Synergistic inhibition of lung cancer cell lines by (−)-epigallocatechin-3-gallate in combination with clinically used nitrocatechol inhibitors of catechol-O-methyltransferase

Sarah C. Forester and Joshua D. Lambert*

Center of Excellence for Plant and Mushroom Foods for Human Health, Department of Food Science, The Pennsylvania State University, University Park, PA 16802, USA

*To whom correspondence should be addressed. Department of Food Science, The Pennsylvania State University, 332 Food Science Building, University Park, PA 16802, USA. Tel: +1 814-865-5223; Fax: +1 814-863-6132; Email: jdl134@psu.edu

(−)-Epigallocatechin-3-gallate (EGCG) has exhibited been studied for lung cancer inhibitory activity in vitro and in animal models, but it is rapidly methylated and inactivated by catechol-O-methyltransferase (COMT). Entacapone and tolcapone, COMT inhibitors, are used to mitigate the symptoms of Parkinson’s disease. We investigated the synergistic effects of entacapone/tolcapone and EGCG against lung cancer cell lines in culture. EGCG, entacapone and tolcapone inhibited the growth of H1299 human lung cancer cells (IC50 = 174.9, 76.8 and 29.3 μM, respectively) and Cl−13 murine lung cancer cells (IC50 = 181.5, 50.7 and 19.7 μM, respectively) as single agents following treatment for 72 h. Treatment with 1:10, 1:5, 1:2.5 and 1:1 combinations of EGCG and tolcapone or entacapone resulted in synergistically enhanced growth inhibition. The growth inhibitory effect of the combinations was mediated by induction of intracellular oxidative stress, cell cycle arrest and increased nuclear translocation of nuclear factor-κB. Methylation of EGCG was dose dependently inhibited by entacapone and tolcapone (IC50 = 10 and 20 μM, respectively) in a cell-free system, and both compounds increased the intracellular levels of unmethylated EGCG. Treatment of mice with EGCG in combination with tolcapone increased the bioavailability of EGCG and decreased the methylation of plasma norepinephrine; no apparent liver or behavioral toxicity was observed. In conclusion, the combination of EGCG and entacapone/tolcapone synergistically inhibited the growth of lung cancer cells in culture, and the mechanistic basis for this synergy is likely due in part to inhibition of COMT with resultant increase in the levels of unmethylated EGCG.

Introduction

Lung cancer is the second most common cancer in the USA (1). Despite considerable effort to develop new treatments, the overall 5 year survival rate for lung cancer is 15.6%, with prognosis worsening with the stage of disease (1). Green tea (Camellia sinensis, Theaceae) has been investigated for its preventive and therapeutic effects against lung cancer both in laboratory and human studies (2).

Animal model studies have shown that green tea, green tea extract and (−)-epigallocatechin-3-gallate (EGCG) can inhibit lung carcinogenesis (3–11). For example, green tea (6 g tea solids/l of water) given to mice for 16 weeks as the only source of drinking fluid reduced lung tumor multiplicity in 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK)-treated AJ mice. The reduction in tumor multiplicity was accompanied by increased apoptosis in lung adenomas (4). Supplementation with Polyphenon E (5 g/l, containing 60% EGCG) had similar effects in the same model of lung tumorigenesis, with reduced cell proliferation and lower levels of c-Jun and Erk1/2 (extracellular signal-regulated kinase 1/2) phosphorylation in adenocarcinomas (12). Other studies have reported that inhibition of lung carcinogenesis by green tea in vivo is accompanied by decreases in parameters including tumor multiplicity, upregulation of p53 and downregulation of Bcl-2 (B-cell lymphoma 2) (13).

Although typically considered an antioxidant, EGCG has been reported to induce oxidative stress in a number of lung cancer cell lines and this effect appears to underlie some of the observed in vitro anti-lung cancer effects (14). For example, EGCG induced apoptosis in the H661 lung cancer cell line, and this effect was related to EGCG hydrogen peroxide. EGCG-mediated apoptosis was counteracted by inclusion of exogenous catalase (15). The induction of oxidative stress by EGCG observed in vitro has also been observed in vivo. EGCG (0.1–0.5% in the diet) inhibited growth of lung tumors in H1299 human lung cancer xenograft-bearing nu/nu mice (16). Tumor growth inhibition was associated with increased levels of tumor cell apoptosis, increased levels of 8-hydroxy-2′-deoxyguanosine and increased phosphorylation of histone 2A variant X (γH2AX). The anticancer activities of EGCG against lung cancer in vitro and in vivo appear to be mediated at least in part by reactive oxygen species (ROS).

The bioavailability of EGCG is relatively low and it is extensively metabolized to methylated, glucuronidated and sulfated metabolites in vivo (17). Catechol-O-methyltransferase (COMT) metabolizes EGCG to 4′-O-methyl-EGCG (MeEGCG) and 4′,4″-di-O-methyl-EGCG (DiMeEGCG) (Figure 1) (18), both of which have significantly less biological activity than the unmethylated EGCG (19). Some evidence suggests that inhibition of EGCG methylation may enhance the in vivo biological effects of EGCG and green tea. Landis-Piwowar et al. (20) have reported that MDA-MB-231 breast cancer cells with reduced COMT levels were more susceptible to the growth inhibitory effects of EGCG than cells with higher expression of COMT. In a case–control study of the relationship between tea consumption and breast cancer, tea was found to have a greater protective effect in Asian-American women with one or two copies of the low-activity COMT allele compared with women with two high activity alleles of COMT (21), suggesting enhanced bioavailability of tea polyphenols in women with low-activity COMT. In fact, green tea polyphenols have been shown to be less extensively methylated in humans with low COMT activity (22,23) and were more bioavailable (24) compared with human subjects with high COMT activity.

The nitrocatechols, entacapone and tolcapone (Figure 1) are clinically used COMT inhibitors that are used in the management of Parkinson’s disease (25,26). In this study, we examined whether EGCG in combination with entacapone/tolcapone had enhanced in vitro anticancer activity compared with treatment with the individual compounds against human and murine lung cancer cell lines. We further determined the role of induction of oxidative stress in the anticancer effects of the combinations.

Abbreviations: 3-OMD, 3-O-methyl DOPA; ALT, alanine aminotransferase; COMT, catechol-O-methyltransferase; EGCG, (−)-epigallocatechin-3-gallate; i.e., intragastric; LC–MS, liquid chromatography–mass spectrometry; t-DOPA, 3,4-dihydroxy-L-phenylalanine; MeEGCG, 4′-O-methyl-EGCG; NF-kB, nuclear factor kappa B; Norepi, norepinephrine; ROS, reactive oxygen species; SAM, S-(5′-adenosyl)-L-methionine chloride; SEM, standard error of the mean.

Materials and methods

Chemicals

EGCG (98% pure) was purchased from Quality Phytochemicals LCC (Edison, NJ). Entacapone and tolcapone were purchased from SynFine (Richmond Hill, Canada). The metabolite 3-O-methyl DOPA (3-OMD) was purchased from CacheSyn (Mississauga, Canada). COMT, S-(5′-adenosyl)-L-methionine chloride (SAM) and 3,4-dihydroxy-L-phenylalanine (t-DOPA)

© The Author 2013. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com
were purchased from Sigma Chemical Co. (St Louis, MO). 6-Carboxy-2,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (H₂DCFDA) was obtained from Invitrogen (Carlsbad, CA). All other chemicals were of the highest grade commercially available.

**Inhibition of COMT**

Methylation reactions were carried out by incubating EGCG (10 μM) with COMT (25 U) and SAM (75 μM) in 10 mM Tris–HCl buffer (pH 7.4) containing dithiothreitol (1.25 mM), MgCl₂ (1.5 mM), catalase (30 U/ml) and superoxide dismutase (5 U/ml) in the presence or absence of entacapone or tolcapone (0–30 μM) for 30 min at 37°C. The reaction was terminated by addition of 20 μl of perchloric acid. After centrifugation at 10,000g, the samples were analyzed by liquid chromatography–mass spectrometry (LC–MS) as described previously (18).

**Cell culture**

H1299 human (American Type Culture Collection, Manassas, VA) and CL-13 murine (a gift from Dr Steven A.Belinsky, The Lovelace Respiratory Research Institute, Albuquerque, NM) lung cancer cells were maintained in RPMI-1640 media (Cellgro, Manassas, VA), supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma) under a 5% CO₂ atmosphere at 95% relative humidity. IMR-90 cells (American Type Culture Collection) were cultured in similar conditions with the exception of Dulbecco’s modified Eagle’s media. Cells were subcultured by trypsinization to maintain growth in the log phase.

**Determination of COMT specific activity**

Cells were grown in 75 cm² flasks and total cell fractions were harvested by scraping into lysis buffer and centrifugation. t-DOPA (1 μM final concentration) was incubated with 0.1 mg protein from either cell line in the presence of SAM (75 μM), in 10 mM Tris–HCl buffer [containing dithiothreitol (1.25 mM), MgCl₂ (1.5 mM), pH 7.4] for 30 min at 37°C. The reaction was terminated by addition of 200 μl of 75% methanol. After centrifugation at 10,000g, the production of 3-OMD was measured by LC–MS using a previously described method (27), with the following modifications. The mobile phase consisted of 30% methanol and 3-OMD was detected in negative mode using single ion monitoring (210 m/z).

**Cell viability assay**

Cells were plated (5000 cells per well) in 96-well plates and allowed to attach overnight. Cells were then treated with EGCG, entacapone or tolcapone (10–100 μM) for 24, 48 and 72 h. Cells were also treated with multiple combinations of EGCG/entacapone or EGCG/tolcapone (1:10, 1:5, 1:2.5 and 1:1 molar ratios). All cell treatments included catalase (30 U/ml) and superoxide dismutase (5 U/ml) to stabilize EGCG in the cell culture medium and minimize production of exogenous ROS (28). Cell viability was determined spectrophotometrically after incubation using the thiazolyl blue tetrazolium bromide (MTT) assay (λ = 540 nm).

**Cell cycle progression**

Cells (10⁴) were plated in 75 cm² flasks and allowed to attach for 48 h in 0.1% serum media. Cells were then treated with EGCG, entacapone, tolcapone, EGCG + entacapone or EGCG + tolcapone (25 μM each or 25 + 25 μM combinations) in serum-complete media for 24 h (H1299) or 72 h (CL-13). The cells were then harvested by trypsinization and fixed with 70% methanol in phosphate-buffered saline. Cells were then stained with 40 μg/ml propidium iodide and 0.5 mg/ml RNase in phosphate-buffered saline for 30 min at 37°C. Cell cycle analysis was performed using a Coulter XL-MCL flow cytometer with FCS Express software (De Novo Software, Los Angeles, CA).

**Western blot analysis**

Cells [10⁴ in 25 cm² flasks (for total protein) or 3 x 10⁶ in 75 cm² flasks (for nuclear protein)] were plated and allowed to attach for 24 h. Cells were then treated with EGCG, entacapone, tolcapone, or EGCG + entacapone or EGCG + tolcapone (25 μM each or 25 + 25 μM combination) in serum-complete media. Cell fractions were harvested by scraping into lysis buffer and centrifugation.
Lung cancer growth inhibition by EGCG and COMT inhibitors

Nuclear fractions were prepared as described previously (29). Proteins (30 µg) were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and probed with primary antibodies (Cell Signaling Technology, Danvers, MA) for cyclin D1 (1:1000 dilution), nuclear factor kappa B (NF-κB) (1:2000 dilution), β-actin (1:1000 dilution) and Histone H3 (1:2000 dilution) overnight at 4°C. After incubation with a fluoroscence-labeled secondary antibody (LI-COR Biosciences, Lincoln, NE), proteins were imaged with an Odyssey imaging system (LI-COR).

**Intracellular EGCG levels**

Cells were plated in 6-well plates (1.5 x 10^5 per well) and allowed to attach for 24 h. Cells were then treated with EGCG (100 µM), EGCG + entacapone (100 + 100 µM) or EGCG + tolcapone (100 + 100 µM) in serum-complete media. After 30 min, the cells were scraped into 0.2% ascorbic acid in water, sonicated and centrifuged at 14 000 g for 10 min (4°C). The supernatant was combined with an equal volume of cold methanol to precipitate protein and centrifuged at 14 000 g for 10 min (4°C). The supernatant was then filtered and analyzed for EGCG and its methyl metabolites by an established LC–MS method (18). Concentrations were normalized for protein content of cell lysates.

**Intracellular oxidative stress**

Cells were plated in 25 cm² flasks (2.5 x 10^5 cells) after attatching for 24 h. H1299 and CL-13 cells were treated for 24 and 72 h, respectively, with EGCG, entacapone, tolcapone, EGCG + entacapone and EGCG + tolcapone (5 µM each or 5 + 5 µM combinations for H1299 cells and 25 µM each or 25 + 25 µM combinations for CL-13 cells) in serum-complete media. The cells were then stained with 5 µM H$_2$DCFDA in media for 30 min at 37°C and harvested by trypsinization. The cells were pelleted, resuspended in fresh media and analyzed by flow cytometry.

**Extracellular ROS production**

CL-13 cells were plated in 96-well plates (5 x 10^3 cells) per well) allowed to attach for 24 h. Cells were then treated with EGCG, entacapone, tolcapone, EGCG + entacapone and EGCG + tolcapone (100 µM each or 100 + 100 µM combinations) for 6, 24, 48 and 72 h. At each time point, 90 µl of culture media was combined with 10 µl methanol and 20 µl Ferrous Oxidation–Xylenol Orange assay solution (1 mM xyl-enol orange, 2.5 mM ferrous sulfate, 1 M sorbitol, in 250 mM sulfuric acid) in a new 96-well plate. The plate was mixed briefly by shaking and incubated in the dark at room temperature for 30 min. The concentration of hydroperoxides in the media was determined spectrophotometrically at 540 nm.

**Animals and treatments**

Changes in liver chemistry. Male CF-1 mice (4 weeks old) were purchased from Charles River (Wilmington, MA) and maintained on 12 h light/dark with standard chow and water provided ad libitum. Mice were housed in gang cages (n = 10 per cage) on corn cob bedding. All experiments were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University (IACUC #28962). Following a 2 week acclimation period, mice were separated into groups based on body weight and fasted for 12 h (8 p.m. – 8 a.m.) prior to treatment. Mice were given EGCG (100 mg/kg, intragastric [i.g.]), alone or in combination with tolcapone (30 mg/kg, i.g.). All solutions were made in 5% ethanol in water. After dosing, mice had ad libitum access to water but were kept without food for the duration of the experiment. Mice were killed at t = 12 and 24 h.

Changes in EGCG bioavailability and norepinephrine metabolism. In a separate experiment, SCID mice (4 weeks old) were maintained on an AIN 76A standard diet supplemented with tolcapone (0.05% wt/wt), EGCG (0.15% wt/wt) or a combination of the two agents (0.05 + 0.15% wt/wt). Mice were killed after 9.5 weeks of ad libitum access to water and treatment diet. Blood was collected via cardiac puncture from anaesthetized mice. Blood was centrifuged at 3200 g for 15 min at 4°C and plasma and frozen at −80°C until analysis.

**Statistical analysis**

All values are reported as means ± standard error of the mean (SEM). Isobolograms were generated from IC$_{50}$ values determined using the MTT assay and graphed under the line of additivity (31). IC$_{50}$ values were determined by non-linear regression using Graph Pad software package version 5 (La Jolla, CA). Two-tailed Student’s t-tests were performed to determine statistical significance of intracellular EGCG concentrations and COMT specific activity.

One-way analysis of variance with Tukey’s post-test was used to determine statistical significance of ROS, cell cycle and western blotting quantitation. A P value <0.05 was considered statistically significant.

**Results**

**Cell viability assay**

Both EGCG/entacapone and EGCG/tolcapone combinations synergistically reduced H1299 (Figure 1A and C) and CL-13 (Figure 1B and D) cell viability, compared with treatment with single compounds as measured by isobologram analysis. The EGCG/tolcapone combination was the most potent. The IC$_{50}$ value of EGCG was reduced from 174.9 to 25 µM in H1299 cells when combined with 13.2 µM tolcapone. The IC$_{50}$ value of EGCG was reduced from 181.5 to 25 µM in CL-13 cells by combination with 12.9 µM tolcapone. Growth curves suggest that the combination treatments over 72h inhibited cell proliferation rather than inducing cytotoxicity (Figure 1E and F). IMR-90 fibroblast cells were significantly (P < 0.05) less sensitive to the combination treatments compared with the two cancer cell lines, with the exception of the EGCG/entacapone combination in the H1299 cell line (Figure 1G).

**Cell cycle progression**

Treatment with equimolar combinations of EGCG and tolcapone (25 + 25 µM) or EGCG and entacapone (25 + 25 µM) induced cell cycle arrest in both H1299 and CL-13 cells (Figure 2). In H1299 cells, this arrest was in the G$_0$/G$_1$ phase (Figure 2A), whereas in the CL-13 cells, arrest was in the G$_2$/M phase (Figure 2B). The nitrates, chelots, but not EGCG, also caused a significant (P < 0.05) arrest in the cell cycle at G$_2$/M, in the H1299 human cell line. The arrest, however, was less dramatic compared with combination-induced cell cycle arrest.
Fig. 3. Effects of cotreatment with EGCG and entacapone or tolcapone on the expression of proliferation and survival-related proteins in (A) H1299 and (B) CL-13 cell lines in culture. Cells were treated with either single (25 μM) or combination treatment (25 μM each) for 6h for cyclin D1 and cyclin A, and 24h for cyclin B1 and NF-κB expression. Results are shown as means ± SEM of proteins of interest normalized by expression of housekeeping proteins and controls (n = 3–4). An asterisk indicates combinations that were significantly different from single treatments (P < 0.05). E, entacapone; T, tolcapone.
Cyclin expression
Proteins involved in cell cycle control were monitored in this study by western blot. EGCG combined with entacapone or tolcapone reduced the expression of cyclin D1 in both cell lines upon treatment with 25 μM as single treatments or in combination (Figure 3). In contrast, treatment with the single agents had no effect of expression of this cell cycle regulator. No treatment effect was observed on the expression of cyclin A or cyclin B1 except for the EGCG/tolcapone in the H1299 cell line.

Nuclear translocation of NF-κB
Treatment with EGCG in combination either tolcapone or entacapone reduced nuclear levels of NF-κB in both cell lines, but had no effect on total cellular NF-κB (Figure 3). Treatment with entacapone or tolcapone, but not EGCG, as single agents had similar but less pronounced effects on nuclear levels of NF-κB (Figure 3).

ROS production and intracellular oxidative stress
Cotreatment of H1299 and CL-13 cells with tolcapone/entacapone and EGCG significantly increased the intracellular levels of ROS in both cell lines as measured by flow cytometry (Figure 4A and B). In contrast, the single agents did not significantly affect intracellular ROS in the CL-13 cells but did increase levels in the H1299 cells. Incubation of EGCG in combination with tolcapone and entacapone also increased the levels of ROS in the cell culture medium, although the effect was significant only in the presence of tolcapone (Figure 4C).

Enzymatic inhibition of COMT
Entacapone and tolcapone dose dependently inhibited methylation of EGCG by COMT (IC₅₀ = 10 and 20 μM, respectively) in cell-free system (Figure 5A and B). MeEGCG was the primary methylated product produced by COMT in the presence of SAM, yet a small amount of DiMeEGCG was formed. Inhibition of EGCG methylation by entacapone (10 μM) and tolcapone (20 μM) was time dependent with 19 and 26% inhibition at 30 min, respectively (Figure 5C and D).

Determination of intracellular EGCG and COMT specific activity
Cotreatment of cells with EGCG and entacapone or tolcapone for 30 min increased the intracellular levels of unmethylated EGCG in both cell lines compared with treatment with EGCG alone (Figure 5E). In H1299 cells, the unmethylated fraction increased from 35.7 to 80.4% and to 96.9% in the presence of entacapone and tolcapone, respectively. In CL-13 cells, the fraction of unmethylated EGCG was much higher when EGCG was used a single agent compared with H1299 cells, and only a small increase is observed following cotreatment with entacapone or tolcapone (Figure 5E). Analysis of COMT activity in both H1299 and CL-13 cells showed that the former cells had 25% higher methylation activity toward l-DOPA than the latter cells (Figure 5F).

Modulation of ALT and Norepi methylation in vivo
Co-treatment with dietary tolcapone dose dependently increased plasma levels of total EGCG in mice (Figure 6A). Plasma ALT levels in mice dosed with tolcapone (30 mg/kg body weight) alone or combined with EGCG (100 mg/kg body weight) were unchanged compared with vehicle control-dosed mice (Figure 6B). In a separate experiment, methylation of plasma Norepi was decreased by 52% in mice that were maintained on a diet that contained tolcapone in combination with EGCG (Figure 6C). The treatments also did not alter the appearance of gross liver morphology in either experiment.

Discussion
In this study, we report for the first time that the combination of EGCG with the clinically useful COMT inhibitors, entacapone and tolcapone, synergistically inhibited the proliferation of two lung cancer cell lines in vitro. Inhibition of cell proliferation was accompanied by an increase in intracellular oxidative stress, an inhibition of cell cycle progression and an inhibition of NF-κB nuclear translocation. Although the effect was not as pronounced, the treatments did inhibit growth of the normal cell line, which may be due to the relatively high concentrations chosen for this study.

The general safety of consuming nitrocatechols with green tea polyphenols remains to be fully examined. Tolcapone has been shown
to be an uncoupler of mitochondrial respiration and toxic to human neuroblastoma cells in culture. Entacapone did not exhibit toxic effects against this cell line (32). Although nitrocatechols may have toxic effects towards specific cell lines in culture, we observed that tolcapone was well tolerated by mice. Although tolcapone did have an effect on Norepi methylation, it did not effect plasma ALT levels or alter liver morphology.

We initially hypothesized that the cotreatment with EGCG and nitrocatechol COMT inhibitors would result in increased intracellular levels of unmethylated EGCG and resultant improvements in

---

Fig. 5. Inhibition of COMT-mediated methylation of EGCG by entacapone and tolcapone. Both concentration- (A and B) and time-dependent (C and D) inhibition by entacapone and tolcapone, respectively, were examined upon incubation with COMT in a cell-free system. Effects of entacapone or tolcapone on intracellular levels of EGCG (E) were determined in the H1299 and CL-13 lung cancer cell lines. Cells were incubated for 30 min with EGCG (100 µM) alone or in combination with an equal concentration of entacapone or tolcapone. Comparison of COMT specific activity (F) was determined in H1299 and CL-13 cell lines. t-DOPA (1 µM) was incubated with 0.1 mg of cellular protein in the presence of SAM for 30 min. Specific activity was calculated based on the formation of 3-OMD (µM). Results are shown as means ± SEM (n = 2–4). An asterisk indicates combinations that were significantly different from EGCG treatment and that cell line-specific activities are significantly different (P < 0.05).

Fig. 6. (A) Effect of tolcapone on the bioavailability and potential hepatotoxicity of EGCG and the methylation of Norepi. (B) CF-1 mice (n = 6) were given a single dose of either tolcapone (30 mg/kg, i.g.), EGCG (100 mg/kg, i.g.) or the combination. ALT levels were unchanged in mice killed after 12 and 24 h compared with vehicle control mice. (C) SCID mice (n = 10) were given a standardized diet supplemented with tolcapone (0.05% wt/wt), EGCG (0.15% wt/wt) or the combination for 9.5 weeks. Plasma levels of Me-Norepi were decreased in mice that received the combination treatment. An asterisk indicates a significantly different change compared with controls (P < 0.05). E, entacapone; T, tolcapone.
Lung cancer growth inhibition by EGCG and COMT inhibitors

Anticancer activity. Previous studies have suggested that the methylated metabolites of EGCG have reduced anticancer activity. For example, Fang et al. (19) have reported that both 4′-O-methyl EGCG and 4,4′-O-methyl EGCG have reduced inhibitory potency against DNA methyltransferase in a cell-free system compared with EGCG. Conversely, decreased expression of COMT has been reported to enhance the growth inhibitory activity of EGCG in breast cancer cell lines in culture (20) and to be associated with an enhancement of EGCG-mediated protective effects against breast cancer in human observational studies (21). Indeed, we observed that both tolcapone and entacapone could inhibit the methylation of EGCG in a cell-free system and increase the unmethylated fraction of EGCG in both CL-13 and H1299 cells. The effect in the former cell line was much less pronounced than in the latter, because CL-13 cells have reduced COMT activity compared with H1299 cells. The fact that synergistic growth inhibition is observed in both lines suggests that inhibition of COMT activity is not the only mechanism underlying these synergistic effects.

In addition to their inhibitory activity with regard to EGCG methylation, both nitrocatechols also had growth inhibitory properties as single agents, tolcapone being more potent than entacapone. The effects of tolcapone may be the result of its ability to bind to mitochondrial respiratory proteins (33), thereby causing mitochondrial uncoupling (34). That being said, there is a dearth of literature on the potential anticancer activity of these compounds. Given the widespread clinical use of these compounds, further studies on their potential anticancer effects are warranted.

It has been shown that the inhibition of cell proliferation and associated markers of proliferation can be mediated by EGCG-generated ROS. Previously, EGCG has been shown to cause increased intracellular oxidative stress in the H1299 cell line both in culture and as xenograft tumors on immunocompromised mice (16). In this study, we found that EGCG as a single agent increased intracellular oxidative stress in the H1299 cell line but not in the CL-13 cell line. In combination with tolcapone or entacapone, however, there was a dramatic increase in intracellular oxidative stress in both cell lines. Further studies are needed to determine if these effects are relevant in vivo.

In this study, the combination treatments had greater than additive effects on cell cycle of both cell lines. Interestingly, the effects were different between cell lines with H1299 cells arresting in G0/G1 phase, whereas CL-13 cells arrested in G2/M. H1299 cell growth was arrested to a greater extent and at an earlier time point by the combinations compared with the CL-13 cells. Intracellular ROS was also induced with lower combination treatments and at an earlier time point in the H1299 cells compared with the CL-13 cell line. This suggests that the H1299 cells were more sensitive to oxidative stress induced by the combinations, and that early ROS generation may cause growth arrest at a similar time. Oxidative stress has also been linked to growth arrest in G2/M (35), which can explain the combination-induced G2 phase growth arrest in the CL-13 cell line. The differences in phase of growth arrest between the two cell lines could be due to differences in upstream targets, differences in doubling time or another species-specific difference.

We found that the EGCG/nitrocatechol combination treatments decreased expression of cyclin D1 in both cell lines but did not affect cyclin A or cyclin B1. Based on this, we would have predicted to observe arrest at the G1/S interphase in both cell lines. Others, however, have reported that downregulation of cyclin D1 is associated with G1/M phase arrest. Manohar et al. (36) have previously reported that P27Kip-1, an inhibitor of hypoxia inducible factor-1, can inhibit cell cycle progression at the G2/M transition and that this inhibition is associated with a decrease in the expression of cyclin D1.

Translocation of the transcription factor NF-κB to the nucleus is a key prosurvival signal and is frequently increased in cancer cells as a means of resistance to chemotherapy. In the present study, we found that combination treatment with EGCG and tolcapone or entacapone had a greater than additive inhibitory effect on nuclear translocation of NF-κB. Previous studies, both in vitro and in vivo, have reported that green tea polyphenols can modulate NF-κB. For example, green tea polyphenols have been previously shown to reduce NF-κB in lung samples derived from diethylnitrosoamine-treated Swiss albino mice (37). EGCG has also been shown to downregulate the gene NF-κB inducing kinase (NIK) (38). Interestingly, we found that treatment with EGCG as a single agent did not affect nuclear NF-κB levels in contrast to these previous reports. It is possible that the concentrations of EGCG used in this study were insufficient to have an effect as a single agent.

Although the synergistic effects in the present study are due in part to inhibition of localized methylation of EGCG in cancer cell lines, it is anticipated that coadministration of EGCG and entacapone or tolcapone to animal models (or human subjects) should alter the pharmacokinetic profile of EGCG by inhibiting COMT activity in plasma and tissues. This is hypothesized to result in not only and increased intracellular levels of unmethylated EGCG, but also an increase in circulating levels of unmethylated EGCG, which would be anticipated to result in further enhancement of cancer preventive activity.

In conclusion, this study found for the first time that the clinically used COMT inhibitors, entacapone and tolcapone, can synergistically enhance the antiproliferative effects of EGCG against lung cancer cells in vitro. This synergy appears to be in part to inhibition on EGCG methylation and increase in intracellular levels of increased intracellular EGCG. Mechanistically, the combination enhanced intracellular oxidative stress and inhibited nuclear translocation of NF-κB. Future work should focus on the modulation of green tea polyphenol bioavailability by COMT inhibitors, and determining the anticancer effects of the combinations in vivo.

Funding
American Institute for Cancer Research (10A102 to J.D.L.).

Acknowledgements
We wish to thank Ryan J.Elias for LC–MS use. We would also like to thank Nicole Zembower, Susan Magargee, Ruth Nisly and Ningchun Xu at the Pennsylvania State University Flow Cytometry Core Facility.

Conflict of Interest Statement: None declared.

References


Received January 23, 2013; revised September 20, 2013; accepted October 11, 2013