Published OnlineFirst November 9, 2012; DOI: 10.1158/0008-5472.CAN-12-2633

Polo-like Kinase 1: A Potential Therapeutic Option in Combination with Conventional Chemotherapy for the Management of Patients with Triple-Negative Breast Cancer

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Abstract

Breast cancers are composed of molecularly distinct subtypes with different clinical outcomes and responses to therapy. To discover potential therapeutic targets for the poor prognosis-associated triple-negative breast cancer (TNBC), gene expression profiling was carried out on a cohort of 130 breast cancer samples. Polo-like kinase 1 (PLK1) was found to be significantly overexpressed in TNBC compared with the other breast cancer subtypes. High PLK1 expression was confirmed by reverse phase protein and tissue microarrays. In triple-negative cell lines, RNAi-mediated PLK1 depletion or inhibition of PLK1 activity with a small molecule (BI-2536) induced an increase in phosphorylated H2AX, G_2–M arrest, and apoptosis. A soft-agar colony assay showed that PLK1 silencing impaired clonogenic potential of TNBC cell lines. When cells were grown in extracellular matrix gels (Matrigel), and exposed to BI-2536, apoptosis was observed specifically in TNBC cancerous cells, and not in a normal cell line. When administrated as a single agent, the PLK1 inhibitor significantly impaired tumor growth *in vivo* in two xenografts models established from biopsies of patients with TNBC. Most importantly, the administration of BI-2536, in combination with doxorubicin + cyclophosphamide chemotherapy, led to a faster complete response compared with the chemotherapy treatment alone and prevented relapse, which is the major risk associated with TNBC. Altogether, our observations suggest PLK1 inhibition as an attractive therapeutic approach, in association with conventional chemotherapy, for the management of patients with TNBC. *Cancer Res; 73(2); 813–23. ©2012 AACR.*

Introduction

Breast cancer is a heterogeneous disease with different subgroups characterized by specific clinical outcomes and responses to therapy (1). Triple-negative breast cancers (TNBC) are characterized by a lack of expression of estrogen and progesterone receptors (ER/PR) and a lack of HER2 overexpression (2). TNBCs account for 15% of breast cancers and are associated with African-American ethnicity, younger age, and poorer outcome when compared with the other breast cancer subtypes: luminal A (LA), luminal B (LB), and HER2+/ER− (HER2; ref. 2). TNBC show a high rate of *TP53* mutation (3), are associated with a "BRCAness" phenotype (4), and are highly proliferative, genetically unstable, poorly differentiated, and often grade 3 carcinomas (5). In contrast to HER2 and luminal carcinomas, which can be treated with targeted therapy such as anti-HER2 monoclonal antibodies or hormonal therapies, respectively, there is no available targeted therapy for patients with TNBC who are managed exclusively with conventional chemotherapy. Although they show high rates of objective initial response, the majority of patients do not display a complete response and have a poorer prognosis than those within other breast tumor subgroups due to high rates of recurrence (6). Because of this unfavorable prognosis and the lack of targeted therapy, there is currently an intensive search for identifying therapeutic targets for TNBC (7).

Polo-like kinase 1 (PLK1) is the best-characterized member of the human PLK family (PLK1-5) of serine/threonine protein kinases, which is involved in various functions such as mitotic entry, spindle assembly, and DNA damage response (8). In

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-12-2633

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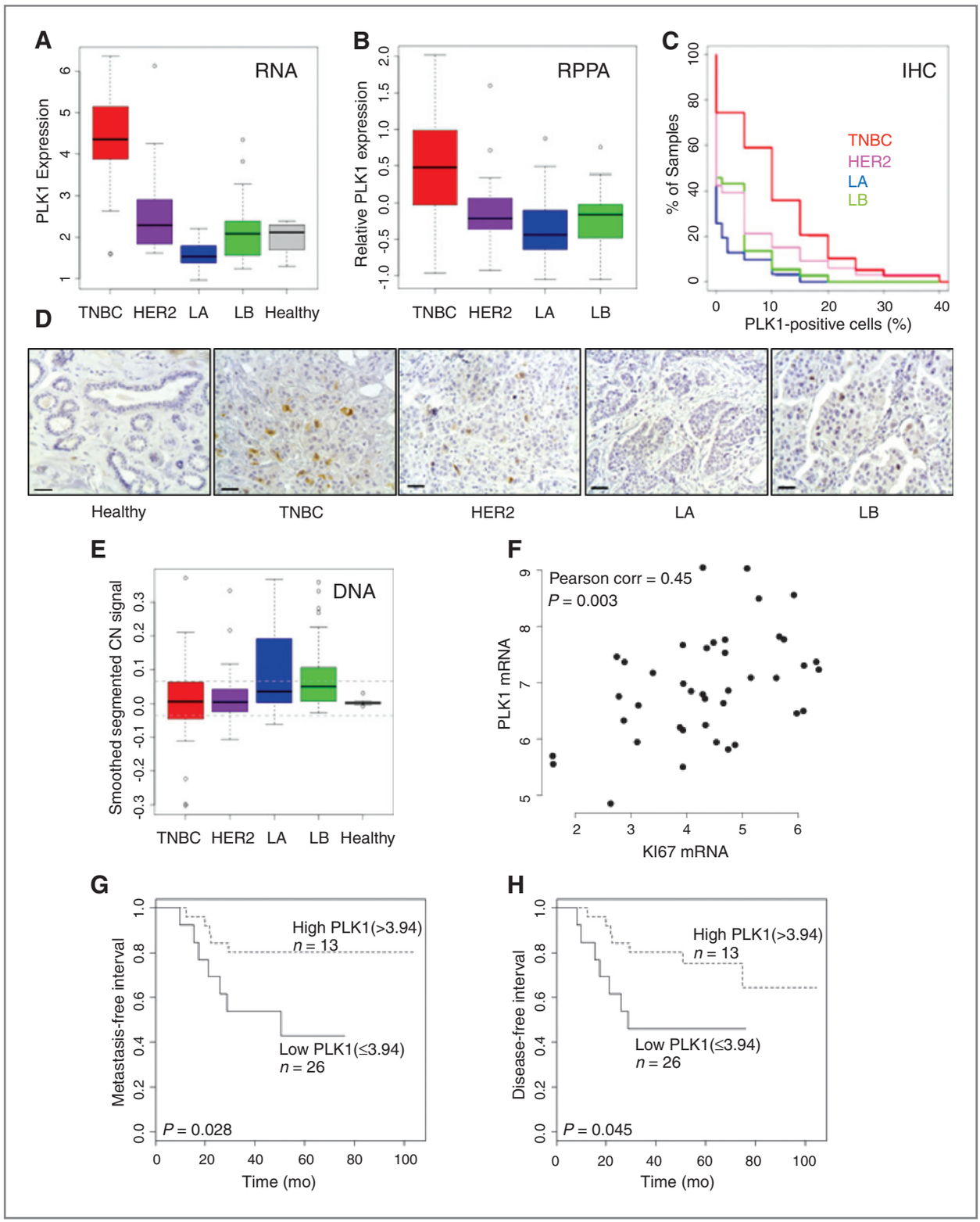

Figure 1. PLK1 is overexpressed in TNBC. PLK1 expression was measured in TNBC, HER2, LA, LB, and healthy breast tissues. A, mRNA PLK1 levels were determined by microarray analysis. PLK1 protein level was evaluated by reverse phase protein array (B) and immunohistochemistry (C). Protein and mRNA relative quantifications were logarithmic transformed and illustrated by box plots. C and D, PLK1 expression was analyzed on tissue microarray. The graph represents for each subtype the percentage of samples having more than a given percentage of stained cells (C). PLK1 displayed a cytoplasmic and a

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normal tissues, PLK1 is only found in actively proliferative tissues. PLK1 is overexpressed in diverse tumors including breast cancer, and is associated with poor prognosis (9–13). High PLK1 levels have been found in TNBC (9, 14), and PLK1 has recently been proposed to be a potential therapeutic target for TNBC based on a protein kinase siRNA library screen and *in vitro* assays (15). Besides proliferation, the overexpression of PLK1 in mouse NIH3T3 fibroblasts transforms the cells, making them capable to form tumors when injected in mice (16). PLK1 depletion/inhibition triggers apoptosis in various cancer cell lines, and impairs tumor growth in mice (17). PLK1 depletion/inhibition preferentially kills cancer cells compared with normal cells (18–20) and p53-defective cancer cells compared with p53 wild-type cancer cells (21–23). Several PLK1 inhibitors have been developed; some of them, such as BI-2536, have been evaluated in clinical trials (8, 24–27).

We conducted RNA microarrays on a cohort composed of tumor samples, healthy breast tissues, and triple-negative cell lines. PLK1 was overexpressed in TNBC compared with healthy tissues and to the other breast cancer subtypes. We examined the effects of PLK1 depletion (RNAi) and inhibition (BI-2536) on the behavior of triple-negative cell lines and in human TNBC-derived xenograft models. *In vitro* or *in vivo*, these experiments showed a beneficial effect of targeting PLK1 in TNBC.

Materials and Methods

Human samples, clinical, and microarray data

Our cohort was composed of 35 LA, 40 LB, 46 TNBC, 33 HER2, 18 normal breast tissues, and 14 TNBC cell lines. Experiments were conducted in agreement with the Bioethic Law No. 2004–800 and the Ethic Charter from the French National Institute of Cancer (INCa), and after approval of the ethics committee of our Institution. Immunohistochemistry with PLK1 antibodies (Cell Signaling Technology) was conducted as described (28). Clinical data were collected and disease-free interval was defined as the time from the diagnosis of breast cancer to the occurrence of a locoregional, distant, or controlateral recurrence. DNA (Affymetrix SNP6.0), RNA (Affymetrix U133 plus 2.0), and RPPA microarrays were conducted as described (28).

Cell culture, RNA interference, PLK1 inhibitor, and cellular assays

Cell lines were purchased in 2006 and 2008 from the American Type Culture Collection, and cultured as described (28). The cell lines have been characterized by DNA and RNA microarrays (year 2009). Cells were transfected with 20 nmol/L of either a control RNAi (AllStars) or 2 distinct PLK1 RNAi (PLK1#6, PLK1#7; all from Qiagen), or incubated with a PLK1 inhibitor (BI-2536; Selleck Chemicals). Cell proliferation was determined by MTT (Sigma). Caspase 3/7 activity was determined by the Caspase-Glo 3/7 luminescent assay (Promega) or by Western blot analysis as described (28). Cell-cycle analysis was carried out with FACScalibur (Becton Dickinson) using Cellquest software (Becton Dickinson) to determine cellular DNA content. For soft-agar colony formation, 0.35% agar containing RNAi-transfected MDA-MB-468 and HCC70 cells was overlaid onto precast 0.5% bottom agar and incubated for 4 weeks. Colonies were visualized after MTT staining. Three-dimensional (3D) cell culture was conducted with Matrigel (BD Biosciences) as described (29). Briefly, MDA-MB-468 and MCF10A were grown in the 3D on-top assay for 7 and 10 days, respectively. Cells were then treated with BI-2536, and cell viability was determined 3 days later using the WST1 Cell Proliferation Assay Kit (Millipore).

Mice, compounds, treatment, and tumor growth measurement

Mice were purchased from Charles River. Their care and housing were conformed to the institutional guidelines as put forth by the French Ethical Committee. Human TNBC breast cancer xenograft models (HBCx-10 and HBCx-24) were established as previously detailed (30–32). HBCx-10 represents a xenograft model of doxorubicin + cyclophosphamide (DC)-induced complete remission and tumor recurrence (30, 31), whereas HBCx-24 does not respond to DC (not shown). Mice-bearing human breast cancer xenografts were treated intraperitoneally with BI-2536 (Selleck Chemicals, 20 mg/kg) twice a week alone or combined with doxorubicin (Teva Pharmaceuticals, 2 mg/kg) and cyclophosphamide (Baxter, 100 mg/kg) at days 1, 22, and 43. Tumor growth was evaluated with a caliper twice a week.

Statistical analysis

The R software was used for statistical analyses (33). Pearson correlation was used to estimate an association between 2 variables. For cellular assays and *in vivo* experiments, *P* values were calculated using the Student *t* test.

Additional experimental details are available in the Supplemental Material.

Results

PLK1 is overexpressed in triple-negative breast cancer

To discover potential therapeutic targets for the poor prognosis-associated TNBC, a gene expression profiling was carried out on a cohort of 130 breast cancers. PLK1 was expressed at higher levels in TNBC (mean $\log_2 = 4.36$) compared with HER2 (mean $\log_2 = 2.6$; $P = 2.8 \times 10^{-10}$), LA (mean $\log_2 = 1.56$; $P < 2 \times 10^{-16}$), LB (mean $\log_2 = 2.16$; $P = 7.6 \times 10^{-13}$), and normal tissues (mean $\log_2 = 1.97$; $P = 1.1 \times 10^{-9}$; Fig. 1A). PLK1 protein levels, measured by reverse phase protein array, were higher in

nuclear localization. Scale bar, 30 μm (D). E, PLK1 analysis at the DNA level. The smoothed segmented copy number (CN) signal is presented in boxplots, with dashed lines indicating the thresholds retained to call CN gains and losses. F, correlation between PLK1 mRNA and Ki67 mRNA within the TNBC subtype. Each tumor is represented by a solid circle. Kaplan–Meier curves of metastasis-free interval (G) and disease-free interval (H) for TNBC divided into tertiles according to their PLK1 expression by comparing the combined 2 top tertiles (>3.94) with the lowest tertile (≤3.94) of PLK1 mRNA expression. Being in the lowest tertile was associated with a higher risk of developing metastasis (HR = 4.05, 95%CI = 1.17–14.05, $P = 0.028$, log-rank test) and disease (HR = 3.41, 95% CI = 1.03–11.26, $P = 0.045$, log-rank test).

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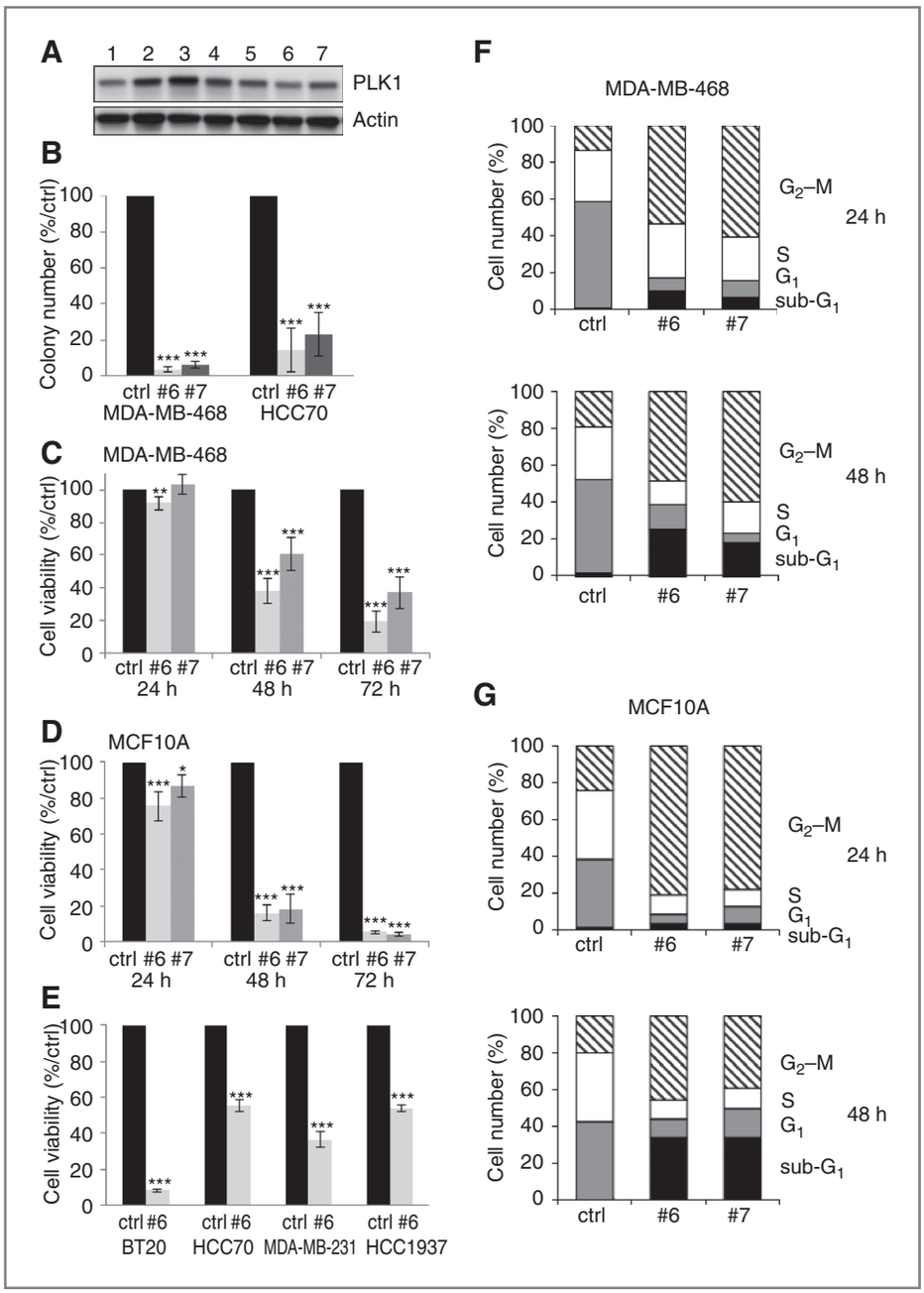

Figure 2. PLK1 depletion impairs triple-negative cell viability and affects cell cycle. A, PLK1 expression was analyzed by Western blot analysis in BT20 (1), HCC38 (2), HCC1937 (3), HCC70 (4), MDA-MB-468 (5), MDA-MB-231 (6), and MCF10A (7). Actin was used as a control. B–G, cell lines were transfected with control (black bars) or 2 different PLK1 RNAi (#6 and #7) MDA-MB-468 and HCC70 (B) were grown on soft agar medium for 4 weeks. Colonies were stained and counted. C–E, cell viability was assessed by MTT assay 24, 48, and 72 hours after transfection (C and D) or only 72-hour posttransfection (E). B–E, data are mean ± SD from at least 3 independent experiments. *P* values are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). F and G, cell-cycle analysis. MDA-MB-468 (F) and MCF10A (G) were incubated with propidium iodide 24 or 48 hour posttransfection. Flow cytometry was carried out to determine cellular DNA content. Cells (%) within the different cell-cycle phases are presented. F and G, the data are from a single experiment done twice with similar results.

TNBC compared with HER2 ($P = 2.2 \times 10^{-5}$), LA ($P = 8.1 \times 10^{-8}$), and LB ($P = 4.5 \times 10^{-8}$; Fig. 1B). These results were confirmed by immunohistochemistry showing a higher percentage of cells expressing PLK1 in TNBC compared with HER2 ($P = 0.016$), LA ($P = 2.5 \times 10^{-5}$), and LB ($P = 0.001$; Fig. 1C). PLK1 staining was observed both in the cytoplasm and the nucleus in TNBC samples, and not in healthy breast tissues (Fig. 1D). The higher expression of PLK1 in TNBC did not result from an increase in *PLK1* DNA copy number (Fig. 1E). PLK1 mRNA levels correlated positively with the proliferation marker Ki67 mRNA within the TNBC subgroup (Pearson correlation = 0.45; $P = 0.003$; Fig. 1F). PLK2 was found to be expressed at lower levels in TNBC compared with the other breast cancer subtypes (HER2: $P = 8.9 \times 10^{-9}$, LA: $P = 1.0 \times 10^{-11}$, LB: $P = 6.6 \times 10^{-7}$) and normal tissues ($P = 2.0 \times 10^{-4}$; Supplementary Fig. S1A), and this was associated with a loss of *PLK2* DNA copy number (Supplementary Fig. S1B). PLK3 gene expression was not significantly different between the different groups (Supplementary Fig. S1C and D). PLK4 presented a pattern of expression similar to that of PLK1, with higher expression in TNBC compared with HER2 ($P = 1.6 \times 10^{-5}$), LA ($P = 6.0 \times 10^{-6}$), LB ($P = 1.2 \times 10^{-3}$), and normal breast tissues ($P = 4.8 \times 10^{-3}$;

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Supplementary Fig. S1E and F). PLK5 was not detected in our study. In conclusion, transcriptomic data showed that TNBC overexpressed both PLK1 and PLK4, and underexpressed PLK2. These results suggest that PLK1 and PLK4 may represent attractive therapeutic targets for this subgroup of breast cancer. We then focused on PLK1 because several PLK1 inhibitors have been evaluated in clinical trials (8, 27, 34, 35).

The lowest tertile of PLK1 expression is associated with poor clinical outcome in triple-negative breast cancer

We investigated the relationship between PLK1 expression and prognosis within our TNBC subcohort. As there was no consensual prognostic threshold, patients with TNBC were initially divided into tertiles based on their PLK1 mRNA expression levels. As the second and third top tertiles were prognostically favorable (not shown), they were combined and considered as high for PLK1 expression (PLK1 >3.94) and compared with the low tertile (PLK1 $\leq$3.94). Being in the lowest tertile was significantly associated with a higher risk of developing metastasis (Fig. 1G) and disease (Fig. 1H) than being in the 2 highest tertiles.

RNAi-mediated PLK1 depletion impairs clonogenicity and viability in triple-negative cell lines

PLK1 expression was analyzed in a panel of triple-negative cell lines including the "normal" MCF10A cells (Fig. 2A). PLK1 silencing using 2 different RNAi significantly impaired HCC70 and MDA-MB-468 to form colonies in a soft agar assay (Fig. 2B), indicating that PLK1 was required for anchorage-independent growth. Proliferation assays upon RNAi-mediated PLK1 depletion were then conducted. MDA-MB-468 viability was impaired after RNAi transfection (Fig. 2C), and more pronounced effects were observed in MCF10A (Fig. 2D). PLK1-depletion inhibited

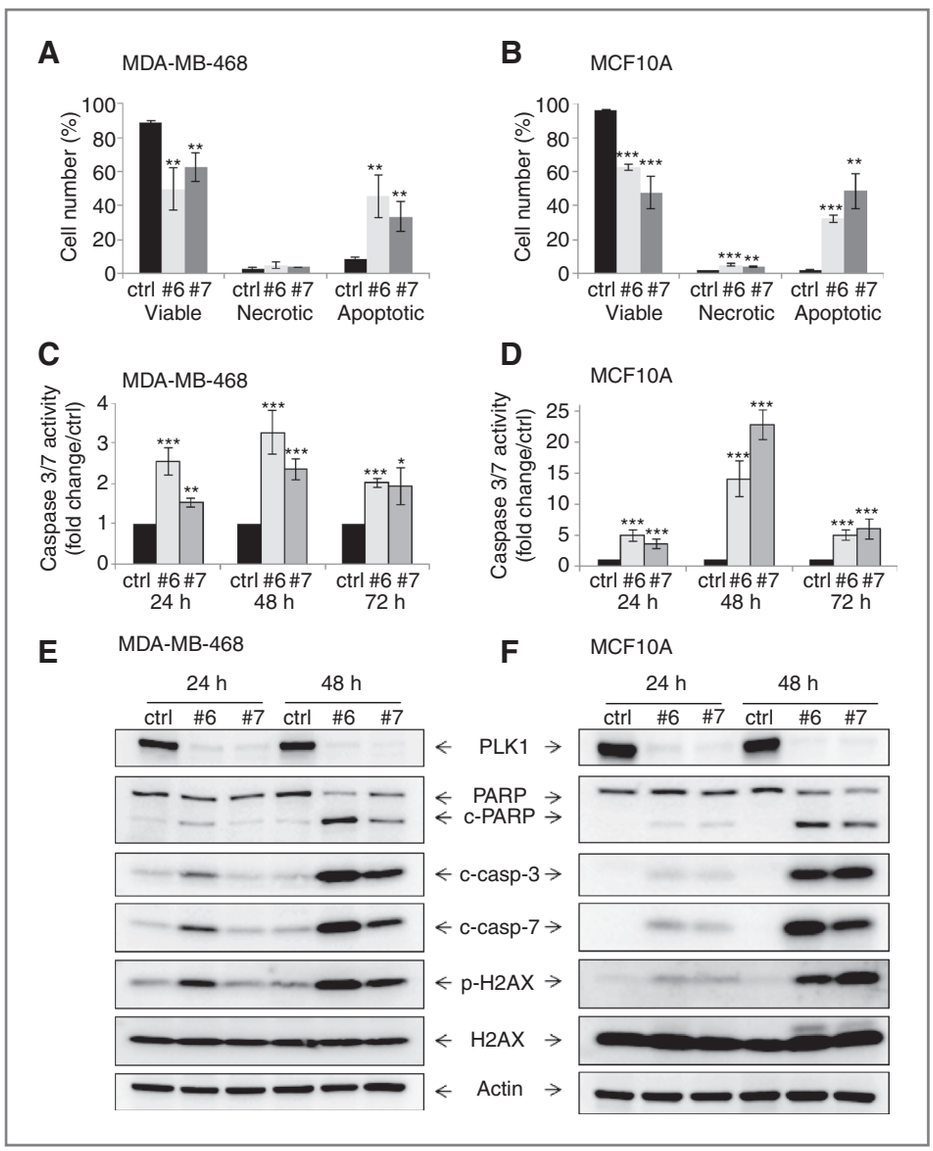

Figure 3. RNAi-mediated PLK1 depletion induces apoptosis in triple-negative cell lines. MDA-MB-468 (A, C, and E) and MCF10A (B, D, and F) were transfected with control (ctrl) and PLK1 RNAi (#6 and #7). A and B, Annexin V binding assay. Percentage of viable, necrotic, and apoptotic cells were measured 48-hour posttransfection. C and D, caspase 3/7 activity assay. E and F, Western blot analysis was conducted with antibodies recognizing H2AX, phosphorylated H2AX, and the cleaved form of PARP, caspase 3, and caspase 7. PLK1 depletion was verified using PLK1 antibodies. Actin was used as a loading control. A–D, data are mean $\pm$ SD from at least 3 independent experiments. E and F, results are from a single experiment, representative of 3 (MDA-MB-468) or 2 (MCF10A) different experiments.

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the proliferation of all tested TNBC cell lines (Fig. 2E). Our data with MCF10A were in contrast with those suggesting that these "normal" cells were less sensitive to PLK1 depletion (18, 19). However, MCF10A cells were the cells growing the fastest with a doubling time in 96-well plates as follows: MCF10A (16 hours), MDA-MB-231 (25 hours), MDA-MB-468 (40 hours), HCC1937 (40 hours), HCC70 (50 hours), and BT20 (60 hours; not shown). Therefore, this result is not surprising, considering the function of PLK1 in mitosis.

RNAi-mediated PLK1 depletion induces DNA breaks, G_2–M arrest, and apoptosis in triple-negative cell lines

We next analyzed by fluorescence-activated cell sorting (FACS) the effect of PLK1 depletion on cell-cycle progression. After RNAi transfection, MDA-MB-468 and MCF10A arrested in G_2–M, and the percentage of cells with sub-G_1 DNA content increased suggesting that PLK1-depleted cells undergo apoptosis (Fig. 2F and G). Annexin V staining experiments showed that PLK1 silencing induced apoptosis in MDA-MB-468 and MCF10A (30%–40%; Fig. 3A and B). Caspases 3/7 were activated in both cell lines after PLK1 RNAi transfection (Fig. 3C and D), and this was confirmed by Western blot analysis with the appearance of their cleaved forms, and the cleaved form of their substrate Poly(ADP-ribose)polymerase (PARP; Fig. 3E and F). PLK1 depletion induced H2AX phosphorylation, reflecting the presence of DNA breaks (Fig. 3E and F).

BI-2536–mediated PLK1 inhibition induces apoptosis and impairs viability in triple-negative cell lines

We next analyzed the effect of BI-2536, a PLK1 inhibitor, on the behavior of triple-negative cell lines. BI-2536 impaired cell viability in the nanomolar range (Table 1). In contrast to the results obtained after RNAi-mediated PLK1 depletion, MCF10A cells were less sensitive to PLK1 inhibition compared with the other cell lines. BI-2536 treatment of MDA-MB-468 and MCF10A induced apoptosis in a dose-dependent manner (Fig. 4A and B). Caspase 3/7 activity was detected at 10 nmol/L BI-2536 in both cell lines, and at 5 nmol/L only in MDA-MB-468 (Fig. 4A and B). Western blot analysis confirmed these results, with the detection of cleaved caspases 3, 7, and 8 and PARP after cell treatment with 10 nmol/L BI-2536 (Fig. 4C and D). Again, 5 nmol/L BI-2536 induced the cleavage of PARP and caspases only in MDA-MB-468 (Fig. 4C and D). Phosphorylated H2AX was induced by BI-2536 at 5 and 10 nmol/L in MDA-MB-468, but only at 10 nmol/L in MCF10A (Fig. 4C and D). Overall, these results indicated that BI-2536 induced apoptosis at lower concentrations in MDA-MB-468 compared with MCF10A.

Table 1. BI-2536 impairs triple-negative cell viability

	BI-2536 [IC_{50}] = nmol/L
MCF10A	7.3 ± 1.9
MDA-MB-231	3.5 ± 0.9
MDA-MB-468	2.7 ± 0.8
HCC70	2.0 ± 0.2
HCC1937	1.7 ± 1.0
BT20	1.4 ± 0.4

NOTE: Cell lines were treated with various BI-2536 concentrations and cell viability was measured using an MTT assay. Half maximal inhibitory concentrations (IC_{50}) were determined from dose–response curves.

BI-2536–mediated PLK1 inhibition specifically affects malignant cells in three-dimensional culture

BI-2536 was tested in triple-negative cell lines grown in a more physiologic context, in 3D culture in Matrigel (29). Under these conditions, nontransformed mammary epithelial cell lines, such as MCF10A, recapitulate epithelial morphogenesis by forming acinar structures within 10 days of culture, and then stop to grow, whereas cancer cells, such as MDA-MB-468, exhibit disorganized structures and continue to proliferate (36). BI-2536, added to these structures once formed, was effective on MDA-MB-468, and had no effect on MCF10A (Fig. 4E). This may be explained by the fact that MCF10A does not express PLK1 once the acini are formed (Fig. 4F).

BI-2536–mediated PLK1 inhibition impairs tumor growth in human triple-negative breast cancer xenograft models

To evaluate the antitumoral effects of PLK1 inhibition *in vivo*, BI-2536 was administrated in 2 breast cancer xenograft models established from patients with TNBC (30, 32). BI-2536, administrated alone, induced a dramatic tumor growth inhibition (TGI) in both models ($P < 0.0001$) with a TGI of 99% (Fig. 5A) and 85% (Fig. 5B). No body weight loss was observed (not shown). Three of 8 (37.5%) mice showed a complete response (CR) at day 24 in the HBCx-24 model (not shown). No CR was observed with the HBCx-10 model, and tumor relapsed after BI-2536 withdrawal (Fig. 5B and Table 2).

BI-2536 in combination with chemotherapy impairs tumor relapse in a human triple-negative breast cancer–derived xenograft model of tumor recurrence

We next addressed whether inhibition of PLK1 could impair tumor relapse after conventional chemotherapy, a major issue for patients with TNBC. We had the opportunity to test *in vivo* this hypothesis using the HBCx-10 model, which represents a model of chemotherapy-induced complete remission and tumor recurrence (31). A combination of DC, used for the management of TNBC patients, led first to CR; however, the tumor relapsed after stopping DC treatment (Fig. 5B, Table 2). The combination of DC with BI-2536 induced 100% CR, which were observed earlier than CR induced by DC treatment alone (Fig. 5B and Table 2). BI-2536 + DC combination impaired tumor relapse in contrast to DC alone (Table 2). At day 200, 4 mice were still tumor-free and could be considered as cured (Table 2). Altogether, these data suggested that, *in vivo*, PLK1 inhibition in combination with conventional chemotherapy is more efficient to achieve CR and, most importantly, impaired tumor relapse.

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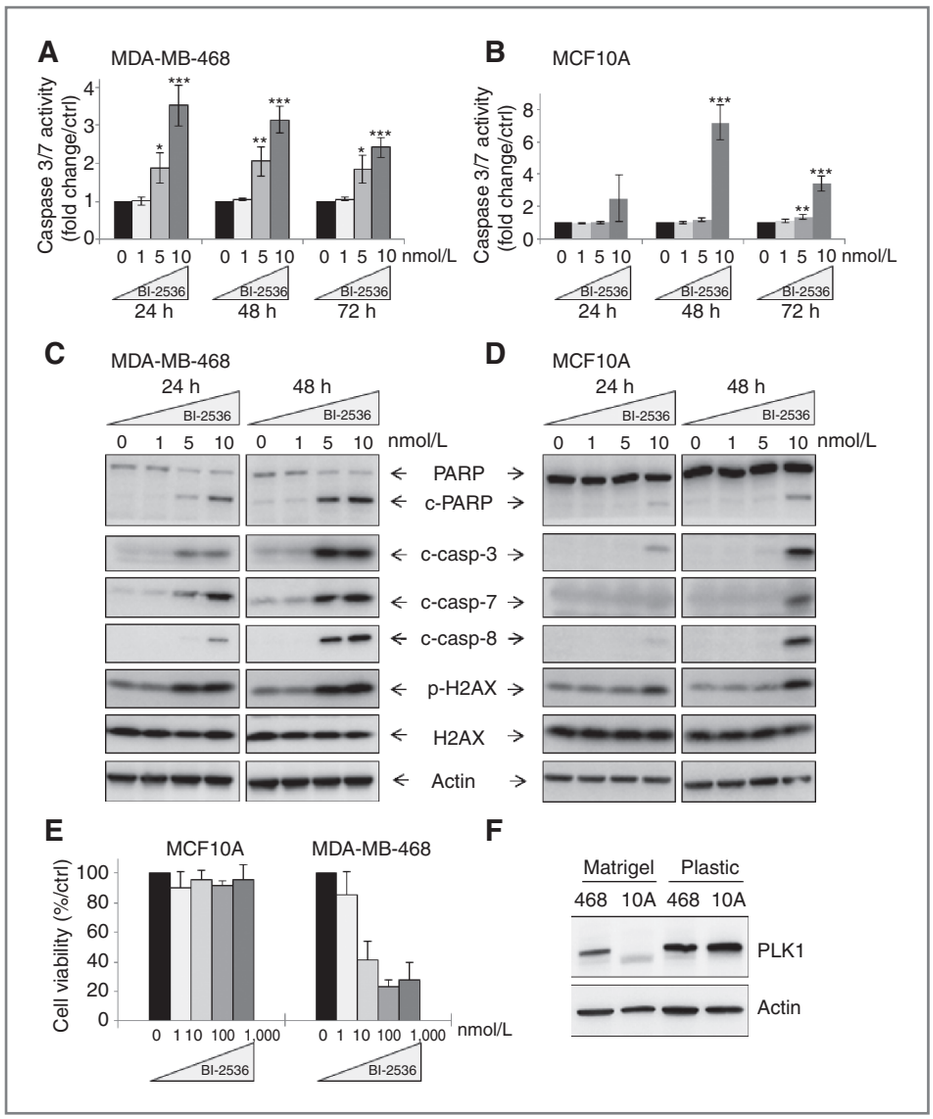

Figure 4. BI-2536–mediated PLK1 inhibition induces apoptosis in triple-negative cell lines, and specifically affects malignant cells in 3D culture. MDA-MB-468 (A and C) and MCF10A (B and D) were treated with dimethyl sulfoxide (DMSO) or various concentrations of BI-2536 (1, 5, and 10 nmol/L). Apoptosis was evaluated by caspase 3/7 assay (A and B) or Western blot analysis as in Fig. 3E and F (C and D). A and B, data are mean ± SD from at least 3 independent experiments. C and D, results are from a single experiment, representative of 3 different experiments (apart for 5 nmol/L BI-2536: experiment done twice). E and F, MDA-MB-468 cells were grown in Matrigel for 7 days to form grape-like disorganized structures and MCF10A for 10 days to form acini round 3D well-organized structures. Cells were then treated with various concentrations of BI-2536 (or DMSO). Three days later, cells were analyzed directly in plate (E) or collected for Western blot analysis (F). Cell viability was determined using a WST1 Cell Proliferation Assay Kit (E). Results are presented as % cell growth compared with DMSO-treated cells. The data are mean ± SD from 3 independent experiments Western blot analysis (F), PLK1 expression in MDA-MB-468 (468) and MCF10A (10A) cultured in 3D (Matrigel) or 2D (plastic). Actin was used as a loading control. Data shown are representative of 2 different experiments.

Table 2. *In vivo* efficacy of BI-2536 alone or combined with DC in HBCx-10

Treatment	CR, % (CR mice/total mice)		
Number of mice	**Day 33**	**Day 120**	**Day 200**
Control	0%	—	—
($n = 9$)	(0/9)		
BI-2536	0%	0%	—
($n = 9$)	(0/9)	(0/7)	
DC	29%	0%	—
($n = 7$)	(2/7)	(0/6)	
DC + BI-2536	100%	100%	100%
($n = 9$)	(9/9)	(5/5)	(4/4)

NOTE: For abbreviations and legend, see Fig. 5B.

Discussion

Treatment of patients with TNBC remains a major challenge for oncologists. Although they respond well to the current therapeutic strategies based on conventional chemotherapies, they represent a large proportion of breast cancer death because of a high recurrence rate. Alternative treatments are therefore needed to improve survival of these patients.

We found that both PLK1 and PLK4 are overexpressed in TNBC, and may represent potential therapeutic targets. We focused on PLK1 as small-molecule inhibitors have been evaluated in clinical trials. We confirmed previous analysis showing that PLK1 belongs to a list of 16 kinases overexpressed in TNBC compared with LA (14). We validated this TNBC-specific high PLK1 expression at a protein level using RPPA and immunohistochemistry techniques. High PLK1 protein expression has been reported in breast cancers (10, 13), specifically in TNBC (9), and shown to be associated with poor prognosis (9). We also found that high PLK1 expression is associated with

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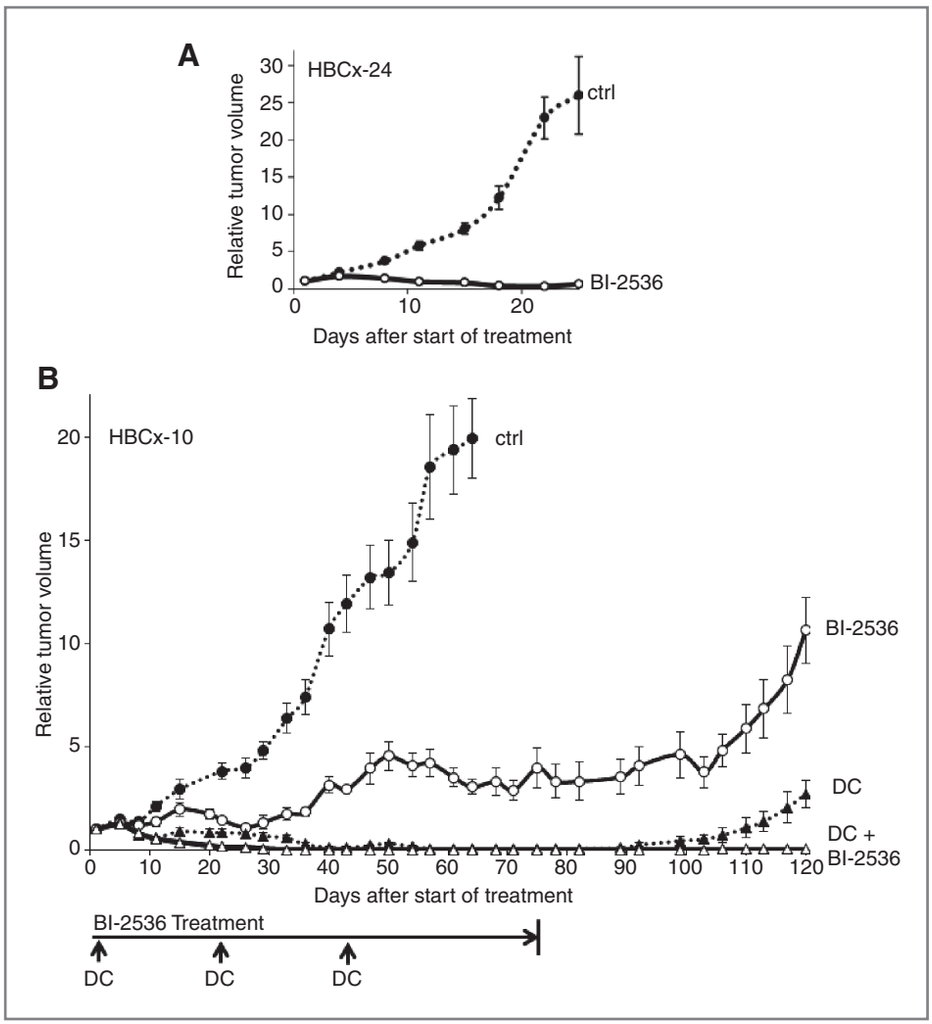

Figure 5. BI-2536 in combination with chemotherapy impairs tumor relapse in TNBC-derived xenograft model. A, BI-2536 (20 mg/kg) was injected intraperitoneally in a TNBC xenograft mice model (HBCx-24) twice a week until day 24 ($n = 8$ mice). Control group received drug-formulating vehicle only ($n = 8$ mice). B, BI-2536 was administrated intraperitoneally in another TNBC xenograft mice model (HBCx-10), alone at 20 mg/kg (BI-2536), or in combination with DC (2 mg/kg doxorubicin + 100 mg/kg cyclophosphamide; DC + BI-2536) until day 75, and stopped because of 4 unexpected and unexplained deaths in the combined group (absence of body-weight changes between groups, not shown). DC was administered at day 1, 22, and 43. Control mice were treated with BI-2536 vehicle. BI-2536 ($n = 9$ mice), DC ($n = 7$ mice), DC + BI-2536 ($n = 9$ mice), and control ($n = 9$ mice). Tumor volume was measured with calipers. Growth curves were obtained by plotting relative tumor volume mean versus time.

poor prognosis within the entire population; this could be simply reflecting the high expression of PLK1 in the poor prognosis-associated TNBC subtype. In fact, we found that, within the TNBC subtype, high PLK1 expression was associated with a better prognosis. This may be explained by the fact that low PLK1-expressing tumors respond worse to conventional chemotherapy, possibly due to their lowest proliferative rates.

PLK1 is the most investigated PLK family member and has been pointed out as an oncology target due to its overexpression in several tumors (12, 17). Therefore, many studies have analyzed the effects of PLK1 inhibition/depletion in various cancer cells (17). In agreement with those studies, we observed that PLK1 depletion/inhibition had similar effects in triple-negative cell lines: increase of DNA breaks, G_2–M arrest, and apoptosis as well as inhibition of cell viability and tumorigenicity. We found that RNAi-mediated PLK1 silencing and BI-2536-mediated PLK1 inhibition promoted similar cellular effects. Nevertheless, we noticed some differences. When compared with TNBC cell lines, the "normal" MCF10A were most sensitive to PLK1 silencing (RNAi) but less sensitive to PLK1 inhibition (BI-2536). BI-2536 inhibits the activity of all PLKs, suggesting that the inhibition of several PLKs, compared with a specific silencing of PLK1, could differentially affect cancer cells. On the other hand, knocking down the expression may have different consequences compared with the inhibition of the kinase activity. Inhibiting the activity of PLK1 may be more specific to cancer cells than knocking down its expression, which abolishes not only its kinase activity but also interactions with its cellular partners. PLK1 is essential for mitosis entry and it is not surprising that its inhibition/depletion impairs viability of cultured cells. However, it is interesting to point out that the consequences of PLK1 knockdown are not only related to the proliferative status of cells. Indeed, BT-20, the cancer cells of our panel with the lowest proliferative rate are the most sensitive to PLK1 inhibition and silencing. These cells may have some characteristics that render them more sensitive to PLK1 inhibition. The depletion of the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) induces an elevated expression of PLK1 in prostate cancer cells, and PTEN-null prostate cells are more sensitive to PLK1 inhibition compared with PTEN wt prostate cells (37). PTEN loss, which is a hallmark of TNBC (28, 38, 39), may therefore confer a higher sensitivity to PLK1 inhibition to

Published OnlineFirst November 9, 2012; DOI: 10.1158/0008-5472.CAN-12-2633

this subgroup of breast cancer; however, this feature is shared by triple-negative cell lines (28) and could not account for the high sensitivity of BT20 [which in fact express low PTEN levels compared with the other triple-negative cell lines with no detectable PTEN (28)] to PLK1 inhibition. PLK1 has been found to form a synthetic lethal pair with mutated Ras (40), but Ras is not mutated in human breast cancer and in the triple-negative cell lines we analyzed in this article, with the exception of MDA-MB-231 (41, 42).

Antitumoral effects of BI-2536 have been observed *in vivo* in xenograft models derived from various cell lines (25). In more relevant preclinical models established from human TNBC, we observed that BI-2536, administrated alone, impaired tumor growth. However, we noticed that the tumor relapsed after the treatment was stopped. We had the opportunity to evaluate the effect of BI-2536 on tumor recurrence, the major issue for patients with TNBC, by using a xenograft model characterized with high sensitivity to anthracycline-based chemotherapy followed by tumor relapse (31). In combination with DC used in clinic for the management of patients with TNBC, BI-2536 led to a faster CR compared with DC alone, and most importantly impaired relapse after the treatments were stopped. Although BI-2536 has been shown to be well tolerated (25), some mice died during the combination treatment for unknown reasons. However, no relapse was observed still 200 days after the beginning of the experiment for the surviving mice. Further experiments should be carried out to analyze whether impaired relapse could still be observed at lower doses of BI-2536, to avoid toxicity, in combination with DC. Our *in vivo* results suggest that the BI-2536 + DC association was capable to eradicate all the cancer cells. The tumor-initiating cells, which are resilient to chemotherapy and radiation, are thought to be responsible for tumor relapse. Recently, RNAi and small-molecule kinase inhibitors screens identified PLK1 as a protein kinase that targets the tumor-initiating cells from breast cancer (15) and neuroblastoma (43).

Because of the high sequence homology between the different PLK, PLK1 inhibitors are not specific to PLK1 (44). Therefore, this may be a concern as these kinases seem to have different expression patterns and nonoverlapping functions; the lack of selectivity of PLK1 inhibitors may result in undesired effects (8, 17). Indeed, PLK2 may act as a tumor suppressor *in vivo* (17), and we show that the *PLK2* gene is specifically lost in TNBC. The inhibitor used in our study, BI-2536, inhibits PLK1 (IC_{50} = 0.83 nmol/L), PLK2 (IC_{50} = 3.5 nmol/L), and PLK3 (IC_{50} = 9.0 nmol/L; 25). New compounds targeting specifically PLK1 within the PLK family may avoid the undesired effects of the nonselective PLK1 inhibitors.

Several PLK1 inhibitors have been evaluated in phase I/II clinical trials (26, 27, 34, 35). BI-2536 phase I trials revealed that the drug was well tolerated with minor antitumor responses (27, 45). Two phase II trials have shown that BI-2536 monotherapy had limited antitumor activity (46, 47). Only 14 patients with breast cancers were enrolled in one of the trials with no reported information regarding their molecular subtypes (46). Patients with TNBC have increased pathologic CR rates compared with non-TNBC, and those with pathologic complete response have excellent survival. However, patients with residual disease after neoadjuvant chemotherapy have significantly worse survival (6). Although clinical trials with a PLK1 inhibitor used as a single agent did not show high antitumor activity in different types of cancers, we suggest that PLK1 may represent a promising therapeutic target in combination with conventional chemotherapy, in particular for the treatment of patients with TNBC. Indeed, TNBC express high levels of PLK1, and the association of a PLK1 inhibitor with a conventional chemotherapy treatment impairs tumor relapse *in vivo* in a recurrence-prone TNBC xenograft model.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acknowledgments

The authors thank Martine Yann and Dr. Bernard Asselain for the clinical data; Dr. Marika Pla and colleagues for animal facilities support; Dr. Xavier Sastre-Garau, his colleagues and patients for the human breast tumor samples; Dr. Fabien Reyal for normal breast tissues; Benoit Albaud, Caroline Hego, and Cecile Reyes from the platform of Molecular Biology Facilities; and Amélie Brisson and Drs. Céline Baldeyron and Sylvie Maubant for critical reading of this manuscript.

Grant Support

This work was supported by Institut de Recherches Servier and Institut Curie. L.D. Koning is supported by a grant from Cancéropôle Ile de France.

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

Received July 12, 2012; revised October 1, 2012; accepted October 15, 2012; published OnlineFirst November 9, 2012.

References

1. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature 2000;406:747–52.

2. Metzger-Filho O, Tutt A, de Azambuja E, Saini KS, Viale G, Loi S, et al. Dissecting the heterogeneity of triple-negative breast cancer. J Clin Oncol 2012;30:1879–87.

Published OnlineFirst November 9, 2012; DOI: 10.1158/0008-5472.CAN-12-2633

3. Manie E, Vincent-Salomon A, Lehmann-Che J, Pierron G, Turpin E, Warcoin M, et al. High frequency of TP53 mutation in BRCA1 and sporadic basal-like carcinomas but not in BRCA1 luminal breast tumors. Cancer Res 2009;69:663–71.
4. Turner N, Tutt A, Ashworth A. Hallmarks of 'BRCAness' in sporadic cancers. Nat Rev Cancer 2004;4:814–9.
5. Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. N Engl J Med 2010;363:1938–48.
6. Liedtke C, Mazouni C, Hess KR, Andre F, Tordai A, Mejia JA, et al. Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. J Clin Oncol 2008;26:1275–81.
7. Pal SK, Childs BH, Pegram M. Triple negative breast cancer: unmet medical needs. Breast Cancer Res Treat 2011;125:627–36.
8. Lens SM, Voest EE, Medema RH. Shared and separate functions of polo-like kinases and aurora kinases in cancer. Nat Rev Cancer 2010;10:825–41.
9. King SI, Purdie CA, Bray SE, Quinlan PR, Jordan LB, Thompson AM, et al. Immunohistochemical detection of Polo-like kinase-1 (PLK1) in primary breast cancer is associated with TP53 mutation and poor clinical outcom. Breast Cancer Res 2012;14:R40.
10. Weichert W, Kristiansen G, Winzer KJ, Schmidt M, Gekeler V, Noske A, et al. Polo-like kinase isoforms in breast cancer: expression patterns and prognostic implications. Virchows Arch 2005;446:442–50.
11. Rizki A, Mott JD, Bissell MJ. Polo-like kinase 1 is involved in invasion through extracellular matrix. Cancer Res 2007;67:11106–10.
12. Strebhardt K, Ullrich A. Targeting polo-like kinase 1 for cancer therapy. Nat Rev Cancer 2006;6:321–30.
13. Wolf G, Hildenbrand R, Schwar C, Grobholz R, Kaufmann M, Stutte HJ, et al. Polo-like kinase: a novel marker of proliferation: correlation with estrogen-receptor expression in human breast cancer. Pathol Res Pract 2000;196:753–9.
14. Finetti P, Cervera N, Charafe-Jauffret E, Chabannon C, Charpin C, Chaffanet M, et al. Sixteen-kinase gene expression identifies luminal breast cancers with poor prognosis. Cancer Res 2008;68:767–76.
15. Hu K, Law JH, Fotovati A, Dunn SE. Small interfering RNA library screen identified polo-like kinase-1 (PLK1) as a potential therapeutic target for breast cancer that uniquely eliminates tumor-initiating cells. Breast Cancer Res 2012;14:R22.
16. Smith MR, Wilson ML, Hamanaka R, Chase D, Kung H, Longo DL, et al. Malignant transformation of mammalian cells initiated by constitutive expression of the polo-like kinase. Biochem Biophys Res Commun 1997;234:397–405.
17. Strebhardt K. Multifaceted polo-like kinases: drug targets and anti-targets for cancer therapy. Nat Rev Drug Discov 2010;9:643–60.
18. Liu X, Lei M, Erikson RL. Normal cells, but not cancer cells, survive severe Plk1 depletion. Mol Cell Biol 2006;26:2093–108.
19. Lansing TJ, McConnell RT, Duckett DR, Spehar GM, Knick VB, Hassler DF, et al. *In vitro* biological activity of a novel small-molecule inhibitor of polo-like kinase 1. Mol Cancer Ther 2007;6:450–9.
20. Raab M, Kappel S, Kramer A, Sanhaji M, Matthess Y, Kurunci-Csacsko E, et al. Toxicity modelling of Plk1-targeted therapies in genetically engineered mice and cultured primary mammalian cells. Nat Commun 2011;2:395.
21. Degenhardt Y, Greshock J, Laquerre S, Gilmartin AG, Jing J, Richter M, et al. Sensitivity of cancer cells to Plk1 inhibitor GSK461364A is associated with loss of p53 function and chromosome instability. Mol Cancer Ther 2010;9:2079–89.
22. Guan R, Tapang P, Leverson JD, Albert D, Giranda VL, Luo Y. Small interfering RNA-mediated Polo-like kinase 1 depletion preferentially reduces the survival of p53-defective, oncogenic transformed cells and inhibits tumor growth in animals. Cancer Res 2005;65:2698–704.
23. Sur S, Pagliarini R, Bunz F, Rago C, Diaz LA Jr, Kinzler KW, et al. A panel of isogenic human cancer cells suggests a therapeutic approach for cancers with inactivated p53. Proc Natl Acad Sci U S A 2009;106:3964–9.
24. Olmos D, Swanton C, de Bono J. Targeting polo-like kinase: learning too little too late? J Clin Oncol 2008;26:5497–9.
25. Steegmaier M, Hoffmann M, Baum A, Lenart P, Petronczki M, Krssak M, et al. BI 2536, a potent and selective inhibitor of polo-like kinase 1, inhibits tumor growth *in vivo*. Curr Biol 2007;17:316–22.
26. Frost A, Mross K, Steinbild S, Hedbom S, Unger C, Kaiser R, et al. Phase I study of the Plk1 inhibitor BI 2536 administered intravenously on three consecutive days in advanced solid tumours. Curr Oncol 2012;19:e28–35.
27. Mross K, Frost A, Steinbild S, Hedbom S, Rentschler J, Kaiser R, et al. Phase I dose escalation and pharmacokinetic study of BI 2536, a novel Polo-like kinase 1 inhibitor, in patients with advanced solid tumors. J Clin Oncol 2008;26:5511–7.
28. Marty B, Maire V, Gravier E, Rigaill G, Vincent-Salomon A, Kappler M, et al. Frequent PTEN genomic alterations and activated PI3K pathway in basal-like breast cancer. Breast Cancer Res 2008;10:R101.
29. Lee GY, Kenny PA, Lee EH, Bissell MJ. Three-dimensional culture models of normal and malignant breast epithelial cells. Nature Methods 2007;4:359–65.
30. Marangoni E, Vincent-Salomon A, Auger N, Degeorges A, Assayag F, de Cremoux P, et al. A new model of patient tumor-derived breast cancer xenografts for preclinical assays. Clin Cancer Res 2007;13:3989–98.
31. Marangoni E, Lecomte N, Durand L, de Pinieux G, Decaudin D, Chomienne C, et al. CD44 targeting reduces tumour growth and prevents post-chemotherapy relapse of human breast cancers xenografts. Br J Cancer 2009;100:918–22.
32. Reyal F, Guyader C, Decraene C, Lucchesi C, Auger N, Assayag F, et al. Molecular profiling of patient-derived breast cancer xenografts. Breast Cancer Res 2012;14:R11.
33. R Development Core Team (2011). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.
34. Olmos D, Barker D, Sharma R, Brunetto AT, Yap TA, Taegtmeyer AB, et al. Phase I study of GSK461364, a specific and competitive Polo-like kinase 1 inhibitor, in patients with advanced solid malignancies. Clin Cancer Res 2011;17:3420–30.
35. Jimeno A, Li J, Messersmith WA, Laheru D, Rudek MA, Maniar M, et al. Phase I study of ON 01910.Na, a novel modulator of the Polo-like kinase 1 pathway, in adult patients with solid tumors. J Clin Oncol 2008;26:5504–10.
36. Kenny PA, Lee GY, Myers CA, Neve RM, Semeiks JR, Spellman PT, et al. The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. Mol Oncol 2007;1:84–96.
37. Liu XS, Song B, Elzey BD, Ratliff TL, Konieczny SF, Cheng L, et al. Polo-like kinase 1 facilitates loss of PTEN tumor suppressor-induced prostate cancer formation. J Biol Chem 2011;286:35795–800.
38. Shah SP, Roth A, Goya R, Oloumi A, Ha G, Zhao Y, et al. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. Nature 2012;486:395–9.
39. Stemke-Hale K, Gonzalez-Angulo AM, Lluch A, Neve RM, Kuo WL, Davies M, et al. An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer. Cancer Res 2008;68:6084–91.
40. Luo J, Emanuele MJ, Li D, Creighton CJ, Schlabach MR, Westbrook TF, et al. A genome-wide RNAi screen identifies multiple synthetic lethal interactions with the Ras oncogene. Cell 2009;137:835–48.
41. Torbett NE, Luna A, Knight ZA, Houk A, Moasser M, Weiss W, et al. A chemical screen in diverse breast cancer cell lines reveals genetic enhancers and suppressors of sensitivity to PI3K isotype-selective inhibition. Biochem J 2008;415:97–110.
42. Hollestelle A, Elstrodt F, Nagel JH, Kallemeijn WW, Schutte M. Phosphatidylinositol-3-OH kinase or RAS pathway mutations in human breast cancer cell lines. Mol Cancer Res 2007;5:195–201.
43. Grinshtein N, Datti A, Fujitani M, Uehling D, Prakesch M, Isaac M, et al. Small molecule kinase inhibitor screen identifies polo-like kinase 1 as a target for neuroblastoma tumor-initiating cells. Cancer Res 2011;71:1385–95.
44. de Carcer G, Manning G, Malumbres M. From Plk1 to Plk5: functional evolution of polo-like kinases. Cell Cycle 2011;10:2255–62.
45. Hofheinz RD, Al-Batran SE, Hochhaus A, Jager E, Reichardt VL, Fritsch H, et al. An open-label, phase I study of the polo-like kinase-1 inhibitor,

Published OnlineFirst November 9, 2012; DOI: 10.1158/0008-5472.CAN-12-2633

BI 2536, in patients with advanced solid tumors. Clin Cancer Res 2010;16:4666–74.

46. Schoffski P, Blay JY, De Greve J, Brain E, Machiels JP, Soria JC, et al. Multicentric parallel phase II trial of the polo-like kinase 1 inhibitor BI 2536 in patients with advanced head and neck cancer, breast cancer, ovarian cancer, soft tissue sarcoma and melanoma. The first protocol of the European Organization for Research and Treatment of Cancer (EORTC) Network Of Core Institutes (NOCI). Eur J Cancer 2010;46: 2206–15.
47. Sebastian M, Reck M, Waller CF, Kortsik C, Frickhofen N, Schuler M, et al. The efficacy and safety of BI 2536, a novel Plk-1 inhibitor, in patients with stage IIIB/IV non–small cell lung cancer who had relapsed after, or failed, chemotherapy: results from an open-label, randomized phase II clinical trial. J Thorac Oncol 2010;5:1060–7.

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Polo-like Kinase 1: A Potential Therapeutic Option in Combination with Conventional Chemotherapy for the Management of Patients with Triple-Negative Breast Cancer

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Cancer Res 2013;73:813-823. Published OnlineFirst November 9, 2012.