

Growth inhibition of estrogen receptor positive human breast cancer cells by Taheebo from the inner bark of *Tabebuia avellandae* tree

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Abstract. Selective estrogen receptor (ER) modulators are used as a therapy for ER⁺ clinical breast cancer, but they exhibit adverse effects. Herbal medicines may provide an alternative or complementary approach. Taheebo, extracted from the inner bark of the *Tabebuia avellandae* tree found in the Brazilian Amazon, exhibits selective anti-proliferative effects in carcinoma cell lines. The present study identifies the mechanistic leads for the inhibitory effects of Taheebo. Human breast carcinoma derived ER⁺ MCF-7 cells were used as the model. Aqueous extract of Taheebo was the test compound. Cell cycle analysis, clonogenic assay, and global gene expression profiles were the quantitative parameters. Taheebo treatment resulted in a dose/time-dependent growth inhibition (S phase arrest, reduced clonogenicity) and initiation of apoptosis (chromatin condensation). A 6-h treatment with 1.5 mg/ml Taheebo modulated the gene expression of G2 specific cyclin B1 (-2.0-fold); S phase specific PCNA (-2.0-fold) and OKL38 (+11.0-fold); apoptosis specific GADD-45 family (+1.9-5.4-fold), Caspases (+1.6-1.7-fold), BCL-2 family (-1.5-2.5-fold), estrogen responsive ESR1 (-1.5-fold), and xeno-biotic metabolism specific CYP 1A1 (+19.8 fold) and CYP 1B1 (+7.9-fold). The anti-proliferative effects of Taheebo correlate with down-regulated cell cycle regulatory and estrogen responsive genes, and up-regulated apoptosis specific and xeno-biotic metabolism specific genes. These data validate a rapid mechanistic approach to prioritize efficacious herbal medicines, thereby complementing the existing endocrine therapy for breast cancer.

Introduction

It is well recognized that ~80% of sporadic clinical breast cancers express estrogen receptor (ER) and that endocrine therapy with or without chemotherapy is a common treatment

option (1,2). However, long-term treatment with chemo-endocrine therapy is frequently associated with adverse toxicity that compromises patient compliance (3,4).

In recent years complementary and alternative approaches utilizing herbal medicines are being extensively used independently, or as adjuvants to chemo-endocrine therapy in an effort to reduce therapy related toxicity and enhance efficacy (3,5-8). However, long-term safety, efficacy and toxicity of herbal medicines and their interactions with conventional therapeutic regimen remain to be established.

Taheebo, a naturally occurring constituent of the inner bark of the *Tabebuia avellandae* tree found in the Brazilian Amazon, has been used for over 1,500 years in South America to treat a variety of health conditions (9-11). In the USA, Taheebo is available as a nutritional supplement under the name Pau d'Arco tea. However, the mechanism(s) of action, molecular targets of efficacy and long-term toxicity profile of Taheebo remain to be identified.

The human breast carcinoma derived MCF-7 cell line represents a widely used preclinical cell culture model for hormone responsive ER⁺ clinical breast cancer. This model has been extensively utilized in *in vitro* (cell culture) as well as *in vivo* (xeno-transplant) approaches to evaluate the efficacy of selective estrogen receptor modulators and aromatase inhibitors for preventive/therapeutic intervention of hormone responsive ER⁺ clinical breast cancer (12,13).

The experiments in the present study utilized the MCF-7 cell culture model to examine the growth inhibitory effects of Taheebo and identify possible mechanistic leads for its efficacy. The phenomenological and mechanistic data generated from this study provide evidence that the growth inhibitory effects of Taheebo are associated with down-regulated expression of several cell cycle regulatory and estrogen responsive genes, and with up-regulated expression of apoptosis specific genes and of xeno-biotic metabolism specific genes. Taken together, the outcome of this study validates the present experimental approach for a mechanism based evaluation of the efficacy of plant derived medicinal products for therapy of hormone responsive ER⁺ breast cancer.

Material and methods

ER⁺ human mammary carcinoma MCF-7 cell line. The ER⁺ human mammary carcinoma derived MCF-7 cell line was

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obtained from ATCC, Manassas, VA. The cell line was maintained and propagated in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 μ g/ml bovine insulin and 1% penicillin-streptomycin mixture as recommended by the supplier. Routinely, cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, and were subcultured at about 70% confluency.

Dose response of Taheebo. Lyophilized powder of non-fractionated Taheebo was provided by Taheebo Japan Co. Ltd. (Osaka, Japan). This powder was dissolved in double distilled water at a concentration of 500 mg/ml to make the stock solution. This stock solution was serially diluted in the MEM culture medium to obtain various concentrations of Taheebo ranging from 1.5, 0.75, 0.5, 0.25, 0.125 and 0.05 mg/ml for dose response experiments. The initial dose response of a 6-day duration was conducted at the concentration range of 0.05-0.5 mg/ml. The viable cell counts were obtained using a hemocytometer after trypan blue staining and were confirmed using a coulter counter. For the experiments on cell cycle analysis, induction of apoptosis/necrosis and clonogenicity Taheebo was evaluated at the concentration of 0.75 mg/ml. For micro-array based gene expression profiles and electron microscopy Taheebo was evaluated at a concentration of 1.5 mg/ml for durations of 6 and 24 h, respectively.

Cell cycle progression. For cell cycle analysis, controls and Taheebo treated cultures were trypsinized and washed with PBS. The cell suspensions were fixed in 75% ethanol and stained with propidium iodide (PI). The fluorescence assisted cell sorting was performed using a flow cytometer (BD FACS Canto, Becton Dickinson), and percent distribution of cell population in G0/G1, S, and G2/M phases of cell cycle were calculated using the multicycle software. The data were expressed as G1:S+G2/M ratio.

Electron microscopy for apoptosis and necrosis. MCF-7 cells treated with 1.5 mg/ml of Taheebo for 24 h and parallel non-treated controls were fixed with 2.5% Glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature, rinsed in the same buffer several times, post-fixed in 1% osmium tetroxide solution, dehydrated in serial ethanol dilutions and embedded in Poly-Bed araldite media. Ultra-thin sections (65-70 nm) were then counterstained with uranyl acetate and lead citrate and viewed under a Philips-410 transmission electron microscope (Phillips, Eindhoven, Holland).

Clonogenic assay. This assay was performed using a protocol similar to the one previously published (14). Briefly, MCF-7 cells were pre-treated with Taheebo at a concentration of 0.75 mg/ml for 72 h. The treated cells were trypsinized, washed and re-plated at a seeding density of 1,000 cells per flask. The untreated control cells were also re-plated at an identical seeding density. Both sets of cultures were maintained without any further treatment for 8 days. Percentage of survival was calculated as (colony number in treated group/colony number in control group) x 100.

Micro-array analysis for global gene expression profile. Cells (1.0x10⁶ cells per flask) were plated in T25 flasks 24 h

prior to treatment. Treatment with Taheebo was performed for 6 h at a dose of 1.5 mg/ml. RNA was isolated following the protocol for RNeasy kit (Qiagen Inc., Chatsworth, CA). The RNA was quantified using a spectrophotometric assay by absorbance at 260 nm. The integrity of RNA was evaluated by, i) 260:280 absorbance ratio, ii) 28S:18S intensity ratio, and iii) RNA integration number (RIN) using the Bioanalyzer (Agilent Technologies). RNA was amplified using a low input linear amplification kit according to the process outlined by the manufacturer (Agilent Technologies). Amplified target cRNA (1-5 μ g) was labeled with either cyanine-5 or cyanine-3 using ULS RNA Fluorescent Labeling Kit (Kreatech Biotechnology, Amsterdam, The Netherlands), according to the manufacturer's protocol. Concentration of labeled cRNA and the label incorporation was determined with a Nanodrop-1000 spectrophotometer. All labeling and post-labeling procedures were conducted in an ozone-free enclosure to ensure the integrity of the label. Labeled materials were setup for fragmentation reaction as described by the Agilent Technology Protocol processing manual and hybridized overnight in a rotating oven at 65°C in an ozone-free room. Whole Human Genome slide 4x44K format (Agilent Technologies) was used. From each labeled sample, 825 ng were co-hybridized using Sure-Hyb chambers (Agilent Technologies). Wash conditions used were as outlined in the Agilent Technologies processing manual, and the arrays were scanned using Agilent scanner 2505B. Feature Extraction from Agilent Technologies was used to extract the micro-array data. Further analysis was performed using Rosetta luminator software by MOgene, LC, St. Louis, MO.

Real-time PCR for micro-array data validation. The RNA was used to synthesize complementary DNA using TaqMan one-step RT-PCR master Mix Reagents (Applied Biosystems, Foster City, CA) with Taq Man probe-primers set (Applied Biosystems, Foster City, CA).

Real-time PCR was used to verify the micro-array data for six modulated genes using the following Taq Man probes and primers for individual genes (Applied Biosystems, Foster City, CA). DUSP 4 (NM_001394, Assay ID Hs 00154826_m1), DUSP 10 (NM_007207, Assay ID Hs 00200527_m1), GTSE 1 (NM_016426_m1, Assay ID Hs 00212681_m1), Cyclin B1 (NM_031966, Assay ID Hs 00259126_m1), CYP 1A1 (NM_000499, Assay ID Hs 00153120_m1) and OKL 38 (NM_013370, Assay ID Hs 00203539_m1). Briefly, the reverse transcription protocol used 1 μ g of total RNA and random hexamer method. The fold change in expression of each gene was calculated using the $\Delta\Delta$ CT method with GAPDH mRNA as the internal control (15).

Statistical analysis. Mean data with their deviations ($\pm$ SEM) are presented from independent triplicate sets of experiments. P-values were calculated using two sample t-test and p<0.05 was considered significant.

The statistical analysis of the data from micro-array was performed on three independent sets of experiments using Agilent and Rosetta software (MOgene, LC). Differentially expressed genes were considered as average of three sets of experiments. Modulations of ≥ 1.5 -fold change (log ratio $\geq +0.176$ or ≤ -0.176) in the Taheebo treated group compared

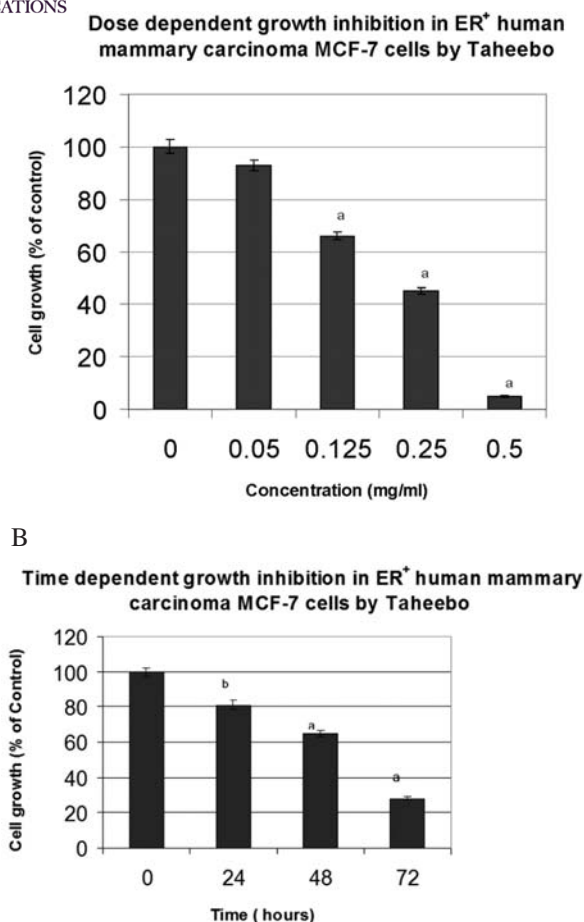


Figure 1. Dose-dependent growth inhibition of ER⁺ MCF-7 cells by Taheebo. (A) Inhibition of growth in response to treatment with 0.05-0.5 mg/ml of Taheebo. Data expressed as inhibition of cell growth (% control). Mean $\pm$ SEM, n=3 per treatment group. P<0.001 compared to control. (B) Short term dose response to growth inhibition by 0.75 mg/ml Taheebo at 24, 48, and 72 h post-seeding. Data expressed as inhibition of cell growth (% control). Mean $\pm$ SEM, n=3 per treatment group. P<0.01 compared to control.

to the untreated control group were considered statistically significant (p<0.01).

Results

Dose response of Taheebo on ER⁺ MCF-7 cells. The data presented in Fig. 1A exhibits dose-dependent growth inhibition of MCF-7 cells in response to treatment with Taheebo. The IC₅₀ concentration extrapolated from growth curves after a 6-day treatment schedule was ~0.25 mg/ml.

The short-term dose response of high dose (0.75 mg/ml) Taheebo at 24, 48 and 72 h post-seeding demonstrated 20, 35 and 72% growth inhibition relative to that of controls (Fig. 1B). These data identified the optimal concentration of Taheebo for subsequent experiments.

To identify minimum effective, maximum cytostatic and toxic concentrations of Taheebo, a time-dependent kinetics of viable cell number was performed. This experiment revealed that a 24-h treatment with 1.5 mg/ml of Taheebo resulted in a statistically non-significant 15% increase in the viable cell number relative to the initial seeding density of 1.0×10^5 cells.

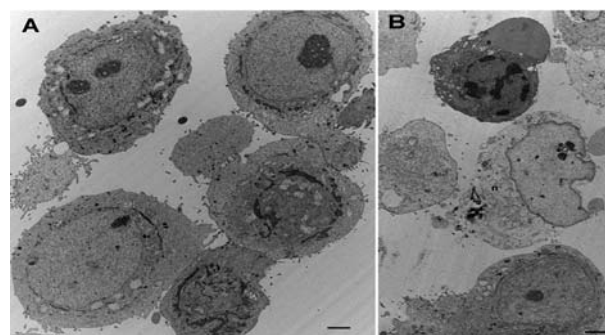


Figure 2. Transmission electron microscopy based cellular morphology of apoptotic and necrotic cells. (A) Untreated control. (B) treated with 1.5 mg/ml of Taheebo for 24 h. Note the presence of peripheral condensation and central fragmentation of nuclear chromatin in the apoptotic cell, and the presence of membrane blebbing and cytoplasmic lysosomes in the necrotic cells.

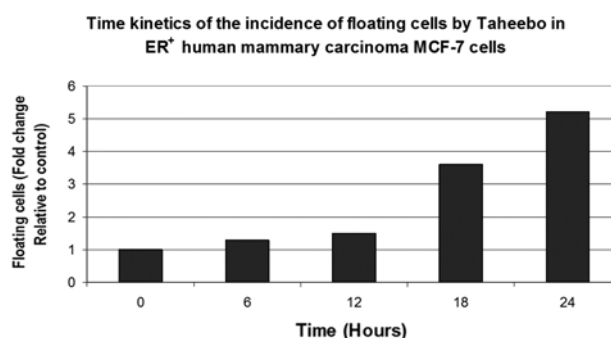


Figure 3. Time-dependent increase in non-adherent (floating) cells in response with the treatment of 1.5 mg/ml of Taheebo.

In contrast, a 48-h treatment with 1.5 mg/ml Taheebo resulted in a 94% decrease in the viable cell number relative to the initial seeding density. Thus, the 24-h treatment was defined as non-effective, while the 48-h treatment was defined as toxic.

Cell cycle regulation of ER⁺ MCF-7 cells by Taheebo. The time-dependent alteration in cell cycle regulation of MCF-7 cells in response to treatment with 0.75 mg/ml Taheebo is presented in Table I. The data expressed as the G1:S+G2/M ratio demonstrate a time-dependent progressive decrease associated with the S phase arrest of cells.

Induction of apoptosis/necrosis in ER⁺ MCF-7 cells by Taheebo. The effect of Taheebo on cell viability was also evaluated by transmission electron microscopy based cellular morphology (Fig. 2A and B), and by the incidence of non-viable floating cells at 6, 12, 18, and 24 h of duration (Fig. 3). The electron microscopy data show the ultra-structural characteristics of the Taheebo treated cultures revealing the presence of apoptotic cells characterized by the presence of peripheral condensation of the chromatin and membrane 'blebbing' (Fig. 2B), and of necrotic cells characterized by the presence of numerous lysosomes in the cytoplasm (Fig. 2B). Furthermore, the data presented in Fig. 3 reveal a time-dependent increase in the incidence of a non-adherent (floating) cell population.

Table I. Cell cycle regulation in ER⁺ human mammary carcinoma MCF-7 cells by Taheebo.^a

Treatment	Dose (mg/ml)	Duration (h)	G1 (%)	S+G2/M (%)	Ratio	Modulation (% control)
None		24	53.2	46.5	1.1	-
Taheebo	0.75	24	59.9	39.9	1.5	+36.4
None		48	63.1	36.7	1.7	-
Taheebo	0.75	48	64.9	35.0	1.8	+5.9
None		72	67.5	32.3	2.1	-
Taheebo	0.75	72	60.9	39.1	1.5	-28.6

^aData expressed as arithmetic means from independent determinations in triplicate.

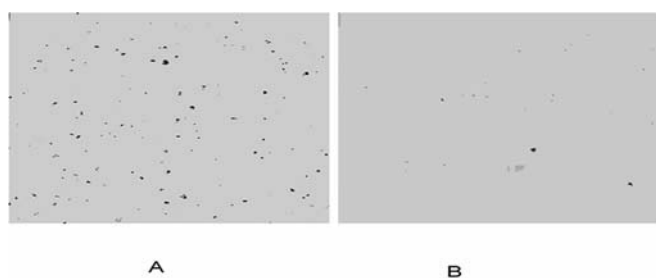


Figure 4. Inhibition of clonogenic growth of ER⁺ MCF-7 cells in response to 72 h of pre-treatment with 0.75 mg/ml of Taheebo. (A) Colonies in the untreated control. (B) Colonies in the Taheebo treated cultures at day 8 post-seeding.

Long-term growth inhibition of ER⁺ MCF-7 cells by Taheebo.

The persistence of long-term growth inhibition in response to treatment with Taheebo was examined in the experiment presented in Fig. 4A and B. The colony counts obtained at the end of 11 days (3 day pre-treatment and 8 day clonogenic assay) revealed that relative to control cultures, mean colony number 419.0 ± 6.4 (Fig. 4A), the Taheebo treated cultures exhibited 88% inhibition mean colony number 50.3 ± 2.9 , $p < 0.001$ (Fig. 4B), in the number of colonies formed.

Modulation of global gene expression profile of ER⁺ MCF-7 cells by Taheebo. The data presented in Table II examine the modulatory effect of Taheebo on global gene expression profiles in ER⁺ MCF-7 cells. In Taheebo treated cells from among multiple genes functioning in the regulation of cell cycle progression, down-regulation of S phase specific PCNA, GSTE1 and OKL38, and G2 specific cyclin B and cdc2 resulted in a >1.5-fold reduction relative to that in untreated controls. In contrast, among multiple genes functionally involved in cellular apoptosis, the GADD45 gene family members and inactivators of the anti-apoptotic BCL-2, such as BIM, HRK/BH3 and BBC3 were notably up-regulated to >1.5-fold, relative to those in untreated controls. Furthermore, tumor necrosis factor receptor super family (TNFRSF) genes responsible for apoptosis, and Calpain family genes responsible for necrosis were also up-regulated. Genes responsible for estrogen responsiveness such as ESR1 for ER- α were down-regulated, while genes such as CYP 1A1 and CYP 1B1, responsible for xeno-biotic metabolism, including that of estradiol were significantly up-regulated.

Comparison of the data on gene expression by microarray and real time PCR

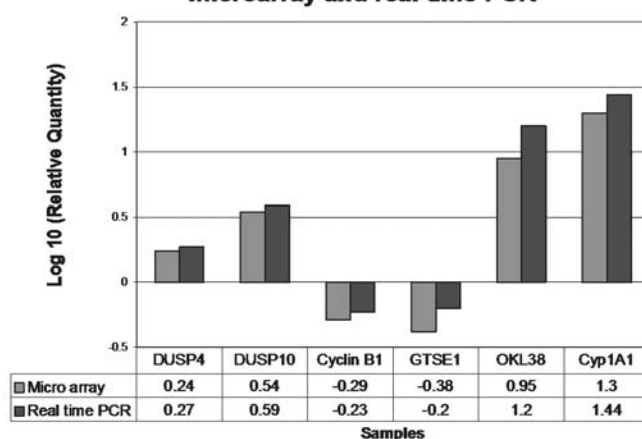


Figure 5. A comparison in modulation of gene expression using the microarray and real-time PCR assays.

The extent of modulation in the expression of selected genes was also confirmed using real-time PCR assay (Fig. 5).

Discussion

It is generally recognized that hormone responsive ER⁺ clinical breast cancers represent ~80% of all sporadic cancer and exhibit a positive response to endocrine based therapeutic interventions (1,2). However, long-term endocrine therapy with selective estrogen receptor modulators is frequently associated with reduced patient compliance, in part due to acquired drug resistance, or adverse systemic toxicity. These limitations emphasize a need to identify efficacious novel agents with minimal long-term toxicity.

The present study utilized the well-established human breast carcinoma derived ER⁺ MCF-7 cell culture model to examine the therapeutic efficacy of a natural plant extract, Taheebo, and to identify potential mechanistic leads for its efficacy on ER⁺ clinical breast cancer.

The phenomenological evidence for the growth inhibitory effect of Taheebo on ER⁺ MCF-7 cells is supported by the data that demonstrate a dose- and time-dependent inhibition of growth in response to treatment with Taheebo. These growth curves also identified optimum maximally cytostatic treatment for Taheebo in the present model system.



Gene	Gene bank no.	Fold Change		Log Ratio	
		up	down	Log Ratio	Function
Cell cycle regulatory genes					
GTSE1	NM_016426		2.38	-0.376	Homo sapiens G2 and S phase expressed 1 (GTSE1). DNA damage response. Signal transduction by p53 class mediator resulting in cell cycle arrest.
PCNA	NM_002592		1.96	-0.29	Homo Sapiens proliferating cell nuclear antigen (PCNA), regulation of cell cycle; DNA replication
Cyclin B1	NM_031966		1.96	-0.29	Regulation of cell cycle at G2/M
CyclinE1	NM_001238		1.6	-0.2	Regulation of cell cycle at G1/S transition.
CyclinE2	NM_057749		3.22	-0.5	Regulation of cell cycle at G1/S transition.
cdc2	NM_001786		2.56	-0.4	Cell division cycle 2.
Cyclin A2	NM_001237		2.77	-0.44	Mitotic G2 arrest. Regulators of CDK kinases. This cyclin binds and activates CDC2 and cdK2 at G1/S and G2/M transitions.
RFC4	NM_002916		1.56	-0.19	Replication factor C4
cdc20	NM_001255		1.85	-0.27	Cell division cycle homolog 20.
Chk1	NM_005197	1.61		0.2	Checkpoint suppressor 1.
CCRK	NM_178432	1.84		0.26	Cell cycle related kinase (CCRK); p42.
cdk6	NM_001259		2.0	-0.296	Cyclin dependent kinase 6.
cdk2	NM_001798		1.50	-0.18	Cyclin dependent kinase 2.
p18	NM_001262		1.88	-0.275	Cdk-4 inhibitor, p18.
POLE2p59	NM_002692		1.81	-0.26	ε DNA polymerase activity DNA replication etc.
EXO1	NM_003686		1.51	-0.182	DNA binding exonuclease (5' to 3') activity, interacts with Msh2 which is involved in Mismatch repair.
OKL38	NM_013370	11.03		1.04	Homo sapiens pregnancy-induced growth inhibitor, negative regulation of cell growth (akaBDG1)
MCM6	NM_005915		1.639	-0.21	Minichromosome maintenance deficient 6 DNA replication related function.
cdc25A	NM_001789		1.75	-0.24	Regulate cyclin dependent protein kinase act. cdc25A DUSP control entry into and progression through S phase by dephosphorylating sites on cyclinE-cdk2 (Thr14,Tyr15).
AURKA	NM_198433		1.85	-0.27	Cell cycle regulated kinase that appears to be involved in microtubule formation/chromosome seggregation and stabilizes spindle pole etc.
cdc20	NM_001255		1.85	-0.27	It interacts with multiple checkpoints in cell cycle. It is involved for nuclear movement prior to anaphase and chromosome segregation.
SKP2	NM_032637		1.96	-0.29	S phase kinase associated protein 2; It is an element for cycA-cdk2 S phase kinase
POLA2	NM_002689		1.89	-0.276	DNA directed polymerase-α; DNA replication.
HCAP-G	NM_022346		1.69	-0.226	Homo sapiens chromosome condensation protein G. Mitotic cell cycle, chromosome condensation.
cdc7	NM_003503		1.85	-0.267	Negative regulation of cell proliferation. G1/S transition. cDc7 activity is essential for initiation of DNA replication.
AMID	NM_032797	2.62		0.42	Homo Sapiens apoptosis inducing factor (AIF) like mitochondrion - associated inducer of death (AMID).
Caspase4	NM_033306	1.62		0.21	
caspase 7	NM_033339	1.652		0.22	
caspase8	NM_033356	1.643		0.21	Transcript variant C , caspase 8.
AATK	AK131529	1.8		0.25	Apoptosis associated tyrosine kinase.
PAWR	NM_002583	1.47		0.17	PRKC, apoptosis WT1 regulator.
GADD45G	NM_006705	3.47		0.54	GADD 45 family A,B,G are responsible for growth suppression, induction of apoptosis through inhibition of NF-κB, up-regulation of c jun N terminal kinase (JNK).
GADD45B	NM_015675	1.92		0.28	
GADD45A	NM_001924	5.41		0.73	

Table II. Continued.

Gene	Gene bank no.	Fold Change		Log Ratio	
		up	down	Log Ratio	Function
TP53INP1	NM_033285		1.47	-0.17	Its transcription is activated by p53. It is responsible for G1 arrest and p53 mediated apoptosis.
CCAR1	NM_018237		1.55	-0.19	
Bcl-2 Family					
BCL-2	NM_000633		1.53	-0.19	
HRK/BH3	NM_003800	3.75		0.57	Activator of apoptosis. HRK regulates apoptosis through inactivating BCL2 and BCLXL but not with the death promoting Bcl2 related protein BAX, BAK or BCLXs. HRK interacts with (BH3) domain with BCL2/BCL-XL.
Bcl2like11 (BIM)	NM_138622	2.156		0.33	<i>Homo Sapiens</i> apoptotic like facilitator. BCL-2 like 11.
BBC3	NM_014417	3.08		0.49	<i>Homo Sapiens</i> BCL-2 binding component 3 also known as PUMA.
Athanogene1	NM_004323	1.758		0.245	BCL2 associated athanogene1. It binds and enhances anti-apoptotic effects of BCL2 and represents a link between growth factor receptors and apoptotic mechanism.
Athanogene3	NM_004281	1.6			
Tumor necrosis factor receptor super family genes					
TNRSF1A	NM_001065	2.44		0.387	TNFRSF family leads to apoptosis through MAPK/JNK and/or through Fas -associated death domain (FADD).
TNFR SF6	NM_000043	1.67		0.22	
TNFRSF21	NM_014452	1.677		0.22	
Calpain family					
CAPN12	NM_144691	1.6		0.2	Calpains, together with the lysosomal aspartyl proteases mediate necrotic cell death.
CAPN10	NM_023083	1.5		0.176	
CAPN2 (m/II)	NM_001748	1.46		0.17	
Dual specific phosphatase (DUSP) family genes					
MAP2K6	NM_002758		-4.786	-0.68	Extracellular signal regulated (ERK) kinase.
MAP3K1/MEKK1	ENST0000026477		-1.5	-0.18	Activates other MAP kinase signalling pathway.
MAP3K2/MEKK2	NM_006609		-1.47	-0.18	Phosphorylates and activates I κ B kinase.
MAP3K10	NM_002446	1.64		0.21	Induction of apoptosis via JUN pathway.
MAP3K14	NM_003954	1.8		0.25	NF- κ B inducing kinase.
DUSP2	NM_004418		2.63	-0.42	Dual specific phosphatase 2.
DUSP8	NM_004420	2.2		0.34	Dual specific phosphatase 8.
DUSP4	NM_001394	1.75		0.24	Dual specific phosphatase 4.
DUSP10	NM_007207	3.53		0.547	Dual specific phosphatase 10.
DUSP5	NM_004419	2.02		0.3	Dual specific phosphatase 5.
DUSP 13	NM_001007271	2.0		0.3	Dual specific phosphatase 13.
DUSP1	NM_004417	1.8		0.25	Dual specific phosphatase 1.
DUSP 16	NM_0030640	2.62		0.41	Dual specific phosphatase 16.
Estrogen responsive genes					
ESR1	NM_000125		1.6	-0.2	Estrogen receptor- α .
CYP1A1	NM_000499	19.77		1.3	
CYP1B1	NM_000104	7.95		0.9	

^aModulation in gene expression. Fold change represents comparison between Taheebo treated and untreated control group. Values for fold change represent means from independent triplicate sets of experiments. Genes in bold have been verified by real-time PCR.



SPANDIDOS PUBLICATIONS experiments designed to identify possible phenom-

enoads for growth inhibition revealed that a short-term treatment of up to 72 h is associated with a progressive time-dependent decrease in G1:S+G2/M ratio due to a modest S phase arrest. This is consistent with a progressive time-dependent increase in the incidence of non-adherent (floating) cells. These data suggest that regulation of cell cycle progression and induction of cellular apoptosis/necrosis represent susceptible mechanistic pathways that are responsible for the observed effects of Taheebo.

The experiment was designed to examine the long-term effects of Taheebo using the clonogenic assay revealed that withdrawal of Taheebo retained its inhibitory effects for at least 8 days. These data identify possible leads that dose fractionation achieved by discontinuous treatment reduces long-term toxicity without compromising the efficacy.

The experiment utilizing micro-array based global gene expression profiling identified important leads for possible molecular targets and mechanistic pathways critical for the efficacy of Taheebo. Overall, the micro-array assay examined the gene expression profiles of 43,376 genes, using the 44K gene chip. About 1,600 genes (3.7%) exhibited down-regulated expression, 1,900 genes (4.4%) exhibited up-regulated expression, and 40,000 genes (92.2%) remained essentially unchanged. It is clear from the data obtained from selected genes relevant to growth, proliferation, apoptosis and metabolism of ER⁺ MCF-7 cells in the culture that Taheebo modulates the expression of multiple genes that are involved in these biological processes. The modulation in gene expression profiles essentially correlated with the cell cycle regulation and induction of initial stages of cellular apoptosis. Thus, with regard to S phase arrest, it is noteworthy that genes such as PCNA and GTSE1 were down-regulated. These genes play a role in DNA synthesis and replication at the S phase of the cell cycle (16). In addition, the GADD 45 family of genes was also substantially up-regulated in response to the treatment with Taheebo. This gene family is involved in the regulation of growth, DNA repair, and induction of apoptosis (17). Interestingly, OKL38 gene, also known as the human bone marrow stromal cell derived growth inhibitor (BDGI), exhibited a robust 12-fold up-regulation. This gene is known to induce S phase arrest with subsequent initiation of apoptosis (18,19). The genes responsible to inactivate anti-apoptotic BCL-2, such as BIM, HRK/BH3 and BBC3 exhibited 2.16-, 3.75-, and 3.08-fold up-regulation, respectively. These genes are also known to arrest the cells in the S phase and initiate apoptosis (19,20). These data on the modulated expression of OKL38, BIM, HRK/BH3 and BBC3 genes taken together, suggest that initiation of the apoptotic process by Taheebo is in part due to reduced function of the anti-apoptotic BCL-2 gene. In this context it is also noteworthy that consistent with the presence of necrotic cells in Taheebo treated cultures, the Calpain family genes (21) were up-regulated to >1.5-fold, relative to those in untreated controls.

Regarding the effect of Taheebo on ER signaling, it was observed that Taheebo treatment up-regulated the dual specific phosphatase (DUSP) gene family is responsible for negative regulation of MAPK signaling (22-24). These data indicate that Taheebo suppresses the proliferation of ER⁺ MCF-7 cells via

its effect on the ligand independent AF-1 domain that is critical for the ER signaling pathway. Consistent with the possible efficacy of Taheebo via ER signaling, observed down-regulation of cyclin A and cdk2 is suggestive of an inhibition of phosphorylation of ER N-terminal AF-1 domain (25,26). This interpretation provides proof of a principle mechanistic lead for the possible anti-estrogenic effects of Taheebo components at high cytostatic doses via the ER signaling pathway.

With regard to estrogen responsive genes, it is noteworthy that Taheebo treatment resulted in 1.6-fold down-regulation of ESR1, the gene for ER- α , while ESR2, the gene for ER- β , remained essentially unaltered. Although, CYP 1A1 and CYP 1B1 genes are primarily involved in xeno-biotic metabolism, they are also responsible for cellular metabolism of estradiol leading to the generation of C2-, C4-, and C16 α -hydroxylated metabolites capable of modulating the growth of MCF-7 cells (27-29). Similar to the MCF-7 cells, modulation of cellular metabolism of estradiol was noted in the 184-B5/HER cell culture model for ER/HER-2⁺ pre-invasive comedo ductal carcinoma *in situ* (30).

All the targets and pathways discussed above are well recognized in the growth, cellular proliferation and tumorigenicity of ER⁺ MCF-7 cells (12,13). Furthermore, experimental modulation in these pathways in response to treatment with selective estrogen receptor modulators is associated with growth inhibition of MCF-7 cells both in *in vitro* cell cultures as well as in *in vivo* xeno-transplant models for tumorigenicity via multiple mechanisms (12,13,22,23,25,26). These data taken together, suggest that the mode of action of Taheebo is similar to that of a selective estrogen receptor modulator. In this context it is noteworthy that published studies with Tamoxifen on ER⁺ MCF-7 cells demonstrated that in addition to the ER-dependent mechanism, the selective estrogen receptor modulator Tamoxifen alters cellular metabolism of estradiol to favor the generation of anti-proliferative metabolites (31-33), and influence cell cycle progression in MCF-7 cells via cdk-4/6 and p53-dependent pathways (12,13,34).

With regard to the identification of molecular target(s) and mechanistic pathway(s) for the efficacy of Taheebo, it needs to be recognized that non-fractionated aqueous extract of Taheebo used in the present study contains a mixture of several mechanistically distinct constituents including furanone-naphthoquinones, quinines, naphthoquinones and flavonoids (35-37). Naphthofurandione, a major component of Taheebo, has selective anti-tumor effects on a variety of human carcinoma derived cell lines (35-38). It is therefore, conceivable that the efficacy for growth inhibition by Taheebo, as evidenced by the present data, is due to interactive effects of multiple water soluble components from Taheebo.

In conclusion, the outcome of the present study validates a human tissue derived cell culture approach to evaluate the efficacy of non-fractionated herbal medicinal extracts and to identify clinically translatable mechanistic leads for alternative therapy of ER⁺ clinical breast cancer.

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