RESEARCH ARTICLE

# Tocotrienols activity in MCF-7 breast cancer cells: Involvement of ERβ signal transduction

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The term Vitamin E is utilized to describe eight molecules, subdivided into two groups, tocopherols and tocotrienols (TTs). It has been shown that specific TTs affect the growth of several lines of tumour cells, and that this activity is not shared by tocopherols. In agreement with these observations, a TTs-rich fraction from palm oil (PTRF) was reported to inhibit proliferation and induce apoptosis in several cancer cells. However, the molecular mechanism involved in TTs activity is still unclear. We have recently proposed that TTs pro-apoptotic activity involves estrogen receptor beta (ERβ) signalling. In this study, we report that, in MCF-7 breast cancer cell, expressing both ERα and ERβ, PTRF treatment increases ERβ nuclear translocation, as demonstrated by immunofluorescence experiments and significantly inhibits ERα expression (−458.91-fold of change) and complete disappearing of the protein from the nucleus. Moreover, PTRF treatment induces ER-dependent genes expression (macrophage inhibitory cytokine-1, early growth response-1 and Cathepsin D) which is inhibited by the ER inhibitor, ICI 182.780, and induces DNA fragmentation. Finally, cDNA-array experiments suggest that the activation of specific pathways in cells treated with γ-TT with respect to α-TT. Our data suggest a novel potential molecular mechanism for TTs activity.

**Keywords:**
Apoptosis / Estrogen nuclear receptor / Frizzled 1 / Macrophage inhibitory cytokine-1 / Tocopherols

## 1 Introduction

The term ''Vitamin E'' usually refers to a family composed of α-, β-, γ-, and δ-tocopherols and corresponding four tocotrienols (TTs). The biological role of vitamin E has been initially referred to its ability to affect the resorption-gestation performance in rodents but, so far, the real biological functions in humans have not been fully understood, and have been generally attributed to a non-specific antioxidant activity [1]. More recently, non-antioxidant activities have been reported for both tocopherols (TOCOs) and TTs with a special concern to their specific ability to affect gene expression and cell response [2].

Recent studies have suggested that TTs have specific functions and activities, distinct from those identified for TOCOs. TTs have been reported to suppress the enzymatic activity responsible for cholesterol synthesis in the liver [3], and to lower both total cholesterol and the LDL fraction. α-TT has been reported to be specifically able to prevent neuro-degenerative processes by regulating specific mediators of cell death [4]. Moreover, it has been demonstrated that oral supplementation of TTs protects against stroke and

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**Abbreviations: Bmp-4,** bone morphogenetic protein 4; **EGR-1,** early growth response-1; **ER,** estrogen receptor; **ERE,** estrogen-responsive element; **MCM,** minichromosome maintenance; **MIC-1,** macrophage inhibitory cytokine-1; **PTRF,** Tocotrienol-rich-fraction from palm oil extract; **SERCA,** sarcoplasmic/endoplasmic reticulum calcium ATPase; **SFRP,** secreted fizzled-related protein; **TOCO,** tocopherol; **TT,** tocotrienol

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this phenomenon was linked to their ability to reach brain tissues [5]. Previous reports by others and by our laboratory indicated that TTs can suppress cell growth and induce apoptosis in several cell lines of murine and human cancer [6, 7].

Although the molecular mechanisms underlying these potentially beneficial effects of TTs are still poorly known, some studies performed in our laboratories based on cDNA-array methods, revealed that a TT-rich fraction from palm oil (PTRF) induces a significant reduction of cell proliferation both *in vitro* in cultured breast cancer cells [8, 9] and *in vivo* in tumours induced in athymic mice by the inoculation of human breast cancer cells [10]. This approach led us to identify a set of genes transcriptionally modulated by PTRF and involved in cell cycle control. On the basis of *in silico* and *in vitro* binding experiments coupled with cell culture studies, we have more recently suggested that the effects of specific TT (γ- and δ-TT forms) on gene expression is at least in part mediated by the ability to bind to estrogen receptor beta (ERβ) in cultured MDA-MB-231 cells [7], a cell line expressing ERβ but not ERα. Such interaction results in the nuclear translocation of ERβ and the activation of specific genes containing an estrogen-responsive element (ERE) in their promoter. Finally, in agreement with other authors [11], we showed a pro-apoptotic activity of TTs in the same cell line by observing caspase-3 activation and genomic DNA fragmentation [7].

A number of cell-based and animal models have conclusively demonstrated that an intricate interplay among ERα and ERβ activities exist. ERα and ERβ are products of different genes and exhibit tissue- and cell-type specific expression and have specific roles in estrogen-dependent action *in vivo* [12, 13]. Several tissues express both receptors and when co-expressed, usually ERβ exhibits an inhibitory action on ERα-mediated gene expression and in many instances opposes the actions of ERα. The molecular mechanisms regulating the relative expression of both ERs and their direct or indirect interactions to determine each other's function have been proposed to play an important role in different pathological processes, but are still largely unknown [14].

The aim of this study was to investigate the effect of the PTRF, in a cell line expressing both ER isoforms, the MCF-7 cell line, where ERs mediated cell signaling is the result of a fine tuning and balance between ERα and ERβ activity. Moreover, cDNA-array experimental approach, allowed the identification of novel candidate pathways possibly involved in the modulation of cellular signaling by specific TT forms.

## 2 Materials and methods

### 2.1 Chemicals

PTRF was obtained from Sime Darby Plantation (Malaysia) and purified as described previously [15]. The final purity of Vitamin E in PTRF was 95–99% and typically contained 32% α-TOCO, 25% α-TT (α-T3), 29% γ-TT (γ-T3) and 14% δ-TT (δ-T3).

Purified TTs were provided by Dr. Hiroyuki Yoshimura of the Eisai Food and Chemical (Tokyo, Japan). Purity was 99% near for all TTs. Pure α-TOCO was purchased by Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of PTRF and TTs were stored at −20°C in aliquots and diluted to the desired concentration in DMSO.

The non-specific ER antagonist ICI 182.780 was purchased from Tocris (Ballwin, MO, USA).

### 2.2 Cells lines and treatments

MCF-7 human breast cancer cells were obtained from the American Tissue Culture Collection (Manassas, VA, USA). Cells were grown in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% foetal bovine serum (Sigma-Aldrich), Pen/Strep (Invitrogen Life Science, CA, USA), 2 mM glutamine (Sigma-Aldrich) and 10% non-essential aminoacid (MEM, Sigma-Aldrich).

Before any experimental session, cells were synchronized in $G_1/G_0$ by starvation in serum-free medium for 3 days. Once synchronized, $5.0 \times 10^5$ cells were seeded onto multi-well plates in phenol red-free RPMI 1640 and, where appropriate, incubated with ICI 182.780 ($10^{-5}$ M in ethanol) for 30 min.

PTRF or α-TOCO was added to the culture medium for 24 and 48 h or only 24 h, depending on the type of experiment.

Final PTRF concentration in culture media was set at 8 μg/mL, to standardize this study according with our previous reports [8]. α-TOCO concentration was 2.56 μg/mL. Moreover, concentrations of purified TTs are 2 μg/mL (β-TT) and 2.32 μg/mL (γ-TT). Concentrations reported for TTs and α-TOCO are related to the percentage present in the PTRF mixture. Concentrations of TTs used in the experiments were in the micromolar order, which is a concentration that can be achieved in the human serum, as described previously [16]. Control cells were treated with the same volumes of DMSO and/or ethanol vehicle alone.

### 2.3 Nuclear localization of ERβ and ERα

About $1 \times 10^5$ cells were treated with PTRF or α-TOCO, in the Lab-Tek Chamber Slides™ system (Nalge Nunc International, Rochester, NY, USA), for 24 h according to the previous article [7]. Cells were incubated with 20 μL of the diluted (1:20) ERβ or (1:50) ERα primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and stained with 100 μL DAPI to counterstain the nucleus. A Zeiss Axioskop II (Carl Zeiss AG, Oberkochen, Germany) microscope with appropriate filters was used. Images were collected and processed using the SPOT software.

### 2.4 RNA isolation and real-time PCR measurements

Total RNA was extracted from cells using TRI Reagent™ (Sigma-Aldrich), according to the manufacturer's instructions with some minor modifications [7]. Primers (Table 1) corresponding to selected genes were designed with Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA). Real-time PCR were performed using the SuperScript™ Platinum® SYBR® Green One-Step kit (Invitrogen) according to the previous article [7]. The $C_t$ values for each target and reference genes were obtained and their difference was calculated ($\Delta C_t$). For normalization purpose, an identical set of reaction was prepared using primer specific for β-actin. Quantitative differences in the cDNA target among samples were determined using the mathematical model of Pfaffl [17] as described in the previous article [7].

### 2.5 Protein extraction and Western blot

Cells were lysed in RIPA buffer and samples were submitted to electrophoresis as described by Comitato *et al.* [7] and then incubated overnight at 4°C with a 1:500 dilution of rabbit early growth response-1 antibody (EGR-1; Santa Cruz Biotechnology), 1:500 Cathepsin D rabbit antibody (Santa Cruz Biotechnology), 1:250 Macrophage Inhibitory Cytokine-1 goat antibody (MIC-1; Novus Biologicals, Littleton, CO, USA), 1:500 bone morphogenetic protein 4 (Bmp-4; Santa Cruz Biotechnology), 1:400 ERα (Santa Cruz Biotechnology), 1:1000 ERβ (Santa Cruz Biotechnology) and 1:1000 α-tubulin mouse antibody (MP Biomedicals, Irvine, CA, USA). After washing with TPBS, membranes were incubated for 1 h at RT with 1:2000 goat anti-mouse or goat anti-rabbit or 1:5000 donkey anti-goat peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Specific spots were detected by chemiluminescence reagents ECL Plus (Amersham Pharmacia Biotech, Piscataway, NJ) and visualized by autoradiography by high-performance chemiluminescence film (Amersham Biosciences, Buckinghamshire, UK).

### 2.6 DNA laddering

DNA fragmentation was assessed according to Gooch and Yee [18] in cells after 24 h treatment with PTRF and α-TOCO. Following the isolation, DNA was electrophoresed in 1.5% agarose gels containing ethidium bromide and visualized by UVIpro Bronze acquisition system (UVITEC, Cambridge, UK).

### 2.7 RNA extraction and cDNA-array experiment

After 24 h of TTs treatment, total RNA was extracted from cells using RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The isolated RNA samples were sent to ServiceXS BV (Leiden, The Netherlands) where they were processed according to Affymetrix protocols.

Briefly, RNA concentration was determined by absorbance at 260 nm, and quality and integrity was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies). Next, 2 μg of high quality total RNA was used with the Affymetrix Eukaryotic One-Cycle Target Labeling and Control reagents to generate biotin-labelled anti-sense cRNA. The quality of the cRNA was checked using the Agilent 2100 bio-analyzer.

The labelled cRNA was hybridized to the NuGO Affymetrix Human Genechip NuGO_Hs1a520180 (custom designed by the European Nutrigenomics Organization NuGO, consisting of 23 941 probesets including 71 control probesets, for details see http://blog.bigcat.unimaas.nl/~martijn/NuGO/).

Cell intensity File (*.cel) for each GeneChip processed are generated using Command Console Software. Three biological replicates were generated for each experimental condition. Microarrays statistical analysis was performed using oneChannelGUI R package [19], using a custom CDF file for NuGO_Hs1a520180 (based on Entrez Gene, version 10.0.0; available *via* http://nugo-r.bioinformatics.nl/NuGOR.html).

Raw signal intensity were normalized using Express method with robust multi array as background correction and keeping the remaining values to their default values.

Differentially expressed genes analysis was performed using ''compute linear model fit'' function in oneChannelGUI and then computing the ''contrasts'' with the analogous function of oneChannelGUI inherited from affylmGUI [20]. The differentially expressed genes are chosen setting as threshold a delta fold change at least 1 and with 0.05 *p*-value after Benjamini–Hochberg correction.

This approach provided expression values for 2770 (γ-TT *versus* Control) and 1168 (α-TT *versus* Control) genes differentially expressed, respectively.

**Table 1.** List of genes considered, GenBank identification code and sequence of primers utilized for real-time PCR

| Gene | GenBank | Forward | Reverse |
|---|---|---|---|
| MIC-1 | NM_004864 | 5′TGGTGCTCATTCAAAAGACCG3′ | 5′GTGGAAGGACCAGGACTGCTC3′ |
| EGR-1 | NM_001964 | 5′CTCCACAGGGCTTTCGGAC3′ | 5′GAGAGGGAGGACTTGGCTCTG3′ |
| Cathepsin D | NM_001909 | 5′CTGTGAGGCCATTGTGGACAC3′ | 5′CAGCTTGTAGCCTTTGCCTCC3′ |
| Bmp-4 | NM_001202 | 5′GCCGTCATTCCGGACTACAT3′ | 5′GGGCGCTCAGGATACTCAAG3′ |
| β-Actin | NM_001614 | 5′AGAAGGATTCCTATGTGGGGG3′ | 5′CATGTCGTCCCAGTTGGTGAC3′ |

### 2.8 Network analysis

A global network for all the genes differentially expressed in our set of comparison was built using MIMI plug-in [21] of Cytoscape [22], selecting as query parameter: input gene and nearest neighbourhood. This resulted in a network of around 6000 genes on which we have mapped microarray expression data for retrieve only the subset of genes differentially expressed in one comparison as described previously.

Over-representation of KEGG pathway was computed using DAVID [23] web server (http://david.abcc.ncifcrf.gov/home.jsp). The selected pathways are chosen according to a *p*-value below 0.05.

### 2.9 Statistics and data presentation

All data are presented as the means $\pm$ SE of at least three independent experiments. Statistical analysis was performed with ''R software'' from ''R Foundation for Statistical Computing'' (Vienna, Austria). Real-time data were analyzed by one-way ANOVA with repeated measures followed by the Bartlett and Fligner–Killeen test for homogeneity of variance. Dunnett *post hoc* test was used to evaluate difference among multiple conditions. About $p \leq 0.05$ were considered to be statistically significant.

Figures and tables present one out of at least three independent experiments providing similar results or the mean ($\pm$ SE) of at least three experiments, respectively.

## 3 Results and discussion

TTs are attracting increasing scientific interest as candidate for specific biological actions, well beyond their antioxidant activity, affecting cellular function and survival.

PTRF is a standardized mixture obtained from palm oil. It contains about 70% w/w of TTs together with α-TOCO and carotenoids and it has been often utilized as a tool to study the biological effects of TTs. We initially considered the effect of PTRF in inhibiting breast cancer cell growth [24, 25]. In these early reports, the observation that PTRF pro-apoptotic activity in breast cancer cells was not associated with a specific ERs expression profile, led to the conclusion that PTRF activity was independent of estrogen-related signaling. However, we have recently reported a direct interaction of PTRF and purified TTs with ERβ, by combining results obtained with software-based docking simulations, *in vitro* estrogen (E2) displacement assays and immunocytochemistry and by evaluating ERE-dependent gene expression in the MDA-MB-231 cell line, expressing only Erβ [7].

As mentioned in Section 1, an intricate interplay between ERα and ERβ activities has been demonstrated in a number of experimental models. However, the final effect of the ligand–receptor interaction in the presence of the co-expression of both ER isoforms is not obvious in different circumstances, and warrants further investigation. ERα and ERβ belong to the nuclear receptors superfamily and more specifically to the family of steroid receptors acting as ligand-regulated transcription factors [26]. There are several complex relationships between the two receptors. On a simplification attempt, such complex relationship has been considered as a ''*yin/yang*'' like interaction. In fact, ERα and ERβ have different biological functions, as indicated by their different expression patterns [14]. In particular, as mentioned above, ERβ appears to act as a dominant regulator of estrogen signalling, and when co-expressed with ERα, it causes a concentration-dependent reduction in ERα-mediated transcriptional activation [12, 27]. However, it has been argued that the ERβ-dependent antagonism of ERα-mediated responses does not represent a general mechanism in ER signalling, as it could be restricted to a limited number of genes [28]. Therefore, on the basis of these observations, it is possible to speculate that TTs binding to ERβ, modulate a specific cell response that also results in the down-regulation of ERβ expression inducing a shift of ERα/ERβ balance in favour of ERβ, which can be responsible for the reduction of MCF-7 tumourigenicity [13]. Consistent with this view, E2 has been reported to increase cell proliferation and to cause tumour formation in MCF-7 cells expressing higher levels of ERα than ERβ [14].

A number of evidences suggest that there are several distinct pathways by which estrogens, *via* ERs, can regulate a wide spectrum of biological processes [29]. In the classical model of ER action, ligand-activated ERs specifically bind DNA at EREs through their DNA-binding domains and bring co-regulators to the transcription start site. In order to identify which pathways were activated, we tested the expression of a selected small set of ERE-containing genes (namely, MIC-1, EGR-1 and Cathepsin D) in MCF-7 cells, at baseline and after PTRF treatment, in the presence of ICI 182.780, a specific ERs inhibitor. This set of genes was selected from a database previously identified using a gene array approach [10]; moreover, they were also considered and analyzed in our previous article on MDA-MB-231 cells [7].

Therefore, the aim of this study was to investigate the molecular pathways activated by treatment with either PTFR or specific TTs, leading to apoptosis in breast tumour cells expressing both ER isoforms. To this purpose, we have utilized MCF-7 human breast cancer cells, expressing ERα and ERβ at the level of both RNA transcript and protein [30].

Our first aim was to confirm our previous observations of an agonist interaction of TTs with ERβ. In agreement with the previous reports [31], in baseline conditions, the localization of ERβ in MCF-7 cells is predominantly cytoplasmatic (Fig. 1). After 24 h of treatment with PTRF, an evident nuclear staining is visible, indicating a significant translocation of the receptor inside the nucleus. On the other hand, at the same time point, ERα which is normally located in the

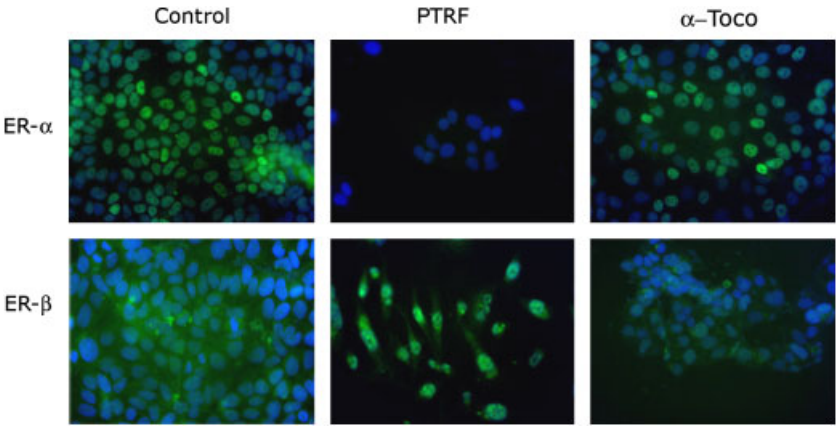



**Figure 1.** Cells were treated for 24 h with PTRF (8 μg/mL) or α-TOCO (2.56 μg/mL). PTRF treatment was associated with ERβ strong nuclear staining and total disappearance of nuclear ERα. On the other hand, no difference was observed in cells treated with α-TOCO in respect to control. One out of the three independent experiments yielding similar results is shown.

nucleus [32], is no more visible. Moreover, in agreement with our previous results obtained on MDA-MB-231 cells [7], α-TOCO treatment of MCF-7 is not associated with any significant changes of ERα and ERβ localization, with respect to control cells. These results indicate that PTRF, but not α-TOCO, induces the nuclear transfer of ERβ also in the presence of ERα, suggesting a specific role of ERβ as mediator of PTFR effects. Moreover, in cells treated with γ-TT and δ-TT, but not α-TT, we have observed the same trend of localization associated to PTRF of ERα and ERβ (data not shown).

In MCF-7 cells, PTRF treatment induces a significant increase of the expression of MIC-1 and EGR-1 mRNAs, after 24 and 48 h of treatments (Figs. 2A and B, respectively). Unexpectedly, Cathepsin D mRNA is down-regulated after 24 h and slightly up-regulated after 48 h of PTRF treatment. Pre-treatment with ICI 182.780 partially prevents the modulation of all genes. As expected, the expression of an ERE-lacking gene, Bmp-4, utilized as a ''negative control'' to exclude the possibility of a non-specific activation of gene expression by PTRF, remains unchanged in all the experimental conditions.

Protein levels are consistent with the amount of mRNA measured: MIC-1 and EGR-1 levels were significantly increased by PTRF treatment (Fig. 3) while remained stable at the baseline level in the presence of ICI 182.780 pre-treatment (Fig. 3). Surprisingly, and in disagreement with mRNA levels, Cathepsin D protein expression increased. According to the unchanged mRNA expression, the protein levels of the ERE-lacking gene Bmp-4 were not affected by treatments. ICI 182.780 treatment alone was not associated to any effects.

Our data indicate that the effects of PTRF on gene expression modulation in MCF-7 cells are due, at least in part, to its interaction with ERβ transcriptional pathway. ICI 182.780 is a well-known specific estrogen antagonist blocking the majority of pathways mediated by ERα and ERβ [33]. The inhibition of the effects of PTRF in the presence of this inhibitor, as demonstrated by Real-time PCR and Western blot analyses, clearly indicates that ERβ specifically mediates PTRF effects.

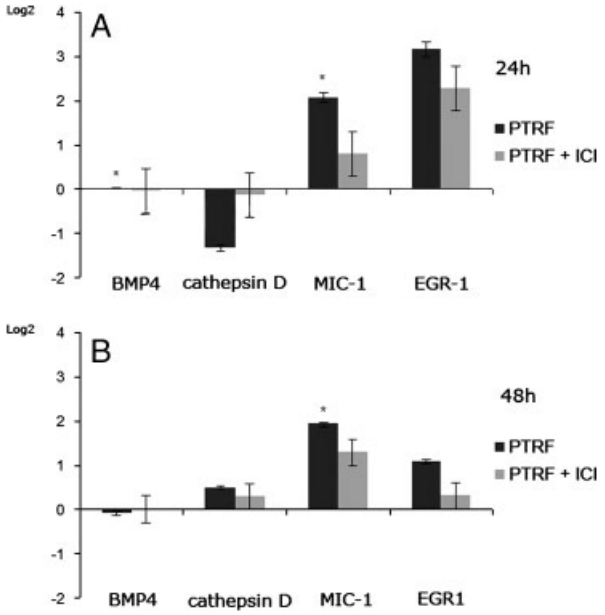



**Figure 2.** Expression of Bmp-4, EGR-1, MIC-1 and Cathepsin D genes in MCF-7 cells treated for 24 and 48 h with 8 μg/mL PTRF. Where appropriate, cells were pre-treated with ICI 182.780 for 30 min. Gene expression was analyzed by real-time quantitative PCR and results were log transformed (logarithm 2) in order to obtain data symmetrically distributed. Statistical significance was calculated by Dunnett *post hoc* test ($p = * \leq 0.05$).

Moreover, the treatment with PTRF is associated with alterations of the morphology of MCF-7 cells. We observed a loosening of cellular spindle shaped morphology with cells becoming smaller and rounded, detaching and flattening. Trypan blue staining indicates that the majorities of floating cells are not necrotic and still alive (data not shown), suggesting that the cells might have started apoptosis. In order to determine whether PTRF may induce apoptosis, we extracted DNA from adherent and non-adherent cells and then subjected it to agarose gel electrophoresis. Figure 4 shows that DNA fragmentation occurs in MCF-7 cells treated with PTRF. This effect is in agreement with previously

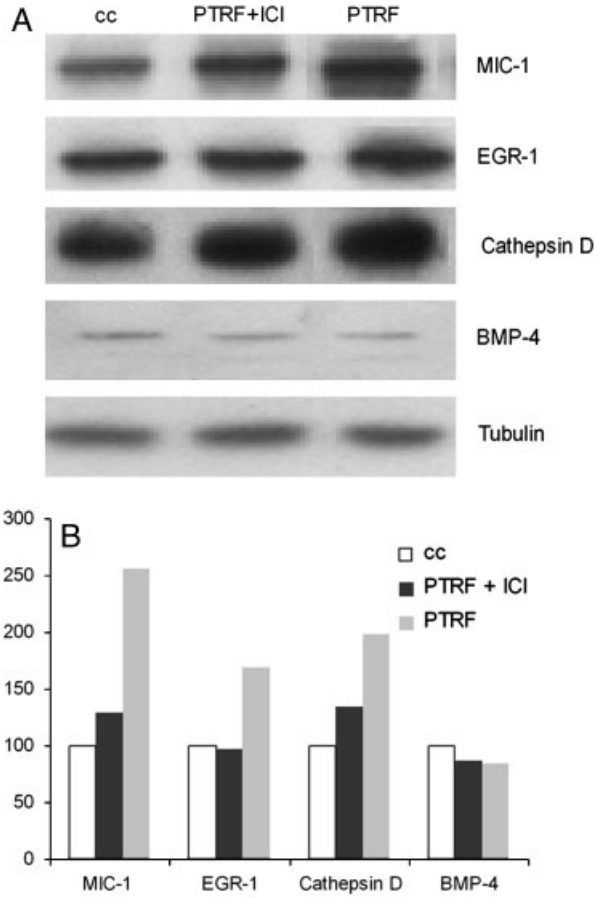



**Figure 3.** Total proteins from MCF-7 cells treated for 48 h with 8 μg/mL PTRF. Where appropriate, cells were pre-treated with ICI 182.780 for 30 min.

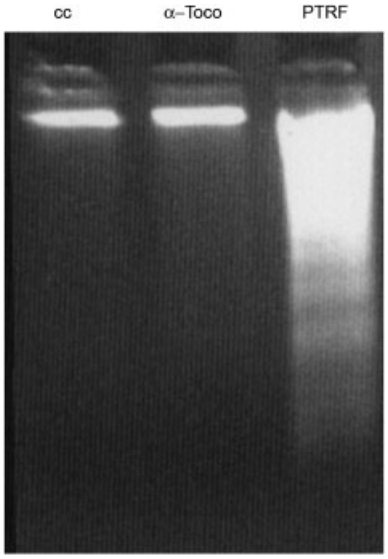



**Figure 4.** DNA laddering in MCF-7 cells treated for 24 h with PTRF (8 μg/mL) or α-TOCO (2.56 μg/mL) and then DNA was extracted and electrophoresed in 1.5% agarose gels containing ethidium bromide and visualized by UVIpro Bronze acquisition system (UVITEC). The typical apoptotic laddering was observed only in PTRF-treated cells.

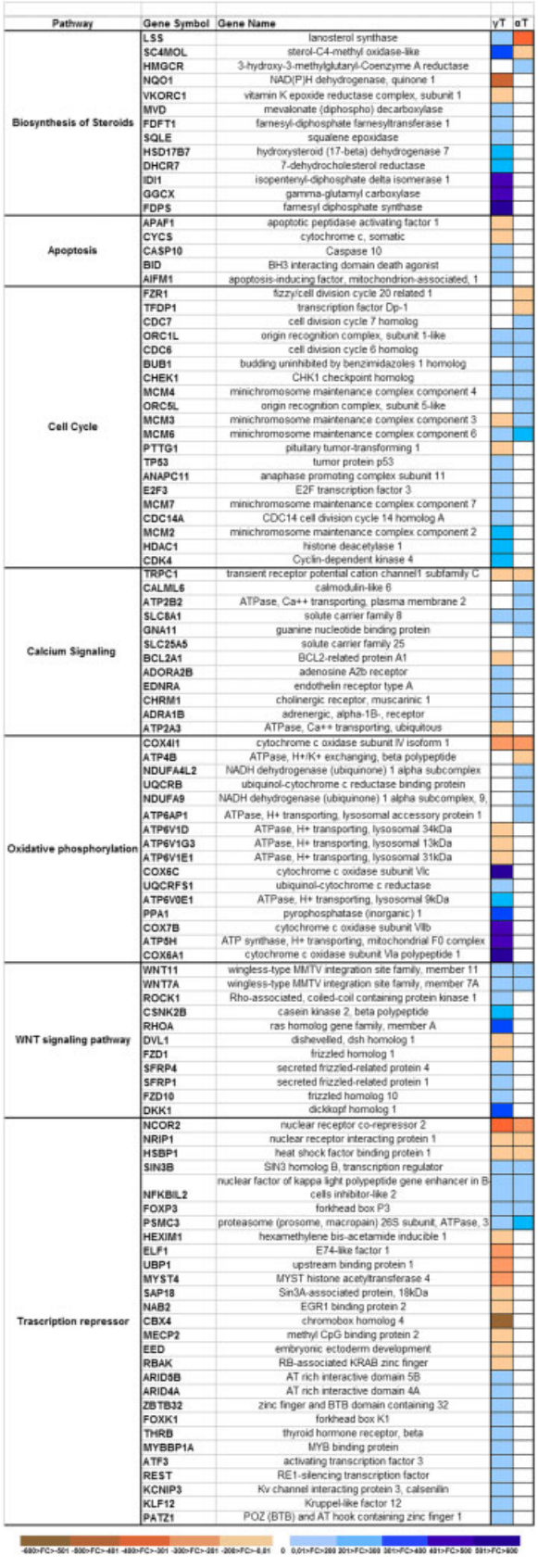
published articles by other authors that showed evident pro-apoptotic [34–36] and inhibitory effects on cell growth [24, 37] either by purified TTs or by PTRF.

In our previous study on MDA-MB-231 breast cancer cells, we have showed that the activity of PTRF mainly relied on γ- and δ-TT, but not on α-TT and α-TOCO [7]. We therefore hypothesized that γ- and δ-TT could be the real effectors of PTRF activity also in MCF-7. In order to corroborate this hypothesis, cDNA-array-based experiments were performed, utilizing RNA extracted from MCF-7 cells treated with purified TTs. This approach confirmed the Real-time PCR experiments and, in addition, showed a strong down-regulation of ERα (ESR1) mRNA expression, in agreement with immunocytochemistry assay. Moreover, this approach allowed the identification of specific cellular signalling networks associated with TTs effects. Among others, the network analysis performed comparing γ-TT and α-TT treatments *versus* control samples revealed the activation of specific molecular pathway, in cells treated with γ-TT but not with α-TT (Fig. 5). In particular, we observed a significant up-regulation of mRNAs encoding for enzymes involved in the steroid biosynthesis pathway, and the modulation of several repressors of transcriptional factors: namely the up-regulation of forkhead box K1, forkhead box P3, retinoblastoma-binding protein 1, ARID domain-containing protein 5B, and NF-kappa-B inhibitor-like protein 2 and the down-regulation of E74-like factor 1, hexamethylene bis-acetamide-inducible protein 1, methyl CpG-binding protein 2, heat shock factor binding protein 1 and nuclear receptor co-repressor 2. The modulation of the expression of these genes has been associated to the development of mammary carcinoma [38, 39], suggesting that they play a role in the adaptability of cancer cells to pro-apoptotic stimuli.

Using a mini plug-in of Cytoscape software, we built up the network of the specific genes regulated by PTFR. The network analysis revealed that some of the repressors of the transcription factors, NRIP1, THR, hexamethylene bis-acetamide-inducible protein 1 and methyl CpG-binding protein 2, directly modulate ERα mRNA expression [39–42], suggesting a role for these molecules in the down-regulation of ERα observed in MCF-7 treated with γ-TT.

cDNA arrays also indicate that Wnt genes are affected by TT treatment. Wnts regulate a variety of cellular activities, including proliferation, migration, polarity and gene expression [43] and they have been proposed to be implicated in tumour formation in different organs [44]. Interestingly, we observed a strong decrease of frizzled-1 mRNA, one of the main seven trans-membrane receptors of Wnt molecules [45]. The expression of frizzled-1 receptors has been reported to be usually up-regulated in breast cancer [46]. Moreover, we found an up-regulation of genes encoding for secreted frizzled-related protein-1 and -4 (SFRP-1



| Pathway | Gene Symbol | Gene Name | γT | αT |
|---|---|---|---|---|
| Biosynthesis of Steroids | LSS | lanosterol synthase | | |
| | SC4MOL | sterol-C4-methyl oxidase-like | | |
| | HMGCR | 3-hydroxy-3-methylglutaryl-Coenzyme A reductase | | |
| | NQO1 | NAD(P)H dehydrogenase, quinone 1 | | |
| | VKORC1 | vitamin K epoxide reductase complex, subunit 1 | | |
| | MVD | mevalonate (diphospho) decarboxylase | | |
| | FDFT1 | farnesyl-diphosphate farnesyltransferase 1 | | |
| | SQLE | squalene epoxidase | | |
| | HSD17B7 | hydroxysteroid (17-beta) dehydrogenase 7 | | |
| | DHCR7 | 7-dehydrocholesterol reductase | | |
| | IDI1 | isopentenyl-diphosphate delta isomerase 1 | | |
| | GGCX | gamma-glutamyl carboxylase | | |
| | FDPS | farnesyl diphosphate synthase | | |
| Apoptosis | APAF1 | apoptotic peptidase activating factor 1 | | |
| | CYCS | cytochrome c, somatic | | |
| | CASP10 | Caspase 10 | | |
| | BID | BH3 interacting domain death agonist | | |
| | AIFM1 | apoptosis-inducing factor, mitochondrion-associated, 1 | | |
| Cell Cycle | FZR1 | fizzy/cell division cycle 20 related 1 | | |
| | TFDP1 | transcription factor Dp-1 | | |
| | CDC7 | cell division cycle 7 homolog | | |
| | ORC1L | origin recognition complex, subunit 1-like | | |
| | CDC6 | cell division cycle 6 homolog | | |
| | BUB1 | budding uninhibited by benzimidazoles 1 homolog | | |
| | CHEK1 | CHK1 checkpoint homolog | | |
| | MCM4 | minichromosome maintenance complex component 4 | | |
| | ORC5L | origin recognition complex, subunit 5-like | | |
| | MCM3 | minichromosome maintenance complex component 3 | | |
| | MCM6 | minichromosome maintenance complex component 6 | | |
| | PTTG1 | pituitary tumor-transforming 1 | | |
| | TP53 | tumor protein p53 | | |
| | ANAPC11 | anaphase promoting complex subunit 11 | | |
| | E2F3 | E2F transcription factor 3 | | |
| | MCM7 | minichromosome maintenance complex component 7 | | |
| | CDC14A | CDC14 cell division cycle 14 homolog A | | |
| | MCM2 | minichromosome maintenance complex component 2 | | |
| | HDAC1 | histone deacetylase 1 | | |
| | CDK4 | Cyclin-dependent kinase 4 | | |
| Calcium Signaling | TRPC1 | transient receptor potential cation channel1 subfamily C | | |
| | CALML6 | calmodulin-like 6 | | |
| | ATP2B2 | ATPase, Ca++ transporting, plasma membrane 2 | | |
| | SLC8A1 | solute carrier family 8 | | |
| | GNA11 | guanine nucleotide binding protein | | |
| | SLC25A5 | solute carrier family 25 | | |
| | BCL2A1 | BCL2-related protein A1 | | |
| | ADORA2B | adenosine A2b receptor | | |
| | EDNRA | endothelin receptor type A | | |
| | CHRM1 | cholinergic receptor, muscarinic 1 | | |
| | ADRA1B | adrenergic, alpha-1B-, receptor | | |
| | ATP2A3 | ATPase, Ca++ transporting, ubiquitous | | |
| Oxidative phosphorylation | COX4I1 | cytochrome c oxidase subunit IV isoform 1 | | |
| | ATP4B | ATPase, H+/K+ exchanging, beta polypeptide | | |
| | NDUFA4L2 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex | | |
| | UQCRB | ubiquinol-cytochrome c reductase binding protein | | |
| | NDUFA9 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9, | | |
| | ATP6AP1 | ATPase, H+ transporting, lysosomal accessory protein 1 | | |
| | ATP6V1D | ATPase, H+ transporting, lysosomal 34kDa | | |
| | ATP6V1G3 | ATPase, H+ transporting, lysosomal 13kDa | | |
| | ATP6V1E1 | ATPase, H+ transporting, lysosomal 31kDa | | |
| | COX6C | cytochrome c oxidase subunit VIc | | |
| | UQCRFS1 | ubiquinol-cytochrome c reductase | | |
| | ATP6V0E1 | ATPase, H+ transporting, lysosomal 9kDa | | |
| | PPA1 | pyrophosphatase (inorganic) 1 | | |
| | COX7B | cytochrome c oxidase subunit VIIb | | |
| | ATP5H | ATP synthase, H+ transporting, mitochondrial F0 complex | | |
| | COX6A1 | cytochrome c oxidase subunit VIa polypeptide 1 | | |
| WNT signaling pathway | WNT11 | wingless-type MMTV integration site family, member 11 | | |
| | WNT7A | wingless-type MMTV integration site family, member 7A | | |
| | ROCK1 | Rho-associated, coiled-coil containing protein kinase 1 | | |
| | CSNK2B | casein kinase 2, beta polypeptide | | |
| | RHOA | ras homolog gene family, member A | | |
| | DVL1 | dishevelled, dsh homolog 1 | | |
| | FZD1 | frizzled homolog 1 | | |
| | SFRP4 | secreted frizzled-related protein 4 | | |
| | SFRP1 | secreted frizzled-related protein 1 | | |
| | FZD10 | frizzled homolog 10 | | |
| | DKK1 | dickkopf homolog 1 | | |
| Trascription repressor | NCOR2 | nuclear receptor co-repressor 2 | | |
| | NRIP1 | nuclear receptor interacting protein 1 | | |
| | HSBP1 | heat shock factor binding protein 1 | | |
| | SIN3B | SIN3 homolog B, transcription regulator | | |
| | NFKBIL2 | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 2 | | |
| | FOXP3 | forkhead box P3 | | |
| | PSMC3 | proteasome (prosome, macropain) 26S subunit, ATPase, 3 | | |
| | HEXIM1 | hexamethylene bis-acetamide inducible 1 | | |
| | ELF1 | E74-like factor 1 | | |
| | UBP1 | upstream binding protein 1 | | |
| | MYST4 | MYST histone acetyltransferase 4 | | |
| | SAP18 | Sin3A-associated protein, 18kDa | | |
| | NAB2 | EGR1 binding protein 2 | | |
| | CBX4 | chromobox homolog 4 | | |
| | MECP2 | methyl CpG binding protein 2 | | |
| | EED | embryonic ectoderm development | | |
| | RBAK | RB-associated KRAB zinc finger | | |
| | ARID5B | AT rich interactive domain 5B | | |
| | ARID4A | AT rich interactive domain 4A | | |
| | ZBTB32 | zinc finger and BTB domain containing 32 | | |
| | FOXK1 | forkhead box K1 | | |
| | THRB | thyroid hormone receptor, beta | | |
| | MYBBP1A | MYB binding protein | | |
| | ATF3 | activating transcription factor 3 | | |
| | REST | RE1-silencing transcription factor | | |
| | KCNIP3 | Kv channel interacting protein 3, calsenilin | | |
| | KLF12 | Kruppel-like factor 12 | | |
| | PATZ1 | POZ (BTB) and AT hook containing zinc finger 1 | | |

-600>FC>-501 -500>FC>-401 -400>FC>-301 -300>FC>-201 -200>FC>-8.61 0 0.01>FC>200 201>FC>300 301>FC>400 401>FC>500 501>FC>600

and SFRP-4). SFRPs represent a family of extracellular factors that antagonizes Wnt activies [47]. An up-regulation of SRFP1 and SFRP4 has been associated to apoptosis [48, 49] whereas their down-regulation has been observed in breast cancer [49]. It has been recently reported that the activation of ER-independent Wnt/β-catenin signalling by estrogen in uterine epithelial cells during the early growth phase, significantly contributes to the ER-dependent late growth response [50]. Moreover, Yokota *et al.* identified SFRP1 as an estrogen-inducible gene in stromal cells [51].

At single gene level, we observed a strong down-regulation of MCM3 gene, one of the members of the hexameric minichromosome maintenance (MCM) complex. An incomplete functionality of the hexameric MCM complex has been reported to prevent the unwinding of DNA double helix during the S-phase of mitosis [52]. The protein encoded by this gene is involved in the initiation of eukaryotic genome replication and is usually over-expressed in several types of tumour [53]. Moreover, we detected a modulation of cell cycle pathway following γ-TT treatment. In particular, we observed a down-regulation of pituitary tumour-transforming gene together with an up-regulation of tumour protein p53 gene. Similarly to MCM3, pituitary tumour-transforming gene is usually over-expressed in breast cancer cells [54] and encodes for human securin, a protein that interacts with p53 blocking its interaction with DNA and thus inhibiting its ability to induce cell death [55].

Moreover, focusing to the $Ca^{2+}$-related signalling pathways, we observed a strong down-regulation of the expression of BCL2A1, a negative modulator of mitochondrial apoptosis, and of ATP2A3 gene, which encodes for a sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA)-3 responsible for resorption of cytosolic calcium inside the endoplasmatic reticulum [56]. It is well known that high levels of cytosolic $Ca^{2+}$ act as a mitochondrial pro-apoptotic signal. In fact, in normal conditions the equilibrium between cytosolic and endoplasmatic reticulum calcium levels is maintained by SERCA pumps combined with proteins Bax/Bcl2 balance modulating the release of cytocrome c from mitochondria. Accordingly, low expression of SERCA and Bcl2 result in high level of cytosolic $Ca^{2+}$ [57].

◀ **Figure 5.** Over-represented KEGG pathways and GO biological processes in γ- and α-TTs *versus* control cDNA arrays experiments. From all the expressed genes, according to array analysis, were chosen only genes with a delta FC value (expr(treatment)-expr(Control)) at least 1 and a *p*-value adjusted for Benjamini–Hochberg correction of 0.05. The entire set of differentially expressed genes in treatments *versus* control was used as input for DAVID web server. Only the biological processes with a *p*-value below 0.05 were considered as over-represented. We also illustrated delta FC values for treatments *versus* controls (see text for more details).

Finally, as demonstrated by immunocytochemistry experiments, γ-TT but not α-TT, activates apoptosis in MCF-7. cDNA-array data confirm that α-TT does not activate any pro-apoptotic gene. On the other hand, γ-TT down-regulates cytocrome *c* gene expression and activates the apoptosis inducing factor–mitochondrion associated. Moreover, γ-TT modulates different genes involved in mitochondrial phosphorylation, including different subunits of the cythocrome *c* oxidase. In particular, the subunit IV (COX4l1) is down-regulated by 276 folds in comparison to control. The decrease of the subunit IV has been demonstrated to disassembly the enzyme complex and to compromise membrane potential, leading to a decrease of ATP levels and to a sensitization of the cells to apoptosis [58]. Therefore, on the basis of these observations, we can hypothesize that the molecular mechanism underlying γ-TT induced apoptosis in MCF-7 cells is, at least in part, mitochondria-driven.

In general, we remark that the biological processes specifically modulated by γ-TT, but not α-TT, share many similarities with gene expression profiling of estrogen-regulated genes in MCF-7 breast cancer cells after treatment with anti-estrogens drugs [59].

On the basis of these results and previously published data [7], we can hypothesize that γ-TT induction of specific metabolic pathways and of apoptosis act through an ERβ-associated signal transduction pathway. However, the involvement of different transduction pathways in TTs effects cannot be excluded. Further studies are needed to fully understand the molecular mechanisms underlying TT effects.

## 4 Concluding remarks

These data confirm our previous original observation indicating an estrogenic activity of specific TTs forms and corroborate the notion that TTs biological activity is specific, distinct from TOCOs and not ''restricted'' to their antioxidant capacity [60, 61]. Moreover, the evidence that TT do not share a significant number of biological activities with the related TOCOs, also suggests that the possibility to pull them apart from the ''heterogeneous'' family of Vitamin E, and to propose new roles and functions for this class of molecules in human health and disease.

Moreover our observations may have a pharmacological concern. In the last years, the estrogenic therapy has been mainly based on the targeting of ERα and ERβ; and in particular, a number of studies addressed a protective role of ERβ against breast cancer development. Therefore, the detection of molecules acting as specific target for ERα and ERβ would open up to original ''nutritional management'' opportunities and possibly to novel therapeutic approaches.

All these observation strongly suggest that the molecules of nutritional interest eliciting ERβ activity can act as possible tumour suppressors. Our observations could open new avenues for a specific role of TTs in regulating gene expression and in modulating the growth of breast cancer cell and of other tumour in part or totally dependent on estrogen signals.

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