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. In order to discover potential therapeutic targets for the poor prognosis-associated triple-negative breast cancer (TNBC), gene expression profiling was performed on a cohort of 130 breast cancer samples. Polo-like kinase 1 (PLK1) was found to be significantly over-expressed in TNBC compared to 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, G2/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 TNBC patient's biopsies. Most importantly, the administration of BI-2536, in combination with doxorubicin + cyclophosphamide chemotherapy, led to a faster complete response compared to 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.
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 human epithelial growth factor receptor-2 (HER2) overexpression (2). TNBC account for 15% of breast cancers and are associated with African American ethnicity, younger age and poorer outcome when compared to the other breast cancer subtypes: luminal A (LA), luminal B (LB), and HER2+/ER- (HER2) (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 III 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 does 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 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...
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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 to normal cells (18-20) and p53-defective cancer cells compared to 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 performed RNA microarrays on a cohort composed of tumor samples, healthy breast tissues and triple-negative cell lines. PLK1 was overexpressed in TNBC compared to 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 performed 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. IHC with PLK1 antibodies (Cell Signaling Technology) was performed 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 contralateral recurrence. DNA (Affymetrix SNP6.0), RNA (Affymetrix U133 plus 2.0) and RPPA microarrays were performed 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 nM 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 Caspase-Glo® 3/7 luminescent assay (Promega) or by Western-blotting 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 4 weeks. Colonies were visualized after MTT staining. Three-dimensional cell culture was performed 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 two variables. For cellular assays and in vivo experiments, p values were calculated using the Student’s t test.

Additional experimental details are available in the Supplemental material.
Results

PLK1 is overexpressed in triple-negative breast cancer

In order to discover potential therapeutic targets for the poor prognosis associated TNBC, a gene expression profiling was performed on a cohort of 130 breast cancers. PLK1 was expressed at higher levels in TNBC (mean log2=4.36) compared to HER2 (mean log2=2.6) (p=2.8×10^{-10}), LA (mean log2=1.56) (p<2×10^{-16}), LB (mean log2=2.16) (p=7.6×10^{-13}) and normal tissues (mean log2=1.97) (p=1.1×10^{-9}) (Fig. 1A). PLK1 protein levels, measured by reverse phase protein array, were higher in TNBC compared to HER2 (p=2.2×10^{-5}), LA (p=8.1×10^{-8}) and LB (p=4.5×10^{-8}) (Fig. 1B). These results were confirmed by IHC showing a higher percentage of cells expressing PLK1 in TNBC compared to HER2 (p=0.016), LA (p=2.5×10^{-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 to the other breast cancer subtypes (HER2: p=8.9×10^{-9}, LA: p=1.0×10^{-11}, LB: p=6.6×10^{-7}) and normal tissues (p=2.0×10^{-4}) (Fig. S1A), and this was associated with a loss of PLK2 DNA copy number (Fig. S1B). PLK3 gene expression was not significantly different between the different groups (Fig. S1C-D). PLK4 presented a pattern of expression similar to that of PLK1, with higher expression in TNBC compared to HER2 (p=1.6×10^{-5}), LA (p=6.0×10^{-6}), LB (p=1.2×10^{-3}) and normal breast tissues (p=4.8×10^{-3}) (Fig. S1E-F). PLK5 was not detected in our study. In conclusion, transcriptomic data showed that TNBC over-expressed both PLK1 and PLK4, and under-expressed 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, TNBC patients 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 to 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 two 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 performed. MDA-MB-468 viability was impaired after RNAi transfection (Fig. 2C), and more pronounced effects were observed in MCF10A (Fig. 2D). PLK1-depletion inhibited 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 follow: MCF10A (16h), MDA-MB-231
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(25h), MDA-MB-468 (40h), HCC1937 (40h), HCC70 (50h), BT20 (60h) (not shown). Therefore, this result is not surprising, considering the function of PLK1 in mitosis.

**RNAi-mediated PLK1 depletion induces DNA breaks, G2-M arrest and apoptosis in triple-negative cell lines**

We next analyzed by FACS the effect of PLK1 depletion on cell-cycle progression. After RNAi transfection, MDA-MB-468 and MCF10A arrested in G2-M, and the percentage of cells with sub-G1 DNA content increased suggesting that PLK1-depleted cells undergo apoptosis (Fig. 2F-G). Annexin V staining experiments showed that PLK1 silencing induced apoptosis in MDA-MB-468 and MCF10A (30-40%) (Fig. 3A,B). Caspases 3/7 were activated in both cell lines after PLK1 RNAi transfection (Fig. 3C,D), and this was confirmed by Western blotting with the appearance of their cleaved forms, and the cleaved form of their substrate Poly(ADP-ribose)polymerase (PARP) (Fig. 3E,F). PLK1 depletion induced H2AX phosphorylation, reflecting the presence of DNA breaks (Fig. 3E,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 were less sensitive to PLK1 inhibition compared to the other cell lines. BI-2536 treatment of MDA-MB-468 and MCF10A induced apoptosis in a dose dependent manner (Fig. 4A,B). Caspase 3/7 activity was detected at 10 nM BI-2536 in both cell lines, and at 5 nM only in MDA-MB-468 (Fig. 4A,B). Western-blotting confirmed these results, with the detection of cleaved caspases 3, 7, 8 and PARP after cell treatment with 10 nM BI-2536 (Fig. 4C,D). Again, 5 nM BI-2536 induced the
cleavage of PARP and caspases only in MDA-MB-468 (Fig. 4C,D). Phosphorylated H2AX was induced by BI-2536 at 5 nM and 10 nM in MDA-MB-468, but only at 10 nM in MCF10A (Fig. 4C,D). Overall, these results indicated that BI-2536 induced apoptosis at lower concentrations in MDA-MB-468 compared to MCF10A.

**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 physiological context, in three-dimensional (3D) culture in Matrigel (29). Under these conditions, non-transformed 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 anti-tumoral 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 out 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, 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 doxorubicin + cyclophosphamide (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, 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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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 due to 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 to LA (14). We validated this TNBC-specific high PLK1 expression at a protein level using RPPA and IHC 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 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, G2-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 to TNBC cell lines, the “normal” MCF10A were the most sensitive to PLK1 silencing (RNAi) but the less sensitive to PLK1 inhibition (BI-2536). BI-2536 inhibits the activity of all PLKs, suggesting that the inhibition of several PLKs, compared to a specific silencing of PLK1, could differentially affect cancer cells. On the other hand, knocking-down the expression may have different consequences compared to 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 to 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 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 to 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 paper, with the exception of MDA-MB-231 (41, 42).
Anti-tumoral 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 to 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).

Due to the high sequence homology between the different polo-like kinases, PLK1 inhibitors are not specific to PLK1 (44). Therefore, this may be a concern since these kinases appear to have different expression patterns and non-overlapping 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 (IC₅₀=0.83nM), PLK2 (IC₅₀=3.5nM) and
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PLK3 (IC$_{50}$=9.0nM) (25). New compounds targeting specifically PLK1 within the PLK family may avoid the undesired effects of the non-selective 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 the trial 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.
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References


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

<table>
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<tr>
<th>Cell Line</th>
<th>[IC₅₀] (nM)</th>
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<tr>
<td>MCF10A</td>
<td>7.3 ± 1.9</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>3.5 ± 0.9</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>2.7 ± 0.8</td>
</tr>
<tr>
<td>HCC70</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>HCC1937</td>
<td>1.7 ± 1.0</td>
</tr>
<tr>
<td>BT20</td>
<td>1.4 ± 0.4</td>
</tr>
</tbody>
</table>

Cell lines were treated with various BI-2536 concentrations and cell viability was measured using an MTT assay. Half maximal inhibitory concentrations (IC₅₀) were determined from dose-responses curves.
Table 2. **In vivo** efficacy of BI-2536 alone or combined with DC in HBCx-10

<table>
<thead>
<tr>
<th>Treatment</th>
<th>complete response (%)</th>
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<th>Day 120</th>
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<tr>
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<td>(number of mice)</td>
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<tr>
<td>control (n=9)</td>
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<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>BI-2536 (n=9)</td>
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<tr>
<td>DC + BI-2536 (n=9)</td>
<td>100% (9/9)</td>
<td>100% (5/5)</td>
<td>100% (4/4)</td>
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Abbreviations and legend: see Fig.4B
Figure 1. **PLK1 is overexpressed in triple-negative breast cancer**

PLK1 expression was measured in TNBC, HER2, LA, LB and healthy breast tissues. (A) mRNA PLK1 levels were determined by microarray analysis. (B-C) PLK1 protein level was evaluated by reverse phase protein array (B) and IHC (C). Protein and mRNA relative quantifications were logarithmic transformed and illustrated by box plots. (C-D) PLK1 expression was analyzed on TMA. 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 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. (G-H) 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 (hazard ratio=4.05, 95% confidence intervals=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).

Figure 2. **PLK1 depletion impairs triple-negative cell viability and affects cell cycle**

(A) PLK1 expression was analyzed by Western-blotting in BT20 (1), HCC38 (2), HCC1937 (3), HCC70 (4), MDA-MB-468 (5), MDA-MB-231 (6), 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) (B) MDA-MB-468 and HCC70 were grown on soft agar medium for 4 weeks.
Colonies were stained and counted. (C-E) Cell viability was assessed by MTT assay 24h, 48h and 72h after transfection (C,D) or only 72h post-transfection (E). (B-E) Data are mean ± Standard Deviation (SD) from at least 3 independent experiments. P values are indicated (* p<0.05, ** p<0.01, *** p<0.001). (F-G) Cell cycle analysis. MDA-MB-468 (F) and MCF10A (G) were incubated with propidium iodide 24 or 48h post-transfection. Flow cytometry was carried out to determine cellular DNA content. Cells (%) within the different cell-cycle phases are presented. (F-G) The data are from a single experiment done twice with similar results.

**Figure 3. RNAi-mediated PLK1 depletion induces apoptosis in triple-negative cell lines**

MDA-MB-468 (A,C,E) and MCF10A (B,D,F) were transfected with control (ctrl) and PLK1 RNAi (#6 and #7). (A,B) Annexin V binding assay. Percentage of viable, necrotic and apoptotic cells were measured 48h post-transfection. (C,D) Caspase 3/7 activity assay. (E,F) Western blotting performed 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 ± SD from at least 3 independent experiments. (E-F) Results are from a single experiment, representative of 3 (MDA-MB-468) or 2 (MCF10A) different experiments.

**Figure 4. BI-2536 mediated PLK1 inhibition induces apoptosis in triple-negative cell lines, and specifically affects malignant cells in three-dimensional culture**

MDA-MB-468 (A,C) and MCF10A (B,D) were treated with DMSO or various concentrations of BI-2536 (1, 5, 10 nM). Apoptosis was evaluated by caspase 3/7 assay (A-B) or Western-blotting as in Figure 3E-F (C-D). (A-B) Data are mean ± SD from at least 3 independent experiments. (C-D) Results are from a single experiment, representative of 3 different experiments (apart for 5nM BI-2536: experiment done twice). (E-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-blotting (F). (E) Cell viability was determined using a WST1 Cell Proliferation Assay Kit. Results are presented as % cell growth compared to DMSO-treated cells. The data are mean ± SD from 3 independent experiments (F) Western-blotting: 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.

Figure 5. BI-2536 in combination with chemotherapy impairs tumor relapse in triple-negative breast cancer 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, due to 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, 43. Control mice were treated with BI-2536 vehicle. BI-2536 (n=9 mice), DC (n=7 mice), DC + BI-2536 (n=9 mice), control (n=9 mice). Tumor volume was measured with calipers. Growth curves were obtained by plotting relative tumor volume mean vs time.
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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