Anti-proliferative effects of Chinese herb *Cornus officinalis* in a cell culture model for estrogen receptor-positive clinical breast cancer

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Received January 21, 2011; Accepted September 22, 2011

DOI: 10.3892/mmr.2011.617

Abstract. Selective estrogen receptor modulators and a combination of mechanistically distinct chemotherapeutic agents represent conventional therapeutic interventions for estrogen receptor-positive (ER^+) clinical breast cancer. Long-term treatment with these agents is associated with acquired tumor resistance and other adverse side effects that impact on patient compliance. Herbal medicines are being widely used in complementary and alternative medicine. However, long-term safety and efficacy of the use of herbal medicines, as well as their interaction with conventional endocrine and chemotherapeutic drug regimens remain largely unknown. The present study utilized a human cell culture model for ER^+ clinical breast cancer to examine the potential therapeutic efficacy of an aqueous extract prepared from the fruit of popular Chinese herb *Cornus officinalis* (CO), also known as *Fructus cornii*. The human mammary carcinoma-derived MCF-7 cell line represented the model. Status of anchorage-independent growth and cellular metabolism of 17β-estradiol (E_2) represented the quantitative end-point biomarkers for efficacy. MCF-7 cells adapted for growth in serum-depleted medium (0.7% serum, <1 nM E_2) retained their endocrine responsiveness as evidenced by growth promotion by physiological levels of E_2, and growth inhibition by the selective ER modulator tamoxifen at the clinically achievable concentrations. Treatment of MCF-7 cells with CO resulted in inhibition of E_2-stimulated growth in a dose-dependent manner. Similarly, CO treatment also produced a dose-dependent progressive reduction in the number of anchorage-independent colonies, indicating effective reduction of the carcinogenic risk. Treatment of MCF-7 cells with CO at a maximally effective cytostatic concentration resulted in a 5.1-fold increase in the formation of the anti-prolifertive E_2 metabolite 2-hydoxyestrone (2-OHE_1), a 63.6% decrease in the formation of the pro-mitogenic metabolite 16α-hydroxestrone (16-αOHE_1) and a 9.1% decrease in the formation of mitogenically inert metabolite estrone (E_3). These alterations led to a 14.5-fold increase in the 2-OHE_1:16α-OHE_1, and a 3.3-fold increase in the E_3:16α-OHE_1 ratios. These data validate a rapid cell culture-based mechanistic approach to prioritize efficacious herbal medicinal products for long-term animal studies and future clinical trials on ER^+ clinical breast cancer.

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Key words: breast carcinoma, Chinese herb, estradiol metabolism

Introduction

Invasive breast cancer remains one of the leading causes of mortality in the US. The American Cancer Society projections for invasive breast cancer incidence and mortality for 2012 estimate 230,480 newly diagnosed invasive breast cancer cases and 39,520 invasive breast cancer-related deaths (1). These projections emphasize a persistent need to identify new modalities for prevention/therapy of invasive breast cancer.

Conventional chemo-endocrine therapy with selective estrogen receptor modulators with or without combination chemotherapy using mechanistically distinct cytotoxic drugs continues to represent the treatment of choice for hormone responsive estrogen receptor-positive (ER^+) clinical breast cancer. However, these modalities are frequently associated with acquired tumor resistance and/or adverse systemic toxicity, compromising long-term patient compliance (2,3).

Complementary and alternative medicine, using herbal medicinal products, has acquired wide application in non-responsive patients for a potential to reduce chemo-endocrine therapy-related toxicity (4-7). Most of the herbal medicinal products are available from health food stores as general health-improving nutritional supplements, and also from herbal medical practitioners. However, evidence for long-term safety and efficacy of herbal medicines remains to be systematically documented and, therefore, is currently equivocal.

Several herbal medicinal preparations have been noted to sensitize tumor cells to radiation therapy, enhance clinical efficacy of LAK/IL-12 based immunotherapy in cancer patients and represent an accessory modality of treatment for

malignant tumor, immuno-deficiency and for the prevention of cancer radiation/chemotherapy-associated side effects (8-10). In this study, we investigated the anti-proliferative action on the human mammary carcinoma MCF-7 cells of the fruit of *Cornus officinalis* (CO), also known as *Fructus cornii* (FC), a popular Chinese herb of a nutritional nature. CO represents a major ingredient herb in some well-known traditional Chinese herbal mixtures.

The human mammary carcinoma-derived ER^+ MCF-7 cell line represents a well-recognized pre-clinical model for hormone responsive clinical breast cancer. This model has been extensively used both as a cell culture approach, as well as an *in vivo* xenotransplant approach for pre-clinical efficacy studies on synthetic ER modulators and inhibitors of estrogen biosynthesis (11).

The present study utilized the MCF-7 cell culture model to examine the cellular and endocrine effects of CO that are relevant to growth inhibition of human breast cancer cells. The data generated from this study demonstrate that low doses of non-fractionated aqueous extract from CO exhibit progressive cytostatic growth arrest and reduction of carcinogenic risk in 17β-estradiol (E_2)-stimulated ER^+ MCF-7 cells. Furthermore, treatment of MCF-7 cells with CO at a maximally effective cytostatic concentration alters the cellular metabolism of E_2 via distinct metabolic pathways. These data taken together validate the present cell culture model as a rapid mechanism-based approach to screen natural herbs for their therapeutic efficacy and prioritize promising lead agents for subsequent animal studies and clinical trials for breast cancer therapy.

Materials and methods

Cell line. The ER^+ human breast carcinoma MCF-7 cell line was originally obtained from the Michigan Cancer Foundation (Detroit, MI, USA). These cells were cultured in DME/F12 medium supplemented with 7% heat-inactivated fetal calf serum and recommended additives (12).

For the present experiments, MCF-7 cells were adapted for growth in serum-depleted medium by maintaining the cultures in the tissue culture medium supplemented with 0.7% serum for at least 5 passages. These stock cultures were routinely maintained in DME/F12 medium supplemented with 0.7% serum in an humidified atmosphere of 95% air:5% CO_2 at 37˚C and were sub-cultured at 1:4 split at ~80% confluency.

Growth parameters. The population doubling time was determined during the exponential growth phase by obtaining the viable cell counts from triplicate flasks at 24, 48, 72 and 96 h post-seeding of $1.0x10^5$ cells per flask, and the mean values from four time points were used to determine the population doubling times. Saturation density was determined from the viable cell number at day 7 post-seeding of $1.0x10^5$ cells, and the data were expressed as the means ± SD, n=6 per treatment group.

Anchorage-independent colony formation. This assay was performed according to a previously published protocol (12). Briefly, 1,000 MCF-7 cells per 2 ml were suspended in the culture medium containing 0.33% agar. Cell suspension (2 ml) was overlaid on a basement of 0.6% agar in each well of 6-well cluster plates. The cultures were maintained at 37˚C in a humidified atmosphere of 95% air:5% CO_2 for 21 days. The cultures with developed anchorage-independent colonies were then fixed in Cornoy's fluid and the colony count in each well was determined microscopically at x10 magnification. The data were expressed as the means ± SD, n=12 per treatment group.

Chemical reagents. E_2 and tamoxifen (TAM) were obtained from Sigma Chemical Co. The stock solutions (100 μM) of these compounds were prepared in 100% ethanol and serially diluted in the culture medium to obtain the final working solutions of 20 nM for treatment.

Aqueous extract of CO. To prepare the aqueous extract of CO, 20 g of the CO fruit was boiled in 200 ml of de-ionized water until the volume was reduced to 100 ml, and the supernatant was collected (Aqueous Extract #1). The resultant residue was further boiled in 100 ml of water until the volume was reduced to 50 ml (Aqueous Extract #2). The two supernatants, Extract #1 (100 ml) and Extract #2 (50 ml), were combined and concentrated by boiling until the volume was reduced to 25 ml. These combined extracts were centrifuged at 5,000 rpm at room temperature for 10 min. The resultant supernatant (20 ml) was collected and stored as stock solution at -20˚C in 5 ml aliquots. These stock solutions were appropriately diluted in DME/F12 medium to obtain the working concentrations.

Dose response of CO. For the dose response experiments, MCF-7 cells were seeded at the initial density of $1.0x10^5$ cells/25 cm^2 in T-25 flasks. Treatment with CO at 0.01, 0.05, 0.1, 0.5, 1.0 and 2.0% doses was initiated at day 1 post-seeding and continued up to day 7 post-seeding, with a medium change every 48 h. At the end of the treatment schedule, the cultures were trypsinized and trypan blue excluding viable cell counts were obtained. The cell viability data from these dose response experiments were used to identify minimum effective and maximum cytostatic concentrations of CO relative to the initial seeding density of MCF-7 cells. The data were expressed as viable cell number means ± SD, n=6 per treatment group.

Sample preparation for cellular metabolism of E_2. The MCF-7 cells at day 1 post-seeding were treated with appropriate maximally effective cytostatic concentration of CO in the presence of 20 nM E_2 for 48 h and the medium was analyzed for E_2 metabolites following published methods (13). Briefly, a 5-ml aliquot of the medium was diluted 1:1 with sodium acetate buffer (pH 4.65) and 20 μl of β-glucuronidase (110,200 U/ml; Sigma). This solution was incubated at 37˚C for 24 h to de-conjugate the steroids. After the addition of deuterated E_2 as an internal standard (14,15), each sample was thoroughly vortexed. Two volumes of chloroform were added to the samples and the resulting mixture was vortexed and centrifuged. The chloroform layer was removed and reduced to dryness using a vacuum equipped centrovap console (Labconco, Inc., St. Louis, MO, USA).

Each sample was derivitized by adding 10 μl of dry pyridine and 40 μl of bis (trimethylsilyl) trifluoroacetamide (BSTFA), vortexed and allowed to react at room temperature

Table I. Endocrine responsiveness of ER^+ human mammary carcinoma MCF-7 cells.

Treatment	Concentration	Biomarker		
		Population doubling (h)[a]	Saturation density ($x10^5$)[b]	Anchorage-independent colonies[c]
Serum	0.7%	33.0	11.8±0.9[d]	16.7±3.4[g]
E_2	20 nM	28.5	17.9±1.6[e]	37.2±2.1[h]
TAM	20 nM	35.9	3.9±0.6[f]	7.7±0.4[i]

[a]Determined during the exponential growth phase. [b]Viable cell number at day 7 post-seeding of $1.0x10^5$ cells. [d-f]Means ± SD, n=6 per treatment group. [c]Number of colonies at day 21 post-seeding of 1,000 cells/well. [g-i]Means ± SD, n=12 per treatment group. [d<e, d>f, g<h, g>i]Data analyzed by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison test (α=0.05).

overnight. One microliter of each sample was injected into the GC-MS apparatus without further treatment.

GC-MS conditions for analysis of E_2 metabolites. Select E_2 metabolites were analyzed on an Agilent 6980N gas chromatograph equipped with an Agilent 5973 mass selection detector, an Agilent 7683 injector and an HP GI701CA MSD Chemstation. The injection port was equipped with a split/splitless capillary inlet system and a silanized glass insert. The temperature of the injection port was maintained at 300˚C. The GC-MS interface was maintained at 270˚C and the ion source was maintained at 280˚C. The ionization energy was 70eV. The carrier gas was helium at a flow rate of 1 ml/min. Separations of metabolites were carried out using a Hewlett-Packard Ultra 2 capillary column with cross-linked 5% phenyl-methyl silicone (25 m x 0.2 mm x 0.33 μm film thickness). The oven temperature was increased from 60 to 260˚C at 40˚C/min, then at 1˚C/min to 280˚C (13-15).

Under selected ion monitoring, the following mass ions and GC elution times of trimethylsilylated estrogens were routinely monitored: Estrone (E_1) m/z 342, 15.90 min; E_2 m/z 416, 16.40 min; deuterated E_2 m/z 420, 16.40 min; 2-hydroxyestrone (2-OHE_1) m/z 430, 18.47 min; 4-hydroxyestrone (4-OHE_1) m/z 430, 18.92 min; 16α-hydroxyestrone (16α-OHE_1) m/z 286 and 430, 19.37 min; and estriol (E_3) m/z 504 and 345, 20.76 min. The other E_2 metabolites, 2-hydroxyestradiol (2-OHE_2) and 4-hydroxyestradiol (4-OHE_2), were monitored using the mass and base ions m/z 504 and 373 at 19.06 and 20.15 min, respectively, in a second run using the same parameters as above. Deuterated estradiol (2, 4, 16α, 16β-^{2}H4) was synthesized in our laboratory according to the method of Dehennin *et al* (14), and was used as the internal standard. The individual metabolites were quantified using a six point calibration curve (range 1-50 ng). The data were expressed as ng metabolite per 10^6 cells.

Modulation of E_2 metabolism. In the cellular metabolism of E_2, E_1 functions as a common precursor for the formation of 2-OHE_1 and 16α-OHE_1, and the pro-mitogenic 16α-OHE_1 is converted in to the mitogenically inert proximate metabolite E_3. Therefore, to accurately evaluate the modulation in E_2 metabolism, the 2-OHE_1:16α-OHE_1 and E_3:16α-OHE_1 ratios were considered. The 2-OHE_1:16α-OHE_1 ratio was calculated by dividing values of 2-OHE_1 by those of 16α-OHE_1. To determine the proportion of total 16α-OHE_1 that is converted to the mitogenically inert E_3, the E_3:16α-OHE_1 ratio was calculated by dividing the values of E_3 by those of 16α-OHE_1 + E_3.

Statistical analysis. Experiments with E_2 and TAM were performed using n=6 flasks per treatment group. The experiment for dose response of CO to determine cytostatic growth arrest was performed using n=6 flasks per treatment group, while that for the effect of CO on anchorage-independent growth was performed with n=12 wells per treatment group using 6-well plates. The experiment for E_2 metabolism was performed using n=3 flasks per treatment group.

The significance of differences between the control and experimental data points for individual experiment was analyzed by the 2-sample t-test using the Prism 4.0 statistical software (Graph Pad Software, Inc.). The p-values for these data were further analyzed by one-way ANOVA with Dunnett's Multiple Range Test (α=0.05), correcting for multiple comparisons among control group and multiple treatment groups.

Results

Growth of MCF-7 cells in serum-depleted culture medium. The experiment designed to examine the persistence of endocrine responsiveness of MCF-7 cells in serum depleted culture conditions is presented in Table I. Relative to the cells maintained in 0.7% serum, those treated with E_2 exhibited a 13.6% decrease in population doubling time, a 51.7% increase in saturation density and a 122.7% increase in the number of anchorage-independent colonies. By contrast, the cells treated with TAM exhibited a 8.8% increase in population doubling time, a 66.9% decrease in saturation density and a 53.9% decrease in the number of anchorage-independent colonies.

Dose response of CO. The experiment presented in Table II identifies the growth inhibitory profile of CO on E_2-stimulated MCF-7 cells. The treatment of E_2-stimulated MCF-7 cells with CO resulted in progressive dose-dependent cytostatic growth arrest of 8.3, 35.7 and 75.1%, respectively, relative to that observed in control cells treated with E_2 alone. Thus, the data generated from this experiment identified 0.01% as minimum effective, and 0.1% as maximum cytostatic concentrations for CO, relative to the initial seeding density of MCF-7 cells. Treatment with the higher dose of 0.5% CO, however, resulted

Table II. Cytostatic growth arrest of ER+ human mammary carcinoma MCF-7 cells by *Cornus officinalis* (CO).

Treatment	Concentration	Viable cell no. ($x10^5$)[a]	Inhibition (% control)
E_2	20 nM	16.1±1.6[b]	-
E_2 + CO	0.01%	14.8±1.5[b]	8.3
	0.05%	10.3±1.0[c]	35.7
	0.10%	4.0±0.7[d]	75.1
	0.50%	0.5±0.1[e]	96.9

[a]Determined at day 7 post-seeding of $1.0x10^5$ cells. [b-d]Means ± SD, n=6 per treatment group. [b>c, b>d, b>e]Data analyzed by Dunnett's multiple comparison test (α=0.05).

Table III. Inhibition of anchorage-independent colony formation in ER+ human mammary carcinoma MCF-7 cells by *Cornus officinalis* (CO).

Treatment	Concentration	No. of colonies[a]	Inhibition (% control)
E_2	20 nM	38.0±2.6[b]	-
E_2 + CO	0.01%	24.7±1.6[c]	35.0
	0.05%	17.0±1.1[d]	55.3
	0.10%	4.4±0.5[e]	88.4

[a]Determined at day 21 post-seeding of 1,000 cells/well. [b-e]Means ± SD, n=12 per treatment group. [b>c, b>d, b>e]Data analyzed by Dunnett's multiple comparison test (α=0.05).

Table IV. Effect of *Cornus officinalis* (CO) on 17β-estradiol (E_2) metabolism in ER+ human mammary carcinoma MCF-7 cells.

Treatment	Concentration	E_2 metabolite[a]			
		E_1	2-OHE_1	16α-OHE_1	E_3
E_2	20 nM	7.8±0.6[b]	0.9±0.2[d]	2.2±0.1[f]	0.22±0.03
CO	0.1%	14.4±1.9[c]	5.5±1.3[e]	0.8±0.2[g]	0.20±0.04

[a]Nanograms per 10^6 cells. Means ± SD, n=3 per treatment group. The metabolites were determined at 48 h of incubation with E_2 alone or with E_2 + CO. [b,c]p=0.03; [d,e]p=0.01; [f,g]p=0.04.

in a viable cell number that was lower than the initial seeding density, thus demonstrating a cytotoxic response.

Efficacy of CO for inhibition of anchorage-independent growth. The experiment presented in Table III was designed to examine the effect of CO on the number of anchorage-independent colonies. The data generated from this experiment demonstrated that the E_2-stimulated increase in the number of colonies was progressively decreased to 35.0, 55.3 and 88.4% in response to the treatment with 0.01, 0.05 and 0.10% CO, respectively.

Effect of CO on estradiol metabolism. The experiment presented in Table IV examined the effect of CO on the cellular metabolism of E_2. The treatment with CO resulted in a 5.1-fold increase in the anti-proliferative E_2 metabolite 2-OHE_1, a 63.6% decrease in the pro-mitogenic metabolite 16α-OHE_1 and a non-significant 9.3% decrease in the mitogenically inert metabolite E_3 formation, relative to that in the E_2-treated controls. Other biologically active metabolites, such as 4-OHE_1, 4-OHE_2 and 2-OHE_2, remained essentially unaltered in the CO-treated group relative to those in the E_2-treated control group.

Modulation of estradiol metabolism. The data presented in Table V examined the effect of CO on the 2-OHE_1:16α-OHE_1 ratio. Relative to the E_2-treated controls that exhibited a ratio of 0.44±0.11, CO-treated cells exhibited a ratio of 6.84±0.47, resulting in a 14.5-fold increase. The data presented in Table VI examined the effect of CO on the E_3:16α-OHE_1 ratio. Relative to the E_2-treated control cells that exhibited a ratio of 0.09±0.02, CO-treated cells exhibited a ratio of 0.39±0.11, resulting in a 3.3-fold increase.

Discussion

Hormone responsive ER+ clinical breast cancer is traditionally treated with selective ER modulators, such as TAM, and/or

Table V. Effect of *Cornus officinals* (CO) on the ratio of 2-hydroxyestrone (2-OHE_1) and 16α-hydroxyestrone (16α-OHE_1).

Treatment	Concentration	2-OHE_1:16α-OHE_1 ratio[a]
E_2	20 nM	0.44±0.11[b]
CO	0.1%	6.84±0.47[c]

[a]Means ± SD, n=3 per treatment group. Data analyzed by 2-sample t-test compared to the data from the E_2-treated control group. [b,c]p=0.001.

Table VI. Effect of *Cornus officinalis* (CO) on the ratio of estriol (E_3) and 16α-hydroxyestrone (16α-OHE_1).

Treatment	Concentration	E_3:16α-OHE_1 ratio[a]
E_2	20 nM	0.09±0.02[b]
CO	0.1%	0.39±0.11[c]

[a]E_3/(16α-OHE_1 + E_3). Means ± SD, n=3 per treatment group. Data analyzed by the 2-sample t-test compared to the data from the E_2-treated control group. [b,c]p=0.01.

cytotoxic chemotherapeutic drugs, such as combinations of alkylating agents, anti-folates, nucleic acid anti-metabolites, anthracyclins and micro-tubule inhibitors (2,3,16). These treatment options are frequently associated with acquired tumor resistance and adverse systemic toxicity, compromising long-term patient compliance. Herbal medicinal products with potential to enhance therapeutic efficacy and reduce toxicity represent an attractive treatment option in complementary and alternative medicine. The long-term safety, efficacy and lack of adverse reactions of herbal medicines with conventional chemo-endocrine therapy is, however, largely unknown.

Human carcinoma-derived cell culture models, such as the ER^+ MCF-7 cell line, provide a rapid mechanistic approach to obtain clinically translatable data (11,12). MCF-7 cells adapted for growth in chemically defined serum-depleted medium (0.7% serum, <1 nM E_2) exhibited a positive growth regulatory effect of E_2 at physiologically relevant levels. By contrast, treatment with the prototypic selective ER modulator TAM at the clinically achievable pharmacological concentration resulted in growth inhibition. These data are consistent with the previous observations that *in vivo* MCF-7 cell transplants in the athymic 'nude' mouse xenotransplant model require E_2 for tumor formation, and that systemic administration of TAM inhibits tumor growth (11,16).

Anchorage-independent growth represents a specific and sensitive *in vitro* surrogate end point biomarker for tumorigenic cell lines (11,12,17-20). In accord, it is noteworthy that E_2 treatment resulted in an increased number of anchorage-independent colonies, while TAM treatment resulted in decreased colony number. These data on anchorage-independent growth together with those on modulatory effects of E_2 and TAM on cell viability provide evidence for persistent endocrine responsiveness of MCF-7 cells adapted for growth in serum-depleted medium.

The data with CO for induction of cytostatic growth arrest and reduction of carcinogenic risk at relatively low doses taken together identify valuable phenomenological leads for the efficacy of CO on ER^+ MCF-7 cells. The maximally effective low dose may thus provide an acceptable toxicity profile for future animal studies or clinical trials. In this context, it is noteworthy that ER^+ MCF-7 cells are also responsive to the growth inhibitory effects of non-fractionated aqueous extracts prepared from other plant products, such as *Lycium barbarum* and *Tabebuia avellandae*, and these extracts appear to operate via several mechanisms, including modulation of cellular E_2 metabolism, as well as altered expression of several target genes for cell cycle progression, cellular apoptosis and xenobiotic metabolism (21,22).

During the Cyp450-mediated oxidative metabolism of E_2, this mitogenic hormone is converted via C17-oxidation to E_1. The intermediate metabolite E_1 functions as a common precursor either for the formation of 2-OHE_1 via C2-hydroxylation, or for the formation of 16α-OHE_1 via C16α-hydroxylation (23-28). These metabolites have documented distinct growth modulating effects on MCF-7, or other human mammary carcinoma-derived cell lines. In accord, it is noteworthy that treatment of mouse mammary epithelial cells or human mammary carcinoma MCF-7 cells with E_2 and 16α-OHE_1 resulted in increased anchorage-dependent and anchorage-independent growth, while treatment with 2-OHE_1 resulted in reduction in these parameters (29-31). Additionally, in the MCF-7 xenograft model these treatments produced similar modulations in the tumor size (31). Thus, 2-OHE_1 has potent anti-proliferative effect, while 16α-OHE_1 functions as a pro-mitogenic metabolite (26-33). Data generated from the present study, measuring cellular metabolism of E_2, clearly demonstrated that treatment of MCF-7 cells with CO at the maximally effective cytostatic concentration predominantly affected the C2-hydroxylation pathway, up-regulating the formation of anti-proliferative 2-OHE_1.

Overall, the data on cytostatic growth arrest and on modulation of cellular metabolism of E_2 by non-fractionated aqueous extract from CO fruit essentially extend and confirm our recent preliminary observations (32), and thereby identify a mechanistic lead for the efficacy of CO in ER^+ human mammary carcinoma MCF-7 cells.

It is well established that Cyp450-dependent conversion of pro-mitogenic E_2 to mitogenically inert E_3 is accomplished predominantly via the well-documented E_2-E_1-16α-OHE_1-E_3 pathways (23-26). Thus, in the present study a 14.5-fold increase in the 2-OHE_1:16α-OHE_1 ratio in response to the treatment with CO was predominantly due to a specific increase in 2-OHE_1 formation. In addition, treatment with CO resulted in a 3.3-fold increase in the E_3:16α-OHE_1 ratio. This alteration was predominantly due to a specific decrease in 16α-OHE_1 formation. Thus, these E_2 metabolite ratios may represent unique and novel mechanistically distinct biomarkers that quantify alterations in the mitogenecity of E_2.

Since E_1 functions as a common precursor for the formation of 2-OHE_1 and 16α-OHE_1 (23-28), and because individual E_2 metabolites exhibit distinct growth modulating effects (26-33), the ratios of 2-OHE_1:16α-OHE_1 and E_3:16α-OHE_1 may represent modulatable endocrine biomarkers for carcinogenic risk and, therefore, may identify novel mechanistic quantitative

parameters for the efficacy of therapeutic/preventive agents. In this context, it is noteworthy that our previous studies have demonstrated that human mammary epithelial cells transformed by the clinically relevant HER-2 oncogene, as well as ER^+ MCF-7 and ER^- MDA-MB-231 human mammary carcinoma cell lines exhibit a modulatable 2-OHE_1:16α-OHE1 ratio that is up-regulated in response to treatment with the naturally occurring phytochemical indole-3-carbinol (34-36).

It is well recognized that traditional herbal medicine uses several herbal preparations in combination, and that individual herbal preparations may contain several biologically active components playing distinct roles in their combined activity. Thus, although non-fractionated aqueous extract from CO fruit exhibits growth inhibitory effects in the present study, the identity of water soluble components that may be responsible for the observed effects and their putative molecular/mechanistic targets remain currently unknown. In this context, it should be noted that methanolic or aqueous extracts from several distinct species of CO have documented anti-proliferative, anti-oxidant and anti-inflammatory properties (37,38).

In conclusion, the data generated in the present study have identified distinct phenomenological and mechanistic leads for the efficacy of CO. Thus, the outcome of this study has validated a rapid mechanism-based approach to prioritize efficacious herbal medicinal products for the treatment of ER^+ clinical breast cancer.

Acknowledgements

Major funding for this study was provided by philanthropic contributions to the American Foundation for Chinese Medicine, from Peter Cheney, Suzanne Hoyt, the family of Daniel and Kathleen Mezzalingua, the family of Hakan and Marie Ledin and by the Issac and Laura Perlmutter Fund. The study is dedicated to the memory of Laurie Mezzalingua (1968-2009).

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