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Reference Profile Correlation Reveals Estrogen-like Trancriptional Activity of Curcumin

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Key Words

Phytoestrogen • Breast cancer • Gene expression profiling • Reference profile correlation • Chemoprevention

Abstract

Background: Several secondary metabolites from herbal nutrient products act as weak estrogens (phytoestrogens), competing with endogenous estrogen for binding to the estrogen receptors and inhibiting steroid converting enzymes. However, it is still unclear whether these compounds elicit estrogen dependent transcription of genes at physiological concentrations. Methods: We compare the effects of physiological concentrations (100 nM) of the two phytoestrogens Enterolactone and Quercetin and the suspected phytoestrogen Curcumin on gene expression in the breast cancer cell line MCF7 with the effects elicited by 17-β-estradiol (E2). Results: All three phytocompounds have weak effects on gene transcription; most of the E2 genes respond to the phytoestrogens in the same direction though to a much lesser extent and in the order Curcumin > Quercetin > Enterolactone. Gene regulation induced by these compounds was low for genes strongly induced by E2 and similar to the latter for genes only weakly regulated by the classic estrogen. Of interest with regard to the treatment of menopausal symptoms, the survival factor Birc5/survivin and the oncogene MYBL1 are strongly induced by E2 but only marginally by phytoestrogens. Conclusion: This approach demonstrates estrogenic effects of putative phytoestrogens at physiological concentrations and shows, for the first time, estrogenic effects of Curcumin.

Introduction

Many breast cancer risk factors are related to cumulative exposure to estrogens [1] including hormone replacement therapy [2, 3]. Contact to environmental pollutants with estrogenic activity has also been associated with a slightly elevated breast cancer risk [4] and geographical or apparently ethnic differences in breast cancer incidence have been attributed to diets containing natural compounds with weak estrogenic activity called phytoestrogens [5].

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Phytoestrogens are plant derived substances that have estrogenic activity. They bind to the estrogen receptor with an affinity 1000-10000 fold lower than that of the endogenous hormone and initiate estrogen-dependent transcription [6]. According to their chemical structures phytoestrogens are classified into six main groups: flavones, flavonones, isoflavones, coumestans, lignans, and stilbenes.

Phytoestrogens have been proposed for chemoprevention and as a substitute of hormone replacement therapy [5], because it can be assumed that they compete with the endogenous hormone for binding to the estrogen receptor, thereby reducing the proliferative effects of endogenous estrogens. However, in the absence of endogenous hormone, phytoestrogens could stimulate proliferation of breast cancer cells and contribute to breast cancer progression. Hence, the question of whether phytoestrogens are safe substitutes of hormone replacement therapy is still open.

In addition to receptor dependent functions there is growing evidence that phytoestrogens reduce local production of estrogens by inhibiting the activity of key steroidogenic enzymes involved in the synthesis of estradiol (E2) [7, 8]. Phytoestrogens could diminish local estrogen levels by inhibiting their converting enzymes thereby reducing the proliferation stimulus and thus protect against breast cancer progression.

The estrogenicity of a compound is reflected by its effect on the growth of estrogen-responsive breast cancer cells that can be measured *in vitro* by a series of assays [9, 10]. Growth-promoting effects have been observed for genistein and other isoflavones such as daidzein and biochanin A [11] only at low doses (= 10 μM) in ER-positive MCF7 and T47D cells whereas at higher doses, growth and survival of both ER-positive and ER-negative (MDA-MB-231 and -435) cells were inhibited [12-20]. The flavones quercetin and apigenin inhibit E2-induced DNA synthesis and proliferation of ER-positive and ER-negative breast cancer cells [21-23]. Lignans have also been investigated for their effects on cell growth. At low doses enterolactone stimulated but at concentrations above 10 μM, it inhibited proliferation of MCF7 cells [24, 25].

Hormone replacement therapy (HRT) is usually prescribed to reduce the incidence of cardiovascular and coronary heart diseases or bone fractures in post-menopausal women. In 2002, the initial results of a long-term randomized controlled trial of HRT (Women's Health Initiative [WHI]) showed that, after 5 years of combined estrogen and progestogen therapy in an older population, there was a significant reduction in fractures, no overall cardiovascular benefit, and an increased rate of breast cancer [3]. The increased cancer risk appears to dissipate after ending HRT [26]; this has caused a decrease of the use of HRT in the United States which in turn has contributed to a decline of breast cancer incidence [27]. New approaches for treatment of menopausal symptoms are therefore highly desirable. Natural plant-derived phytoestrogens show a weak estrogenicity and could therefore be an alternative to HRT although it is not clear whether they are efficacious and safe.

Here we addressed phytoestrogen effects on gene transcription using complex microarrays in order to establish i) whether estrogenic effects can be observed at a physiological concentration, ii) whether eventual estrogenic effects are generalized to all estrogen responsive genes and iii) whether the extent of regulation is similar for all estrogen responsive genes. We tested three molecules belonging to three different classes of plant derived secondary metabolites: Curcumin is a polyphenol [28] derived from the plant curcuma longa used as a spice component (curry), Enterolactone is a lignan present in flax and sesame seeds and in many vegetables and berries [29] and Quercetin is a flavonoid present in apples, in tea, in grapes and in many vegetables [30]. Quercetin and Enterolactone but not Curcumin are known phytoestrogens. However it is already known that this polyphenol is able to displace > 85% of estradiol binding to its receptor as shown by 3H-estradiol ligand binding assays [31].

Many *in vitro* studies have been performed with rather high phytoestrogen concentrations (10 μM and above) that are unlikely to be achieved *in vivo* and it is not clear whether the effects on gene transcription observed under these conditions have any clinical relevance. For this reason we decided to use a concentration of 100 nM that has been measured in the breast cyst fluid of untreated women [32].

We show, through the correlation with an estrogen reference gene expression profile, that the phytoestrogens, including Curcumin, have estrogenic effects on gene transcription even at low, physiological concentrations.

Materials and Methods

Cell culture and treatment

MCF7 cells were obtained from ICLC cell line collection (Genoa, Italy) and grown at 37 °C in a humidified atmosphere of 5 % CO_2 in DME medium supplemented with 10 % fetal calf serum and 1 % L-glutamine solution (200 mM). Medium was

changed every two days. Before the start of the experiments, cell cultures were kept for 10 days in phenol red free DMEM (Seromed, Berlin, Germany) supplemented with 10 % dextran coated charcoal treated fetal calf serum (Seromed, Berlin, Germany). In this period, medium was changed each other day and cell cultures were rinsed three times with PBS. Cells were treated for 72 h with 1nM 17-β-estradiol (E2), 100 nM Curcumin, 100 nM enterolactone or 100 nM quercetin (all from Sigma, St. Louis, USA) in ethanol (stock concentration = 100 μM) or with vehicle alone (control).

Preparation of RNA and cRNA

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA synthesis was performed using T7-$(dT)_{24}$ oligo primers and the Custom SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, Irvine, CA, USA). Double stranded cDNAs were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), ethanol precipitated, and used to prepare cRNAs using the Bioarray High Yield RNA Transcription Kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. cRNAs were purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany), controlled by agarose gel electrophoresis and subjected to fragmentation for 35 min. at 94 °C in fragmentation buffer (40 mM Tris-acetate pH 8.1, 100 mM CH_3COOH, 30 mM $Mg(CH_3COO)_2x4H_2O$).

GeneChip microarray analysis and data normalization

Labeled cRNA was used for screenings of GeneChip Human Genome U133plus2 arrays (Affymetrix, Santa Clara, CA, USA). The experiment consisted of 3 biological replicates. Hybridization and scanning was performed on the Affymetrix platform. Data were normalized following the GCRMA procedure [33] of Bioconductor 2.3.1 [34] (http://www.bioconductor.org). Statistically significant expression changes were determined using permutation tests (SAM, [35] (http://www-stat.stanford.edu/~tibs/SAM/). Genes regulated at least two fold in comparison to untreated controls were considered. The delta value was set to return a median false significant number of zero. Annotations were obtained through the DAVID database (http://david.niaid.nih.gov/david/beta/index.htm) [36].

Reference profile correlation

Gene expression profiles of phytoestrogen treated cells were compared to 17-β-estradiol treated cells. For this purpose, significantly E2-regulated genes (E2 responsive genes) were identified through class comparison using SAM as described above. Expression values of E2 responsive genes in E2 and phytoestrogen treated cells were analyzed by Spearman correlation and plotted. P and r-values were calculated. Since Spearman correlation is non-parametric no r^2-values were available.

Quantitative RT-PCR

Expression data validation was performed by quantitative real-time RT-PCR using the RNA extracted from drug- or mock-treated cells as described previously. Expression data were normalized on GAPDH and on RNA polymerase II (RPII) gene expression data obtained in parallel. Expression changes were calculated using the mean value of normalizations obtained using GAPDH and RPII genes as house keeping genes.

Preparation of Cellular Extracts

Cells were washed 3 times with PBS and collected by scraping and centrifuged. Lysis buffer (10 mM Na_3PO_4; 0.4M NaCl; 0.2 % Triton X-100) was added to the pellets and the mixture was sonicated. After centrifugation for 15 min at 15000xg the supernatant containing the soluble proteins was collected and either analyzed immediately or stored at -20 °C.

Protein Determination

Protein concentrations were determined by the BCA protein assay (Pierce, Oud-Beijerland, Netherlands) with bovine serum albumin as the standard.

Western Blots

Lysates from human MCF7 breast cancer cells treated with 1nM E2 or different concentrations of Curcumin (100 nM, 1 μM, 10 μM and 25 μM) and non-treated control cells were analyzed using an antibody against MYBL1 (Sigma Deisenhofen, Germany). Equal amounts of protein were subjected to SDS-PAGE and the intracellular amount of β-actin was analyzed as loading control (antibody from Sigma Deisenhofen, Germany). Additionally, the amount of protein blotted onto the membranes was visualized with Ponceau red before blocking. Following electrophoretic separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, proteins were electro-blotted on nitrocellulose membranes (Whatman, Brentford, UK). The membranes were blocked in 5 % non-fat milk (Merck, Darmstadt, Germany) overnight at 4 °C. The first antibody was incubated for 1 h at room temperature. Thereafter, membranes were washed in tris buffered saline with Tween buffer, and a further incubation was carried out with a peroxidase-conjugated antibody (Dako, Hamburg, Germany) for 1 h at room temperature. The enhanced chemiluminescence system was used for visualization of the protein bands as recommended by the manufacturer (GE Healthcare, Little Chalfont, UK). Semi-quantitative evaluation of the bands was performed by densitometric analysis with the ImageJ software provided by the NIH (http://rsb.info.nih.gov/ij/).

Results

Estrogen Response Reference Profile in the human breast cancer cell line MCF7

As a first step we generated a gene expression profile of the estrogen receptor positive breast cancer cell line MCF7. We used a concentration of 1 nM that is considered to correspond to physiological levels and is used in most *in vitro* studies. The choice of the time point of 72 hours was based on the consideration that post-menopausal women, the likely users of phytoestrogens, have a relatively stable, non ovarian endogenous estrogen

Table 1. Estrogen response reference profile (E2 genes). Gene expression of MCF7 cells after 72 hours of treatment with 1nM 17-β-estradiol was analyzed by microarray hybridization. Normalized expression data were analyzed by Significance Analysis of Microarray (SAM). The 25 most up- and down-regulated genes that are statistically significantly (false discovery rate (FDR) = 0%) regulated by 17-β-estradiol are listed, expression values are given as fold change.

Expression of estradiol responsive genes relative to untreated controls			
Affymetrix Probeset ID	Gene Symbol	Description	Fold change
25 Top up-regulated genes			
209687_at	CXCL12	CHEMOKINE (C-X-C MOTIF) LIGAND 12	280.99
228554_at	NA	DKFZP58	172.11
238047_at	RP13-102H20.1	HYPOTHETICAL PROTEIN FLJ30058	143.96
205239_at	AREG	AMPHIREGULIN (SCHWANNOMA-DERIVED GROWTH FACTOR)	98.39
213906_at	MYBL1	V-MYB MYELOBLASTOSIS VIRAL ONCOGENE HOMOLOG	97.23
203963_at	CA12	CARBONIC ANHYDRASE XII	92.80
202376_at	SERPINA3	SERPIN PEPTIDASE INHIBITOR, CLADE A	91.33
210735_s_at	CA12	CARBONIC ANHYDRASE XII	74.44
205470_s_at	KLK11	KALLIKREIN 11	72.16
228241_at	AGR3	BREAST CANCER MEMBRANE PROTEIN 11	58.55
206115_at	EGR3	EARLY GROWTH RESPONSE 3	51.95
220038_at	SGK3	SERUM/GLUCOCORTICOID REGULATED KINASE FAMILY 3	47.47
205380_at	PDZK1	PDZ DOMAIN CONTAINING 1	43.30
204508_s_at	CA12	CARBONIC ANHYDRASE XII	41.31
231120_x_at	PKIB	PROTEIN KINASE INHIBITOR BETA	34.54
227627_at	SGK3	SERUM/GLUCOCORTICOID REGULATED KINASE FAMILY 3	31.86
244745_at	RERG	RAS-LIKE, ESTROGEN-REGULATED, GROWTH INHIBITOR	31.26
226117_at	TIFA	TRAF-INTERACTING PROTEIN	28.27
201739_at	SGK1	SERUM/GLUCOCORTICOID REGULATED KINASE	26.45
220651_s_at	MCM10	MCM10 MINICHROMOSOME MAINTENANCE DEFICIENT 10	23.86
202240_at	PLK1	POLO-LIKE KINASE 1 (DROSOPHILA)	23.73
210297_s_at	MSMB	MICROSEMINOPROTEIN, BETA	23.56
25 most downregulated genes			
238476_at	C5ORF41	ADULT RETINA PROTEIN	0.17
229130_at	LOC285535	HYPOTHETICAL PROTEIN LOC285535	0.15
226382_at	LOC283070	HYPOTHETICAL PROTEIN LOC283070	0.14
239050_s_at	NA	BF514509	0.14
201983_s_at	EGFR	EPIDERMAL GROWTH FACTOR RECEPTOR	0.14
241376_at	NA	AV685992	0.13
234331_s_at	FAM84A	FAMILY WITH SEQUENCE SIMILARITY 84, MEMBER A	0.13
225956_at	C5ORF41	ADULT RETINA PROTEIN	0.12
202728_s_at	LTBP1	LATENT TRANSFORMING GROWTH FACTOR BETA BP 1	0.11
221986_s_at	KLHL24	KELCH-LIKE 24 (DROSOPHILA)	0.11
242868_at	EPAS1	ENDOTHELIAL PAS DOMAIN PROTEIN 1	0.10
231941_s_at	MUC20	MUCIN 20	0.09
236576_at	NA	N63005	0.09
228256_s_at	C5ORF26	TIGA1	0.09
225915_at	CAB39L	CALCIUM BINDING PROTEIN 39-LIKE	0.09
203354_s_at	PSD3	PLECKSTRIN AND SEC7 DOMAIN CONTAINING 3	0.08
201280_s_at	DAB2	DISABLED HOMOLOG 2	0.07
209201_x_at	CXCR4	CHEMOKINE (C-X-C MOTIF) RECEPTOR 4	0.07
226158_at	KLHL24	KELCH-LIKE 24 (DROSOPHILA)	0.06
207016_s_at	ALDH1A2	ALDEHYDE DEHYDROGENASE 1 FAMILY, MEMBER A2	0.05
226622_at	MUC20	MUCIN 20	0.05
221218_s_at	TPK1	THIAMIN PYROPHOSPHOKINASE 1	0.05
205220_at	GPR109B	G PROTEIN-COUPLED RECEPTOR 109B	0.04
1556190_s_at	PRNP	PRION PROTEIN (P27-30)	0.04
219768_at	VTCN1	V-SET DOMAIN CONTAINING T CELL ACTIVATION INHIBITOR 1	0.03

production and therefore, effects of a long term estrogen exposure are more relevant. On the other hand, estrogens strongly induce proliferation in MCF7 cells and very long exposures would provoke cell density dependent differences between E2 treated and control cells. At 72 hours, the immediate proliferative burst induced by estrogen stimulation has faded out and a stable proliferation rate has been reached. Gene expression was assessed by microarray hybridization, in order to identify genes that respond to the treatment with 17-β-estradiol. We compared profiles obtained from E2 treated MCF7 cells with those from untreated cells. We performed a significance analysis of microarrays using the permutation test, SAM, that yielded 334 significantly (false discovery

Table 2. Effects of 100 nM Curcumin on gene expression in MCF7 cells. Gene expression of MCF7 cells after 72 hours of treatment with 100nM of Curcumin was analyzed by microarray hybridization. Fold change values as compared to untreated controls are shown for and Curcumin (FC_CUR) and estradiol (FC_E2). All genes with a p-value (Curcumin versus control, student´s T-Test) < 0.05 and a fold change value >1.5 are shown.

Effects of 100nM Curcumin on Gene Expression in MCF7 Cells					
Affy ID	*Gene symbol*	*Description*	*p-value*	*FC_CUR*	*FC_E2*
213906_at	MYBL1	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	0.0192	2.71	100.64
224428_s_at	CDCA7	cell division cycle associated 7	0.0147	2.05	13.05
1559966_a_at	NA	NA	0.0120	2.01	1.54
214519_s_at	RLN2	relaxin 2	0.0481	1.92	6.32
202094_at	BIRC5	baculoviral IAP repeat-containing 5 (survivin)	0.0035	1.87	16.47
207828_s_at	CENPF	centromere protein F, 350/400ka (mitosin)	0.0205	1.84	6.02
218585_s_at	DTL	denticleless homolog (Drosophila)	0.0136	1.79	10.80
234987_at	SAMHD1	SAM domain and HD domain 1	0.0343	1.78	2.73
205046_at	CENPE	centromere protein E, 312kDa	0.0447	1.76	21.09
226712_at	SSR1	signal sequence receptor	0.0332	1.76	1.50
219000_s_at	DCC1	NA	0.0205	1.74	11.67
203438_at	STC2	stanniocalcin 2	0.0311	1.69	2.53
202134_s_at	WWTR1	WW domain containing transcription regulator 1	0.0123	1.69	1.72
228711_at	ZNF37A	zinc finger protein 37a (KOX 21)	0.0340	1.68	1.59
225655_at	UHRF1	ubiquitin-like, containing PHD and RING finger domains, 1	0.0022	1.65	7.06
220651_s_at	MCM10	MCM10 minichromosome maintenance deficient 10	0.0230	1.64	23.92
234863_x_at	FBXO5	F-box protein 5	0.0072	1.64	9.15
218859_s_at	C20orf6	chromosome 20 open reading frame 6	0.0469	1.63	1.78
223413_s_at	LYAR	NA	0.0445	1.62	5.92
205440_s_at	NPY1R	neuropeptide Y receptor Y1	0.0320	1.62	5.80
1554696_s_at	TYMS	thymidylate synthetase	0.0082	1.61	7.65
1553269_at	ZNF718	zinc finger protein 718	0.0467	1.58	2.14
205283_at	FCMD	Fukuyama type congenital muscular dystrophy (fukutin)	0.0428	1.58	1.58
204983_s_at	GPC4	glypican 4	0.0119	1.58	0.81
208859_s_at	ATRX	alpha thalassemia/mental retardation syndrome X-linked	0.0466	1.56	0.96
229304_s_at	MLF1IP	MLF1 interacting protein	0.0161	1.55	4.79
225664_at	COL12A1	collagen, type XII, alpha 1	0.0312	1.54	3.24
219654_at	PTPLA	protein tyrosine phosphatase-like, member A	0.0024	1.54	1.35
209409_at	GRB10	growth factor receptor-bound protein 10	0.0276	1.52	1.04
219959_at	MOCOS	molybdenum cofactor sulfurase	0.0221	1.51	4.71
219544_at	FLJ22624	NA	0.0277	1.50	5.67
1555495_a_at	SDCCAG10	serologically defined colon cancer antigen 10	0.0365	1.50	1.64

rate = 0 %) regulated probesets corresponding to 262 genes for 17-β-estradiol consistent with the known strong effects of the steroid on estrogen receptor α expressing cells. Most of these genes have been described to be regulated by estrogen. Table 1 shows the expression values relative to untreated control cells for estradiol. In the following we refer to this list as the estrogen response reference profile (E2 genes). The validation of the estrogen response of several of these genes by real time PCR yielded fully concordant data (data not shown).

Expression analyses after treatment with phytoestrogens

In parallel we treated MCF7 cells with the phytoestrogens Quercetin and Enterolactone and the suspected phytoestrogen, Curcumin, for 72 hours. The phytoestrogen concentration of 100nM corresponds to the maximal concentration observed for Enterolactone in breast cyst fluid [32] that is two orders of magnitude lower than the concentrations used in most *in vitro* studies of phytoestrogens. Applying the same statistical approach to the comparison of each single phytoestrogen and the control profile, no significant gene regulation events could be identified. The effects of Curcumin on gene expression in MCF7 cells are shown in Table 2 and presented as fold change over control cells.

Reference profile correlation

We noticed that many genes that were regulated by E2 more than twofold in a statistically significant manner were regulated in the same direction yet to a much lesser extent by Curcumin (Fig. 1A). Viceversa, most Curcumin regulated genes are regulated in the same direction by E2 (Fig. 1B). Genes regulated by Quercetin and Enterolactone also showed this tendency (data not shown).

87 %, 50 % and 79 % of the E2 genes were regulated in the same direction by Curcumin, Enterolactone and Quercetin, respectively, and the mean extent of regulation was about one tenth of that observed for estradiol (Table 3). The expression change of these

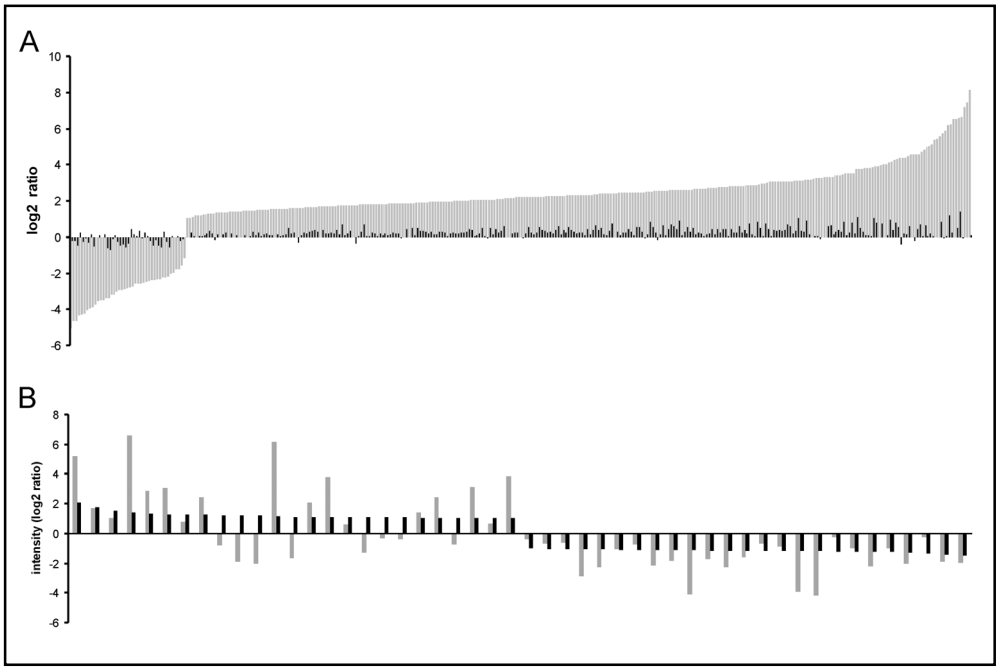

Fig. 1. Expression of Curcumin responsive genes in 17-β-estradiol treated cells. Gene expression of MCF7 cells after 72 hours of treatment with 1 nM E2 or 100 nM Curcumin was analyzed by microarray hybridization. Expression values obtained form Curcumin (black bars) and 17-β-estradiol (grey bars) treated cells are shown for all estradiol responsive genes (panel A) and for all Curcumin responsive genes (panel B). Expression values are indicated as Log2 ratios over untreated controls (1 = two fold induction, -1 = twofold decrease).

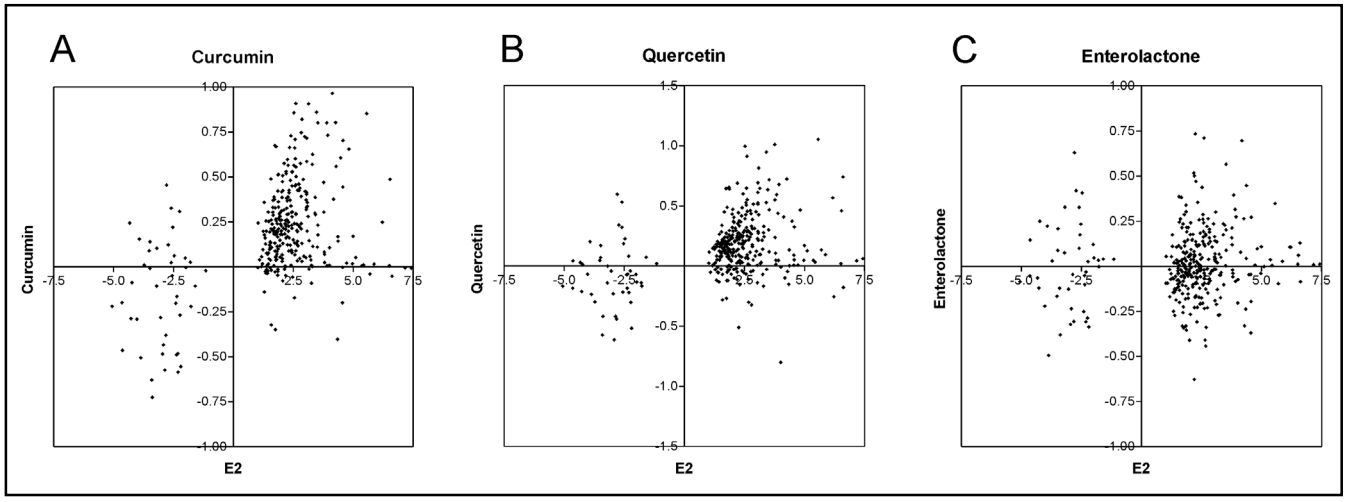

Fig. 2. Reference profile correlation. Spearman correlation of expression values obtained for E2 genes after treatment with 1nM 17-β-estradiol (abscissas) with those obtained after treatment with 100 nM of Curcumin, Quercetin or Enterolactone (ordinates) as indicated. Values are shown in log2 scale. Most of the genes are in the first and third quadrants that contain genes regulated in the same direction by the two compounds. This correlation is strongest for Curcumin and weak for Enterolactone.

genes after phytoestrogen treatment is low and on its own not statistically significant. We therefore wished to know whether there is a significant correlation between the effects of 17-β-estradiol and phytoestrogens, independent of the extent of regulation. We performed Spearman correlation analyses on the expression data limited to the E2 genes. These correlations were significant for all three phytoestrogens in the order of Curcumin ($r=0.3941$, $p<0.0001$) > Quercetin

	Estrogenic Effects of Phytoestrogens	
	Genes regulated in the same direction as by estradiol (%)	Extent of regulation as compared to estradiol (fold)
Curcumin	87	0.13
Enterolactone	50	0.09
Quercetin	79	0.11

Table 3. Regulation of estradiol responsive genes by Curcumin, Quercetin and Enterolactone.

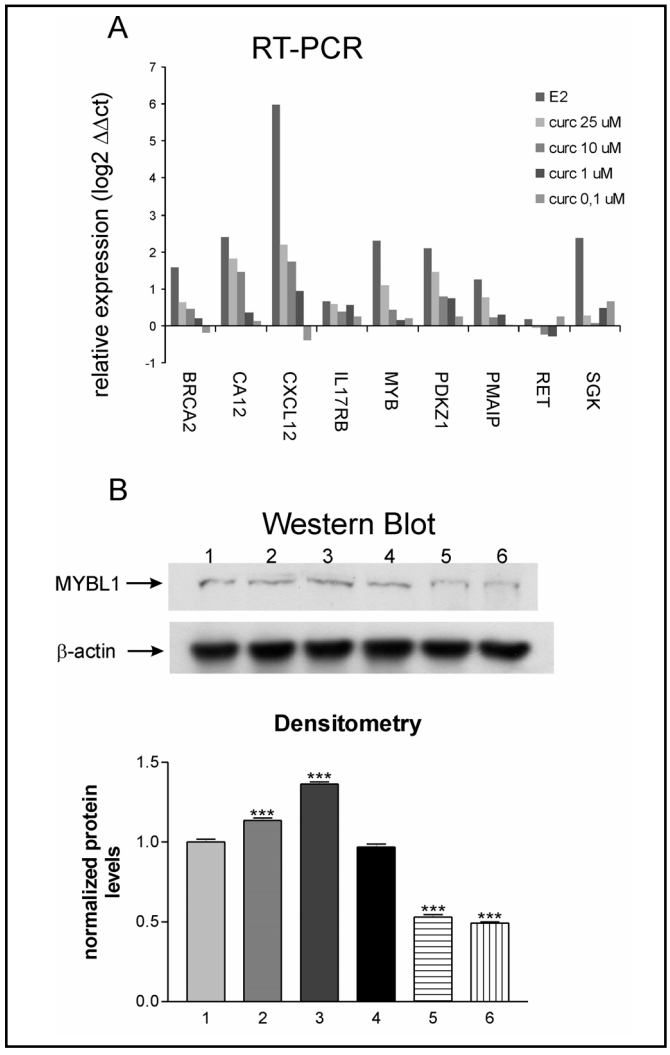

Fig. 3. Real time PCR and Western Blot validation of Curcumin effects on estrogen responsive genes. (A) Expression data are indicated as log2 of the expression values normalized on the expression values of three housekeeping genes analyzed in parallel (*G6PD*, *GAPDH* and *RPII)*. E2 indicates expression values in 17-β-estradiol treated cells (positive control). (B) Expression of MYBL1 (upper panel) was analyzed on protein level by Western Blots of lysates from E2 and Curcumin treated MCF7 cells in comparison to un-treated cells. Lanes 1: un-treated cells, lanes 2: cells treated with 1nM E2; lanes 3: cells treated with 100 nM Curcumin, lanes 4: cells treated with 1 μM Curcumin, lanes 5: cells treated with 10 μM Curcumin, lanes 6: cells treated with 25 μM Curcumin. Expression levels were normalized to β-actin (lower panel) by densitometric analysis (middle).

(0.3105, $p<0.0001$) > Enterolactone ($r=0.1377$, $p=0.0118$) (Fig. 2).

Validation of gene expression by real time PCR and Western Blot

Since Curcumin yielded the most significant estrogen-like effects although it had not yet been classified as a phytoestrogen we validated the effect on gene expression by quantitative RT-PCR. We assumed that the phytoestrogenic effect of Curcumin would be more pronounced at higher concentrations. We tested concentrations of 100 nM to 25 μM (25 μM is the concentration used in most *in vitro* studies of Curcumin).

We selected a small series of genes with different fold change values (maximum: CXCL12, 281 fold induced, minimum: IL17RB, 8 fold induced) after E2 treatment from the list of E2 genes (Table 1) for validation. In the selection we did not consider the extent of regulation by Curcumin. Fig. 3A shows that all of the eight estrogen regulated genes tested are also up-regulated by Curcumin in a dose dependent manner. BRCA2 and CXCL12 appear slightly downregulated by Curcumin at the lowest concentration (100 nM) but clearly upregulated by the higher concentrations. SGK shows a biphasic behavior. It is most likely that the reproducibility between microarray data and RT-PCR data drops at low concentrations since the extent of regulation is very limited and hidden by the intrinsic variability of the measurements. Thus, the reference profile correlation approach can identify estrogenic effects despite the intrinsic noise of the data.

In order to verify Curcumin effects at protein level, we have added Western blot analysis of one of the most strongly Curcumin modulated genes, MYBL1. Our results from protein analysis confirm the gene expression data although Curcumin at higher concentrations shows the opposite effect, which we attribute to its known anti-proliferative and pro-apoptotic functions at higher concentrations (Fig. 3B). Although the effect of Curcumin at a concentration of 100nM on the regulation of estradiol induced genes is only tiny, this effect is significant if one compares the estrogen effects with those elicited by Curcumin on all estradiol-regulated genes.

Differential effects of the phytoestrogens on E2 genes

As expected, the effects of 17-β-estradiol were much stronger although the steroid was used at a concentration 100 fold lower than that used for the phytoestrogens. However, the ratio of the fold change values for 17-β-estradiol versus control in comparison to that of the phytoestrogens versus control is not the same for all the genes analyzed. Apparently, strongly estradiol-

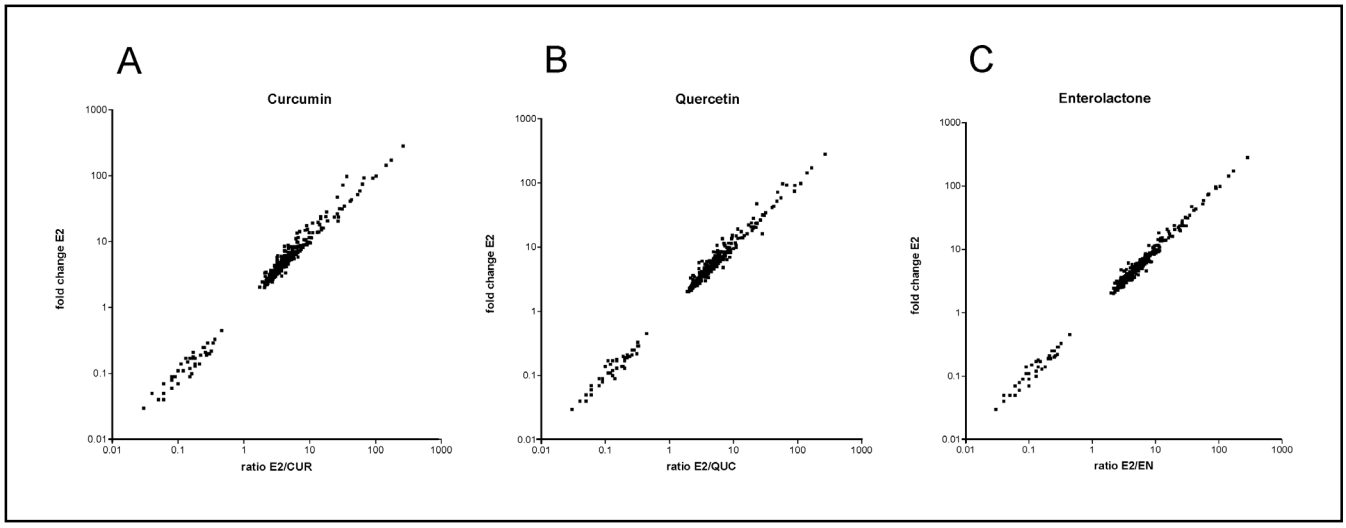

Fig. 4. Correlations between the regulation extents. Analysis of the correlation between the extent of responsiveness to 17-β-estradiol (fold E2, ordinates) and the ratio between the extent of responsiveness to 17-β-estradiol and responsiveness to the phytoestrogens (fold E2/CU, E2/QC, E2/EL, abscissas).

induced genes respond to a lesser extent to the three phytoestrogens tested whereas only weakly estrogen responsive genes are affected to a similar extent by the herbal analogs. The ten genes that are most strongly upregulated by E2 are on average 103 fold more induced by E2 as compared to Curcumin whereas the ten most weakly E2 upregulated genes are only 2 fold increased by E2 as compared to the polyphenol. We wished to know whether there is a specific relation between the extent of responsiveness to 17-β-estradiol and to the phytoestrogens. In Fig. 4, the ratios between the fold changes (17-β-estradiol versus control divided by phytoestrogen versus control) are plotted over the 17-β-estradiol fold change values. It becomes clear that there is an almost perfect linear correlation for all three phytoestrogens. The power of the phytoestrogens was low for genes strongly regulated by 17-β-estradiol and similar to the latter for genes only weakly regulated by the classic estrogen. The linear correlation is determined by the fact that the phytoestrogens have quantitative similar effects on all genes whereas E2 has drastically different effects on these genes.

Functional analysis of phytoestrogen induced genes

The ideal molecule for hormone replacement therapy would maintain the protective effects that estrogens exert on bones, the cardiovascular system and the brain but not the cancer promoting effects on proliferation and apoptosis. We showed that the phytoestrogens tested exert bland estrogenic effects on most E2 genes yet to various extents as compared to E2. We therefore asked whether these differences affect genes with different functions. For this purpose we analyzed which functional categories of genes (Gene Ontology) are enriched among the 50 genes with the highest difference in induction between 17-β-estradiol and Curcumin treated cells in comparison to the whole list of genes that are regulated in the same direction by the two compounds and then repeated the

Over-represented functional gene classes				
upper 50				
Category	Term	Count	%	PValue
SP_PIR_KEYWORDS	signal	7	17.50%	0.013
UP_SEQ_FEATURE	signal peptide	7	17.50%	0.035
lower 50				
Category	Term	Count	%	PValue
SMART_NAME	SM00350:MCM	4	8.33%	0.009
UP_SEQ_FEATURE	domain:MCM	4	8.33%	0.009
INTERPRO_NAME	IPR001208:MCM	4	8.33%	0.015

Table 4. Analysis of enrichment of gene annotation categories. Gene ontology categories of the 50 genes with the highest and the lowest ratio of induction by E2 versus Curcumin were compared with those of the E2 genes in order to identify functional gene classes that are significantly over-represented in these two subgroups of differential (phyto)estrogen responsiveness as compared to all estrogen responsive genes. Hits with $p<0.05$ are shown. MCM complex is defined as a hexameric protein complex required for the initiation and regulation of DNA replication.

analysis for the genes with the lowest difference (upper 50 and lower 50, Table 4) using DAVID annotation tools. The analysis revealed a significant enrichment of signal peptide encoding genes among the fifty genes with the highest difference and minichromosome maintainance genes among the lower fifty genes. It is therefore conceivable that the estrogenic effect of Curcumin is less pronounced on estrogen induced protein secretion. In fact, the inflammatory chemokine CXCL12 is the most differentially regulated gene in the list. However, Curcumin apparently maintains estrogenic activity on the initiation of replication where MCM proteins are required. Among the upper 50 genes we also detected the oncogenic transcription factors v-MYB and a member of the E2F family (E2F7) as well as the survival factor BIRC5/survivin but also the tumor suppressor BRCA2. In addition to the MCM genes other replication related genes such as flap-structure specific endonclease, FEN1, and DNA-topoisomerase 2a, TOP2A, are found among the lower 50 genes.

Discussion

Here we analyze the effects on gene expression exerted by two putative estrogenic substances isolated from plants, Quercetin and Enterolactone, that have been proposed as substitutes for hormone replacement therapy (for a review see [5]) plus the polyphenol Curcumin, which is suspected to exert estrogenic activity. Such phytoestrogens are of major interest especially since hormone replacement therapy has been reported to increase breast cancer risk [3, 37]. Identification of compounds that have the beneficial effects of estrogens on bone and cardiovascular homeostasis and that can reduce the menopausal symptoms is an active area of research. Substitutes for classic hormone replacement therapy might also increase breast cancer risk through the activation of estrogen signaling. Phytoestrogens that are proposed as such substitutes must therefore thoroughly be characterized with regard to their estrogenic effects. On the other hand, for many of the proposed compounds, conclusive evidence of their estrogenic activity and methods to quantify the "estrogenicity" are still missing.

In order to identify the potentially estrogenic effects of Enterolactone, Quercetin and Curcumin in breast cancer cells we analyzed in a first approach all genes that are regulated by 17-β-estradiol (E2). If a compound has estrogen-like activities it is expected to mimic estrogen effects on gene transcription though the effect will be much weaker. The range of the expression change that we observed in response to E2 in the human estrogen-sensitive breast cancer cell line MCF7 ranges from 2 fold to 281 fold. It is as yet unknown how these drastically different effects are obtained. Transcription factor binding sites, the long and short range chromatin structure, the actual sequence, possible cytosine methylation and the availability of transcriptional co-activators and -repressors may contribute to the actual responsiveness of a gene [38-40].

All three compounds tested at the physiological concentration of 100 nM regulate many E2 genes in the same direction like 17-β-estradiol yet the extent of regulation is very limited. The comparison between untreated controls and phytoestrogen treated cells does not yield statistically significant gene regulation events. However, the gene expression profile elicited by the phytoestrogens shows a significant correlation with the profile in response to 17-β-estradiol. Interestingly, Curcumin showed the strongest effects and the most significant correlation. Estrogenic effects of this compound have been described [28] although polyphenols are usually not considered as phytoestrogens.

Curcumin has been described to inhibit nuclear factor kappa B (NFκB) activation [41] and we have recently shown that this also holds true for breast cancer where the polyphenol reduces the number of metastases formed by estrogen receptor (ER) negative MDA-MB-231 cells in a mouse model of hematogenous metastasis through the inhibition of NFκB mediated expression of matrix metalloproteinases and chemokines [42, 43].

The ER is able to trans-repress NFκB [44] and this might interfere with the effects of Curcumin on NFκB regulated but not E2 genes. On the other hand, trans-repression of estrogen signaling by NFκB has also been reported [45]. This is however unlikely to contribute to the estrogenic effects of the polyphenol since NFκB affects the ERβ that is not expressed or expressed at very low levels in MCF7 cells. Hence, the activation of E2 genes by Curcumin most probably constitutes an ERα mediated estrogen-like effect.

When we compared the relation between the responsiveness to E2 with that to the phytoestrogens we observed that there is an intriguing relation: The stronger a gene is regulated by estrogen the higher is the fold difference between the estrogen effect over the phytoestrogen effect. This correlation is linear. We would have expected a nonlinear relation with the genes belonging to distinct groups, as it would be the case if

there was a correlation with the number of estrogen responsive elements (EREs) present in the promoter. The estrogen receptors homodimerize upon ligand binding [46] and the hormone also induces a conformational change in the receptor proteins that is needed for co-activators to bind to the receptor-DNA complex [47]. Different extents of gene activation by the ligand bound estrogen receptors are likely due to different promoters being bound by different receptor-co-activator combinations [38, 39]. The stronger the effect of estrogen the more it depends on co-activators. If the phytoestrogens were unable to recruit co-activators while inducing dimerization and DNA-binding, their activity would correspond to the low, co-activator independent activation obtained by the free ER dimers. This would explain the linear dependence between activation by E2 and the ratio between E2 and the phytoestrogens.

The decision whether the phytoestrogens analyzed might be safe substitutes for hormone replacement therapy would be straightforward using our approach if the genes with a presumed protective effect were in the group of relative strong phytoestrogen effects and the genes with presumed influence on the breast cancer risk were in the opposite group where phytoestrogens yield only very weak effects as compared to estradiol. The most frequent functional classes of estrogen regulated genes are related to cell proliferation. The analysis of annotation category enrichment comparing genes were the difference between the response to E2 and Curcumin is highest to those where it is lowest does not yield conclusive evidence of functional differences since several replication related genes are similarly regulated by estradiol and Curcumin. However, the anti-apoptotic gene BIRC5/survivin and two oncogenic transcription factors are in the uppermost quintile of genes strongly regulated by estradiol but weakly responsive to phytoestrogens. These genes certainly contribute to the breast cancer risk and as a matter of fact, BIRC5/survivin and the MYBL1 homologue MYBL2 are included in the recurrence score developed by Paik and co-workers [48]. We have recently shown that expression of the estrogen induced cytokine CXCL12 in human breast cancer strongly correlates with disease free survival [49]. Induction of CXCL12 could therefore be protective yet Curcumin only weakly induces the chemokine.

Here we compared effects on gene expression elicited by weakly active compounds with the values obtained after treatment of cells with a strong lead compound E2. The analysis is limited to those genes that are statistically significantly regulated by the latter. This approach allows correlations of gene expression changes between the lead and test compounds even when the effect of test compounds on gene expression is not strong enough to yield statistically significant gene regulation events. We feel that this approach may prove useful for many other weak but potentially interesting compounds, especially in the field of chemoprevention where long term treatments with non toxic and often weakly effective compounds is sought.

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