of AMG 900 activity. We observed polyploidy in a vast majority of tested cell lines within 5 days of treatment with AMG 900. The onset of polyploidy was independent from sensitivity to AMG 900 or p53 functional status. The presence of polyploidy after the treatment with AMG 900 in both wt *TP53* and p53-deficient cell lines is in agreement with previous studies with pan-Aurora kinase inhibitors (VX-680, danusertib) or Aurora B inhibitor ZM447439 [12, 48, 49]. However, induction of apoptosis was observed in every *TP53* mutant, AMG 900-highly sensitive cell line we tested. Apoptosis induction was less common in less sensitive, wild-type *TP53* cell lines. These findings of an increased apoptosis in TP53 LOF cell lines corroborate the results of the study conducted by Gizatullin et al. [39], who found that cell lines with a compromised p53/p21

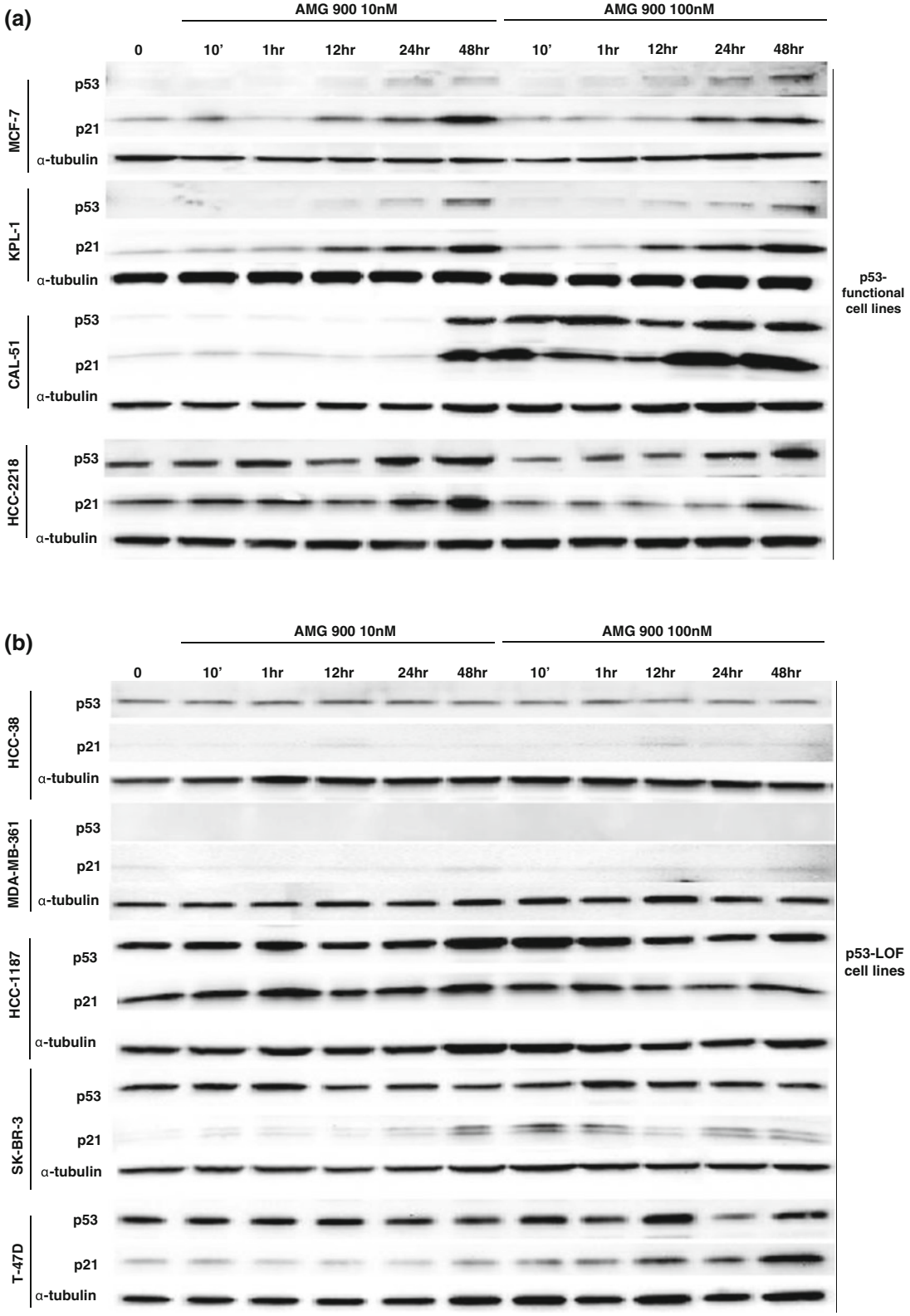

Fig. 2 p53 and p21 time course analysis with AMG 900. p53/p21 protein expression time course Western blot experiment was performed in a subset of **a** p53-functional cell lines with either wt *TP53* (MCF-7, KPL-1, CAL-51) or *TP53* mutation that does not lead to LOF (HCC-2218), or **b** cell lines with *TP53*-LOF mutations (HCC-38, MDA-MB-361, HCC-1187, SK-BR-3, T-47D). Cells were treated with 10 or 100 nM AMG 900 for 10 min to 48 h. Significant increases in p53 and p21 expression were observed 24 and 48 h after treatment with AMG 900. These effects were more pronounced in cell lines with functional p53. α-tubulin was used as a loading control

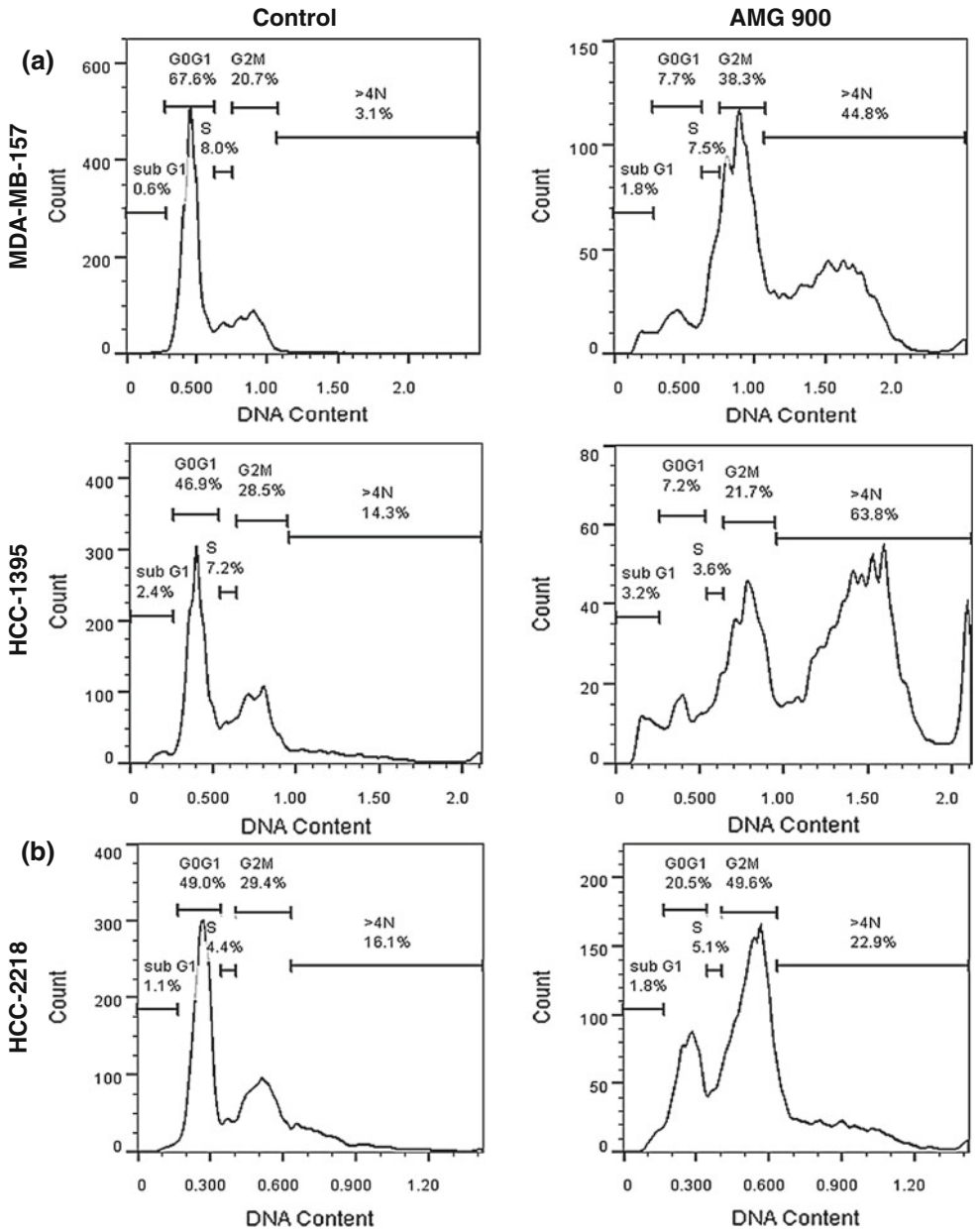

Fig. 3 AMG 900 induced ≥4 N DNA accumulation. The effects of AMG 900 on cell cycle were determined by flow cytometry in a subset of cell lines with a variable sensitivity to AMG 900 and p53 functionality. Cells were treated with 10 nM of AMG 900 for 24 h to 5 days. Examples of cell lines where AMG 900 caused **a** ≥4 N DNA accumulation, **b** 4 N DNA accumulation

postmitotic checkpoint function are more likely to undergo apoptosis after treatment with VX-680 than cells with intact checkpoint function. Furthermore, Kaestner et al. [9] have shown that the p53-dependent postmitotic G1 checkpoint was not required for the induction of apoptosis after treatment with a selective Aurora B inhibitor ZM447439 but was required after treatment with Aurora kinase A inhibitor MLN8054.

In this study, we did not attempt to determine the specific causal effects of inhibition of individual Aurora kinases on cell cycle regulation and apoptosis. Payton et al. [25] have shown previously that AMG 900 effectively blocks the autophosphorylation of Aurora A and B, as well as the phosphorylation of histone H3, a proximal substrate of Aurora B. Pan-Aurora kinase inhibitors generally induce cellular phenotypes that are compliant with perturbed Aurora B function [50]. Similar phenotype findings were previously reported for AMG 900, as evidenced by aborted cell division without a prolonged mitotic arrest [25].

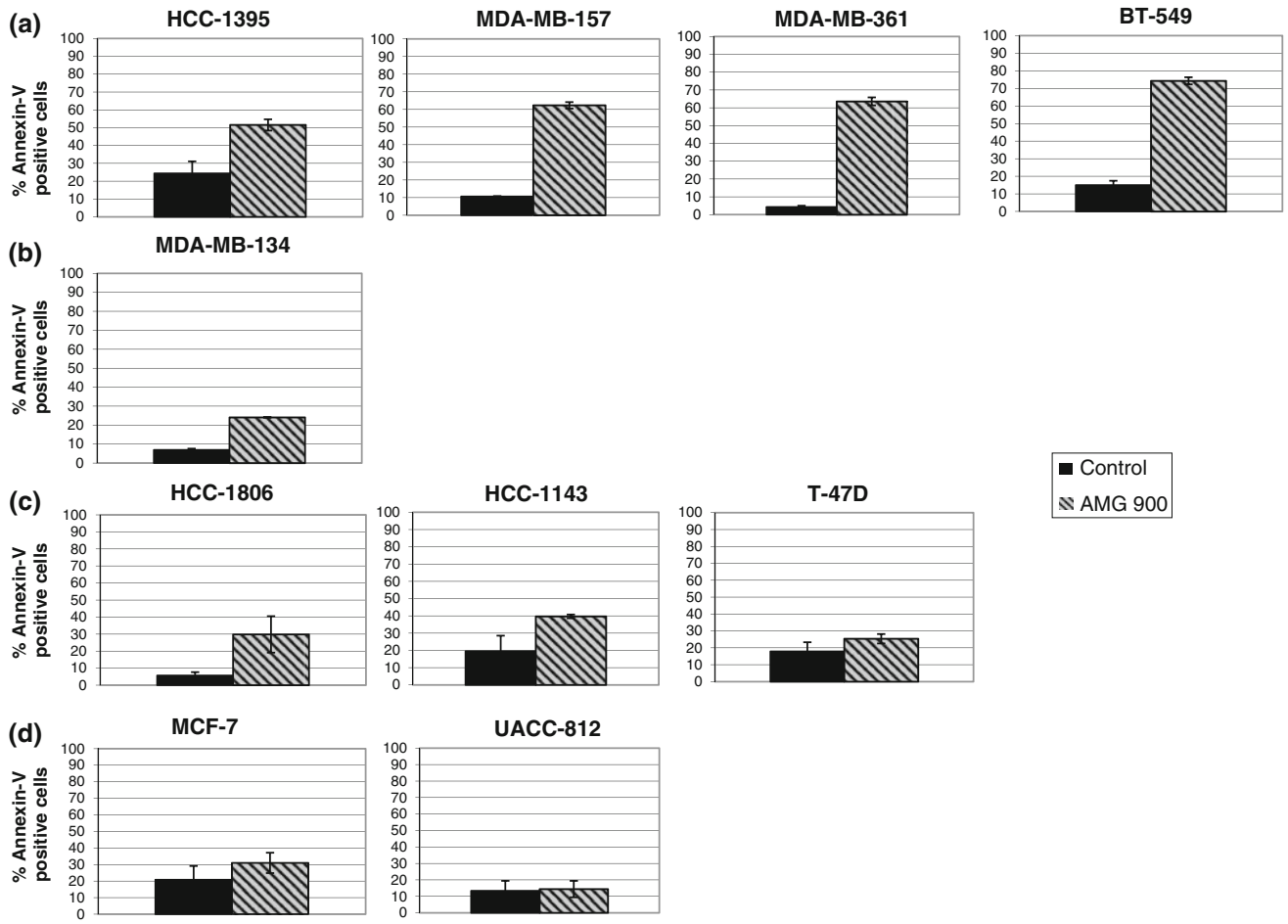

Fig. 4 The effects of AMG 900 on apoptosis. The effects of AMG 900 on apoptosis were analyzed by flow cytometry in a subset of cell lines with variable sensitivity to AMG 900 and p53 functionality. Cells were treated with 10 nM of AMG 900 for 5 days. **a** Highly sensitive *TP53*-LOF cell lines showed highest amounts of AMG 900-induced apoptosis (Annexin-V positive cells). **b** Highly sensitive p53-functional MDA-MB-134 cell line and **c** less sensitive *TP53*-LOF cell lines showed less induction of apoptosis. **d** Less sensitive p53-functional cell lines show a minimal or no induction of apoptosis after treatment with AMG 900

A time-course Western blot experiment was conducted in a subset of cell lines with variable sensitivity to AMG 900 and p53/p21 status. Significant increases in p53 protein expression accompanied with an induction of p21 protein expression were observed 24–48 h post-treatment with AMG 900 in cell lines with functional p53. Conversely, these effects on p53/p21 expression were not as prominent in cell lines with dysfunctional p53. The up-regulation of p53 and p21 following Aurora kinase inhibition has been reported previously [8, 25, 38, 51], and further confirms the functional interactions between Aurora kinases and the p53/p21 complex.

These data lead us to hypothesize that the functional p53/p21 complex may play a role in preventing cells with AMG 900-induced polyploidy from undergoing apoptosis. This mechanism could explain the differential response to treatment observed in our proliferation experiments, where *TP53* LOF mutant cell lines displayed more lethality at 10 nM AMG 900 as well as more induction of apoptosis. We further hypothesize that the nearly ubiquitous induction of polyploidy by AMG 900 may underlay the consistent and strong growth inhibition we observed (as evidenced by the low IC_{50} values).

In summary, we have found that AMG 900 is highly active in breast cancer cell lines and identified biomarkers that predict for response to this compound in vitro. Specifically, we have described the novel observation that there is a strong association between p53/p21 functional status and response to Aurora kinase inhibition in breast cancer. This observation requires clinical validation incorporating *TP53* somatic mutation analysis and/or p21 expression to identify those patients most likely to benefit from treatment.

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Conflict of interest The study was funded in part by Amgen; stock ownership in Amgen (Judy Dering, Dennis J. Slamon), consultant/advisory role in Genentech, and Sanofi-Aventis (Dennis J. Slamon). Other authors have no conflict of interest.

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