



(–)-Epigallocatechin-3-gallate down-regulates EGFR, MMP-2, MMP-9 and EMMPRIN and inhibits the invasion of MCF-7 tamoxifen-resistant cells

Fulvia FARABEGOLI*¹, Alessio PAPI† and Marina ORLANDI†

*Department of Experimental Pathology, University of Bologna, Bologna, Italy, and †Department of Biology, University of Bologna, Bologna, Italy

Synopsis

The activation of the EGFR (epidermal growth factor receptor) signalling pathway is one of the key mechanisms underlying the development of resistance to tamoxifen in breast cancer patients. As EGCG [(–)-epigallocatechin-3-gallate], the most active catechin present in green tea, has been shown to down-regulate EGFR, we studied the effects of 10–100 µg/ml EGCG treatment on growth and invasion in a breast carcinoma cell line resistant to tamoxifen [MCF-7Tam (MCF-7 breast carcinoma cell line resistant to tamoxifen) cells] and parental MCF-7. A dose-dependent down-regulation of EGFR mRNA expression and protein level occurred after 50 µg/ml EGCG treatment of MCF-7Tam cells. EGFR molecules on the plasma membrane surface of MCF-7Tam cells significantly decreased. EGFR phosphorylation (Tyr-992, Tyr-1045 and Tyr-1068) was higher in MCF-7Tam than in MCF-7 and it was reduced by EGCG treatment. ERK (extracellular regulated kinase) and phospho-ERK p42/44 were also down-regulated by EGCG treatment and *in vitro* cell growth and invasion decreased. MMP-2 (matrix metalloproteinase-2) and MMP-9, which are implicated in cell invasion and metastasis, and EMMPRIN (extracellular matrix metalloproteinase inducer), a glycoprotein able to activate MMPs, were significantly reduced after 50 µg/ml EGCG treatment. In keeping with this, TIMP-1 (tissue inhibitor of metalloproteinases-1) and TIMP-2, which down-regulate MMPs, increased after EGCG treatment. Altogether, the present data demonstrated that EGCG could attenuate the tamoxifen-resistant phenotype of MCF-7Tam cells. EGCG could stop MCF-7Tam cell growth and *in vitro* invasion through down-regulation of EGFR and other molecules implicated in aggressive biological behaviour. The present data support the hypothesis that EGCG is an interesting molecule to be investigated in tamoxifen-resistant breast carcinoma.

Key words: breast carcinoma, tamoxifen, (–)-epigallocatechin-3-gallate (EGCG), invasion, tissue inhibitor of metalloproteinases-1 (TIMP-1), receptor tyrosine kinase

INTRODUCTION

The development of breast tumours resistant to anti-oestrogens is one of the major obstacles in the therapy of breast carcinomas. The failure of endocrine-targeted therapies can be due to the activation of novel signalling pathways that circumvent the effects of anti-oestrogens. Emerging data indicate that altered expression and/or modification of several growth factor receptors and downstream signalling molecules correlate with tamoxifen resistance: in particular, EGFR (epidermal growth factor receptor), HER2

(human epidermal growth factor receptor type 2) and IGF-1R (insulin-like growth factor-1 receptor) signalling pathways are often elevated in non-responsive tumours that exhibit either *de novo* or acquired resistance [1–3]. The activity of kinases that function downstream of these receptors such as ERK1/2 (extracellular regulated kinase 1/2), p38, PKB (protein kinase B; also called Akt) and PAK1 (p21-activated kinase-1) is also increased [4]. Altogether, these modifications are also associated with the development of a more aggressive phenotype. Many of the molecular pathways that are involved in circumventing anti-oestrogen-induced blocks in cell proliferation and survival are

Abbreviations used: DAPI, 4',6-diamidino-2-phenylindole; EGCG, (–)-epigallocatechin-3-gallate; EGFR, epidermal growth factor receptor; EMEM, Eagle's minimal essential medium; EMMPRIN, extracellular matrix metalloproteinase inducer; EMT, epithelial-to-mesenchymal transition; ER, oestrogen receptor; ERK1/2, extracellular regulated kinase 1/2; FBS, fetal bovine serum; Grb2, growth-factor-receptor-bound protein 2; HER2, human epidermal growth factor receptor type 2; FBS, fetal bovine serum; MCF-7Tam cell, MCF-7 breast carcinoma cell line resistant to tamoxifen; α -MEM, minimum essential medium α ; MMP, matrix metalloproteinase; PKB, protein kinase B; RTK, receptor tyrosine kinase; RT-PCR, reverse transcription-PCR; TBS, Tris-buffered saline; TIMP-1, tissue inhibitor of metalloproteinases-1.

¹ To whom correspondence should be addressed (email fulvia.farabegoli@unibo.it).



also implicated in increased migration and metastasis of breast cancer cells. Genotype and phenotype changes occur in the development of the tamoxifen-resistant phenotype. MCF-7 cells selected for tamoxifen resistance showed signalling perturbations associated with the EMT (epithelial-to-mesenchymal transition): elongated, fibroblast-like phenotype that lacked characteristic cell-cell contacts, enhanced basal motility and an invasive phenotype were associated with increased expression of extracellular matrix proteins, down-regulation of E-cadherin, disruption of adherens junctions complexes, accumulation of β -catenin and NF- κ B (nuclear factor κ B) in the nucleus and transcription of target genes such as *c-myc* and cyclin D1 [5,6]. Treatment of these cells with the pharmacological inhibitors gefitinib and AZD0530 inhibited migration and invasion, indicating that the invasive phenotype of these resistant cells was dependent on EGFR and c-Src respectively [5–7]. Molecules able to down-regulate one or more of these molecular pathways are therefore interesting for the aim of attenuating the malignant phenotype emerging with resistance to tamoxifen.

Many reports demonstrated that EGCG [(–)-epigallocatechin-3-gallate], the most important catechin present in green tea, could down-regulate many molecular signalling pathways, including EGFR and HER2 [8–11].

We therefore used EGCG on an MCF-7 tamoxifen-resistant cell line developed in our laboratory and we investigated the relation between EGCG and EGFR and molecules implicated in cell motility and invasion.

MATERIALS AND METHODS

Cell lines

MCF-7 cell line was obtained from A.T.C.C. (Manassas, VA, U.S.A.) and grown in E-MEM (Eagle's minimal essential medium) supplemented with 10% (v/v) FBS (fetal bovine serum), 100 units/ml penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate (all from Cambrex, Bioland, MA, U.S.A.). MCF-7Tam (MCF-7 breast carcinoma cell line resistant to tamoxifen) cell line was developed by growing MCF-7 cells in α -MEM (minimum essential medium α) without Phenol Red, supplemented with 10% charcoal-stripped FBS, 100 units/ml penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate (all from Cambrex); 10^{-7} M 4-OH-tamoxifen (Sigma-Aldrich, St. Louis, MO, U.S.A.) was added to the medium for 1 year before starting the experiments, as previously reported [12].

MTT assay

MCF-7 and MCF-7Tam cells were seeded on a 96-well microtitre plate (1×10^4 cells per well) overnight, in E-MEM (MCF-7) or in α -MEM with 10^{-7} M 4-OH-tamoxifen (MCF-7Tam). The next day, the cells were incubated with different concentrations of 10–100 μ g/ml EGCG (Sigma-Aldrich) for 24, 48 and 72 h. Cell growth experiments were conduc-

ted in triplicate. The effect of EGCG on cell growth was examined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma-Aldrich] assay. Briefly, 10 μ l of MTT solution (5 mg/ml) was added to each well to make a final volume of 110 μ l and incubated for 3 h at 37°C. The supernatant was aspirated, and the MTT-formazan crystals formed by metabolically viable cells were dissolved in 200 μ l of DMSO. After a 1 h incubation in DMSO, the attenuation (*D*) at 570 nm was detected and recorded by means of an ELISA reader (Becton Dickinson, Boston, MD, U.S.A.).

Western-blot analysis

MCF-7 and MCF-7Tam cells (40 000 cell/cm²) were plated and treated with EGCG (10–100 μ g/ml). The cells were detached and were collected by centrifugation at 300 *g* for 10 min. The pellets were resuspended in lysis buffer [20 mM Tris/HCl, pH 7.5, 0.5 mM EDTA, 0.5% Triton X-100 and 5 mM Na₃VO₄ (sodium orthovanadate)] and sonicated on ice, in the presence of protease inhibitors. Protein concentration was determined by the method of Lowry [12a]. Cell lysates (50 μ g of protein per lane) were size fractionated on SDS/7.5–12% polyacrylamide gel prior to transfer to Hybond TM-C Extra membranes (GE Healthcare) by standard protocols. Membranes were blocked for 2 h with 5% (w/v) non-fat dried skimmed milk powder in TBS (Tris-buffered saline; 0.2 M Tris/HCl, 0.5 M NaCl and 1% Tween 20, pH 7.6) at room temperature (22°C). The membranes were incubated overnight at 4°C with the following primary antibodies: anti-MMP-2 (matrix metalloproteinase-2), anti-MMP-9 or anti-TIMP-1 (tissue inhibitor of metalloproteinases-1) or anti-TIMP-2 (Santa Cruz Biotechnology), or anti-EMMPRIN (extracellular matrix metalloproteinase inducer; Zymed Labs) or anti-EGFR (Lab Vision; Fremont, CA, U.S.A.) or anti-ERK p42/44 (ERK1/2) and anti-phospho-ERK p42/44 (Cell Signaling Technology) diluted 1:500, anti-phospho-EGFR Tyr-1068, phospho-EGFR Tyr-1045 and phospho-EGFR Tyr-992 (Cell Signaling Technology) diluted 1:300. Anti-rabbit or anti-mouse peroxidase-conjugated antibodies were diluted 1:1000 in TBS containing 5% dry milk and used as secondary antibodies. The proteins were detected by luminol (GE Healthcare). Bands were quantified by using a densitometry images analysis software (Image Master VDS; Pharmacia Biotech, Uppsala, Sweden). Actin (Sigma-Aldrich) was used as a control.

Toluidine Blue staining

MCF-7 and MCF-7Tam cells were grown on a coverslip in 3.5 mm Petri dishes and fixed in 4% Formalin (Carlo Erba, Milan, Italy) for 15 min. After washing twice in PBS and once in water, the cells were incubated (10 min) with a solution of 0.5% Toluidine Blue in sodium tetraborate (1%), prepared and filtered 24 h before use. The cells were rinsed with double-distilled water several times, air-dried and mounted with mounting medium (Diapath; Martinengo, Bergamo, Italy). The samples were visualized with a Nikon (Nikon Italy, Florence, Italy) epifluorescence microscope (Eclipse E600W) equipped with a 100 kV lamp and

filters for FITC and DAPI (4',6-diamidino-2-phenylindole). Pictures were taken by a PanFluor $\times 100$ objective with 0.50–1.25 lens aperture, recorded by a Nikon digital camera (DXM 1200F) and elaborated by ATC-2U software (Nikon).

Immunofluorescence

MCF-7 and MCF-7Tam cells were grown on a coverslip in 3.5 mm Petri dishes and fixed in cold methanol (4°C) for 10 min. The mouse monoclonal anti-EGFR antibodies Ab-12 at 1:800 (for both the extracellular and the cytoplasmic domain) and Ab-5 at 1:500 (specific for the extracellular domain; both from Lab Vision) were used. Anti-mouse FITC-conjugated secondary antibody (Sigma) was added 1:800 to the cells for 1 h in the dark at 37°C. Nuclei were counterstained by DAPI (200 mg/ml) dissolved 1:500 in DABCO (1,4-diazadicyclo[2.2.2]octane; both from Sigma–Aldrich) and added to the samples. The edges were sealed by rubber cement and the samples were kept in the dark. Samples were visualized with a Nikon (Nikon Italy) epi-fluorescence microscope (Eclipse E600W) equipped with a 100 kV lamp and filters for FITC and DAPI. Pictures were taken by a PanFluor $\times 60$ objective with 0.50–1.25 lens aperture, recorded by a Nikon digital camera (DXM 1200F) and elaborated by ATC-2U software.

FACS

Mouse monoclonal EGFR Ab-5 antibody (Lab Vision) specific for the extracellular domain was used to detect and estimate the EGFR molecules present on the plasma membrane of control and EGCG-treated MCF-7Tam cells. Cells were plated on 3.5 mm Petri dishes. The antibody was dissolved in sterile PBS (1:800). *In vivo* incubation (1 h) was followed by two washings in PBS and fixation in 1% Formalin in PBS for 15 min. Then, the samples were incubated with the secondary antibody (1:800 anti-mouse FITC-conjugated) (Sigma) for 30 min at 37°C. After washing in PBS the cells were evaluated by a FACSCalibur™ (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

Zymography

MMP-2 and MMP-9 activities of MCF-7Tam and MCF-7 cells were determined by gelatin zymography. Cells were seeded: after 18 h they were washed with PBS and replaced in serum-free medium (α -MEM, control) or serum-free α -MEM medium with EGCG for 24 h. The cells were centrifuged (300 g for 10 min) and the protein concentration was determined. Then, 10 μ g of proteins per lane was added to the sample buffer [1 M; Tris/HCl, pH 6.8, 2% SDS and 10% (v/v) glycerol] and the buffer was loaded on to an SDS/10% polyacrylamide gel containing 1 mg/ml gelatin (Sigma). After electrophoresis, SDS was removed from the gel by washing twice with 2.5% Triton X-100 for 1 h. After a brief rinse, the gel was incubated at 37°C for 18 h in a buffer (pH 7.6) containing 100 mM Tris/HCl, 10 mM CaCl₂ and 20 mM NaCl. The gel was stained with 1% Coomassie Brilliant Blue R250 for 2 h and then treated with destaining solution (40% methanol, 10% acetic acid and 50% distilled water). The MMP

activities, indicated by clear bands of gelatin digestion on a blue background, were quantified by using a densitometry images analysis software (Image Master VDS).

Invasion assay

MCF-7Tam and MCF-7 cell invasion was determined using a Boyden chamber (NTG, Milan, Italy) with a polycarbonate membrane of 8.0 μ m pore size (NTG). The filter was coated with 12.5 μ g of Matrigel (Sigma–Aldrich). Control and 24 h EGCG-treated cells were seeded on to the upper part of each chamber (4×10^5 cell per well in 800 μ l of serum-free α -MEM medium). Chemoattractant (200 μ l of MEM with 10% FBS) was added to the lower part of each chamber. After incubation for 12 h at 37°C, non-migratory cells on the upper surface of the filter were wiped with a cotton swab, whereas the migrated cells on the lower surface of the filter were fixed and stained with Toluidine Blue (Sigma–Aldrich). The number of invasive cells on the lower surface of the membrane was counted in five random fields. The total numbers of migrating cells under control and EGCG treatment conditions were statistically compared.

RNA extraction and RT-PCR (reverse transcription-PCR)

RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform method of Chomczynski and Sacchi [13] with some modifications. RT-PCR reaction was performed using the cMaster RT plus PCR system kit according to the supplier's instructions (Eppendorf, Hamburg, Germany). The following primers were used: EGFR forward, 5'-CTCACGCAGTTGGGCACTTT-3', reverse, 5'-TCATGGGCAGCTCCTTCAGT-3'; β -actin was used as an internal control; the sequences of the primers were: forward, 5'-GGCATCGTGATGGACTCCG-3', reverse, 5'-GCTGGAAGGTGGACAGCGA-3'. The annealing temperature was 58°C. The β -actin primers were put together with EGFR primers into the same tube.

Statistical analysis

Experiments were conducted three times unless otherwise specified. Data have been expressed as means and S.D. (\pm S.E.M.). Statistically significant differences in the means were assessed by ANOVA followed by the Bonferroni's multiple comparison test or the two-tail Student's *t* test, as appropriate, using GraphPad Prism 5.1 statistical software package. The level for accepted statistical significance was $P < 0.05$.

RESULTS

EGFR expression in MCF-7 and MCF-7Tam cells

MCF-7 cells were grown in a medium deprived of oestrogens and Phenol Red and in the presence of 10^{-7} M 4-OH-tamoxifen

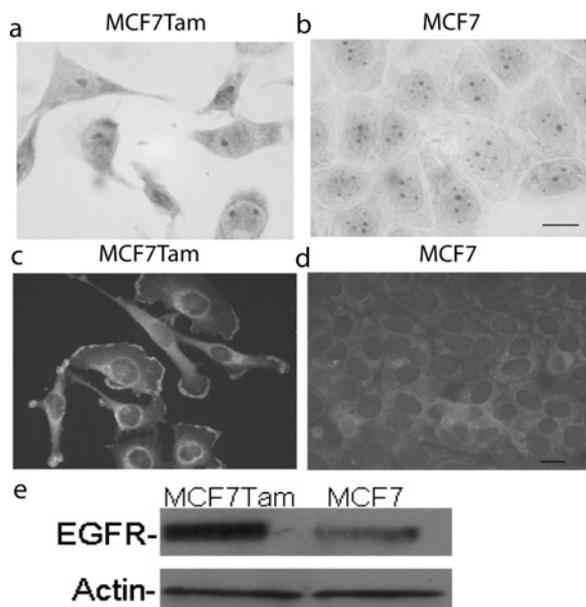


Figure 1 MCF-7-Tam and MCF-7 cells showed different morphology and different levels of EGFR expression

The cells were cultured on coverslips, fixed in 4% formalin, stained with Toluidine Blue (0.1%) and observed by a Nikon optical microscope with a $\times 100$ objective. MCF-7Tam cells (a) showed a fibroblast-like morphology, whereas MCF-7 cells (b) presented a roundish shape and very tight cell–cell junctions. Scale bar, 30 μm . (c, d) Immunofluorescence detection of EGFR in MCF-7Tam and MCF-7 cells. The cells, cultured on coverslips, were fixed in cold methanol and incubated with a monoclonal antibody against EGFR followed by an anti-mouse FITC-conjugated antibody. The samples were analysed by a Nikon optical microscope equipped with a $\times 60$ objective. Pictures were taken by using the same exposition time, in order to assure that the different fluorescence intensity was only due to the intrinsic characteristics of the samples. Scale bar, 30 μm . EGFR expression was scarce in MCF-7 cells (d) and restricted to the cytoplasm. In contrast, MCF-7Tam cells showed bright EGFR staining (c) in both the cytoplasm and plasma membrane. (e) Western-blot analysis of MCF-7Tam and MCF-7 cells. Western-blot analysis showed a fainter and thinner band in MCF-7 cells than in MCF-7Tam cells.

for 1 year before starting the experiments, as previously reported [12]. Tamoxifen treatment induced a strong selection of MCF-7 cells: MCF-7 cells sensitive to tamoxifen treatment underwent cell death, whereas a part of the MCF-7 cells became resistant and acquired the ability to grow and duplicate in the presence of the drug. We named these cells MCF-7Tam cells. Together with the ability to proliferate and grow in the presence of tamoxifen, MCF-7Tam cells also displayed an altered morphology reminiscent of an EMT, as already described [6–8]. As shown in Figure 1(b), MCF-7 cells grew in a monolayer with tight cell–cell contact and they gave rise to roundish colonies. In contrast, MCF-7Tam cells lacked cell–cell contact. They showed an elongated, fibroblast-like phenotype (Figure 1a) and ruffled membranes observed in actively spreading cells.

EGFR expression was poorly detectable in the cytoplasm of MCF-7 cells by immunofluorescence (Figure 1d), whereas bright EGFR immunostaining was present in both the cyto-

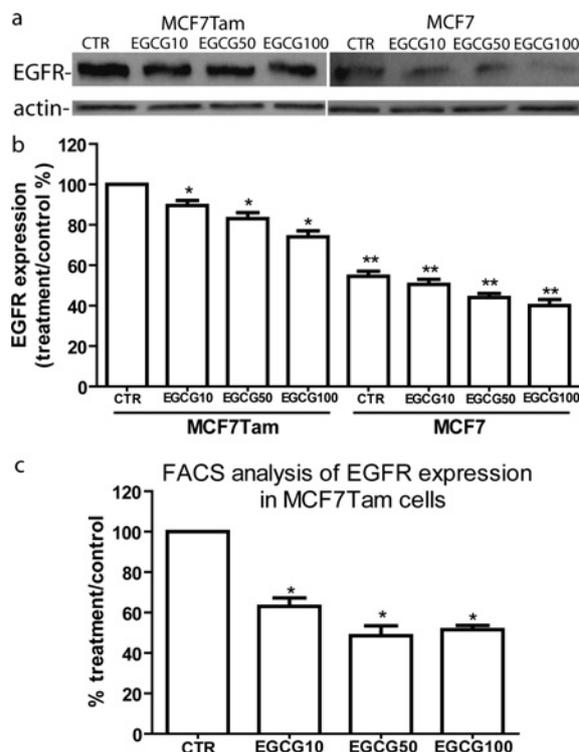


Figure 2 Western-blot and FACS analyses of EGFR expression in MCF-7Tam and MCF-7 cells treated with EGCG

EGFR protein level was assessed by Western blot in cell lysate of MCF-7Tam and MCF-7 cells treated with 10–100 $\mu\text{g}/\text{ml}$ EGCG for 24 h (a). Densitometry data are expressed as percentage of treated samples with respect to the control (CTR) (b). EGFR expression in MCF-7Tam cells was assumed as 100% with respect to all the other samples, including untreated MCF-7 cells, in order to compare the different levels of EGFR expression in the two cell lines and the corresponding decrease after EGCG treatment. EGCG reduced EGFR in both MCF-7Tam and MCF-7 cells. The means with S.E.M. are shown. * $P < 0.05$, ** $P < 0.01$ indicate significant difference with respect to the control. FACS analysis of EGFR expression in MCF-7Tam cells (c). MCF-7Tam cells were treated with 10–100 $\mu\text{g}/\text{ml}$ EGCG for 24 h. Mouse anti-EGFR external domain monoclonal antibody (1:800) was used on control (CTR) and EGCG-treated cells and detected by a secondary FITC-conjugated anti-mouse antibody (1:800). FACS analysis detected an EGCG dose-dependent decrease in EGFR expression. The means with S.D. are shown. * $P < 0.05$, ** $P < 0.01$ indicate significant difference with respect to the control.

plasm and the plasma membrane of MCF-7Tam cells (Figure 1c). With respect to MCF-7, MCF-7Tam cells expressed EGFR to a high extent, as also demonstrated by Western-blot analysis (Figure 1e).

When EGCG was given to MCF-7 and MCF-7Tam cells, a gradual decrease in EGFR was found by Western blotting in both groups of samples (Figures 2a and 2b). FACS analysis of the cell-surface-associated EGFR in MCF-7Tam cells showed a dose-dependent decrease, statistically significant after 10 $\mu\text{g}/\text{ml}$ EGCG treatment for 24 h (Figure 2c). EGCG treatment also suppressed the activity of the EGFR signalling pathway leading to cell proliferation, as demonstrated by decreased expression of phospho-EGFR Tyr-992 and Tyr-1068 (see Supplementary Figure

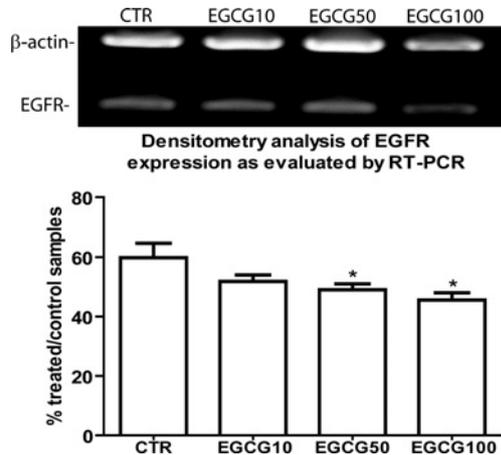


Figure 3 RT-PCR analysis of EGFR expression in MCF-7Tam cells treated with 10–100 µg/ml EGCG for 24 h

One-step RT-PCR was performed on RNA extracted from control and treated MCF-7Tam cells. PCR product was loaded on to a 1% agarose gel stained with ethidium bromide. Densitometry analysis was reported as the β -actin/EGFR ratio of treated samples with respect to control samples. A dose-dependent decrease in EGFR expression was detectable and it was statistically significant after 50 µg/ml EGCG treatment for 24 h. * $P < 0.05$ indicates significant difference with respect to the control.

S1 at <http://www.bioscirep.org/bsr/031/bsr0310099add.htm>). Phospho-EGFR Tyr-1045, which is involved in the direct interaction of Grb2 (growth-factor-receptor-bound protein 2)-mediated Cbl-induced ubiquitination of EGFR and subsequent endocytosis, showed a modest increase after 10 and 50 µg/ml EGCG treatment and decreased after 100 µg/ml EGCG treatment (Supplementary Figure S1). Loss of EGFR after EGCG treatment found by Western blotting and FACS was also mirrored by RT-PCR: dose-dependent down-regulation of EGFR transcription occurred in MCF-7Tam cells. EGFR gene transcription decrease was statistically significant after 50 µg/ml EGCG treatment for 24 h (Figure 3). In MCF-7 cells, EGFR down-regulation was also detectable, although to a lesser extent than in MCF-7Tam cells (results not shown).

ERK p42/44 activity, viability and invasion in MCF-7 and MCF-7Tam cells

EGFR represents one of the most important signalling pathways able to induce survival and proliferation in the course of treatment with tamoxifen and in many other human cancers. The ERK and PI3K (phosphoinositide 3-kinase)–PKB signalling pathways function downstream of EGFR: the continuous activity of the EGFR is essential for the phosphorylation of the ERK1/2 downstream signalling molecules, which have been shown to be essential for cell proliferation. We investigated whether the EGFR signalling pathway was active in both MCF-7Tam and MCF-7 cells by ERK and phospho-ERK p42/44 analysis. We also asked whether EGCG treatment was able to down-regulate ERK and phospho-ERK molecules and consequently reduce

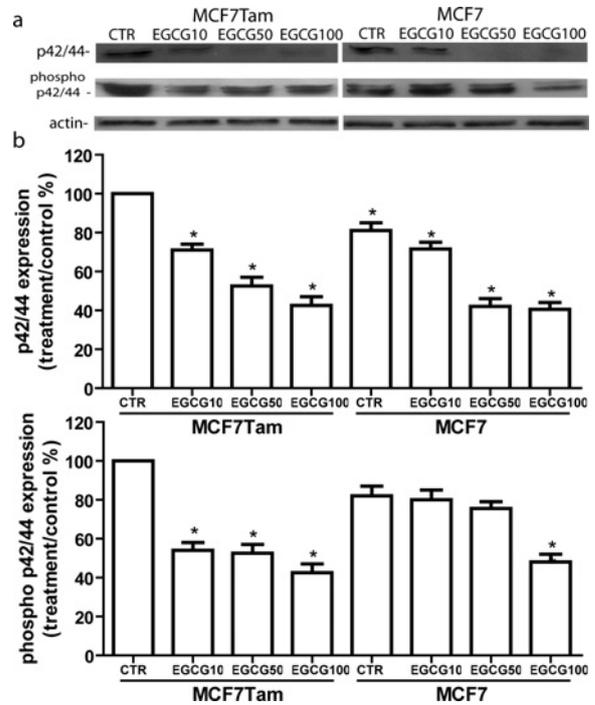


Figure 4 ERK and phospho-ERK p42/44 Western-blot analysis in MCF-7Tam and MCF-7 cells after EGCG treatment for 24 h

ERK p42/44 expression in control (CTR) and EGCG-treated MCF-7Tam and MCF-7 cells (a). The results are expressed as percentage of treated samples with respect to control samples. ERK p42/44 and phospho-ERK p42/44 expression in MCF-7Tam control cells was assumed as 100% with respect to all the other samples, including untreated MCF-7 cells, in order to compare the different levels of expression in the two cell lines and the corresponding relative decrease after EGCG treatment. Phospho-ERK p42/44 expression in control (CTR) and EGCG-treated MCF-7Tam and MCF-7 cells (b). EGCG treatment was significantly more effective in MCF-7Tam cells than in MCF-7 cells. The means with S.E.M. are shown. * $P < 0.05$ indicates significant difference with respect to the control.

cell proliferation. ERK p42/44 expression was higher in MCF-7Tam than in MCF-7 cells (Figure 4a) and EGCG treatment decreased ERK p42/44 expression to a similar level in the two cell lines. In contrast, the effect of EGCG treatment was remarkably greater in MCF-7Tam cells than in MCF-7 cells (Figure 4b). In fact, phospho-ERK p42/44 expression decreased significantly in MCF-7Tam cells after 10 µg/ml EGCG treatment for 24 h, whereas in MCF-7 cells, only 100 µg/ml EGCG treatment was significantly effective.

Accordingly, loss of EGFR expression and ERK-phospho-ERK p42/44 down-regulation were associated with reduced cell growth. MCF-7 and MCF-7Tam cells were treated with various concentrations of EGCG for 24, 48 and 72 h. At the end of the treatment, cells were subjected to an MTT assay to determine the cell viability. As shown in Figures 5(a) and 5(b), MCF-7Tam cell treatment with high EGCG concentrations (50 and 100 µg/ml) caused a remarkable cytotoxic effect, which developed earlier and was greater in MCF-7Tam cells than in MCF-7 cells. After 50 µg/ml EGCG treatment for 72 h, MCF-7Tam cell growth was

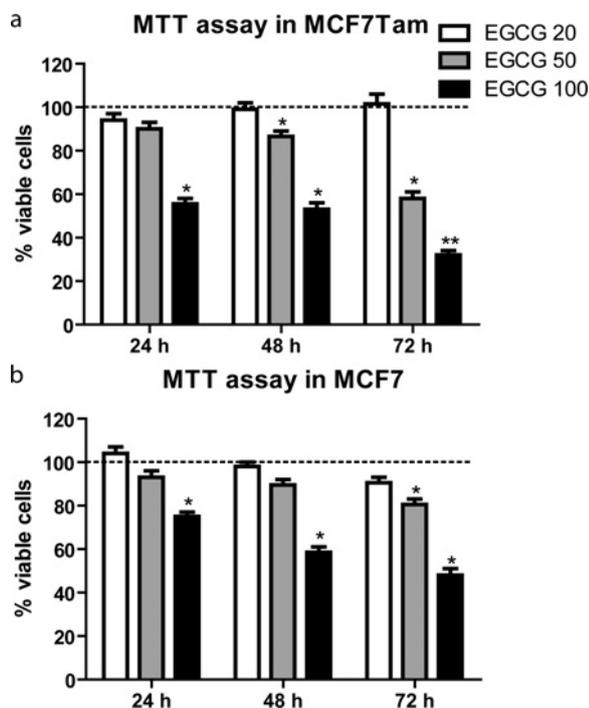


Figure 5 MTT assay in MCF-7Tam and MCF-7 cells treated with 20–100 µg/ml EGCG for 24, 48 and 72 h
Dose-dependent cytotoxic effect after EGCG treatment developed earlier and, at high dose, was greater in MCF-7Tam cells than in MCF-7 cells. Data are expressed as percentage of treated samples with respect to the control (CTR). * $P < 0.05$, ** $P < 0.01$ indicate significant difference with respect to the control.

reduced to 60% with respect to control cells and a further decrease in cell growth to 33% was found after 100 µg/ml EGCG treatment for 72 h. MCF-7 cell growth was inhibited to a lesser extent after EGCG treatment (Figure 5b): in fact, 50 µg/ml EGCG treatment for 72 h reduced MCF-7 cell growth by 84%. A further inhibitory effect on cell growth was detected after 100 µg/ml EGCG treatment (49%).

A cell invasion assay was performed by incubating MCF-7Tam and MCF-7 cells with EGCG at various concentrations for 24 h. We observed a dose-dependent reduction of invasive cells at a minimum of 10 µg/ml EGCG treatment. The inhibition of migration was statistically significant at the concentration of 50 µg/ml EGCG, whereas 100 µg/ml EGCG treatment reduced MCF-7Tam cell invasion by more than 50% (Figures 6a and 6b). In contrast, MCF-7 cells showed little cell motility, presumably due to MCF-7 strong cell–cell contact.

MMP-2, MMP-9, TIMP and EMMPRIN expressions in MCF-7 and MCF-7Tam cells

We hypothesized that the invasion inhibitory effect of EGCG might be due to the suppression of MMP-2 and MMP-9 activities, two molecules involved in a wide range of proteolytic events,

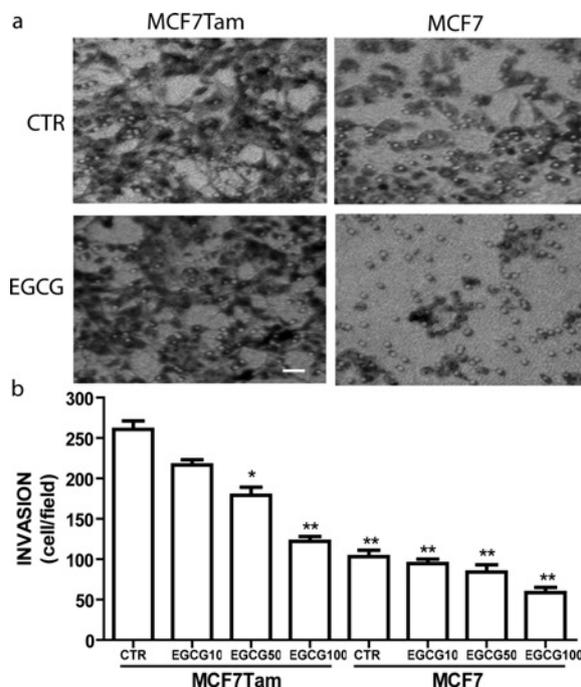


Figure 6 Cell invasion assay in MCF-7Tam and MCF-7 cells
Effect of 10–100 µg/ml EGCG treatment on matrigel invasion of MCF-7Tam and MCF-7 cells (a) as detected by using a Boyden chamber. After 12 h migration through the matrigel-coated membranes, the cells were fixed, stained, photographed and counted under light microscopy. MCF-7 cells showed a limited attitude to invasion with respect to MCF-7Tam cells. The effect of 100 µg/ml EGCG treatment on MCF-7Tam and MCF-7 cells is shown (a). Data are expressed as the number of cells for a field for treatment (b). The means (\pm S.E.M.) for three independent experiments are shown. Scale bar, 100 µm. * $P < 0.05$, ** $P < 0.01$.

including tumour growth, tumour cell migration and metastasis. MMP-2 and MMP-9 were less expressed in MCF-7 cells than in MCF-7Tam cells (Figure 7a). We analysed MMP-2 and MMP-9 expression by Western blotting in control and EGCG-treated MCF-7Tam and MCF-7 cells and we found that EGCG inhibited MMP-2 and MMP-9 expression in a dose-dependent manner in both the cell lines. As shown in Figures 7(a) and 7(b), the inhibitory effect of EGCG on MMP-2 and MMP-9 expression was statistically significant after 50 µg/ml administration. The zymography analysis of MMP-2 and MMP-9 activities in MCF-7Tam and MCF-7 cells treated with 50 and 100 µg/ml EGCG for 24 h clearly showed a dose-dependent decreased activity (Figure 7c). MMP-2 and MMP-9 decreased activity was statistically significant after 50 µg/ml EGCG treatment for 24 h.

MMP activity is controlled by endogenous inhibitors, the TIMPs. Changes in TIMP levels directly affect MMP activity since they have the ability to form tight 1:1 complexes with the active MMP enzymes. Particularly, TIMP-1 regulates MMP-9, and TIMP-2 regulates MMP-2. Therefore we evaluated TIMP-1 and TIMP2 expression after EGCG treatment (Figure 8a). A significant dose-dependent TIMPs increase was detected only in MCF-7Tam cells. TIMP-1 and TIMP-2

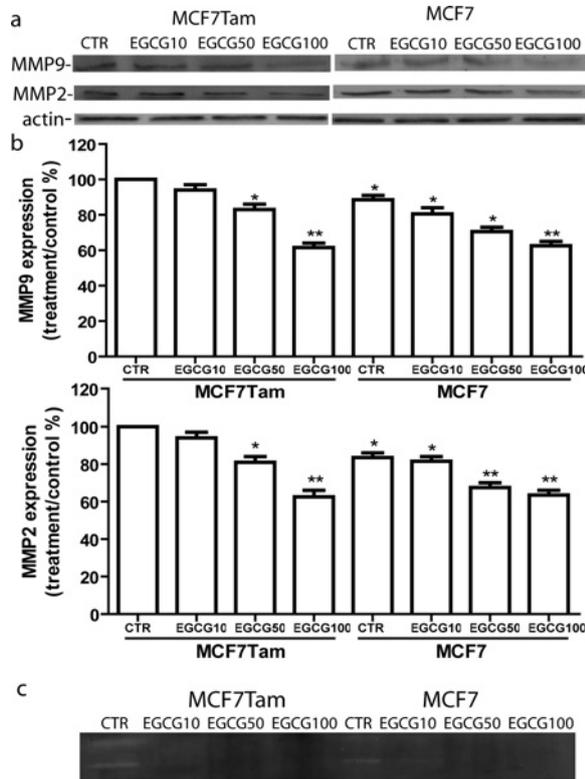


Figure 7 Effect of 10–100 µg/ml EGCG treatment on MMP-9 and MMP-2 protein levels as assessed by Western-blot and zymography analyses

Cell lysates of MCF-7Tam and MCF-7 cells were used for Western-blot analysis after 10, 50 and 100 µg/ml EGCG treatment. MMPs decrease occurred to a comparable extent in the two cell lines. Densitometry data (b) are expressed as percentage of treated samples with respect to control samples. MMPs expression in MCF-7Tam control cells was assumed as 100% with respect to all the other samples, including untreated MCF-7 cells, in order to compare the different levels of expression in the two cell lines and the corresponding relative decrease after EGCG treatment. The means with S.E.M. are shown. * $P < 0.05$, ** $P < 0.01$ indicate significant difference with respect to the control. The effect of 10–100 µg/ml EGCG treatment for 24 h on MMP9 and MMP2 activity was evaluated by zymography in MCF7Tam and MCF-7 cells (c). The gelatin zymogram showed the MMP9 (83 kDa) and MMP2 (62 kDa) form in serum-free conditioned media. After 50 and 100 µg/ml EGCG treatment the bands were not detectable.

expression increased in MCF-7Tam cells after 50 µg/ml EGCG treatment and 100 µg/ml EGCG treatment respectively. In contrast, EMMPRIN, a transmembrane glycoprotein that is highly expressed on the surface of malignant tumour cells and that induces production of MMPs, was highly expressed in MCF-7Tam cells and was significantly reduced after EGCG treatment (Figure 9b); MCF-7Tam cells expressed EMMPRIN to a large extent with respect to MCF-7 cells, but after 100 µg/ml EGCG treatment for 24 h, EMMPRIN was reduced to a level comparable with MCF-7 cells (Figure 9b). In MCF-7 cells, EGCG treatment at the highest dose (100 µg/ml) scarcely reduced EMMPRIN expression.

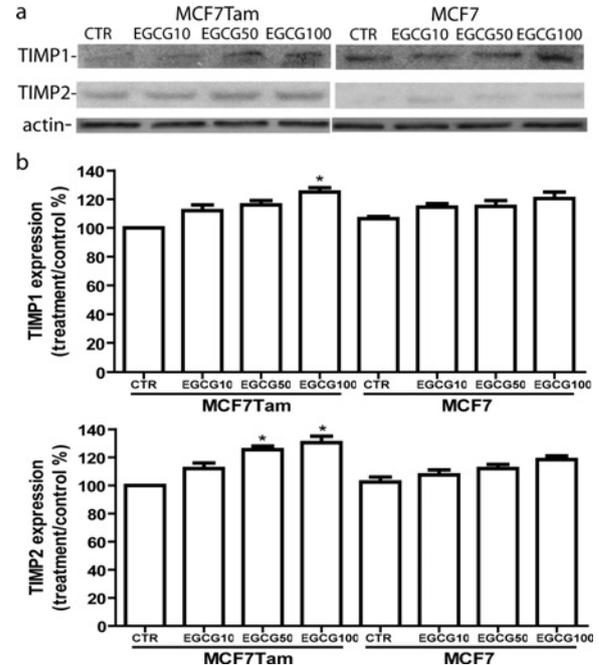


Figure 8 Effect of 10–100 µg/ml EGCG treatment for 24 h on TIMP-1 and TIMP-2 protein levels, as assessed by Western-blot analysis in cell lysate of MCF7Tam and MCF-7 cells

(a) TIMPs increase was only statistically significant in MCF-7Tam cells. Densitometry data are expressed as percentage of treated samples with respect to the control (CTR) (b). TIMPs expression in MCF-7Tam control cells was assumed as 100% with respect to all the other samples, including untreated MCF-7 cells, in order to compare the different levels of expression in the two cell lines and the corresponding relative decrease after EGCG treatment. The means with S.E.M. are shown. * $P < 0.05$ indicates significant difference with respect to the control.

DISCUSSION

The present results represent the first report about the effects of EGCG on a breast carcinoma cell line resistant to tamoxifen; we developed and used this cell line as a model to better understand the molecular mechanisms underlying the EGCG effects. In the present study, MCF-7Tam cells were established by long-term exposure of oestrogen-dependent MCF-7 cells to tamoxifen [12]. In agreement with other reports, MCF-7Tam cells displayed elevated levels of EGFR, increased motility and a fibroblast-like phenotype [6,7]. Previously, we have also demonstrated that pS2 transcription, which is considered a good indicator of an active oestrogen signalling pathway, was down-regulated in our MCF-7Tam cells [12].

The development of tamoxifen-resistant tumours is one of the major obstacles in the endocrine therapy of breast carcinoma. A lot of data support the hypothesis that it occurs via activation of novel signalling pathways that circumvent the effect of anti-oestrogens. We found that the EGFR signalling pathway was activated to a greater extent in MCF-7Tam cells

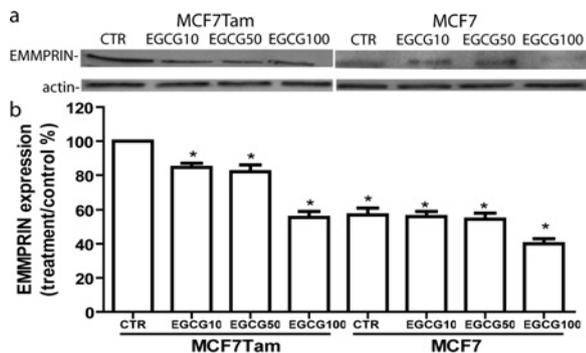


Figure 9 Effect of 10–100 $\mu\text{g/ml}$ EGCG treatment for 24 h on EMMPRIN protein levels, as assessed by Western-blot analysis in MCF-7Tam and MCF-7 cells

MCF-7Tam cells showed a greater amount of EMMPRIN than MCF-7 cells (a). A significant decrease in EMMPRIN expression was found after a 24 h EGCG treatment (b). Densitometry data are expressed as percentage of treated samples with respect to the control (CTR). EMMPRIN expression in MCF-7Tam control cells was assumed as 100% with respect to all the other samples, including untreated MCF-7 cells, in order to compare the different levels of expression in the two cell lines and the corresponding relative decrease after EGCG treatment. The means with S.E.M. are shown. * $P < 0.05$ indicates significant difference with respect to the control.

than in MCF-7 cells and EGCG was able to down-regulate it. Particularly, phosphorylated Tyr-992 was undetectable in MCF-7 cells, as already reported [14], whereas it was highly expressed in MCF-7Tam cells, together with increased phosphorylation of Tyr-1068: both were reduced by EGCG treatment. Tyr-992 phosphorylation results in the activation of PLC γ (phospholipase C γ)-mediated downstream signalling [15] and phosphorylated Tyr-1068 binds Grb2, a crucial molecule for the EGF-induced Ras/MAPK (mitogen-activated protein kinase) signalling pathway, able to induce breast cancer cell motility and invasion [16]. On the basis of these results we considered that the increased sensitivity of MCF-7Tam cells with respect to MCF-7 cells to EGCG treatment could be due to not only the down-regulation of the ER (oestrogen receptor) signalling pathway that occurred after tamoxifen treatment, but also the activation of alternative signalling pathways that made MCF-7Tam cells able to survive even in the presence of the drug.

These findings support the hypothesis that in the absence of the ER signalling pathway, EGFR might be one of the driving molecules for MCF-7Tam cells survival and proliferation and that EGCG was able to down-regulate it. Cell proliferation and cell motility were inhibited, and the activity of molecules strongly implicated in local invasion and metastasis (MMP-2, MMP-9, EMMPRIN and TIMPS) was modulated.

The effect of EGCG and green tea catechins on EGFR has already been described in many *in vitro* and *in vivo* models [8,9,17]. Emerging data indicate that EGCG exerts anticancer effects, at least in part, through inhibition of activation of the specific RTKs (receptor tyrosine kinases) [10]. EGCG seemed to alter the lipid rafts and to inhibit the homo- and heterodimerization of EGFR, inactivating the signalling pathway [18].

As a consequence, the EGFR is internalized and degraded and the signalling pathway is interrupted. In keeping with these studies, we also found a significant and dose-dependent loss of EGFR on the plasma membrane after EGCG treatment. Low-dose EGCG treatment increased the phosphorylation of Tyr-1045, directing EGFR to lysosomal degradation, therefore reducing the quantity of EGFR present on the plasma membrane [19].

The alteration of the lipid rafts may also explain why EGCG can inhibit the activation of other membrane-associated RTKs: this ability might play a critical role in the anticancer effects of green tea catechins. In contrast, several studies supported the hypothesis that EGCG can directly inhibit the tyrosine kinase activity of EGFR [20] and there is evidence that EGCG can directly target downstream intracellular signalling molecules. The numerous molecular targets of the green tea catechins have been the subject of various reviews [20,21] but, taken together, the numerous studies in the literature suggest that the antitumour effects of EGCG and green tea catechins may be due to binding to numerous and various cell targets. This characteristic is not a limit but an advantage, conferring on this molecule potential utility towards different types of cancer [22].

In the present study, we also found that EGCG was effective in inhibiting the *in vitro* invasion of MCF-7Tam cells and that this event was related to down-regulation of MMPs and EMMPRIN, molecules implicated in local invasion and metastasis. The relation between down-regulation of EGFR and MMPs inhibition has been demonstrated in oral squamous cell carcinoma after ZD1839 (Iressa) treatment, which selectively inhibited the EGFR tyrosine kinase activity. Down-regulation of EGFR was associated with a reduction of MMP-2 and MMP-9 activities [23]. Evidence that linked EGFR and MMP-2 and -9 regulation was provided for thyroid carcinoma [24], glioma [25], head and neck squamous cell carcinomas [26] and ovarian cancer [27]. In association with MMPs and the PAR (plasminogen activator receptor), EGFR contributes to form structures that mediate migration through the basal membrane. We cannot exclude that the effect of EGCG on MMPs could also be direct, as suggested by many studies on different human cell lines and animals [28–31].

The decreased *in vitro* matrigel invasion after EGCG treatment was associated with MMP-2 and -9 reduced expression. As already demonstrated, EGCG inhibits MMPs activity, leading to accumulation of non-activated MMP-2 at the cell surface [31]. In the present study, we observed increased expression of both TIMPs, but the effect on TIMP-2 was significant after 50 $\mu\text{g/ml}$ EGCG treatment for 24 h, whereas the increase in TIMP-1 was significant after 100 $\mu\text{g/ml}$ EGCG treatment. These findings contribute toward explaining the impaired *in vitro* invasion after EGCG treatment. Pro-MMP-2 activation needs TIMP-1 and -2 contributions. The active forms of MMPs subsequently activate the remaining pro-MMPs. Inactivation of the physiological function of MMPs, or even pro-MMPs, is accomplished by non-covalent TIMP binding [32]. Besides growth factors, such as Her2/neu, cytokines and hormones, MMP-2 expression is also dependent on EMMPRIN. In the present study we found that EMMPRIN expression was very high in MCF-7Tam with respect to MCF-7 cells, and we found that EGCG could reduce

EMMPRIN expression to a level present in MCF-7 cells. Then, the final inhibitory effect on MMP-2 and -9 activity was due to both TIMP-1 and -2 up-regulation and EMMPRIN down-regulation. Blocking MMP-2 secretion and activation during breast carcinoma development may decrease metastasis: a low level of MMP-2 is linked to a favourable prognosis in patients with hormone receptor-negative tumours, usually associated with high risk [33]. Our results contribute to showing that the anticancer effect of EGCG occurred via multiple cellular and molecular mechanisms and they also support the hypothesis that EGCG may be useful in inhibiting and/or preventing metastasis.

In the present study, we have also investigated the effect of EGCG on MCF-7 cells, the parental cell line used to develop the MCF-7Tam cells. EGFR, ERK and phospho-ERK p42/44 expression was higher in MCF-7Tam cells than in MCF-7 cells, as well as cell migration and EMMPRIN expression. Differences concerning the expression of MMPs and TIMPs were less pronounced in the two cell lines. EGCG treatment was demonstrated to be effective in both cell lines, but the most significant effects were detected in the MCF-7Tam cell line. On the basis of these findings, we remark that EGCG was able to attenuate a certain number of cell features acquired during the development of tamoxifen resistance.

So far, no study has been published about the effect of down-regulation of EGFR in a breast carcinoma cell line resistant to tamoxifen, but a potent cytotoxic effect was observed when green tea and tamoxifen were combined on MCF-7, ZR75 and T47D human breast cancer cells *in vitro* and *in vivo* [34]. Interestingly, the combination of tamoxifen and EGCG increased the toxicity in MDA-MB-231 cells, a breast carcinoma cell line that lacks ER α , and this effect was obtained by down-regulating EGFR and mTOR (mammalian target of rapamycin) signalling pathways [35,36]. As a complement of the aforementioned results about the effects of EGCG and tamoxifen, the present data support that EGCG can be effective in down-regulating the expression of some of the most implicated molecules that confer increased malignancy on tamoxifen-resistant breast carcinoma cells. EGCG might be a valuable molecule to associate with tamoxifen even in the case of resistant disease. As demonstrated by Zhang et al. [37], the combined treatment of squamous cell carcinoma of the head and neck with EGCG and the EGFR-TKI (EGFR-tyrosine kinase inhibitor) erlotinib revealed a synergistic antitumour effect. As green tea is cheap, safe and healthy, its use ought to be encouraged and better investigated: EGCG might be a useful molecule in preventing the drug-resistance development and in attenuating the drug-resistant phenotype.

The effects of EGCG described in the present study and in many other studies were obtained by treating cells with relatively high EGCG concentrations, which could hardly be reached by single dose administration in humans. Studies on EGCG bioavailability reported a plasma EGCG concentration of up to 4.4–7.4 $\mu\text{mol/l}$: instability of EGCG at the intestinal pH of 8.5, the balance of transporters involved in EGCG influx and efflux and the phase II enzymes involved in its transformation are factors that would contribute to its low bioavailability [38,39]. To overcome the limits of bioavailability of EGCG, or the potentially

toxic effects at high doses, Siddiqui et al. [40] recently developed a nanoparticle-mediated delivery technique that enhanced the bioavailability of EGCG and might limit any unwanted toxicity. Moreover, we cannot deny the obvious fact that the low EGCG concentration after oral administration was demonstrated to be effective in inhibiting or delaying cancer development in rodents [41]. Finally, we cannot exclude that the combinations of various phytochemicals, or phytochemicals and anticancer drugs at low doses, which might exert synergistic anticancer effects, might be more effective and less toxic.

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