Modulation of Biomarkers by Chemopreventive Agents in Smoke-exposed Rats

Alberto Izzotti, Roumen M. Balansky, Francesco D’Agostini, Carlo Bennicelli, Steven R. Myers, Clinton J. Grubbs, Ronald A. Lubet, Gary J. Kelloff, and Silvio De Flora

Department of Health Sciences, University of Genoa, Via A. Pastore 1, I-16132 Genoa, Italy [A. I., R. M. B., F. D’A., C. B., S. D. F.]; Department of Pharmacology and Toxicology, University of Louisville, Louisville, Kentucky 40292 [S. R. M.]; Chemoprevention Center, University of Alabama, Birmingham, Alabama 35294-3361 [C. J. G.]; and National Cancer Institute, Rockville, Maryland 20892 [R. A. L., G. J. K.]

ABSTRACT

Chemoprevention opens new perspectives in the prevention of cancer and other chronic degenerative diseases associated with tobacco smoking, exploitable in current smokers and, even more, in exsmokers and passive smokers. Evaluation of biomarkers in animal models is an essential step for the preclinical assessment of efficacy and safety of potential chemopreventive agents. Groups of Sprague Dawley rats were exposed whole body to a mixture of mainstream and sidestream cigarette smoke for 28 consecutive days. Five chemopreventive agents were given either with drinking water (N-acetyl-L-cysteine, 1 g/kg body weight/day) or with the diet (1,2-dithiole-3-thione, 400 mg; OPZ, 400 mg; phenylethyl isothiocyanate, 500 mg; and 5,6-benzoflavone, 500 mg/kg diet). The monitored biomarkers included: DNA adducts in bronchoalveolar lavage cells, tracheal epithelium, lung and heart; oxidative damage to pulmonary DNA; hemoglobin adducts of 4-aminobiphenyl and benzo(a)pyrene-7,8-diol-9,10-epoxide; micronucleated and polynucleated alveolar macrophages and micronucleated polychromatic erythrocytes in bone marrow. Exposure of rats to smoke resulted in dramatic alterations of all investigated parameters. N-Acetyl-L-cysteine, phenylethyl isothiocyanate, and 5,6-benzoflavone exerted a significant protective effect on all alterations. 1,2-Dithiole-3-thione was a less effective inhibitor and exhibited both a systemic toxicity and genotoxicity in alveolar macrophages, whereas its substituted analogue Oltipraz showed limited protective effects in this model. Interestingly, combination of N-acetyl-L-cysteine with Oltipraz was the most potent treatment, resulting in an additive or more than additive inhibition of smoke-related DNA adducts in the lung and hemoglobin adducts. These results provide evidence for the differential ability of test agents to modulate smoke-related biomarkers in the respiratory tract and other body compartments and highlight the potential advantages in combining chemopreventive agents working with distinctive mechanisms.

INTRODUCTION

Worldwide tobacco smoke is the leading avoidable cause of cancer and other chronic degenerative diseases (1–3). Prevention of this plague is chiefly based on antismoke campaigns, which have been successful in attenuating the epidemic of lung cancer mortality in the male population of several countries (4, 5). A growing interest is centered on protection of the host organism either by means of dietary interventions or by using chemopreventive drugs (4). Chemoprevention may provide a complementary preventative approach in current smokers as well as an important strategy for lowering the risk in exsmokers and in passive smokers.

Evaluation of biomarkers in animal models is an essential step for the preclinical assessment of efficacy and safety of potential chemopreventive agents (6). In previous studies, we investigated the optimal conditions needed for the time course formation and persistence of Hb⁴ adducts⁵ oxidative damage to pulmonary DNA (7) and DNA adducts in organs (lung, heart, liver, bladder, and testis), tissues (dissected tracheal epithelium), and cells (BAL cells, mainly represented by PAM) of Sprague Dawley rats exposed whole body to ETS (7). ETS is a form of indoor air pollution resulting from the mixture of sidestream smoke and that portion of mainstream smoke that is released into indoor air by actively smoking individuals.

On the basis of these results, we designed a study aimed at evaluating the relative efficacy of several chemopreventive agents on a variety of smoke-related biomarkers in rats. Test agents included: (a) 1,2-D3T; (b) its substituted analogue OPZ, formerly used as a drug for schistosomiasis treatment; (c) PEITC, which is found at relatively high levels in watercress; (d) 5,6-BF, a synthetic flavonoid that interacts with the Ah receptor; and (e) NAC, an analogue and precursor of GSH. All agents were given p.o., either as dietary supplements (1,2-D3T, OPZ, PEITC, and 5,6-BF) or in drinking water (NAC). A treatment regimen combining OPZ and NAC was also assayed. The monitored biomarkers included: DNA adducts in BAL cells, tracheal epithelium, lung, and heart; oxidative damage to pulmonary DNA, evaluated by measuring 8-OH-dG levels; Hb adducts of 4-ABP and BPDE; cytogenetic damage, assessed by measuring the frequency of MN and PN PAM and of MN PCE in bone marrow. The present studies provide evidence for the differential ability of various test agents to modulate ETS-related markers of biologically effective dose and early biological damage. Furthermore, the study with NAC and OPZ highlights the potential of combining chemopreventive agents with distinctive mechanisms of action.

MATERIALS AND METHODS

Animals. A total of 64 male Sprague Dawley rats, aged 8 weeks, were purchased from Harlan Italy (Correzzana, Milan, Italy). The animals were housed at the University of Genoa in a climatized environment at a temperature of 22 ± 1°C, relative humidity of 50 ± 5%, ventilation accounting for 15 air renewal cycles/h, and with a 12-h light-dark cycle. The rats were acclimatized for 7 days during which they were maintained on Teklad IRM Rat/Mouse Diet (Harlan Italy) and were given drinking water ad libitum. Their weights at the time of exposure to ETS are reported in Table 1. Animal care was in accordance with Italian and institutional guidelines.

Exposure to Cigarette Smoke. The rats were divided into eight groups, each composed of eight animals. One group was kept in a filtered air environment (sham-exposed rats), whereas the remaining rats were exposed whole body to the smoke generated by Kentucky 2R1 reference cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY), having a declared content of 4.46 mg of total particulate matter and 2.45 mg of nicotine each. Before use, the cigarettes were kept for 48 h in a standardized atmosphere humidified with a mixture of 70% glycerol and 30% water. A mixture of sidestream smoke (89%) and mainstream smoke (11%),…

Received 5/22/00; accepted 1/16/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by National Cancer Institute Master Agreement N01-CN-75008.
2 Present address: National Center of Oncology, Sofia-1756, Bulgaria.
3 To whom requests for reprints should be addressed, at Department of Health Sciences, University of Genoa, Via A. Pastore 1, I-16132 Genoa, Italy. Phone: 39-010-3538500; Fax: 39-010-3538504; E-mail: sfd@unige.it.

4 The abbreviations used are: Hb, hemoglobin; BAL, bronchoalveolar lavage; PAM, pulmonary alveolar macrophages; ETS, environmental tobacco smoke; 1,2-D3T, 1,2-dithiole-3-thione; OPZ, Oltipraz or 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione; PEITC, phenylethyl isothiocyanate; 5,6-BF, 5,6-benzoflavone; NAC, N-acetyl-L-cysteine; GSH, reduced glutathione; 8-OH-dG, 8-hydroxy-2’-deoxyguanosine; 4-ABP, 4-aminobiphenyl; 4’-ABP, 4’-fluoroaminobiphenyl; BPDE, benz(o)pyrene-7,8-diol-9,10-epoxide; DRZ, diagonal radioactive zone; GC-MS, gas chromatography-mass spectroscopy.

5 S. R. Myers et al., manuscript in preparation.

Downloaded from cancerres.aacrjournals.org on September 23, 2017. © 2001 American Association for Cancer Research.
mimicking an exposure to ETS, was produced by using a smoking machine (model TE-10; Teague Enterprises, Davis, CA), where each smoldering cigarette was puffed for 2 s, once every min for a total of eight puffs, at a flow rate of 1.05 liters/min to provide a standard puff of 35 cm³ (8). The machine was adjusted to burn five cigarettes at one time, had a ydivided in two 3-h rounds via a cannula inserted into the trachea. A portion of BAL cells from each lungs of each rat with two 10-ml aliquots of cold (4°C) 0.15M NaCl infused puncture and fractionated into hematocrit and plasma, which were stored

cytogenetic analyses. The trachea, lungs, and heart were collected. The ciliated epithelium of the trachea was accurately scraped. All cells, tissues, and organs were stored at −80°C until use.

DNA Purification. Heart, lungs, and tracheal epithelium from each rat and BAL cells pooled within each group of rats were thawed and homogenized in a Potter-Elvehjem apparatus at 4°C in 250 mm sucrose, 5 mm DTT, 50 mm Tris-HCl (pH 7.6). DNA was isolated by solvent extraction using an automatic extractor (Genepure 341; Applied Biosystems, Foster City, CA) according to the method of Gupta (9), with some minor modifications as described previously (10). Due to the low amount of material available for each sample, DNA extraction from tracheal epithelium and BAL cells was achieved by using the same procedure, except that 30 μl of Quik-Precip (Edge-Biosystems, Gaithersburg, MD) were added during precipitation with alcohol. DNA was eluted with water. Purity of DNA was checked by spectrophotometric analysis (10).

Detection of DNA Adducts. Aliquots of 6 μg of DNA were assayed for the presence of DNA adducts by butanol extraction, as described previously (11). Each sample was labeled with 64 μCi of carrier-free [γ-32P]ATP (ICN Biochemicals, Irvine, CA), with a specific activity of ≥7000 Ci/mmol. TLC was carried out on sheets of polyethyleneimine (Macherey & Nügel, Düren, Germany) according to standard procedures (11). Based on the results of a previous study (7), 7 m urea and 3 m lithium formate (System A) were used in D3. However, in some experiments (see “Results”), we generally used isopropanol:4 M ammonium hydroxide (1:1, v/v; System B). Autoradiography was performed by using a 32P InstantImager Electronic Autoradiographic System equipped with InstantQuant software (model A2024; Packard, Meriden, CT). The relative adduct labeling index (cpm adduct/cpm normal nucleotides) was calculated, and DNA adduct levels in each sample were expressed as DNA adducts/10⁶ nucleotides. Due to the different amounts of available DNA, the samples were assayed either in two separate experiments (individual tracheal epithelia and pooled BAL cells) or three separate experiments (individual hearts and lungs). A 7R,8S,9R-trihydroxy-10R-(N'-deoxyguanosin-3'-phosphate)-7,8,9,10-tetrahydrobenzo(a)pyrene standard (National Cancer Institute Chemical Carcinogen Reference Standard Repository, Midwest Research Institute, Kansas City, MO) was used as a positive quality control to check the labeling efficiency in each experiment. On the whole, 528 32P postlabeling analyses were performed.
Detection of Oxidative DNA Damage. Oxidative damage was evaluated in lung DNA samples pooled within each experimental group. A $^{32}$P postlabeling procedure was used, as previously described (7, 12). As previously discussed (12), this procedure specifically detects $32$-OH-$d$G, which is the only modified nucleotide retained in chromatograms developed in acidic medium. To avoid artifacts that may result from radiation-induced oxidation of guanine, a selective hydrolysis of $7R,9S,9S$-trihydroxy-10R-(N$_2$-deoxyguanosyl)-3' ,6'-phosphate)-7,8,9,10-tetrahydrobenzo(a)pyrene was achieved by treating depolymerized DNA with 90% trifluoroacetic acid before the $^{32}$P postlabeling reaction (12). The results were expressed as $32$-OH-$d$G/10$^7$ nucleotides and were generated in two separate experiments, each one assessing duplicate-pooled DNA samples.

Detection of Hb Adducts. Hb adducts of 4-ABP and BPDE were evaluated at the University of Louisville by testing packaged RBC. 4-ABP and 4'-F-ABP were purchased from Fluka Chemika-Biochemika (Ronkonkoma, NY) and Sigma-Aldrich Chemical Co. (Milwaukee, WI), respectively. Trimethylamine in hexane was prepared by adding 1 g of trimethylamine hydrochloride (Fluka) to 2 ml of water, neutralizing the solution with NaOH, and extracting with 10 ml of hexane. The internal standard, 4'-F-ABP, was recrystallized from dichloromethane-hexane and used to prepare a stock solution of 25 ng/ml in 0.1 N HCl which was stored at 4°C. Pentafluoropropionic anhydride (PF-ABP) were purchased from Fluka Chemika-Biochemika (Ronkonkoma, NY) and Sigma-Aldrich Chemical Co. (Milwaukee, WI), respectively. Triethylamine was purchased from Fluka. The RBC were washed three times with 0.9% NaCl and lysed by the addition of 15 ml of ice cold deionized water and 2 ml of toluene with vigorous shaking. After 15 min, the lysate was removed by centrifugation at 10,000 g for 20 min to remove cellular debris. Hb concentrations were determined by measuring the absorbance at 415 nm (oxyHb; extinction coefficient, 125 mm$^{-1}$ cm$^{-1}$).

4-ABP adducts were measured by GC-MS (13). Before extraction the Hb samples were spiked by adding 400 pg of the internal standard 4'-F-ABP. The Hb solution was made 0.1 M in NaOH and incubated for 2 h at room temperature. The hydrolysat was extracted twice with 15 ml of methylene chloride (Fluka) to 2 ml of water, neutralizing the solution with NaOH, and extracting with 10 ml of hexane. The internal standard, 4'-F-ABP, was recrystallized from dichloromethane-hexane and used to prepare a stock solution of 25 ng/ml in 0.1 N HCl which was stored at 4°C. Pentafluoropropionic anhydride (PF-ABP) were purchased from Fluka. The RBC were washed three times with 0.9% NaCl and lysed by the addition of 15 ml of ice cold deionized water and 2 ml of toluene with vigorous shaking. After 15 min, the lysate was removed by centrifugation at 10,000 g for 20 min to remove cellular debris. Hb concentrations were determined by measuring the absorbance at 415 nm (oxyHb; extinction coefficient, 125 mm$^{-1}$ cm$^{-1}$).

Modulation of $^{32}$P-postlabeled DNA Adducts. The autoradiographic patterns were similar in BAL cells, tracheal epithelium, lung, and heart. In particular, up to four discrete spots could be distinguished in sham-exposed rats; two spots, close to the origin of the chromatogram, were identified as c1 and c2; two spots, characterized by a greater mobility, were identified as c1 and c2 (not shown). Spot 1 was detected in all cell types, whereas spot 3 was detectable only in the lung of sham-exposed rats. In ETS-exposed rats, 3 spots (1–3) were evident and consistently detected, and spots c1 and c2 were replaced by a massive DRZ (Fig. 1A).

Table 3 summarizes the results obtained in the eight experimental groups. In sham-exposed rats, the levels of $^{32}$P-postlabeled DNA adducts ranked as follows: heart > lung > BAL cells > tracheal epithelium. Exposure to ETS resulted in a marked increase of spots 1, 2, and 3 in the appearance of a DRZ. The ratio of total DNA adduct levels in ETS-exposed versus sham-exposed rats was 14.5 in the tracheal epithelium, 10.3 in BAL cells, 8.5 in lung, and 6.5 in heart.

With the exception of OPZ, all tested chemopreventive agents were successful in inhibiting the formation of ETS-related DNA adducts to a significant extent (Table 3). Table 4 shows at a glance the effects produced by chemopreventive agents on the monitored end points. The modulation of DNA adducts in BAL cells was least evident after the statistical analysis, because the necessity to work with pooled samples lowered the degrees of freedom. In any case, it is clear from both Table 3 and Table 4 that OPZ failed to affect DNA adduct formation in all types of cells. 1,2-D3T was ineffective in BAL cells but significantly decreased total DNA adducts and DRZ in the lung and tracheal epithelium; in addition, it inhibited spot 2 in the heart. PEITC, 5,6-BF, NAC, and its combination with OPZ displayed a potent and broad-spectrum inhibition of the ETS-induced nucleotide alterations.

Modulation of DNA adducts by chemopreventive agents was cal-
cuated as \[100 - (C - A/B - A) \times 100\], where \(A\) is DNA adduct levels in sham-exposed rats, \(B\) in ETS-exposed rats without chemopreventive agents, and \(C\) in ETS-exposed rats with each chemopreventive agent. The resulting rank of potency in inhibiting ETS-related total DNA adduct was as follows: BAL cells, OPZ + NAC (75.3%) > NAC (72.7%) > 5,6-BF (67.9%) > PEITC (57.6%) > 1,2-D3T (46.1%) > OPZ (14.7%, not significant); tracheal epithelium, OPZ + NAC (65.4%) > NAC (63.0%) > 5,6-BF (62.6%) > PEITC (59.1%) > 1,2-D3T (42.4%) > OPZ (17.1%, not significant); lung cells, OPZ + NAC (75.6%) > 5,6-BF (71.1%) > NAC (59.6%) > PEITC (52.9%) > 1,2-D3T (23.6%) > OPZ (4.9%, i.e., a nonsignificant increase); heart, 5,6-BF (60.2%) > PEITC (46.3%) > OPZ + NAC (41.9%) > NAC (36.2%) > 1,2-D3T (25.2%) > OPZ (10.2%, not significant).

Thus, combination of NAC with OPZ was consistently more efficient than NAC alone, but only in the lung inhibition of total DNA adduct levels by OPZ + NAC was it greater than the sum of the effects produced individually by the OPZ/NAC combination. Indeed, DNA adduct levels in the lung of ETS-exposed rats receiving OPZ + NAC (8.5 ± 3.6 adducts/10^8 nucleotides; see Table 3) were not only much lower than in rats receiving OPZ alone (25.5 ± 5.2, \(P < 0.001\)) but also appreciably lower than in rats receiving NAC alone (12.1 ± 3.4); this difference was statistically significant (\(P = 0.05\)).

The effects of exposure to ETS and treatment with chemopreventive agents on DNA adduct levels were quite similar in the different types of investigated cells. In fact, DNA adduct levels in the lung, tracheal epithelium, BAL cells, and heart among the eight experimental groups were highly correlated, as follows: lung versus tracheal epithelium: total adducts, \(r = 0.955\), \(P < 0.001\); DRZ, \(r = 0.977\), \(P < 0.001\); spot 1, \(r = 0.358\), not significant; spot 2, \(r = 0.877\), \(P < 0.01\); spot 3, \(r = 0.860\), \(P < 0.01\); versus BAL cells: total adducts, \(r = 0.893\), \(P < 0.01\); DRZ, \(r = 0.990\), \(P < 0.001\); spot 1, \(r = 0.583\), not significant; spot 2, \(r = 0.818\), \(P < 0.01\); spot 3, \(r = 0.568\), not significant; lung versus heart: total adducts, \(r = 0.828\), \(P < 0.01\); DRZ, \(r = 0.855\), \(P < 0.01\); spot 1, \(r = 0.795\), \(P < 0.05\); spot 2, \(r = 0.710\), \(P < 0.05\); spot 3, \(r = 0.843\), \(P < 0.01\); tracheal epithelium versus BAL cells: total adducts, \(r = 0.988\), \(P < 0.001\); DRZ, \(r = 0.985\), \(P < 0.001\); spot 1, \(r = 0.660\), not significant; spot 2, \(r = 0.858\), \(P < 0.01\); spot 3, \(r = 0.639\), not significant; tracheal epithelium versus heart: total adducts, \(r = 0.945\), \(P < 0.001\); DRZ, \(r = 0.922\), \(P < 0.001\); spot 1, \(r = 0.539\), not significant; spot 2, \(r = 0.856\), \(P < 0.01\); spot 3, \(r = 0.958\), \(P < 0.001\); BAL cells versus heart: total adducts, \(r = 0.893\), \(P < 0.01\); DRZ, \(r = 0.864\), \(P < 0.01\); spot 1, \(r = 0.527\), not significant; spot 2, \(r = 0.824\), \(P < 0.05\); spot 3, \(r = 0.589\), not significant.

Comparison of Two Chromatographic Conditions for Evaluating Inhibition of DNA Adducts in the Lung by Oltipraz and/or N'-Acetyl-l-cysteine. The observed effects of NAC and OPZ on smoke-induced formation of DNA adducts, as described in the previous section, were opposite to those found in another laboratory (see “Discussion”). This prompted us to perform additional experiments aimed at assessing whether these discrepant findings may be ascribed to the different conditions followed for the TLC separation of 32P-postlabeled DNA adducts. When using System A, which was the one used in all other experiments of the present study, the data (not shown) were fully comparable with those reported in Table 3.

DNA adduct patterns in the lung, as detected by using System B, were different from those detected by using System A, in that no DRZ was visible, and four discrete spots were detected, the chromatographic positions of which did not correspond to the three spots detected with System A (Fig. 1). Total DNA adduct levels in the lung of ETS-exposed rats were 15 times lower than those detected with System A, and inhibition patterns were also completely different. In fact, when using System B, NAC treatment did not affect DNA adduct levels and patterns, whereas OPZ produced an attenuation of DNA adducts (data not shown). A further decrease in DNA adduct levels was produced by combined treatment with OPZ and NAC, which significantly decreased both total DNA adducts and spot 1. Furthermore, the combined NAC/OPZ treatment significantly decreased total DNA adducts (\(P < 0.05\)) as compared with treatment with OPZ alone as well as total DNA adducts (\(P < 0.01\)), spot 1 (\(P < 0.05\)), and spot 2 (\(P < 0.01\)) as compared with treatment with NAC alone (data not shown). NAC did not inhibit at all total DNA adducts, OPZ produced a 35.3% inhibition, and OPZ + NAC produced a 58.8% inhibition. Therefore, although individually the two drugs yielded opposite effects under the two chromatographic conditions, their combined treatment consistently yielded more than additive effects.

Modulation of Oxidative DNA Damage. Oxidative DNA damage was evaluated in lung cells by measuring 8-OH-dG levels. The results reported in Table 3 are means ± SD of the values recorded in two separate experiments, each one assaying in duplicate lung DNA pooled within each group of rats. Exposure of rats to ETS resulted in a 2.2-fold increase of 8-OH-dG as compared with sham-exposed rats. As also shown in Table 4, all tested chemopreventive agents were successful in preventing the ETS-induced oxidative damage of pulmonary DNA, to such an extent that 8-OH-dG levels were comparable with those observed in sham-exposed rats.

The profiles of 8-OH-dG levels in lung DNA in the eight treatment groups were poorly correlated with DNA adduct levels in the same cells. The only significant correlation was between 8-OH-dG and DRZ (\(r = 0.720, P < 0.05\)), which indicates a parallel effect of exposure to ETS and treatment with chemopreventive agents on this marker of oxidative DNA damage and the nucleotide alterations contributing to formation of DRZ.

Modulation of Hb Adducts. The results of Hb adduct analyses are summarized in Table 3, where the data are reported as means ± SD within the eight rats composing each experimental group. The samples from each rat were run either in seven (BPDE-Hb) or eight (4-ABP-Hb) replicates. Exposure of rats to ETS resulted in powerful increases of both 4-ABP-Hb adducts (46.9-fold) and BPDE-Hb adducts (29.6-fold). All chemopreventive agents produced significant decreases of ETS-induced Hb adducts (Tables 3 and 4). In general, inhibition of 4-ABP-Hb adducts was more pronounced than inhibition of BPDE-Hb adducts. In particular, the rank of potency in inhibiting ETS-related 4-ABP-Hb adducts, calculated as indicated for DNA adducts, was: OPZ + NAC (68.0%) > 5,6-BF (52.3%) > PEITC (50.8%) > NAC (42.7%) > 1,2-D3T (33.8%) > OPZ (33.4%). The rank for BPDE-Hb adducts was:
OPZ + NAC (54.8%) > NAC (28.6%) > 1,2-D3T (27.7%) > 5,6-BF and PEITC (24.8%) > OPZ (24.2%).

Thus, combination of NAC with OPZ was by far the most effective treatment, yielding a 68.0% inhibition of 4-ABP adducts versus 76.1% resulting from the sum of the individual effects, and an obstacle for OPZ. In the absence of any chemopreventive agent. In ETS-related alterations, and 1,2-D3T even enhanced to a significant extent from the sum of the individual effects, and an obstacle for OPZ. In the absence of any chemopreventive agent. In ETS-related alterations, and 1,2-D3T even enhanced to a significant extent.

**DISCUSSION**

Whole-body exposure of Sprague Dawley rats to ETS consistently resulted in significant alterations of all of the investigated parameters, which covered a range of early biological modifications, from molecular damage to cytogenetic alterations. Promutagenic lesions were strongly enhanced in smoke-exposed rats, as shown by 6.5- to 15-fold increases of total DNA adduct levels in lung, heart, tracheal epithelium, and BAL cells. Moreover, the levels of two specific HB adducts related to smoke exposure (BPDE and 4-ABP) were increased 30- to 47-fold. The intensity of oxidative DNA damage (8-OH-dG levels) in lung cells as well as cytogenetic alterations both in the lower respiratory tract (PAM) and in bone marrow (PCE) were also significantly increased after exposure of rats to ETS, but to a less dramatic extent. These results are in agreement with the conclusions of our previous studies concerning the formation and stability of ETS-related DNA adducts in various rat tissues (7), induction of 8-OH-dG in lung cells (7), formation of 4-ABP- and BPDE-Hb adducts, and induction of cytogenetic damage in smoke-exposed rodents (reviewed in Ref. 17). In the same papers, we thoroughly discussed the possible pathogenetic meaning of these biological alterations. Taken together, these results converge in providing further evidence that cigarette smoke is a powerful inducer of genotoxic damage in multiple organs of exposed rodents, which contrasts with difficulties encountered in reproducing smoke-related lung carcinogenicity in animal models (3, 18).

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Sham</th>
<th>ETS</th>
<th>ETS + OPZ</th>
<th>ETS + 1,2-D3T</th>
<th>ETS + NAC</th>
<th>ETS + NAC + OPZ</th>
<th>PEITC</th>
<th>5,6-BF</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA adducts in BAL cells&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spot 1</td>
<td>0.7 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.1 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.0 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.1 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.9 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.8 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.5 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.0 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spot 2</td>
<td>0.0 ± 0.0</td>
<td>0.6 ± 0.2</td>
<td>0.4 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.1 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.1 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spot 3</td>
<td>0.0 ± 0.0</td>
<td>0.5 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.3 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.3 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.1 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.4 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>2.2 ± 0.6</td>
<td>26.8 ± 3.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23.3 ± 3.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.9 ± 3.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.0 ± 1.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.6 ± 2.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.7 ± 3.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.0 ± 2.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Whole-body exposure of Sprague Dawley rats to ETS consistently resulted in significant alterations of all of the investigated parameters, which covered a range of early biological modifications, from molecular damage to cytogenetic alterations. Promutagenic lesions were strongly enhanced in smoke-exposed rats, as shown by 6.5- to 15-fold increases of total DNA adduct levels in lung, heart, tracheal epithelium, and BAL cells. Moreover, the levels of two specific HB adducts related to smoke exposure (BPDE and 4-ABP) were increased 30- to 47-fold. The intensity of oxidative DNA damage (8-OH-dG levels) in lung cells as well as cytogenetic alterations both in the lower respiratory tract (PAM) and in bone marrow (PCE) were also significantly increased after exposure of rats to ETS, but to a less dramatic extent. These results are in agreement with the conclusions of our previous studies concerning the formation and stability of ETS-related DNA adducts in various rat tissues (7), induction of 8-OH-dG in lung cells (7), formation of 4-ABP- and BPDE-Hb adducts, and induction of cytogenetic damage in smoke-exposed rodents (reviewed in Ref. 17). In the same papers, we thoroughly discussed the possible pathogenetic meaning of these biological alterations. Taken together, these results converge in providing further evidence that cigarette smoke is a powerful inducer of genotoxic damage in multiple organs of exposed rodents, which contrasts with difficulties encountered in reproducing smoke-related lung carcinogenicity in animal models (3, 18).

The five chemopreventive agents, administered p.o., and the com-
bination of two of them were evaluated only in conjunction with exposure to ETS and at one dose for each agent. Agents and doses were selected based on our previous studies, data in the literature, and past or ongoing National Cancer Institute-sponsored studies (see, e.g., Refs. 19–32). OPZ and 1,2-D3T were administered at doses close to their maximum tolerated doses. NAC was used at a relatively high albeit nontoxic dose. PEITC was used at a dose that was highly effective in prior studies with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (19).

Under our experimental conditions, the two dithiolethiones administered to ETS-exposed rats displayed some adverse effects. In particular, 1,2-D3T was frankly toxic, as shown not only by the impaired food intake but especially by the striking lack of body weight gain. In particular, 1,2-D3T was frankly toxic, as shown not only by the impaired food intake but especially by the striking lack of body weight gain. In particular, 1,2-D3T was frankly toxic, as shown both by the impaired food intake but especially by the striking lack of body weight gain.

Table 4 Summary of the effects produced by chemopreventive agents on ETS-induced biomarkers

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>ETS + OPZ</th>
<th>ETS + DTT</th>
<th>ETS + NAC</th>
<th>ETS + NAC + OPZ</th>
<th>ETS + PEITC</th>
<th>ETS + BF</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA adducts in BAL cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spot 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spot 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spot 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA adducts in the tracheal epithelium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spot 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spot 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spot 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA adducts in the lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spot 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spot 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spot 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA adducts in the heart</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spot 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spot 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spot 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidative DNA damage in lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-OH-dG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin adducts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-ABP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPDE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytogetic effects</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MN PAM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MN PAM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MN PCE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*↑, significant increase compared with rats exposed to ETS only; ↓, significant decrease compared with rats exposed to ETS only; —, no significant variation.

It is well documented that dithiolethiones possess antigenotoxic and anticarcinogenic properties in a variety of experimental test systems (6, 21, 22). Among other mechanisms, these compounds act as monofunctional inducers of phase II enzymes (21). In the present study, OPZ alone failed to significantly affect the ETS-related formation of DNA adducts and induction of cytogenetic damage, whereas it significantly attenuated the pulmonary oxidative DNA damage and 4-ABP-Hb and BPDE-Hb adducts. Apart from its systemic toxicity and genotoxicity to PAM, 1,2-D3T significantly decreased ETS-induced total DNA adducts and DRZ in BAL cells, tracheal epithelium, and lung and total adducts, DRZ and spot 2 in the heart, oxidative damage to lung DNA, and Hb adducts to 4-ABP and BPDE.

The three remaining chemopreventive agents, i.e., 5,6-BF, PEITC, and NAC, were the most potent inhibitors of all of the investigated alterations resulting from exposure of rat to ETS. 5,6-BF is a broad-spectrum inducer of Phase I and Phase II activities involved in the metabolism of xenobiotics (23). We previously showed that this agent is a profound inhibitor of 7,12-dimethylbenz(a)anthracene-induced DNA adducts and mammary carcinogenesis in rats (24). PEITC, like other organic isothiocyanates, inhibits Phase I enzymes involved in carcinoigen activation and at the same time induces Phase II enzymes that accelerate cellular disposal of activated carcinogens (25). This effect has been also reported to occur with typical components of cigarette smoke, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (26). It has been postulated that enzyme induction is signaled by free isothiocyanates, the intracellular concentrations of which are inversely related to cellular GSH stores because isothiocyanates are readily and reversibly converted to dithiocarbamates (27). Either *per se* or as a precursor of intracellular cysteine and GSH, NAC is a weak inducer of both Phase I and Phase II enzymes and exerts antigenotoxic and anticarcinogenic effects through a variety of mechanisms, among which are nucleophilicity and scavenging of reactive oxygen species (28, 29). NAC has previously been found to significantly attenuate cytogenetic alterations in Sprague Dawley rats (30) and C57BL × DBA/2 F1 mice (17) as well as adducts to either mitochondrial DNA (31) or nuclear DNA (10, 11, 28, 32) in various tissues of Sprague Dawley rats exposed whole body to mainstream cigarette smoke.
In general, there was a close parallelism and a significant correlation in the modulation of total DNA adducts, DRZ, and the three individual spots among the four types of cell populations. Formation of DNA adducts in the mixed cell lung population, in tracheal epithelium, and in BAL cells reflects an increased risk of developing lung cancer and perhaps certain chronic obstructive pulmonary diseases, whereas formation of DNA adducts in perennail cardiac myocytes has tentatively been associated with smoke-related cardiomyopathies (33). However, both PEITC and NAC (34) and a mixture of PEITC and benzyl isothiocyanate (35) failed to inhibit the weak tumorigenic response induced in the lung of A/J mice exposed to ETS for 5 months, followed by 4 months of recovery in filtered air. A good parallelism and a significant correlation also occurred in the modulation of 4-ABP-Hb and BPDE-Hb adducts, which in turn were both highly correlated with total DNA adducts, DRZ, and the three individual spots in all investigated cell types. GSH and Hb compete for reaction with nitrosobiphenyl, a reactive metabolite of 4-ABP that covalently binds Hb (36), which explains the observed protective effect of precursors of intracellular GSH, such as NAC, which even in nonsmoking humans has been shown to lower 4-ABP-Hb adduct levels (37). These patterns should be taken into account in the design and choice of biomarkers in Phase II chemopreventive trials in smokers.

The efficiency of NAC in inhibiting DNA adducts, contrasting with the lack of protection by OPZ, deserves particular comments. In fact, opposite results have been obtained in another laboratory, in which NAC failed to inhibit the formation of lipophilic DNA adducts in tissues of Sprague Dawley rats exposed whole body to sidestream smoke (38), whereas OPZ was effective in the same model (39). The results of comparative analyses provided evidence that the above discrepancies are methodological and namely depend on the chromatographic conditions used in D3 for the separation of 32P-postlabeled DNA adducts. The problem is that, as evaluated in the comparative analyses reported in a previous study (7) and confirmed herein, System B yields a much lower amount of DNA adducts than System A and fails to detect the massive DRZ which is the expression of the multitude of DNA-binding agents present in cigarette smoke. Because System B underestimates the overall genotoxic potential of cigarette smoke, evaluation of the efficacy of chemopreventive agents by using this procedure is made only toward a few selected components of this complex mixture. In our experimental model, NAC was much more effective than OPZ in modulating all ETS-related biomarkers. In humans, NAC administration was found to decrease DNA adduct levels in BAL cells of Dutch smokers (40), and decreased the load of smoke-related urinary mutagens in the majority of the Italian subjects thus far examined (41). In contrast, OPZ failed to modulate the excretion of urinary mutagens in Chinese smokers. Therefore, on the whole, the data available in both humans and experimental animals suggest that NAC compares favorably with OPZ in attenuating smoke-related biomarkers. Nevertheless, the findings that administration of OPZ to rats inhibits smoke-induced lipophilic DNA adducts in the lung, as shown by Arif et al. (39) and confirmed in this study, and that OPZ can block in vitro alterations of human fetal lung DNA caused by cigarette smoke condensate (42) deserve further attention on some possible protective role of OPZ in smoke carcinogenesis.

An interesting issue was the outcome of the combination of NAC (given with drinking water) and OPZ (given with the diet) in modulating the monitored biomarkers, due to the distinctive and possibly complementary mechanisms of action of these drugs (22, 28, 29). A positive interaction between NAC and OPZ was observed for DNA adducts in the lung and both 4-ABP- and BPDE-Hb adducts.

In conclusion, the results of the present study indicate that chemopreventive agents working with different mechanisms of action can prevent a variety of biomarkers involved in early stages of the smoke-related pathogenesis of lung cancer and possibly of other chronic degenerative diseases in which alterations of these biomarkers can be detected (33, 43). The positive interactions observed by associating an aminothiol and a dithiolthione are encouraging in the framework of the expanding field of combined chemoprevention.

REFERENCES


Modulation of Biomarkers by Chemopreventive Agents in Smoke-exposed Rats

Alberto Izzotti, Roumen M. Balansky, Francesco D'Agostini, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/6/2472

Cited articles
This article cites 35 articles, 11 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/6/2472.full#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/61/6/2472.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.