NF-κB pathway inhibitors preferentially inhibit breast cancer stem-like cells

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Abstract  Accumulating evidence indicates that breast cancer is caused by cancer stem cells and cure of breast cancer requires eradication of breast cancer stem cells. Previous studies with leukemia stem cells have shown that NF-κB pathway is important for leukemia stem cell survival. In this study, by using MCF7 sphere cells as model of breast cancer stem-like cells, we evaluated the effect of NF-κB pathway specific inhibitors on human breast cancer MCF7 sphere cells. Three inhibitors including parthenolide (PTL), pyrrolidinedithiocarbamate (PDTC) and its analog diethyldithiocarbamate (DETC) were found to preferentially inhibit MCF7 sphere cell proliferation. These compounds also showed preferential inhibition in term of proliferation and colony formation on MCF7 side population (SP) cells, a small fraction of MCF7 cells known to enrich in breast cancer stem-like cells. The preferential inhibition effect of these compounds was due to inhibition of the NF-κB activity in both MCF7 sphere and MCF7 cells, with higher inhibition effect on MCF7 sphere cells than on MCF7 cells. PDTC was further evaluated in vivo and showed significant tumor growth inhibition alone but had better tumor growth inhibition in combination with paclitaxel in the mouse xenograft model than either PDTC or paclitaxel alone. This study suggests that breast cancer stem-like cells could be selectively inhibited by targeting signaling pathways important for breast cancer stem-like cells.

Keywords  Breast cancer stem-like cells · Side population cells · NF-κB · Sphere cells · Xenograft

Abbreviations
PTL Parthenolide
PDTC Pyrrolidinedithiocarbamate
DETC Diethyldithiocarbamate
SP Side population
ABC ATP-binding cassette

Introduction
Breast cancer is the most frequent malignancy among women in Western countries, with an incidence in the U.S. of 111 cases per 100,000 woman-years (wy) and a mortality rate of 24 deaths per 100,000 wy [1]. Although the mortality of breast cancer has been decreasing [1, 2], which was believed to be the result of widespread mammography screening and the implementation of adjuvant therapy with tamoxifen and polychemotherapy [3, 4], breast cancer still is the most fatal disease for women in Western countries [1].

In 2003, Clarke and colleagues demonstrated that a highly tumorigenic subpopulation of breast cancer stem cells expressing CD44⁺CD24⁻ surface marker in clinical specimen had the capacity to form tumors with as few as one hundred cells whereas tens of thousands of the bulk cells did not [5]. Recently, accumulating evidence indicates that breast cancer is originated from breast cancer stem cells, a rare population within breast tumor [5, 6]. Since the current cancer drugs, which are developed extensively based on their activity to inhibit bulk replicating cancer stem cells.
cells, may not be able to eliminate the cancer stem cells effectively, which have been demonstrated in a variety of tumors [7–13]. It is conceivable that improved breast cancer treatment requires eradication of cancer stem cells [6–8, 14, 15].

Although the breast cancer stem cells were initially identified in primary patient samples, cancer stem cells from patient samples are limited for breast cancer stem cell research because of the limited source. Meanwhile, some breast cancer cell lines were reported to harbor potential cancer stem-like cells. For instance, by using a sphere culture technique, MCF7 sphere cells were found to enrich breast cancer stem-like cells expressing CD44+CD24− [16]. Another approach to enrich breast cancer stem-like cells is to isolate the side population (SP) cells by flow cytometry [17, 18]. SP cells were first defined in hematopoietic system [19]. Although the detailed mechanism for the generation of the SP phenotype is still unknown, it is believed that some ATP-binding cassette (ABC) transporters including ABCG2/BCRP, ABCB1/MDR1 and ABCA3, might be involved in pumping out the fluorescent dye Hoechst 33342, causing the SP phenotype [20–22]. Based on the property to pump out Hoechst dye, SP cells could be isolated from different breast cancer cell lines, such as human breast cancer cell line MCF7 and SKBR3 [17, 18, 20]. Patrawala and colleagues reported that MCF7 SP cells had higher tumorigenicity than non-SP cells [18], which indicates that MCF7 SP cells enrich breast cancer stem-like cells.

In this study, based on the leukemia stem cell research, we intended to study the role of NF-κB pathway in breast cancer stem-like cell using MCF7 sphere cells and SP cells as models. Leukemia stem cells (LSC), the first cancer stem cell defined in the early 1990s [14, 23, 24], was the main cancer stem cell model for studying biology of cancer stem cells in the past decade. Leukemia stem cell study has benefited solid tumor stem cell study. For example, it was demonstrated the NF-κB activity was selectively inhibited in breast cancer stem-like cells with known NF-κB pathway inhibitors. Our data indicated that PTL, PDTC and its analog DETC could preferentially inhibit LSC cells [25–27]. Here we first tested the sensitivity of breast cancer stem-like cells with known NF-κB pathway inhibitors. Our data indicated that PTL, PDTC and its analog DETC could preferentially inhibit both MCF7 sphere cells and SP cells, suggesting that these compounds were capable of preferentially inhibiting breast cancer stem-like cells. The mechanism was demonstrated to be mediated through inhibition of the NF-κB activity. In particular, these compounds could selectively inhibit the NF-κB activity better in MCF7 sphere cells than in MCF7 bulk cells. PDTC was further evaluated in the mouse xenograft model and found to be effective in inhibiting tumor growth alone and achieved a better tumor inhibition in combination with paclitaxel than PDTC or paclitaxel alone in vivo.

Materials and methods

Cell culture

Human breast cancer cell line MCF7 cells were obtained from ATCC (American Type Culture Collection). Cells were grown in DMEM medium (Invitrogen) supplemented with 0.1% fetal bovine serum (Invitrogen), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen), in a 37°C incubator containing 5% CO2. Sphere cell culture was performed according to the published protocol with some modifications [16, 28]. Briefly, single cells were plated in ultralow attachment plates (Corning, NY) at a density of 20,000 viable cells/ml in primary culture and 1,000 cells/ml in passages. Cells were grown in a serum-free mammary epithelial growth medium without bovine pituitary extract (MEGM, BioWhittaker), but supplemented with B27 (Invitrogen), 20 ng/ml EGF and 20 ng/ml bFGF (BD Biosciences). In order to passage sphere cells, spheres were collected into 15 ml tube and allowed to settle for 15 min. Supernatant was removed. Sphere cells were dissociated enzymatically with 0.05% trypsin, 0.5 mM EDTA (Invitrogen) and mechanically by a glass Pasteur pipette. The cells obtained from dissociation were passed through a 40-μm sieve and analyzed microscopically for single cells and subjected to the following experiments.

Hoechst 33342 staining and flow cytometry analysis/sorting of SP cells

To identify and sort SP and non-SP fractions, cells were washed with PBS and detached from the culture dish with trypsin and EDTA, pelleted by centrifugation, and resuspended in 37°C DMEM containing 2% FBS at 1 × 10^6 cell/ml. Cell staining was performed according to the protocol [29] with slight modification. The cells were then incubated with Hoechst 33342 (Sigma) at 5 μg/ml either alone or in combination with 50 μM verapamil (Sigma) for 90 min at 37°C. Following staining, the cells were spun down and resuspended in HBSS (Invitrogen, Carlsbad, CA) containing 1 μg/ml propidium iodide and maintained at 4°C for flow cytometry analysis/sorting. Cell analysis and sorting were performed on a Moflo flow cytometer (Dako Cytomation, Fort Collins, CO, USA) equipped with a Coherent Enterprise II laser emitting MLUV at 351 nm and blue 488 nm lines. The Hoechst 33342 emission was first split using a 610dsp filter and then the red and the blue...
emissions were collected through a 670/30 nm and a 450/65 nm bandpass filters, respectively.

Cell proliferation assay

To test the sensitivity of MCF7 bulk cells and sphere cells to specific compounds, both MCF7 bulk cells and sphere single cells were seeded at concentration of $3 \times 10^4$ cells/ml in 96-well plates. After overnight incubation, serial concentrations of tested compounds were added. Each concentration was repeated three times. These cells were incubated in a humidified atmosphere with 5% CO$_2$ for 3 days. Then, 20 $\mu$l MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma) solution (4.14 mg/ml) was added to each well and incubated at 37°C for 4 h. The medium was removed and formazan was dissolved in DMSO and the optical density was measured at 590 nm using a Bio-assay reader (Bio-Rad, USA). The growth inhibition was determined using: Growth inhibition = (control’s O.D. – sample’s O.D.)/control’s OD.

To test the drug sensitivity of SP cells, both MCF7 SP and non-SP cells were sorted into 96-well culture plates at 500 cells per well and incubated in DMEM medium at 37°C in an incubator containing 5% CO$_2$ for 24 h. Then, cells were treated with compounds at concentration as indicated in the text. After 72 h exposure to the tested agents, proliferation of the cells was determined by using a fluorescence-based cell proliferation assay (CyQUANT Cell Proliferation Assay Kit, Molecular Probe). Fluorescence signal was detected by a Bio-assay reader (Bio-Rad, USA) according to the manufacturer’s instructions.

Quantification of NF-$\kappa$B activation

Trans-AM NF-$\kappa$B assay, an enzyme-linked immunosorbent assay (ELISA)—based method (Trans-AM NF-$\kappa$B; Active Motif, Carlsbad, CA) was used for NF-$\kappa$B activity quantification according to the manufacturer’s instruction. Briefly, cell nuclear extracts were placed in 96-well plates coated with an oligonucleotide containing the NF-$\kappa$B consensus sequence, and the presence of active NF-$\kappa$B was detected by using antibodies specific for p50 subunits that are not complexed to I$\kappa$B and thus are able to bind the consensus sequence. A horseradish peroxidase (HRP)—conjugated secondary antibody is used to quantify NF-$\kappa$B binding by conversion of an applied chromogenic substrate.

Colonial formation assay

The colony formation assay was carried out in 35 mm dishes. Briefly, both SP and non-SP cells were plated in 35 mm dishes at 5,000 cells/well in 0.35% top agar in culture medium over a 0.5% agar layer. For compound testing group, compounds were added into the top agar at concentrations as indicated. Plates were further incubated in cell culture incubator for 12 days until colonies were large enough to be visualized. Colonies were stained with 0.01% Crystal Violet for 1 h and counted. Experiments were done in triplicate.

Nuclear extract preparation

Nuclear extracts were prepared according to the protocol by ActiveMotif Company (Active Motif, Carlsbad, CA). Cells were washed and collected in ice-cold PBS/PIB buffer and resuspended in 10 ml of ice-cold hypotonic buffer containing 20 mM HEPES (pH7.5), 5 mM NaF, 10 $\mu$M Na$_2$MoO$_4$, 0.1 mM EDTA. PBS/PIB buffer was prepared by adding 0.5 ml of PIB (125 mM NaF, 250 mM $\beta$-glycerophosphate, 250 mM para-nitrophenyl phosphate (PNPP) and 25 mM NaVO$_3$) to 10 ml of 1× PBS prior to use. Following a 15 min incubation on ice, 50 $\mu$l of a 10% Nonidet P-40 solution was added and mixed by gentle pipetting. Nuclei were then pelleted by centrifugation at 14,000 × g for 30 s, washed with the hypotonic buffer and resuspended in 50 $\mu$l ice-cold complete lysis buffer (Active Motif, Carlsbad, CA). After the nuclear lysates were centrifuged, supernatants were collected for quantification of NF-$\kappa$B activation. The protein concentrations in the supernatants were determined using the BCA protein assay kit (Pierce, Rockford, IL).

Antitumor activity of PDTC in tumor xenograft model

Female athymic nude mice (NCR-nu/nu, NCI) were housed under specific pathogen-free conditions. The in vivo experiments were performed in accordance with the guidelines of our institute. For mice in MCF7 xenograft study, the mice were given injections of $\beta$-estradiol (Sigma) dissolved in pure sesame oil (0.1 mg per 0.05 ml sesame oil per mouse, subcutaneously) 1 day before the injection of MCF7 cells and then at weekly intervals [30, 31]. Mice were inoculated subcutaneously with $2 \times 10^7$ MCF7 cells. When the tumor volumes reached 100–200 mm$^3$, the mice were randomly divided into groups as indicated such that each group harbored tumors of a similar size. Each group included 5 mice. Stock solution of
Paclitaxel was prepared by dissolving the drug in a vehicle solution (EtOH:cremophor, 50:50 v/v). PDTC was dissolved in saline. Paclitaxel alone and paclitaxel in combination with PDTC stock solution were mixed with physiologic saline or saline containing PDTC (10:90 v/v). A dosing solution (200 µl) was intravenously injected, over 1 min via a tail vein. The PDTC dose was 60 mg/kg, and the paclitaxel dose was 10 mg/kg. Tumor measurements were done twice a week using traceable digital vernier calipers (Fisher). The tumor volumes were determined by measuring the length (l) and the width (w) and calculating the volume using the formula $V = lw^2/2$.

Statistical analysis

The growth inhibition effect was compared by Student’s $t$ test. $P < 0.05$ was considered statistically significant.

Results

PTL, PDTC and DETC preferentially inhibit MCF7 sphere cell proliferation

It has been shown that NF-κB pathway specific inhibitors, including MG-132, PTL and PDTC could selectively inhibit leukemia stem cell proliferation [25–27]. To study the sensitivity of breast cancer stem-like cell to NF-κB pathway inhibitors, 11 compounds targeting different steps of the NF-κB pathway, including antioxidants Curcumin [32]; PDTC [33]; DETC [34]; Quercetin [35], NF-κB phosphorylation inhibitors Sulfasalazine [36]; Sulindac [37]; Ibuprofen [38]; PTL [39] and NF-κB degradation inhibitors MG-132 [40]; Cyclosporin A [41]; Genistein [42], were tested in this study. MCF7 sphere cells were used as a model of breast cancer stem-like cells [16, 43]. Among all the inhibitors, antioxidants which inhibit NF-κB activation including PDTC and its analog DETC, and NF-κB phosphorylation inhibitors PTL were shown to preferentially inhibit sphere cell proliferation. As shown in Fig. 1, PTL, PDTC and DETC at 1 µM inhibited MCF7 sphere cell growth by 33.2, 50.8 and 52.2%, respectively, but did not show obvious growth inhibition effect on MCF7 bulk cells. In contrast, cancer drug paclitaxel (2.5 nM) inhibited sphere cells by 6.1%. These data indicate that, unlike paclitaxel that act primarily on replicating bulk MCF7 cells, PTL, PDTC and DETC selectively inhibited MCF7 sphere cell proliferation over MCF7 bulk cells.

PTL, PDTC and DETC could preferentially inhibit MCF7 SP cell proliferation and colony formation

Apart from sphere cells cultured from MCF7 cells, side population (SP) cells isolated from MCF7 cells were also found to enrich breast cancer stem-like cells, which showed higher colony formation ability in vitro and in vivo tumorigenicity than non-SP fraction [18]. MCF7 SP cells were shown to contain 1.2% SP cells, which could significantly be blocked by ABC transporter inhibitor verapamil (0.12%) (Fig. 2a). It was of interest to test whether the various NF-κB inhibitors that are preferentially active against sphere cells as above have similar effect on MCF7 SP cells. For this purpose, both SP and non-SP cells were sorted out by flow cytometry as indicated by trapezoids on the left (R5) and right (R8), respectively (Fig. 2a), and seeded into 96 well plates at 500 cells/well. After overnight incubation, both SP and non-SP cells were treated with PDTC, DETC and PTL for 3 days. Cell proliferation was determined using a fluorescence-based cell proliferation assay as indicated above. Interestingly, unlike cancer drug paclitaxel (2.5 nM), which inhibited MCF7 SP cells by 39.3%, and non-SP cells by 52.2%, all the three compounds showed preferential inhibition for the MCF7 SP cells over the non-SP cells (Fig. 2b). PTL (5 µM) inhibited MCF7 SP cell growth by 95.2% and non-SP cells by 66.1% (Fig.2b). Both PDTC and DETC also showed higher ability to inhibit both SP and non-SP proliferation. As shown in Fig. 2b, 1 µM of PDTC and DETC preferentially inhibited MCF7 SP cells by 86.3 and 94.5%, but inhibited non-SP cells by 74.4 and 71.9%, respectively. Similarly, PTL (5 µM)
inhibited colony formation of MCF7 SP cells by 38.7% but 8.7% for non-SP cells (Fig. 2c). In contrast to the above NF-κB inhibitors, the control cancer drug paclitaxel showed the reverse inhibition effect, that is, paclitaxel inhibited MCF7 SP and non-SP colony formation by 15.4 and 39.2%, respectively.

Taken together, these data indicate that, unlike cancer drug paclitaxel, PTL, PDTC and DETC could preferentially inhibit MCF7 SP cell proliferation and colony formation over non-SP cells.

PTL, PDTC and DETC preferentially inhibit NF-κB activity in MCF7 sphere cells

To investigate the mechanism by which breast cancer stem-like cells are more sensitive to these inhibitors, we compared the NF-κB activity in sphere cells to the bulk MCF7 cells under the same treatment. An ELISA-based method (Trans-AM NF-κB; ActiveMotif, Carlsbad, CA) was used to quantify the NF-κB activation because of its high sensitivity to detect and quantify NF-κB activation compared to the more traditional method EMSA [44] or Western blot or gel shift assay. Although there is no significant difference in the basal level of NF-κB activity in MCF7 bulk cells and MCF7 sphere cells (data not shown), all the three compounds, PTL, PDTC and DETC, reduced the NF-κB activity within MCF7 sphere cells but had less effect for MCF7 bulk cells (Fig. 3). As shown in Fig. 3, when treated with 5 μM PTL, PDTC and DETC for 24 h, NF-κB activity in MCF7 sphere cells was decreased by 94.6, 61.8 and 70.1%, respectively. However, the treatment only inhibited the NF-κB activity within MCF7 cells by 38.8, 33.6 and 47.3%, respectively (Fig. 3). These data suggest that the preferential inhibition effects of PTL, PDTC and DETC were caused by inhibition of the NF-κB activity.

Effects of PDTC on tumor growth in nude mice

PDTC was chosen for further investigation in this study. To provide evidence that PDTC specifically inhibited cancer stem-like cells within MCF7 cells, we treated MCF7 cells with PDTC (5 μM) for 24 h and examined the SP fraction within MCF7 cells by flow cytometry. Interestingly, PDTC significantly decreased the SP fraction within the MCF7 cells. As shown in Fig. 4a, the SP fraction within MCF7 cells was only 0.27% after treatment, compared with 1.20% in control MCF7 cells (Fig. 4a). Since PDTC specifically inhibited breast cancer stem-like cells, it is possible that combination of PDTC with clinical drug like paclitaxel could have enhanced effects on treatment of breast cancer. To test this, we performed both in vitro and in vivo experiments to compare the combination effect of PDTC with clinical drug paclitaxel with either drug alone. Indeed, combination of PDTC with paclitaxel showed synergistic...
effect on MCF7 sphere cells in vitro. PDTC alone at the concentrations of 0.5 and 1 μM inhibited MCF7 sphere cell growth by 44.8 and 58.8% whereas paclitaxel alone inhibited MCF7 sphere cell growth by 15.6% (Fig. 4b). In contrast, the combination of PDTC with paclitaxel showed better growth inhibition effects than either PDTC or paclitaxel alone. As shown in Fig. 4b, combinations of paclitaxel (1 μM) with PDTC (0.5 μM) and with PDTC (1 μM) inhibited MCF7 sphere cells by 60.7 and 89.6%, respectively. We further evaluated the antitumor effect of PDTC in MCF7 nude mouse xenograft model. As shown in Fig. 4c, PDTC alone showed certain antitumor effect in vivo. When combined with paclitaxel, PDTC produced a better inhibitory effect on tumor growth than either PDTC or paclitaxel alone (Fig. 4c). DETC, which is an analog of PDTC, was also tested in the xenograft model and found to have similar growth inhibitory effects as PDTC (data not shown).

Discussion

Although the breast cancer stem cells are the first solid tumor stem cells identified in 2003 [5], breast cancer stem cell research is still in its early stage and a feasible model is needed for breast cancer stem cell research. Currently, most cancer stem cells were identified in primary patient samples, which is limited for cancer stem cell research because of their limited supply. In contrast, cancer stem cells from cell lines could be a promising model for cancer stem cell research because of its unlimited availability and easy handling. Very recently, sphere cells and side population cells isolated or cultured from human breast cancer cell line MCF7 were reported to enrich breast cancer stem-like cells [16, 18], which have higher tumorigenicity and can be used as a model for breast cancer stem cell study [43]. Besides the model, new approaches are also needed to target breast cancer stem cells. Leukemia stem cell research established the original paradigm of cancer stem cells and has shed light on the study to identify and characterize solid tumor stem cells [45–47]. Importantly, it may also suggest the direction for elimination of solid tumor stem cells. For instance, one promising approach to eliminate leukemia stem cell is to target signaling pathways important for leukemia stem cell survival or self-renewal [48–50]. Some signaling pathways, including the PI3K pathway, NF-κB pathway, mTOR signaling and PTEN signaling, were found to be preferentially important for leukemia stem cell survival or/and self-renewal, which could be selectively targeted for elimination of leukemia stem cells [25–27, 51, 52].

In this study, by using MCF7 sphere cells as a model, we investigated the effect of NF-κB pathway inhibitors on breast cancer stem-like cells. Interestingly, NF-κB pathway inhibitors, including PTL, PDTC and DETC, were found to preferentially inhibit MCF7 sphere cell proliferation over parental MCF7 cells. PTL is a compound extracted from Tanacetum parthenium [53] and is known to inhibit the NF-κB pathway by preventing IkBa degration [54], inhibiting IkB kinase b [55] and alkylating of p65 [56]. PDTC and DETC are known to be antioxidants which inhibit the NF-κB pathway through blocking activation of nuclear factor kappa B (NF-kappa B) [33] and also inhibiting the IKK activity [57] and IxB-κ [58]. Like their effect on breast cancer stem-like cells revealed in this study, both PTL and PDTC could preferentially inhibit leukemia stem cell [26, 27]. Cancer drug paclitaxel is different from PTL,
PDTC and DETC, in that it had better activity for bulk MCF7 cells than for MCF7 sphere cells. In addition, all the three compounds, unlike paclitaxel, also showed a preferential inhibitory effect on colony formation of MCF7 SP cells. Since both MCF7 sphere cells and SP cells are known to enrich in breast cancer stem-like cells [16, 18], these data indicate that the three compounds, PTL, PDTC and DETC, which are different from common cancer drug paclitaxel, could preferentially inhibit breast cancer stem-like cells. It is interesting to note that while there is no apparent difference in the basal level of NF-κB activity of the MCF7 sphere cells and bulk cells, treatment with PTL, PDTC and DETC preferentially inhibited the NF-κB activity in the sphere cells over bulk cells (Fig. 3). NF-κB is known to be a suppressor of apoptosis, and it is conceivable that its role in the more quiescent cancer stem-like sphere cells is more important than in the bulk cells. The mechanism of action of PTL, PDTC and DETC for MCF7 sphere cells is related to the inhibition of NF-κB activity. These compounds caused greater inhibition of the NF-κB activity in MCF7 sphere cells than in MCF7 bulk cells, suggesting that the NF-κB pathway might be preferentially vulnerable in MCF7 sphere cells than the MCF7 bulk cells. Further studies are needed to determine what step in the NF-κB pathway is more important in sphere cell survival.

To substantiate the in vitro activity of PDTC on breast cancer stem-like cells, we evaluated PDTC alone and in combination with paclitaxel in the mouse xenograft model. PDTC alone showed significant inhibition effect on tumor growth. Interestingly, when combined with paclitaxel, PDTC had a higher inhibition on tumor growth than paclitaxel or PDTC alone (Fig. 4c). DETC, an analog of PDTC, also showed similar tumor growth inhibition effect in vivo as PDTC (data not shown). Our results are consistent with the previous finding that PTL in combination with docetaxel could reduce metastasis and improve survival in a xenograft model of breast cancer [59]. Taken together, this study indicates that it is possible to inhibit breast cancer stem cells by targeting the NF-κB pathway. Future studies are needed to investigate other signaling pathways for breast cancer stem cells that may be exploited for development of new drugs that target cancer stem cells for improved treatment of breast cancer.

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