Reduction of the putative $CD44^+CD24^-$ breast cancer stem cell population by targeting the polyamine metabolic pathway with PG11047

Helena Cirenajwis[a], Sandra Smiljanic[a], Gabriella Honeth[b], Cecilia Hegardt[b], Laurence J. Marton[c] and Stina M. Oredsson[a]

Cancer stem cells (CSCs) are considered to be of particular concern in cancer as they possess inherent properties of self-renewal and differentiation, along with expressing certain genes related to a mesenchymal phenotype. These features favour the promotion of tumour recurrence and metastasis in cancer patients. Thus, the optimal chemotherapeutic treatment should target the CSC population, either by killing these cells and/or by inducing their transition to a more differentiated epithelial-like phenotype. Experiments were carried out on the trastuzumab-resistant human epidermal growth factor receptor 2-overexpressing breast cancer cell line JIMT-1 to unravel the chemotherapeutic effects of the polyamine analogue [^1N,^{12}N]bis(ethyl)-*cis*-6,7-dehydrospermine (PG11047) and of the polyamine biosynthetic inhibitor 2-difluoromethylornithine (DFMO) on the $CD44^+CD24^-$ CSC population. Furthermore, effects on the properties of self-renewal and epithelial/mesenchymal markers were also investigated. Treatment with PG11047 reduced the $CD44^+CD24^-$ subpopulation of JIMT-1 cells by approximately 50%, inhibited and/or reduced self-renewal capability of the CSC population, decreased cell motility and induced expression of mesenchymal to epithelial transition-associated proteins that are involved in promoting an epithelial phenotype. By contrast, DFMO slightly increased the $CD44^+CD24^-$ subpopulation, increased cell motility and the level of mesenchymal-related proteins. DFMO treatment reduced the self-renewal capability of the CSC population. Both PG11047 and DFMO reduced the expression of the human epidermal growth factor receptor 2 protein, which is correlated to malignancy and resistance to trastuzumab in JIMT-1 cells. Our findings indicate that treatment with PG11047 targeted the CSC population by interfering with several stem cell-related properties, such as self-renewal, differentiation, motility and the mesenchymal phenotype. *Anti-Cancer Drugs* 21:897–906

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[a]Department of Biology, Division of Cell and Organism Biology, [b]Department of Oncology, Clinical Sciences, Lund University, Lund, Sweden and [c]Progen Pharmaceuticals, Palo Alto, California, USA

Correspondence to Helena Cirenajwis, Institution of Biology, Sölvegatan 35A, SE-223 62 Lund, Sweden
Tel: +4646 2224587; fax: +4646 2224425;
e-mail: helena.cirenajwis@cob.lu.se

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Introduction

More than 25 years have passed since the first discovery of human $CD34^+CD38^-$ leukaemia initiating cells [1]. Since then, tumour-initiating cells or cancer stem cells (CSCs) have been found in a variety of solid tumours, including colon [2,3], prostate [4,5], brain [6] and breast cancer [7]. In breast tumours, a $CD44^+CD24^{-/low}ESA^+$ $Lineage^-$ subpopulation was originally identified as the tumourigenic (tumour-initiating) fraction, based on the enhanced ability of these cells to form tumours in non-obese diabetic/severe combined immunodeficiency mice when injected at a very low number [7]. Human breast cancer cell lines contain $CD44^+CD24^{-/low}ESA^+$ cells that have stem cell properties including anchorage-independent growth at clonal densities (self-renewal) and the ability to reconstruct the parental cell fractions, along with in-vivo tumourigenicity [8,9]. The $CD44^+CD24^-$ phenotype is also correlated with the enhanced expression of proinvasive genes and the ability to form distant metastasis [10–12]. In addition, tumourigenicity of prospective breast CSCs has been linked to the expression of α-6 integrin [13], β-1 integrin [14] and aldehyde dehydrogenase 1 [15,16], along with the presence of a $Hoechst^{low}$ population [17].

The CSC properties of proinvasiveness and the ability to form distant metastasis are related to the mesenchymal phenotype [18]. This mesenchymal phenotype may also persist in the CSC progeny. The epithelial to mesenchymal transition (EMT) is a phenomenon that normally occurs during early embryonic development to allow epithelial-like epiblast cells to adjust to a mesenchymal phenotype important for migration [19,20]. Later in development, the cells undergo the reverse process, a mesenchymal to epithelial transition (MET). Putative CSCs are positive for a number of markers associated with an EMT process, such as increased expression of vimentin, N-cadherin, Akt kinase activity, Snail, Slug and Twist [21–23].

Human epidermal growth factor receptor 2, also referred to as HER2, or ErbB2, has been implicated in the role of regulating the CSC population by promoting

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tumourigenesis and invasion associated with Akt phosphorylation [24]. The *HER2* gene is amplified in 20–30% of human breast cancers and is associated with aggressive metastatic disease [25]. The humanized anti-HER2 antibody trastuzumab (Herceptin) has been used to treat HER2 overexpressing breast cancers. However, one-third of the patients fail to respond and many of the initial responders eventually show disease progression [26]. Thus, the development of strategies for treating trastuzumab-resistant tumours is evident. The major issue in using conventional chemotherapy is the recurrence of tumours as a result of resistance, a property likely resident within the small CSC population [27–29]. A goal in future cancer treatments is to identify treatment regimes that target the CSC population.

A new group of compounds being tested in clinical cancer trials are the polyamine analogues [30]. One of these is the conformationally restricted spermine analogue [^{1}N,^{12}N]bis-(ethyl)-*cis*-6,7-dehydrospermine (PG11047) [31], which is currently in clinical trials in patients with advanced cancer as a monotherapy and in combination with conventional chemotherapeutic drugs [32]. PG11047 is efficiently taken up by the natural polyamine transport system and accumulates in the cell to high concentrations. Inside the cell it exerts feedback inhibition on the synthesis of the natural polyamines putrescine, spermidine and spermine. In addition, it activates the polyamine catabolic enzyme spermidine/spermine N^1-acetyltranseferase, which results in cellular depletion of the natural polyamines. The cell is left with high concentrations of PG11047, a molecule that cannot take over the function of the natural polyamines and in fact may modulate normal polyamine functions. As a result, cell proliferation ceases and apoptosis may be induced [33]. Acting as a single agent, PG11047 treatment has shown to efficiently inhibit growth of human lung and prostate cancer *in vitro* and *in vivo* [34,35]. PG11047 treatment potentiated the antitumour activity of bevazicumab and cisplatin in preclinical models of lung and prostate cancer [35]. Recently, Kuo *et al.* [32] reported on a 13-gene transcription expression profile that correlated to PG11047 sensitivity in breast cancer cell lines.

Another chemotherapeutic drug that targets the polyamine biosynthetic pathway is 2-difluoromethylornithine (DFMO), which acts by inhibiting ornithine decarboxylase [36]. DFMO treatment very efficiently inhibits the growth of cells in culture, but has so far not proven to be effective as a single agent in cancer treatment for several reasons [37]. However, DFMO and sulindac treatment was shown to be chemopreventive in the development of sporadic colorectal adenomas [38] and may be efficient in combination with other chemotherapeutic drugs [37].

Testing novel chemotherapeutic drugs on cancer cell lines permits for rapid identification of effects on putative CSCs [8]. In this study, we have investigated how treatment with either PG11047 or DFMO affected the putative CSC population, described here as $CD44^+$ $CD24^-$, in the trastuzumab-resistant HER2-overexpressing cell line JIMT-1. We investigated markers related to mesenchymal and epithelial phenotypes, the self-renewal properties of these cells and cell migration after treatment. Our findings show that treatment with PG11047 reduced the $CD44^+CD24^-$ subpopulation by 50%, inhibited the self-renewal capacity of the cells, decreased cell motility and induced the expression of cytokeratin 18 (CK18) related to an epithelial phenotype, while reducing mesenchymal-associated proteins. DFMO treatment inhibited the self-renewal properties of the cells; however, it also showed some less beneficial effects.

Materials and methods

Material

Dulbecco's modified Eagle's medium/nutrient mixture Ham's F12 medium, foetal calf serum (FCS), nonessential amino acids, penicillin/streptomycin, Nonidet P-40 and phosphate-buffered saline (PBS) tablets were purchased from VWR (Lund, Sweden). Tissue culture plastics were purchased from Nunc (Roskilde, Denmark). Insulin, hydrocortisone, Accutase, ribonuclease A, propidium iodide, bovine serum albumin (BSA), 7-aminoactinomycin D and poly(2-hydroxyethyl methacrylate) (polyHEMA) were purchased from Sigma (Stockholm, Sweden). Epithelial growth factor and B27 were purchased from Invitrogen (Stockholm, Sweden). Mammary epithelial growth medium was purchased from Cambrex (Walkersville, Maryland, USA) and basic fibroblastic growth factor from R&D Systems (Minneapolis, Minnesota, USA). Antibodies used for western blot and immunofluorescence microscopy against human HER2 (CB11), vimentin (RV202), CK18 (DC10), Akt1 (BDI111) and phosphorylated Akt1 (p-Akt1) (104A282), were purchased from Abcam (Cambridge, UK), whereas the CD44 antibody (F10-44-2) was purchased from Serotec (Oxford, UK). Secondary horseradish peroxidase-conjugated antibodies used for western blot were purchased from DAKO (Glostrup, Denmark). Antibodies used for flow cytometry included CK18-fluorescein isothiocyanate (FITC) (B23.1 DC10) from Abcam, and CD44-FITC (G44-26), CD24-phycoerythrin (PE) (ML5), along with PE-conjugated or FITC-conjugated mouse IgG1 isotype controls (MOPC-21) from Becton Dickinson (Stockholm, Sweden). Enhanced chemiluminescence detection reagents were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Precast NuPage Novex Bis–Tris SDS polyacrylamide gels and iBlot Transfer Stacks were purchased from Invitrogen. PG11047 (formerly CGC-11047 and SL-11047) was provided by Progen Pharmaceuticals (Palo Alto, California, USA). DFMO was purchased from Ilex-Oncology (San Antonio, Texas, USA). Paclitaxel was donated by Sophie Manner, Department of Chemistry, Lund University, Sweden.

Cell line and culturing conditions

The human breast carcinoma cell line JIMT-1 (ACC589) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cells were routinely cultured in Dulbecco's modified Eagle's medium/nutrient mixture Ham's F12 medium with the addition of 8% FCS, nonessential amino acids (1 mmol/l), insulin (10 μg/ml), penicillin (100 U) and streptomycin (100 μg/ml). The cells were routinely passaged twice a week from a cell density of 110 000 cells/cm^2 to a cell density of 30–40 000 cells/cm^2. In all experiments, the seeding cell density was 16–18 000 cells/cm^2, unless otherwise stated.

Multicellular mammospheres were derived from the single cells when seeded at clonal density (1000 cells/ml) in suspension, essentially as described by Dontu *et al.* [39]. PolyHEMA-coated hydrophobic Petri dishes (2.5 cm in diameter; 5 mg/ml polyHEMA in 95% ethanol; 2.2 ml/Petri dish allowed to dry slowly for 3 days at 37°C) were used for mammosphere formation. Mammospheres were cultured in serum-free mammary epithelial growth medium including hydrocortisone, insulin and GA-1000, additionally supplemented with B27, 20 ng/ml epithelial growth factor and 20 ng/ml basic fibroblastic growth factor. Bovine pituitary extract was excluded from the medium.

Substances

PG11047 was diluted in PBS to give a stock solution of 4 mmol/l. DFMO was dissolved in Millipore water to a concentration of 495 mmol/l after adjusting the pH to 7.2. The PG11047 and DFMO stock solutions were sterile-filtered and used in the experiments at a final concentration of 10 μmol/l and 1 mmol/l, respectively. Paclitaxel was diluted in dimethyl sulfoxide to a 100 mmol/l stock solution, and further diluted in PBS to give a 100 μmol/l working solution.

Cell surface markers identified by flow cytometry

Cells were harvested using Accutase and identified based on their expression of the cell surface markers CD44 and CD24 using a FACSCalibur instrument (Becton Dickinson, Erembodegem, Belgium). In brief, the cells were incubated with the monoclonal antibodies CD44-FITC and CD24-PE and the isotype controls for 15 min on ice and thereafter washed with PBS containing 1% FCS. To exclude the dead cells in the analysis, 7-aminoactinomycin D was added to the samples. Analysis was carried out using the FlowJo software (TreeStar, Ashland, USA).

Cytokeratin 18 identified by flow cytometry

Cells were harvested using trypsin (0.05%)/EDTA (1 mmol/l) and fixed in ice-cold 70% ethanol for 10 min at −20°C. The cells were blocked in 1% BSA in PBS before incubation with a FITC-conjugated antibody against human CK18 for 40 min at room temperature. The cells were washed with PBS containing 1% BSA and 0.05% Tween 20 throughout the preparation. Finally, CK18 expression was analysed using a FACSCalibur instrument and quantified using the FlowJo software.

Cell death

After 96 h of treatment with DFMO or PG11047, both the detached and attached cells were collected and fixed in ice-cold 70% ethanol. Trypsin (0.05%)/EDTA (1 mmol/l) was used to dislodge the attached cells. The cells were washed with PBS and DNA staining was performed using propidium iodide (100 μg/ml) in the presence of ribonuclease A (100 μg/ml) and Nonidet P-40 (0.6%). The samples were run on an Ortho Cytoron Absolute flow cytometer (Ortho Raritan, New Jersey, USA) and dead cells represented by the sub-G_1 fractions were determined using the MultiCycle software (Phoenix Flow System, California, USA).

Immunofluorescence microscopy

Cells were plated on poly-L-lysine-coated glass slides and treated with PG11047 for 96 h. After fixation with 3.7% paraformaldehyde (in PBS) for 15 min and subsequent washing in PBS, the cells were permeabilized with 0.2% Triton X-100 and blocked with 1% FCS in a single step. The cells were incubated with a primary antibody against human vimentin overnight at 4°C. After washing, the cells were incubated for 1 h with the Alexa Fluor 488 goat antimouse antibody (Invitrogen). Slides were counterstained with bisbenzimide and finally washed in PBS before mounting. Fluorescence-labelled cells were photographed using an Olympus/Nikon epifluorescence microscope (Olympus Optical Co. Ltd., Japan) equipped with a digital camera (Nikon Imaging Japan Inc., Japan). Each slide was photographed at randomly chosen areas (at least 10 areas). The fluorescence of all the images was analysed using the ImageJ software and the final images shown were made using Adobe Photoshop 8.0.1 (Adobe Systems Incorporated, San Jose, California, USA).

Mammosphere forming assay

Cells were treated for 96 h with either PG11047 or DFMO and thereafter reseeded at a low density under the anchorage-independent culture conditions described above. After 10 days of culturing, the mammospheres formed were photographed and manually counted in an inverted phase-contrast microscope. The percentage of sphere-initiating cells was defined as the number of counted mammospheres in relation to the number of seeded cells.

Digital holographic cell motility assay

Cell motility was analysed using a novel technique of three-dimensional holographic imaging [40]. Cells were seeded at a cell density of 10 000 cells/cm^2 and treated with either PG11047 or DFMO for 96 h. Digital photos were taken every 5 min for 4 h and time-lapse movies were constructed to track cell motility. An area for imaging was selected randomly. The total distance of

movement of individual cells was determined using HoloStudio version 1.8 (Phase Holographic Imaging AB, Lund, Sweden) in combination with the MatLab version 7.1 (Mathworks, Natick, Massachusetts, USA) software.

Western blot

Cells were harvested with Accutase and further diluted in the sample buffer [62.5 mmol/l Tris–HCl (pH 6.8), 20% glycerol, 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 0.025% bromophenol blue] before sonication. The samples were immediately boiled for 6 min and stored at −20°C until analysis. Aliquots equivalent of 100 000 cells were loaded per lane and separated on 4–12% acrylamide Bis–Tris gels using the NuPAGE Electrophoresis System (Invitrogen) and transferred to nitrocellulose membranes using the iBlot Dry Blotting System (Invitrogen). The membranes were blocked in either 5% dry-milk (nonphosphorylated proteins) or 5% BSA (phosphorylated proteins) and incubated with primary antibodies against HER2, CD44, CK18, Akt1 or p-Akt1. After incubation with a peroxidase-conjugated secondary antibody, the membranes were exposed to enhanced chemiluminescent solution to detect the protein bands. Data was collected and analysed using the Quantity One software (Bio-Rad, Hercules, California, USA). The intensities of the bands were determined by densitometric scanning.

Regarding p-Akt1 and Akt1, the densitometric values were used to calculate a ratio. p-Akt1 and Akt were detected on different western blots. Initially, p-Akt1 and Akt1 in the treated cells were calculated as percentage of control of the respective membrane. The ratio between p-Akt1 and Akt1 was set to 1 for the control cultures (100%/100%). The percentage of control for p-Akt1 and Akt1 for the treated cells were also used to calculate a ratio. A ratio above 1 implies an increase in p-Akt1 and a ratio below 1 implies a decrease in p-Akt1 compared with the control.

Statistics

Significance was evaluated using a two-tailed unpaired Student's *t*-test.

Results

The $CD44^+CD24^-$ subpopulation decreased after treatment with PG11047

To investigate the effect of treatment with either PG11047 or DFMO on cell proliferation (Fig. 1a), cell death (Fig. 1b) and on the putative breast CSC population, that is, the $CD44^+CD24^-$ subpopulation (Figs 1c–e), JIMT-1 cells were grown in the absence or presence of either substance alone, and examined every 24 h for up to 96 h using cell counting and flow cytometry.

Treatment with either PG11047 or DFMO significantly reduced ($P < 0.05$) cell proliferation after 48 h itself (Fig. 1a). PG11047 treatment was more efficient than DFMO treatment, and the cells completely stopped proliferating in the former after 72 h. The major effect of DFMO or PG11047 treatment was seen after 96 h when the cell numbers were 74.9% and 56% of the cell number in the control, respectively. The decreased cell proliferation was not accompanied by increased cell death (Fig. 1b).

After 72 h of treatment, the number of $CD44^+CD24^-$ cells comprising 22% of the total population in the control was significantly reduced ($P < 0.05$) to 11% in the presence of 10 μmol/l PG11047, whereas treatment with 1 mmol/l DFMO increased the $CD44^+CD24^-$ subpopulation to 27% (Fig. 1c). Even with 24 and 48 h of treatment with PG11047, reduction of the $CD44^+_{CD24-}$ subpopulation was observed (Fig. 1d). The major effect of the treatment occurred after 72 and 96 h (not shown), and there was no significant difference between these time points. In addition, the commonly used anticancer drug paclitaxel was examined for its effect on the $CD44^+CD24^-$ subpopulation after 72 h of treatment as a comparison. Treatment with 1 mmol/l DFMO or 100 nmol/l paclitaxel increased the number of $CD44^+CD24^-$ cells by 10% and 27%, respectively (Fig. 1e), whereas treatment with 10 μmol/l PG11047 reduced the $CD44^+CD24^-$ subpopulation by 55%.

In conclusion, the effects seen on the putative breast CSC population in the cultures treated with either DFMO or PG11047 can be interpreted as treatment-induced phenotypic changes rather than eradication of specific cell populations.

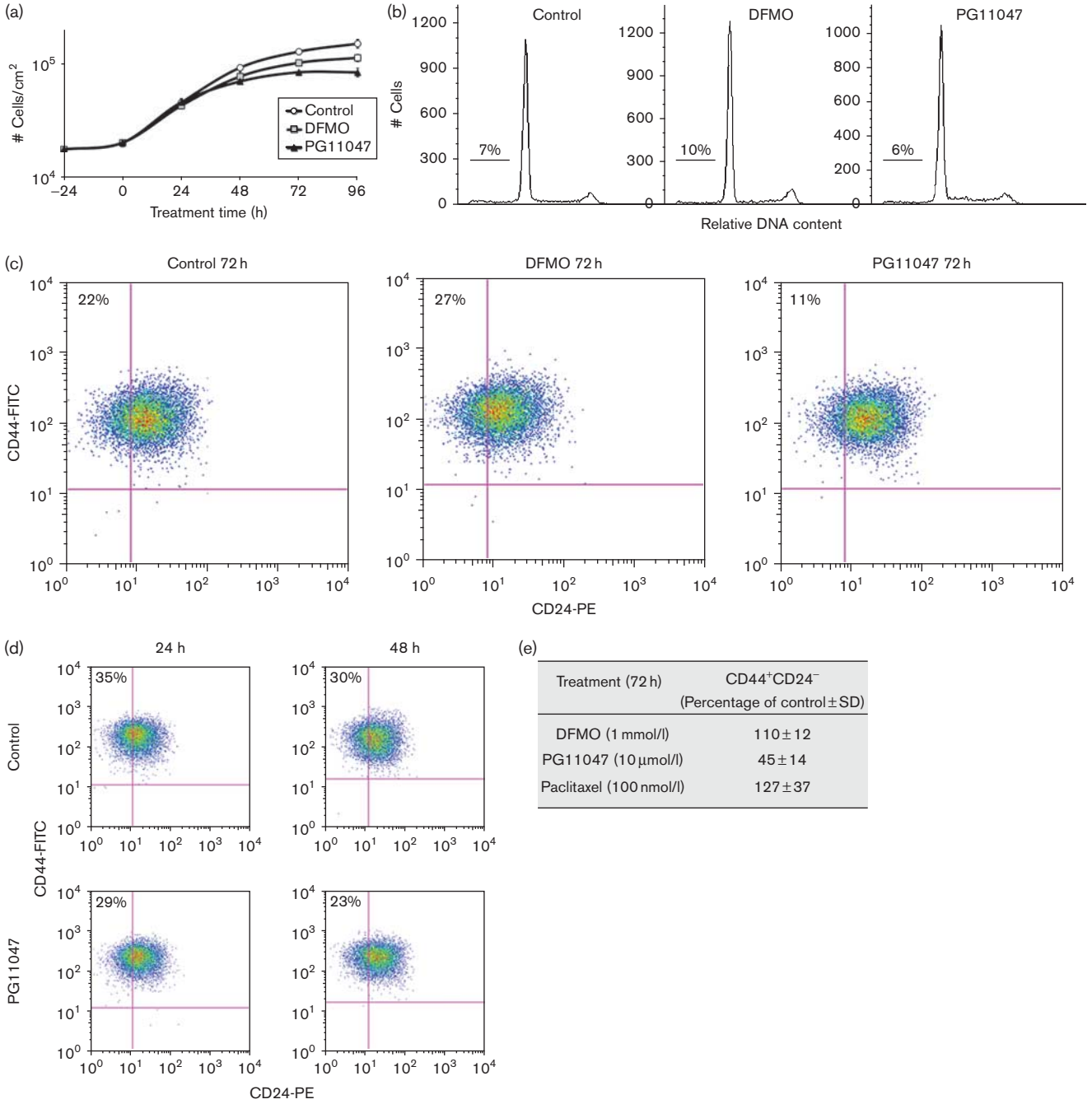
Decreased expression of proteins related to malignancy and the mesenchymal phenotype

Our data show that treatment with either PG11047 or DFMO induced phenotypic changes in JIMT-1 cells (Fig. 2a). The DFMO-treated cells attained a stretched out fibroblastic morphology, whereas the PG11047-treated cells generally were larger and more granular compared with the control. On the basis of recent findings suggesting that the $CD44^+CD24^-$ stem cell population expresses proteins related to a mesenchymal phenotype [21], we decided to study various proteins related to this process and proteins related to malignancy.

HER2 overexpression is related to aggressive behaviour in breast cancer and, thus, treatment regimes lowering HER2 should be beneficial. Korkaya *et al.* [41] showed that the expression of HER2 was related to the expression and activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway. In this study, the expression of HER2 was significantly decreased after treatment with either DFMO or PG11047 for 72 and 96 h, the major difference being noted after 96 h (Fig. 2b).

Treatment with PG11047 also decreased the expression of CD44 and p-Akt1, whereas the expression of nonphosphorylated inactive Akt1 (Akt1) was slightly increased (Fig. 2b). The reverse effects were seen after DFMO treatment. The ratio between the active p-Akt1 and

Fig. 1



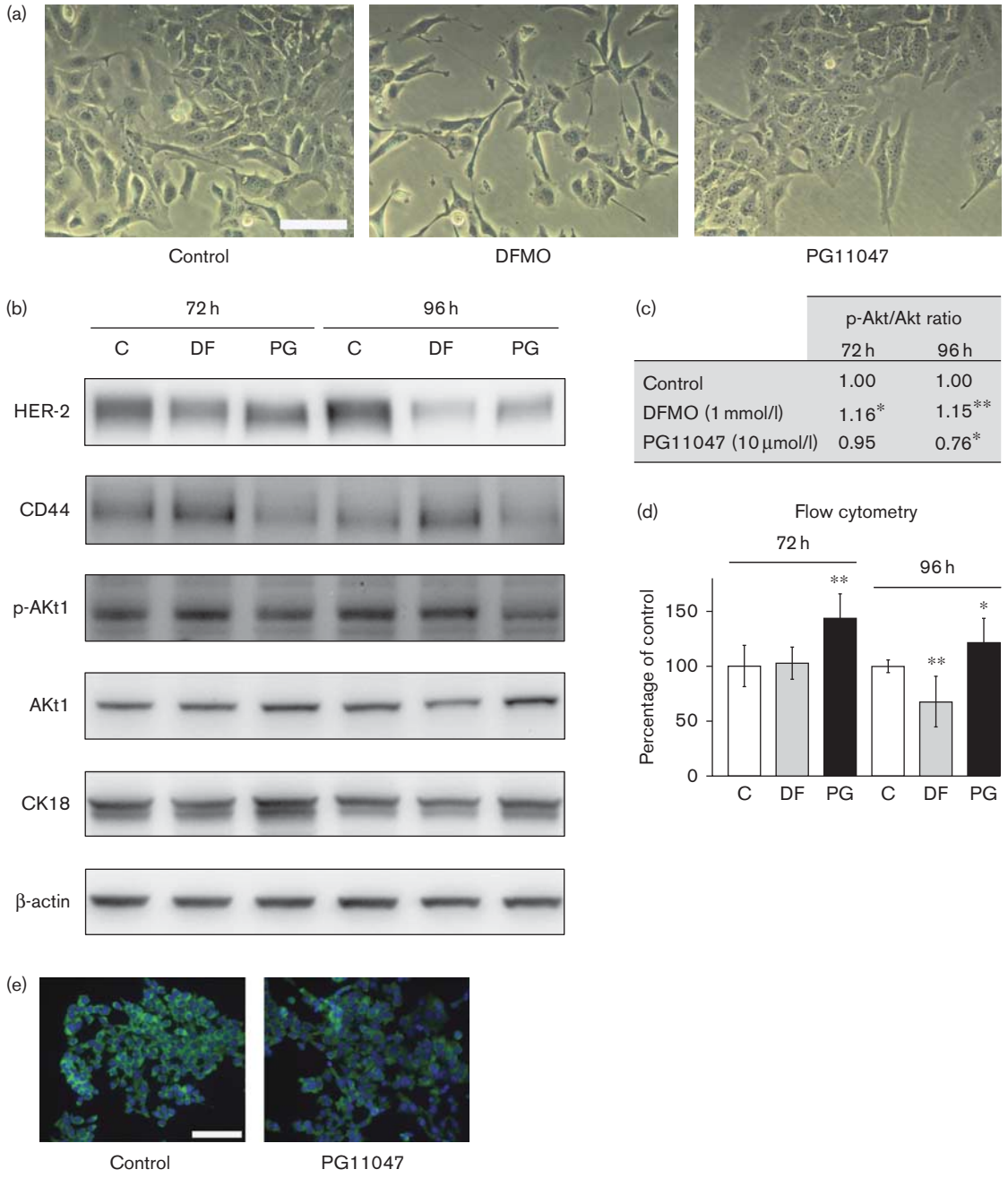
(e)

Treatment (72 h)	$CD44^+CD24^-$ (Percentage of control ± SD)
DFMO (1 mmol/l)	110 ± 12
PG11047 (10 μmol/l)	45 ± 14
Paclitaxel (100 nmol/l)	127 ± 37

[^1N,^{12}N]Bis(ethyl)-*cis*-6,7-dehydrospermine (PG11047) treatment reduced the $CD44^+CD24^-$ population in the human epidermal growth factor receptor 2 overexpressing JIMT-1 breast cancer cell line. Cells were seeded and treated with either 10 μmol/l PG11047 or 1 mmol/l 2-difluoromethylornithine (DFMO) for 24–96 h [addition of the compound 24 h after seeding, that is, at time 0 shown in (a)]. Cell proliferation was determined by cell counting (a) and cell death (percentage of cells in sub-G_1) was evaluated using flow cytometry (b). Effect of the treatments on the cancer stem cells population was evaluated using flow cytometry after the staining of cells with CD44-fluorescein isothiocyanate (FITC) and CD24-phycoerythrin (PE) antibodies (c–e). (d) Control and PG11047-treated cells after 24 and 48 h of treatment. (e) Cells were treated with 100 nmol/l paclitaxel for 72 h as a comparison with PG11047-treated or DFMO-treated cells. Data from one representative experiment is shown in (a–d) from a minimum of two independent experiments with $n = 6–9$. A summary of two experiments is shown in (e). Mean ± standard deviation (SD), $n = 6–9$.

inactive Akt1 was calculated based on densitometric scanning as described earlier (Fig. 2c). A ratio above 1 implies an increase in p-Akt1 and a ratio below 1 implies a decrease in p-Akt1 compared with the control. Thus, treatment with DFMO increased the ratio from 1 to 1.16 (72 h) and 1.15 (96 h), indicating that there was an increase

Fig. 2



Effect of treatment on the expression of proteins related to mesenchymal and epithelial phenotypes. JIMT-1 cells were treated with either 10 μmol/l [^{1}N,^{12}N]-bis(ethyl)-*cis*-6,7-dehydrospermine (PG11047) or 1 mmol/l 2-difluoromethylornithine (DFMO) (addition of compound 24 h after seeding) for 96 h and the cells were photographed in the inverted phase-contrast microscope (a). The expression of human epidermal growth factor receptor 2 (HER2), CD44, cytokeratin 18 (CK18), p-Akt1 and Akt1 was investigated after 72 and 96 h of treatment using western blot (b). Beta-actin was used as a loading control. The bands of p-Akt1 and Akt1 were densitometrically scanned and the ratios were calculated (c). CK18 expression was also investigated using flow cytometry after the labelling of cells with CK18-fluorescein isothiocyanate antibodies (d). Vimentin was detected with immunofluorescence microscopy (e). Green, vimentin. Blue, bisbenzimide-stained cell nuclei. Bar = 100 μm. Data are from two independent experiments with $n = 3–6 \pm$ standard deviation. Significance was evaluated using a two-tailed unpaired Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

in p-Akt1 as compared with the control. Treatment with PG11047 reduced the ratio from 1 to 0.95 (72 h) and 0.76 (96 h), indicating that there was a decrease in p-Akt1 as compared with the control.

The expression of the luminal epithelial marker CK18 was investigated using western blot and flow cytometry. Treatment with PG11047 significantly increased CK18 expression to 144% and 121% of the control after 72 h and

96 h, respectively (Fig. 2b and d). After 96 h of DFMO treatment, there was a significant decrease in the expression of CK18 (Fig. 2b and d).

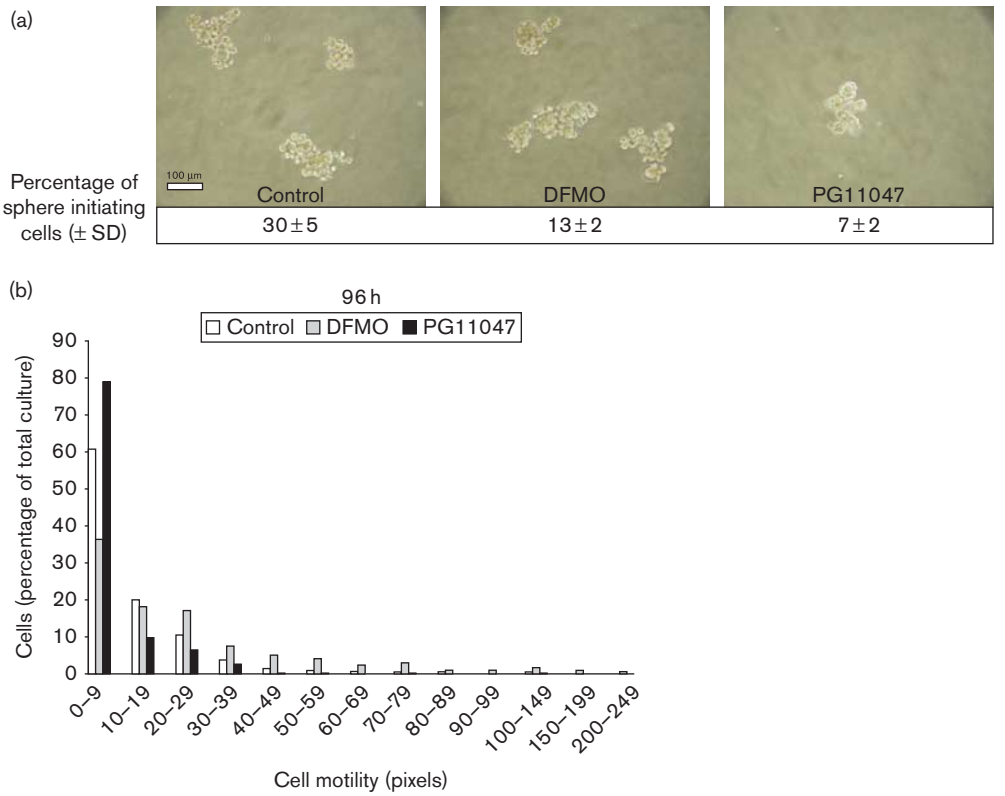
The mesenchymal marker vimentin was investigated with immunofluorescence microscopy in the control and PG11047-treated cells (Fig. 2e). The expression of vimentin was significantly reduced (40%, $P < 0.05$) in the cells treated with PG11047 for 96 h.

In summary, treatment with PG11047 decreased the protein expression of HER2, CD44, vimentin, along with the p-Akt1/Akt1 ratio, whereas the expression of CK18 increased. Treatment with DFMO decreased HER2, whereas CD44 and the p-Akt1/Akt1 ratio increased. The expression of CK18 decreased after DFMO treatment.

Treatments targeting polyamine metabolic pathways affect stem cell-renewal and cell motility in the breast CSC population

The long-time survival of a tumour requires that CSCs undergo a process of self-renewal to maintain the CSC population. Finding a chemotherapeutic drug that can interfere with the CSC renewal pathway would likely result in a better prognosis for cancer patients. To determine whether treatment with DFMO or PG11047 had an impact on self-renewal and on early progenitor cell proliferation, adherently grown JIMT-1 cells treated for 96 h with either PG11047 (10 μmol/l) or DFMO (1 mmol/l) were reseeded at a clonally low density in the absence of drugs and cultured as mammospheres for 10 days, as recently described by Dontu *et al.* [39]. The mammospheres formed are supposed to be clonally derived from single stem/progenitor cells, defined here as sphere-initiating cells, and not from cellular aggregation. We found that untreated cultures comprised 30% sphere-initiating cells, whereas treatment with DFMO or PG11047 significantly decreased ($P < 0.01$) this number to 13% and 7%, respectively (Fig. 3a). In addition, the size of the mammospheres formed from the PG11047-treated monolayer cells was generally smaller as compared with those from the untreated or DFMO-treated cultures. In summary, this may indicate that treatment with either PG11047 or DFMO affects the self-renewal process of CSCs.

Fig. 3



Effects of [^{1}N,^{12}N]-bis(ethyl)-*cis*-6,7-dehydrospermine (PG11047) or 2-difluoromethylornithine (DFMO) treatment on mammosphere formation and cell motility. JIMT-1 cells were treated with either 10 μmol/l PG11047 or 1 mmol/l DFMO for 96 h and reseeded at clonal density under anchorage-independent conditions (a). The number of formed mammospheres was determined by manual counting using a phase-contrast microscope after 10 days of incubation. Data is from two independent experiments [$n = 4 \pm$ standard deviation (SD)]. Cell motility was analysed after 96 h of treatment using digital holographic imaging (b). Digital photos were taken every 5 min for 4 h and time-lapse movies were constructed to track cell motility. Note that the cells were divided into different categories of movement shown on the x-axis. One pixel = 0.77 μm. One representative experiment is shown in the figure from three independent experiments with $n = 3$.

CSCs have been shown to exhibit properties of increased motility and invasiveness, which are part of the metastatic process. Treatment that could render cancer cells less prone to migration would likely improve cancer patient survival. Digital holography was used to study cell motility of the JIMT-1 cells treated with either 10 µmol/l PG11047 or 1 mmol/l DMFO for 96 h (Fig. 3b). There were significantly ($P < 0.01$) more cells in the low motility category (0–9 pixels) in the PG11047-treated cultures as compared with the control, whereas DFMO treatment significantly ($P < 0.01$) increased cell motility.

Discussion

Optimally, chemotherapeutic treatment of metastatic diseases should target the CSC population along with the remaining tumour cells (bulk cells). Inherent properties of resistance among the CSC population likely explain why conventional therapies fail to eradicate these cells and why tumour relapses frequently occur. Recent studies performed by Mani *et al.* [21] and Gupta *et al.* [42] suggest that the CSCs may have developed mesenchymal properties through the process of EMT. The CSC population can be diminished either by killing these cells and/or by inducing a more differentiated epithelial-like phenotype, that is, the reverse process of EMT involving a transition from a MET phenotype. In this study, we found that PG11047 treatment interfered with CSC self-renewal, cell motility and the process of EMT, whereas DFMO showed more modest effects.

The JIMT-1 cell line was established from a pleural metastasis of a breast cancer patient clinically resistant to trastuzumab [43]. As HER2 overexpression has been directly correlated with an enhanced number of CSCs [24,44], we used the JIMT-1 cell line as our experimental model system. We found that this cell line contains approximately 22–35% $CD44^+CD24^-$ putative CSCs, and that treatment with PG11047 reduced this population by approximately 50%. As there was no cell death, the reduction of the $CD44^+CD24^-$ population was most likely because of the PG11047-induced changes in gene expression. This is interesting in view of the observation that specific polyamine analogues are potent inhibitors of lysine-specific demethylase and have been shown to stimulate the reexpression of aberrantly silenced tumour-suppressor genes in treated colon cancer cells [45]. Treatment with DFMO in general does not result in cell death; however, treatment with PG11047 has been shown to induce cell death in a breast cancer cell line [33]. In contrast to PG11047 treatment, DFMO treatment resulted in an increased $CD44^+CD24^-$ population; however, it was not increased to the same extent as in the cultures treated with paclitaxel. Paclitaxel treatment has also been shown to increase the number of $CD44^+CD24^-$ cells by other investigators [28]. To the best of our knowledge, the only other compound besides PG11047 that reduces a putative $CD44^+/CD24^{-/low}$ breast CSC population is the antibacterial drug salinomycin, which also reduces the mRNA expression of CSC genes [42]. Thus, we decided to investigate how the level of proteins related to malignancy and HER2 expression were affected in the JIMT-1 cells treated with either PG11047 or DFMO.

It was earlier shown that the response to trastuzumab in HER2-overexpressing sensitive cell lines involved a reduction of the CSC population by decreasing HER2, which subsequently inhibited the PI3K/Akt pathway (downregulation of phospho-Akt) [24,43]. This phenomenon was not found in the trastuzumab-resistant cell lines, suggesting a participatory role for these proteins in the mechanism of trastuzumab resistance [24,43]. When trastuzumab-resistant HER2-overexpressing JIMT-1 cells were treated with PG11047, a response was observed that resembled that seen in trastuzumab-sensitive cell lines, that is, a decrease in HER2 expression and a decrease in p-Akt1, implying an inhibition of the PI3K/Akt pathway. This may be a promising observation for patients with trastuzumab-resistant tumours. In addition, DFMO treatment decreased the HER2 level, but did not decrease the p-Akt1 expression; rather, there was an increased expression of the protein. This might be coupled with the increased CD44 level found in the DFMO-treated JIMT-1 cells. CD44 has been shown to be involved in the activation of the PI3K/Akt pathway [46]. The reduced CD44 level in the PG11047-treated JIMT-1 cells may have contributed to the decreased p-Akt1 and HER2 levels.

As EMT may be a part of the process in the formation of CSCs that exhibit migratory and invasive properties, a chemotherapeutic treatment that reverses the process is likely to be beneficial. Our data showed that CK18, which is expressed in luminal epithelial cells, was increased in JIMT-1 cells after PG11047 treatment, suggesting a MET induction [47]. A downregulation of CK18 is directly related to the progression of breast cancer in humans, suggesting that an increase in CK18 expression suppresses tumour progression [48]. The mesenchymal protein vimentin was reduced in the cells treated with PG11047, also pointing to an MET induction.

A 13-gene transcriptional marker set, developed using a panel of breast cancer cell lines, has been suggested as a predictor of the response to PG11047 treatment [32]. It was also shown that a high p-Akt1 level predicted sensitivity, whereas high levels of Akt and CK18 predicted resistance [32]. This may seem contradictory to our results; however, the JIMT-1 cell line was not included in their study and thus it is not possible to compare the high and low levels.

An implicit property of CSCs is the ability to self-renew. The reduction in the number of mammospheres formed in the PG11047-treated cultures was greater than the reduction in the $CD44^+CD24^-$ population. The difference was even more remarkable for the DFMO-treated cultures

in which there was an increase in the $CD44^+CD24^-$ population (10%), but the number of mammospheres was reduced by 57%. Our data suggest that both DFMO and PG11047 interfere with the self-renewal pathway. Although DFMO treatment resulted in an increased $CD44^+CD24^-$ population, this did not result in an increase in cells with self-renewal properties. There is intensive research regarding the exact identity of the breast CSCs. Most likely, all $CD44^+CD24^-$ cells are not CSCs and presumably only a subpopulation of these cells can be identified as true CSCs using additional markers [49].

It has been suggested that PG11047 affects pathways involved in aspects of motility and EMT [32]. As discussed above, PG11047 treatment of JIMT-1 cells resulted in changes in protein expression pointing to a MET-like process. Along with these findings, treatment with PG11047 reduced cell motility. This observation may be correlated with the down-regulation of CD44 [50]. Contrary to PG11047 treatment, DFMO treatment clearly enhanced overall cell motility in JIMT-1 cells. Currently we have no explanation for this observation, which is in contrast to studies showing decreased cell motility of DFMO-treated cells [51–53]. However, it may be related to an increase in the expression of CD44 in the DFMO-treated cells.

Future research will focus on combining PG11047 (CSC selective and MET inducing) with conventional chemotherapeutic drugs that are cytotoxic, and with targeted therapies.

Conclusion

Our data show that treatment of trastuzumab-resistant HER2-overexpressing JIMT-1 cells with either PG11047 or DFMO reduced the rate of cell proliferation and decreased the self-renewing capacity of the treated cells, as expressed by the significantly reduced mammosphere-forming ability of single cells. In addition, PG11047 treatment resulted in a decreased $CD44^+CD24^-$ putative CSC population and decreased cell movement. Furthermore, PG11047-treated cells expressed higher levels of proteins that are related to an epithelial phenotype and lower levels of those proteins that are related to a mesenchymal phenotype. Although DFMO treatment decreased the self-renewal properties in JIMT-1 cells, there was an increase in the $CD44^+CD24^-$ putative CSC population and the cells migrated at a higher rate. DFMO treatment resulted in a mesenchymal-like phenotype. Our results suggest that PG11047, which decreased the CSC population size and self-renewal ability and cell motility, should be combined with a commonly used chemotherapeutic drug.

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