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Research Article

(-)-Epigallocatechin-3-Gallate Modulates the Activity and Expression of P-Glycoprotein in Breast Cancer Cells

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Abstract

Breast cancer is the most common cancer in women worldwide. Chemotherapy is successful in many instances but some patients do not respond satisfactorily. Multi-drug resistance is considered the major cause of failure in chemotherapy treatment. It has been found said resistance is associated with the over expression of type ABC transporters, mainly P-glycoprotein (P-gp). It is now known that P-gp could be modulated by compounds such as green tea and its polyphenols, including (-)-Epigallocatechin-3-gallate (EGCG). The aim of this study was to evaluate the effect of EGCG on P-gp gene and protein expression, and its activity in breast cancer cell line. We found that EGCG does cause cell death at concentrations higher than100 μM,can induce apoptosis with low concentrations and necrosis with high ones. EGCG inhibited Pgp protein expression in a dose-dependent manner, 60% with 10μM EGCG. However, no changes were observed in gene expression. We also observed EGCG also inhibited P-gp activity in breast cancer cells, 20% and 90% at doses of 10 and 100 μM respectively. Our results suggest that EGCG could improve the efficacy of breast cancer treatment by increasing the accumulation of chemotherapeutic drugs in cancer cells by blocking P-gp function.

INTRODUCTION

Breast cancer is the most common cancer in women. This disease directly affects approximately 12% of women at some stage of their life-time [1]. Approximately 1.3 million women are diagnosed with breast cancer annually across the world and approximately 465,000 die from the disease [2]. Chemotherapy resistance is a serious complication in the treatment of advanced breast cancer [3].

Multiple drug resistance (MDR) in tumor cells has been related to the expression of transport proteins that alter cellular drug transport and distribution [4]. MDR-related proteins include P-glycoprotein (P-gp), Breast cancer resistance protein (BCRP), multi drug resistance associated protein (MRP), and the lung resistance related protein (LRP) [5]. P-gp is a 150–180-kDa membrane phospho-glycoprotein encoded by the MDR1 gene, which functions as an energy-dependent drug transporter with broad specificity; P-gp is expressed in the apical membrane of many secretary cell types where the normal function involves the excretion of drugs and their metabolites [6]. P-gp activity is of great clinical importance in non-cancer-related drug therapy due to its wide-ranging effects on the absorption and excretion of a variety of drugs. P-gp confers a multi-drug resistance (MDR) phenotype to cancer cells that have developed resistance to chemotherapy drugs [7].

In recent years much effort has been directed at the identification of chemo sensitizers (MDR reversers or modulators) in order to modulate P-gp mediated multidrug resistance. Most chemo sensitizers directly block P-gp activity by binding to chemotherapeutic drug binding sites, or to other modulator binding sites that cause inhibition of drug binding or transport. The other approach for MDR modulation is down-regulation of P-gp protein expression [8]. For these reasons, the discovery and evaluation of new molecules that can function as therapy or co-therapy to reverse or reduce the multidrug resistance phenotype (MDR) is most important.

Recently, several studies have documented the ability of chemo preventive phytochemicals to increase the sensitivity of cancer cells to anticancer drugs [9,10]. (–)-epigallocatechin gallate (EGCG), a major component in green tea extracts, has a wide range of biological and pharmacological activities, including

antioxidant, antimutagenic, and anticarcinogenic activities [11]. Current studies about the effect of EGCG on breast cancer cells have been mainly focused on its anti-cancer properties, obtaining favorable results; however, there is not studies about the effect of this phytochemical on drug transporters in non-resistant breast cancer cell. The purpose of this study was to evaluate the effects of EGCG on expression and activity of P-glycoprotein in breast cancer cells.

MATERIALS AND METHODS

(−)-epigallocatechingallate (EGCG) was purchased from Sigma Aldrich Co., and the purity was determined to be ≥ 95% by HPLC. Stock solution of EGCG (5mg/ml) was dissolved in phosphate buffered saline (PBS). Aliquots were stored at -20°C.

MCF-7 cells and culture conditions

MCF-7 cells and HepG2 cells (ATCC, Manassas, VA) were cultured in DMEM (GIBCO, USA), with 10% FBS (GIBCO, USA) and 50 µg/ml gentamycin. The cultures were maintained in a humidified 5% CO_2 incubator at 37°C. Cells were sub-cultured every 3-4 days to maintain logarithmic growth.

Cell viability

Cell viability was determined using MTS assay (CellTiter 96® AQueous One Solution kit, Promega, USA). Briefly, for cell viability, MCF-7 cells were seeded into 96-well plate at a density of 10,000 per well and incubated for 24 h at 37°C and 5% CO_2.The culture medium was replaced by a fresh one supplemented with different concentration of EGCG (0.01, 0.1, 1, 10, 100, and 1000 µM) and incubated for 24 h. *In vitro* studies that endeavor to mimic *in vivo* effects generally used EGCG at concentrations higher than 10µM and even as high as 200 µM, which are physically un-achievable in the human body. Physiological concentrations of EGCG in human plasma reach between 0.1 and 1µM, and may approach 7µM with supplements (Zeng *et al*., 2014). After treatment, the medium was gently removed and replaced with 20 µl of the MTS reagent and incubated at 37°C for 1 h. Absorbance was detected at 490 nm with a Microplate Reader (VICTOR™ 3X Multilabel Plate Reader, Perkin Elmer, USA). Untreated cells served as non-treatment control cell viability. The results represented a percentage of the relative viability of cells against the untreated control.

Assessment of cell death

The assessment of cell death was carried out using the acridine orange and ethidium bromide staining as qualitative assay as described previously [12]. Briefly, MCF-7 cells were seeded into 6-well plates (250,000/well) and incubated for 24 h at 5% CO_2 and 37°C.Culture medium was replaced with fresh media containing EGCG at 0.1, 1, 10, 100, 250 and 500µM and the cells were then incubated for another 24 h. After washing thoroughly with PBS, 250 µl of a mixture of 100 µg/ml acridine orange/ 100 µg/ml ethidium bromide (Sigma Aldrich, USA) was added to the each well. The cells were then incubated at room temperature for 10 seconds and observed under a fluorescence microscope. Images of fluorescently stained cells were photographed with an Olympus digital camera. The data represents the average number of live, apoptotic or necrotic cells over at least 15 images for each treatment. Cells incubated in culture medium were used as a non-treated control. 1µl/ml of 30% H_2O_2 served as apoptosis control [13] and smashed cells were used as necrosis control. Acridine orange is a vital dye and will stain both live and dead cells. Ethidium bromide will stain only cells that have lost membrane integrity. Live cells will appear uniformly green. Early apoptotic cells will stain green and contain bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells will also incorporate ethidium bromide and therefore stain orange, but, in contrast to necrotic cells, the late apoptotic cells will show condensed and often fragmented nuclei. Necrotic cells stain orange, but have a nuclear morphology resembling that of viable cells, with no condensed chromatin [12].

Analysis of P-gp expression by Immunofluorescence microscopy

For immunofluorescence, MCF-7 cells were grown on glass slides in 6-well culture plates (250,000 cells per plate) and treated with EGCG at 0.01µM to 1000 µM for 24 h. After treatment the cells were fixed for 30 min in 3.7% ice-cold paraformaldehyde. Fixed cells were washed 3 times with PBS and incubated overnight at 4°C with the mouse monoclonal anti-P-gp antibody (1:250), diluted in PBS-BSA 3%. After incubation with primary antibody, cells were washed 3 times with PBS, and incubated with secondary antibody Alexa Fluor® 568 Goat Anti-Mouse IgG (1:100) in the dark for 2 h at room temperature. Cell nuclei were counterstained with Hoechst solution (1µg/ml in PBS) for 5 min at room temperature. After washing with PBS, cells were mounted on glass slides using glycerol 10%. Negative controls for each treatment that were processed without primary antibody showed negligible background fluorescence. P-gp immunofluorescence expression for each sample was quantified using Image J version 1.44 software.

Analysis of P-gp expression by Western blot analysis

MCF-7 cells were seeded into 60 mm culture dishes at a density of 500, 000 cells and treated with EGCG at 0.01µM to 100µM. After treatment, cells were washed twice with ice cold PBS and total cell lysates. Whole-cell lysis buffer consisted of 200 ml Igepal, 200 ml 10% sodium dodecyl sulphate, 10 ml 1mM dithiothreitol and 9.59 ml phosphate buffered saline, with addition of 1x protease inhibitor cocktail tablet (Roche, UK). For immunoblotting, 75µg of total protein extract was mixed with Laemmli sample buffer (Bio Rad Laboratories, USA). Proteins were separated on 8% PAGE (180 V for 1 h) and transferred onto polyvinylidine difluoride membranes (Millipore, U.S.A.) overnight. Membranes were washed briefly with TBS-T (Tris-buffered saline and 0.1% Tween-20, pH 7.6) and blocked with PBS containing 5% milk powder and 0.05% Tween 20for 1 h at room temperature. After washing three times for 15 min (0.05% Tween in PBS), the membranes were incubated overnight at 4°C with the primary, mouse anti-human antibody C219 against P-gp (Calbiochem, USA)1 µg/ml in BSA 2.5% and 0.05% Tween-20. As loading control, β-actin goat monoclonal antibody (Santa Cruz Biotechnology, USA) was used with a dilution of 1:500. After the first incubation, the membrane was washed three times for 15 min and then incubated with the secondary; horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin-G

(Cell signaling Technology, USA) diluted 1:2500 in 5% milk powder and 0.05% Tween-20. Secondary antibody incubation was performed for 1 h at room temperature. Membranes were washed, and P-gp and β-actin detection was performed with the enhanced chemiluminescence system (Super Signal West Femto Chemioluminiscence substrate, Termo scientific, U.S.A.). HepG2 cell extracts were used as positive control.

Analysis of mRNA MDR1 expression

Total RNA was extracted from cell lines using Direct-zol RNA miniprep (Zymo Research, R2050) following manufacturer's protocol. For RT-PCR, SuperScript III One-Step RT-PCR Platinum Taq (Invitrogen, USA) was used to amplify a 325pb for MDR1 exon 1 and a 496 bp for GAPDH control. The RT-PCR One-Step reaction was carried out at 50 ºC for 30 min, then 2 min at 94 ºC for denaturing, followed by 35 cycles of 15 s at 94 ºC, 35 s at 59 ºC and 45 s at 68 ºC, with a final 1 min at 68 ºC, using the following pair of primers: sense 5´-ACCATGGATCTTGAAGGGGACC-´3 and antisense 5´-CCTCCAGATTCATGAAGAACCC-´3 for MDR1 exon 1; and forward 5´- CAAGGTCATCCATGACAACTTTG-3´and reverse GTCCACCACCCTGTTGCTGTAG 3´ for GAPDH control. RT-PCR products were separated in 1.5% agarose gel, stained with ethidium bromide and visualized under ChemiDoc™ XRS+ imagen system (BioRad, USA).

Analysis of P-gp activity

MCF-7 cells were seeded at 100 000 cells/well in 12-well plates and cultured for 3 days. At confluence, cells were treated with EGCG at 0.1 µM to 100 µM for 24 h. MCF-7 cells treated with P-gp inhibitor Verapamil (100 µM) were used as positive control. After treatment cells were incubated with 10µM Rhodamine 123 (R-123) for 2h at 37°C. R-123 accumulation was stopped by washing the cells 3 times with cold PBS and the cells were lysed with 0.1% Triton X-100 at room temperature. Fluorescence of R-123 in cell lysates was measured using a Microplate Reader (VICTOR™ X Multilabel Plate Readers) at a wavelength of 485 nm for excitation and 538 nm for emission.

Statistical analysis

The data were represented as the mean ± SD of 3 independent experiments conducted by cuatriplicate. The data was statistically analyzed using the SPSS 10.0 software (SPSS Inc., Chicago, ILL., U.S.A.), the *t-test* and ANOVA. Differences were considered significant if the *P*-value was less than 0.05.

RESULTS

Figure 1 shows the effect of EGCG on cell viability in breast cancer human cells. As we can see, EGCG did not modify the number of breast cancer cells when concentrations of 0.01 to 10 µM were added. When cells were treated with 100 µM EGCG, a 20% reduction in the number of cells was observed, but this was not statistically significant. However, when cells were treated with 1000 µM EGCG, a significant reduction in the number of cells (90%, $p<0.05$) was observed. For this reason we decide to evaluate the effect of EGCG in MCF-7 cells in a wide concentration range.

To better understand the reduction on the cell viability in MC-7 cells by EGCG, we decided to characterize the cell death induced

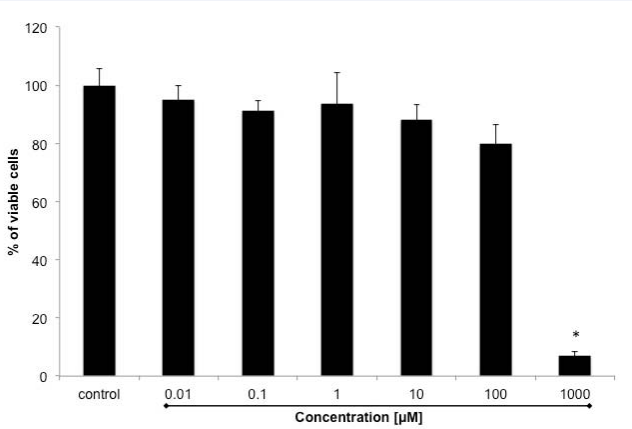

Figure 1 Effect of EGCG on cell viability on MCF-7 cells.
Cells were exposed in cultured medium with different concentrations of EGCG for 24 h. Results are expressed as percentage of cell viability as compared to control group. Data are presented as the mean ± SD of at least three independent experiments. *p <0.05 as compared with control group

by Acridine orange/ethidium bromide (AO/EtBr) double staining used to differentiate between apoptotic and necrotic cells by means of control for each cell death. AO/EtBr staining revealed the presence of around 10% more of apoptotic cells in MC-7 cells when exposed for 24 h to 250 µM (Figure 2, yellow cells) but, at the same concentration, a low number of necrotic cells were also observed (Figure 2, red cells). Moreover, cells exposed to 500 µM EGCG showed a great number of necrotic cells (Figure 2, red cells) and only some scattered alive cells were observed. Therefore, we decided to treat cells with EGCG at concentrations of 0.1, 1, 10 and 100 µM for studying the Pgp expression.

For evaluating the expression of P-gp we used immunofluorescence stain and Western blot methods. The immunofluorescence microscopic analysis confirmed the presence of P-gp in MCF-7 cells. High level of P-gp was observed by the presence of a red stain to the membrane and cytoplasm. (Figure 3) shows the relative level of P-gp (red fluorescence) in MCF-7 cells in control and treated cells. To further assess the localization of P-gp, we performed a double stain with Alexa Fluor® 568 and Hoechst solution in order to identify cytoplasm and nuclei. P-gp appeared localized to the plasma membrane and cytoplasm. Following treatment with EGCG at 0.1, 1, 10 and 100 µM, we observed a reduction in P-gp expression in MCF-7 cells in a dose-respondent manner. A slight reduction of P-gp expression was observed with 1 µM EGCG, but a clear reduction was observed with 100 µM EGCG (Figure 3).

Whole cells were lysed and equal amounts of total protein lysates were subjected to SDS-PAGE and Western blot analysis. Densitometric analysis revealed that EGCG at 0.1 and 1 µM did not change the P-gp protein expression in whole cell lysates of MCF-7 cells (Figure 4,5). In contrast, we found significant changes in P-gp protein expression in those whole cells lysates treated with 10 µM (25%, $p<0.05$). The most important effect was observed in whole cells lysates of MCF-7 cells treated with 100 µM EGCG, where a reduction of 50% in P-gp expression ($p<0.05$) was observed.

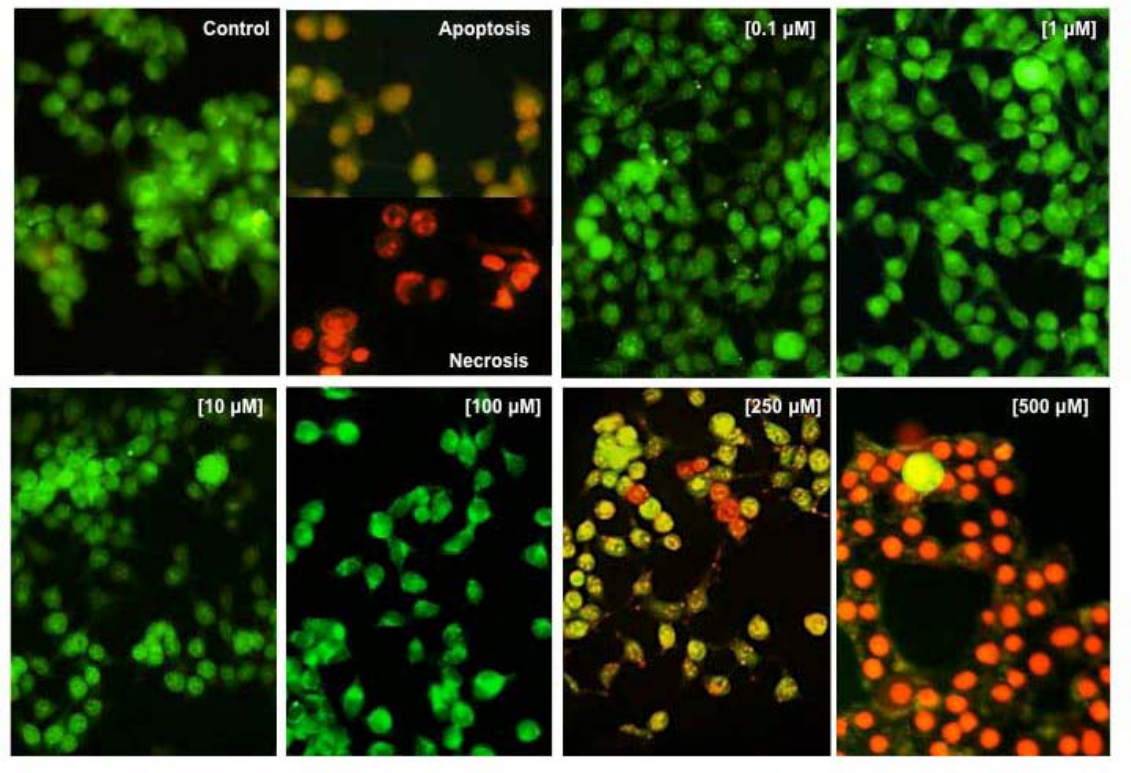

Figure 2 **Cell death induced by EGCG in MCF-7cells.**
Cells were treated with EGCG (0.1 to 500 µM) for 24 h and stained with AO/EtBr staining and analyzed using fluorescence microscopy (100X). Cells exposed to 1µL/mL of 30% H_2O_2 for 2 h were used as apoptosis control, smashed cells were used as necrosis control,and non-treated cells were used as negative control. These are representative results of at least three independent experiments (n=3).

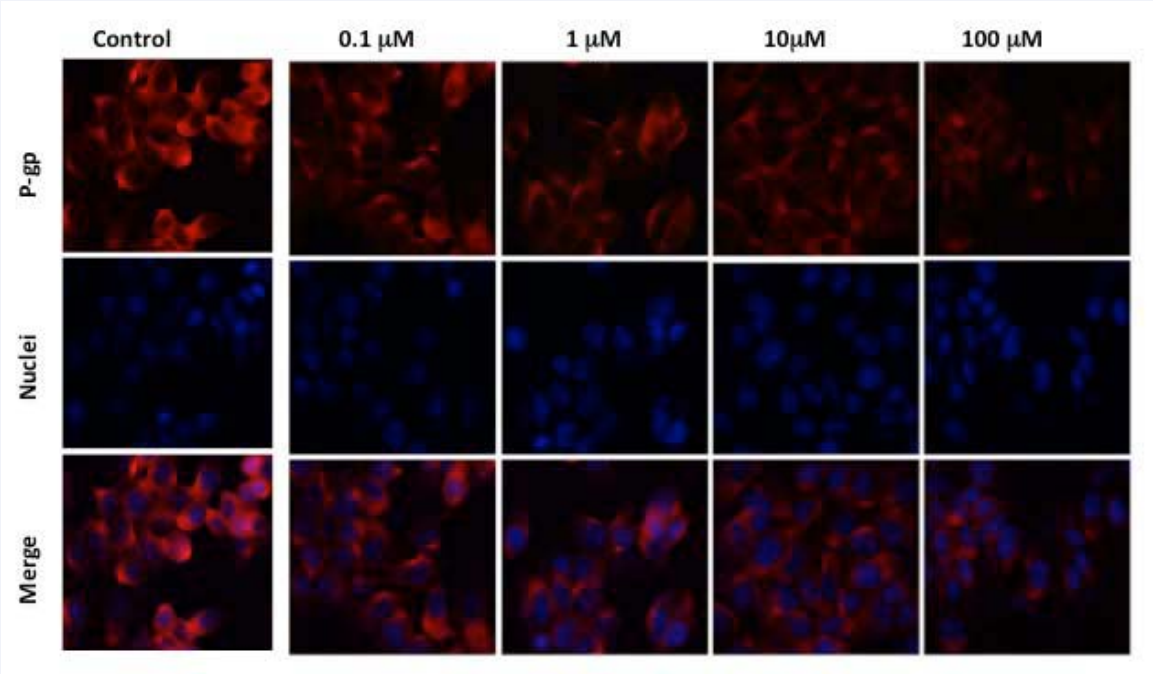

Figure 3 **Analysis of the effect of EGCG on P-gp expression in MCF-7cells.**
Cells were treated with EGCG (0.1 to 100 µM) for 24 h and incubated overnight at 4°C with the mouse monoclonal anti-P-gp antibody (1:250) (100X). Cell nuclei were counterstained with Hoechst solution (1µg/ml) for 5 min at room temperature. These are representative results of at least three independent experiments (n=3).

To assess whether EGCG could modulate P-gp gene expression (MDR1), we carried out a RT-PCR analysis. The experiment in Figure 6 shows the effect of EGCG treatment for 24 h on MDR1 gene expression in MCF-7 cells. As expected, a basal expression of MDR1 gene expression in MCF-7 cells with no treatment was observed. When the MCF-7 cells were treated with EGCG at 0.1, 1, 10, and 100μM no obvious changes in gene expression were observed.

To further study the changes of P-gp transport by EGCG, the efflux of R-123 was measured as a direct representation of P-gp transport (Figure 5). R-123, a member of the rhodamine family of flurone dyes, has been used to examine membrane transport by the ABCB1 transporters. Therefore, an efflux experiment was performed in order to verify that EGCG affects P-gp activity and not the membrane's permeability. MCF-7 cells were loaded with the fluorescent dye, washed and the R-123 remaining in the cells was then monitored in the presence or absence of P-gp modulators. Verapamil (100 μM), an inhibitor of P-gp, increased the R-123 in MCF-7 cells. No changes in R-123 content were observed when cells were treated with 0.1 and 1 μM of EGCG. However, the R-123 remaining in cells increased in a dose dependent manner in MCF-7 cells treated with 10 and 100 μM EGCG, suggesting that EGCG decreased the efflux of R-123 by blocking P-gp activity.

DISCUSSION

Green tea is one of most consumed beverages in the world. It contains many ingredients thought to promote health such as polyphenolic flavonoids, of which EGCG is the major constituent [14]. Previous studies have demonstrated that EGCG induces cytotoxicity because it may cause DNA damage, inhibits and/or poisons the enzyme to o I and II, and induces intracellular production of superoxide and hydrogen peroxide when used in large doses [15]. EGCG-induced apoptosis was evidenced by nuclear condensation, increased protein levels of activated caspase-3, down-regulation of gelsoin and tropomyosin-4 (Tm-4), and up-regulation of tropomyosin-1(Tm-1) in MCF-7 cells [16]. Our results agree with this; we found deeply cytotoxic effects in MCF-7 cells treated at 1000 μM EGCG. Besides, our present study found EGCG-induced apoptosis when cells were treated with 250 μM; however, the presence of scattered necrotic cells at this same concentration were observed, and with a concentration of 500 μM EGCG, most of the cells went into necrosis. It has been reported that EGCG induces cell death by apoptosis [17]. It was previously reported that treatment of human MCF-7 cells with EGCG has a dose-dependent effect on ROS generation and intracellular ATP levels in MCF-7 cells, leading to either apoptosis or necrosis [18].

The regulation of P-gp expression has been widely investigated

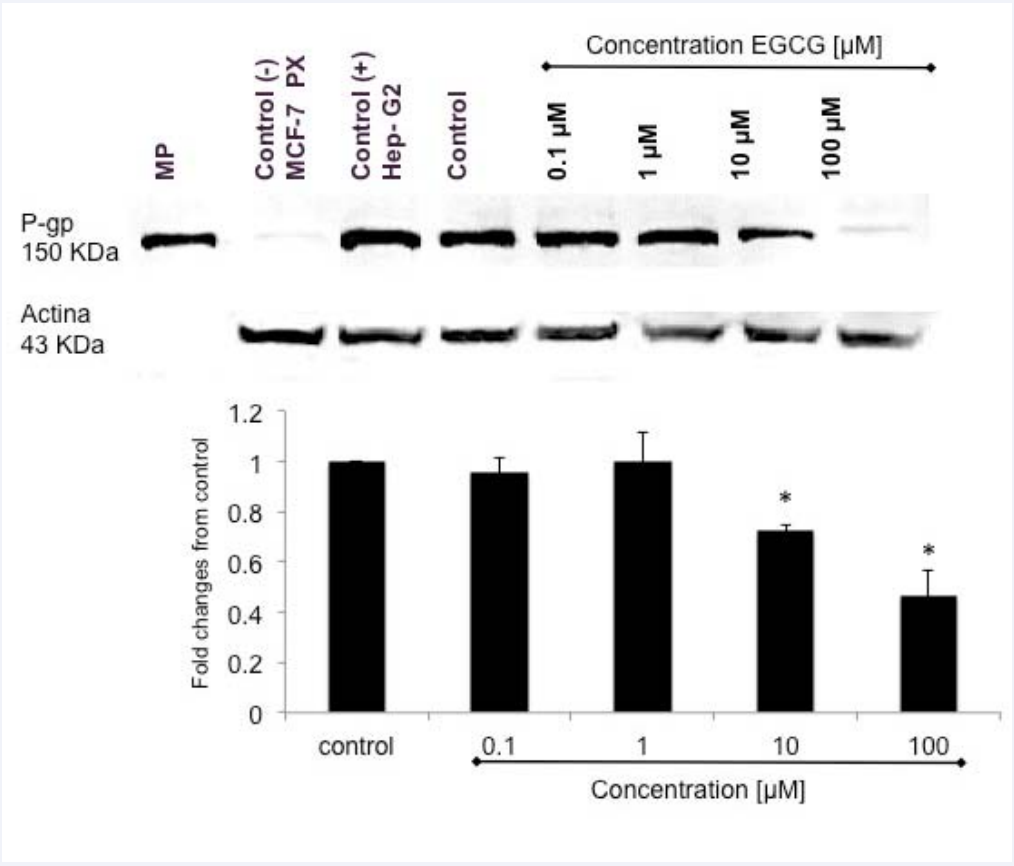

Figure 4 Representative Western blotting of total protein extracts fromMCF-7cells treated with EGCG.
Cells were treated with EGCG (0.1 to 100 μM). Cell lysates were collected separated with 7% SDS-PAGE, and immunoblotted with anti-P-Glycoprotein (C219) and actin antibodies. Densitometric analyses give the results of triplicate analyses for this individual experiment, which is representative of others that were also conducted. $p<0.05$ as compared with control.

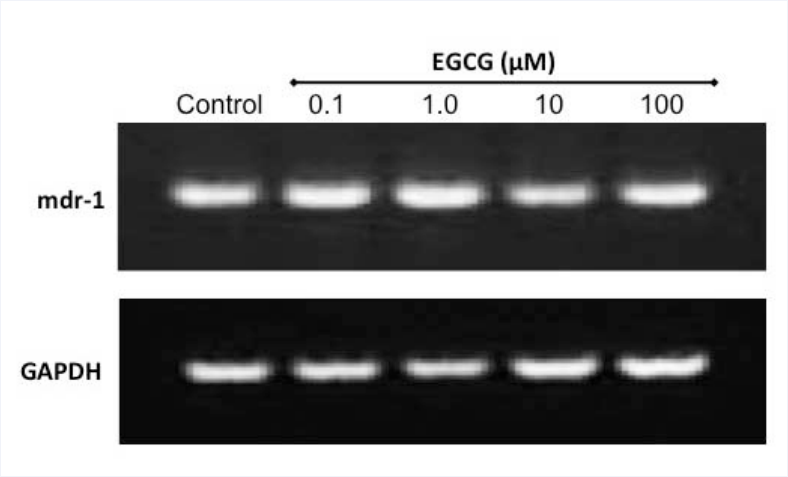

Figure 5 **Expression of mRNA for MDR1 and GAPDH in MCF-7 cell lines after EGCG treatment**
(a) Bands for MDR1 (expected size 325 bp)
(b) Bands for GAPDH (expected size 496 bp)
Line 1, negative control, lines 2 to 5: 0.1, 1.0, 10 and 100 µM of EGCG. Representative gel from 3 independent experiments

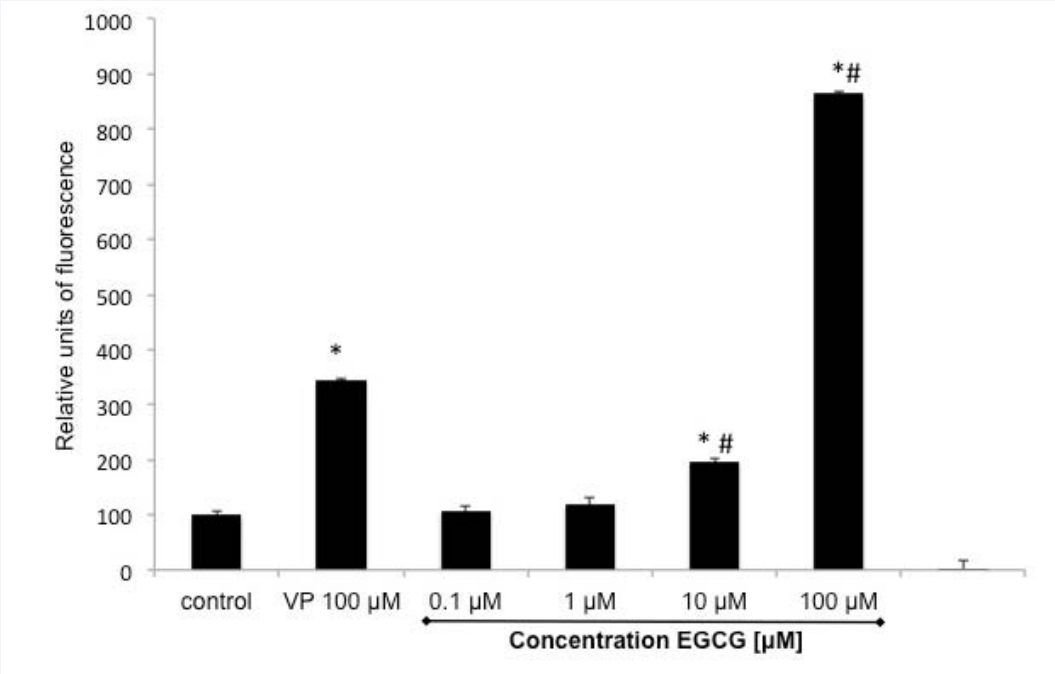

Figure 6 **Effects of EGCG on the relative accumulative amounts of rhodamine 123 in MCF-7 cells**
The accumulation of rhodamine 10µM Rhodamine 123 for 2h at 37°C in the presence of verapamil (VP 100 µM) or EGCG (0.1 to 100 µM). Data are presented as the mean ± SD of at least three independent experiments.
*p <0.05 as compared with control group; # p<0.05 as compared with 100 µM verapamil (VP)

to better understand its pathophysiological implications. In particular, it has been shown that P-gp expression is regulated through transcriptional and post-transcriptional mechanisms and by various endogenous and environmental stimuli that evoke stress response [19]. In addition, localization of the drug transporter P-glycoprotein (P-gp) to the plasma membrane is thought to be the only contributor of Pgp-mediated multidrug resistance (MDR). However, the immunofluorescence results showed that the primary P-gp localization in MCF-7 cells was observed to the plasma membrane and cytoplasm, suggesting that P-gp expressed in intracellular organelles contributeto drug resistance. In fact, Yamagishi et al. demonstrated that intracellular P-gp is localized to lysosomes in KBV1 (+P-gp) cells. Using confocal microscopy P-gp colocalizes with lysosomal-associated membrane protein 2 (LAMP2, a well characterized lysosomal marker, arrows). This lysosomal P-gp was demonstrated to

be functional because DOX accumulation in this organelle was prevented upon incubation with the established Pgp inhibitors valspodar or elacridar or by silencing Pgp expression with siRNA [20].

The present study found that MCF-7 cells treated with EGCG reduced P-gp protein expression in a dose-dependent manneras was observed by Yamagishi. However, the dramatic P-gp decrease found after EGCG treatment by immunofluorescence and western blot was not related to gene transcription down-regulation. The effects of EGCG on P-gp gene and protein expression are under discussion. It has been reported that EGCG induces a down-regulation of MDR1 in BEL7404/ADM and BEL7402/5-FU cells [21]. In addition, it has also been observed that EGCG attenuated the mRNA and protein levels of P-gp in a dose-dependent manner in the human pancreatic carcinoma cell line PANC-1 [22]. However, in KB-A1 cells, which have been shown to express P-gp at a high level on their plasma membrane. The effect of EGCG treatment showed no obvious change of MDR-1 gene expression in KB-A1 cells [8]. We speculated that EGCG does not modified the expression or function of transcriptional factors which recognize MDR-1 promoter, for instance YB-1, that in the human breast cancer cell line, MCF7, relates the nuclear localization of YB-1 to the activation of MDR1 [23]. But this hypothesis should be answer with further studies. We speculated that the transcriptional and post-transcriptional regulation of P-gp by EGCG might depend on cell line and tissue, as well as concentration. Interestingly, YB-1 was also observed in intrinsically resistant human breast tumors in which high levels of P-gp were present. This is the case for MCF-7 WT cells, which express high levels of P-gp and EGCG treatment has been shown relevant effects on P-gp protein repression and inhibition [23].

Reports showing P-gp activity modulation by flavonoids are quite numerous. Whereas some flavonoids such as kaempferide were shown to inhibit P-glycoprotein-mediated transport processes by directly interacting with the vicinal ATP- and steroid-binding sitesin *E. coli* JM109 cells, others, like (-) epicatechin from green tea, were shown to activate P-gp through a heterotropic allosteric mechanism in NIH3T3-G185 cells[24]. This effect of green tea polyphenols is controversial since Jodo in et al. showed that several of these polyphenols inhibit the photo labeling of P-gp by 75% and increase the accumulation of rhodamine123 threefold in the multidrug-resistant cell line CHR C5[25]. In present results we found an inhibitory effect of P-gpactivity in MCF-7 cells by EGCG, and this is the first study that shows that EGCG can modulate the P-gp activity in non- resistant breast cancer cells. The inhibitory effect of EGCG on P-gp was also observed in Caco-2 cells, MDCKII and T84 cells [26]. Kitagawa et al. reported that EGCG inhibits the efflux of P-gp substrates, verapamil and quercetin in KB-C2 cells [27]. However, the inhibitory effect of P-gp activity has also been associated with an increase of AUC of several drugs [28-30]. Therefore, EGCG might be a potential agent for modulating the bioavailability of P-gp substrates inthe intestine and the multidrug resistance phenotype associated with the expression of this transporter.

MCF-7 cells express MDR1 and also Breast cancer resistance protein (BCRP). A recent study in MDCK-II/BCRP cells (established line of canine kidney cells transfected with human BCRP) showed that all green tea components (EGCG included) had only marginal effect on BCRP-mediated dasatinib uptake in BCRP-expressing cells [31], suggesting that EGCG is not able to modulate the function of BCRP transporter

CONCLUSIONS

Our results suggest that EGCG could improve the efficacy of breast cancer treatment by increasing the accumulation of chemotherapeutic drugs in cancer cells by blocking P-gp function. Further studies are required to evaluate *in vivo* the effect of polyphenols from green tea or the catech in EGCG on the multidrug resistance associated with P-gp during chemotherapy for breast cancer.

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