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Epigallocatechin-3-gallate augments the therapeutic effects of benzo[a]pyrene-mediated lung carcinogenesis

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Abstract

Our previous study found curcumin and vitamin E to have protective effects against benzo[a]pyrene (BaP) exposure in human normal lung epithelial BEAS-2B cells. The first objective of the present study was to determine whether epigallocatechin-3-gallate (EGCG) elicited the same response. Co-treatment with 5 μ M BaP and 20 μ M EGCG in BEAS-2B promoted a significant reduction in cell viability and greater G2/M cell cycle arrest, induction of ROS, and reductions in BaP-induced CYP1A1/CYP1B1/COMT, EGFR, p-Akt (Ser473), p-p53 (Thr55), and survivin mRNA/protein expression, as well as an increase in p-p53 (Ser15). Based on these findings, the second objective was to extend the investigation by developing a novel BaP-transformed BEAS-2B cell line, BEAS-2B_{BaP}, to examine the effects of EGCG when co-administered with gefitinib, an EGFR tyrosine kinase inhibitor. Cell colony formation assay demonstrated *in vitro* tumorigenic potential of BEAS-2B_{BaP}, which had an overexpression of EGFR. Viability testing revealed gefitinib co-treatment with EGCG resulted in more cell death compared to gefitinib alone. Co-treated cells had greater reductions in gefitinib-induced CYP1A1/CYP1B1, EGFR, cyclin D1, p-Akt (Ser473), and survivin mRNA/protein expression, as well as an increase in p-p53 (Ser15). Therefore, EGCG was found to promote greater cytotoxicity to BEAS-2B co-treated with BaP and BEAS-2B_{BaP} upon gefitinib co-treatment through regulating metabolism enzymes and signaling pathways involving EGFR and p53. These findings suggest that EGCG did not act as a protective compound in BEAS-2B after acute BaP exposure, but has the potential to be a useful adjuvant chemotherapeutic compound when coupled with gefitinib for chemosensitization.

Keywords

EGCG; BaP; gefitinib; EGFR; lung carcinogenesis

Introduction

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer. It is estimated that 80% of lung cancer deaths are attributed to cigarette smoking⁽¹⁾ and the

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polyaromatic hydrocarbon, benzo[a]pyrene (BaP), is the most potent carcinogen ⁽²⁾. The metabolic conversion of BaP by cytochrome P450 (CYP) enzymes to its ultimate carcinogen, BaP-7,8-diol-9,10-epoxide (BPDE), results in phase II detoxification or the formation of BPDE-DNA adducts ⁽³⁾. BaP also stimulates reactive oxygen species (ROS) when it is metabolized to BaP quinones ⁽⁴⁾. The persistent formation of DNA adducts and ROS contribute to the initiation of lung carcinogenesis ⁽⁵⁾.

Patients with advanced or metastatic NSCLC undergo epidermal growth factor receptor (EGFR) genetic testing to determine if there is an EGFR-positive mutation. Activating EGFR mutations are found in 50% of Asians and 10% of non-Asians ⁽⁶⁾. Patients with EGFR-positive mutations respond well to the EGFR tyrosine kinase inhibitor (TKI), such as gefitinib, but eventually develop acquired resistance to the drug ⁽⁷⁾. Although gefitinib greatly improves the tumor response rate and prolongs the patient's progression-free survival, it does not increase overall survival ⁽⁸⁾, so new therapeutic options are desirable.

Green tea has been found to exhibit antimutagenic, anticarcinogenic, and antitumor properties in *in vitro* and *in vivo* models ⁽⁹⁾, and clinical trials involving cancers of the lung, breast, and prostate are ongoing ⁽¹⁰⁾. Epigallocatechin-3-gallate (EGCG) demonstrates the greatest health advantages, such as inhibiting cell growth ⁽¹¹⁾, inducing cell cycle arrest ⁽¹²⁾, stimulating apoptosis ⁽¹³⁾, and inhibiting oncogene expression ⁽¹⁴⁾, so the study of EGCG is of great significance.

Gefitinib acts through the inhibition of PI3K-Akt and MEK-ERK signaling pathways in patients harboring EGFR mutations, and through this treatment tumor cells undergo apoptosis, but the mechanisms involved aren't clearly characterized ⁽¹⁵⁾. The present study found EGCG to promote cytotoxicity in normal lung epithelial cells co-treated with BaP after acute exposure. Co-treatment induced ROS formation, G2/M arrest, a greater reduction in phase I and II enzymes, reductions in EGFR, p-Akt (Ser473), p-p53 (Thr15), and survivin, as well as an upregulation in p-p53 (Ser15). A novel BaP-transformed lung cell line, BEAS-2B_{BaP}, was developed in order to test the possible therapeutic effects of EGCG in combination with gefitinib. The cells displayed greater colony number and area, and higher EGFR expression, indicating possible carcinogenesis initiation. EGCG promoted gefitinib-induced cytotoxicity in BEAS-2B_{BaP} cells. Gefitinib and EGCG co-treated cells also had significant changes in phase I/II enzymes, EGFR, cyclin D1, p-Akt (Ser473), p-p53 (Ser15), and survivin compared to gefitinib alone.

Experimental Procedures

Chemicals

BaP (Sigma, MO, USA), gefitinib (Santa Cruz Biotechnology, CA, USA), and EGCG (95%, Sigma, MO, USA) were all dissolved in dimethylsulfoxide (DMSO, MO, USA) to make 39.6 mM, 50 mM, and 50 mM stock solutions, respectively. LHC-9 growth medium, penicillin/streptomycin, and fetal bovine serum (FBS) were purchased from Thermo Scientific (PA, USA). Hyclone (1×) porcine trypsin used for subculture was purchased from GE Healthcare Life Sciences (UT, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from USB Corporation (CA, USA) and

trypan blue was from Sigma (MO, USA). Dichlorofluorescein diacetate (DCFDA) was purchased from Sigma (MO, USA) and phosphate buffered saline (1×PBS) was purchased from Gibco Life Technologies (NY, USA). RNeasy® Blood and Tissue Mini kits were purchased from QIAGEN (MD, USA). One-step real time-polymerase chain reaction (RT-PCR) kits with SYBR® green were purchased from Bio-Rad (CA, USA). Primers were purchased from Eurofins (Luxembourg) and Western blot antibodies were purchased from Santa Cruz (CA, USA), Abcam (MA, USA), and GE Healthcare (NJ, USA). Radioimmunoprecipitation (RIPA) lysis buffer was from Santa Cruz Biotechnology (CA, USA) and the enhanced chemiluminescence (ECL) kit was purchased from GE Healthcare (NJ, USA).

Cell culture

Authenticated normal human bronchial epithelial cell line, BEAS-2B (CRL-9609), was purchased from the American Type Culture Collection (ATCC). Upon receipt from ATCC, cell stocks were prepared and cells were used for experiments within five passages and the cells were cultured following previous studies^(16, 17). A novel BaP-transformed BEAS-2B cell line, named BEAS-2B_{BaP}, was created by treating BEAS-2B cells with 5 µM of BaP for fifteen continuous generations (once per week). Therefore, BEAS-2B_{BaP} cells passaged less than 4 months were used for this study.

Cell viability assays

For the trypan blue exclusion assay, BEAS-2B cells were seeded into 48-well plates at a density of 3×10^4 cells per well and treated for 24 h. The cells were trypsinized, suspended in a 2× dilution factor of trypan blue dye, and then viable cells were counted using a light microscope and a hemocytometer. The following equation was used to calculate the percent cell viability: number of live cells/total number of cells × 100. The MTT assay was implemented to quantify BEAS-2B_{BaP} cell viability upon 24 and 48 h treatment following a previous study⁽¹⁸⁾. Absorbance was measured using a Synergy 4 BioTek microplate reader (VT, USA). This experiment was performed independently in triplicate, with a total of six replicates per treatment in each experiment. Percent cell viability was calculated using the following equation: mean absorbance of test wells/mean absorbance of control wells × 100.

Analysis of cell cycle by flow cytometry

Flow cytometry was employed to observe the effect of EGCG on BaP-induced cell cycle arrest after 24 h in BEAS-2B cells following our previous study⁽¹⁹⁾. The treatments included vehicle control (0.06% DMSO), BaP (5 µM), EGCG (20 µM), and BaP+EGCG (5 µM BaP and 20 µM EGCG). Approximately 5×10^3 events were captured and the percentage of cells in G0/G1, S, and G2/M phases of the cell cycle were defined with GuavaSoft (Millipore Corporation, MA, USA) software⁽¹⁷⁾. All treatments were run in triplicate and each experiment was repeated three times.

ROS production

As previously described, DCFDA was used to measure ROS activity in BEAS-2B and BEAS-2B_{BaP} cells⁽¹⁹⁾. Cells were seeded into 96-well plates at 5×10^3 cells per well and

treated for 2, 4, 8, 12, and 24 h in three independent experiments. ROS fluorescence was then measured using a Synergy 4 BioTek microplate reader (VT, USA). The fluorescence in treated cells at each time point was compared to its respective control. Experiments were performed independently in triplicate.

Soft agar colony assay

Following our previous study ⁽¹⁷⁾, BEAS-2B and BEAS-2B_{BaP} cells were grown using the soft agar colony assay and fed every 3 days with LHC-9 culture medium for a total of 11 days. At the cessation of the study, the colonies were stained with 0.005% crystal violet and then visualized and scored using an image analyzer in conjunction with a microscope. The reported number of colonies and the colony area were calculated from six random areas per well and then averaged. Lung adenocarcinoma A549 cell line was used as a positive control (data not shown).

RNA isolation and quantitative real-time PCR (qRT-PCR)

Following the RNeasy[®] plus mini kit manual instructions, total RNA was isolated from 1×10^6 BEAS-2B and BEAS-2B_{BaP} cells treated in 6-well plates for 24 h. Total RNA (50 ng) was amplified using a one-step RT-PCR kit with SYBR[®] green following manufacturer instructions and a previous study ^(17, 19). Melt curve analysis was used to determine the specificity of the PCR products, and glyceraldehyde 3-phosphate (GAPDH) served as the housekeeping gene. The Ct values of the genes of interest were normalized with GAPDH and the 2^{-Ct} method was employed to calculate the fold-change in gene expression. Experiments were performed independently in triplicate. Non-template controls were included for every primer tested. Primer sequences are included in Table 1.

Western blot

BEAS-2B and BEAS-2B_{BaP} cells were treated for 24 h and Western blot was conducted following previous studies ^(17, 19). The intensities for all the bands of interest were quantified using ImageJ software and normalized by tubulin.

Statistical analyses

Factorial analysis of variance (ANOVA) was performed to test the effects of BaP, EGCG, and/or gefitinib on cell cytotoxicity and ROS production. One-way ANOVA was used to determine the difference in soft agar assay and gene/protein expression between groups followed by a Fisher's LSD test. All analyses were performed using SPSS 13.0 software and ImageJ software was used to determine protein changes in the Western blot bands. Differences at $P < 0.05$ were considered statistically significant.

Results

EGCG increased BaP-induced cytotoxicity

Cell viability upon EGCG treatment (0–40 μ M) after 24 and 48 h was determined using the trypan blue assay in BEAS-2B cells (data not shown). Five μ M BaP was used based on our previous study ⁽¹⁹⁾ and the viability of BEAS-2B cells co-treated with BaP and EGCG (0–20

μM) for 24 h was tested using the trypan blue exclusion assay (Figure 1A). BaP alone significantly decreased the number of viable cells when compared to the control ($P<0.01$) and cells co-treated with BaP+EGCG had a significant decrease in viability compared to BaP alone ($P<0.01$). EGCG-promoted BaP-induced cell cytotoxicity in BEAS-2B cells was further confirmed in HBEC3-KT cell line (ATCC CRL-4051, a normal human bronchial epithelial cell, data not shown).

EGCG increased BaP-induced G2/M arrest

Flow cytometry analysis determined that BEAS-2B cells treated with BaP and/or EGCG for 24 h displayed a significant decrease in cells in the G0/G1 phase when compared to the control ($P<0.05$) (Table 2). BaP+EGCG co-treatment promoted a significant reduction in cells in G0/G1 compared to BaP alone ($P<0.01$). There were no significant changes to S phase, but a significant increase in the number of BaP- and EGCG-treated cells were measured in G2/M phase compared to the control ($P<0.05$). Co-treatment with BaP+EGCG resulted in a significant increase in G2/M phase compared to the BaP-treated cells ($P<0.05$).

EGCG promoted BaP-induced ROS

EGCG alone promoted minimal changes to ROS formation compared to the control at all observed time points (Figure 1B). Maximum ROS formation was observed in BEAS-2B cells treated with BaP alone or BaP+EGCG for 4 h. Cells treated with BaP for 4 h and BaP+EGCG for 2, 4, and 8 h promoted greater ROS production compared to the control ($P<0.05$). At 2, 4, and 8 h, BaP+EGCG also had significantly greater ROS formation when compared to BaP alone ($P<0.05$). EGCG-promoted BaP-induced ROS in BEAS-2B cells was further confirmed in HBEC3-KT cell line (data not shown).

EGCG reduced BaP-induced metabolism and promoted cell cytotoxicity through signaling pathways

CYP1A1 and CYP1B1 had significant changes in mRNA expression upon treatment (CYP1A2 and CYP3A4 data not shown). A significant increase in CYP1A1 mRNA expression in cells treated with BaP and BaP+EGCG compared to the control was observed ($P<0.01$) (Figure 2A). BaP caused a 10,949% increase and BaP+EGCG resulted in a 6,927% increase in mRNA expression. BaP+EGCG treatment promoted a significant decrease in CYP1A1 expression compared to BaP alone ($P<0.01$). When CYP1B1 expression was measured, a 486% and 223% increase in BaP- and BaP+EGCG-treated cells was observed compared to the control ($P<0.05$). A significant decrease occurred in BaP+EGCG-treated cells when compared to BaP-treated cells ($P<0.05$). Catechol-O-methyl transferase (COMT) showed significant changes, unlike glutathione peroxidase 1 (GPX1) and superoxide dismutase 2 (SOD2) (data not shown). EGCG and BaP+EGCG treatment resulted in a 82% and 79% decrease in gene expression, respectively, when compared to the control ($P<0.05$). A significant reduction occurred in cells treated with BaP+EGCG compared to BaP ($P<0.05$).

EGFR and survivin mRNA expression was measured in BaP- and/or EGCG-treated BEAS-2B cells (Figure 2B). EGFR expression significantly decreased to 63%, 75%, and 52% in BaP-, EGCG-, and BaP+EGCG-treated cells when compared to the control ($P<0.01$).

Survivin expression significantly decreased to 85% and 71% in BEAS-2B cells treated with EGCG and BaP+EGCG, respectively, when compared to the control ($P<0.01$). BaP+EGCG co-treatment promoted a significant decrease in survivin expression compared to BaP alone ($P<0.01$).

A reduction in EGFR protein expression was observed in cells treated with EGCG (35%) and BaP+EGCG (23%) compared to the control and BaP treated cells, respectively (Figure 2C&2D). No protein changes among treatments were observed when measuring total Akt and p53. When p-Akt (Ser473) was measured, a large decrease in protein expression occurred in BaP+EGCG co-treated cells compared to the other treatments (58% and 85% compared to the control and BaP, respectively). BaP+EGCG treatment (1,889%) promoted a large increase in protein expression, followed by EGCG (1,222%) and BaP treatment (950%) compared to the control when p-p53 (Ser15) was measured. Reductions in p-p53 (Thr55) expression occurred in BaP- (43%) and BaP+EGCG-treated (42%) cells compared to the control.

Establishment of BEAS-2B_{BaP}

BEAS-2B cells were exposed to 5 μ M BaP for 15 passages, once per week continuously. Untreated BEAS-2B cells retained a normal morphology characterized by a smaller, polygonal and cuboidal shape ⁽²⁰⁾, while transformed cells were larger and rotund, which has been observed in another chemically-transformed BEAS-2B cell line (Figure 3A) ⁽²¹⁾. A significant increase in colony number and area was observed in BEAS-2B_{BaP} cells compared to BEAS-2B cells (Number: 79 ± 5.3 for BEAS-2B, 95 ± 2.6 for BEAS-2B_{BaP}; Area: 169 ± 15.2 for BEAS-2B, 286 ± 39.6 for BEAS-2B_{BaP}, $P<0.01$; BEAS-2B_{BaP} were $>10\times$ smaller than positive control A549, data not shown) (Figures 3B&3C). EGFR mRNA expression was upregulated to 184% in BEAS-2B_{BaP} compared to BEAS-2B ($P<0.05$) (Figure 3D). Cyclin D1 was measured and a significant 1,648% increase occurred in BEAS-2B_{BaP} ($P<0.01$). Western blot analysis confirmed the upregulation of EGFR in BEAS-2B_{BaP} cells compared to BEAS-2B cells (Figure 3E).

EGCG increased gefitinib-induced cytotoxicity

The individual effects of gefitinib (Gef) and co-treatment of Gef with EGCG after 24 and 48 h was tested using MTT assay (Figure 4). Based on published reports, Gef cytotoxicity was tested at 0, 2, 4, 8, 16, and 32 μ M ^(22, 23). After 24 h of treatment, Gef-treated BEAS-2B_{BaP} cells underwent significant cytotoxicity starting at 4 μ M to 32 μ M compared to the control ($P<0.05$). Cells co-treated with Gef and EGCG for 24 and 48 h showed significant cytotoxicity compared to Gef alone ($P<0.01$).

EGCG modulated gefitinib-induced phase I & II metabolism

BEAS-2B_{BaP} cells were treated with control, 20 μ M EGCG, 4 μ M Gef, 4 μ M Gef+EGCG, 16 μ M Gef, and 16 μ M Gef+EGCG, and mRNA expression was analyzed (Figure 5A). CYP1A1 expression was significantly decreased to 46% in cells treated with EGCG for 24 h compared to the control ($P<0.01$). Additionally, 483%, 136%, and 603% increases in 4 μ M Gef, 4 μ M Gef+EGCG, and 16 μ M Gef mRNA expression were found to be significant compared to the control ($P<0.01$). Co-treatment with EGCG promoted a significant decrease

of CYP1A1 in Gef+EGCG-treated cells compared to Gef alone ($P<0.01$). CYP1B1 expression was reduced to 52%, 42%, and 51% in EGCG-, 4 μ M Gef+EGCG-, and 16 μ M Gef+EGCG-treated cells and it was increased to 172% and 178% in 4 μ M Gef and 16 μ M Gef treatments compared to control ($P<0.01$). A significant decrease in CYP1B1 in the co-treated cells compared to the Gef-alone ($P<0.01$) occurred. There were no significant changes in CYP2D6 and CYP3A4 mRNA expression (data not shown). BEAS-2B_{BaP} cells treated with EGCG, 4 μ M Gef, 4 μ M Gef+EGCG, 16 μ M Gef, and 16 μ M Gef+EGCG decreased to 71%, 43%, 77%, 77%, and 63% in uridine 5'-diphospho-glucuronosyltransferase 1A1 (UGT1A1) expression, respectively, when compared to the control ($P<0.05$). A significant decrease between 16 μ M Gef and 16 μ M Gef+EGCG treatments was observed ($P<0.05$). There were no observed changes to mRNA UGT1A7, SOD2, GPX1, and glutathione S-transferase P (GSTP1) expression at any of the treatments tested (data not shown).

EGCG promoted gefitinib-induced cell death through regulating signaling pathways

EGFR mRNA expression decreased to 64%, 21%, and 20% upon EGCG, 4 μ M Gef+EGCG, and 16 μ M Gef+EGCG treatment compared to the control ($P<0.05$). Cells treated with 4 μ M Gef+EGCG and 16 μ M Gef+EGCG had a significant decrease in EGFR expression compared to their respective Gef treatment ($P<0.01$). Cyclin D1 mRNA expression was downregulated to 56%, 28%, 14%, 29%, and 15% in cells treated with EGCG, 4 μ M Gef, 4 μ M Gef+EGCG, 16 μ M Gef, and 16 μ M Gef+EGCG, respectively, compared to the control ($P<0.01$) (Figure 5B). Co-treated cells demonstrated a significant downregulation in cyclin D1 compared to their respective Gef treatment ($P<0.01$). EGCG treatment promoted a 14%, 24%, and 40% decrease in survivin expression in the EGCG-, 4 μ M Gef+EGCG-, and 16 μ M Gef+EGCG-treated cells compared to the control ($P<0.01$). Survivin mRNA expression significantly decreased in co-treated cells compared to their respective Gef treatment ($P<0.01$).

EGFR protein expression in cells co-treated with Gef+EGCG revealed a remarkable downregulation of EGFR compared to Gef alone (64% in 4 μ M Gef+EGCG vs. 4 μ M Gef and 51% in 16 μ M Gef+EGCG vs. 16 μ M Gef) (Figure 5C&5D). Gefitinib treatment decreased p-Akt (Ser473) expression compared to the control (69% in 4 μ M gefitinib and 55% in 16 μ M Gef), and a more pronounced reduction was observed after co-treatment with Gef+EGCG (57% in 4 μ M Gef+EGCG vs. 4 μ M Gef and 33% in 16 μ M Gef+EGCG vs. 16 μ M Gef). There were no observable changes in total p53 expression, but p-p53 (Ser15) was upregulated in the co-treated cells compared to cells treated with Gef alone (496% in 4 μ M Gef+EGCG vs. 4 μ M Gef and 753% in 16 μ M Gef+EGCG vs. 16 μ M Gef). Co-treatment results in a reduction in survivin, especially for the 16 μ M Gef+EGCG treatment (30%) compared with 16 μ M Gef treatment alone. Finally, COMT protein expression was decreased after EGCG treatment (58%) compared with control, and co-treatment promoted noticeable decreases compared to Gef alone-treated cells (66% in 4 μ M Gef+EGCG vs. 4 μ M Gef and 34% in 16 μ M Gef+EGCG vs. 16 μ M Gef).

Discussion

All studies used EGCG at 20 μ M due to its biological application and lack of individual cytotoxicity⁽¹⁸⁾. BaP promoted significant G2/M arrest, and it is widely accepted that BaP exposure can cause DNA damage, so G2/M arrest allows for DNA repair prior to mitosis to prevent the propagation of mutated cells⁽²⁴⁾. Either G1 or G2/M arrest induction upon EGCG treatment has been observed in esophageal⁽²⁵⁾ and lung⁽¹²⁾ cancer cell lines. Cells co-treated with BaP and EGCG had a significant induction of ROS compared to the BaP-treated cells after 2, 4, and 8 h. Hwang *et al.* found that colon cancer cells treated with EGCG for 6 h induced ROS formation, so the same phenomena could have occurred in the present study⁽²⁶⁾. EGCG has been shown to exhibit both antioxidative and prooxidative effects in different cell lines and in *in vivo* studies under varying experimental conditions^(27, 28), but more studies in this context would be worthwhile.

Our phase I metabolism findings are consistent with well-established literature that asserts the induction of CYP1A1 and CYP1B1 by BaP^(29, 30). EGCG has also been shown to be a formidable inhibitor of CYP1A1 and other phase I substrates⁽³¹⁾, as seen in the present study. By inhibiting phase I metabolism of BaP in BEAS-2B cells, EGCG could trigger BaP to be metabolized *via* different routes in an attempt to decrease the number of DNA damaging BPDE adducts (data not shown), but despite this effort, other cellular processes foster EGCG-promoted cell death in the co-treated cells. The phase II enzyme COMT was also decreased upon EGCG co-treatment. COMT is heavily involved in the detoxification/inactivation of a variety of compounds and EGCG has been found to inhibit COMT activation *in vitro*⁽³²⁾. Therefore, the inhibition of CYP1A1/1B1 and COMT by EGCG could retard BaP metabolism, thereby increasing ROS production, resulting in greater cytotoxicity.

In the present study, EGCG promoted greater inhibition of EGFR and its downstream target Akt and these findings are consistent with other studies in cancer cells⁽³³⁾. Although there were no changes to total Akt protein expression, p-Akt (Ser473) expression was reduced in the cells treated with EGCG, which is expected since the trend is similar to that of EGFR. Upregulation of total Akt has also been characterized as an important regulator of carcinogenesis in numerous cancers⁽³⁴⁾, but the data surrounding the role of p-Akt is equivocal⁽³⁵⁾.

BaP is known to induce the tumor suppressor protein, p53, which is often mutated in NSCLC, which negates the DNA repair and apoptotic role of p53^(36, 37). While there were no observable changes to total p53 protein expression, p-p53 (Ser15) increased in co-treated cells whereas p-p53 (Thr55) decreased. Many studies, including ours, have found that cancer cells nullify p53 by exporting it from the nucleus to the cytoplasm, where it loses its ability to promote cell cycle arrest and apoptosis^(17, 38). Phosphorylation of p53 at Thr55⁽³⁹⁾ and Ser15^(19, 40) play an important role in the nucleocytoplasmic shuttling of p53. Therefore, the observed increase in p-p53 (Ser15) and downregulation of p-p53 (Thr55) indicates p53 activation and functioning as a transcription factor in the nucleus after BaP and EGCG exposure, to further induce G2/M arrest and cytotoxicity. Furthermore, EGCG and co-

treatment of BEAS-2B cells had a significant reduction in survivin expression, which has been seen in breast cancer cells upon EGCG treatment ⁽⁴¹⁾.

Our newly developed BEAS-2B_{BaP} cells served as a novel model due to EGFR overexpression and *in vitro* tumor-like properties, revealed by the anchorage independent growth assay. The concentrations of gefitinib used were based on our cytotoxicity studies and those by other groups ⁽⁴²⁾. EGCG and gefitinib co-treatment exerted greater cytotoxic effects in BEAS-2B_{BaP} cells. While BaP greatly contributed to ROS formation in BEAS-2B cells with EGCG, gefitinib did not induce ROS formation in BEAS-2B_{BaP} cells (data not shown); suggesting the mediation of ROS *via* EGCG could be contingent on the cell line and/or treatment.

It has been reported that CYP3A4 is the major phase I enzyme involved in the hepatic metabolism of gefitinib ⁽⁴³⁾ along with CYP3A5, CYP2D6, and CYP1A1, with a finite amount of CYP1A2 and CYP1B1, in human supersomes ⁽⁴⁴⁾. The present study found CYP1A1 and CYP1B1 to be the predominant phase I enzymes involved in gefitinib metabolism in BEAS-2B_{BaP} cells. Alfieri *et al.* found CYP1A1 to be the primary enzyme involved in gefitinib metabolism in NSCLC cells ⁽⁴⁵⁾. Negligible gefitinib-induced CYP1B1 induction has also been established in liver and intestinal microsomes ⁽⁴⁴⁾. Both CYP1A1 and CYP1B1 expression in BEAS-2B_{BaP} cells were significantly reduced by EGCG treatment, so it is possible that EGCG-induced phase I metabolism inhibition promotes greater intracellular gefitinib activity in the cells, thereby compounding cytotoxicity. UGT1A is involved with the glucuronidation of compounds and it is found predominately in the liver, with minimal extrahepatic expression ⁽⁴⁶⁾. Both gefitinib ⁽⁴⁷⁾ and EGCG ⁽⁴⁸⁾ treatment have been found to promote UGT1A1 inhibition in human liver microsomes, which is similar to the findings of the present study. Minimal UGT1A expression has been measured in the lungs ⁽⁴⁶⁾, which the present study confirmed. Additionally, COMT protein expression significantly decreased with co-treatment as well. Therefore, co-treatment with EGCG resulted in an attenuation of phase I and II enzymes, which could promote greater cytotoxicity by slowing the elimination of gefitinib from the cell and prolonging its intracellular effects.

EGFR inhibition was more pronounced in BEAS-2B_{BaP} cells co-treated with gefitinib and EGCG. Cyclin D1, a downstream effector of EGFR, was inhibited by gefitinib, which was further augmented by EGCG co-treatment, presumably as a result of the EGCG-induced inhibition of EGFR ⁽⁴⁹⁾. As a downstream effector of EGFR, co-treatment resulted in a concerted inhibition of p-Akt (Ser473). Our data are in agreement with a previous study demonstrating EGCG's inhibitory effects on the tyrosine kinase activity of EGFR in epidermoid carcinoma cells ⁽⁵⁰⁾. Moreover, the synergistically increased p-p53 (Ser15) expression and downregulation of survivin in BEAS-2B_{BaP} cells after gefitinib and EGCG co-treatment provide further explanation of the observed cytotoxicity data. However, some discrepancies between mRNA and protein expression were observed in EGFR and survivin after gefitinib and/or EGCG treatment. Previous studies have demonstrated that the correlation between mRNA levels and protein products of specific genes vary widely ^(51, 52). It is possible that different regulations of expression in mRNA and/or protein levels, especially post-transcriptional, translational, and protein degradation regulation, might

contribute significantly to these findings ⁽⁵²⁾. Nevertheless, our findings demonstrate the possible chemotherapeutic potential of gefitinib and EGCG co-treatment.

In summary, EGCG produced significant cytotoxicity to tumor-like BEAS-2B_{BaP} cells when co-treated with gefitinib and it is possible that gefitinib and EGCG work through the aforementioned metabolism and cell signaling pathways to have a synergistic effect on cell death in BEAS-2B_{BaP} cells. NSCLC is the number one cause of cancer-related death globally and with >10% of patients exhibiting EGFR mutations ⁽¹⁾, increasing the sensitivity of EGFR TKIs while simultaneously reducing the incidence of acquired or secondary resistance is of great interest to the advancement of treatment options. Future studies examining the role of EGCG in gefitinib-resistant cell lines would be valuable and possibly provide greater insight into the mechanisms involved in the current study. Finally, it appears that EGCG could play a pivotal role in the treatment of cells harboring EGFR overexpression/activation, and better characterization of its individual mode of action and even possible therapeutic roles in conjunction with gefitinib in NSCLC patients is valuable.

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Figure 1A **Figure 1B**

Figure 1. The effects of BaP, EGCG, and BaP+EGCG on cytotoxicity and ROS production
(A) BEAS-2B cells were treated with BaP (5 µM) and/or EGCG (0–20 µM) for 24 h and cell viability was measured with trypan blue assay. Experiments performed in triplicate yielded similar results. Values are represented as means $\pm$ SD, $n=6$. **(B)** DCFDA assay was used to measure ROS formation. Data are expressed as means $\pm$ SD of six replicates. BaP: 5 µM BaP; EGCG: 20 µM EGCG; and BaP+EGCG: 5 µM BaP and 20 µM EGCG. Bars bearing

the letter 'a' indicate no significant differences compared to control, those labeled 'b' denote a significant difference when compared to the control, and 'c' denotes a significant difference when BaP+EGCG is compared to BaP alone.

Figure 2A **Figure 2B**

Figure 2C **Figure 2D**

Figure 2. qRT-PCR and Western blot in BEAS-2B cells upon BaP and EGCG treatment
(A) Quantitative RT-PCR analysis of CYP1A1, CYP1B1, and COMT gene expression was measured in BEAS-2B cells treated with BaP and/or EGCG for 24 h. **(B)** Quantitative RT-PCR analysis of EGFR and survivin gene expression was measured in BEAS-2B cells treated with BaP and/or EGCG for 24 h. Relative gene expression values were calculated following the $\Delta\Delta C_t$ method. Data are expressed as means $\pm$ SD of three replicates. **(C)** Western blot of EGFR, Akt, p-Akt (Ser473), p53, p-p53 (Ser15), and p-p53 (Thr55) protein expression in BEAS-2B cells. BaP: 5 μ M; EGCG: 20 μ M; and BaP+EGCG: 5 μ M BaP and 20 μ M EGCG. **(D)** The intensity of each band was semi-quantified using ImageJ software and each treatment was normalized using tubulin and compared to the control. Bars bearing

the letter 'a' indicate no significant differences compared to control, those labeled 'b' denote a significant difference when compared to the control, and 'c' denotes a significant difference when BaP+EGCG is compared to BaP alone.

Figure 3A **Figure 3B**

Figure 3C **Figure 3D**

Figure 3E

Figure 3. Effects of BaP on BEAS-2B transformation (BEAS-2B_{BaP} cells)

(A) Cell morphology of BEAS-2B cells after 15 generations of 5 μ M BaP exposures. (B) Colonies of BEAS-2B and BEAS-2B_{BaP} cells as shown by anchorage-independent growth assay. (C) Colony number and area of BEAS-2B and BEAS-2B_{BaP} cells. Values are represented as means $\pm$ SD, n=6. (D) Quantitative RT-PCR analysis of EGFR and cyclin D1 was measured in BEAS-2B and BEAS-2B_{BaP} cells. Relative gene expression values were calculated following the Δ Ct method. (E) Western blot of EGFR in BEAS-2B and BEAS-2B_{BaP} cells. Bars bearing the letter 'a' indicate no significant differences compared to BEAS-2B, and those labeled 'b' denote a significant difference when compared to BEAS-2B.

Figure 4. The cytotoxic effects of gefitinib (Gef) and/or EGCG on BEAS-2B_{BaP} cells
BEAS-2B_{BaP} cells were treated with Gef (0–32 μM) and EGCG (20 μM) for 24 and 48 h and cell viability was determined using MTT assay. Experiments performed in triplicate yielded similar results. Values are represented as means ± SD, n=6.

Figure 5A **Figure 5B**

Figure 5C **Figure 5D** **Figure 5. qRT-PCR and Western blot in BEAS-2B_{BaP} cells treated with gefitinib (Gef) and EGCG**

(A) Quantitative RT-PCR analysis of CYP1A, CYP1B1, and UGT1A1 gene expression in BEAS-2B_{BaP} cells after 24 h Gef and/or EGCG (20 μ M) treatment. (B) Quantitative RT-PCR analysis of cyclin D1, EGFR, and survivin gene expression in BEAS-2B_{BaP} cells after 24 h Gef and/or EGCG (20 μ M) treatment. Relative gene expression values were calculated following the Δ Ct method. Data shown are means $\pm$ SD of three replicates. (C) Western blot of EGFR, p-Akt (Ser473), p53, p-p53 (Ser15), survivin, and COMT expression in

BEAS-2B_{BaP} cells after 24 h Gef and/or EGCG (20 μ M) treatment. **(D)** The intensity of each band was semi-quantified using ImageJ software and each treatment was normalized using tubulin and compared to the control. Bars bearing an 'a' indicate no significant difference compared to the control, 'b' is significantly different from the control, 'c' is a significant difference between 4 μ M Gef and 4 μ M Gef+EGCG, and finally, 'd' is a significant difference between 16 μ M Gef and 16 μ M Gef+EGCG.

Table 1

Forward and reverse primers of genes used in qRT-PCR

Primer	Forward primer (5' to 3')	Reverse primer (5' to 3')
GAPDH	GGTGGTCTCCTCTGACTTCAACA	GTTGCTGTAGCCAAATTCGTTGT
CYP1A1	TGGTCTCCCTTCTCTACACTCTTGT	ATTTTCCCTATTACATTAAATCAATGGTTCT
CYP1A2	AACAAGGGACACAACGCTGAAT	GGAAGAGAAACAAGGGCTGAGT
CYP1B1	CACTGCCAACACCTCTGTCTT	CAAGGAGCTCCATGGACTCT
CYP2D6	CCTACGCTTCCAAAAGGCTTT	AGAGAACAGGTCAGCCACCACT
CYP3A4	CTTCATCCAATGGACTGCATAAAT	TCCCAAGTATAAACTCTACACAGACAA
UGT1A1	GAAGGTCATGTGATCTGAATGAGA	TTTTGTCTGGCTGTTCCCACT
UGT1A7	GCACTTCGCAATGGTGCC	GTGGTCGTAGTCATGCCAGA
COMT	TCCTTCTCAACTGCCATTCC	CCTTAGCGTTAGCGTCCGTC
SOD2	GCTGACGGCTGCATCTGTT	CCTGATTGGACAAGCAGCAA
GSTP1	GCCCTACACCGTGGTCTATT	GGTCTCCGTCCTGGAACCTTG
GPX1	TATCGAGAATGTGGCGTCCC	TCTTGGCGTTCTCCTGATGC
EGFR	AGGACCAAGCAACATGGTCA	CCTTGCAGCTGTTTTCACCT
Survivin	TGCCCCGACGTTGCC	CAGTTCTTGAATGTAGAGATGCGGT
Cyclin D1	AACTACCTGGACCGCTTCCT	CCACTTGAGCTTGTTACCA

Table 2

Effects of EGCG on BaP-induced cell cycle in BEAS-2B cells

	G0/G1	S	G2/M
Control	63.3±2.34 ^a	11.5±1.23 ^a	23.1±1.53 ^a
BaP	52.3±1.34 ^b	13.7±1.58 ^a	32.7±2.35 ^b
BaP+EGCG	43.4±0.08 ^{b,c}	14.9±1.57 ^a	39.5±1.54 ^{b,c}
EGCG	55.7±0.52 ^b	12.3±0.84 ^a	29.6±0.02 ^b

Data are represented as mean $\pm$ SD (n=3). Mean values within the same row containing different superscript letters indicates significance among the treatment groups following a One-way ANOVA and a Fisher's LSD test ($P < 0.05$). Values bearing the letter 'a' indicate no significant differences compared to control, those labeled 'b' denote a significant difference when compared to the control, and 'c' denotes a significant difference when BaP+EGCG is compared to BaP alone.