Dasatinib sensitises triple negative breast cancer cells to chemotherapy by targeting breast cancer stem cells

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BACKGROUND: Patients with triple negative breast cancer (TNBC) exhibit poor prognosis and are at high risk of tumour relapse, due to the resistance to chemotherapy. These aggressive phenotypes are in part attributed to the presence of breast cancer stem cells (BCSCs). Therefore, targeting BCSCs is a priority to overcoming chemotherapy failure in TNBCs.

METHODS: We generated paclitaxel (pac)-resistant TNBC cells which displayed higher sphere forming potential and percentage of BCSC subpopulations compared to the parental cells. A screen with various kinase inhibitors revealed dasatinib, a Src kinase family inhibitor, as a potent suppressor of BCSC expansion/sphere formation in pac-resistant TNBC cells.

RESULTS: We found dasatinib to block pac-induced BCSC enrichment and Src activation in both parental and pac-resistant TNBC cells. Interestingly, dasatinib induced an epithelial differentiation of the pac-resistant mesenchymal cells, resulting in their enhanced sensitivity to paclitaxel. The combination treatment of dasatinib and paclitaxel not only decreased the BCSCs numbers and their sphere forming capacity but also synergistically reduced cell viability of pac-resistant cells. Preclinical models of breast cancer further demonstrated the efficiency of the dasatinib/paclitaxel combination treatment in inhibiting tumour growth.

CONCLUSIONS: Dasatinib is a promising anti-BCSC drug that could be used in combination with paclitaxel to overcome chemoresistance in TNBC.
decreased percentage of ALDH+ BCSCs in basal-b subtype of breast cancer.\textsuperscript{18} As BCSCs are the driving cells for chemotherapy resistance, we evaluated the role of dasatinib in targeting BCSCs and its ability to overcome chemotherapy resistance. We found that pac-resistant cells displayed higher BCSC sphere forming capacity and higher active Src kinase level compared to parental cells and showed that dasatinib treatment completely abolished these effects. We also found dasatinib to block pac-induced Src activation as well as BCSC sphere formation capacity and expansion in parental TNBC cells. Moreover, we demonstrated that the combination of dasatinib and paclitaxel synergistically reduced cell viability of pac-resistant cells in vitro and significantly inhibited breast tumour growth in vivo, suggesting the dasatinib/ paclitaxel combination treatment as a potential therapy for overcoming chemotherapy resistance in TNBC patients.

**MATERIALS AND METHODS**

All experimental protocols and procedures were performed in accordance to McGill University regulations. All experimental protocols and procedures were approved by McGill University.

**Drugs**

LY294002, SB431542, Gefitinib, Dasatinib, Rapamycin and palbociclib were purchased from Sigma-Aldrich. JNK inhibitor II and PD98059 were bought from Calbiochem.

**Cell culture**

SUM159 cells were grown in F-12 HAM’S serum, with 5% foetal bovine serum (FBS), 5 μg/ml insulin and 1 μg/ml hydrocortisone. Paclitaxel-resistant SUM159 cells were generated by exposure to six to eight cycles of treatments with 10 nM paclitaxel.

**Cell viability assay**

Cells were seeded into 96-well plates (5,000 cells/well) and treated with a dose range of drugs. After 48 h, cells were incubated with PrestoBlue\textsuperscript{TM} Cell Viability Reagent (Thermo Fisher Scientific) for 1 h at 37 °C. Plates were then subjected to fluorescence reading (excitation: 535 nm, emission: 615 nm) using a microplate reader. IC50 drug values were determined using GraphPad Prism 6.0 (GraphPadSoftware, Inc., San Diego, CA) and the combination index (CI) was determined with the CompuSyn 1.0 software.

**Tumoursphere assay**

Cells were seeded in ultra-low-attachment 24-well plate (10,000/ well) and cultured in serum-free HAM’S F12 medium supplemented with 10 ng/ml EGF, 10 ng/ml bFGF and 1×B27 (Invitrogen). For treatment, each inhibitor was added at IC50 value. After 7 days, numbers of tumourspheres (> 60 μm diameter) were counted using ImageJ software and sphere-forming efficiency (SFE) was calculated using the following equation: SFE (%) = (no. of spheres)/(no. of cells plated) × 100%.

**Flow cytometry**

Monolayer cells were dissociated with trypsin and blocked in prechilled 2% FBS in PBS for 30 min at 4 °C. Cells were filtered through 70 μm cell strainer and counted. For each sample, 250,000 cells were co-stained with PE-conjugated anti-CD24 and APC-conjugated anti-CD44 or stained with APC-conjugated anti-MUC1 antibodies (BD Bioscience, San Diego, USA) for 30 min on ice. As negative and positive controls, we used non-stained and single-stained cells, respectively. Cells were analysed using Accuri C6 flow cytometer (BD Biosciences) and FlowJo software (Tree Star Inc.).

ALDEFLUOR\textsuperscript{TM} Kit (Stemcell Technologies) was used to analyse Aldehyde dehydrogenase (ALDH) enzyme activity, according to the manufacturer’s protocol. A total of 10\textsuperscript{6} cells were incubated with 5 μl ALDH substrate (Bodipy-aminoacetaldehyde) for 45 min at 37 °C. For the negative control, an aliquot of each sample was incubated with diethylaminobenzaldehyde (DEAB). Analysis were performed using Accuri C6 flow cytometer.

**Real-time PCR**

Real-time PCR analysis of CDH2, FN1, SNAI1, ZEB1, TP63, ACTA2 and GAPDH were performed using SsoFastTM EvaGreen\textsuperscript{®} Supermix (Bio-Rad) as described previously.\textsuperscript{15} Primer sequences of CDH2, FN1, SNAI1, TP63, MUC1, ZEB1, ACTA2 and GAPDH were the same as previously used.\textsuperscript{19,20}

**Western blot**

Western blot assay was performed as previously described.\textsuperscript{19,20}

**3D Matrigel cell culture**

A total of 4,000 SUM159-P cells in 100 μL growth medium were seeded into poly-D-lysine coated 8-well culture slides coated with 100 μL growth factor reduced Matrigel\textsuperscript{®} (BD Biosciences). Cells were maintained in growth medium with 5% Matrigel\textsuperscript{®} for 48 h for colonies outgrowth. The morphology of the colonies was evaluated after 72 h, following dasatinib treatment. Formed colonies were fixed with 4% paraformaldehyde, permeabilised using 0.3% Triton and stained with Alexa Fluor\textsuperscript{®} 488 anti-F-actin antibody (Invitrogen), overnight at 4 °C, followed by 4’,6-diamidino-2-phenylindole (DAPI) staining. Slides were mounted with SlowFade\textsuperscript{®} Gold antifade reagent. Confocal analysis was conducted using a Zeiss LSM 510 Meta Axiover confocal microscope with 63× objective.

**In vivo studies**

All animal experiments were conducted in accordance with protocols approved by the McGill University Health Center. 1 × 10\textsuperscript{6} SUM159 and SUM159-P cells were implanted in the mammary gland of NOD SCID IL2gammar\textsuperscript{-} knockout (NSG) mice. One week after, mice transplanted with SUM159 cells were divided into two groups (6 mice/group) and treated with (i) vehicle (20% cremophor + 20% ethanol + 60% dextrose, 10% w/v), (ii) paclitaxel (20 mg/kg, once a week). Mice transplanted with SUM159-P cells were randomised into four groups (6 mice/group) and treated with (i) vehicle (20% cremophor + 20% ethanol + 60% dextrose, 10% w/v), (ii) paclitaxel (20 mg/kg, once a week), (iii) dasatinib (10 mg/kg, daily), (iv) paclitaxel + dasatinib. Tumour volume were measured using a caliper and mice were weighted every 3 days and determined according to the formula: (L^2*W)/2. Upon tumours reaching 700–1100 mm\textsuperscript{3} in volume, mice were sacrificed and tumours collected for further analyses.

**Dissociation of breast tumour xenograft**

1 × 10\textsuperscript{6} SUM159 and SUM159-P cells were transplanted in the contralateral mammary glands of NSG mice model. Upon reaching 800 mm\textsuperscript{3} in size, primary mammary tumours were collected, cross-cut into small pieces and minced using scalps. Each tumour tissue was digested using 5 ml collagenase (nine parts of DMEM/ F12 medium mixed with one part of 10X collagenase/hyaluronidase) and incubated at 37 °C for 2 h. Cells were centrifuged and incubated with 1 ml Trypsin/EDTA for 5 min. Digestion was stopped by adding 10 ml 2% FBS/HBSS. Cells were filtered through 40 μM cell strainers, centrifuged, washed in 2% FBS/ HBSS, counted and subjected to flow cytometry analysis.

**Immunohistochemistry staining and scoring**

Primary tumours were fixed in 10% formalin, embedded and sectioned. Lab Vision\textsuperscript{TM} UltraVision\textsuperscript{™} LP Detection kit (Thermo Fisher Scientific) was used to perform immunohistochemistry staining. Tumour slides were deparaffinised and rehydrated through a descending series of alcohol then washed four times with buffer. Antigen retrieval was induced through incubation in 10 mM citrate buffer (pH 6.0) at 90 °C for 20 min. To reduce nonspecific background, slides were incubated in hydrogen...
peroxide Block for 10 min and Ultra V Block for another 5 min. Slides were washed and stained with rabbit polyclonal anti-CD44 antibody (Abcam) at room temperature for 1 h. Slides were incubated in HRP Polymer at room temperature for another 15 min. A mixture of DAB Plus Chromogen and DAB Plus Substrate was applied for detection. Slides were counterstained with hematoxylin and covered using mounting medium.

Mean percentage of IHC staining based on a visual semi-quantitative scoring was performed by two pathologists. Membranous and cytoplasmic staining patterns were observed in the viable tumour cells; however, the average percentage of membranous staining was higher than cytoplasmic one. Necrotic and apoptotic parts were excluded. Staining intensity of both the membranes and cytoplasm was assessed separately according to a graded scoring system of 1 (faint to weak, at ×40 magnification), 2 (weak to moderate at ×40), 3 (moderate at ×20) and 4 (strong at ×4). The total percentage of both membranous and cytoplasmic staining was used for the assessment of CD44 protein expression in tumour cells.

Statistical analyses
Student’s t-test or one-way ANOVA was used to evaluate significance between groups. At least three independent experiments were performed and P < 0.05 was considered significant.

RESULTS
Paclitaxel resistance increases the breast cancer stem cell content
SUM159PT (hereafter referred to as SUM159), a metastatic human breast cancer cell line derived from a patient with anaplastic breast carcinoma was used in our study, as this cell line is initially sensitive to paclitaxel and has been shown to contain functional cancer stem cell populations.\(^1,2\) We generated paclitaxel-resistant SUM159 cells (SUM159-P) from parental SUM159, using 6–8 cycles of paclitaxel (10 nM) treatment. Each cycle consisted of two days’ drug treatment and two days’ recovery by culturing cells in drug-free media. Cytotoxic effects of paclitaxel on SUM159 and SUM159-P cells were compared by performing PrestoBlue cell viability assay. As shown in Fig. 1a, SUM159 cells are sensitive to paclitaxel with an IC50 value of 3 nM, whereas SUM159-P cells are highly resistant to paclitaxel with an IC50 value of 50 nM (17-fold higher than the parental cells). No change was observed in cell morphology between the parental and paclitaxel-resistant cells (Fig. 1b).

Recent studies indicated that chemotherapy-treated breast cancer patients displayed increased percentage of BCSCs.\(^2\) We analysed breast cancer tissue samples from Kordje dataset of Oncomine database (www.oncomine.com) and found that paclitaxel-related taxane (docetaxel) treatment of breast cancer led to an increase in stemness/differentiation markers (ALDH1A3 and CD44) (Fig. 1c), while expression of luminal differentiation markers (MUC1 and EpCAM) were decreased (Fig. 1d). We then investigated whether chemotherapy resistance was associated with an increase in BCSCs in SUM159-P cells, using in vitro tumoursphere formation assay, a standard method for assessing CSC numbers. This assay measures the capacity of cells to form three-dimensional spheres in suspension cultures and reflects their ability to self-renew.\(^3\) As shown in Fig. 1e and f, SUM159-P cells displayed higher sphere forming potential as reflected by the increased SFE compared to the parental SUM159 cells. We then measured ALDH activity and stem cell markers CD24 and CD44 levels in SUM159 and SUM159-P cells. We found SUM159-P cells to exhibit significantly higher percentage of ALDH+ (10.3%) and CD24low/CD44high (33.9%) BCSCs compared to SUM159 cells (7.5% and 24.0%, respectively; Fig. 1g–j). Moreover, we compared the IC50 value for paclitaxel in both CD24low/CD44high BCSCs and CD24+CD44+ non-BCSCs isolated from SUM159 cells and found that the BCSC population is more resistant to paclitaxel treatment with a higher value of IC50 compared to non-BCSCs (Fig. 1i). Together, these results indicate that chemotherapy resistance of SUM159-P cells is associated with higher amount of BCSCs and increased sphere forming ability.

To further extend these findings in vivo, we performed xenograft fat pad transplantation of SUM159 and SUM159-P cells in the contralateral mammary glands of NSG mice.\(^25\) Upon reaching 800 mm\(^3\) in size, primary mammary tumours were harvested, dissociated into single cells, and assessed for CD24low/CD44high BCSC population, using flow cytometry. The BCSC population was markedly increased in SUM159-P derived tumours (14.8%) compared with the parental SUM159 tumours (5.6%) (Fig. 1k, l), indicating that paclitaxel-resistant breast cancer cells exhibit enhanced stemness properties, both in vitro and in vivo.

Dasatinib potently eliminates pac-resistant BCSCs
To determine whether paclitaxel-resistant TNBCs are therapeutically treatable, we performed a drug screen with a library of kinase-targeting agents (Table 1). All selected targets are activated in TNBCs and critical for regulating the tumorigenesis processes, thus representing potential therapeutic targets for TNBC.\(^26\) Importantly, these kinases have been shown to promote cancer stemness in various models of solid tumours.\(^15,19,20,31–34\) IC50 doses were determined by cell viability assay in bulk SUM159-P cells (Fig. S2A). We then tested these inhibitors for their ability to modulate BCSC sphere forming capacity, using tumoursphere formation assays. As shown in Fig. 2a, three inhibitors (SB431542, JNK inhibitor II, PD98059) showed moderate effects in decreasing the number of tumourspheres in SUM159-P cells (~40% reduction); while another four compounds (LY294002, Gefitinib, Rapamycin, Palbociclib) showed stronger effects (50–80% reduction). Interestingly, dasatinib reduced tumoursphere-forming efficiency by more than 90% and inhibited BCSC sphere forming capacity to a greater degree than any other inhibitors. We next assessed the effects of the different inhibitors on the BCSC CD24low/CD44high sub-population, using flow cytometry. As shown in Fig. 2b and Fig. 2b, LY294002, SB431542 and Rapamycin had mild to moderate effects on eliminating CD24low/CD44high BCSC sub-population (~35% reduction), while treatment with Gefitinib, PD98059 and palbociclib showed stronger effects and decreased the proportion of this BCSC subpopulation by 52%, 53 and 68%, respectively. Notably, the percentage of CD24low/CD44low sub-population was reduced by 95 and 87% after treatment with dasatinib and JNK inhibitor II, respectively. Altogether, dasatinib was most efficiently inhibiting BCSC sphere formation and significantly reduced the CD24low/CD44low BCSC subpopulation. To further confirm the effects of dasatinib on BCSC properties, we assessed its effects on another BCSC sub-population, namely ALDH+ cells. As shown in Fig. 2c, dasatinib treatment resulted in a strong and significant threefold decrease in the proportion of ALDH+ cells, further demonstrating the efficacy of dasatinib in reducing BCSC populations in chemotherapy-resistant breast cancer cells.

Dasatinib suppresses BCSCs through inhibition of paclitaxel-induced Src kinase activation
Having shown that dasatinib drastically inhibited BCSC sphere forming capacity and expansion in pac-resistant cells, we next investigated the underlying mechanisms by examining the phosphorylation status of Src kinase, the main target for dasatinib. Western blot analysis showed that SUM159-P cells exhibit higher amount of BCSCs and increased sphere forming ability.
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Fig. 1 Paclitaxel resistance is related to stem-like properties. a Cell viability inhibition by different doses of paclitaxel in SUM159 and paclitaxel-resistant SUM159 cells (SUM159-P). The IC50 values of paclitaxel after 48 h of treatment were determined in both cell lines. b Phase-contrast microscopic images showed cell morphology of SUM159 and SUM159-P cells. c, d mRNA expression levels of ALDH1A3, CD44, MUC1 and EPCAM in breast cancer patients from the Korde dataset from Oncomine (www.oncomine.com) (n = 21, 18, 21, at 0-, 1-, 4-cycle of docetaxel, respectively). e, f Representative images of SUM159- and SUM159-P-derived tumour spheres. The number of tumour spheres (> 60 µm diameter) was counted and sphere forming efficiency (SFE) was calculated. g, h Flow cytometry analysis of ALDH+ BCSCs in SUM159 and SUM159-P cells. DEAB, a specific ALDH inhibitor, was used as a control to determine the ALDH activity. The percentage of ALDH+ populations is graphed. i, j Flow cytometry analysis of CD24lowCD44high BCSCs in SUM159 and SUM159-P cells. CD24lowCD44high population was gated based on high 50% of CD44+ population and low 50% of CD24− population. The percentage of CD24lowCD44high population is indicated. k, l the percentage of CD24lowCD44high population in cells dissociated from SUM159 and SUM159-P derived tumours. Data are graphed as box and whisker plot, n = 3
stemness in SUM159-P cells could originate from inhibition of pac-induced Src activation resulting from long-term paclitaxel treatments.

BCSCs are intrinsically resistant to conventional treatment and can survive through chemotherapy, giving rise to a population of drug-resistant cancer cells. We hypothesised that short-term treatment with paclitaxel could eliminate the bulk tumour cells and select for the preferential outgrowth of drug-resistant BCSCs. Parental SUM159 cells were treated or not with 10 nM paclitaxel for 4 days, allowed for recovery in drug-free media for 4 days and subjected to tumoursphere assay. As shown in Fig. 3b-f, although paclitaxel treatment decreased total cell number of SUM159 cells, it significantly increased the number of tumoursphere-forming cells, indicative of an increased BCSC sphere forming activity. Short-term exposure of SUM159 cells to paclitaxel also increased the percentage of ALDH+ BCSC population from 8.6% to 12.7% (Fig. 3c, d) and did not significantly change the percentage of CD24low/CD44high BCSC subpopulation (Fig. 5s), demonstrating the specific expansion of ALDH+ BCSCs following short term chemotherapy treatment. To address the role and involvement of Src, cells were then treated with dasatinib. While paclitaxel increased phospho-Src levels (Tyr416) in SUM159 cells, dasatinib completely abolished both basal and pac-induced Src phosphorylation (Fig. 3e). Moreover, dasatinib not only markedly reduced the tumoursphere formation and the percentage of ALDH+ cells in parental SUM159 cells, but also blocked pac-induced breast cancer stemness (Fig. 3b-d). Together, these results suggest that pac-increased BCSC sphere formation and expansion are mediated at least in part through Src kinase activity. Besides targeting pac-induced BCSCs enrichment, dasatinib treatment also enhanced the cytotoxic effect of paclitaxel on SUM159 bulk cells (Fig. 3f), suggesting the depletion of both CSCs and non-CSCs after concomitant treatment with dasatinib and paclitaxel.

Dasatinib induces mesenchymal-epithelial transition and differentiation of chemo-resistant cells

The enrichment of cancer stem cells has been shown to correlate with the induction of epithelial-mesenchymal transition (EMT) in breast cancer cells. Having found that dasatinib prevented BCSC sphere forming capacity and expansion in pac-resistant cells, we sought to investigate whether dasatinib could disrupt the mesenchymal phenotype of these cells. Interestingly, the morphology of SUM159-P cells changed from spindle-like cell shape into cohesive clusters with round cellular morphology after 4 days' treatment with dasatinib, suggesting a mesenchymal-epithelial transition (MET) (Fig. 4a). In accordance with these morphological changes, dasatinib led to decreased mRNA expression of mesenchymal markers, including N-cadherin (CDH2), Fibronectin (FN1), Snail (SNAI1), ZEB1 and basal markers TP63, alpha smooth muscle actin (ACTA2) in SUM159-P cells (Fig. 4b). Furthermore, when examined at the protein level, dasatinib treatment led to increased epithelial marker (E-cadherin) expression and decreased expression of the two mesenchymal makers (Fibronectin, Snail) (Fig. 4c), further indicating that dasatinib contributes to the differentiation of chemo-resistant breast cancer cells by inducing MET.

EMT is a mechanism by which differentiated tumour cells transit to stem-like cancer cells. Having found dasatinib to induce MET in SUM159-P cells, we examined its effect on phenotypic differentiation. We found increased expression of the luminal differentiation marker MUC1 after dasatinib treatment (Fig. 4d) as well as a fourfold increase in the MUC1+ cell population (Fig. 4e, f). Given that dasatinib suppressed BCSC properties and altered the phenotype of chemo-resistant cells to a more differentiated epithelial type in 2D culture, this prompted us to examine whether dasatinib treatment could also lead to the formation of acinar-like structure of SUM159-P cells in three-dimensional (3D) culture. For this, we performed a 3D cell culture on Matrigel to study the patterns of epithelial differentiation in vitro. Indeed, this 3D culture better represents morphology and tumour growth in vivo compared to monolayer cell cultures as it mimics cell–cell and cell–matrix interactions in the tumour microenvironment. In this model, more differentiated cancer cells form acinar-like structures, round spheroids with hollow lumen inside. The more invasive cancer cells, however, form large, poorly differentiated spheroids, or aggressively invading structures. Briefly, SUM159-P cells were cultured in Matrigel for 5 days in the presence or absence of dasatinib and then subjected to immunofluorescence staining of F-actin and Dapi. F-actin staining was performed to better visualise the cell margins. Interestingly, SUM159-P cells formed invasive branching morphogenesis, characterised by the migration of cells through the surrounding Matrigel. In contrast, dasatinib treatment resulted in the formation of round, acinar-like structures characterised by smoothing of the outer layer and development of a central lumen (Fig. 4g). Taken together, these findings indicate that inhibition of Src activity by dasatinib resulted in phenotypic differentiation of chemo-resistant cancer cells in both 2D and 3D cultures.

Dasatinib-induced differentiation sensitises BCSCs to chemotherapy

Differentiated cancer cells are more sensitive to conventional chemotherapy than cancer stem cells. Indeed, when analysing 508 breast cancer tissue samples (169 sensitive patients and 339 insensitive patients in Booser dataset from R2: Genomics Analysis and Visualisation Platform [http://r2.amc.nl]), we found a positive correlation between chemotherapy sensitivity and high expression levels of differentiation markers (CD24, EpCAM) and luminal markers (E-cadherin, KRT8, KRT18, MUC1) (Fig. 5a). Having shown that dasatinib induced epithelial differentiation in BCSC-enriched SUM159-P cells, we hypothesised that dasatinib treatment could sensitise the chemo-resistant BCSCs to paclitaxel. To test this hypothesis, SUM159-P cells were treated with DMSO, dasatinib, paclitaxel alone or in combination and subjected to tumoursphere formation assay. Parental SUM159 cells were used as an experimental control. SUM159-P cells exhibited higher numbers of tumoursphere compared to parental SUM159 cells, confirming a higher sphere forming capacity of BCSCs in SUM159-P. Paclitaxel alone didn’t affect the tumoursphere formation while dasatinib significantly decreased the BCSC sphere forming ability in SUM159-P. Paclitaxel treatment could sensitise chemo-resistant BCSCs to paclitaxel. To test this hypothesis, SUM159-P cells were treated with DMSO, dasatinib, paclitaxel alone or in combination and subjected to tumoursphere formation assay. Parental SUM159 cells were used as an experimental control. SUM159-P cells exhibited higher numbers of tumoursphere compared to parental SUM159 cells, confirming a higher sphere forming capacity of BCSCs in SUM159-P. Paclitaxel alone didn’t affect the tumoursphere formation while dasatinib significantly decreased the BCSC sphere forming ability in SUM159-P cells, as indicated by the significantly decreased sphere forming efficiency (SFE) (Fig. 5b, c). Notably, a combination treatment of dasatinib and paclitaxel showed even stronger effect than dasatinib alone and eliminated nearly all the tumoursphere-forming cells (Fig. 5b, c). Thus, these results suggest that dasatinib treatment could sensitise chemo-resistant BCSCs to paclitaxel.

We further evaluated the effects of dasatinib and paclitaxel on CD24low/CD44high and ALDH+ BCSC subpopulations. As shown in Fig. 5d-g, we found that paclitaxel alone did not reduce CD24low/CD44high nor ALDH+ BCSC subpopulations, compared to the DMSO treatment. Dasatinib, on the other hand, decreased the

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**Table 1. Inhibitors in the drug screen and their targets**

| Inhibitor       | Major target(s) |
|-----------------|------------------|
| LY294002        | PI3K/AKT         |
| SB431542        | TGFÎ²RI         |
| Gefitinib       | EGFR             |
| Dasatinib       | BCR-Abl, Src     |
| Rapamycin       | mTOR             |
| JNK inhibitor   | JNK              |
| PD98059         | MEK              |
| Palbociclib     | CDK4/6           |

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**Fig. 2** Dasatinib potently kills BCSCs in SUM159-P cells. **a** SUM159-P cells were treated in the presence of either DMSO or IC50 doses of kinase inhibitors and subjected to tumoursphere formation assay. The SFE was calculated and graphed. **b** SUM159 and SUM159-P cells treated with either DMSO or IC50 doses of kinase inhibitors were subjected to flow cytometry analysis. The percentage of CD24lowCD44high populations was indicated and graphed. **c** SUM159 and SUM159-P cells were treated with either DMSO or IC50 dose of dasatinib. ALDEFluor assay was performed and percentage of ALDH+ cells were determined by flow cytometry.
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Fig. 3 Dasatinib suppresses BCSC through down-regulation of paclitaxel-induced Src kinase activation. **SUM159 cells treated with DMSO and SUM159-P cells treated with DMSO, paclitaxel (10 nM), dasatinib (1 uM) were subjected with western blot analysis using anti-phospho-Src (Tyr 416), anti-Src and anti-β-actin antibodies. **Tumourspheres were derived from SUM159 cells treated with paclitaxel, dasatinib or in combination. The number of tumourspheres and SFE were determined. **ALDEFluor assay was conducted in SUM159 cells treated with paclitaxel, dasatinib or in combination for 4 days. The percentage of ALDH+ cells was indicated and graphed. **SUM159 cells were treated with paclitaxel, dasatinib or in combination for 2 days. Total cell lysates were analysed for phospho-Src (Tyr 416), Src and β-actin by western blot (e). Cell growth inhibition was measured by PrestoBlue assay (f).

proportion of both CD24low/CD44high and ALDH+ BCSCs. Combination treatment of dasatinib/paclitaxel produced maximal reduction in CD24low/CD44high and ALDH+ BCSC cell numbers, compared with either control-, dasatinib, or paclitaxel-treatment conditions. These results are consistent with our data using tumoursphere formation assays and further support our hypothesis that Src kinase inhibition by dasatinib sensitises BCSCs to the chemotherapy drug paclitaxel.
Combination of dasatinib and paclitaxel potently eliminates breast cancer cells. BCSCs display resistance to conventional chemotherapy and drive tumour relapse after initial response to chemotherapy. Thus, specific eradication of BCSCs would provide an attractive solution to address the ineffectiveness of traditional breast cancer therapies. The combined use of BCSC-targeting agents with chemotherapy may target both BCSC and non-BCSC populations and offer a promising therapeutic strategy for chemotherapy-resistant patients. Having shown that the combination of dasatinib
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Fig. 5  Dasatinib-induced differentiation sensitises BCSCs to chemotherapy. a mRNA expression levels of the indicated genes in breast cancer patients, Booser dataset from 'R2: Genomics Analysis and Visualisation Platform [http://r2.amc.nl]'. b–g SUM159-P cells were treated with paclitaxel (10 nM), dasatinib (1 uM) or in combination. SUM159 cells were used as a control. b, c Tumoursphere formation assay was performed and the SFE was calculated. d, f Flow cytometry analysis was conducted and the percentage of CD24lowCD44high population was assessed. e, g ALDEFluor assay was conducted and percentage of ALDH+ cells were quantified by flow cytometry.
and paclitaxel decreased the fraction of chemotherapy-resistant BCSCs and their sphere forming capacity, we hypothesised that this combination treatment could enhance cytotoxicity of dasatinib and paclitaxel alone and reverse the paclitaxel resistance in SUM159-P cells. SUM159-P cells were treated with dasatinib and paclitaxel in an equi-effective fashion based on their IC50 values for 2 days and assessed for cell viability. Cytotoxicity of dasatinib/paclitaxel combination treatment in SUM159-P was significantly higher than dasatinib or paclitaxel single-drug exposure. The CI–isobologram indicates that the combination of dasatinib and paclitaxel was synergistic in a wide range of fractions affected (Fa) value (Fa 0.2–Fa 0.97) (Fig. 6a). We also determined the cytotoxic effects of dasatinib, paclitaxel and combination therapy on the isolated CD24+CD44+ non-BCSCs fraction of SUM159-P and found that the concomitant dasatinib therapy enhanced the cytotoxic effect of paclitaxel in these cells (Fig. 6b). These results,
combined with the data in Fig. 5 suggest that the combination of dasatinib and paclitaxel has a broad effect on suppressing cell survival of chemo-resistant TNBC cells, via eliminating both BCSCs and non-BCSCs.

We further examined the efficacy of dasatinib/paclitaxel combination treatment in reducing tumour volume in a preclinical model of breast cancer, using orthotopic xenografts in NSG mice. NSG mice were inoculated with SUM159 and SUM159-P cells (1 × 10^6 cells per mouse) in the left and right mammary fat pad, respectively. One week following tumour cell transplantation, these mice were randomly divided into four groups (6 mice per group) and treated with vehicle, paclitaxel (20 mg/kg, once a week), dasatinib (10 mg/kg) alone or in combination for up to 6 weeks. Treatment of parental cells-derived tumours with paclitaxel efficiently reduced the tumour volume to a significant extent (P < 0.05) decrease at week 7, compared to the vehicle-treated group. As opposed, paclitaxel treatment of the SUM159-P resistant cells-derived tumours did not significantly reduce the tumour burden (Fig. 6c). Interestingly, while the dasatinib treatment suppressed SUM159-P tumour growth compared with vehicle alone (P < 0.05), the combination of dasatinib and paclitaxel induced near-complete tumour regression (P < 0.01), with four out of six mice showing no tumour at all (Fig. 6d and e). Altogether, our data indicate that dasatinib significantly improves the efficacy of paclitaxel in eliminating chemo-resistant breast cancer cells both in vitro and in vivo.

Dasatinib sensitises BCSCs to paclitaxel in vivo

We next explored whether dasatinib/paclitaxel combination also led to a reduction of BCSCs in vivo. For this, we examined the stemness marker CD44 expression levels, using IHC, in tumours collected out of SUM159 and SUM159-P xenografts. As indicated in Fig. S4, both membranous and cytoplasmic staining were analysed and scored by two independent pathologists. The score of staining intensity and the percentage of stained area were analysed. The total percentage of membrane and cytoplasmic CD44 staining was calculated in each treatment group.

Taken together, these data suggest the dasatinib/paclitaxel combination as a promising therapeutic to target both BCSC and non-BCSC populations and to therefore overcome chemotherapy resistance in breast cancer. Our studies provide preclinical evidence of the future combination use of dasatinib and paclitaxel to treat chemo-resistant breast cancer patients and to improve better outcomes in TNBC patients.

DISCUSSION

Triple-negative breast cancers display worse patient outcomes than any other breast cancer molecular subtypes, with significant shorter disease-free survival and overall survival times. Although showing an initial response to taxane- and platin-based primary chemotherapy, TNBC can develop tumour recurrences and metastases due to intrinsic and acquired chemo-resistance. The chemotherapy failure in TNBC has been partly attributed to the enrichment of BCSCs. Our study revealed that chemotherapy treatment stimulates BCSC self-renewal and expansion both in vitro and in vivo and further confers adaptive resistance, suggesting the needs to target BCSCs in chemo-resistant TNBCs. We also uncovered a novel function of Src kinase in regulating chemotherapy-induced BCSCs. Moreover, our in vitro and in vivo results highlighted that dasatinib can synergistically reestablish chemo-resistant TNBCs to paclitaxel through targeting BCSCs.

We found chemo-resistant TNBCs to exhibit increased BCSC numbers (CD24^low^CD44^high^ and ALDH^+^ BCSCs) and increased sphere forming capacity. Moreover, using xenografts in NSG mice, we found chemo-resistant TNBC-derived tumours to also contain higher percentage of BCSCs. These results suggest that the long-term treatment of paclitaxel-induced the expansion of BCSCs and conferred adaptive resistance to chemotherapy in TNBC. This is consistent with previous studies revealing an enriched cancer stem cell-like gene signature in neoadjuvant chemotherapy-treated breast tumours or a link between increased BCSC percentages and intrinsic chemotherapy resistance. We showed that short term (4 days) exposure of TNBCs to paclitaxel also increased BCSC numbers and their tumoursphere formation ability. This suggests that short term paclitaxel treatment only kills bulk tumour cells and imposes a strong selection for drug-resistant BCSC survival and expansion. Our results, combined with previous studies, emphasise the need to identify agents that efficiently eliminate BCSCs in stem-like cells enriched chemo-resistant TNBCs.

We also found dasatinib to efficiently reduce BCSC numbers through inhibition of pac-induced Src activation in both parental and chemo-resistant TNBCs. This is consistent with previous work showing upregulation of the stemness marker CD44 and activated Src in tumours from chemotherapy-treated mice. Thus, based on these observations, blocking Src activation appears as an attractive strategy to eliminate BCSCs in chemo-resistant breast cancer.

The induction of EMT in neoplastic mammary epithelial cells has been shown to enrich cells with stem-like properties.
Residual breast cancers following conventional chemotherapy not only contain higher percentage of BCSCs, but also display mesenchymal features. Recently, researchers have been trying to identify selective inhibitors of BCSCs using high-throughput screening of compounds with EMT-specific toxicity. We found dasatinib treatment of chemoresistant TNBCs to decrease BCSC numbers and induce a reversed EMT phenotype and phenotypic changes from spindle-like cell shape to cohesive clusters with round cellular morphology. Several studies have linked Src activation and EMT in pancreatic and ovarian cancers. Interestingly, a recent study suggested that activation of Lyn, another dasatinib target, could in fact regulate EMT and breast cancer cell invasion. Thus, it will be interesting to further study the molecular mechanisms underlying these effects and further characterise the connection between dasatinib, SFK activation and EMT in breast cancer.

Recently, differentiation therapy has been shown to be an attractive way to force CSCs to differentiate terminally and therefore control tumour progression. Lombardo et al. found that bone morphogenetic protein 4 (BMP4), which promotes terminal differentiation of colorectal cancer stem cells via induction of Wnt signalling, could increase their sensitivity to chemotherapy. Another study showed that all-trans-retinoic acid treatment induced BCSC differentiation, leading to a shrinkage of the BCSC population. We show that dasatinib treatment leads to the formation of acinar-like structure of chemo-resistant TNBCs in 3D Matrigel culture and to the increased expression of luminal differentiation markers, indicating that dasatinib could induce a phenotypic differentiation of BCSC-enriched chemo-resistant cells. Thus, dasatinib could prove useful to induce phenotypic differentiation of breast cancer stem cells.

It has been established that differentiated cancer cells are more sensitive to chemotherapy. In this study, we found that dasatinib treatment not only induced epithelial differentiation of BCSC-enriched chemo-resistant cells but also sensitised these cells to paclitaxel. We further found that dasatinib and paclitaxel had a synergistic effect on reducing cell viability of chemoresistant cells in vitro and in reducing tumour growth in vivo. This is consistent with findings from clinical trials, where single dasatinib treatment did not show significant antitumour activity in patients with metastatic breast cancer, while a recent phase I clinical trial in metastatic breast cancer showed that dasatinib plus weekly paclitaxel treatment proved more effective and produced a partial response in 4 of 13 patients (31%), including 4 patients previously treated with taxanes. Thus, our study has several translational implications. First, it reveals that chemotherapy treatment triggers BCSC sphere forming capacity and expansion and further confers adaptive resistance, highlighting the needs to target BCSCs in chemo-resistant TNBCs. Secondly, we uncovered a new role for Src kinase in mediating chemotherapy-induced BCSC properties and found dasatinib as a potent blocker of cancer stemness, further highlighting the potential clinical value of the SFK inhibitor, dasatinib as an efficient anti-stemness cancer drug in TNBCs. Thirdly, although dasatinib is well-tolerated clinically applicable Src inhibitor, the results from phase II clinical trials of dasatinib monotherapy on solid tumours are discouraging. One possible explanation for this issue in breast cancer was that patients were unselected in the clinical trials of dasatinib. Given the role of Src kinase in paclitaxel resistant BC, the activation of Src kinase may serve as a response biomarker for the dasatinib and paclitaxel combination treatment in TNBCs. Last, our in vitro and in vivo results indicate that dasatinib can sensitize refractory breast cancer cells to paclitaxel. Although a similar trend was observed in the phase I clinical trial (Bristol-Myers Squibb Study CA180194) in metastatic breast cancer, the underlying mechanisms remained unclear. We show here that dasatinib, in fact induces epithelial differentiation of chemo-resistant TNBCs and that combination of dasatinib and paclitaxel led to the near-complete elimination of BCSCs. Thus, our data could explain the observed results from the Bristol-Myers Squibb phase I trial. In the future, it will be interesting to also investigate the dasatinib involvement in other BCSC-related chemo-resistance mechanisms, including the expression of drug export proteins, induction of quiescence, activation of stemness signalling pathway.

Our study highlights the potential clinical utility of dasatinib/paclitaxel combinatorial treatment to overcome chemotherapy resistance and to improve better outcomes in TNBC patients. Several previous studies also tested the use of dasatinib in combination with other drugs as potential therapeutic strategies in different types of breast cancer. For instance, combination of Src inhibitor and anti-oestrogen therapy effectively stimulated cancer cell cycle arrest and suppressed Src inhibitor resistance in ER+ breast cancer. Moreover, for HER2 overexpressing breast cancer, although Src inhibitor alone did not show effects in treating trastuzumab or lapatinib-resistant tumours, targeting Src signalling pathway significantly sensitised these resistant tumours to anti-HER2 treatments. These studies, combined with our present findings in TNBCs highlight the potential benefit of using Src inhibitor, especially in combinatorial regimens for treating different types of breast cancers.

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AUTHOR CONTRIBUTIONS

J.T. designed, performed experiments, analysed data and drafted the manuscript. F.A.R. was involved in performing PCR and western blot experiments. M.D. contributed to designing and performing experiments. A.M. assisted in 3D cell culture. B.K., I.Y.H. and K.B. were involved in IHC analysis. S.A. and B.J. assisted in designing experiments and editing the manuscript. J.T. was involved in research design, data interpretation, supervision of the project and drafting the manuscript.

ADDITIONAL INFORMATION

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