Abstract. Potential mechanisms for the synergistic cytotoxicity elicited by epigallocatechin gallate (EGCG) (25 μM) and 4-hydroxytamoxifen (4-OHT) (1 μM) in MDA-MB-231 human breast cancer cells were investigated. The role of apoptosis was determined using chromatin condensation and Annexin-V staining. Condensed chromatin was visible following 24 h of combination treatment while flow cytometry experiments demonstrated that apoptosis was 2-fold greater following 36 h of combination treatment compared to EGCG. The temporal appearance of cells in G1-arrest did not correlate with apoptosis and thus was not considered to be a viable mechanism for the enhancement of apoptosis. While 4-OHT was a weak competitive inhibitor of microsomal UGT activity (Ki 95 μM), it did not alter the metabolism of EGCG as the rate of disappearance of EGCG from the media was the same for cells treated with either EGCG or EGCG + 4-OHT. Additionally, the metabolism of EGCG was not shifted toward the production of active methylated metabolites, as neither 4''-MeEGCG nor 4',4''-diMeEGCG (2.5-25 μM) were cytotoxic toward MDA-MB-231 cells. In conclusion, the synergistic cytotoxicity elicited by the combination of EGCG and 4-OHT results from an earlier induction of apoptosis but this was not caused by an increase in G1-arrest or 4-OHT-mediated changes in the metabolism of EGCG.

Introduction

Women with breast cancer that does not contain the α isoform of the estrogen receptor (ERα) are in need of a safe and efficacious drug therapy. In the search for new drugs to target this disease many researchers have advocated multiple drug therapy including the use of natural agents in combination with current cancer drugs (1,2). To this end, our laboratory has previously demonstrated that the combination of epigallocatechin gallate (EGCG) and 4-hydroxytamoxifen (4-OHT) is synergistically cytotoxic toward MDA-MB-231 human breast cancer cells following 7 days of treatment (3). Numerous other groups have also investigated the effect of a combination therapy with tamoxifen. Combinations with tamoxifen examined include: genistein (4,5), tocotrienols (6), taxanes (7), mifepristone (8), 5-deoxy-5-fluorouridine (9), and tiazofurin (10). The combination of tamoxifen and docetaxel synergistically inhibited the growth of two ERα- human breast cancer cell lines, namely MDA-MB-231 and MCF-7Adr (7). Similarly, Shen et al (5) demonstrated synergistic cytotoxicity when MDA-MB-435 cells were treated with a combination of tamoxifen and genistein. Synergism has also been reported in vivo, as complete inhibition of DMBA-induced mammary tumors in rats occurred following treatment with a combination of tamoxifen and genistein. Synergism has also been reported in vivo, as complete inhibition of DMBA-induced mammary tumors in rats occurred following treatment with a combination of tamoxifen and 6-MCDF, an aryl hydrocarbon receptor antagonist (11). Therefore, the identification and subsequent mechanistic studies of drugs that synergize with tamoxifen is an important area of breast cancer research.

The aims of the current study were to determine a time-course for the cytotoxicity elicited by the combination of EGCG and 4-OHT and to elucidate the role of apoptosis in this effect. Since both EGCG and tamoxifen induce apoptosis in various cancer cell lines (12-18), it is likely that the enhanced cytotoxicity is due to a promotion of apoptosis. Likely reasons for an enhancement of apoptosis include changes in the cell cycle as well as changes in the metabolism of EGCG. Since EGCG undergoes extensive glucuronidation (19) and methylation (20), and 4-OHT is extensively glucuronidated (21), alterations in one or both of these pathways could result in an increase in the cytotoxic capacity of EGCG. Therefore, this study will also investigate 4-OHT-mediated changes in the metabolism of EGCG as a mechanism for enhanced apoptosis following combination therapy.

Materials and methods

Chemicals. MDA-MB-231 cells were purchased from ATCC (Manassas, VA). Epigallocatechin gallate (EGCG), 99% purity, was purchased from Cayman Chemical (Ann Arbor, MI). Dulbecco’s modified Eagle’s F-12 media, 4-OHT, trypan blue,
NaHCO₃, PBS, penicillin, streptomycin, sulforhodamine B (SRB), Tris-HCl, poly-L-lysine, ethidium bromide, acridine orange, UDPGA, p-NP, BHT, propidium iodide (PI), DMACA and aluminum oxide were purchased from Sigma Chemical Co. (St Louis, MO). Annexin-V-FLOUS was purchased from Roche (Mannheim, Germany). DMSO, TCA, methanol, acetonitrile, chloroform, diethyl ether, perchloric acid and acetic acid were purchased from BDH chemicals (Poole, England). All other chemicals were of the highest purity commercially available.

Cell cytotoxicity assays. MDA-MB-231 cells were plated in six-well plates (70,000 cells/well) containing 5 ml of Dulbecco's modified Eagle's F-12 media supplemented with 2% NaHCO₃, 100 U/ml penicillin, and 100 mg/ml streptomycin and were allowed to adhere for 24 h. For the cytotoxicity time-course, MDA-MB-231 cells were treated with EGCG (25 μM), 4-OHT (1 μM), a combination of the two or DMSO (0.1%) for 12-48 h. In experiments with methylated metabolites of EGCG, cells were treated with 2.5-25 μM of either 4'-MeEGCG or 4',4''-diMeEGCG for seven days. Cell number was then determined via the sulforhodamine B (SRB) assay as previously described (22) and results are expressed as the percent of control.

Visualization of chromatin condensation. Apoptosis was studied morphologically by staining the cells with a combination of acridine orange and ethidium bromide. Normal cells show chromatin with an organized structure while apoptotic cells have condensed chromatin which appear as a group of spherical beads. Cells (70,000/well) were plated and treated for 12-48 h as described above in 6-well plates which contained sterilized poly-L-lysine-coated coverslips. At the end of the treatment period, coverslips were mounted on glass microscope slides and stained (0.05% acridine orange:0.05% ethidium bromide in isotonic PBS). Cells were visualized using a fluorescent microscope (Zeiss Axioplan) with a primary filter (495 nm) followed by a secondary filter (415 nm). Photographs were taken using a digital camera (Zeiss Axiocam HRc) connected to a computer running Axiosvision software.

Apoptosis via flow cytometry. Apoptosis was determined through the externalization of phosphatidylyserine on the extracellular membrane. Cells (200,000/well) were plated and treated for 6-36 h as described above in 12-well plates. At the end of the treatment period, the cells were harvested, washed and stained with 100 μl of Annexin-V-FLOUS/PI labeling solution. Cells were then incubated on ice for 15 min before analysis on a FACScalibur (Becton-Dickinson). Annexin-V FLOUS and PI were detected in the FL-1 and FL-2 channels, respectively. Data was acquired and analyzed using CellQuest Pro software and is expressed as the number of apoptotic cells as a percent of the total number of cells.

Cell cycle analysis. Flow cytometry was used to analyze DNA content in order to determine cell cycle phases (23,24). Cells (200,000/well) were plated and treated for 12-36 h as described above in 12-well plates. Cells were then harvested, washed and stained with PI in the dark overnight at 4°C. The samples were analyzed via flow cytometry using a FACScalibur.

Figure 1. Time-course of cytotoxicity in MDA-MB-231 cells. Cells were treated with either EGCG (25 μM), 4-OHT (1 μM), EGCG + 4-OHT or DMSO (0.1%) for 12-48 h. Cell number was determined using the SRB assay. Points represent the mean ± SEM of four independent experiments performed in triplicate. Statistics were determined via a two-way ANOVA coupled with a Tukey post-hoc test, where p<0.05 was the minimum requirement for a statistically significant difference. *Significantly decreased from both control and 4-OHT. **Significantly decreased from control.

(Becton-Dickinson) where PI was detected in the FL-2 channel. Data was acquired and analyzed using CellQuest Pro software and is expressed as the percent of control.

UGT catalytic activity. Inhibition of hepatic microsomal UDP-glucuronosyltransferase (UGT) was determined via the rate of p-nitrophenol (p-NP) glucuronidation (25) as we have previously described (26). Inhibition studies were performed using various concentrations of p-NP (0.25-5 mM) and 4-OHT (5-60 μM). The catalytic activity was expressed as nmol/mg/min and the Kᵣ value was then determined via non-linear curve fitting using Prism software.

Concentration of EGCG in the media. The concentration of EGCG in the media was determined by solid phase extraction with aluminum oxide which was then reacted with 4-dimethylaminocinnamaldehyde (DMACA) as described (27). Briefly, cells were treated with EGCG (25 μM) ± 4-OHT (1 μM) and media was collected at various time points (0.5-24 h). One ml of media was mixed with 3 ml of methanol containing 1 mg/ml BHT for 5 min under nitrogen. The samples were centrifuged (2,000 x g for 10 min) and the supernatant was combined with 100 mg of preconditioned alumina and vortexed for 15 min at 4°C under nitrogen. The alumina was washed with 3 ml diethyl ether and detection of EGCG (via a green condensation product) was determined 6 min after the addition of 0.5 ml DMACA. The concentration of EGCG was determined from a standard curve obtained from media spiked with various concentrations of EGCG (0-25 μM). The rate of decay of EGCG from the media was determined using Prism software.

Synthesis of methylated EGCG metabolites. Methylated EGCG metabolites were synthesized according to the procedure described by Meng et al (28). 1HNMR data were identical to that previously reported (28) and HPLC analysis showed both compounds to be >95% pure.

Statistical analysis. All results are expressed as the mean ± SEM of four independent experiments performed in triplicate.
Time-course experiments were analyzed using a two-way ANOVA coupled with a Tukey post hoc test in which $p<0.05$ was the minimum requirement for a statistically significant difference. Analyses that did not involve time were analyzed using a one-way ANOVA coupled with a Tukey post hoc test.

Results

We have previously shown that synergistic cytotoxicity toward MDA-MB-231 cells occurs following treatment with EGCG and 4-OHT for 7 days (3). In order to elucidate the role of apoptosis as a mechanism for this synergism, the first step was to determine the minimum duration of combination treatment required to elicit significant toxicity toward MDA-MB-231 cells. The results demonstrated that 24 h after combination treatment, the cell number was decreased 15±4% compared to control ($p<0.05$), while EGCG did not significantly decrease cell number until 36 h (Fig. 1). The appearance of condensed chromatin was then visualized. After 24 h, chromatin condensation was observed in the combination treatment group, while EGCG and 4-OHT induced chromatin condensation after 36 h and 48 h, respectively (Fig. 2). In order to provide a quantitative and more definitive analysis of apoptosis, FITC-conjugated Annexin-V detection of extracellular phosphatidylserine residues was detected via flow cytometry. After 6 h, the combination treatment significantly increased the proportion of apoptotic cells versus 4-OHT and vehicle treatment (Fig. 3A). After 36 h, the combination group induced apoptosis in 23±4% of cells, while EGCG and 4-OHT induced apoptosis in 13±4% and 4±1% of cells, respectively.

To determine if alterations in cell cycle progression provided a means through which apoptosis was induced, the proportion of cells in each phase of the cell cycle was quantified through the intercalation of DNA with propidium iodide. The combination treatment significantly increased the proportion of cells in the G1-phase versus control after 18, 24 and 36 h, where the proportion of cells in G1-phase was increased by 6±2%, 8±1% and 9±1%, respectively (Fig. 3B). While the number of cells undergoing G1-arrest was significantly increased compared to control, the overall number of cells arrested was small. Therefore, other mechanisms must also be involved.

To determine whether the increase in apoptosis following combination treatment was caused by an inhibition in the metabolism of EGCG by 4-OHT, microsomal inhibition assays were conducted using 4-OHT. The resulting Michaelis-Menten kinetic data was analyzed by non-linear curve fitting, which was then transformed into a traditional Lineweaver-Burk plot. The results demonstrated that 4-OHT is a weak competitive inhibitor of hepatic microsomal UGT activity with a $K_i$ of 95 μM (Fig. 4). To determine whether this weak inhibition...
could result in an increased concentration of EGCG in the media following incubation with EGCG and 4-OHT, the concentration of EGCG in the media was determined. The results demonstrated that the metabolism of EGCG did not change when cells were co-treated with EGCG and 4-OHT compared to EGCG alone (Fig. 5). In order to ascertain whether the metabolism of EGCG was shifted toward the production of an active methylated metabolite, the cytotoxic potential of the two most common methylated metabolites, which occur in vivo, was determined. Neither metabolite, namely 4'-MeEGCG (2.5-25 μM) and 4',4''-diMeEGCG (2.5-25 μM), significantly decreased cell number versus the vehicle control after seven days of treatment (Table I).

**Discussion**

In order to determine the mechanism for the synergistic cytotoxicity elicited by EGCG and 4-OHT, initial experiments focused on the time-course of cytotoxicity and its relationship to the development of apoptosis. Due to the reported activity of tamoxifen and EGCG as single agents, we postulated that the mechanism for the observed synergism between 4-OHT and EGCG in MDA-MB-231 cells was 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 (12,13), while EGCG induces apoptosis in many different cancer cells (14-18). Using both chromatin condensation and flow cytometry, we have shown that apoptosis was elicited to a greater degree and at earlier time points following combination treatment. One other study has investigated the induction of apoptosis by a combination of EGCG and tamoxifen. Specifically, Suganuma et al (29) treated PC-9 lung cancer cells with EGCG (75 μM) and tamoxifen (20 μM). Their results showed a small, but significant, increase in apoptotic cells following treatment with a combination of EGCG and tamoxifen. Our results were more striking as enhanced apoptosis occurred in MDA-MB-231 cells at much lower concentrations of both tamoxifen (1 μM) and EGCG (25 μM). Furthermore, apoptosis occurred earlier and to a greater extent during the first 36 h of combination treatment compared to single treatments. This early event

![Figure 3](image_url)  
**Figure 3.** Quantification of (A) apoptosis and (B) cell cycle progression in MDA-MB-231 cells. Cells were treated with either EGCG (25 μM), 4-OHT (1 μM), EGCG + 4-OHT or DMSO (0.1%) for 12-36 h. Quantification of apoptosis induction was determined via Annexin V/PI staining. Changes in cell cycle progression were determined via PI staining. Both were then analysed via flow cytometry. Points represent the mean ± SEM of four independent experiments performed in triplicate. Statistics were determined via a two-way ANOVA coupled with a Tukey post-hoc test, where p<0.05 was the minimum requirement for a statistically significant difference. 

![Figure 4](image_url)  
**Figure 4.** Lineweaver-Burk Plot of inhibition of UGT activity by 4-OHT. The conjugation of p-NP was used to determine the catalytic activity of UGT. Guinea pig hepatic microsomes were incubated with p-NP (0.25-5 mM) and 4-OHT (5-40 μM) and the rate of p-NP conjugation was expressed as nmol/mg/min. Points represent the mean of four independent experiments performed in triplicate. The type of inhibition and Kᵢ value was determined by non-linear curve fitting using Prism software and the Lineweaver-Burk plot was then constructed.

![Figure 5](image_url)  
**Figure 5.** Concentration of EGCG in the media. Cells were treated with EGCG (25 μM) ± 4-OHT (1 μM) for 0.5-24 h. EGCG concentration was then determined in the media via solid-phase extraction followed by reaction with DMACA. Bars represent the mean ± SEM of four independent experiments performed in triplicate. Statistics were determined via a two-way ANOVA coupled with a Tukey post-hoc test, where p<0.05 was the minimum requirement for a statistically significant difference. None were statistically different. Rate of decay curves were also constructed using Prism software and the rate was not different between the 2 groups.
leads to the synergistic cytotoxicity detected after 7 days of combination treatment (3).

Tamoxifen has also been reported to induce apoptosis when combined with other drugs. 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 G2/M phase of the cell cycle and the degree of DNA laddering. Moreover, the weak inhibition of UGT by 4-OHT did not significantly decrease the metabolism of EGCG. Since the other main metabolic pathway for EGCG is methylation (20), inhibition of UGT could cause an increased production of methylated metabolites. Further support for this comes from the fact that when EGCG is glucuronidated in the 3' position, methylation in the 4' position is inhibited (28). This could be significant because in allergy models methylated derivatives of EGCG were more active than EGCG (32,33). If this also occurred in breast cancer cells then an increase in methylated derivatives could explain the increase in apoptosis. To test this theory, MDA-MB-231 cells were treated with the two major methylated metabolites of EGCG produced in vivo. However, equimolar concentrations of both 4'-Me-EGCG and 4',4''-diMe-EGCG failed to elicit cytotoxicity. This demonstrated that methylated derivatives do not exhibit activity against MDA-MB-231 cells and thus shifting the metabolism toward these metabolites is not a mechanism for enhanced apoptosis. Therefore, other mechanisms must be responsible for this effect.

Because MDA-MB-231 cells do not express a functional form of p53 (34), drug-mediated pro-apoptotic effects must occur via another mechanism. It is likely that the enhanced apoptosis elicited by the combination treatment is caused by overlapping inhibition of signal transduction pathways involved in cell proliferation. These include potential roles for the epidermal growth factor receptor, NFκB and various mitogen activated kinases as EGCG and tamoxifen modulate these pathways (12,35-38). Additional evidence for this theory comes from studies showing that the inhibition of either NFκB activity (39) or MAPK/ERK kinases (40) blocked tamoxifen-induced apoptosis. Therefore, there must be other mechanism(s) responsible for the enhancement of apoptosis.

We have previously postulated that 4-OHT may inhibit the glucuronidation of EGCG which could lead to increased activity from either the parent compound or methylated metabolites (30). A similar effect has been reported in vivo, where plasma levels of EGCG were increased in mice following co-administration of piperine and EGCG and this correlated with a 40% decrease in glucuronidation of EGCG (31). More activity from EGCG could lead to an increase in apoptosis, especially since EGCG produced a greater degree of apoptosis than 4-OHT (Fig. 3A). Since 4-OHT demonstrated weak competitive inhibition of UGT activity in vitro, we then determined if this would lead to a change in the metabolism of EGCG in MDA-MB-231 cells. However, the rate of disappearance of EGCG from the media was not different between EGCG and combination treatment. Therefore, the enhancement of apoptosis following combination treatment does not result from a change in G1-arrest or an alteration in the metabolism of EGCG. The marked decrease in cell number correlated to the synergistic cytotoxicity detected after 7 days of treatment (3). However, the enhanced apoptosis following combination treatment does not result from an increase in G1-arrest or an alteration in the metabolism of EGCG.

### Table I. Cytotoxicity of 4'-Me-EGCG and 4',4''-diMe-EGCG toward MDA-MB-231 cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell number (% of control)</th>
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<tbody>
<tr>
<td>DMSO (0.1%)</td>
<td>100±2</td>
</tr>
<tr>
<td>EGCG (25 μM)</td>
<td>67±3&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>4'-MeEGCG</td>
<td>93±3&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>5 μM</td>
<td>94±3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 μM</td>
<td>93±3&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>15 μM</td>
<td>94±3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>20 μM</td>
<td>91±2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>25 μM</td>
<td>90±2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4',4''-diMe-EGCG</td>
<td>97±10</td>
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<tr>
<td>2.5 μM</td>
<td>98±10</td>
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<tr>
<td>5 μM</td>
<td>96±10</td>
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<td>10 μM</td>
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<td>20 μM</td>
<td>91±2&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>25 μM</td>
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<sup>a</sup>Cells were treated with the compounds for 7 days and cell number was determined by the SRB assay. <sup>b</sup>Values are the mean ± SEM of 4 independent determinations performed in triplicate. <sup>c</sup>Significantly decreased from control, p<0.05 using a one-way ANOVA coupled with a Tukey post-hoc test.
References


