

Targeting drug resistant stem cells in a human epidermal growth factor receptor-2-enriched breast cancer model

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Abstract. Human epidermal growth factor receptor-2 (HER-2) expression in the presence or absence of hormone receptors dictates HER-2-targeted therapy with or without endocrine therapy. These treatment options lead to long-term systemic toxicity, multi-drug resistance and the emergence of therapy-resistant cancer stem cells. These limitations emphasize the need for the development of reliable model systems with which to evaluate stem cell signaling molecules as testable targets for stem cell therapy, and to identify less toxic testable alternatives for therapeutic resistance. In this study, with the aim of identifying such a system, HER-2-positive tumorigenic 184-B5/HER cells were used as a model of HER-2-enriched breast cancer. The epidermal growth factor receptor (EGFR)/HER-2 specific small molecule inhibitor, lapatinib (LAP), was used to select the drug resistant phenotype. Vitamin A derivative, all-trans-retinoic acid (ATRA), and rosemary terpenoid carnosol (CSOL) represented the test agents. The LAP-resistant (LAP-R) phenotype exhibited an upregulated expression of select stem cell specific markers, including tumor spheroids, CD44, NANOG and Oct-4. These data provide evidence for the effective development of a LAP-R cancer stem cell model. ATRA and CSOL inhibited the expression of stem cell-specific markers in the LAP-R phenotype, suggesting that natural products may possess stem cell targeted therapeutic efficacy via the downregulation of select signaling molecules that are functional in cancer stem cells. On the whole, the findings of this study validate a cancer stem cell model which is able to identify natural products as testable alternatives for the treatment of therapy-resistant HER-2-enriched breast cancer.

Introduction

Genetically defined molecular subtypes of clinical breast cancer facilitate the accurate prediction of disease progression and the rational selection of targeted therapeutic options (1). The expression of human epidermal growth factor receptor-2 (HER-2) on a background of estrogen receptor- α (ER- α) and progesterone receptor (PR) positivity or negativity dictates distinct therapeutic options. These conventional therapeutic options include HER-2 selective antibodies and dual specific small molecule inhibitors with or without selective estrogen receptor modulators and aromatase inhibitors (2-4). These treatment options are frequently associated with systemic toxicity, acquired tumor resistance and the emergence of drug-resistant cancer stem cells, favoring the progression of therapy-resistant disease (5). These clinical limitations emphasize the need for the development and molecular characterization of reliable cancer stem cell models, and for the identification of testable alternative agents that display manageable systemic toxicity and exhibit stem cell targeted therapeutic efficacy.

Naturally occurring phytochemicals and herbal extracts possessing minimal systemic toxicity may represent testable alternatives to conventional chemoendocrine therapy for clinical breast cancer (6,7). Published evidence on the present model for HER-2 enriched breast cancer has demonstrated potent anti-proliferative and pro-apoptotic effects of several mechanistically distinct naturally occurring compounds, including phytoalexins (8), isoflavones (9-11), vitamin A derivatives (12,13) and phenolic terpenoids (14,15). This documented evidence for the susceptibility of the present model raises a possibility that natural products may also be effective as testable alternatives for cancer stem cell targeted therapeutic efficacy.

The cancer stem cell-enriched population derived from the primary tumor is characterized by the presence of a tumor-initiating drug-resistant phenotype (16,17). The expression of the stem cell-specific molecular markers, octamer binding transcription factor-4 (Oct-4), sex determining region Y-box-2 (SOX-2) and the DNA binding homeobox nuclear transcription factor, NANOG, are associated with compromised differentiation and overall survival in HER-2-positive clinical breast cancer (18). Drug-resistant stem cell models have been developed from the luminal A, HER-2-enriched and triple-negative molecular subtypes for clinical breast cancer, and the drug-resistant stem cell phenotypes in these models exhibit

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an upregulated cellular expression of select nuclear transcription factors, including Oct-4, NANOG and c-Myc (19-21). Thus, the expression status of transcription factors represents quantitative molecular markers in the developed stem cell models.

The present study utilized a cellular model of human mammary epithelial cells that exhibits tumorigenic transformation due to the targeted expression of the HER-2 oncogene. These tumorigenic cells lack the expression of estrogen and progesterone receptors. Thus, the expression of HER-2 on an ER- α -negative and PR-negative background provides a model for HER-2-enriched breast cancer. The experiments carried out in this study were designed to: i) Develop a drug-resistant stem cell model for HER-2-enriched breast cancer; and ii) examine the stem cell targeted therapeutic efficacy of select natural agents.

Materials and methods

The experimental model used in the current study is described below.

Cells and cell culture. The 184-B5/HER tumorigenic cell line is derived from parental 184-B5 cells that were stably transfected with the HER-2 oncogene (22). The 184-B5/HER cell line was obtained from Professor C.W. Welsch (Michigan State University, East Lansing, MI, USA). This cell line was grown in DME-F12 medium supplemented with 10 ng/ml EGF, 0.5 μ g/ml hydrocortisone, 10 μ g/ml transferrin, 10 μ g/ml insulin and 5 μ g/ml of gentamycin (all from Sigma-Aldrich, St. Louis, MO, USA). The 184-B5/HER cells were routinely maintained in the presence of 200 μ g/ml G418 (Geneticin; Sigma-Aldrich) to eliminate the expression of spontaneous revertants. The cell cultures were maintained at 37°C in humidified atmosphere of 95% air: 5% CO₂, and were sub-cultured at 80% confluency.

Test agents. The small molecule inhibitor, lapatanib (LAP), and all trans-retinoic acid (ATRA) were purchased from Sigma-Aldrich. Carnosol (CSOL) was provided by Nestle Research Center (Lausanne, Switzerland). The stock solutions of LAP, ATRA and CSOL were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at the concentrations of 10 mM. These stock solutions were serially diluted in the culture medium to obtain the final concentrations of 10 μ M LAP, 2 μ M ATRA and 5 μ M CSOL. These final concentrations represented the pre-determined IC₉₀ (maximally cytostatic) concentrations for the 184-B5/HER cells. The treatment for the tumor spheroid assay was of 14 days duration and that for the cellular immuno-fluorescence assays was of 3 day duration.

LAP-resistant (LAP-R) phenotype. To isolate the LAP-R phenotype, parental 184-B5/HER cells were selected in the presence of 10 μ M LAP for approximately 6 months and the surviving cell population was expanded and maintained in the presence of LAP for at least 5 passages prior to the experiments.

Antibodies. The human reactive FITC-conjugated antibodies anti-HER-2 (cat. no. sc-81528), epidermal growth factor receptor (EGFR; cat. no. sc-373746) and IgG (cat. no. SC 2339) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). CD44 (cat. no. ab27285) was purchased from Abcam

(Cambridge, MA, USA). NANOG (cat. no. AP 1486c) and Oct-4 (cat. no. AP 2046a) were purchased from Abgent, Inc. (San Diego, CA, USA). These antibodies were used following the recommended dilutions provided in the technical protocols from the vendors.

Tumor spheroid assay. For this assay, the LAP-R cells were seeded at a density of 100 cells per well in ultra-low adhesion 6-well plates. (Corning/Costar, Corning, NY, USA) in serum-free DME-F12 medium. The culture medium was supplemented with 20 ng/ml EGF, 10 ng/ml basal fibroblast growth factor (FGF), 5 μ g/ml insulin, 1 ng/ml hydrocortisone and 4 μ g/ml heparin sodium (all from Sigma-Aldrich), 1% B27 and 10 ng/ml leukaemia inhibitory factor (LIF; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cultures were maintained at 37°C in humidified atmosphere of 95% air: 5% CO₂ and the spheroids formed at day 14 post-seeding were counted at magnification, x10 under an inverted light microscope. The data were expressed as number of spheroids (mean \pm SD; n=24) per treatment group from quadruplicate experiments.

Cellular immunofluorescence assay. This cellular immunofluorescence assay monitored antibody positivity in cells stained with FITC-conjugated antibodies following the previously published optimized protocol (10,12). Briefly, the cell suspension fixed in 0.25% paraformaldehyde (Polysciences, Warrington, PA, USA), was incubated with 0.1% Triton X-100 (Sigma-Aldrich) on ice for 3 min to permeabilize the cell membrane. The permeabilized cells were washed twice with phosphate-buffered saline (PBS; pH 7.4; Sigma-Aldrich), and stained with appropriate FITC-conjugated antibodies according to the protocol provided by the supplier. The stained cells were monitored for the cellular expression of individual antibody by fluorescence-assisted cell sorting using a flow cytometer. Cells stained with isotype FITC-conjugated IgG represented the negative control. The experimental data were corrected for the fluorescence due to FITC IgG staining, and the status of cellular expression was presented as log mean fluorescence units (FU) per 10⁴ fluorescent events from quadruplicate experiments.

Statistical analysis. Experiments using cellular immunofluorescence assay were performed in quadruplicate per treatment group. Experiments using tumor spheroid assay were performed in quadruplicate per treatment group. The data were presented as the means \pm SD and were analyzed for statistical significance between the control and experimental groups by a two-sample Student's t-test. Data comparing multiple treatment groups were analyzed by one-way analysis of variance (ANOVA) and Dunnett's multiple range test with a threshold of $\alpha=0.05$. All statistical analyses were performed using Microsoft Excel 2013 XLSTAT-Base software. Values of P<0.05 were considered to indicate statistically significant differences.

Results

Inhibition of human epidermal growth factor receptors by LAP. The experiment was designed to examine the efficacy of LAP on the parental 184-B5/HER cells by monitoring the status of the cellular expression of p-EGFR and p-HER-2 (Table I). LAP treatment induced a 57.1 and a 78.2% inhibition in the

Table I. Inhibition of human epidermal growth factor receptors.

Treatment	Concentration	Receptor expression (Log mean FU) ^a	
		p-EGFR	p-HER-2
DMSO	0.1%	11.9±1.4 ^b	51.5±6.2 ^d
LAP	10 μM	5.1±1.8 ^c	11.2±2.3 ^e

^aMean ± SD, n=4 per treatment group determined at day 3 post-seeding of 1.0x10⁵ cells. ^{b-c}P=0.03, ^{d-e}P=0.02; two-sample Student's t-test. FU, fluorescence unit; p-EGFR, phosphorylated epidermal growth factor receptor; pHER-2, phosphorylated human epidermal growth factor receptor-2, DMSO, dimethyl sulfoxide; LAP, lapatinib.

Table II. Stem cell marker status in LAP-R cells.

Phenotype	Tumor spheroid number ^a	CD44 ^b	NANOG ^b	Oct-4 ^b
LAP-S	4.5±1.3 ^c	4.0±0.7 ^e	2.2±0.6 ^g	4.8±0.3 ⁱ
LAP-R	14.8±2.8 ^d	20.8±1.9 ^f	11.8±1.2 ^h	14.2±1.5 ^j

^aDetermined at day 14 post-seeding of 100 cells. Data are the means ± SD, n=4 per treatment group. ^{c-d}P=0.02; two-sample Student's t-test. ^bLog mean FU; mean ± SD, n=4 per treatment group determined at day 3 post-seeding of 1.0x10⁵ cells. ^{e-h}P=0.01, ^{i-j}P=0.02; two-sample Student's t-test. CD44, cluster of differentiation 44; NANOG, DNA binding transcription factor; Oct-4, octamer binding transcription factor-4; LAP-S, lapatinib-sensitive; LAP-R, lapatinib-resistant; FU, fluorescence units.

cellular expression of p-EGFR and p-HER-2, respectively, relative to the cells treated with the solvent DMSO.

Status of stem cell markers in LAP-R cells. The experiment was designed to monitor the tumor spheroid number and the status of the cellular expression of the stem cell markers, CD44, NANOG and Oct-4, in LAP-sensitive (LAP-S) and LAP-R phenotypes. The results are presented in Table II. The LAP-R phenotype exhibited a 2.3-fold increase in tumor spheroid number, a 4.2-fold increase in CD44, a 4.4-fold increase in NANOG and a 1.9-fold increase in Oct-4 expression, respectively, relative to that in the LAP-S phenotype.

Experimental modulation of stem cell markers in LAP-R cells. The experiment that was designed to examine the effects of ATRA and CSOL on the number of tumor spheroids on the LAP-R phenotype is presented in Fig. 1A. Treatment of the spheroid cultures with 2 μM ATRA and 5 μM CSOL resulted in a 69.1 and 80.3% decrease in the spheroid number relative to that in the cultures treated with the solvent DMSO. The effects of ATRA and CSOL on the levels of additional stem cell-specific markers, CD44, NANOG and Oct-4, as regards the LAP-R phenotype were also examined and the results are presented in Fig. 1B. The extent of inhibition of the cellular expression of these markers in response to a treatment with 2 μM ATRA ranged from 71.7 to 81.9%. Treatment with 5 μM CSOL resulted in an inhibition range of 69.0 to 84.4%.

Discussion

The hormone receptor-positive HER-2-expressing breast cancer (luminal B) molecular subtype is treated with HER-2

targeted therapy and conventional endocrine therapy that includes selective estrogen receptors, selective estrogen receptor degraders and aromatase inhibitors (4,5). Hormone receptor-negative HER-2-expressing breast cancer is treated with HER-2 targeted therapy and conventional chemotherapy that includes anthracyclines and taxanes (2). These long-term treatment options are associated with the emergence of drug-resistant cancer stem cells, representing one of the major reasons for the compromised treatment response (17), and thereby, emphasizing the importance of identifying novel, less toxic treatment options for chemo-endocrine therapy-resistant breast cancer. The present study developed a LAP-R stem cell model, and validated the model using ATRA and CSOL as naturally occurring test agents. This approach now facilitates evaluation of growth inhibitory efficacy of additional natural products.

LAP, a dual function small molecule inhibitor of human epidermal growth factor receptors operates by inhibiting the phosphorylation of EGFR and HER-2, as demonstrated in ER-negative, PR-negative, HER-2-positive human breast carcinoma-derived models (23,24). Consistent with these data, the 184/B5/HER cells, in response to treatment with LAP, exhibited a substantial inhibition in the cellular expression of p-EGFR and p-HER-2.

The emergence of drug-resistant phenotypes, tumor spheroid formation and the increased expression of nuclear transcription factors have been demonstrated in stem cell models for select breast cancer subtypes (19-21). Furthermore, the nuclear transcription factors, Oct-4, Kruppel-like factor 4 (Klf-4), SOX-2 and c-Myc, represent essential components for induced pluripotent cell models developed from adult somatic cells (25-27). Thus, these cellular signaling proteins

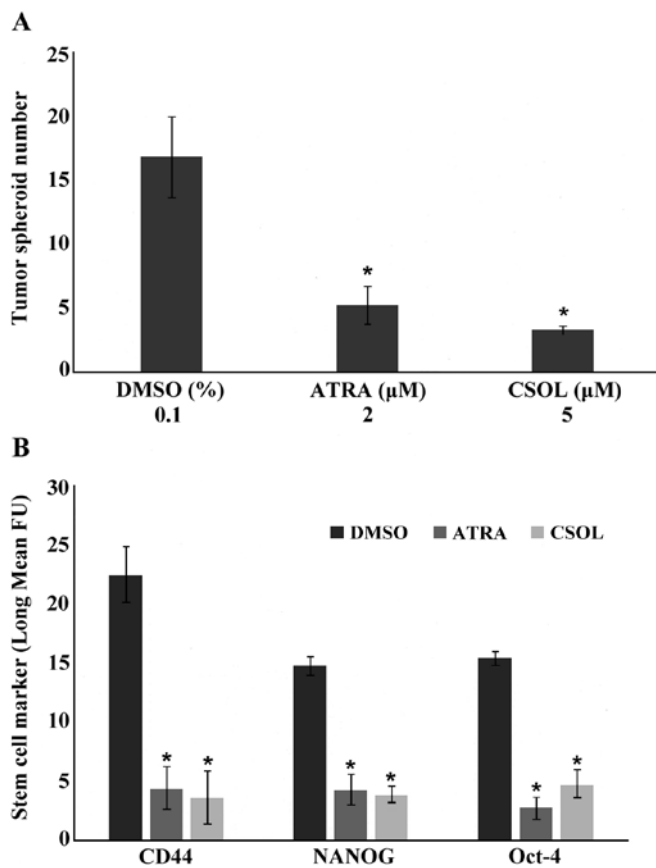


Figure 1. Experimental modulation of stem cell markers by ATRA and CSOL in LAP-R stem cells. (A) Tumor spheroids treated with ATRA and CSOL exhibit the inhibition in the number of tumor spheroids. Results are presented as the means \pm SD, $n=4$ per treatment group. Data analyzed by ANOVA and Dunnett's test. * $P<0.05$, ATRA vs. DMSO and CSOL vs. DMSO. (B) Treatment with ATRA and CSOL downregulated the cellular expression of CD44, NANOG and Oct-4. Results are presented as the log mean FU \pm SD, $n=4$ per treatment group. Data analyzed by ANOVA and Dunnett's test. Statistical significance is indicated as follows: for CD44: 2 μ M ATRA vs. 0.1% DMSO (* $P<0.05$), 5 μ M CSOL vs. 0.1% DMSO (* $P<0.05$); NANOG: 2 μ M ATRA vs. 0.1% DMSO (* $P<0.05$), 5 μ M CSOL vs. 0.1% DMSO (* $P<0.05$); Oct-4: 2 μ M ATRA vs. 0.1% DMSO (* $P<0.05$), 5 μ M CSOL vs. 0.1% DMSO (* $P<0.05$). DMSO, dimethyl sulfoxide; ATRA, all- trans retinoic acid; CSOL, carnosol; CD44, cluster of differentiation; NANOG, DNA binding homeobox transcription factor; Oct-4, octamer binding protein-4; LAP-R, lapatinib LAP-R resistant; FU, fluorescence units; ANOVA, analysis of variance.

represent stem cell selective markers. The data on the status of expression of these markers have demonstrated substantial upregulation in LAP-R cells in comparison to LAP-S cells. Collectively, these data provide evidence for the effective development and stringent characterization of the LAP-R cancer stem cell model.

The vitamin A derivative, ATRA, functions as a potent anti-proliferative and pro-apoptotic agent in HER-2-expressing 184-B5/HER cells, by increasing the cellular expression of the negative growth regulator, retinoic acid receptor- β (RAR- β), and decreasing the cellular expression of the anti-apoptotic BCL-2 protein (12). In addition, ATRA has been shown to inhibit the transcriptional activity of inducible cyclooxygenase-2 (COX-2) via RAR-mediated mechanisms, while CSOL functions via protein kinase C, ERK1/2, Jun terminal kinase and p38-related mitogen-activated protein kinase (MAPK)-dependent pathways (13) to target the transcriptional

activation of inducible COX-2 and of prostaglandin production in 184-B5/HER cells.

Mechanistically distinct naturally occurring phytochemicals have also been demonstrated to target cancer stem cells from multiple organ site cancers. For example, ATRA has been shown to target gastric cancer stem cells by downregulating the expression levels of CD44, aldehyde dehydrogenase-1 (ALDH-1), as well as transcription factors Klf-4 and SOX-2 (28). Sulforaphane, present in broccoli, has been shown to increase drug-mediated cytotoxicity in pancreatic and prostatic cancer stem cells, and to inhibit tumor spheroid formation and ALDH-1. In addition, this natural product inhibits the Notch-1 and nuclear factor- κ B (NF- κ B) pathways (29). The natural polyphenol, quercetin, targets pancreatic cancer stem cells by inhibiting tumor spheroid formation and ALDH-1 expression (30). Furthermore, sulforaphane, acting synergistically with the pan multi-kinase inhibitor, sorafenib, abolishes the stem cell characteristics of pancreatic cancer stem cells by inhibiting tumor spheroid formation, and the ALDH-1 and NF- κ B pathways (31). Recent evidence has also demonstrated that sulforaphane decreases the expression of stem cell markers NANOG, ALDH1A1, Wnt3 and Notch, thereby, inhibiting the growth of triple negative breast cancer stem-like cells (32), and benzyl isothiocyanate, present in cruciferous vegetables, inhibits breast cancer stem-like population via upregulation of the KLF-4-p21^{CIP1} axis (33). Collectively, these data provide evidence that the downregulated signaling molecules may represent molecular targets relevant to stem cell-specific therapeutic efficacy of natural products. Thus, in the present study, the significant inhibition of select stem cell markers, such as tumor spheroids, CD44, NANOG and Oct-4 in LAP-R stem cells treated with ATRA and CSOL, provides evidence for stem cell targeting efficacy of these naturally occurring agents.

In conclusion, the outcomes of the present study provide strong evidence supporting the effective development of a cancer stem cell model for the HER-2-enriched breast cancer subtype. Furthermore, experiments with ATRA and CSOL together have provided valuable mechanistic leads for their stem cell specific efficacy, and thereby, have validated the present experimental approach for the evaluation of stem cell-specific mechanistic efficacy of natural products. However, it needs to be emphasized that to extend clinical translatability of the present approach, reliable stem cell models from patient derived xenografts and organoid cultures from HER-2 targeted therapy resistant breast cancer are essential. These aspects represent promising future directions for the clinical application of patient-derived pre-clinical data (34,35).

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Availability of data and materials

The data used and/or analyzed in this study are available from the corresponding author upon reasonable request.

Author's contribution

NT contributed towards the study conception, experimental design, data analysis, data interpretation and prepared the manuscript for publication.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The author declares that there are no competing interests.

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