Synergistic Inhibition of Lung Tumorigenesis by a Combination of Green Tea Polyphenols and Atorvastatin

Gang Lu,1 Hang Xiao,1 Hui You,1 Yong Lin,2,3 Huanyu Jin,1 Blake Snagaski,1 and Chung S. Yang1,3

Abstract

**Purpose:** The present study investigated the possible synergistic inhibitory effect of a novel combination of polyphenon E (PPE, a standardized green tea polyphenol preparation) and atorvastatin (trade name Lipitor) in a mouse tumorigenesis model and in human lung cancer H1299 and H460 cell lines.

**Experimental Design:** Female A/J mice were given two weekly i.p. injections of 4- (methyl nitrosoamino)-1-(3-pyridyl)-1-butanone (150 mg/kg total dose); 1 week later, mice were treated with PPE (0.25% or 0.5% in drinking fluid), atorvastatin (200 or 400 ppm in diet), or PPE (0.25%) plus atorvastatin (200 ppm) for 16 weeks. The interaction of these two agents was also studied in human lung cancer H1299 and H460 cells.

**Results:** The individual agents, PPE or atorvastatin, were not effective in inhibiting lung tumorigenesis. The low-dose combination of PPE and atorvastatin, however, significantly reduced both the tumor multiplicity and tumor burden (by 56% and 55%, respectively, \( P < 0.05 \)). Iso- bologram analysis of the interaction of the two agents indicated that the combination synergistically decreased tumor multiplicity (\( P = 0.0006 \)) and tumor burden (\( P = 0.0009 \)). The inhibition was associated with enhanced apoptosis and suppressed myeloid cell leukemia 1 (Mcl-1) level in adenoma as determined by immunohistochemistry and Western blots. Treatment with combinations of PPE and atorvastatin also synergistically decreased the number of viable H1299 and H460 cells as determined by isobologram analysis. This synergistic effect was associated with increased apoptosis as determined by the terminal deoxyribonucleotide transferase – mediated nick-end labeling assay. The combination of PPE and atorvastatin was more efficient in reducing the antiapoptotic protein Mcl-1 level and increasing the cleaved caspase-3 and cleaved poly(ADP)-ribose polymerase level than the single-agent treatment.

**Conclusions:** The present work showed that PPE and atorvastatin synergistically inhibited 4- (methyl nitrosoamino)-1-(3-pyridyl)-1-butanone – induced lung tumorigenesis in mice and the growth of lung cancer H1299 and H460 cells, possibly through enhanced apoptosis. The results provide leads for future research on the application of this combination for the prevention and treatment of lung cancer.

**Experimental Design:**

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**Lung cancer** is the leading cause of cancer-related deaths in the United States, and it is also one of the most common cancers worldwide (1). Although new advancements have been made in lung cancer diagnosis and treatment, the overall 5-year survival rate is still less than 5%. The poor lung cancer survival statistics suggest that, in addition to smoking cessation, there is an urgent need for additional approaches for the prevention of this deadly disease. Chemoprevention, defined as the administration of natural or synthetic compounds to inhibit, retard, or reverse the process of carcinogenesis, could be an effective approach to reduce the risk of developing cancer (2, 3). To date, hundreds of natural or synthetic compounds have been found to possess promising cancer chemopreventive actions.

Green tea has been found to have cancer chemopreventive activities and has received a great deal of attention from researchers and the general public. Studies in animals have shown that green tea and its constituents (mostly tea polyphenols and caffeine) inhibit carcinogenesis in the skin, lung, oral cavity, esophagus, stomach, small intestine, colon, liver, prostate, bladder, and other organs (4). Using a 4-(methyl nitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) – induced lung carcinogenesis model, we have shown that administration of green tea extracts or decaffeinated green or black tea extracts (as the sole source of the drinking fluid), before or after NNK treatment, significantly reduced the number of lung tumors formed in A/J mice (5, 6). The tumor
inhibitory activity of (-)-epigallocatechin-3-gallate (EGCG), the most abundant and active polyphenol in green tea, has also been shown when it was given in drinking fluid in A/J mice that received NNK (7). Recently, a standardized green tea polyphenol preparation (PPE, containing 65% EGCG, 25% other catechins, and 0.6% caffeine) has been investigated extensively in terms of its cancer chemopreventive activities. We have shown that 0.5% PPE in drinking fluid, given during weeks 20 to 52 after NNK treatment, significantly inhibits lung tumor progression from adenoma to adenocarcinoma (8). It has also been shown that PPE given at the postinitiation stage significantly lowers both tumor multiplicity and tumor load in a dose-dependent manner in a benzo(a)pyrene-induced lung tumor model (9). The National Cancer Institute has assisted the development of PPE as an agent for human chemoprevention studies. The pharmacokinetics of EGCG has been studied (10). The protective effect of PPE on human cervical lesions has also been shown (11).

Atorvastatin (trade name Lipitor), an inhibitor of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, is a commonly used drug for the treatment of hypercholesterolemia. In addition to inhibiting cholesterol biosynthesis, atorvastatin also inhibits the biosyntheses of farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which are required in higher amounts by malignant cells than normal cells for their growth (12, 13). Increased HMG-CoA activity has been found in various types of tumors, including lung carcinoma, colorectal carcinoma, hepatocellular carcinoma, leukemia, and lymphoma (14–16). In theory, inhibitors of this enzyme would suppress tumor development. Several studies in cell culture have shown the anticancer activities of atorvastatin and other HMG-CoA inhibitors (generally known as statins; refs. 17–25). Some epidemiologic studies have shown the protective effects of statin intake on the risks of lung cancer and other types of cancers (26, 27); however, this relationship is overall inconclusive (28). In animal studies, statins have exhibited inhibitory actions against chemically induced carcinogenesis in the lung, colon, mammary gland, liver, lung, and other organs (29–32).

The concept of using a combination of agents for cancer chemoprevention has recently received much attention. Combinations of two or more chemopreventive agents that have different mechanisms of action have been suggested as a promising strategy to maximize efficacies and minimize toxicities (33, 34). For example, it has been shown that the administration of atorvastatin in combination with celecoxib produced enhanced inhibitory actions against carcinogenesis in both azoxymethane-treated rats and APCMi2 mice models (35, 36).

In this study, we investigated the possible synergistic action of PPE and atorvastatin in the inhibition of NNK-induced lung tumorigenesis in A/J mice. The possible cancer inhibitory action and related mechanisms were also studied in this animal model as well as human lung cancer H1299 and H460 cell lines.

Materials and Methods

Animal treatment. Female A/J mice (4 to 6 wk old) were purchased from The Jackson Laboratory. The animals were fed an ALN-93M diet and maintained at 20 ± 2 °C with a relative humidity of 50 ± 10% and with an alternating 12 h light/dark cycle. They were acclimated in our animal facility for 1 wk and then given two weekly doses of NNK (Chemsyn Science Laboratories, total 150 mg/kg b.w., i.p.) or saline. One week after the second NNK injection, the mice were given PPE alone (0.25% or 0.5% in drinking fluid), atorvastatin alone (200 or 400 ppm in diet), or 0.25% PPE plus 200 ppm atorvastatin for 16 wk. Atorvastatin was provided by the National Cancer Institute. PPE was a gift from Dr. Yukihiko Harai (Mitsui Norin Co., Ltd, Tokyo, Japan). PPE is a standardized green tea polyphenol preparation containing 65% EGCG, 7% epicatechin-3-gallate, 3% epigallocatechin, 9% epicatechin, 3% gallatechin gallate, and 0.6% caffeine. The PPE solution was freshly prepared in deionized water every Monday, Wednesday, and Friday. The control animals were given deionized water. Body weight and food consumption were measured weekly. After a treatment period of 16 wk, the animals were sacrificed by cervical dislocation. The lungs of each animal were removed, inflated, and fixed in 10% buffered formalin. The livers and the omental fat pad were also removed and weighed. Visible tumors (>0.1 mm in diameter) on the surface of the lungs were counted, and the sizes were measured. For histopathologic analysis, the formalin-fixed lungs were embedded in paraffin, dorsal sides facing down, so that most of the tumors were sectioned in 6 serial 5-μm sections. Two sections (each covers all five lobes of the lung) were mounted on every glass slide. Three slides (taken from serial sections; numbers 1, 15, and 30) from each sample were stained with H&E for histopathologic analysis.

Immunohistochemistry. Immunohistochemistry was done on lung tissue sections using specific antibodies to detect the localization and to quantify the levels of the positive stainings. The antibodies used include cleaved caspase-3 (R&D Systems) and Mcl-1 (Santa Cruz Biotechnolog). In brief, antigens were unmasked in antigen unmasking solution (DAKO). Endogenous peroxidase was quenched using 3% H2O2 in distilled water. Sections were then blocked for 1 h at room temperature in PBS containing 3% normal serum and incubated with primary antibody overnight at 4 °C. Biotin-conjugated secondary antibody (1:200) and avidin-biotin peroxidase complex were then applied to the sections. Diaminobenzidine (Sigma) was used as a chromogen. Negative controls were processed in the absence of the primary antibody.

Cell culture and treatment. Human lung cancer H1299 and H460 cell lines were obtained from the American Type Cell Collection and were maintained in RPMI 1640 (Mediatech, Inc.) supplemented with 100 units/mL of penicillin, and 0.1 mg/mL of streptomycin (Sigma-Aldrich) at 37°C with 5% CO2 and 95% air. Cells were kept subconfluent, and the medium was changed every other day. All cells used were between 3 and 30 passages. DMSO was used as the vehicle to deliver atorvastatin and PPE, and the final concentration of DMSO in all experiments was 0.1%.

Assay for viable cells. H1299 or H460 cells were seeded in 96-well plates (1,500 per well). After 24 h, cells were treated with serial concentrations of atorvastatin, PPE, and their combinations in 200 μL of serum complete medium. All experiments were carried out in the presence of superoxide dismutase (5 units/mL) and catalase (30 units/mL) to prevent the autooxidation of EGCG in the culture medium (37). At 24 and 48 h after treatment, cells were subjected to 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay. The medium was replaced by 100 μL fresh medium containing 0.5 mg/mL of MTT (Sigma-Aldrich). After 1 h incubation at 37 °C, the MTT-containing medium was removed, and the reduced formazan dye was solubilized by adding 100 μL of DMSO to each well. After gentle mixing, the absorbance was monitored at 550 nm using a plate reader (TECAN, Phenix Research Products).

Analysis of synergy. The analysis was based on Chou and Talalay’s empirical method (38) with modifications. It was assumed that the dose-response model follows \( \log(F) = \log(d) - \log(D) \), which is a linear regression model with the response \( \log(F) = \log(1 - E) \) and the regressor \( \log(d) \). This model was used for agent 1, agent 2, and the combinations of these two agents with fixed ratios of the doses. \( F \) is the fraction of cells that survived; \( d \) is the dose applied; \( D \) is the IC50 of
Cells (2 × 10⁶) were suspended in 200 μL of 2% paraformaldehyde [in PBS (pH 7.4)] for 60 min at room temperature. After centrifugation (1,500 g, 1 min), the cell pellet was washed and then suspended in 200 μL permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. After washing with PBS twice, cells were resuspended in TUNEL reaction mixture for 60 min at 37°C in a humidified atmosphere in the dark. Cells were then washed twice with PBS before being analyzed using a Beckman Coulter flow cytometer (FC500). Data were processed using AXP acquisition and analysis software.

Western blot analyses. The protein concentration was determined using a BCA protein assay kit (Pierce Chemical). Tissue extracts or cell lysates (denatured at 95°C for 5 min in Laemmli sample buffer) containing 20 to 50 μg protein were subjected to SDS-polyacrylamide gel (Bio-Rad) electrophoresis. The gel was transferred onto a polyvinylidene difluoride membrane (Bio-Rad), and the membrane was incubated with a blocking buffer (Li-Cor Biosciences) for 1 h at room temperature. The membrane was then probed with the respective primary antibody in the blocking buffer at 4°C overnight. After washing with TBS thrice, the membrane was incubated with secondary antibodies conjugated to IR fluorophore, Alexa Fluor 680 (Molecular Probes), or IRdye 800 (Rockland Immunochemicals). The membranes were then scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Statistical analysis. SPSS software was used to perform statistical analyses. For simple comparisons between two groups, the two-tailed Student’s t test was used. One-way ANOVA was used for comparisons among multiple groups. The interaction between the two agents in the cell culture studies was analyzed by the method of Chou and Talalay (38), and the interaction index was used to determine whether the interaction was synergistic, additive, or antagonistic, using the delta method for variance determination. In animal studies, the interaction between two agents (synergistic, additive, or antagonistic) was analyzed by the method of Laska et al. (39) based on isobologram analysis. The means of the responses of a single-agent dose level was calculated through linear interpolations from the responses at the two nearby available dose levels. The variances of the responses were calculated accordingly based on the interpolated mean values.

Results

General health status of experimental animals. During the 16-week chemopreventive period, starting 1 week after the second NNK injection, no significant differences in food or fluid consumption among the groups were found. The body weights of the mice in the atorvastatin alone or atorvastatin and PPE combination treatment group were ~8.2% lower than the NNK control groups (P < 0.05; Fig. 1). The lowered body weight by atorvastatin may be partially due to the lowered body fat weight; the omental fat pad weights were significantly lower in the 200 ppm atorvastatin, 400 ppm atorvastatin, or PPE plus atorvastatin groups (0.26, 0.22, or 0.22 g/mouse, respectively) than the control group (0.36 g/mouse). No signs of toxicity or differences in liver weight (expressed as a percentage of body weight) were found in any of the groups. The atorvastatin–treated groups did not alter plasma alanine transaminase activity.

Inhibition of NNK-induced lung tumorigenesis by PPE, atorvastatin, and their combination. All the NNK-treated mice developed lung tumors at the end of the experiment. The tumors were diagnosed as lung adenomas based on our previous standard (40). Lung adenomas were in a solid, papillary, or mixed growth pattern and are generally composed of well-differentiated cells (Fig. 2A). In slightly larger adenomas, a higher degree of polymorphism was observed. No lung tumors were found in the saline control animals, which did not receive NNK treatment. Data on tumor incidence, multiplicity, and tumor burden were summarized in Table 1. The tumor incidence was slightly lower in the group that received the combination treatment of PPE and atorvastatin compared with all the other NNK-treated groups.
(92% versus 100%, P = 0.47, not shown in the table). Treatment with PPE or atorvastatin alone did not produce a significant effect on tumor multiplicity. The high doses of the PPE or atorvastatin alone, however, both significantly reduced the tumor burden (22% reduction for both 0.5% PPE and 400 ppm atorvastatin groups, P < 0.05) compared with the NNK control group. Low-dose treatments showed no effect. Most interestingly, the combination treatment with PPE and atorvastatin significantly reduced both the tumor multiplicity and tumor burden (by 56% and 55%, respectively, P < 0.05). The inhibitory effect of the combination of the two agents at low doses was significantly stronger than the effects produced by either PPE or atorvastatin at their low or high doses. Isobologram analysis revealed a significant synergy of this combination in the inhibition of tumor multiplicity (P = 0.0006) and tumor burden (P = 0.0009).

**Effects of PPE, atorvastatin, and their combination on cell apoptosis and related molecular changes in the mouse.** Cell apoptosis was determined by immunohistochemistry with anti–cleaved caspase-3 antibody (Fig. 2B). About 0.13% tumor cells were positively stained by the anti–caspase-3 antibody in the NNK control group. Combination of 0.25% PPE and 200 ppm atorvastatin or 0.5% PPE alone significantly increased the apoptotic cells to 0.41% or 0.24% (3.2-fold or 1.9-fold increase, respectively, compared with NNK control group). PPE at 0.25% and 400 ppm atorvastatin also increased cell apoptosis, but with no statistical significance. No difference was produced by the 200 ppm atorvastatin treatment. In the normal lung tissues of the NNK-treated and nontreated mice, the apoptotic index (~0.08%) was not affected by the treatments with the chemopreventive agents.

Consistent with the proapoptotic effect of the combined PPE and atorvastatin treatment was the down-regulation of Mcl-1 (Fig. 2D). Mcl-1 is an antiapoptotic protein in the Bcl-2 family and was overexpressed in the adenoma (Fig. 2D). By immunohistochemistry, the level of Mcl-1 was significantly reduced by the combination of PPE and atorvastatin (45% reduction), whereas PPE (0.5%) or atorvastatin (400 ppm) alone only slightly reduced the Mcl-1 level. Results from Western blots also showed that Mcl-1 was overexpressed in lung tumor samples, slightly decreased by the single treatments with PPE or atorvastatin, but markedly decreased by the combination of the two agents (Fig. 2F).

**Synergistic inhibitory action of atorvastatin and PPE in lung cancer cells.** Treatment of human lung cancer H1299 or H460 cells with PPE or atorvastatin decreased the number of viable cells as measured by the MTT assay, and the combination produced a larger than additive effect (Fig. 3A and B). A similar effect on the reduction of the number of tightly attached cells was also observed under the microscope in H1299 cells (Fig. 3C).

To study the nature of the interaction between PPE and atorvastatin, their concentration-dependent inhibitory effect against H1299 or H460 cells were determined when the agents were used individually and in combinations. In these two cell lines, the concentrations of PPE (based on EGCG equivalent) used were 10, 20, 30, 40, 50, 60, 70, and 80 μmol/L when used alone and were 5, 10, 15, 20, 25, 30, 35, and 40 μmol/L when used in combination with atorvastatin. The concentrations of atorvastatin used were one tenth those of the PPE in H1299 cells and one fifth those of the PPE in H460 cells. The ratios of atorvastatin and PPE (1:10 in H1299 cells and 1:5 in H460 cells) were determined experimentally based on the relative potency of these two cell lines.

We used a linear regression model to construct the median effect plots based on the results from the MTT assay. Interaction index-effect plots were then constructed based on the median effect plots, as described in Materials and Methods. As shown in the median effect plots of H1299 cells (Fig. 4A), the dose-response relationship between the number of viable H1299 cells and treatments with PPE, atorvastatin, and their combination fit the linear regression model at 48 h. The corresponding interaction index–effect plots showed that all the dose pairs tested produced interaction indices lower than
1.0 at both the two time points (Fig. 4B). The error bars in the interaction index-effect plots represented a 95% confidence interval of the interaction index value calculated by the delta ANOVA, which indicated statistical significance of the results with 95% confidence. These results showed a synergistic interaction between PPE and atorvastatin in the growth inhibition of H1299 human lung cancer cells. The synergistic inhibitory effects between PPE and atorvastatin were also observed in reducing viable H460 human lung cancer cells (Fig. 4C and D). Similar results were observed at 24 h in both cell lines (data not shown).

**Cellular and molecular events involved in the inhibitory action.** The effects of the treatments for 72 h on H1299 cell apoptosis were detected by the TUNEL assay (Fig. 5A). The results showed that atorvastatin did not increase the number of TUNEL-positive cells, and PPE caused a moderate increase. However, a combination of these two agents significantly increased the number of TUNEL-positive cells (>8-fold higher than the control). Taken together, our results suggested that PPE and atorvastatin synergistically induce apoptosis as well as the growth inhibition of human lung cancer H1299 cells.

The combination treatment synergistically decreased the level of Mcl-1 at both 24 and 48 hours in both H1299 and H460 cells. Down-regulation of Mcl-1 was associated with increased apoptosis as reflected by the increased levels of cleaved caspase-3 and cleaved PARP, clearly observed at 48 h (Fig. 5B). Again, PPE and atorvastatin seem to act synergistically. Similar results were also observed in H460 cells after the combination treatment of PPE and atorvastatin. This combination synergistically reduced Mcl-1 and increased the cleaved caspase-3 and PARP levels (Fig. 5C).

Cell proliferation–related proteins were also analyzed in H1299 cells by Western blots after treatments with PPE, atorvastatin, and their combination (Fig. 5B). The combination treatment with PPE and atorvastatin significantly decreased the expression levels of CDK4 at both 24 and 48 hours, and it seems

**Table 1.** Effects of treatments of PPE, atorvastatin, or their combination on NNK-induced lung tumorigenesis

<table>
<thead>
<tr>
<th>Group</th>
<th>Mice (n)</th>
<th>Tumor multiplicity</th>
<th>Tumor burden (mm³)</th>
</tr>
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<tbody>
<tr>
<td>Saline control</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NNK control</td>
<td>25</td>
<td>13.8 ± 1.0ab</td>
<td>3.37 ± 0.30a</td>
</tr>
<tr>
<td>NNK + 0.25% PPE</td>
<td>25</td>
<td>13.9 ± 1.0ab</td>
<td>3.62 ± 0.34a</td>
</tr>
<tr>
<td>NNK + 0.5% PPE</td>
<td>24</td>
<td>10.6 ± 1.1ab</td>
<td>2.64 ± 0.36ab</td>
</tr>
<tr>
<td>NNK + 200 ppm ATST</td>
<td>25</td>
<td>16.2 ± 1.4ab</td>
<td>2.77 ± 0.35ab</td>
</tr>
<tr>
<td>NNK + 400 ppm ATST</td>
<td>24</td>
<td>12.0 ± 0.9ab</td>
<td>2.64 ± 0.28ab</td>
</tr>
<tr>
<td>NNK + 0.25% PPE + 200 ppm ATST</td>
<td>25</td>
<td>6.1 ± 0.7c</td>
<td>1.49 ± 0.21c</td>
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NOTE: One week after the second dose of NNK, the mice were treated with PPE (0.25% or 0.5% in drinking fluid), atorvastatin (200 ppm or 400 ppm in diet), or their low-dose combination for 16 wk. Under a dissecting microscope, tumors >0.1 mm were scored. Tumor volumes (mm³) were measured using the formula \[ V = \frac{4}{3}\pi r^3 \] , where \( r \) is the radius of the tumor determined by the mean values of the longest and shortest diameters. Tumor burdens were calculated as the sum of the tumor volume of all tumors in one animal. Values are the mean ± SE. Different superscripts indicate statistical significance by one-way ANOVA analysis (\( P < 0.05 \)).

Abbreviation: ATST, atorvastatin.
to be due to the additive effects of the two agents. For example, at 48 hours, the levels of CDK4 were reduced to 44% by atorvastatin and to 64% by PPE, and to 28% by their combination. This is consistent with the calculation that $0.44 \times 0.64 = 0.28$ according to an additivity model. The CDK6 levels, on the other hand, were reduced by atorvastatin but not by PPE; however, PPE enhanced the effectiveness of atorvastatin, suggesting a synergistic action. The combination treatment also caused a significant decrease in the expression levels of cyclin D1 at 24 and 48 hours, whereas either treatment of PPE or atorvastatin alone did not, suggestive of a synergistic effect. In agreement with the above-mentioned results, the combination treatment caused a strong decrease in the hyper-phospho-Rb levels at both 24 and 48 hours, and this effect seems to be additive.

Discussion

The present studies show that the combination of PPE and atorvastatin can effectively inhibit tumorigenesis in the NNK-induced lung tumor model and the growth of human lung cancer H1299 and H460 cells. Isobologram analysis indicated that the action between these two agents was synergistic in both experimental systems. This is the first demonstration of the effectiveness of such a combination in the inhibition of tumorigenesis and cancer cell growth.

Our results from studies in the NNK-induced lung tumorigenesis model indicate that the inhibition of tumorigenesis was associated with enhanced apoptosis. The low doses of these two agents seem to act synergistically. The down-regulation of Mcl-1 level is consistent with the enhanced apoptosis. The apoptosis induction by 0.5% PPE observed presently is consistent with our previous studies showing that 0.6% green tea extracts in drinking fluid caused a 71% increase in the apoptotic index in NNK-induced lung adenomas (6) and that treatment with 0.5% PPE in drinking fluid for 30 weeks induced a 2.5-fold increase in the apoptotic index in both adenomas and adenocarcinomas (8). We reported in our previous publication that PPE lowered Erk1/2 and C-Jun phosphorylation in NNK-induced lung adenocarcinomas in the tumor progression stage (from adenoma to adenocarcinoma; ref. 8) In the current study, we focused on the postinitiation promotion stage (on the formation of the adenoma), but the effects of the treatments with PPE and atorvastatin on the phosphorylation of Erk1/2 and C-Jun were not observed. Similarly, the treatments did not produce a significant effect on cell proliferation in adenomas as determined by Ki-67 immunohistochemistry (data not shown).

Studies in lung cancer H1299 cell lines also indicate that the anticaner effect is at least partially due to an increase of apoptosis as shown in the TUNEL assay, as well as by the down-regulation of the antiapoptotic protein Mcl-1 and, and the increase of cleaved caspase-3 and PARP levels as determined by Western blots (Fig. 5). Similar changes of apoptotic-associated protein were also observed in H460 cells. Of note is the observed synergistic action of PPE and atorvastatin in reducing the level of Mcl-1, an antiapoptotic protein in the Bcl-2 family. It has been reported that Mcl-1 levels are elevated in human

![Fig. 4. Median effect and interaction index plots of PPE, atorvastatin, or their combinations on H1299 and H460 cells. The cells were seeded (1500 per well) in 96-well plates. At 24 h after seeding, cells were treated with serial concentrations of PPE, atorvastatin, or their combinations with ratios of atorvastatin to EGCG of 1:10 for H1299 cells and 1:5 for H460 cells. After 48 h of treatments, growth inhibition was measured by MTT assay. The interaction index was used to determine additivity, synergy, or antagonism of the combination depending on interaction index = 1, (1, or 1), respectively. Error bars, 95% confidence interval (n = 6). A and C, median effect plots of H1299 and H460 cells. B and D, interaction index plots of H1299 and H460 cells.](www.aacrjournals.org)
lung cancer cell lines, including H1299 cells (41, 42), and in 58% of human non–small-cell lung cancers (43). Over-expression of Mcl-1 was found to prevent lung cancer cell death induced by a variety of proapoptotic stimuli. Depletion of Mcl-1 could directly induce lung cancer cell apoptosis, and reducing Mcl-1 level could sensitize the lung cancer cells to apoptosis induced by cytotoxic agents and ionizing radiation (42). We also observed the synergistic action of PPE and atorvastatin in reducing the levels of CDK6 and cyclin D1 in H1299 cells. These two cell proliferative markers had both been found up-regulated in human non–small-cell lung cancers and lung cancer cell lines (44). In all these cell culture experiments, we added superoxide dismutase and catalase to the culture medium to prevent the autooxidation of EGCG and the actions caused by the reactive oxygen species generated outside of the cells (37). We believe such autooxidation may not occur in the intact lung tissues because they are endowed with autooxidative enzymes such as superoxide dismutase, glutathione peroxidase, and catalase as well as antioxidants such as vitamin C, vitamin E, and glutathione.

Because EGCG is the most active and most abundant constituent in PPE, we believe that most of the activities of PPE are due to EGCG. Although many mechanisms have been proposed for the anticancer actions of EGCG (44), the specific mechanism involved in our experimental system remains to be investigated. Atorvastatin, an inhibitor of HMG-CoA reductase, has been proposed to block the synthesis of farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which results in the inhibition of isoprenylation and membrane localization of small G-proteins, such as Ras and Rho proteins (13). It remains to be determined whether this mechanism plays a role in our experimental system.

The concentration range of EGCG (5–80 µmol/L) that we used was higher than the concentration range of EGCG found in the mouse plasma and lung (4). The concentration range of atorvastatin (0.5–8 µmol/L in H1299 cell and 1-16 µmol/L in H460 cell) was also higher than the human blood levels in the literature (<0.2 µmol/L; ref. 45). Nevertheless, the effective concentrations of chemopreventive agents observed in cell lines are usually higher than the concentrations observed in vivo. The translation of effective concentrations from cell line studies (in which the cells are treated with the agent for a few days) to in vivo studies (in which tissues are exposed to the agent for weeks or months) is a challenging issue and needs further study. The value of these cell line studies should be judged by their usefulness in generating information that can be verified in vivo. We presently showed that the down-regulation of Mcl-1 and enhancement of apoptosis can be shown in both the cell line system and the animal model.

According to the concept of allometric scaling (46, 47), for a mouse that drank 3 mL of 0.25% PPE and ate 2.5 g of diet daily, the dose was 7.5 mg/10.5 kcal = 0.71 mg/kcal, which corresponds to 0.71 × 2,000 = 1,420 mg PPE per day for a person requiring 2,000 kcal/d. This amount of tea polyphenol can be derived from 13 to 15 g of green tea leaves (dry weight) or four to six cups of green tea per day. This is at the higher end in the range of human consumption. A similar calculation shows that 200 ppm atorvastatin for mice corresponds to 88 mg/d for a person requiring 2,000 kcal/d, and this is at the upper end of human therapeutic doses (10-80 mg/d; refs. 48, 49).

Tea is commonly consumed by a large population, and no toxic effects have been reported. Atorvastatin (Lipitor) is a popular cholesterol-lowering drug. The combination of PPE (or tea) and atorvastatin may have a high potential for practical applications in the prevention or treatment of lung cancer. Additional studies are needed to determine whether lower levels of PPE and atorvastatin can prevent lung cancer in animal models and humans.

![Figure 5](image-url)
References


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