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Original article

# (−)-Epigallocatechin-3-gallate downregulates estrogen receptor alpha function in MCF-7 breast carcinoma cells

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**Abstract**

*Background*: (−)-Epigallocatechin-3-gallate (EGCG) is the most active catechin present in green tea, demonstrated to have chemopreventive action and to kill cancer cells selectively. As a previous study found that catechins could compete with 17-β-estradiol for binding to estrogen receptor alpha (ERα), we asked whether EGCG could regulate ERα action. *Methods*: We used MCF-7, a breast carcinoma cell line having a high level of ERα expression. The cells were treated with various EGCG concentrations and cell viability was evaluated by MTT assay. ERα and pS2 expression were analyzed by RT-PCR after RNA extraction. To better define EGCG action in relation to ERα, we studied EGCG cytotoxicity on MCF-7 resistant to tamoxifen (MCF-7tam), MCF-7 treated with $10^{-7}$ M ICI 182,780 for 8 days and on MDA-MB-231, a cell line that lacked ERα by flow cytometry (FCM). *Results*: Both ERα and pS2 mRNA were expressed in samples treated with low EGCG concentration (30 μg/ml). At this concentration, no cell change was detectable. In contrast, pS2 expression was lost in samples treated with 100 μg/ml EGCG for 24 h, indicating ERα alteration. EGCG cytotoxicity was lower when ERα was not present (MDA-MB-231) or inactivated (by tamoxifen or ICI 182,780). *Conclusions*: Functionally active ERα may have a role in EGCG cytotoxicity, increasing the sensitivity to the drug. As higher EGCG concentrations also killed cells resistant to tamoxifen or treated by $10^{-7}$ M ICI 182,780, EGCG ought to be better investigated in breast carcinoma cells treated with drugs targeted to steroid receptors, as a potential complement of therapy.


*Keywords:* Green tea; Chemoprevention; Estrogen receptor signaling; pS2; Endocrine therapy; Tamoxifen; ICI 182,780; MDA-MB-231; RT-PCR; MTT assay

## 1. Introduction

(−)-Epigallocatechin-3-gallate (EGCG) is one of the most important catechins present in green tea (*Camellia sinensis*), a beverage widely diffused and consumed in the Asian population, which shows powerful antioxidant and anti-inflammatory properties. Epidemiological studies indicate that green tea consumption protects against cancer (stomach, lung, colorectal, liver and breast) [1]. In several animal models, EGCG treatment reduced the tumor incidence, the number of tumor masses and metastasis [2,3]. Numerous in vitro studies detected cell cycle arrest and apoptosis after EGCG treatment. EGCG also reduced angiogenesis and both local and distant invasion, and it could inhibit DNA methyltransferas and reactivate methylation-silenced genes in cancer cell lines [4], but the mechanisms underlying green tea effects and the molecular targets are still under investigation [5,6]. Among the numerous molecules proposed to be target of EGCG action, two studies demonstrated that green tea catechins can bind and downregulate ERα and ERβ [7,8]. These findings can explain why various epidemiological studies found a correlation between green tea assumption and low incidence of breast cancer [9]. Estrogens are key molecules in breast cancer development that can influence breast cancer cell

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growth [10]. ERα is a fundamental molecular target of therapy in breast cancer patients expressing the steroid receptors, although a consistent number of treated patients develop resistance to therapy and relapse [11]. We considered that the interaction between EGCG and ERα might be potentially important and may have therapeutic implications. To verify this hypothesis, we used MCF-7 cells, which express a high level of ERα, and we studied ERα and pS2 expression after EGCG treatment. pS2 (also known as Trefoil factor 1, TFF1) is an estrogen-inducible protein [12], widely used as a marker of ERα functional activity. The detection of estrogen target gene expression, like pS2, represents a valuable physiologic assay for tissue specific estrogenicity or antiestrogenicity of a drug [13].

We also asked whether EGCG cytotoxicity changed after inactivation of ERα signaling pathway. To this aim we used two different drugs, tamoxifen and ICI 182,780, having different mechanisms of ERα inactivation. Tamoxifen is the most commonly used drug to treat ER-positive breast cancer patients: it binds to ERα and it blocks the estrogen action, but it also possesses ER-agonist properties. Long-term use of tamoxifen is associated with several important side effects and risk of relapse with resistant disease [14]. ICI 182,780 is a new ER antagonist that destroys the ER signaling pathway by decreasing the level of ERα through their ubiquitinylation and targeting to the proteasome and it is not associated with tamoxifen-like agonist effects. To investigate EGCG cytotoxicity in MCF-7 cells after inactivation of ERα signaling pathway, EGCG was given to MCF-7 cell line resistant to tamoxifen and to MCF-7 cells after 8 days of treatment with $10^{-7}$ M 182,780. As a comparison, we also treated MDA-MB-231 cells, which lack of ERα, with EGCG.

## 2. Materials and methods

### 2.1. Cell lines

MCF-7 and MDA-MB-231 cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in E-MEM and D-MEM, respectively, supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin/streptomycin, 2 mM L-glutamine and 1 mM Na pyruvate (all from Cambrex Bio Science, Rockland, MA, USA). MCF-7tam cell line was developed by growing MCF-7 cells in α-MEM medium (Invitrogen, Carlsbad, CA, USA) without phenol red, supplemented by 10% charcoal-stripped FCS serum, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine and 1 mM Na pyruvate (Cambrex Bio Science, Rockland, MA, USA).

### 2.2. Treatment

$10^{-7}$ M 4-OH tamoxifen (Sigma, St. Louis, MO, USA), dissolved in absolute ethanol and stored at −20 °C, was dissolved in the medium and given to MCF-7 cells for 6 months before starting the experiments. The medium was changed every 5–6 days. $10^{-7}$ M ICI 182,780, also known as fulvestrant (Tocris, Bristol, UK), dissolved in absolute ethanol and stored at −20 °C, was supplemented to MCF-7 cells for 8 days before analysis. EGCG (Sigma, St. Louis, MO, USA) was dissolved in distilled sterile water (10 mg/ml) and stored at −20 °C. Cells were treated with EGCG dissolved in medium at concentrations ranging from 20 to 200 μg/ml for 24 h.

### 2.3. Reverse transcription-PCR (RT-PCR)

RNA was extracted by guanidinium–phenol–isoamylic alcohol method according to Chomczynski and Sacchi [15] with modifications. RT-PCR reaction was performed using the cMaster RT plus PCR system kit according to the instruction of the supplier (Eppendorf, Hamburg, Germany). The following primers were used: ERα, forward 5′-CCG-CTCATGATCAAACGCTCTAAG-3′ reverse 5′-GCCCT-CTACACATTTTCCCTGGTT-3′, which originate a 375 bp DNA fragment [16]. pS2, forward 5′-ATGGCCACCATG-GAGAACAA-3′ and reverse 5′-ATAGAAGCACCAGGG-GACCC-3′ [17], β-actin, forward 5′-GGCATCGTGAT-GGACTCCG-3′, reverse 5′-GCTGGAAGGTGGACAGC-GA-3′. Annealing temperature was 60 °C for ERα and 58 °C for pS2 primers. β-actin primers were used together with ERα or pS2 primers into the same tube.

### 2.4. Immunofluorescence

The cells were grown on coverslip in 3.5 mm Petri dishes, washed by PBS and fixed in 1% formalin in PBS for 15 min. After washings in PBS, the cells were permeabilized by 0.05% Triton X-100 in PBS for 10 min at room temperature, washed again and treated with 10% BSA in PBS for 30 min at 37 °C. The samples were incubated with the rabbit polyclonal antibody against ERα (Ab-16, Labvision Corporation, NeoMarkers, CA, USA), diluted 1:300 in 1% BSA in PBS, for 1 h at 37 °C in a moist chamber. After two washings in PBS, the cells were treated with an anti rabbit-FITC conjugated antibody (MP Biomedicals, Aurora, Ohio, USA) diluted 1:6000 in 1% BSA in PBS for 1 h at 37 °C in a moist chamber. The cells were washed in PBS and distilled water. Then, the coverslips were air-dried and mounted in a solution containing 4′-6-diamidino-2-phenylindole (DAPI) in 1,4 diazabizyclo[2.2.2]octane (DABCO) (both from Sigma, St. Louis, MO, USA). The samples were examined with a Nikon epifluorescence microscope, equipped with a 100 KV lamp and filters for FITC and DAPI.

### 2.5. MTT assay

We used MTT assay to define the EGCG concentration able to induce at least a 20% reduction in cell proliferation

after 24 h treatment. These particular experimental conditions were chosen because we intended to analyze ERα signaling before massive cell death occurred, to avoid misinterpretation of the results. Twenty thousand cells were plated in 96 multiwell plates. EGCG treatment started the day after. At least 4–8 replicas of the same sample at a given EGCG concentration and time were used. After treatment, the medium was replaced with 100 μl of fresh medium and 10 μl MTT (1-(4,5-dimethylthiazol-2-yl)-3-5-diphenilformazan) (Sigma, St. Louis, MO, USA) dissolved in PBS (5 mg/ml), at 37 °C for 4 h. The medium was poured off and replaced by 100 μl of DMSO and let at room temperature for 1 h. The color rapidly changed to purple and the plates were red at 570 nm for evaluation.

### 2.6. Flow cytometry (FCM)

All the FCM analyses were performed using a FACScalibur cytometer (BD, San José, CA, USA) equipped with an argon ion laser tuned at 488 nm. In all analyses, a minimum of 10,000 cells per sample were acquired in the list mode and analyzed with Cell Quest software. The DNA content in MCF-7, MDA-MB-231, MCF-7tam, MCF-7 ICI 182,780 treated cells was assessed using propidium iodide (PI) staining. Cell viability was determined by PI exclusion assay. In this assay, the loss of membrane integrity allows the dye to enter the cell and bind to nucleic acids producing a fluorescent signal in non-viable cells only. Viability was determined as the percentage of cells PI-negative among the total cells. Each sample was allowed to run for 30 s after flow stabilization.

### 2.7. Statistical analysis

Descriptive statistics, such as mean (M), standard deviation (S.D.) and standard error (S.E.), were used to summarize the results. Significant differences between the control and the experimental groups were determined by Bonferroni test. Statistical significance was defined by $p < 0.05$.

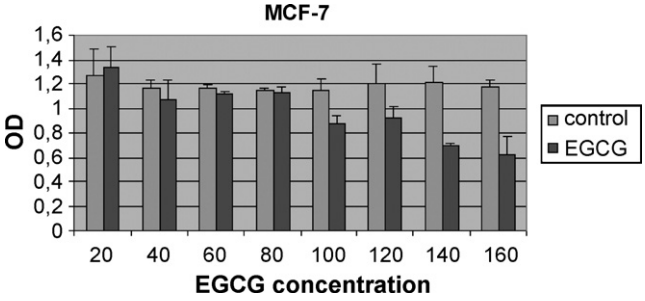


Fig. 1. MTT test on MCF-7-treated with EGCG for 24 h. MCF-7 cells were plated in 96 well plates at the density of 25,000/well. EGCG was added to the medium for 24 h at various concentrations (20, 40, 60, 80, 100, 120, 140 and 160 μg/ml). Control cells were treated with $H_2O$ in E-MEM medium. The bars represent the mean ± S.D. of 4–8 wells, obtained in three different experiments. S.D. is indicated as a thin bar. OD value, which is related with the number of viable cells, decreased after EGCG treatment at concentration higher than 80 μg/ml.

## 3. Results

### 3.1. MCF-7 cell viability following EGCG treatment

At the concentration of 100 μg/ml, the OD value decreased of 24% in treated cells with respect to the control cells (Fig. 1). Treatment with higher concentrations of EGCG (120–160 μg/ml) resulted in higher dose-dependent reduction OD value. Therefore we considered that 100 μg/ml might be an EGCG concentration suitable to our aims.

### 3.2. pS2 expression was lost in MCF-7 cells treated with 100 μg/ml EGCG for 24 h

MCF-7 cells were treated with EGCG at 30 and 100 μg/ml for 2 and 24 h, in order to detect early changes in ERα transcription. After short time treatment (2 h), the cells did not show morphologic alterations suggestive of cell damage and they did not show changes in ERα and pS2 transcription (Fig. 2a). After 24 h of 100 μg/ml EGCG treatment (2c), pS2, a gene downstream ERα pathway that is considered a good indicator of ERα activity, was not expressed. This finding suggested that EGCG might have a mechanism of action similar to $10^{-7}$ M ICI 182,780, a drug that inhibits ERα signaling pathway by accelerated degradation of the receptor. We treated MCF-7 cells with $10^{-7}$ M ICI 182,780 for 8 days, a time considered suitable to induce ERα signaling pathway inhibition and, as expected, we found pS2 loss (Fig. 2c).

Then, we applied immunofluorescence to detect ERα in MCF-7 cells treated with EGCG and with $10^{-7}$ M ICI 182,780 for 8 days to compare them. ERα was present in the nuclei of control MCF-7 cells (Fig. 3a). It was undetectable in MCF-7 cells treated with ICI 182,780 for 8 days (Fig. 3b). In contrast, EGCG-treated cells showed intense nuclear staining (Fig. 3b), a finding indicating that the two drugs do not have the same mechanism of action. Therefore, EGCG can inactivate ERα signaling pathway without affecting both ERα expression and accelerated degradation.

### 3.3. EGCG effect on MCF-7tam, MCF-7 treated with $10^{-7}$ M ICI 182,780 and MDA-MB-231 cells

We intended to verify whether EGCG administration could also be effective in MCF-7 cells resistant to $10^{-7}$ M 4-OH-tamoxifen or having ERα signaling inactivated by $10^{-7}$ M ICI 182,780 treatment. We also included a breast carcinoma cell line, MDA-MB-231, which did not express ERα but was sensitive to EGCG treatment. MCF-7tam was developed by adding $10^{-7}$ M 4-OH tamoxifen to the culture medium. Resistant cells were grown continuously for 6 months before starting the experiments, in order to select

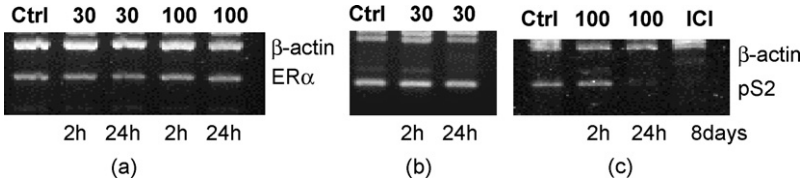


Fig. 2. ERα and pS2 expression in MCF-7 cells treated with EGCG. ERα expression did not change in MCF-7 cells treated with 30 or 100 μg/ml EGCG for 2 and 24 h (a). Both β-actin and ERα band (375 bp) were visible. In MCF-7 cells treated with 30 μg/ml EGCG for 2 and 24 h, pS2 expression was detectable (b). In contrast, pS2 expression was only lost in MCF-7 cells treated with 100 μg/ml EGCG for 24 h and with $10^{-7}$ M ICI 780,181 for 8 days (c).

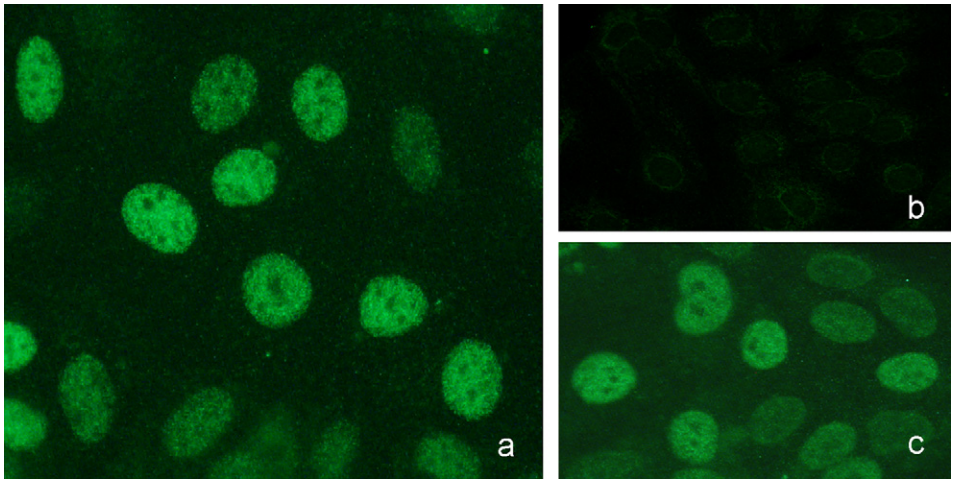


Fig. 3. ERα detection by immunofluorescence. Cells were grown on coverslip, fixed in 1% formalin in PBS, incubated with a rabbit polyclonal antibody anti-ERα and then with a secondary anti-rabbit-FITC antibody. Intense staining was present in the nucleus of MCF-7 control cells (a). After 8 days $10^{-7}$ M ICI 182,780, treatment, staining was not longer visible in MCF-7 cells (b). In contrast, EGCG treatment (100 μg/ml for 24 h) did not change ERα staining pattern (c).

cells having ERα downregulation. As shown in Fig. 4, pS2 transcription was hardly detectable in MCF-7tam cells, whereas the band was clearly visible in normal control MCF-7 cells. In tamoxifen resistant cells, ERα is blocked by the drug, but alternative growth pathways enables breast cancer cells to survive and proliferate. We also used another drug, ICI 182,780, a compound able to increase ERα degradation and inactivate the ERα pathway. $10^{-7}$ M ICI 182,780 was given to MCF-7 cells for 8 days before starting EGCG treatment. After 8 days treatment, pS2 was not expressed (Fig. 2c) and ERα was not detectable by immunofluorescence (Fig. 3c). Then, we treated MCF-7 cells with EGCG for 24 h and we used flow cytometry (FCM) to study the cell viability after treatment. Statistically significant differences between control and treated cells were found only in MCF-7 parental cells (Bonferroni test, $p < 0.05$). Both MCF-7tam cells and ICI 182,780-treated cells showed reduced sensitivity to EGCG. Cell death occurred, but to a lower extent than in MCF-7 cells having a functioning ERα signaling pathway (Fig. 5). Finally, no cell death occurred in MDA-MB-231 cells treated with EGCG at

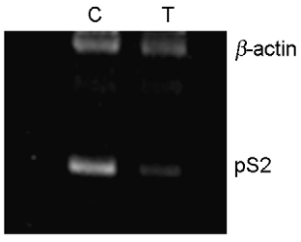


Fig. 4. pS2 expression in MCF-7tam cells. RT-PCR was performed on RNA extracted from normal, control MCF-7 and MCF-7tam cells. Lower band, corresponding to pS2 expression, was detectable in control MCF-7 cells (C), whereas it was hardly detectable in MCF-7tam cells (T).

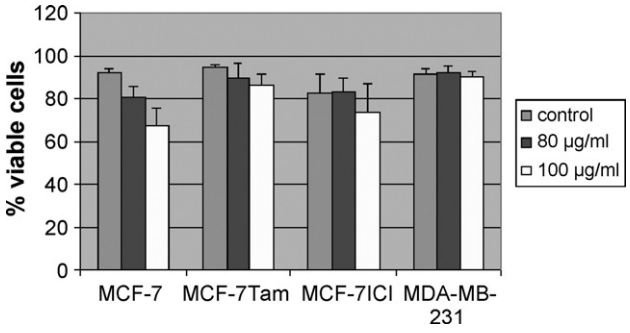


Fig. 5. FCM analysis of MCF-7, MCF-7tam, MCF-7 cells treated with ICI 182,780, for 8 days and MDA-MB-231 cells following EGCG treatment for 24 h. The cells were treated with 80 and 100 μg/ml EGCG for 24 h. Viable cells were detected and evaluated by PI exclusion assay. Each bar represents the media of viable cells percentage counted in three independent experiments, thin bar S.D. Significant difference ($p < 0.05$) between control and treated cells was only found in MCF-7 cells treated with 100 μg/ml. Cell death occurred to a low extent in MCF-7tam and MCF-7 ICI cells, whereas it was not detected in MDA-MB-231 cells at these concentrations.

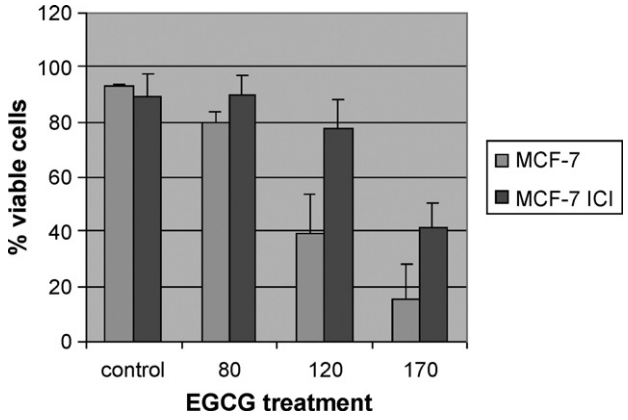


Fig. 6. FCM analysis of MCF-7 and MCF-7 cells treated with $10^{-7}$ M ICI 182,780, for 8 days before EGCG treatment. Effects of low and high EGCG concentrations on MCF-7 cells and MCF-7 treated with $10^{-7}$ M ICI 182,780, for 8 days before EGCG treatment. Each bar represents the media of three independent experiments. Viable cells were detected and evaluated by PI exclusion assay, thin bar: S.D. Bonferroni test was applied to analyze differences among control and treated cells. The difference was significant in MCF-7 cells treated with 120 and 170 μg/ml EGCG. In MCF-7 ICI cells treatment with 170 μg/ml EGCG only showed significant difference in the percentage of viable cells with respect to control cells.

80 and 100 μg/ml. Higher concentration (160 μg/ml) were necessary to elicit cell death.

The treatment of MCF-7 with higher concentrations of EGCG for 24 h dramatically elicited massive cell death. A pervasive lethal effect was also present when EGCG was given to MCF-7 cells previously treated with $10^{-7}$ M ICI 182,780 for 8 days (Fig. 6). Therefore, higher EGCG concentrations were also able to kill MCF-7 cells when the ERα signaling pathway was inactivated. Higher EGCG concentrations were also found to elicit cell death in both MCF-7tam and MDA-MB-231 cells.

## 4. Discussion

In the present study, we found that EGCG treatment downregulated ERα pathway in MCF-7 cells, as demonstrated by loss of pS2 transcription after 100 μg/ml treatment for 24 h. Loss of pS2 was not detectable after 2 h treatment, or after EGCG treatment at a low concentration (30 μg/ml). It only occurred at an early stage of EGCG action, when EGCG was given at a concentration able to induce cell death in a limited number of cells. As 70–80% cells were still viable, these findings suggest that ERα downregulation may have occurred before breast cancer cell death and, possibly, contributed to cause it. We deliberately intended to study the early phases of EGCG action on MCF-7 cells, in order to avoid misinterpretation of the results when cell death is massive as a consequence of high concentration or long time treatment. Interestingly, ERα transcription was detectable in control and treated cells at low and high doses, at 2 and 24 h treatment. The protein was also present in MCF-7 nuclei, suggesting that ERα alteration occurred at an early step of EGCG action. Estrogens are important mitogenic signals for breast cancer cell growth. Survival and proliferation of breast cancer cells are under estrogen influence. Estrogens control several molecules involved in cell cycle regulation and growth [18], up-regulate the anti-apoptotic factor bcl-2 and increase cell survival [19]. In an attempt to treat or prevent breast cancer, patients having functionally active ERα are target of specific therapy based on the strategy to antagonize estrogens [20]. The receptor can be blocked by tamoxifen or functionally inactivated by accelerated degradation by ICI 182,780 administration. We compared the effects of EGCG among control MCF-7 cells, MCF-7 cells treated by tamoxifen for 6 months that had developed drug resistance, MCF-7 cells treated for 8 days with ICI 182,780 that did not show ERα in the nucleus and had lost pS2 expression, and MDA-MB-231 cells, which did not have ERα. The results suggested that when ERα was downregulated, either for receptor block or accelerated degradation, or not present, as in MDA-MB-231 cells, EGCG cytotoxicity was reduced. Therefore, the presence of functionally active ERα might contribute to elicit cell death in breast cancer cells. Higher EGCG concentrations are necessary to kill breast carcinoma cells lacking ERα. Based our findings, we cannot speculate about a molecular mechanism of action of EGCG on cells expressing ERα. Catechins show structure similarity with isoflavones, known to be estrogen antagonist. EGCG and other catechins present in green tea were all found to compete with 17-β-estradiol for binding to ERα and ERβ [7]. EGCG was found to be antiestrogenic for ERα at high doses and co-estrogenic at low dosed for both ERα and ERβ [8]. Green tea decreased levels of ERα in tumors both in vitro and in vivo [21] and interaction between EGCG and ERα signaling has been recently demonstrated [22]. The present results also suggest a possible interaction of EGCG with ERα. Although both EGCG and ICI 182,780 inhibited pS2 expression in MCF-7 cells, they seemed to have different mechanism of action on ERα. We tried to compare the effects of 100 μg/ml EGCG and ICI 182,780 on ERα presence after 8 days of treatment, but no viable cells were present in EGCG-treated samples (data not shown). After 24 h EGCG treatment, MCF-7 cells had intense ERα staining, whereas MCF-7 cells treated with ICI 182,780 for 8 days did not have staining.

The present results disclosed a new aspect of this beverage that may explain why several epidemiologic studies reported a chemopreventive action on breast cancer [2,9,23]. The exact mechanism of green tea catechins action is unknown, although many different molecules and signaling pathways have been demonstrated to be involved and to concur to the final biologic effect [2,4,5]. The molecular targets can be different in various cell lines, having different genetic alterations and biologic characteristics and cell death can be the results of pathways elicited by different molecules. Green tea is a cheap and safe beverage,

able to induce very modest side effects and demonstrated not to damage normal breast cells [24]. EGCG was reported to be cytotoxic to MCF-7 cells at low concentrations, and the combination of EGCG and 4-OH tamoxifen elicited synergistic cytotoxicity in MDA-MB-231 cells, lacking of ERα, at a higher concentration [25]. Based on our current finding, we confirm that EGCG action is particularly effective in breast carcinoma cells having ERα. EGCG treatment can also kill breast carcinoma cells lacking steroid receptors and resistant to drugs currently used in breast cancer therapy. These characteristics make green tea a beverage to be investigated as a complement of other therapeutics in breast carcinoma, particularly in relation to resistance to drugs developed to block or inactivate ERα.

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## Conflict of interest

None.

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