Tamoxifen and epigallocatechin gallate are synergistically cytotoxic to MDA-MB-231 human breast cancer cells

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High concentrations of specific catechins [epigallocatechin gallate (EGCG), epigallocatechin (EGC) and epicatechin gallate (ECG)] inhibit the proliferation of many different cancer cell lines. The aim of this work was to determine if low concentrations of catechins with and without 4-hydroxytamoxifen (4-OHT) co-treatment would cause significant cytotoxicity in estrogen receptor-positive ($ER\alpha^+$) and -negative ($ER\alpha^-$) human breast cancer cells. Therefore, MCF-7, T47D, MDA-MB-231 and HS578T cells were incubated with EGCG, EGC or ECG (5–25 μM) individually and in combination with 4-OHT for 7 days. Cell number was determined by the sulforhodamine B cell proliferation assay. As single agents, none of the catechins were cytotoxic to T47D cells, while only EGCG (20 μM) elicited cytotoxicity in MCF-7 cells. Additionally, no benefit was gained by combination treatment with 4-OHT. $ER\alpha^-$ human breast cancer cells were more susceptible as all three catechins were significantly cytotoxic to HS578T cells at concentrations of 10 μM. In this cell line, combination with 4-OHT did not increase cytotoxicity. However, the most striking results were produced in MDA-MB-231 cells. In this cell line, EGCG (25 μM) produced a greater cytotoxic effect than 4-OHT (1 μM) and the combination of the two resulted in synergistic cytotoxicity. In conclusion, low concentrations of catechins are cytotoxic to $ER\alpha^-$ human breast cancer cells, and the combination of EGCG and 4-OHT elicits synergistic cytotoxicity in MDA-MB-231 cells. *Anti-Cancer Drugs* 15:889–897 © 2004 Lippincott Williams & Wilkins.

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Introduction

Tamoxifen is a non-steroidal selective estrogen receptor modulator (SERM) that is commonly used to treat estrogen receptor-positive ($ER\alpha^+$) breast cancer [1] and is the first drug shown to reduce the incidence of breast cancer in high-risk women [2]. However, tamoxifen also produces numerous side-effects, such as an increased risk of uterine and endometrial cancer [1], liver damage and agranulocytosis [3], hemolytic anemia [3], and erythrocyte hemolysis [4]. Additionally, resistance to tamoxifen therapy can develop following chronic treatment [5,6]. Therefore, new approaches to breast cancer treatment continue to be explored, including the search for both new breast cancer drugs and new combination therapies with tamoxifen.

The development of a combination therapy that increases the efficacy of tamoxifen has been investigated by several studies. For example, the combination of tamoxifen and docetaxel synergistically inhibited the growth of three estrogen receptor-negative ($ER\alpha^-$) cancer cell lines (MDA-MB-231, CEM-VBLr and MCF-7ADr) [7]. Similarly, Shen *et al.* [8] demonstrated synergistic cytotoxicity when MDA-MB-435 cells were treated with tamoxifen and genistein. Synergism has also been reported *in vivo*, as complete inhibition of DMBA-induced mammary tumors in rats was achieved following treatment with both tamoxifen and 6-MCDF, an aryl hydrocarbon receptor antagonist [9]. Therefore, the use of tamoxifen in a combination therapy is an expanding research area that warrants further investigation.

One class of chemicals that has shown anticancer activity in breast cancer models are the polyphenolic catechins [i.e. epigallocatechin gallate (EGCG), epigallocatechin (EGC) and epicatechin gallate (ECG)]. These phytochemicals are cytotoxic toward both $ER\alpha^+$ and $ER\alpha^-$ breast cancer cells [10–13], and induction of apoptosis is a mechanism of action of the catechins [10,13–15]. Importantly, apoptosis does not occur in non-cancerous cells [10,13,16,17]. However, numerous studies have demonstrated that short incubation times with relatively high concentrations of the catechins (50–100 μM) are necessary to cause cytotoxicity [11,13,16,17].

Catechin-mediated cytotoxicity in human breast cancer cells *in vitro* is supported by both epidemiological and

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animal studies. Specifically, Nakachi *et al.* [18] reported that an increased consumption of green tea is associated with a decrease in both the rate of development and recurrence of breast cancer. Additionally, treatment with either green tea extract or purified EGCG has decreased mammary tumor number [19] and weight [12] in rats, and caused the regression of MCF-7 cell implanted tumors in athymic mice [20]. These results suggest that the catechins have characteristics that may be beneficial in the treatment of breast cancer, but detailed information on the minimum dose and duration of catechin treatment needed to cause breast cancer cell cytotoxicity and tumor regression is currently lacking.

The aim of this study was to investigate the cytotoxicity of EGCG, ECG or EGC alone and in combination with tamoxifen in both $ER\alpha^+$ and $ER\alpha^-$ human breast cancer cell lines. Since the majority of the studies with specific catechins have only investigated the effects of high concentrations and short incubation times, we investigated the cytotoxic potential of low concentrations of individual catechins for a longer duration. Furthermore, we also investigated the cytotoxic effect of catechins in combination with 4-hydroxytamoxifen (4-OHT) in human breast cancer cells.

Methods

Chemicals and reagents

EGC, ECG, EGCG, 4-OHT, Dulbecco's MEM media, Dulbecco's modified Eagle's F-12 media, Trypan blue, sodium bicarbonate ($NaHCO_3$), phosphate-buffered saline (PBS), penicillin, streptomycin, insulin, cholera toxin, epidermal growth factor (EGF), hydrocortisone, sulforhodamine B (SRB), Tris–HCl, decon, poly-L-lysine, quantofix test system, ethidium bromide and acridine orange were purchased from Sigma (St Louis, MO). Dimethylsulfoxide (DMSO), trichloroacetic acid (TCA) and acetic acid were purchased from BDH chemicals (Poole, UK). Fetal bovine serum (FBS) and trypsin, were purchased from Life Technologies (Auckland, NZ). All other chemicals were of the highest purity commercially available.

Cell culture

Human breast cancer cells (MCF-7, T47-D, MDA-MB-231 and HS578T) and the non-cancerous MCF-12A cell line were purchased from ATCC (Manassas, VA). All cancerous cell lines were maintained in Dulbecco's MEM media containing 5% FBS, *2%* $NaHCO_3$, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified CO_2-controlled (5%) incubator. MCF-12A cells were maintained in Dulbecco's modified Eagle's F-12 media containing 5% FBS, *2%* $NaHCO_3$, insulin (5 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), EGF (0.02 μg/ml), cholera toxin (0.1 μg/ml) and hydrocortisone (0.5 μg/ml).

Cell growth assays

Cells were plated in six-well plates (70 000 cells/well) containing 5 ml of Dulbecco's modified Eagle's F-12 supplemented with 5% dextran-coated charcoal stripped-FBS, *2%* $NaHCO_3$, 100 units/ml penicillin and 100 μg/ml streptomycin. After 24 h, cancerous cells were treated with EGCG, ECG or EGC individually (5–25 μM) and in combination with 4-OHT. MCF-12A cells were treated with each individual catechin (25 μM). 4-OHT, the active metabolite of tamoxifen, was used because the amount of cytochrome P-450 (CYP-450) enzymes needed to bioactivate tamoxifen is cell line-specific. The concentration of 4-OHT used in the combination experiments was determined from dose–response experiments performed with each cell line. The specific concentrations were 0.01 μM for T47D and MCF-7 cells, 1 μM for MDA-MB-231 cells, and 0.1 μM for HS57T cells. Vehicle control cells were treated with DMSO (1%). All cells were treated for 7 days. At the end of the incubation period, cell number was determined via the SRB assay as previously described [21]. Results are expressed as cell number which was determined from a standard curve obtained for each cell line. All results are the mean ± SE of four independent experiments performed in triplicate.

Determination of hydrogen peroxide generation

The generation of hydrogen peroxide was determined using the Sigma 'Quantofix' hydrogen peroxide test system (Sigma; cat. no. 37206). EGCG, ECG or EGC (5–25 μM) were incubated with the identical media and serum used in the cytotoxicity experiments. Hydrogen peroxide concentrations were determined at 0, 0.25, 0.5, 1, 2, 3, 6, 12 and 24 h following the addition of the individual catechin, and then once every 24 h until day 8. Positive control wells contained known concentrations of hydrogen peroxide (0–750 μM).

Determination of apoptotic cells

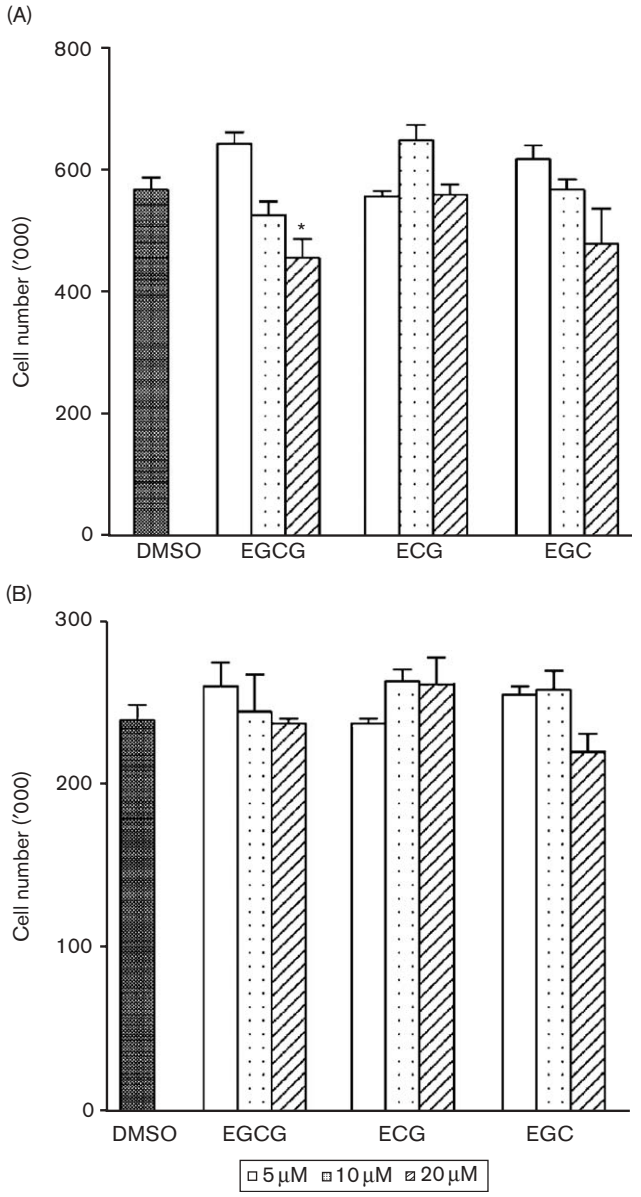
Cell death was studied morphologically by staining with a combination of the fluorescent DNA-binding dyes acridine orange and ethidium bromide. The differential uptake of these two dyes allows the identification of viable and non-viable cells [22]. Both normal and apoptotic nuclei in live cells will fluoresce bright green, while normal and apoptotic nuclei in dead cells will fluoresce orange. Normal nuclei show chromatin with an organized structure, while apoptotic nuclei have highly condensed chromatin which appears as one or a group of spherical beads. Briefly, cells (70 000/well) were plated as described above in a six-well plate which contained sterilized poly-L-lysine-coated coverslips. At 24 h after plating, the cells were incubated for 7 days with 4-OHT (1 μM), EGCG (25 μM), a combination of the two or DMSO (0.1%). On day 8, the media was removed and the coverslips were mounted on glass microscope slides. The cells were stained (0.05% ethidium bromide:0.05% acridine orange in isotonic PBS) and then visualized with

a fluorescent microscope (Zeiss Axioplan) using a primary filter (495 nm) followed by a secondary filter (515 nm). Photographs were taken using a digital camera (Zeiss Axiocam HRc) connected to a Dell Precision 340 computer running Axiovision software.

Statistical analysis

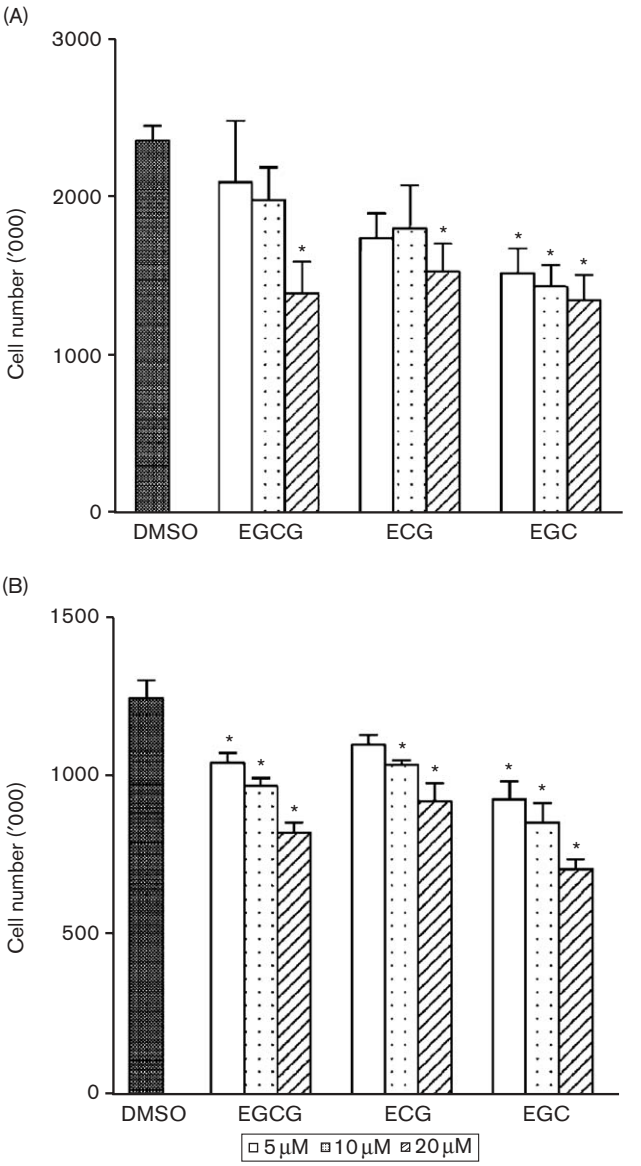
Results were analyzed using an ANOVA coupled with the Student–Newman–Keuls *post-hoc* test. $p < 0.05$ was the minimum requirement for a statistically significant difference.

Fig. 1



Cytotoxicity in ERα$^+$ cells following catechin treatment. (A) MCF-7 or (B) T47D cells were treated with either EGCG, ECG or EGC (5–20 μM) for 7 days. Vehicle control cells were treated with DMSO (0.1%). Cell number was determined on day 8 using the SRB assay. Results are expressed as cell number and are the means ± SE from four independent experiments performed in triplicate. Significance was determined with an ANOVA coupled with the Student–Newman–Keuls *post-hoc* test in which $p < 0.05$ was required for a statistically significant difference. *Significantly decreased compared to DMSO at a level of $p < 0.05$.

Fig. 2



Cytotoxicity in ERα$^-$ cells following catechin treatment. (A) MDA-MB-231 or (B) HS578T cells were treated with either EGCG, ECG or EGC (5–20 μM) for 7 days. Vehicle control cells were treated with DMSO (0.1%). Cell number was determined on day 8 using the SRB assay. Results are expressed as cell number and are the means ± SE from four independent experiments performed in triplicate. Significance was determined with an ANOVA coupled with the Student–Newman–Keuls *post-hoc* test in which $p < 0.05$ was required for a statistically significant difference. *Significantly decreased compared to DMSO at a level of $p < 0.05$.

Table 1 Cell viability of MCF12A cells following catechin treatment

Catechin (25 μM)	Cell no. (% of control)
EGCG	92 ± 3
ECG	97 ± 2
EGC	103 ± 5

Cells were treated for 7 days. Cell number was determined using the SRB assay. Each value represents the mean ± SE from six independent determinations performed in duplicate. Significance was determined with an ANOVA couple with the Student–Newman–Keuls *post-hoc* test in which $p < 0.05$ was required for a statistically significant difference. None were different at this level.

Results

Low concentrations of catechins are cytotoxic to ERα⁻ breast cancer cells

Of the three catechins examined in MCF-7 cells, only EGCG (20 μM) was significantly cytotoxic as cell number was reduced by 20 ± 3% compared to vehicle control following 7 days of treatment (Fig. 1A). However, T47D cells were resistant to the cytotoxic effects of all three catechins, as all treatments failed to alter cell number from control (Fig. 1B). In contrast, the response in ERα⁻ cells was markedly different, as all catechins were significantly cytotoxic to both MDA-MB-231 and HS578T cells. In MDA-MB-231 cells, EGC (5 μM) decreased cell number by 35 ± 4% (Fig. 2A), while 5 μM concentrations of both EGCG and EGC decreased the number of HS578T cells by 16 ± 1 and 25 ± 1%, respectively (Fig. 2B). The cytotoxicity of low concentrations of the three catechins was specific toward cancerous cells as the growth of MCF-12A cells, a human epithelial cell line, was not altered by treatment with either EGCG, EGC or ECG (Table 1).

Catechins do not generate hydrogen peroxide

Catechin-mediated cytotoxicity *in vitro* has been previously attributed to hydrogen peroxide generation caused by a reaction of the polyphenolic catechin with the cell culture media [23]. However, there was no hydrogen peroxide generated by either EGCG, EGC or ECG (5–25 μM) throughout the entire treatment period, while hydrogen peroxide was detected in positive control wells (data not shown).

EGCG and 4-OHT are synergistically cytotoxic to MDA-MB-231 cells

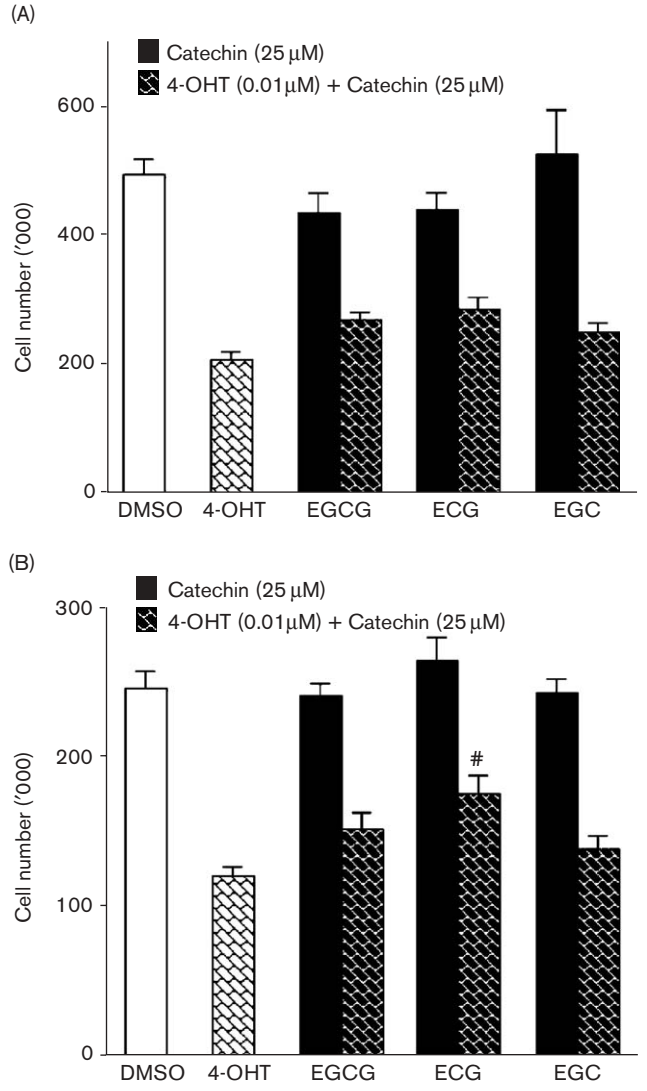
Once the cytotoxicity of low concentrations of catechins was established, their ability to synergize with 4-OHT was then determined. For these experiments, cells were incubated for 7 days with each catechin (25 μM) and 4-OHT (concentration determined from dose–response experiments with each cell line; see Methods for detail). In both MCF-7 and T47D cells, no combination was more effective than 4-OHT (0.01 μM) (Fig. 3A). However, in T47D cells the addition of ECG (25 μM) decreased the cytotoxic potential of 4-OHT as cell number was increased by 31 ± 1% compared to 4-OHT alone (Fig. 3B). In contrast, in MDA-MB-231 cells the combination of EGCG (25 μM) and 4-OHT (1 μM) demonstrated synergistic cytotoxicity (Fig. 4A). Specifically, 4-OHT decreased cell number by 28 ± 11% and EGCG decreased cell number by 49 ± 6%, while only 14 ± 3% of MDA-MB-231 cells remained viable following the combination of the two chemicals. However, synergism did not occur in the other ERα⁻ cell line examined. In HS578T cells, EGC produced a greater cytotoxic effect than 4-OHT. However, the cytotoxicity produced by the combination of EGC + 4-OHT was not greater than that produced by EGC alone (Fig. 4B).

To determine the minimum concentration of EGCG required to elicit synergism, dose–response experiments were performed in MDA-MB-231 cells. When lower concentrations of EGCG (10 and 20 μM) were combined with 4-OHT, cell number was only decreased by 32 ± 3 and 34 ± 2%, respectively (Fig. 5). Therefore, the lowest effective concentration was 25 μM EGCG and 1 μM 4-OHT, as 86 ± 3% of the cells died following 7 days of treatment (Fig. 5). This synergistic effect was visually apparent following acridine orange/ethidium bromide staining. The results demonstrated that following 7 days of treatment 4-OHT caused minimal necrosis (Fig. 6B) and EGCG caused significant cell death via apoptosis (Fig. 6C), while only a very few viable cells remained following combination treatment (Fig. 6D).

Discussion

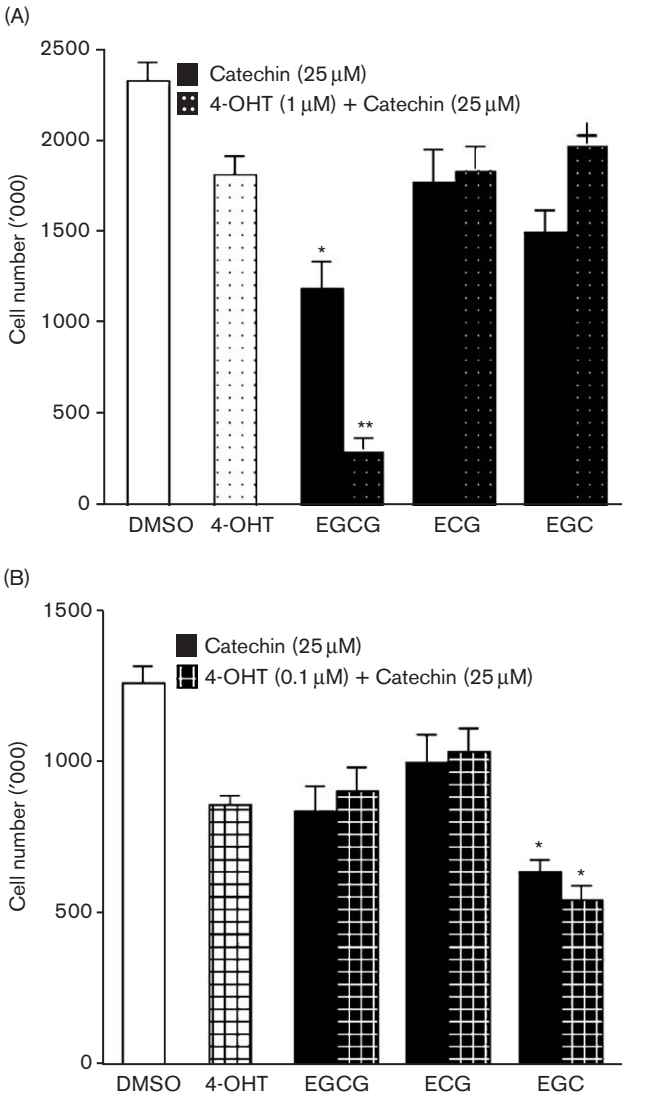
Low concentrations of either EGCG, ECG or EGC were largely ineffective in ERα⁺ cell lines, as only EGCG (20 μM) elicited cytotoxicity in MCF-7 cells. However, 5 μM concentrations of EGC were cytotoxic to both HS578T and MDA-MB-231 cells, while only HS578T cells were susceptible to similar concentrations of EGCG. Additionally, non-cancerous MCF-12A breast epithelial cells remained resistant to the cytotoxic action of the catechins. Therefore, low concentrations of the catechins remained selectively cytotoxic toward cancerous cells and a similar selectivity has been reported by other groups [10,13,15,16,24]. However, most of the previous investigations into the cytotoxic action of the catechins have used short incubation times and concentrations greater than 20 μM. Therefore, the results demonstrating a cytotoxic effect at concentrations as low as 5 μM are extremely promising, particularly as this effect was observed in both of the cell lines that were estrogen insensitive and contained a mutant form of p53 [25]. It has been postulated that EGC could be effective against advanced breast cancers that have a mutant p53 [13] and our results support this theory, but only if the cells are also ERα⁻ as low concentrations of the catechins were not effective in T47D cells which contain a mutant p53, but are ERα⁺ [26].

Fig. 3



Cytotoxicity in ERα$^+$ cells following combination treatment. (A) MCF-7 or (B) T47D cells were treated with either EGCG, ECG or EGC (25 μM), 4-OHT (0.01 μM) or catechin + 4-OHT for 7 days. Vehicle control cells were treated with DMSO (0.1%). Cell number was determined on day 8 using the SRB assay. Results are expressed as cell number and are the means ± SE from four independent experiments performed in triplicate. Significance was determined with an ANOVA coupled with the Student–Newman–Keuls *post-hoc* test in which $p < 0.05$ was required for a statistically significant difference. No treatment was more effective than 4-OHT. #Significantly increased from 4-OHT at a level of $p < 0.05$.

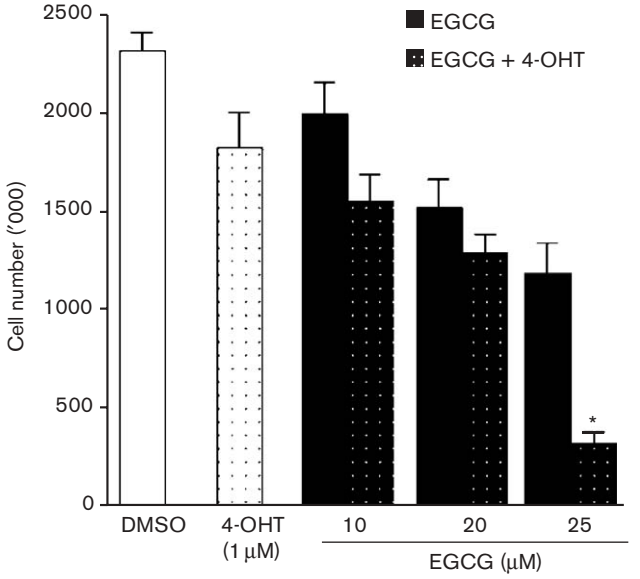
Fig. 4



Cytotoxicity in ERα$^-$ cells following combination treatment. (A) MDA-MB-231 and (B) HS578T cells were treated with either EGCG, ECG or EGC (25 μM), 4-OHT (0.1 μM or 1 μM) or catechin + 4-OHT for 7 days. Cell number was determined on day 8 using the SRB assay. Results are expressed as cell number and are the means ± SE from four independent experiments performed in triplicate. Significance was determined with an ANOVA coupled with the Student–Newman–Keuls *post-hoc* test in which $p < 0.05$ was required for a statistically significant difference. *Significantly decreased compared to 4-OHT at a level of $p < 0.05$. **Significantly decreased compared to either individual treatment at a level of $p < 0.05$.

Importantly, we have also shown that the combination of 4-OHT and EGCG is synergistically cytotoxic to MDA-MB-231 cells. Only two other studies have investigated the cytotoxicity of EGCG in combination with tamoxifen. Specifically, Ishino *et al.* [27] treated cancer cells originating from the tongue and salivary gland (HSC-2 or HSG) with tamoxifen (100 μM) and EGCG (100 μM). In HSC-2 cells there was no increase in the cytotoxicity of tamoxifen and in HSG cells the presence of EGCG decreased the cytotoxicity of tamoxifen. In contrast, Suganuma *et al.* [28] treated PC-9 lung cancer cells with EGCG (75 μM) and tamoxifen (20 μM). Their results showed a small, but significant, increase in apoptosis in cells treated with both EGCG and tamoxifen compared to

Fig. 5



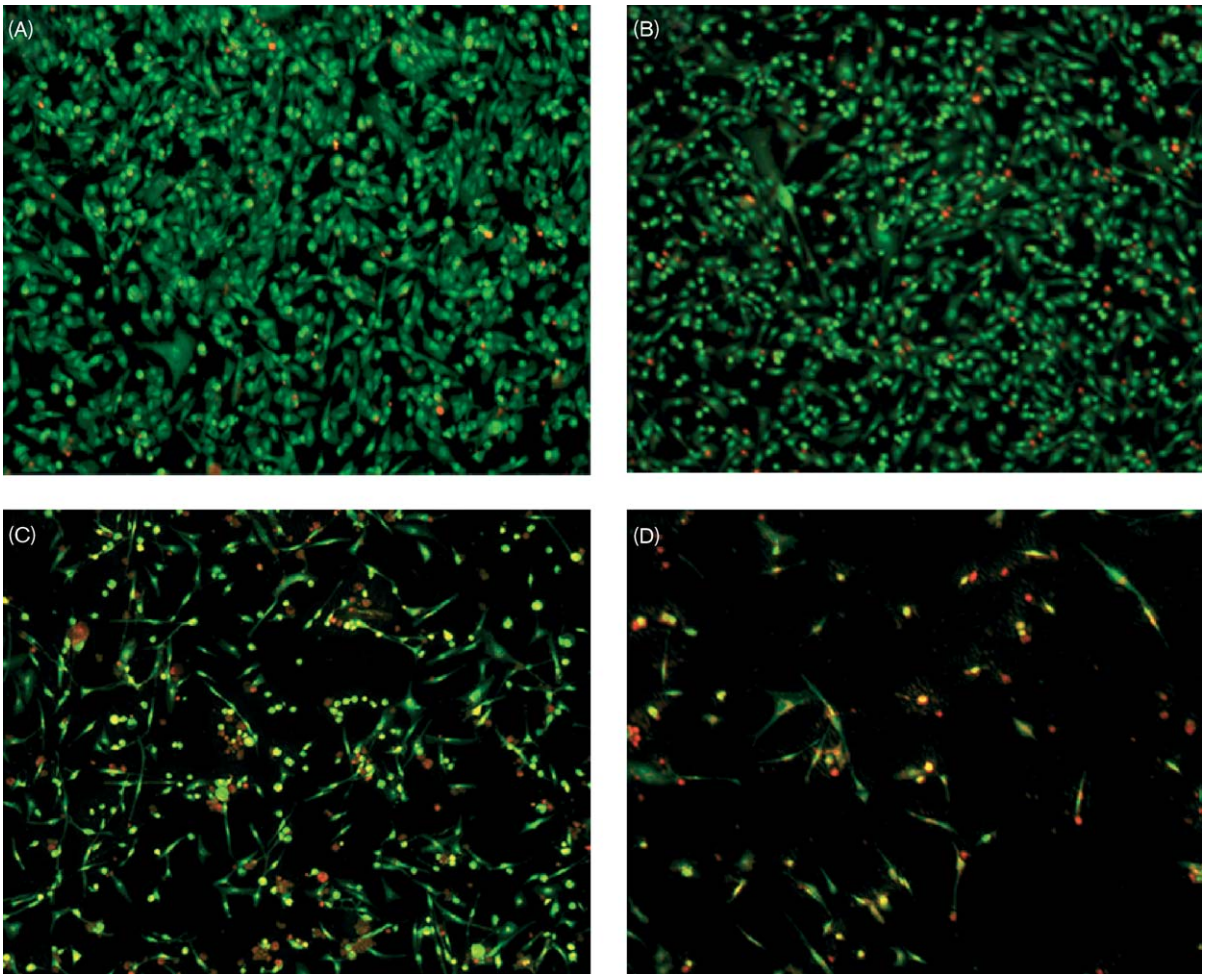
Dose–response of the synergistic cytotoxicity produced by 4-OHT and EGCG. MDA-MB-231 cells were treated for 7 days with 4-OHT (1 μM), EGCG (10–25 μM) or a combination of the two. Vehicle control cells received DMSO (0.1%). Cell number was determined on day 8 using the SRB assay. Results are expressed as percent of control and are the means ± SE from four independent experiments performed in triplicate. Significance was determined with an ANOVA coupled with the Student–Newman–Keuls *post-hoc* test in which $p < 0.05$ was required for a statistically significant difference. *Significantly decreased compared to the respective individual treatments at a level of $p < 0.05$.

either drug alone. Our results were more striking as synergism occurred in MDA-MB-231 cells at much lower concentrations of both tamoxifen (1 μM) and EGCG (25 μM). The concentration of tamoxifen which produced synergism is similar to that used in other investigations which have reported synergistic or additive cytotoxicity in human breast cancer cells [7,8,29,30]. Importantly, the concentration of EGCG required to produce synergistic cytotoxicity is much lower than many *in vitro* studies which have examined the cytotoxicity and/or mechanism of action of EGCG [12,13,16,28,31]. Since the combination of EGCG and 4-OHT is much more cytotoxic to these cells than either agent alone, this could prove to be a viable therapy in breast cancers that are both estrogen insensitive and contain a mutated form of p53. However, other factors are also involved because synergism did not occur in HS578T cells which also contain a mutant p53 and are ERα$^-$ [26].

It has been suggested that the cytotoxicity produced by the catechins *in vitro* is an artifact resulting from hydrogen peroxide generation via a reaction between the individual catechin and the cellular media. However, hydrogen peroxide was not generated when EGCG, EGC or ECG was added to our cell culture system. Long *et al.* [23] reported similar findings, as significant levels of hydrogen peroxide were only generated at catechin concentrations of 50 μM or greater. It is also worth noting that the study by Long *et al.* [23] was conducted with catechins and various commercial medias, but did not include other components routinely used in cell culture such as serum and antibiotics. Our hydrogen peroxide measurements were conducted in cell culture conditions which were identical to the conditions used in the cytotoxicity experiments. Additionally, other experiments have shown that hydrogen peroxide generation is a consequence of the Fenton reaction and thus only occurs in the presence of transition metals [32]. Therefore, we can be confident that the cytotoxicity we have described is not due to hydrogen peroxide generation by the catechins, but is via a separate mechanism.

The mechanism for the observed synergism between tamoxifen and EGCG in MDA-MB-231 cells is likely to involve the enhancement of apoptosis. In addition to the classical anti-estrogenic action of tamoxifen, several studies have demonstrated that this drug causes apoptosis in both ERα$^+$ and ERα$^-$ cell lines and tumors [33,34]. The ability to cause apoptosis in ERα$^-$ cell lines has previously been highlighted as a possible mechanism in combination studies. In MDA-MB-231 cells the co-administration of tamoxifen and docetaxel resulted in a 70% increase in cytotoxicity as compared to either compound alone [7]. The authors concluded that the mechanism of this effect was due to the ability of both compounds to cause apoptosis. The marked decrease in cell number correlated to a synergistic increase in the number of cells remaining in the G_2/M phase of the cell cycle and subsequently the degree of DNA laddering. Therefore, it was postulated that tamoxifen was able to sensitize the cells to the apoptotic effects of docetaxel, facilitating the apoptotic effect. An additional theory highlights the role of the inhibition protein kinase C activity by tamoxifen. In a similar set of experiments, Etreby *et al.* [35] demonstrated an additive cytotoxic effect caused by the combination of tamoxifen (1 μM) and the anti-progestin drug, mifepristone (10 μM), in MCF-7 cells. Interestingly, this increase in cytotoxicity correlated to both an increase in apoptosis and a decrease in protein kinase C activity. This suggests that the synergistic cytotoxic effect of 4-OHT and EGCG observed in our study could also be due to an increase in apoptosis. Many other studies as well as our results from acridine orange/ethidium bromide staining have shown that the catechins induce apoptosis [10,13–16,24] and these compounds have also been reported to decrease protein kinase C activity [36]. This strongly suggests that the catechins would act in a similar manner as docetaxel

Fig. 6



Acridine orange/ethidium bromide staining in MDA-MB-231 cells. Cells were treated with either (A) DMSO (0.1%), (B) 4-OHT (1 μM), (C) EGCG (25 μM) or (D) 4-OHT (1 μM) + EGCG (25 μM) for 7 days. Green cells are viable, orange cells are non-viable and apoptotic cells have condensed chromatin.

or mifepritone when combined with tamoxifen. Additionally, acridine orange/ethidium bromide staining of co-treated cells demonstrated that only a few viable cells remained following 7 days of treatment. This indicates that EGCG-mediated apoptosis was enhanced when the cells were co-treated with 4-OHT and future studies will further define this interaction.

However, other interactions between EGCG and 4-OHT may be important. Since 4-OHT and EGCG are both extensively glucuronidated [37,38], 4-OHT might inhibit the conjugation of EGCG and thus increase the cytotoxicity of EGCG. We have preliminary *in vitro* results which demonstrated that the cytotoxicity of EGCG is inversely correlated to the glucuronide capacity of the cell line [39]. Therefore, inhibition of glucuronidation could increase the concentration of EGCG which would result in an increase in apoptosis.

Overall our results have demonstrated that catechins are effective at much lower concentrations than have been previously reported. Furthermore, these chemicals were cytotoxic in ERα$^-$ cell lines. This is of immense importance as there is currently a lack of viable therapies available to treat this subset of tumors as tamoxifen is regarded as ineffective. However, we have also demonstrated that the combination of EGCG and 4-OHT is cytotoxic to 86% of MDA-MB-231 cells and therefore this drug combination may provide an alternative strategy for the treatment of estrogen-insensitive breast cancer. Future experiments will investigate the efficacy of the

combination treatments in a murine mammary tumor model and determine the mechanism of this effect.

While the synergistic results are promising, our investigation also revealed that ECG (25 μM) impeded the inhibitory actions of tamoxifen (0.01 μM) in T47D cells. A similar effect was observed when oral cancer cells were treated with EGCG and tamoxifen [27]. Therefore, these results suggest that a specific catechin should be administered as opposed to a green tea extract because certain catechins may decrease the efficacy of traditional tamoxifen therapy for $ER\alpha^+$ tumors. However, due to the vast differences that exist between experiments with cell lines and a drug's effect *in vivo* further *in vivo* and *in vitro* investigations are needed before any firm conclusions can be reached.

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