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The combination of raloxifene and epigallocatechin gallate suppresses growth and induces apoptosis in MDA-MB-231 cells

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ABSTRACT

Previous studies have demonstrated that raloxifene induces apoptosis in a variety of cancer cell lines. We aimed to determine if this effect was enhanced by combining raloxifene with epigallocatechin gallate (EGCG). Results demonstrated that EGCG (25 μM) and raloxifene (1–5 μM) produced enhanced cytotoxicity toward MDA-MB-231 breast cancer cells compared to either drug alone following 7 days of treatment. The combination of 5 μM raloxifene and EGCG was the most effective as it decreased cell number by 96% of control, and time-course studies demonstrated that significant cytotoxicity began 36 h after treatment. Potential mechanisms for this effect were then investigated. Flow cytometry experiments demonstrated that apoptosis was significantly increased following 12 h of combination treatment compared to all other treatment groups. A maximal increase in the proportion of cells in the G_1-phase of the cell cycle (116% of control) occurred following 24 h of combination treatment, 12 h after the significant increase in apoptosis, and thus was not considered to be a viable mechanism for the enhancement of apoptosis. While raloxifene was a competitive inhibitor of microsomal UDP-glucuronosyltransferase activity (K_i of 24 μM), it did not decrease 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+raloxifene. Finally, the combination treatment reduced the phosphorylation of EGFR and AKT proteins by 21.2±3.3% and 31.5±1.7% from control, respectively. In conclusion, the synergistic cytotoxicity elicited by the combination of EGCG and raloxifene results from an earlier and greater induction of apoptosis. This is likely to be a result of reduced phosphorylation of EGFR and AKT signaling proteins.

Introduction

Women with breast cancer not containing the estrogen receptor alpha (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 combining current cancer drugs with natural agents (Blagosklonny, 2005; Sarkar and Li, 2006). With this in mind, 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 (Chisholm et al., 2004) and that the combination treatment causes apoptosis to be induced earlier and to a greater degree than either drug alone (Stuart et al., 2007). Raloxifene, a newer selective estrogen receptor modulator (SERM), is similar to tamoxifen as it is an ER antagonist in the breast, thus inhibiting the growth of estrogen-mediated breast cancers (Shafer and Jordan, 2006). A major difference is that tamoxifen can induce uterine cancer (Jordan, 1989), while raloxifene produces fewer side effects (Vogel et al., 2006). Thus, raloxifene has the potential to become the preferred SERM in the treatment and prevention of breast cancer.

It has been demonstrated that raloxifene induces apoptosis in a variety of cancer cells including prostate (Kim et al., 2002a,b; Shazer et al., 2006), uterine leiomyoma (Liu et al., 2007; Palomba et al., 2005), endometrial (Davis et al., 2006), myeloma (Olivier et al., 2006), bladder (Kim et al., 2002c), as well as ERα+ and ERα− breast cancer cells (Werner et al., 2005). Additionally, other groups have shown that the efficacy of raloxifene is increased upon co-administration of 13-*cis* retinoic acid (Anzano et al., 1996). Increasing the efficacy of raloxifene via combination therapy is important because extended exposure to SERMs, such as raloxifene for the prevention of osteoporosis and tamoxifen for the prevention of breast cancer, results in extensive and long-term exposure to these drugs. Therefore, a new approach that would decrease the dose of raloxifene by using it in combination with other agents is worth exploring. Since we have reported synergistic cytotoxicity with the combination of EGCG and 4-OHT we aimed to determine the cytotoxicity of raloxifene toward MDA-MB-231 cells following combination with EGCG. Potential mechanisms for the effects observed in vitro were also examined.

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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 medium, raloxifene, trypan blue, $NaHCO_3$, PBS, penicillin, streptomycin, sulforhodamine B (SRB), Tris–HCl, poly-L-lysine, UDPGA, *p*-NP, BHT, propidium iodide (PI), DMACA, aluminum oxide, NP-40, sodium deoxycholate, sodium chloride, ethylenedinitrilotetraacetic acid (EDTA), acrylamide, bis-acrylamide, glycine, methanol, and ammonium persulfate (APS) were purchased from Sigma Chemical Co. (St. Louis, MO). Annexin-V-FLOUS and Complete Protease inhibitor cocktail tablets were purchased from Roche (Mannheim, Germany). DMSO, TCA, methanol, chloroform, diethyl ether, perchloric acid and acetic acid were purchased from BDH chemicals (Poole, England). Rabbit polyclonal antibodies for EGFR, AKT, VEGF, VEGFR and mouse monoclonal antibodies for pEGFR and pAKT were purchased from BD Biosciences (San Jose, CA). Nitroblue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indoyl phosphate-*p*-toluidine salt (BCIP), avidin–alkaline phosphatase conjugate, Precision plus kaleidoscope SDS-PAGE molecular weight standards, streptavadin and nitrocellulose were purchased from Bio Rad (Hercules, CA). All other chemicals were of the highest purity commercially available.

Cell maintenance

MDA-MB-231 cells were maintained in Modified Eagle's Medium alpha-modification medium (MEM) (pH 7.4) supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.2% $NaHCO_3$. All experiments were conducted on cells that had been passaged 6 times since they were obtained from the ATCC, and were discarded after 15 passages during experimentation.

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 medium supplemented with 2% $NaHCO_3$, 100 U/ml penicillin, and 100 mg/ml streptomycin and were allowed to adhere for 24 h In initial cytotoxicity experiments, cells were treated with EGCG (25 μM) and raloxifene (1–5 μM) for 7 days. For the cytotoxicity and apoptosis time-course studies, MDA-MB-231 cells were treated with EGCG (25 μM), raloxifene (5 μM), a combination of the two or DMSO (0.1%) for 12–48 h. Cell number was then determined via the sulforhodamine B (SRB) assay as previously described (Skehan et al., 1990) and results are expressed as the percent of control.

Apoptosis via flow cytometry

Apoptosis was determined through the externalization of phosphatidylserine on the extracellular membrane. Cells (150,000/well) were plated and treated for 6–48 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 Dickson). Annexin-V FLOUS and PI were detected in the FL-1 and FL-2 channels, respectively. Data were acquired and analyzed using CellQuest Pro software. Results are expressed as the number of apoptotic cells as a percentage of the total number of cells.

Cell cycle analysis

Flow cytometry was used to analyze DNA content in order to determine cell cycle phases (Nicoletti et al., 1991; Zamzami et al., 1995). Cells (150,000/well) were plated and treated for 12–48 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 (Becton Dickson) where PI was detected in the FL-2 channel. Data were acquired and analyzed using CellQuest Pro software. Results are expressed as the number of cells in the G_1-phase of the cell cycle as a percentage of the total number of cells.

UGT catalytic activity

Inhibition of total hepatic microsomal UDP-glucuronosyltransferase (UGT) was determined via the rate of *p*-nitrophenol (*p*-NP) glucuronidation (Fowler et al., 1994) as we have previously described (Bray et al., 2002). Inhibition studies were performed using various concentrations of *p*-NP (0.25–5 mM) and raloxifene (5–40 μM). The catalytic activity was expressed as nmol/mg/min and the K_i 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 (Kivits et al., 1997). Briefly, cells were treated with EGCG (25 μM)±raloxifene (5 μM) and medium was collected at various time points (0.5–24 h). To control for potential internalization of EGCG within the cell, EGCG was added to a parallel set of plates that contained medium but did not contain cells. At each time point, 1 ml of medium was mixed with 3 ml of methanol containing 1 mg/ml BHT for 5 min under nitrogen. The samples were centrifuged (2000 *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 medium spiked with various concentrations of EGCG (0–25 μM). The rate of decay of EGCG from the medium was determined using Prism software.

Preparation of whole cell lysates

MDA-MB-231 cells were plated (200,000 cells/well) in 6-well plates and treated for 18 h. Cells were harvested using a cell scraper, washed and incubated in RIPA buffer (50 mM Tris base, 1% NP-40, 0.25% sodium deoxycholate, 100 mM sodium chloride, 1 mM EDTA and Complete Protease inhibitor cocktail tablets (Roche)), incubated on ice for 15 min, and centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was collected and the protein concentration was determined using the BCA assay.

Western blot analysis

Cell lysates (15 μg) were separated by SDS-PAGE and transferred to nitrocellulose membrane, followed by visualization using enhanced alkaline phosphotase (Biorad).

Statistical analysis

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 in which $p<0.05$ was the minimum requirement for a statistically significant difference.

Results

The combination of EGCG+raloxifene produced significant cytotoxicity toward MDA-MB-231 cells following treatment for 7 days. Maximal cytotoxicity occurred following combination treatment of 25 μM EGCG and 5 μM raloxifene, as cell number was significantly decreased against all other treatment groups (cell number of 56.9±4.6% 67.3±3.7% and 3.9±0.4% of control for EGCG, raloxifene and EGCG+raloxifene, respectively, $p<0.01$) (Fig. 1). In order to elucidate the mechanism for this cytotoxicity, the first step was to determine the time-course required to elicit significant cytotoxicity toward MDA-MB-231 cells. The results demonstrated that 48 h following combination treatment, cell number was significantly decreased compared to all other groups (cell number of 91.0±3.7%, 94.1±3.3% and 72.2±3.5% of control for EGCG, raloxifene and EGCG+raloxifene, respectively, $p<0.01$) (Fig. 2). In order to provide a quantitative analysis of apoptosis, FITC-conjugated Annexin-V detection of extracellular phosphatidylserine residues was performed via flow cytometry. The combination treatment significantly increased the proportion of apoptotic cells versus all other treatment groups after 12, 18, 24, 36 and 48 h (Fig. 3A). Specifically, the greatest increase occurred after 48 h of treatment where the combination group induced apoptosis in 37±5% of cells, compared to 3±0.7%, 16±2% and 8±2% for the control, EGCG and raloxifene groups, 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 propidium iodide with DNA. The combination treatment significantly increased the proportion of cells in the G_1-phase versus all other treatment groups after 18 and 24 h, where the proportion of cells in G_1-phase was increased by 8±1% and 16±1% of control, respectively (Fig. 3B). While the proportion of cells in the G_1-phase of the cell cycle was significantly increased compared to control, this increase was modest. Therefore, other mechanisms for the marked cytotoxicity 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 raloxifene, microsomal inhibition assays were conducted using raloxifene. The resulting Michaelis–Menten kinetic data were analyzed by non-linear curve fitting, which was then transformed into a traditional Lineweaver–Burk plot. The results demonstrated that raloxifene is a relatively weak competitive inhibitor of total hepatic microsomal UGT activity with a K_i of 24 μM (Fig. 4). The concentration of EGCG in the media was determined to ascertain whether this raloxifene-mediated inhibition could alter the metabolism of EGCG. The results demonstrated that the rate of disappearance of EGCG in

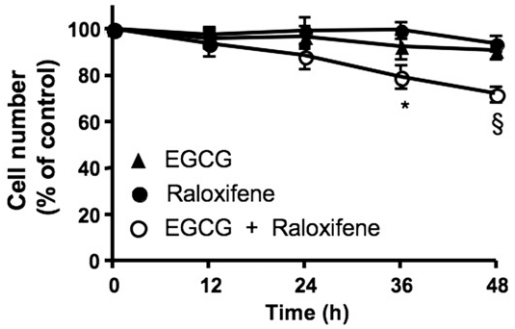

Fig. 2. Time-course of cytotoxicity in MDA-MB-231 cells. Cells were treated with either EGCG (25 μM), raloxifene (5 μM), EGCG+raloxifene or DMSO (0.1%) for 12–48 h. Cell number was determined using the SRB assay. Points represent the mean±SEM of four independent experiments performed in triplicate. Statistics were conducted on the percentage values and 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 DMSO control ($p<0.05$) §significantly decreased from all other groups ($p<0.05$).

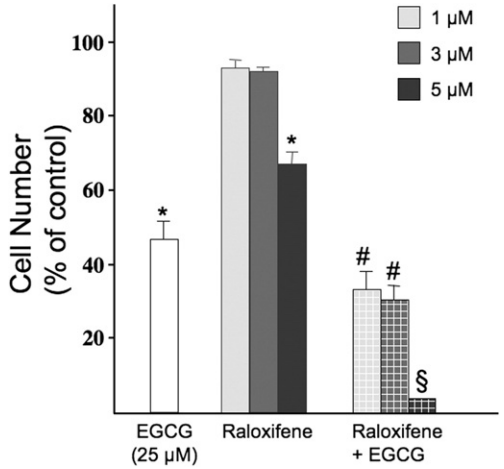

Fig. 1. Cytotoxicity in MDA-MB-231 cells. Cells were treated with either EGCG (25 μM), raloxifene (1–5 μM), EGCG+raloxifene or DMSO (0.1%) for 7 days. Cell number was determined using the SRB assay. Bars represent the mean±SEM of four independent experiments performed in triplicate. Crosshatched bars represent the raloxifene treatment in combination with EGCG treatment. Statistics were conducted on the percentage values and determined via a one-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 DMSO control ($p<0.05$), #significantly decreased from EGCG ($p<0.01$), §significantly decreased from all other treatment groups ($p<0.01$).

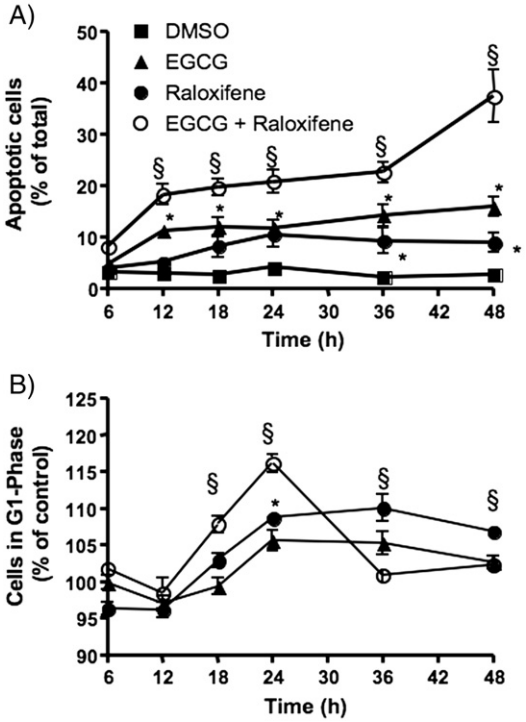

Fig. 3. Quantification of (A) apoptosis and (B) cell cycle progression in MDA-MB-231 cells. Cells were treated with either EGCG (25 μM), raloxifene (5 μM), EGCG+raloxifene or DMSO (0.1%) for 6–48 h. Quantification of apoptosis induction was determined via Annexin-V/PI staining. Changes in cell cycle progression were determined via PI staining. Both were then analyzed 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. *Significantly different compared to control ($p<0.05$), §significantly different compared to all other groups ($p<0.05$).

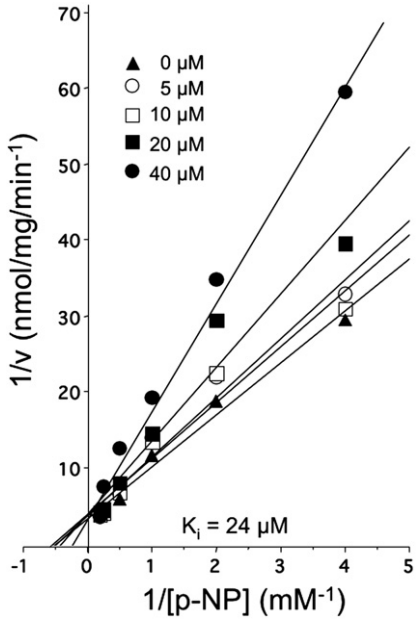

Fig. 4. Lineweaver–Burk plot of inhibition of UGT activity by raloxifene. The conjugation of *p*-NP was used to determine the catalytic activity of UGT. Guinea pig hepatic microsomes were incubated with *p*-NP (0.25–5 mM) and raloxifene (5–40 μM) and the rate of *p*-NP conjugation was expressed as nmol/mg/min.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

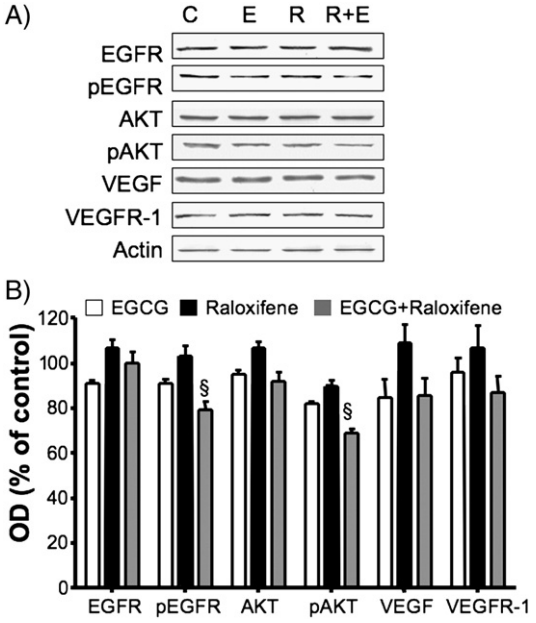

Fig. 6. Protein expression in MDA-MB-231 cells after treatment with EGCG and Raloxifene. Cells were treated with EGCG (25 μM)±raloxifene (5 μM) for 18 h. (A) Representative blots of four independent experiments. (B) Bars represent the mean optical density (OD)±SEM of 4 independent experiments. Data are expressed as a percentage of the control. Statistics were conducted on the percentage values and determined via a one-way ANOVA coupled with a Tukey post-hoc test, where $p<0.05$ was the minimum requirement for a statistically significant difference. §significantly different from all other groups at ($p<0.05$).

the media did not change when cells were co-treated with EGCG+raloxifene compared to EGCG alone (Fig. 5) and thus, the metabolism of EGCG was not decreased by raloxifene.

As no significant differences were observed in the disappearance of EGCG, Western blotting was employed to ascertain if there were any changes in the expression of important cell signaling proteins. The results demonstrated that the combination treatment caused a decrease in the phosphorylation of both EGFR and AKT (Fig. 6), where changes in both of these proteins were significant ($p<0.05$) compared to all other treatment groups (pEGFR expression of 91.5±1.5%, 103.7±4.3% and 79.8±3.3% of the vehicle control for EGCG, raloxifene and combination, respectively; pAKT expression of 82.5±0.9%, 90.3±2.4% and 69.5±1.7% of the vehicle control for EGCG, raloxifene and combination, respectively). No significant changes were observed in the total expression of EGFR, AKT, VEGF or VEGFR (Fig. 6).

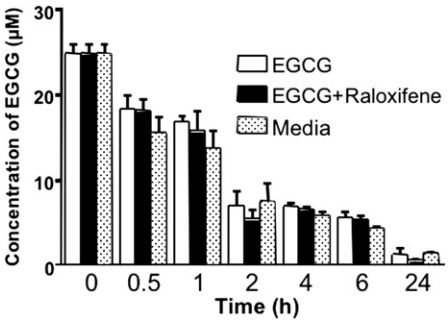

Fig. 5. Concentration of EGCG in the medium. Cells were treated with EGCG (25 μM) ±raloxifene (5 μM) for 0.5–24 h. The medium bar represents wells that only contained EGCG and medium. EGCG concentration was determined in the medium via solid-phase extraction followed by reaction with DMACA. Bars represent the mean±SEM of two 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 3 groups.

Discussion

We have previously shown that the classical SERM tamoxifen elicited significantly enhanced cytotoxicity toward ERα− human breast cancer cells when combined with EGCG (Chisholm et al., 2004) and that this results from an earlier and greater induction of apoptosis following combination treatment (Stuart et al., 2007). This finding prompted us to determine the cytotoxic potential of the combination of raloxifene, another promising SERM, and EGCG. In order to determine the mechanism for the synergistic cytotoxicity elicited by EGCG and raloxifene, initial experiments focused on the time-course of cytotoxicity and its relationship to the induction of apoptosis. Due to the reported activity of raloxifene and EGCG as single agents, we postulated that the mechanism for the observed synergism between raloxifene and EGCG in MDA-MB-231 cells was likely to involve the enhancement of apoptosis. In addition to the classical anti-estrogenic action of raloxifene, several groups have demonstrated that this drug causes apoptosis in a variety of cancer cells (Kim et al., 2002a,b,c; Lui et al., 2007; Olivier et al., 2006; Palomba et al., 2005) including both ERα− and ERα+ cell lines (Werner et al., 2005), while EGCG induces apoptosis in many different cancer cells (Ahmad et al., 1997; Gupta et al., 2000; Huh et al., 2004; Leone et al., 2003; Morre et al., 2000; Roy et al., 2005; Thangapazham et al., 2007). Using flow cytometry, we demonstrated that apoptosis was induced to a greater degree and at an earlier time point following combination treatment, compared to EGCG or raloxifene. Previous studies have also shown that raloxifene induced apoptosis when combined with either estradiol+dihydrodydrogesterone or tibolone, as determined by measuring the apoptotic/proliferation ratio. However, the effect on this ratio was essentially the same when raloxifene was given alone or in combination (Werner et al., 2005). Interestingly, the ratio was markedly decreased following treatment with both

combinations in T47-D cells compared to raloxifene alone. Unfortunately there are no other studies which have directly measured apoptosis in either ERα+ or ERα− breast cancer cells following raloxifene treatment. Clinically, however, raloxifene (60 mg/day) had an antiproliferative but not proapoptotic effect on tumors from ERα+ breast cancer patients (Dowsett et al., 2001). This effect correlated with a 21% decreas in Ki67 protein expression in ERα+ patients but not ERα− patients.

As a potential mechanism for the increase in apoptosis, we analyzed MDA-MB-231 cells for changes in cell cycle progression. The results demonstrated that there was an increase in the proportion of cells in G_1-phase following combination treatment. However, the enhanced apoptosis following EGCG+raloxifene was not likely to be caused by changes in G_1-arrest, since the increase in G_1-phase occurred after the significant increase in apoptosis. We have shown a similar, but slightly weaker, effect following the combination of 4-OHT and EGCG (Stuart et al., 2007). In combination with raloxifene, the overall increase in the proportion of cells in the G_1-phase was greater compared to 4-OHT, but this was still less than a 20% increase over the vehicle control group. Therefore, there must be other mechanism(s) responsible for the enhancement of apoptosis.

We have previously shown that 4-OHT is a very weak inhibitor of UGT, but this was not the cause of the increased cytotoxicity elicited by 4-OHT+EGCG. Furthermore, it was determined that methylated metabolites of EGCG were less cytotoxic than the parent compound (Stuart et al., 2007). However, modulation of EGCG metabolism is possible, as plasma levels of EGCG were increased in mice following co-administration of piperine+EGCG and this correlated with a 40% decrease in glucuronidation of EGCG (Lambert et al., 2004). Therefore, increasing the relative concentration of EGCG could lead to an increase in apoptosis. Since raloxifene demonstrated a stronger competitive inhibition of UGT activity in vitro compared to our previous results with 4-OHT (Stuart et al., 2007), we then determined if this was strong enough to change the metabolism of EGCG in MDA-MB-231 cells. However, the rate of disappearance of EGCG from the medium was not different between EGCG and combination treatment. Therefore, the inhibition of UGT by raloxifene did not significantly decrease the metabolism of EGCG. Since we have already shown that methylated metabolites are not active in MDA-MB-231 breast cancer cells (Stuart et al., 2007), a change in the metabolism of EGCG is not responsible for the enhanced cytotoxicity. Instead, other mechanisms must be responsible for this effect. Interestingly, a recent study conducted by Landis-Piwowar et al. (2007) detailed how altering the structure of EGCG stabilized the compound in physiological conditions. Modifications of the parent compound involved substituting paracetate groups in place of the hydroxyl groups on the B and D-rings of EGCG. This modification yielded an effective EGCG prodrug, which lead to approximately twice the amount of EGCG being recovered from MDA-MB-231 cells after 72 h of treatment. Although we did not make any observations that suggested changes in EGCG metabolism were responsible for the increase in apoptosis seen in combination with raloxifene, the aforementioned paper serves to highlight the importance of metabolism and bioavailability with regard to EGCG mediated cytotoxicity.

Finally, we investigated how the combination of EGCG+raloxifene affected the expression of signaling proteins. We observed decreases in the phosphorylation of both EGFR and AKT proteins, which although modest, were significant against the vehicle control and both EGCG and raloxifene solo treatments (Fig. 6). No significant changes were observed however, in the total expression of EGFR, AKT, VEGF or VEGFR. In the absence of an ER, the MDA-MB-231 cells are known to overexpress the EGFR, an important cell surface receptor involved in cell proliferation and survival (Roos et al., 1986; Biswas et al., 2000). Downstream phosphorylative targets of the EGFR, such as AKT, are critical mediators of the signal transduction initiated by EGFR phosphorylation. Therefore, by decreasing the phosphorylation of the EGFR and AKT the cell is receiving a reduced proliferative/survival signal. The effect of the combination treatment on EGFR and AKT phosphorylation is less than additive over the individual EGCG and raloxifene treatments. Therefore, it is possible that inhibition of overlapping signal transduction pathways involved in cell proliferation and survival leads to the enhanced apoptosis elicited by EGCG+raloxifene. Other downstream targets of the EGFR, which may potentially be involved, include various mitogen activated protein kinases and transcription factors such as NF-κB and AP-1. EGCG is known to modulate these pathways (Chen et al., 2003; Gupta et al., 2004; Thangapazham et al., 2007; Vayalil and Katiyar, 2004), and raloxifene decreased constitutive expression of NF-κB and also decreased the expression of NF-κB-regulated genes (*c-myc*, *hgf*, *pac1*) in JJN-3 and U266 myeloma cells, both ERα+ cells (Olivier et al., 2006). The capacity of raloxifene to modulate these pathways in an ERα− context is unknown. Certainly, raloxifene-mediated modulation of NF-κB transcriptional activity in ERα+ cells could be explained through crosstalk with the ERα. Tamoxifen as been shown to modulate the activity of both JNK and p38 mitogen activated protein kinase pathways, which share common activators with NF-κB, in the absence of the ERα (Mabuchi et al., 2004; Mandelkar et al., 2000). Therefore it is possible that raloxifene may also regulate the same pathways. Further studies are required, however, to fully elucidate the mechanism responsible for this interaction.

Conclusion

EGCG induces apoptosis in MDA-MB-231 cells as a single agent and this effect is significantly enhanced when it is combined with raloxifene. Early induction of apoptosis leads to a significant reduction in cell number after 7 days of treatment. The enhanced apoptosis following combination treatment does not result from an increased proportion of cells in G_1-phase of the cell cycle or an alteration in the metabolism of EGCG. However, the combination treatment significantly reduces the phosphorylation of both EGFR and AKT proteins. Therefore modulating intracellular signaling cascades is a likely mechanism through which the induction of apoptosis is mediated by the combination treatment.

Acknowledgements

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