Inhibiting Aurora kinases reduces tumor growth and suppresses tumor recurrence after chemotherapy in patient-derived triple-negative breast cancer xenografts

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Running title: Aurora kinase targeting triple-negative breast cancer

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

Triple-negative breast cancers (TNBC) have an aggressive phenotype with a relatively high rate of recurrence and poor overall survival. To date, there is no approved targeted therapy for TNBC. Aurora kinases (AK) act as regulators of mammalian cell division. They are important for cell cycle progression and are frequently overexpressed or mutated in human tumors, including breast cancer. In this study we investigated the therapeutic potential of targeting AK in preclinical models of human breast cancers, using a pan-inhibitor of AK, AS703569. In vitro, AS703569 was tested in 15 human breast cancer cell lines. TNBC cell lines were more sensitive to AS703569 than were other types of breast cancer cells. Inhibition of proliferation was associated to cell-cycle arrest, aneuploidy and apoptosis. In vivo, AS703569 administered alone significantly inhibited tumor growth in 7 out of 11 patient-derived breast cancer xenografts. Treatment with AS703569 was associated to a decrease of phospho-histone H3 expression. Finally, AS703569 combined to doxorubicin-cyclophosphamide significantly inhibited in vivo tumor recurrence, suggesting that AK inhibitors could be used both in monotherapy and combination settings. In conclusions, these data indicate that targeting Aurora kinases could represent a new effective approach for TNBC treatment.
INTRODUCTION

Triple-negative breast cancers (TNBC), which lack expression of estrogen receptors (ER), progesterone receptors (PR) and epidermal growth factor receptor 2 (HER2), account for 15% of breast tumors in Europe and an even higher percentage of breast cancer in women of African descent. TNBC tumors have a relatively high rate of recurrence, distant metastases and poor overall survival (1). TNBC is responsible for a large proportion of breast cancer deaths, despite its relatively small proportion among all breast cancers, due to its generally aggressive clinical course. The standard of care is chemotherapy, although recent research suggests a sound rationale for the use of targeted agents with antitumor and/or antiangiogenic activity such as receptor tyrosine kinase inhibitors. The absence of tumor-specific treatment options in this cancer subset underscores the critical need to develop a better understanding of the biology of this disease, as well as to advance treatment strategies for these patients (1).

Signaling pathway abnormalities commonly reported in TNBC involve the regulatory mechanisms of cellular proliferation, differentiation, p21-mediated cell signaling and G1-S checkpoint controls (2, 3).

The Aurora kinase (AK) proteins are serine/threonine kinases that act as regulators of mammalian cell division. Aurora-A localizes to centrosomes/spindle poles and is required for spindle assembly, whereas Aurora-B is a chromosome passenger protein required for phosphorylation of histone H3, chromosome segregation and cytokinesis (4-7).

Aurora A and B have been implicated in tumor formation and progression (6, 8) and are overexpressed in a variety of cell lines (4, 9). Relatively high expression of Aurora A and B has been shown in small patient cohorts in several tumor types, including breast, lung, colon, prostate, pancreas, liver, skin, stomach, rectum, esophagus, endometrium, cervix, bladder, ovary, and thyroid cancers (4-6, 10). In breast cancer, the expression of Aurora kinase A has been found to be predictive of survival in a cohort of more than 600 primary tumors (7).

Given their pivotal role in mitosis and overexpression in cancers, AKs have become attractive targets. A number of inhibitors displaying differential inhibitory activities toward the three family members have since then been developed and utilized to understand the functional role of AK in mitotic progression. AK inhibitors induce tetraploidy and polyploidy as a result of aberrant mitosis. The effect of the AK inhibitors is therefore unique, in that tumor cells do not undergo cell-cycle arrest after drug treatment. Rather, they are catastrophically driven forward and from aberrant mitosis, which leads to cell death.
In vivo studies with several agents targeting AKs have shown promising results, with tumor growth being inhibited in a number of models (8, 11, 12). The therapeutic potential of Aurora-based targeted therapy is also being assessed in clinical trials (13), although none of them have specifically addressed triple-negative breast cancers.

In the current studies, we investigated the efficacy of a pan-Aurora kinase inhibitor, AS703569 (14), in preclinical models of human breast cancers. We report that AS703569 has a potent anti-tumor activity in TNBC cell lines, associated with endoreduplication and apoptosis. This anti-tumor activity was confirmed in vivo using patient-derived breast cancer xenografts.
MATERIALS AND METHODS

Compound
Inhibition of Aurora kinases was achieved using the small-molecule pan-Aurora kinase inhibitor, AS703569 (previously named R763), an orally potent ATP competitive inhibitor. In vitro biochemical assays demonstrated that AS703569 inhibited Aurora kinases A, B, and C with IC50 values of 4.0, 4.8, and 6.8 nM, respectively (Fig1A) (14). The selectivity of AS703569, assessed in a panel of cell-based kinase assays, was previously published (14). In vitro experiments were conducted using dilutions from a 2 mmol/L stock of compound dissolved in DMSO. For in vivo studies AS703569 was suspended in sterile 0.9% NaCl solution and administered by oral gavage.

Human breast cancer cell lines
The following cell lines were obtained from ATCC (American Type Culture Collection): MDA-MB-468, MDA-MB-231, HCC1143, HCC 1395, HCC38, HCC1419, HCC2218, MDA-MB-361, BT474, MCF-7, SK-BR-3, ZR-75-1. All cell lines were authenticated by ATCC and maintained according to the complete growth medium conditions outlined by the ATCC (Manassas, VA) with the exception of MCF-7 (obtained from Barbara Ann Karmanos Cancer Institute, Wayne State University), which was grown in DMEM/F12 with 12mg/ml sodium bicarbonate, 10% Fetal Bovine Serum (FBS), 10mM Hepes, 10ug/ml Insulin (all from Life Technologies, Grand Island, NY) and 10^{-12} M-estradiol (Sigma-Aldrich, St. Louis, MO). The HBCx-17, HBCx-15 and HBCx-8 triple-negative breast cancer cell lines were obtained from 3 patient-derived breast cancer xenografts (15, 16).

Cell proliferation assay
The ATPlite assay was used as a surrogate for detection of cell count/proliferation. Intracellular ATP concentrations were determined using the ATP-lite Luminescence ATP Detection Assay System (Perkin Elmer, Beaconsfield, UK), following manufacturer’s instructions. Briefly, cells were incubated in the presence of serial dilutions of AS703569 (range from 0.001 μM to 100 μM). After 96h in culture, the growth medium was replaced with 100ul phosphate buffered saline (PBS). Cell lysis buffer (50 μl) was added and mixed for 5 min., 50 μl of ATP-lite substrate reagent was then added and mixed for 5 min. After incubating 10 min in the dark, plates were measured for luminescence on a Victor-5 1428.
Multilabel HTS counter (Perkin Elmer, Waltham, MA). Results were displayed as relative light units.

**Cell cycle and apoptosis analysis**

Cells were plated on 6-well tissue culture dishes and allowed to grow overnight to a density of 40-50% confluency. They were then treated with various concentrations of inhibitor and harvested after 48, 72 and 96 hours. At the time of harvest, cells were washed once with PBS, and were trypsinized. Cells were harvested in 1ml of complete medium and pelleted for 5 minutes at 600 x g. Cells were fixed and permeabilized with the addition of 100% ice cold methanol and incubated on ice for 30 minutes. Cells were pelleted and washed twice in 1% bovine serum albumin (BSA; Fisher Scientific, Waltham, MA)/PBS and split into two tubes, one to measure cell cycle by propidium iodide (PI) staining and the other to measure apoptosis by staining of cleaved caspase-3. Cells for cell cycle analyses were resuspended in PI/RNase (Becton Dickenson, Franklin Lakes, NJ) and incubated for 15 minutes at room temperature and then analyzed by flow cytometry using a Guava EasyCyte instrument (Guava, Hayward, CA). Cells to be analyzed for the presence of cleaved caspase-3 were stained with Alexa-488 conjugated cleaved caspase-3 (Asp175) antibody (Cell Signaling Technologies, Beverly, MA) followed by flow cytometric analysis (Guava EasyCyte).

**In vivo efficacy studies**

Female Swiss nude mice, 10-week old, were purchased from Charles River (Les Arbresles, France) and maintained under specific pathogen-free conditions. Their care and housing were in accordance with institutional guidelines as put forth by the French Ethical Committee. Human breast cancer xenografts were established from primary patient’s surgical specimens by grafting tumor fragments into the inter-scapular fat pad of nude mice and maintained through *in vivo* passages as previously described (16). Adriamycin (Doxorubicin) and cyclophosphamide (Endoxan) were purchased from Teva Pharmaceuticals (Paris, France) and Baxter (Maurepas, France), respectively. Docetaxel was purchased from Sanofi (Paris, France). Adriamycin (2 mg/kg), cyclophosphamide (100 mg/kg) and docetaxel (20 mg/kg) were administered by the i.p. route at 3-week intervals.

AS703569 was administered once per week by oral gavage at its maximum tolerated dose (MTD) of 50 mg/kg, which was determined by a tolerance study (Supplement Methods and...
Materials). Inhibition of tumor growth (TGI) and tumor growth delay (GD) were calculated as detailed in Supplementary Methods and Materials.

**Real-Time PCR Amplification**

Extraction of total RNA and synthesis of cDNA were conducted as previously described from 1μg total RNA (17). Ki67 and MDR1 transcripts were quantified using real-time quantitative reverse transcription-PCR. The nucleotide and probe sequences and the conditions for the PCR were previously described for Ki67 and MDR1 (16, 18). Normalization, reference genes and quantification are described in Supplementary Methods and Materials.

Nucleotide, probe sequences and conditions for RT-PCR analysis of AURKA, AURKB, AURKC, PLK1, TBX2, NEK2, MYC and NDC80 genes were described elsewhere (19) and in Supplementary Methods and Materials.

**Pharmacodynamic study and immunohistochemistry of xenograft tumor tissues**

Xenoengrafted tumors HBCx-5, HBCx-10 and HBCx-15 were evaluated for expression of phospho-histone H3 Ser10 (pHisH3) as a marker of AK B activity. Tumor-bearing mice were treated with a single administration of AS703569 (50mg/kg) or buffer vehicle when tumors had reached a volume of approximately 150-200 mm³. Mice were euthanized at 6, 24, 48, 72 h and 21 days post-treatment. Two control groups are included in the kinetics study (named “control 6 h” and “control 72 h”). Four animals were included in each time point. Tumor tissue samples were fixed in neutral-buffered 10% formalin and paraffin embedded using routine protocols with a Sakura Tissue-Tek® VIP® Vacuum Infiltration Processor (Sakura, Torrance, USA) and a Leica EG 1150 Embedding Center (Leica Microsystèmes SAS, Nanterre, France). Further details on TMA techniques, antibodies and morphometry are included in Supplementary Methods and Materials.

**Histological analysis of the inter-scapular fat pad and immunostaining (IHC) of residual tumor cells**

To analyze residual tumor cells after tumor remission, mice were euthanized when tumors had completely regressed (approximately 60 days after the chemotherapy treatment). The interscapular fat pad was excised, fixed and paraffin-embedded. Semi-serial 4μm sections were cut every 250μm until exhaustion of the tissue. Ziehl-Neelsen staining was used to characterize intracellular brown pigments. Ki67 IHC was performed using a rabbit monoclonal anti-human Ki67 antibody (clone SP6 ready-to-use, Spring Bioscience,
Pleasanton, CA) and phosphorylated histone H3 (pHisH3) IHC using a rabbit polyclonal anti-human PH3, 1:100 (Cell Signaling #9701, Ozyme, Saint-Quentin-en-Yvelines Cedex, France). In situ hybridization of a human Alu probe was performed to detect human cells in surrounding mouse tissue. Cell conditioning using citrate buffer 10 mM, pH 6, 95°C for 16 min, incubation and color development were performed in a Ventana Medical Systems DiscoveryXT automate (Illkirch, France). FITC-conjugated Alu probe from Ventana medical system (catalogue number 780-2845) was denatured 8 min at 85°C, hybridized 1H at 50°C and washed twice in 2x SSC 4 min at 45°C. Detection was via a goat biotin-conjugated anti FITC antibody (Jackson ImmunoResearch, Suffolk, UK) and followed by the NBT-BCIP method (BlueMap XT kit) with red counterstaining (Ventana Medical Systems).
RESULTS

AS703569 potently inhibited cell growth of triple-negative breast cancers in vitro

To determine the effects of AS703569 on tumor cell viability, a panel of 15 cell lines, representing the various subtypes of human breast cancers, were treated with increasing concentrations of AS703569 for 96 h. Eight cell lines were triple-negative, 3 cell lines were positive for HER2 and 4 were triple-positive (ER+, PR+ and HER2+). P53 was mutated in 10 cell lines (7 TNBC) and was wild-type (wt) in 5 cell lines (1 TNBC). The 50% inhibitory concentrations of cell viability (IC50) at 96 h, determined by ATPlite assay, are shown in Table 1. The triple-negative cell lines were relatively more sensitive to AS703569, with IC50s between 0.004 and 0.694 μM, whereas the ER+/PR+ and HER2+ cell lines were more resistant to AS703569, with IC50 between 2.52 and 7.37 μM. The difference between these two groups was statistically significant (Fig. 1B; unpaired Student t test, p<0.0001).

AS703569 induces endoduplication and apoptosis

Inhibition of AK B, or both A and B, results in early exit from mitosis without cell division, leading to endoreduplication and polyploidy. The latter is defined as an accumulation in DNA content (>4N). In order to evaluate the association between polyploidy and the anti-tumor activity of AS703569, compound-induced changes in cell cycle progression were investigated in 7 breast cancer cell lines representing different intrinsic sub-types of breast cancer.

Figure 1C depicts representative data in the TNBC cell line MDA-MB-231 and ER+ cell line MCF-7. Consistent with the anti-proliferative data described in Table 1 and Figure 1B, AS703569 caused G2/M arrest and accumulation of DNA content, accompanied by apoptosis, at relatively low concentrations (10 and 100 nM) in the MDA-MB-231 cell line. In the MCF-7 cell line, G2/M arrest and polyploidy were observed at higher concentrations, but led to lower levels of apoptosis compared to that seen in the MDA-MB-231 cells. In both cell lines, polyploidy was observed starting from 48 h, which increased over time to 96 h, similar to the apoptotic effect. To confirm this phenotypic effect of AS703569 in patient-derived models with limited passages, AS703569-induced changes in cell-cycle progression were evaluated in two cell lines derived from the TNBC xenografts used in this study, HBCx-8 and HBCx-17 (Fig. 1C). In both of these cell lines, polyploidy (>4N content) was observed starting at 72 h with 100 nM AS703569. This concentration is known to be achievable in the blood of mice at the dose administered in this study as based on the pharmacokinetic properties of the
compound (data not shown). Endoreduplication was also observed for the AS703569 resistant MDA-MB-361, SKBR-3 and ZR75-1 cell lines (data not shown).

In addition to cell cycle effects, we investigated whether treatment with AS703569 resulted in apoptosis. MDA-MB-231 and MCF-7 cell lines were treated with different concentrations of the compound for up to 96 hours, and cleaved caspase-3 was measured by flow cytometry analysis (Fig. 1D). The percentages of apoptotic cells in both cell lines increased with increasing duration of exposure to AS703569, with a relatively greater percentage of apoptotic cells seen in the TNBC cell line. Similarly, higher percentage of apoptosis was observed in TNBC MDA-MB-468 cells at lower concentrations of AS703569, compared to non-TNBC MDA-MB-361, BT474, SKBR-3 and ZR75-1 cells (data not shown).

**AS703569 significantly inhibited growth of patient-derived breast cancer xenografts.**

To test the effects of Aurora kinase inhibition *in vivo*, the efficacy of AS703569 as a single agent at its maximum tolerated dose (50 mg/kg once a week) was evaluated in 11 human primary breast cancer xenografts (HBCx, 10 of them triple-negative and 1 HER2+), previously characterized for their IHC profile and their response to standard chemotherapies, (15, 16, 20). AS703569 had significant antitumor activity in 7 xenograft models (40%–80% TGI compared with the vehicle-treated control group, *P* < 0.005; responders, Table 2). Five BC xenograft models were resistant or low responder (TGI not significantly different or lower than 40%). No correlation was found with the expression of proteins involved in the cell cycle control such as PTEN, Rb or P53. One HBC xenograft carried both p53 and BRCA2 mutations (15) and showed a moderate response to AS703569 with a TGI of 40% and a T/C growth delay of 2 fold. Importantly, AS703569 was also active in the xenograft models that were resistant to docetaxel (Table 2). An example is illustrated in Figure 2A and Figure 2B: treatment with AS703569 during 6 weeks resulted in TGI of 80% in the HBCx-10 xenograft (Fig. 2A), while treatment by Docetaxel (20 mg/kg given every 3 weeks) didn’t change the HBCx-10 tumor growth curve (Fig. 2B). To determine whether the effect of AS703569 *in vivo* was correlated to the intrinsic expression of AK genes and other mitotic spindle checkpoint genes, RT-PCR analysis were carried out to quantify the expression of AURKA, AURKB, AURKC, PLK1 (polo family protein kinase), TPX2 (microtubule-associated protein homolog), NEK2 (NIMA-related kinase 2) and NDC80 (kinetochore complex component) (Fig. 2C). The expression of the Myc oncogene was also determined as possible factor related to the resistance to AK inhibitors (21). Results showed that AURKA and AURKB genes were
highly expressed (Ct ∼ 26) in all xenograft models, showing increased expression of AURKB in low responder/resistant models compared to responders (Fig. 2C, p = 0.0385, unpaired t-test). AURKC was weekly expressed or not expressed (Ct > 32). The expression of PLK1, TPX2, NEK, NDC80 and MYC genes was relatively high in all models, with no significant differences between responder and resistant xenografts (unpaired t-test).

The AS703569 inhibitor induces a strong and reversible reduction of phosphorylated histone H3 levels in vivo.

To evaluate the pharmacodynamics of AS703569 in vivo, we measured the levels of phosphorylated histone H3 (pHisH3) in 3 BC xenografts as a measure of Aurora kinase B inhibition. The 3 BC xenografts were the responders HBCx-5 and HBCx-10 (HER2+ and TN, respectively) and the non-responder HBCx-15 (TN). Four animals of each BC xenograft were treated with a single administration of AS703569 (50mg/kg) and tumors were collected from mice at various times after treatment: 6, 2, 4, 72 h and 21 days and analyzed by IHC. The kinetics of pHisH3 expression is shown in Figure 3A. In untreated tumors, 8-9 % of tumor cells were stained. Six hours after treatment, pHisH3 immunostaining was decreased dramatically in the 3 xenograft tumors. Twenty-four hours after treatment, pHisH3 expression returned to control levels. No significant treatment effects were observed at time points beyond 24h. An example of the decrease in pH isH3 IHC is shown in Figure 3B: a field from each two independent tumors of the HBCx-10 xenograft, untreated, or at 6h and 24h after treatment. Six hours after the AS703569 inhibitor treatment no positive cells were detected in treated tumors, whereas at the 24h time point pHisH3 expression was similar to that of untreated tumors.

Inhibition of tumor recurrence in a basal-like breast cancer xenograft

We next addressed the question whether Aurora kinase inhibition could prevent tumour recurrence after conventional therapy, using the HBCx-10 xenograft which is sensitive to AC and has a high frequency of tumor relapse (22). In the control group, mice were treated with one administration of AC (adryamicin 2mg/kg, cyclophosphamide 100mg/kg), and by Day 60, 7/10 animals showed complete tumor regression as observed by local palpation (Fig. 4A). In the control group, one mouse was euthanized during tumor remission for histological analysis and 9 animals were followed and tumor regrowth was monitored until ethical euthanasia. In the second group, mice were treated with one administration of AC followed by 8 weeks of treatment with AS703569. Tumor recurrence was seen in two mice, whereas no tumor
regrowth was observed in 5 mice after 305 days. Kaplan-Meier survival analysis (Fig. 4B) demonstrates that mice treated with AC alone (n=10, black line) have decreased survival compared to mice treated with AC and AS703569 (n=7, red line). Histological analysis of the mouse inter-scapular fat pad (group AC alone) euthanized during tumor remission revealed the presence of numerous small islands of tumor cells (Fig. 4C), as confirmed by in situ hybridization with a specific human Alu probe (Fig. 4D and 4E). To determine whether these residual tumor cells were resting or proliferating cells, we performed Ki67 staining, which showed a relatively high proliferation status of the cancer cells (Fig. 4F and 4G). Immunostaining for pHisH3 (Fig. 4H and 4I) revealed that expression in these residual cells was similar to that seen in the HBCx-10 xenograft before chemotherapy (Fig. 3D), indicating Aurora kinase activity in residual cancer cells. One mouse in the AC+ AS703569 group was submitted for histological examination of the inter-scapular fat pad at day 305 (Fig. 4J). The analysis revealed the presence of a small fibrous nodule containing several necrotic and mineralized foci within the fat pad with no residual tumor cells as confirmed by a negative Alu staining (Fig. 4K). Necrotic areas were surrounded by a rim of lipofuscin-containing mouse macrophages (Fig. 4L).
DISCUSSION

As triple-negative breast cancers have high proliferation levels, anti-mitotic agents such as AK inhibitors could represent a new class of targeted therapy. We report here the anti-tumor activity of the pan-Aurora kinase inhibitor (AS703569) in the triple-negative sub-type of breast cancer. In vitro data collected from human breast cancer cell lines showed that TNBC cell lines were more sensitive to the AS703569 inhibitor than the ER/PR or HER2+ cell lines. This sensitivity was not dependent on p53 status, in line with a previous report of in vitro sensitivity of lung, colon and prostate cancer cell lines to AS703569 (14). No correlation with Aurora kinases and Ki67 expression was observed (data not shown). In a previous study, the activity of an AK B inhibitor (AZD1152) in human breast cancer cell lines was reported, with no selective anti-tumor activity for triple-negative cells (23). Our data indicate that both triple-negative and ER+ cell lines exposed to AS703569 showed endoreduplication, while induction of apoptosis seemed to be greater in the triple-negative cells (from 50% to 80%). The mechanisms of the AS703569 poor activity in HER2 or ER+ cells in vitro are unknown and could be related to genes differentially expressed within the panel of breast cancer cell lines. Another possibility regarding the sensitivity of TNBC cell lines to AS703569 may, in part, be due to an off-target effect of the compound.

As for the cell lines, the sensitivity to Aurora kinase inhibition in vivo did not appear to be dependent on p53 mutational status. This relationship is not completely discerned in the literature, with different results being reported depending on tumor cell type and the AK inhibitor employed. Cells compromised for p53-checkpoint function were more likely to induce endoreduplication and apoptosis in response to the inhibitor VX-680 (24). Conversely, leukemia cell lines with wild-type p53 were more sensitive to the AK inhibitor ZM447439 than cell lines with mutant p53, suggesting that a p53-dependent post-mitotic checkpoint may be important in determining cell fate after exposure to AK inhibition (25). This question has not been specifically addressed in the literature for breast cancers. Interestingly, the HBCx-17 xenograft which carries mutations in both p53 and BRCA2 genes (15) was moderately sensitive to AS703569, suggesting that a combination of these factors might not be sufficient to increase sensitivity to AK inhibitors, as it was demonstrated in vitro (26).

General mechanisms of tumor cell resistance to AK inhibitors have not been identified yet, although in vitro studies suggest that mutations of the targeted AKs and overexpression of drug-resistance genes may be involved (27, 28).
The absence or low response observed in 4 xenograft models cannot be related to the PgP expression, as none of the models tested in this study express the MDR1 gene. As Aurora kinases are known to be dramatically up-regulated in highly proliferating cells, the proliferation rate of the tumour (that is, the number of mitotic cells exposed to Aurora-kinase inhibition) could potentially determine sensitivity. Our data indicate that a high proliferation index alone is not sufficient to determine sensitivity to AS703569 and that other genes/pathway may be more important in regulating the response to AKs inhibition. Importantly, AS703569 was effective in models resistant to docetaxel, indicating the therapeutic potential of targeting the Aurora kinase family in this subtype of tumors, especially recurring or advanced breast cancers.

One of the key effector proteins regulated by Aurora B kinase is the RB1 protein (29). However the RB1 protein expression was not related to the in vivo response of the AS703569 inhibitor in this study, which is in line with previous reports showing that RB1 status alone does not correlate with sensitivity to Aurora kinase inhibitors in vivo (21, 30). The finding that AK genes A and B were highly expressed in breast cancer xenografts is consistent with previous works that showed high level of these genes in aggressive breast cancers compared to benign tumors (19). A high AURKB expression was correlated to in vivo response to AS703569. A recent study showed that expression of Aurora A and B in lung cancer cell lines were weak predictors of response to AK inhibitor PF-03814735 (21). Interestingly the same authors reported a higher response to PF-03814735 in lung tumors with amplified or over-expressed MYC. That was not the case in our study, suggesting that the relationship between MYC and the in vivo response to AK inhibitors could be specific to lung cancers.

Consistent with other studies, pHisH3 reduction was rapid and reversible (31, 32). Interestingly, pHisH3 reduction was also observed in a xenograft model that did not respond to AS703569, suggesting that inhibition of AK activity alone may not be sufficient to arrest cell proliferation in resistant tumors and these might escape treatment through alternative pathways. In addition, this result suggests that a decreased expression of pHisH3, the phenotype of Aurora B inhibition, is not predictive of tumor response in vivo.

In the responding tumor xenograft models, tumor growth was not completely abolished, despite the strong inhibition of pHisH3 induced by AS703569. This is consistent with other preclinical studies showing that AK inhibitors alone don’t induce tumor regression in solid cancers (10, 21, 32-34). Treatment with the AK inhibitors PF-03814735 and MK-0457 delayed the growth of colon and ovarian xenografts (32, 33). Similarly, treatment with MK-5108 had anti-tumor activity in the HCT116 and SW48 xenografts, without tumor remissions...
(34). As AK inhibitors have had relatively excellent activity against cancers with high mitotic index such as acute myeloid leukemia and certain lymphomas (35), highly proliferating solid tumors such as TNBC could potentially be sensitive to AK inhibition. Our results indicate that targeting AK is not sufficient to abolish tumor growth in highly proliferating breast cancers, presumably because tumor cells in these tumors may not be addicted to AKs for their proliferation and survival.

To address the question whether targeting Aurora kinases could inhibit tumor recurrence in patient-derived xenograft models, we used a TNBC cancer xenograft characterized by an initial response to AC followed by high frequency of local tumor relapse (22). The positive staining for Ki67 and pHisH3 markers in residual cancer cells that have survived chemotherapy indicates that these cells were still proliferating and had AK activity. AS703569 in combination with an anthracycline-based treatment reduced tumor recurrence. Other studies have reported combination therapies of Aurora kinases inhibitors with cytotoxic anticancer agents, such as docetaxel and cisplatin (34, 36). Although obtained in only one breast tumor model, this preliminary result suggests that targeting Aurora kinases in the adjuvant setting may improve response to anthracyclines in advanced TNBC.

Over a dozen small molecule AK inhibitors have entered clinical development (37, 38). As single agents, the AK inhibitors seem only to have a future in patients with leukemias and lymphomas, while the responses observed in patients with solid tumors have been disappointing, with disease stabilization as the best response. Future studies with AK inhibitors should focus on the possibility of combining these agents with chemotherapy or other targeted anticancer agents.

In summary, we have presented a preclinical study with cancer cell lines and patient-derived tumor xenografts indicating for the first time that targeting AK has an anti-tumor effect in human triple-negative breast cancers.

Acknowledgements
The authors thank Akila Hamini and Narjesse Karboul for providing expert technical assistance.
REFERENCES


**Table 1.** Human breast cancer cell lines characteristics and AS703569 IC50 (μM).

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* p53 mutations were determined by Fasay Assay as previously described (16).
§Rb, PTEN are by Western blotting analysis;
# MDR1 and Ki67 gene expression were determined by quantitative RT-PCR.

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<th>Response to AS703569</th>
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FIGURE LEGENDS

**Figure 1.** A, The chemical structure and *in vitro* potency of AS703569. B, AS703569 IC50 in triple-negative and ER+/PR+ or HER2+ breast cancer cell lines (p<0.0001, unpaired Student t test). C, cell cycle analysis of MDA-MB-231, MCF-7, HBCx-8 and HBCx-17 cell lines at different time points. Endoreduplication is indicated by cell population with > 4n DNA content. D, Caspase 3 flow cytometry in MDA-MB-231 and MCF-7 cells upon incubation with low concentration of AS703569.

**Figure 2.** A, Anti-tumor activity of AS703569 (50mg/kg 1x/week for 6 weeks) in the HBCx-10 xenograft. B, HBCx-10 tumor growth after doxetaxel treatment (20 mg/kg given at day 1 and day 22). C, RT-PCR expression analysis of AURKA, AURKB, AURKC, MYC, PLK1, TPX2, NEK2 and NDC80 genes. Expression values are represented as a ratio to the TBP (tata-binding protein) gene expression.

**Figure 3.** A, Kinetic histogram showing pHisH3 expression (IHC) in tumor sections at different time points after a single administration of AS703569 (n=4 mice for each time point). B, pHisH3 immunostaining in the HBCx-10 xenograft in 2 untreated tumors and 6 and 24H after AS703569 administration.

**Figure 4.** Effects of AS703569 + chemotherapy in the HBCx-10 xenograft. A, Median tumor growth after chemotherapy alone (adriamycin 2mg/kg + cyclophosphamide 100 mg/kg at Day 1) or chemotherapy followed by a 2 months of AS703569 treatment (50mg/kg 1x/week). B, Kaplan-Meier survival analysis of mice treated with chemotherapy alone (n=10, black line) and with chemotherapy and AS703569 (n=7, red line). P < 0.005, Log-rank (Mantel-Cox) Test. C-L, Histological analysis of the interscapular fat pad of a mouse treated with chemotherapy alone (C-I) and of a mouse treated with chemotherapy + AS703569 (J-L). D and E, Alu staining with human specific probes. F and G, Ki67 immunostaining of residual tumor cells. H and J, pHisH3 immunostaining of residual tumor cells. J, histological analysis of the fad pad of the mouse treated with AS703569. K, negative Alu staining. L, Macrophages rich in lipofuscin were found around the necrotic lesion (narrow). Lipofuscins are stained in pink by Ziehl-Neelsen technique.
Figure 1

A

Aurora A (IC50) – 4 nM
Aurora B (IC50) – 4.8 nM
Aurora C (IC50) – 6.8 nM

B

IC50 in breast cancer cell lines

C

MDA-MB-231

DMSO
0.01 μM AS703569
0.1 μM AS703569

MCF-7

DMSO
0.1 μM AS703569
1.0 μM AS703569

C

Cleaved Caspase-3 : MDA-MB-231

D

Cleaved Caspase-3 : MCF-7
Figure 2

A. Effect of the AS703569 treatment on HBCx-10 tumor growth

- untreated
- AS703569 50mg/kg

Days after start of treatment

B. Effects of docetaxel treatment on HBCx-10 tumor growth

- untreated
- Docetaxel 20 mg/kg

Days after start of treatment

C. Expression of Aurora kinases genes

- AURKA
- AURKB
- AURKC

Expression of PLK1, TPX2 and MYC genes

- PLK1
- TPX2
- MYC

Expression of NEK2 and NDC80 genes

- NEK2
- NDC80

Days after start of treatment

responder models
resistant or low responder models
Figure 3

A  Pharmacokinetics of pHisH3 expression (IHC) after a single AS703569 administration

% of positive cells

control  treated 6H  treated 24H  treated 48H  treated 72H  control 72H  treated 21 days

B

untreated  treated 6 H  treated 24 H
Figure 4

A. Tumor volume over time for chemotherapy alone and chemotherapy plus AS703569 treatment.

B. Survival analysis of HBCx10 xenografts treated with chemotherapy alone or chemotherapy plus AS703569.

C-L. Pathological images showing the effects of treatment on tumor morphology.
Molecular Cancer Therapeutics

Inhibiting Aurora kinases reduces tumor growth and suppresses tumor recurrence after chemotherapy in patient-derived triple-negative breast cancer xenografts

Angela Romanelli, Anderson Clark, Franck Assayag, et al.

Mol Cancer Ther Published OnlineFirst September 25, 2012

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-12-0441-T

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