The invention relates to a cigarette filter containing rosemary extract and a method of reducing DNA damage caused by various harmful agents in cigarette smoke. More specifically, the present invention relates to the use of said filter to reduce DNA damage caused by benzo(a) pyrene in human cells by the reduction of the human lung benzo(a) pyrene diol epoxide-dG (BPDE-dG) adduct.
Figure 1

Benzo(a)pyrene (BP)

CYPs, EH → epoxidation

CYPs, ROO$^\cdot$ → epoxidation

(-)-BP-7,8-diol

(+)-anti-BPDE

Guanine

DNA

Stable N2-dG adduct

Mutations within tumor genes (e.g. p53, ras)
Figure 2

\[ y = 1587.5x - 14.122 \]
\[ R^2 = 0.9966 \]
Figure 3

[Chemical structures and reactions depicted in the figure]

CYPs

(-)-BP-7,8-diol

DNA and hydrolysis

(+)-syn-BPDE

BP-ethyl-1

BP-ethyl-2
Figure 4 Upper

DNA binding (pg adducts/mg DNA)

y = 719.08x + 3.146
R^2 = 0.9741

Figure 4 (b)

1/DNA binding (pg adducts/mg DNA)

y = 0.0303x + 0.003
R^2 = 0.9984
Figure 5

The diagram illustrates the adduct relative increase (%) over time after exposure (hours) induced by cigarette smoke (CS) and spontaneous CYP increase. The diagram shows:

- **12 hours**:
  - Increase induced by cigarette smoke (CS)
  - CYP spontaneous increase

- **18 hours**:
  - Increase induced by cigarette smoke (CS)
  - CYP spontaneous increase

- **24 hours**:
  - Increase induced by cigarette smoke (CS)
  - CYP spontaneous increase
Figure 6

DNA adducts [µg adducting DNA] x 10^4

- BP t = 18 h
- BP t + 2h
- BP + Standard CSS t + 2h
- BP + Rosemary filtered CSS t + 2h

CYP spontaneous metabolism after 18h
Figure 6 - Scheme 1

A
1a -- BP 12 h
2a -- BP 14 h
3a -- BP 14 h + Standard CSS
4a -- BP 14 h + Rosemary filtered CSS

B
1b -- BP 18 h
2b -- BP 20 h + Standard CSS
3b -- BP 20 h + Rosemary filtered CSS
4b -- BP 20 h + Rosemary filtered CSS
Figure 1

Benzo(a)pyrene (BP)

CYPs, EH → epoxidation

(-)-BP-7,8-diol

CYPs, ROO* → epoxidation

(+)-anti-BPDE

Guanine

DNA

Stable N²-dG adduct

Mutations within tumor genes (e.g. p53, ras)
Figure 2

DNA binding (pg adducts/mg DNA)

CSS dilution in PBS (times)

y = 1587.5x - 14.122
R² = 0.9966
Figure 3
Figure 4 Upper

\[ y = 719.08x + 3.146 \]
\[ R^2 = 0.9741 \]

Figure 4 (b)

\[ y = 2.0303x + 0.003 \]
\[ R^2 = 0.9984 \]
Figure 5

The figure shows a bar chart illustrating the increase in adduct relative increase over time after exposure to cigarette smoke (CS). The chart includes data points at 12, 18, and 24 hours.

- **12 hours**:
  - Increase induced by cigarette smoke (CS)
  - CYP spontaneous increase

- **18 hours**:
  - Increase induced by cigarette smoke (CS)
  - CYP spontaneous increase

- **24 hours**:
  - Increase induced by cigarette smoke (CS)
  - CYP spontaneous increase

The y-axis represents the adduct relative increase in percentage, with a scale from 0 to 200%.
Figure 6 - Scheme 1

A

1a → BP12 h
2a → BP14 h
3a → BP14 h + Standard CSS
4a → BP14 h + Rosemary filtered CSS

B

1b → BP18 h
2b → BP20 h
3b → BP20 h + Standard CSS
4b → BP20 h + Rosemary filtered CSS
CIGARETTE FILTER CONTAINING ROSEMARY EXTRACT AND A METHOD OF REDUCING DNA DAMAGE CAUSED BY HARMFUL AGENTS IN CIGARETTE SMOKE BY USE OF SAID FILTER

1. FIELD OF THE INVENTION

[0001] The invention relates to a cigarette filter containing rosemary extract and a method of reducing DNA damage caused by various harmful agents in cigarette smoke. More specifically, the present invention relates to the use of said filter to reduce DNA damage caused by benzo(a)pyrenes in human cells by the reduction of the human lung benzo(a) pyrene diol epoxide-dG (BPDE-dG) adduct.

2. BACKGROUND

[0002] The human lung benzo(a)pyrene diol epoxide-dG (BPDE-dG) adduct concentrates in bronchial cells. This adduct now is recognized as a critical event in tumorigenesis by benzo(a)pyrenes. Cigarette smoke is a significant contributor to BPDE-dG formation.

[0003] Tobacco use is by far the most widespread link between exposure to known carcinogens and death from cancer, and is therefore a model for understanding mechanisms of cancer induction. Benzo(a)pyrene (BP) is a highly carcinogenic polycyclic aromatic hydrocarbon (PAH) present in emission extracts, charbroiled food and in small quantity in cigarette smoke, typically less than 10 ng per cigarette. BP is one of more than 60 carcinogens in cigarette smoke that is involved in the aetiology of lung cancer. It is metabolically activated into benzo(a)pyrene 7,8-diol-9,10-epoxide (BPDE) which reacts with DNA predominantly at the N2 position of guanine to produce primarily N2-guanine lesions, e.g. benzo(a)pyrene 7,8-diol-9,10-epoxide-N2-deoxyguanosine (BPDE-dG) adduct. The presence of BPDE-DNA adducts in human tissues has been conclusively established and BPDE-dG adduct concentrated exclusively in bronchial cells and thus implicated in the initiation of human lung cancer.

[0004] Rosemary (Rosmarinus officinalis Labiatae) herb and oil, rosemary extracts, carnosic acid and carnosol are commonly used as spice and flavoring agents in food processing for their desirable flavor and high antioxidant activity.

[0005] However, prior to the instant invention, there was no recognition that the use of a rosemary extract in a cigarette filter would reduce the DNA damage caused in human cells by benzo(a)pyrenes specifically, the human lung benzo(a) pyrene diol epoxide-dG (BPDE-dG) adduct which adduct now is recognized as a critical event in tumorigenesis by benzo(a)pyrenes.

3. SUMMARY OF THE INVENTION

[0006] BP is considered to be a significant carcinogen involved in lung cancer induction in smokers and, as is shown in this study, reactive oxygen species contribute substantially in the formation of the critical lung tumorigenic adduct. While it is both critical to prevent addiction to tobacco and to enhance the efficacy of smoking cessation and reduction programs, these approaches have had little impact. The prevention of the formation of BPDE-dG adduct is one approach which may lead to decreasing lung cancer risk in addicted smokers.

[0007] The invention relates to a cigarette filter containing rosemary extract and a method of reducing DNA damage caused by harmful agents in cigarette smoke. More specifically, the present invention provides for the use of said filter to reduce DNA damage caused by benzo(a)pyrenes in human cells by the reduction of the human lung benzo(a)pyrene diol epoxide-dG (BPDE-dG) adduct.

[0008] It has been found that the amount of (−)-anti-BPDE-dG adduct increases linearly with concentration of cigarette smoke in the presence of (+)-BP-7,8-diol. Catalase and superoxide dismutase inhibit its formation by more than 80%. When MCF-7 cells are treated for 2 hours with the (+)-BP-7,8-diol, cigarette smoke increases dose-dependently the formation of (−)-anti-BPDE-dG and decreases the CYPs dependent formation of (−)-anti-BPDE, the adduct.

[0009] I have treated cells for up to one day with benzo(a)pyrene and then exposed them for 2 hours with cigarette smoke. During these 2 hours, I have discovered that there is twice the increase in the adduct formation in cells treated with cigarette smoke in comparison to levels in non treated cells due to CYPs activity. Thus, I have found that cigarette smoke activates by reactive oxygen species which it contains the second step of benzo(a)pyrene metabolic way leading to the formation of BPDE-dG adduct.

[0010] Cigarette smoke thus may in this way responsible for the formation of the critical lung tumorigenic adduct.

[0011] Finally, I have found that modified cigarette filter containing rosemary extract decreases by more than 70% of the BPDE-dG adducts level due to the cigarette smoke in MCF-7 cells. This discovery, I believe is a significant advance in decreasing lung cancer risk in addicted smokers.

4. BRIEF DESCRIPTION OF THE FIGURES, SCHEME AND TABLE

[0012] FIG. 1. Principal metabolic pathway and DNA binding of the carcinogen benzo(a)pyrene. Benzo(a)pyrene is a tobacco carcinogen that may be converted in vivo enzymatically or by oxygen reactive species to yield DNA-reactive dihydriodiol epoxides. Stereoselective generation of the mutagenic (+)-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydriodio-BP [(−)-anti-BPDE] from (−)-BP-7,8-dihyrdriodio is catalyzed by cytochrome-P450-dependent monooxygenases (P450) or reactive oxygen species. Subsequent reaction of this electrophilic intermediate with genomic DNA produce stable adduct between dihydriodiol epoxide and the excycelic amino group of guanosine. This kind of DNA lesion may be converted into mutations within the following replication cycle unless repair of this adduct is produced.

[0013] FIG. 2. Results obtained by using a cell-free system concomitant with DNA addition: 6 μg calf thymus DNA in 2 ml water was added to 5 ml CSS with different dilutions and reacted for 2 hours at room temperature with (−)-BP-7,8-diol (final concentration 3.6 μM). DNA was hydrolyzed and the released BP-tetrol I were measured as outlined in Materials and Methods.

[0014] FIG. 3. (a) Stereochemistry of BP-7,8-diol epoxidation by peroxyl radicals and cytochrome P450 to reactive species (anti-BPDE and syn-BPDE) that can bind to DNA, and (b) acid hydrolysis of DNA to BP-tetrols measured in this study. The hydrolysis of (−)-anti-BPDE-dG and (−)-syn-BPDE leads to the formation of BP-tetrol 1-2 and BP-tetrol II-1 which however are unstable and are converted into BP-tetrol 1-1 and BP-tetrol 11-2.
FIG. 4. Results obtained using MFC-7 cells. 10×10⁶ cells/150 cm² flask in a total volume of 20 ml were treated for 2 hrs with (+)-BP-7,8-diø (0.2 µM) alone or in presence of different dilutions of CSS DNA was isolated, hydrolyzed, and the released BP-tetrols were measured and the binding levels determined as outlined in Materials and Methods. Two distinct peaks were observed on the chromatograms corresponding to BP-tetrol and BP-tetrol II derived from (−)-anti-BPDE-dG and (+)-syn-BPDE-dG respectively (refs. 32-34). Values represent the means plus Std of two independent experiments with 3-4 HPLC runs: FIG. 4a, upper panel, increases of (−)-anti-BPDE-dG adducts with CSS dilution; FIG. 4b, lower panel, increases of 1/(+)-syn-BPDE-dG adduct with CSS dilution.

FIG. 5. BPDE-dG binding in MCF-7 cells after exposure to BP 2.5 µM or BP 2.5 µM CS solution (dilution 20 times) for the time indicated. Cigarette smoke solution was added the last 2 hours during the exposure to BP (Scheme 1). Analysis of BPDE-dG was performed as described in Materials and Methods. Values represent the means of two independent experiments with 4-6 HPLC runs plus Std. The BPDE-dG value were 11.7 ± 0.5 (means ± d.) g of adducts per mg DNA after 12 hours of incubation and 17.6 ± 0.4, 26.1 ± 0.9 after hours and 24 hours respectively. The CYP spontaneous metabolism increased theses values to 17.2 ± 0.5, 27.8 ± 0.8 and 42.2 ± 1.0 two hours after the reference time at 12, 18 and 24 hours respectively. The addition of cigarette smoke solution (CSS) induced a much more dramatic change during the same two hours period leading to a final BPDE-dG value of 36.9 ± 1.2, 56.7 ± 0.9 and 80.2 ± 1.2 (means ± d.) g of adducts per mg DNA at 12, 18 and 24 hours respectively.

FIG. 6. BPDE-dG binding in MCF-7 cells after exposure to BP 2.5 µM or BP 2.5 µM CS solution obtained from standard filter and filter containing rosemary extract. The cigarette smoke solution was added for the last 2 hours during the exposure to BP for the time indicated (Scheme 1). Analysis of BPDE-dG was performed as described in Materials and Methods. The HPLC runs show that there is only one peak on chromatograms which correspond to BP-tetrol I derived from (+)-anti-BPDE-dG. Values represent the means of two independent experiments with 4-6 HPLC runs plus Std.

Scheme 1. MCF-7 cells were treated with BP for 12 hrs and 18 hrs following with cigarette smoke for another 2 hours together with BP (experiments A and B respectively). The goal of this experiment was to activate at different times the BP to produce BP-7,8-diø and after treat the cells with cigarette smoke and follow its effect during the last 2 hours. The control represents cells treated only with BP.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% BPDE-dG relative to standard CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard CSS</td>
<td>100</td>
</tr>
<tr>
<td>+Superoxide Dismutase SOD (20 µg)</td>
<td>16</td>
</tr>
<tr>
<td>+Catalase (4 µg)</td>
<td>12</td>
</tr>
<tr>
<td>+Inactivated Catalase (4 µg)</td>
<td>100</td>
</tr>
<tr>
<td>Romarin filtered CSS instead Standard CSS</td>
<td>42</td>
</tr>
</tbody>
</table>

The standard CSS system includes 2 ml calf thymus DNA (3 mg/ml), 600 µl 30 µM (+) BaP-7,8-diø and 5 ml of diluted CSS with PBS (1:19) at pH 7.4, and was incubated at room temperature for 2 h. Each value was obtained from three independent experiments performed in duplicate. The average error was about 12% in each duplicate experiment. The BPDE-dG value of the standard was 56±5.3 (mean±s.d.) adducts per mg DNA.

5. DETAILED DESCRIPTION

Cigarette smoking is causally associated with a large number of human cancers. Tobacco use is by far the most widespread link between exposure to known carcinogens and death from cancer, and is therefore a model for understanding mechanisms of cancer induction.

Benzo(a)pyrene (BP) is a highly carcinogenic polycyclic aromatic hydrocarbon (PAH) present in emission exhausts, charbroiled food and in small quantity in cigarette smoke, typically less than 10 mg per cigarette. BP is one of more than 60 carcinogens in cigarette smoke that is involved in the etiology of lung cancer. It is metabolically activated into benzo(a)pyrene-7,8-diol-9,10-epoxide (BPDE) which reacts with DNA predominantly at the N₂-position of guanine to produce primarily N₇-guanine lesions, e.g. benzo(a)pyrene-7,8-diol-9,10-epoxide-N₇-deoxyguanosine (BPDE-dG) adduct.

The presence of BPDE-DNA adducts in human tissues has been conclusively established and BPDE-DG adduct concentrated exclusively in bronchial cells and is thus implicated in the initiation of human lung cancer.

This carcinogen is metabolized by phase I enzymes to a large number of metabolites including phenols, arene oxides, quinones, dihydrodiols, and diol epoxides. An overview of BP metabolic way leading to the formation of (+)-anti-BPDE-dG adduct is presented in FIG. 1.

In more detail, the ultimate carcinogen (+)-anti-BPDE is formed from BP by two rounds of cytochrome P450-mediated oxidation. The first step of this oxidation leads preferentially to (−)-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene ([−]-BP-7,8-diø). The diol is further oxidised primarily to the highly mutagenic (+)-7,8-dihydronitroso-7,9,10-oxa-7,8,9,10-tetralhydro-BP ([+]-anti-BPDE). Numerous studies have clearly identified the [(+)-anti-BPDE] as the primary carcinogenic metabolite of BP exhibiting enhanced mutagenic activity in vitro and in vivo. Most previous studies of genetic variation in metabolism of lung carcinogens have focused on metabolic activation by various cytochromes P450s, although expression of these enzymes in lung is generally low. The activation of BP-7,8-diø by lung epithelial cells is not caused solely by classic CYPs/GSTs dependent biotransformation processes, but also involves several metabolic routes other than CYPs. These include, lipoxygenase, lipid-peroxidation products and peroxidase-dependent pathways, COX-1 and COX-2.

Increasing evidence suggests the causal significance of tobacco free radicals in lung cancer induction in smokers. Each puff of smoke forms over 10 trillion free radicals present in smoke, which may contribute to both tumor initiation as well as promotion of various forms of human cancer caused by repeated attacks from ROS on cellular macromolecules. The major free radical species are postulated to be an equilibrium mixture of semiquinones, hydroquinones and quinones. It is suggested that this free radical complex causes redox cycling that generates superoxide anion from molecular oxygen and leads to the formation of hydrogen peroxide and hydroxyl radical. These reactive species cause DNA nicking.
and single-strand breaks in DNA of cultured rodent and human cells. Quinone-associated redox cycling may also be involved in these effects; hydroquinone and catecholestibels to play a major role.

[0026] I have discovered that cigarette smoke can activate by its oxygen generated radicals the second step of the BP metabolic pathway leading to the formation of BPDE-dG adduct, presumably by metabolism of the formed in the cells (−)-BP-7,8-diol to (−)-anti-BPDE-[Fg]. I have also discovered that this activation is at least twice higher than this obtained with CYPs machinery.

[0027] Further, I have discovered that ROS from the cigarette smoke may be in part responsible for the increased BPDE-dG adduct formation.

[0029] I have also found that a filter containing a formulated rosemary powder can reduce considerably the BPDE-dG level due CS oxygen generated radical, and that my discoveries can be used for cigarette filters which reduce the formation of carcinogenic BPDE-dG adduct in bronchial epithelial cells.

[0030] My invention provides (i) a means for determining the relative contribution of ROS in cigarette smoke on the activation of BP-7,8-diol in comparison with cytotoxic PGE4; (ii) a means for establishing whether cigarette smoke’s ROS promotes the carcinogenic process by contributing to the metabolism of BP-7,8-diol resulting in an increase in the formation of the critical lung BPDE-dG; (iii) a filter containing a scavenger of cigarette free radicals to significantly decrease the formation of BPDE-dG; and (iv) use of said filter to significantly decrease the function of BPDE-dG adducts.

7. EXAMPLES

[0031] Chemicals. Protease K (EC 3.4.21.64, from Trichoderma album) was purchased from Sigma (St. Louis, Mo.), RNase T1 (EC 3.1.21.3, from Aspergillus oryzae) and RNase (DNase free, heterogeneous mixture of ribonucleases from bovine pancreas) were obtained from Boehringer Mannheim (Mannheim, Germany). Phosphate-buffered saline (PBS) contained 3.0 mM KCl, 1.5 mM KH2PO4, 10 mM NaCl, 8.0 mM Na2HPO4 (pH 7.4), HPLC-grade water, MeOH, H2O, and ethanol for extraction from E. Merck, Darmstadt, Germany. If not stated otherwise, the other chemicals were purchased from Sigma (L’Isle d’Abeau Chesnes, France), Boehringer Ingelheim (Heidelberg, Germany) and Boehringer Mannheim (Mannheim, Germany). All BP metabolite standards were obtained from National Cancer Institute, Chemical Carcinogen Reference Standard Repository MidWest Research Institute (Kansas City, Mo.).

[0032] Apparatus. High-performance liquid chromatography (HPLC) was carried out with a Hewlett-Packard high pressure isocratic and gradient systems (Hewlett Packard, Germany) equipped with a Shimadzu RF-10A XL fluorescence detector linked to a Hewlett-Packard integrator.

[0033] Preparation of Cigarette Smoke/PBS Solution (CSS). Smoking was performed according to Pryor et al. without the Cambridge filter. Essentially the same smoke collecting method has been used earlier by Nakayama et al. The smoke from burning 8 cm of one cigarette (Marlboro) during 3.8 min with the help of constant vacuum generated from a water pump was bubbled through 10 ml of phosphate-buffered saline (PBS) solution which traps both the gas-phase and tar cigarette smoke chemicals. As there were no water-insoluble tar compounds present on the walls of the wash bottles, a major part of the water-soluble compounds from the smoke of a single cigarette was contained in the 10 ml PBS solution. This aqueous solution named cigarette smoke solution (CSS) was reacted immediately with exogenous DNA or added to MCF-7 cells in culture in the presence of benzo(a)pyrene or its proximate metabolite (+)-BaP-7,8-diol. Different dilutions of CSS were used (see below).

[0034] Incorporation of a Rosemary Powder Extract into the Cigarette Filter. The filter of the conventional cigarette was removed and 40 mg of a rosemary powder extract, was introduced in the place free from the filter near to the cigarette itself. After this operation, the filter was reinstalled. The effect of this filter was evaluated by mass spectrometry. Briefly the cigarette smoke was bubbled in an organic solution containing 3,5,5-tetramethyl-1-pyrroline-N-oxide (TPMPD) a spin trap adduct. The amount of hydroxyl radical adduct was then quantified using liquid chromatography, mass spectrometry. Under the smoking conditions used a 30% decrease in the hydroxyl radical was observed.

[0035] Reaction of Exogenous DNA with (+)-BP-7,8-Diol in Presence of Diluted Cigarette Smoking Solution (CSS). 2 ml calf thymus DNA (3 mg/ml) was added to 5 ml diluted 20 times CSS and reacted for 2 hours at room temperature with (+)-BP-7,8-diol (final concentration 3.6 µM) according to the following reaction:

DNA+(-)-BP-7,8-diol+CSS→(-)-anti-BPDE-N-dG

The level of the resulting (−)-anti-BPDE-dG adduct was measured (see below). As control an experiment without CSS was performed.

[0036] Cell Culture Conditions and Treatment. The human mammary carcinoma cell line MCF-7 was grown in 150-cm² cell culture flasks in a total volume of 20 ml minimal essential medium E-MEM supplemented with 10% FCS, 15 mM Heps buffer, and antibiotics (200 units/ml penicillin, 200 µg/ml streptomycin, and 25 µg/ml ampicillin). Cells were maintained and treated at 37°C in 5% CO₂/95% air atmosphere.

[0037] After MCF-7 cells had covered 90% of the surface area of the flasks, (2-3 days after splitting of a confluent culture), the medium was replaced with 20 ml of fresh medium containing 10% serum. Twenty four hours later, near-confluent cells e.g. more than 90% of cells which were treated with DMSO alone or with carcinogen (see below) dissolved in DMSO and cigarette smoke/PBS (CSS see above). The final concentration of DMSO did not exceed 0.1% of the incubation volume. Control samples included in each incubation set were treated with DMSO alone.

[0038] a) Treatment of MCF-7 Cells with (+)-BP-7,8-Diol and Cigarette Smoke. Cells were treated for 2 hours with (−)-BP-7,8-diol (0.2 µM) alone or in presence of different dilutions of CSS. The (+)-BaP-7,8-diol was activated by ROO's generated from the CSS and cell CYP's to form (−)-anti-BPDE-dG and (+)-syn-BPDE-dG respectively. Their levels were measured by the formation of BP-tetrol I-1 and BP-tetrol 11-2 (see below and FIG. 3).

[0039] b) Time/Dose Exposure Experiments with BP. To characterize time/dose exposure to BP and BPDE-dG level, cells (1x10⁶ cells/150 cm² flask, total volume of 20 ml) were treated with medium containing final concentration of 1.25, 2.5 and 5.0 µM each for 6, 12, 18 and 24 hours (two flasks/dose/time point). BPDE-dG adduct formed in the cells increase linearly in a dose- and time-dependent manner as to
was shown also by others (30). On the basis of the results obtained we choose 2.5 μM as working concentration for BP.

**[0040]** Treatment of MCF-7 Cells with BP and Cigarette Smoke. To see the effect of CS concentration, the experience of scheme N° 1 was performed with different CSS dilutions (1:79; 1:39; 1:19; 1:9 vol/vol). On the basis of the results obtained from this experience I choose 1:19 (vol/vol) dilution as working CS concentration. Thus, the cells (see above “Cell Culture and Treatment”) were treated with BP (2.5 μM) and CSS (dilution 1:19 vol/vol) according to Scheme I (See FIG. 6 – Scheme 1).

**[0041]** All incubation sets were repeated 2-3 times with duplicate samples. At the end of treatment, cells were examined microscopically for morphological changes, then harvested by trypsination with 0.05% trypsin-EDTA (0.05% trypsin, 0.14 M NaCl, 3 mM KCl, 0.1 M Na₂HPO₄, 1.5 mM KH₂PO₄, 0.5 mM EDTA). After addition of an equal volume of medium containing 10% FCS, the cells were centrifuged at 1000 g, washed three times with PBS, and the cell pellet then stored frozen at -20°C. The viability of the cells treated with BP and cigarette smoke or (+)-BP 7,8-diol and cigarette smoke was roughly 90% at the time of harvesting as determined by a Trypan blue exclusion assay. The doses used did not show any cytotoxicity as measured by lactate dehydrogenase activity assay (ELISA Kit, Boehringer, Mannheim).

**[0042]** DNA Preparation and Hydrolysis. DNA isolation from MCF-7 cell pellets was carried out by treatment with RNase, proteinase K, saltation procedure (31) and chloroform. Briefly, the cell pellets were resuspended in EDTA-sodium dodecyl sulfate (SDS) buffer [10 mM Tris buffer, 1 mM Na₂EDTA, 1% SDS (w/v), pH 8] incubated for 1 h at 37°C with RNase T1 (2000 U/ml) and RNase A (DNase free; 100 μg/ml) on a shaker (100 rpm). Then proteinase K (300 μg/ml) was added and the incubation continued overnight at 37°C. After digestion, 6M NaCl was added to have final concentration of 1M followed by a centrifugation at 10000 g. DNA in the supernatant was precipitated with 2 vol. ethanol, washed with 70%, 100% ethanol, ether, dried and dissolved in 10 mM Tris buffer. Again, RNase A (100 μg/ml) and RNase T1 (2000 U/ml) were added and the solution incubated at 37°C for 1 h. followed by proteinase K (100 μg/ml) for another 2 hours at 37°C. The solution was extracted once with chloroform and the solution was made 1M NaCl. DNA was precipitated with 2 vol. cold ethanol.

**[0043]** The portion of DNA to be hydrolyzed was rinsed with 100% ethanol to remove unbound BP-tetrols. The DNA, free of unbound BP-tetrols, was dissolved in water and the DNA concentration was determined by A₂₆₀nm. The purity was ascertained by the ratios at A₂₆₀/A₂₈₀ and A₂₆₀/A₃₂₅. The amount of DNA for analysis was hydrolyzed as described previously by incubation at 90°C for 4 hours in a final concentration of 0.1 N HCl. This releases tetrols (FIG. 3) from BPDE-DNA adducts with >90% recovery. The volume of the hydrolysate for injection was made 700 μl containing 5-10 μg DNA.

**[0044]** Determinations of BPDE-N₂-dG adduct level. The adduct levels were determined by HPLC-ESI-MS/MS as previously described [32,33] using r-c-e-9,10-tetrahydroxyl-7,8,9,10-tetrahydrobenzo(a)pyrene (BP-tetrol II-1) as an internal standard [34]. The hydrolysate was loaded onto a Pre-column module (HID-Germany) containing 5 μm L-column, reverse-phase material (Nucleosil 100) equilibrated with 10% MeOH and washed for 20 min with 12 ml 10% MeOH. Subsequently, the pre-column was switched by a Valco Instruments switching valve to flow over a 4.6 mm×25 cm 5 μm C18 reverse-phase (Nucleosil 100) analytical column (Alltech GmbH, Unterhaching, Germany). The products obtained by hydrolysis were eluted with the following MeOH/H₂O gradient: 50%, 0-17 min; 50 to 60%, 17-32 min; 60%, 32-42 min; 60 to 100%, 42-57 min. Retention times of the BP tetrots were: BP tetrot 1-1 (trans-anti-BP-tetrol) (35.2 min); BP-tetrol II-1 (trans-syn-BP-tetrol), internal standard (36.9 min); BP-tetrol II-2 (cis-syn-BP-tetrol) (42.5 min). Fluorescence was assessed at an excitation wavelength of 344 nm and emission wavelength of 398 nm. As I did not detect the formation of BP-tetrol II-1 in separate analysis of MCF-7 samples, I used it as internal standard (2 pg added to each HPLC run) for verification of the relative retention time. The detection limit was 0.5 pg of BP tetrol I-1 and BP-tetrol II-1. The level of each BP-tetrol was determined by using a standard curve generated from the fluorescence peak area of authentic BP-tetrol standard analyzed just before the analysis of MCF-7 samples. The BP-tetrol-I-1 detected is derived after hydrolysis of (+)-anti-BPDE-DNA adduct. The hydrolysis of (+)-anti-BPDE-dG leads to the formation of BP-tetrol I-2, which however is unstable and is converted in BP-tetrol I-1 (FIG. 3) (38). Thus, the level of the formed (+)-anti-BPDE-dG was measured by the quantity of BP-tetrol I-1 found on HPLC runs. Based on the finding that BPDE reacting with DNA produce primarily BPDE-N₂-dG (7), I assumed that BP-tetrol-I-1 level corresponds to this of BPDE-N₂-dG. The level of BPDE binding to MCF-7 DNA was quantified in duplicates. The adduct level was calculated from the equation 1 pmol/mg DNA/3.125=1 adduct per 10⁶ nucleotide. The HPLC runs were quantitatively reproducible, and variability between the two assays was lower than 5%.

**[0045]** The mechanism of mutagenicity by BP is sufficiently well defined and used as a “molecular signature” to establish the causal nature between particular genetic events in development of tumors and carcinogenic exposure (the “smoking gun”). The “BP molecular signature” has major implication for pinpointing the tobacco smoke as the cause of human lung cancer, and for the elaboration of specific strategies to minimize tobacco smoking, or introduce preventive measures. Specific agents used in cancer chemoprevention appear to act by inhibiting carcinogen damage to DNA, mutagenesis, tumor promotion and/or tumor progression.

**[0046]** I have explored the relative role of CS on the biotransformation of BP-7,8-diol to BPDE capable of forming stable DNA adduct in human cells. Numerous studies have demonstrated that stable PAH-DNA adducts can lead to mutations through mis-incorporation of nucleotides or deletion. CS is an aerosol of complex chemical composition containing both organic and inorganic compounds, of which 4800 have been identified so far. Both vapor phase and particulate phase of smoke are known to possess free radicals. While the gas phase radicals are generally short-lived, the radicals in the particulate phase are relatively stable and consist of a hydroquinone, semiquinone, quinone complex, this complex is an active redox system capable of reducing molecular oxygen to produce superoxide, eventually leading to hydrogen peroxide and hydroxyl radicals. In addition, at least 60 different CS carcinogens have been implicated in tumor initiation and promotion: the most potent carcinogens agent contained in CS are BP and NNK (4-(methylamino)-1(3-pyridyl)-1-butanone).

**[0047]** The Effect of Cigarette Smoke on (+)-anti-BPDE-dG Using a Cell-Free System Concomitant with DNA Adduc-
tion. To elucidate the mechanism of BPDE-dG formation dependent from active oxygen generated from cigarette smoke, I looked for this adduct in cell-free in vitro system coinoculum with DNA adduction. The CSS solution containing the gas-phase and tar cigarette smoke radicals was immediately reacted with DNA in the presence of ( + )-BP-7,8-diol (see protocol above). The results from this experiment show that CSS can oxidize the ( - )-BP-7,8-diol to ( - )-anti-BPDE which in turn form the ( - )-anti-BPDE-dG adduct. The amount of ( - )-anti-BPDE-dG increased linearly and dose dependently (see Fig. 2).

Previously it was found that large amounts of active oxygen such as $\mathrm{H}_2\mathrm{O}_2$ and $\mathrm{O}_3^-$ were generated from cigarette smoke after trapping the smoke in PBS. This active oxygen generated from cigarette smoke might be responsible for the observed formation of ( - )-anti-BPDE-dG. To verify this, I checked the effect of catalase and superoxide dismutase (SOD) on the ( - )-anti-BPDE-dG produced, and found that both enzymes inhibited the formation of the adduct. Inactivated catalase showed no effect (Table 1). From these results I concluded that cigarette smoke could oxidize ( + )-BP-7,8-diol, thus forming ( - )-anti-BPDE-dG, and that such capacity can be explained mainly by the action of oxygen generated from cigarette smoke.

The Effect of Cigarette Smoke on ( - )-anti-BPDE-dG Adduct Formed in MCF-7 Cells Treated with ( + )-BP-7,8-diol. Two independent pathways have been shown to participate in the metabolism of BP-7,8-diol to BPDE (Fig. 3). The cytochrome P450 dependent metabolism of the ( + )-enantiomer leads preferentially to ( + )-syn-BPDE whereas the pathway involving haem-containing proteins in conjunction with a peroxide (e.g. lipid peroxide) preferentially results in ( - )-anti-BPDE. The ( - )-BP-7,8-diol on the other hand, may be metabolized by both pathways and results in the formation of ( + )-anti-BPDE, the ultimate form of BP, and ( + )-syn-BPDE. The different pathways can be distinguished by HPLC analysis since the tetrals derived from anti- and syn-BPDE respectively are clearly separated under my conditions.

To investigate further the role of cigarette smoke dependent epoxidation of ( + )-BP-7,8-diol leading to the formation of ( - )-anti-BPDE that form with DNA ( - )-anti-BPDE-dG adduct, human mammary cell line MCF-7 was used. The reason for which I used MCF-7 cells to see the effect of cigarette smoke ROS on the activation of ( + )-BP-7,8-diol was that these cells have little peroxidase activity. The cells were treated with the ( + )-BP-7,8-diol, a stereotaxic probe which can distinguish the adducts formed by ROS and CYPs dependent ways (Fig. 3). Two distinct peaks were observed on the chromatograms corresponding to BP-tetrol I and BP-tetrol II derived from ( - )-anti-BPDE-dG and ( + )-syn-BPDE-dG respectively (refs 32-34). Cigarette smoke increased linearly and dose dependently the ROS dependent formation of ( - )-anti-BPDE-dG (Fig. 4a) and decreased the CYPs dependent formation of ( + )-syn-BPDE-dG adduct measured by the formation of BP-tetrol II. This decrease is also dose dependent and the inverse of DNA adducts increased linearly with cigarette smoke concentration (46). The inhibitory effect of CSS on CYPs dependent formation of ( + )-syn-BPDE-dG and increased formation of ( - )-anti-BPDE-dG adduct confirm the role of the oxygen generated from the cigarette smoke in the formation of ( - )-anti-BPDE-dG adduct.

Other studies showed that induced CYPs activity was impaired by the oxidative challenge. The mechanism underlying such a phenomenon could be a down-regulation of cytochrome P4501A1 gene. Having little peroxidase activity may lead MCF cells under “stress” conditions to increased DNA damage and reduced repair capacity. Consequently this may cause an increase of BPDE-DNA adduct independently from BP-7,8-diol activation.

The Effect of Cigarette Smoke on BPDE-dG Adduct Formed in Cells Treated with BP. Previous studies with MCF-7 cell cultures revealed that these cells possess inducible P4501B1 and P4501A1 activity. The presence of P450 catalyzed metabolic turnover of BP and the absence of detectable peroxidase activity in MCF-7 cells, allowed the evaluation of the role of cigarette smoke oxygen radicals on BP activation in human cell cultures. MCF-7 cells have high CYP1A1 enzyme activity for the metabolic activation of BP leading to the formation of ( - )-BP-7,8-diol and consequently to ( + )-anti-BPDE-dG (Fig. 1). The level of adduct formation at 6 hours was considerably lower than that observed after 12 and 24 hours of exposure. After treatment with 2.5 $\mu$M BP for 6 hours approximately 2000 pg adducts per pg DNA were formed, whereas more than 11000 pg and more than 20000 pg adducts per pg DNA were present after 12 hours and 24 hours respectively (Wilcoxson Rank Sum Test p = 0.0022).

The cells were treated for 12 and 18 hours with BP to induce the formation of ( - )-BP-7,8-diol which is substrate for ROS. Indirect confirmation for the preferentially formation of ( - )-BP-7,8-diol is the absence of BP-tetrol II derived from syn-BPDE on HPLC runs which precursor is ( + )-BP-7,8-diol (Fig. 3). The cells were then exposed for 2 hours with CSS of cigarette smoke together with BP. The HPLC runs show that there is only one peak on chromatograms which correspond to BP-tetrol I derived from ( + )-anti-BPDE-dG. The difference between cells treated with CSS and those non treated (controls) is presented on Fig. 5. As mentioned above the cell line used in this study maintained the capability to lower the CYP1A1 expression after oxidative challenge by CSS. The suppression of cytochrome P450 presumably lowers activation of BP to ( - )-BP-7,8-diol and ( + )-anti-BPDE. Thus the increased difference by CSS is due to the increased metabolism of ( - )-BP-7,8-diol by ROS generated from CSS. Wilcoxson Rank Sum Test gives p = 0.0022 for treated with CSS vs controls for 14 hours and 20 hours respectively. Recently Dramatic damage by BP of DNA occurs in human bronchial epithelial cells forming BPDE-g adduct which could be considered as “critical” for the initiation of human lung cancer bronchial epithelial cells. Thus, active oxygen species generated in cigarette smoke could play an important role in the formation of this “critical” adduct in bronchial epithelial cells (Fig. 1).

The Effect of Filter Containing Rosemary Extract on the Formation of BPDE-dG Adduct. Rosemary (Rosmarinus officinalis) Labiatae herb and oil are commonly used as spice and flavoring agents in food processing for its desirable flavor and high antioxidant activity. Topical application of rosemary extract, carnosol or ursolic acid to mouse skin inhibited the covalent binding of benz(a)pyrene to epidermal DNA, tumor initiation by 7,12-dimethylbenz(a)anthracene (DMBA), TPA-induced tumor promotion, ornithine decarboxylase activity and inflammation. Rosemary extracts were proved to be efficient not only in the promotion phase but also in the initiation phase. Rosemary extracts, carnosic acid and carnosols extremely inhibit phase I enzyme, CYP 450 activities and induce the expression of the phase II enzyme, glutathione S-transferase (GST) and quinone reductase.
activities. Carnosol inhibits nitric oxide (NO) production in activated macrophage. The antioxidant property had been referred to as the mechanistic basis of their protective effects.

With the aim of removing free radicals and reactive oxygen species in the cigarette smoke a small amount of rosemary powder was incorporated in a standard filter (see Materials). The decrease in free radicals in the condensate induced by the filters incorporating a rosemary extract was estimated by the quantitation of the hydroxyl radical content of CSS with a spin trap (TMDP) using LC-ESI-MS/MS. Under the smoking conditions used a 30% decrease in the hydroxyl radical was observed. Due to the efficiency of this filter to reduce the level of the free radicals in cigarette smoke, as compared to a comparable standard Marlboro filter without the additive, 1 compared the effect of CS passed through this filter in comparison to the standard filter on the formation of BPDE-dG using MCF-7 cells.

The results presented in FIG. 6 were obtained when the MCF-7 cells were treated with BP. Two groups of experiments were performed (A and B). The cells were treated with BP for 12 and 18 hours respectively following with CSS from the two filters for another 2 hours together with BP (Scheme 1). To evaluate the CYPs dependent increase of the adduct during these last 2 hours, two controls for each group were performed: 12 and 14 hours for group A, 18 and 20 hours for group B. The CSS from the standard filter double the binding level obtained for 14 and 20 hours.

However, the rosemary filter strongly impedes the increase obtained by the standard filter, more than 70% in the two groups (FIG. 6). The modified filter scavenges ROS and consequently decreases the activation of (∼)BP-7,8-diol (FIG. 3). Aside from the reduction of the free radicals, rosemary powder may have also other mechanisms to reduce BPDE-dG formation.

Using whole rosemary extract (6 μg·mL⁻¹) also inhibits CYP1A1 activity and DNA adduct formation by 80% after 6 hours co-incubation with 1.5 μM BP in human bronchial epithelial cells (REAS-2B). Thus, using filters which decrease the amount of the free radicals to reduce the formation of the critical tumorigenic adduct are a significant benefit for the addicted smokers.

My inventive rosemary cigarette filter therefore is a promising candidate for chemopreventive programs with the aim to reduce BPDE-dG in bronchial epithelial cells.

It will be understood that the above description of the present invention is susceptible to various modifications, changes and adaptations, and the same are intended to be comprehended within the meaning and range of equivalents of the appended claims. The most obvious modification, for example, is the use of various gel materials as the electroresponsive composition of matter.

It will thus be seen that the objects set forth above, among those made apparent from the preceding description, are efficiently attained and, since certain changes may be made in carrying out the above method (process) without departing from the spirit and scope of the invention, it is intended that all matter contained in the above description shall be interpreted as illustrative and not in a limiting sense.

It is also to be understood that the following claims are intended to cover all of the generic and specific features of the invention herein described and all statements of the scope of the invention which, as a matter of language, might be said to fall there between.

What is claimed is:

1. A method for reducing human lung benzo(a)pyrene diol epoxide-dG (BPDE-dG) adducts from cigarette smoke generated by smoking a cigarette having a filter, said method comprising the steps of passing the cigarette smoke through the filter wherein said filter is impregnated by an extract of a plant from the Labiatae family wherein said extract comprises polyphenol compounds or derivatives thereof.

2. The method according to claim 1, wherein the plant is rosemary.

3. The method according to claim 1 wherein the extract is generated by extraction with an alcoholic solvent or an aqueous alcoholic solvent.

4. A method for reducing human lung benzo(a)pyrene diol epoxide-dG (BPDE-dG) adducts from cigarette smoke generated by smoking a cigarette having a filter, said method comprising the steps of passing the cigarette smoke through the filter wherein said filter is impregnated by a mixture comprising at least one polyphenol compound or its derivative.

5. The method according to claim 4, wherein the mixture comprises at least one polyphenol compound or a derivative thereof selected from the group consisting of carnosol, rosmarinic acid, and carnosic acid.

6. The method according to claim 5, wherein the mixture comprises carnosol, carnosic acid, rosmarinic acid, and rosmarinol.

7. The method according to claim 6, wherein the mixture comprises carnosic acid or carnosol.

8. The method according to claim 7, wherein the filter comprises from 0.5 g to 0.1 mg of at least one polyphenol compound or its derivative.

9. The method according to claim 7, wherein the filter comprises 0.01 g of at least one polyphenol compound or its derivative.

10. The method according to claim 7, wherein the at least one polyphenol or its derivative is coupled to a polymeric carrier or is in a microcapsule matrix or is added to the fibers of a filter.