Published in final edited form as:
Cancer Lett. 2008 June 28; 265(1): 135–147. doi:10.1016/j.canlet.2008.02.017.

The influence of diesel exhaust on polycyclic aromatic hydrocarbon-induced DNA damage, gene expression and tumor initiation in Sencar mice in vivo

Lauren A. Courter[a,*], **Andreas Luch**[b], **Tamara Musafia-Jeknic**[a], **Volker M. Arlt**[c], **Kay Fischer**[d], **Robert Bildfell**[d], **Cliff Pereira**[e], **David H. Phillips**[c], **Miriam C. Poirier**[f], and **William M. Baird**[a]

a*Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR 97331, USA*

b*Federal Institute for Risk Assessment, Departments of Product Safety & Scientific Services/ZEBET, Berlin, Germany*

c*Institute of Cancer Research, Section of Molecular Carcinogenesis, Sutton, Surrey SM2 5NG, United Kingdom*

d*College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331, USA*

e*Department of Statistics, Oregon State University, Corvallis, OR 97331, USA*

f*Carcinogen-DNA Interactions Section, National Cancer Institute, NIH, Bethesda, MD 20892, USA*

Abstract

The carcinogenic effects of individual polycyclic aromatic hydrocarbons (PAH) are well established. However, their potency within an environmental complex mixture is uncertain. We evaluated the influence of diesel exhaust particulate matter on PAH-induced cytochrome P450 (CYP) activity, PAH-DNA adduct formation, expression of certain candidate genes and the frequency of tumor initiation in the two-stage Sencar mouse model. To this end, we monitored the effects of treatment of mice with diesel exhaust, benzo[*a*]pyrene (BP), dibenzo[*a,l*]pyrene (DBP), or a combination of diesel exhaust with either carcinogenic PAH. The applied diesel particulate matter (SRM_{1975}) altered the tumor initiating potency of DBP: a statistically significant decrease in overall tumor and carcinoma burden was observed following 25 weeks of promotion with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), compared with DBP exposure alone. From those mice that were treated at the beginning of the observation period with 2 nmol DBP all survivors developed tumors (9 out of 9 animals, 100%). Among all tumors counted at the end, 9 carcinomas were detected and an overall tumor incidence of 2.6 tumors per tumor-bearing animal (TBA) was determined. By contrast, co-treatment of DBP with 50 mg SRM_{1975} led to a tumor rate of only 66% (19 out of 29 animals), occurrence of only 3 carcinomas in 29 animals and an overall rate of 2.1 tumors per TBA ($P = 0.04$). In contrast to the results with DBP, the tumor incidence induced by 200 nmol BP was

Author to whom correspondence and reprint requests should be addressed: William M. Baird, Department of Environmental & Molecular Toxicology, Oregon State University, Agricultural and Life Sciences 1007, Corvallis, OR 97331, Phone: 541-737-1886, Fax: 541-737-0497, Email: william.baird@oregonstate.edu.

[*] *Present Address: Center for Research on Occupational and Environmental Toxicology, Oregon Health* & Science University, Portland, OR 97239, USA.

found slightly increased when co-treatment with SRM_{1975} occurred (71% vs. 85% after 25 weeks). Despite this difference in tumor incidence, the numbers of carcinomas and tumors per TBA did not differ statistically significant between both treatment groups possibly due to the small size of the BP treatment group. Since bioactivation of DBP, but not BP, predominantly depends on CYP1B1 enzyme activity, SRM_{1975} affected PAH-induced carcinogenesis in an antagonistic manner when CYP1B1-mediated bioactivation was required. The explanation most likely lies in the much stronger inhibitory effects of certain PAHs present in diesel exhaust on CYP1B1 compared to CYP1A1. In the present study we also found molecular markers such as highly elevated *AKR1C21* and *TNFRSF21* gene expression levels in tumor tissue derived from animals co-treated with SRM_{1975} plus DBP. Therefore we validate microarray data as a source to uncover transcriptional signatures that may provide insights into molecular pathways affected following exposure to environmental complex mixtures such as diesel exhaust particulates.

Keywords

DNA adducts; carcinogenesis; diesel exhaust; PAH; cytochrome P450

1. Introduction

Diesel engine particulate exhaust contributes to air pollution, thus posing an environmental health risk for humans. Epidemiological studies indicate increases in morbidity and mortality due to air pollution [1,2]. For instance, an increased risk of lung cancer [3] and elevated levels of genotoxicity in various tissues [4–6] have been observed upon exposure to particulate matter. Polycyclic aromatic hydrocarbons (PAHs), which are large and co-planar compounds and products of incomplete combustion of fossil fuels, make up a substantial component of ambient air pollution. Similar to heterocyclics, quinones, and aldehydes, PAHs are also found to be adsorbed to diesel exhaust particulate matter [7,8].

Benzo[*a*]pyrene (BP) and dibenzo[*a*,*l*]pyrene (DBP) are well-studied carcinogenic PAHs found in many environmental complex mixtures, including tobacco smoke, automobile exhaust, and air pollution [9,10]. BP and DBP require metabolic activation in order to exert their carcinogenic potential [11]. In general, three distinct mechanistic pathways are involved in metabolic activation of PAHs to certain extents: (i) the monooxygenation route leading to diol-epoxides (i) or *o*-quinones (ii), and the radical cation pathway (iii). The source of activation for most carcinogenic PAHs are the cytochrome P450 (CYP) enzymes, in particular the forms CYP1A1 and CYP1B1 [12,13]. CYP enzymes convert PAHs into arene oxides and, after intermediate formation of dihydrodiols, into ultimate electrophilic and genotoxic diol-epoxides. Covalent DNA adduct formation by PAH diol-epoxides that may occur subsequently is potentially capable of inducing mutations in tumor suppressor genes and proto-oncogenes [14–16]. It has been shown that mutations in such critical target genes are associated with tumor formation in model organisms and increased carcinogenic risk in humans [17].

Since carcinogenic risk associated with individual PAH exposure is well characterized [18] concern exists about the regulation of PAH emissions and the potential threat to human health. Although PAHs are typically found in complex mixtures, much of the PAH research has focused on the effects of individual, potent carcinogenic member compounds such as BP or DBP. Due to difficulties and uncertainties in scientific evaluation, limited research has been done on binary or artificial mixtures [19–24], and to a lesser extent on complex mixtures [25–27]. These previous studies have focused primarily on the genotoxic effects of PAHs (e.g. DNA adduct formation) and metabolic activation catalyzed by CYP enzymes. In addition, other studies investigated the effects of environmental complex mixtures on cellular gene expression patterns through the use of high-density oligonucleotide microarrays [27–29]. In the present

study, we evaluated the influence of the presence of diesel exhaust particulate matter on CYP-mediated PAH activation, PAH-DNA adduct formation, PAH-induced gene expression and tumor initiation in Sencar mice in vivo.

2. Materials and methods

2.1. Chemicals and reagents

Caution: BP and DBP are potent carcinogens and should be handled according to National Cancer Institute guidelines. SRM_{1975} (organic extract of diesel particulate matter) is a known mutagen and should be handled with the same precautions. Solutions of PAHs and SRM_{1975} were prepared and used under subdued light.

The Standard Reference Material 1975 (SRM_{1975}), which is a dichloromethane extract of diesel exhaust particulate matter collected from an industrial diesel-powered forklift, was obtained from the National Institute of Standards and Technology (Gaithersberg, MD). A detailed description of the components of SRM_{1975} can be found at the following website: https://srmors.nist.gov/certificates/1975.pdf. BP and DBP were purchased from Chemsyn Science Laboratories (Lenexa, KS). Proteinase K (EC 3.4.21.64; from *Tritirachium album*), 7-ethoxyresorufin (ERES), resorufin (RES), and NADPH were purchased from Sigma (St. Louis, MO). Tris-equilibrated phenol, RNase T1 (EC 3.1.21.3; from *Asperigillus oryzae*) and RNase (DNase free, a heterogenous mixture of ribonucleases from bovine pancreas) were obtained from Boehringer Mannheim (Indianapolis, IN).

2.2. Tumor initiation

Here we utilized the Sencar mouse two-stage skin tumorigenesis model. Sencar mice are susceptible to chemical carcinogens and thus particularly sensitive to tumor initiation [30]. The initiation and promotion stages are clearly distinguished. Mice were obtained from NCI-Frederick Cancer Research and Development Center (Frederick, MD). During the resting phase of their hair-growth cycle (at 6–7 weeks of age), the dorsal area of female mice was shaved two days prior to treatment with toluene (vehicle control), SRM_{1975}, BP, and DBP alone, or SRM_{1975} plus BP or DBP. There were six exposure groups with six mice per group. Mice were housed in polycarbonate cages (3 mice per cage) and fed with Teklad rodent diet (No. 8604, Harlan) and water ad libitum. They were maintained at 22°C on a standard 12 hour light/dark cycle with 40–60% relative humidity. Animals were housed and cared for in accordance with the Institute of Laboratory Animal Resources (ILAR, 1996), Commission of Life Sciences, National Research Council document entitled, *Guide for the Care and Use of Laboratory Animals*. Topical treatments were carried out as follows: 10 mice with 200 μL toluene, 10 mice with 200 nmol (50.4 μg) BP, 10 mice with 2 nmol (0.6 μg) DBP, 35 mice with 50 mg SRM_{1975}, 35 mice with 50 mg SRM_{1975} plus 200 nmol BP, and 35 mice with 50 mg SRM_{1975} plus 2 nmol DBP. The doses applied were chosen based on the tumorigenic potency of compounds observed in previous investigations [26,31,32], or on the genotoxic effects previously observed with similar diesel exhaust extracts [33]. Two weeks after initiation, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) at 1 μg/200 μL acetone per mouse was topically administered twice-weekly for 25 weeks, and mice were examined weekly for the occurrence of skin papillomas. Following necropsy, tumors were confirmed by routine histopathology techniques by the Integrative Health Sciences Facilities and Service Cores of the Environmental Health Sciences Center at Oregon State University.

2.3. DNA isolation from mouse epidermis

Following acclimatization, mice were shaved and treated with SRM_{1975}, PAHs, or SRM_{1975} plus either PAH. Twenty-four hours after treatment, the mice were killed by cervical dislocation, and the epidermal cells were harvested as described by Slaga et al [34]. Briefly,

Nair® depilatory cream was applied to the shaven dorsal area to remove residual fur and skin was rinsed off with cold water. Harvested skins were submerged in a 58°C water bath for 30 s and placed in water on ice, followed by removal of the epidermal cells by scraping with a razor blade. The epidermal cells from six mice per treatment group were pooled, placed in 500 μL of 0.025 M EDTA/0.075 M NaCl buffer, and stored in −80°C for subsequent DNA isolation or used immediately for microsome preparation.

The DNA was isolated as described [35]. Briefly, the pooled mouse epidermal samples from each treatment group were homogenized in a glass homogenizer containing EDTA, SDS buffer [10 mM Tris, 1 mM Na_2EDTA, 1% (w/v) SDS, pH 8]. Homogenates were treated with 50 units/mL RNase, DNase-free and 1000 units/mL RNase T1 at 37°C for 1 hour, followed by treatment with 500 μg/mL proteinase K at 37°C for 1 hour. DNA was extracted with 25:24:1 phenol:chloroform:isoamyl alcohol, precipitated with 100% ethanol and dissolved in sterile double-distilled water.

2.4. ^{32}P-postlabeling of DNA adducts

DNA adducts were measured for each DNA sample using both the nuclease P1 digestion and butanol extraction enrichment versions of the ^{32}P-postlabeling [36]. Briefly, DNA samples (4 μg) were digested with micrococcal nuclease (120 mUnits) and calf spleen phosphodiesterase (40 mUnits), enriched and labeled as reported. Chromatographic conditions for thin-layer chromatography (TLC) on polyethylene imine-cellulose (PEI-cellulose, Macherey-Nagel Düren, Germany) were as follows [37,38]: D1, 1.0 M sodium phosphate, pH 6; D3, 4 M lithium-formate, 7 M urea, pH 3.5; D4, 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8. After chromatography TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, IL, USA) and DNA adduct levels (RAL, relative adduct labeling) were calculated from adduct cpm, the specific activity of [γ-^{32}P]ATP and the amount of DNA (pmol of DNA-P) used. An external BP diol-epoxide-DNA standard was used for identification of DNA adducts detected in experimental samples [39]. Results were expressed as DNA adducts/10^8 nucleotides.

2.5. Microsome preparation

Microsomes were prepared as previously described [40], with minor modifications. The epidermal cells were harvested as described above; however, the heat treatment was carried out at 52°C instead of 58°C. Epidermal samples were homogenized with a steel homogenizer containing microsomal homogenization buffer [0.25 M K_2PO_4, 0.15 M KCl, 10 mM EDTA, and 0.25 mM phenylmethlysulfonylfluoride (PMSF)] and centrifuged at 15,000 × g for 20 min at 4°C. The supernatant was centrifuged at 100,000 × g for 90 min at 4°C, and the pellet was resuspended in microsome dilution buffer [0.1 M KH_2PO_4, 20% glycerol, 10 mM EDTA, 0.1 mM DTT and 0.25 mM PMSF]. Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL).

2.6. Ethoxyresorufin O-deethylase (EROD) assay

Fifty micrograms of microsomal protein were added to 1 μM 7-ethoxyresorufin (ERES) in 200 μL 0.1 M Tris-HCl buffer (pH 7.8) per well in a black 96 well plate (E&K Scientific, Campbell, CA). NADPH was added to each well and the fluorescence was measured on a Spectra MAX Gemini plate reader (Molecular Devices, Sunnyvale, CA). The kinetic assay was monitored over 15 min at 37°C with an excitation wavelength at 535 nm and emission wavelength at 585 nm. Each sample was assayed in triplicate over three separate experiments. The amount of resorufin produced was calculated from the fluorescence of concentration standards. Ten micrograms of microsomal protein isolated from Chinese hamster ovary (CHO) cells over-expressing human CYP1A1 were used as positive control.

2.7. Quantitative real-time PCR analysis

Relative quantitation with real-time reverse transcriptase PCR (qRT-PCR) was performed using an ABI Prism 7000 Sequence Detection System (PE Applied Biosystems, Foster City, CA), according to manufacturer's instructions. RNA was isolated from skin tumor tissue or from skin tissue treated with toluene (solvent control). Since only low-quality RNA was obtained from DBP-induced tumor tissue, qRT-PCR could not be conducted in this exposure group. The cDNA was synthesized by reverse transcription of 10 μg RNA (Advantage RT-for-PCR Kit; Clontech-Takara Bio, Mountain View, CA). The cDNA preparation was then diluted 1:50 and 1.5 μL was used as template to run qRT-PCR in a 50 μL reaction mixture applying 20X Assays-on-Demand gene expression primers and probes (PE Biosystems) for all genes analyzed (assay identification number; GenBank accession number): *CYP1A1* (Mm00487218_m1; NM_009992), *CYP1B1* (Mm00487229_m1; NM_009994), *ALDH3A1* (Mm00839312_m1; NM_008562), *AKR1C21* (Mm00472624_m1; NM_029901), *NQO1* (Mm00500821_m1; NM_008706), *LGALS8* (Mm00497776_m1; NM _018886), *CYR61* (Mm00487498_m1; NM_010516), *MCL1* (Mm00725832_s1; NM_008562), *TNFRSF21* (Mm00446361_m1; NM_178589), *FOS* (Mm00487425_m1; NM_010234), and the house-keeping gene *GAPDH* (Mm99999915_g1; NM_001001303). qRT-PCR consisted of initial denaturation for 10 min at 95°C, 40 cycles at 95°C for 15 sec, and 50°C for 1 min. Each sample (obtained from one exposure group) was assayed in triplicate and the cycle threshold values were normalized to *GAPDH* and the fold-change was calculated using the $2^{-\Delta\Delta C}T$ method [41].

2.8. Statistical analysis

Mortality and final tumor rates were compared between treatments with two-sided Fisher's exact tests (FET) utilizing the Exact statement in the SAS FREQ procedure. Numbers of tumors per tumor-bearing animal (TBA) were compared between treatments with the Wilcoxon rank test utilizing the Exact statement in the SAS NPAR1WAY procedure. The time-until-tumor data were compared between treatments with survival analysis methods. The Kaplan-Meier Log rank (LR) test was used to compare treatments non-parametrically utilizing the SAS LIFETEST procedure. Treatments were also compared semi-parametrically with Cox proportional hazard regression utilizing the SAS PHREG procedure. For all of the analyses above, prior to treating the mouse as the individual independent unit, it was first confirmed that there was little evidence of any cage effects ($P = 0.33$). In each case, this was done by comparing cages within each treatment with the analysis method appropriate for the response (FET, Wilcoxon, LR, or Cox regression). Comparison of PAH-DNA adduct levels, EROD and qRT-PCR results between treatment groups were carried out using ANOVA. Tumor analyses were conducted within version 9.1 of the SAS System for Windows (SAS Institute, Inc., 2003, Cary, NC). PAH-DNA adduct, EROD and qRT-PCR statistical analyses were carried out using GraphPad Prism software 4.00 for Windows (GraphPad Software, San Diego, CA).

3. Results

3.1. Effect of SRM_{1975} on PAH-induced tumor initiation

In order to determine the effects of diesel exhaust on BP- and DBP-mediated tumor initiating activity, we analyzed time-until-tumor formation, tumor incidence, and the number of tumors per TBA in response to the treatment of mice with carcinogens in the presence or absence of SRM_{1975}. Table 1 compiles the data on the tumor initiating activity of the two carcinogenic PAHs tested each alone or on co-exposure with SRM_{1975}, following 25 weeks of promotion with TPA. SRM_{1975} alone was a weak tumor initiator: just one mouse out of 26 survivors (3.8%) has developed one papilloma at the end of the observation period (week 25; Table 1, Fig. 1). In contrast, papillomas first emerged at weeks 10, 4, 3, and 2 in mice treated with BP, SRM_{1975} plus BP, DBP, and SRM_{1975} plus DBP, respectively (Fig. 1*A*, 1*B*).

The effect of SRM_{1975} on the tumor initiating activity of BP in mouse skin is illustrated in Fig. 1*A* and Table 1. Mice treated with SRM_{1975} plus BP started to develop tumors after 4 weeks of promotion, about 6 weeks earlier compared to mice treated with BP alone (Fig. 1*A*). Standard statistics that assume proportional hazards give little evidence of a difference between SRM_{1975} plus BP and BP ($P = 0.22$, LR and Cox regression) due to the different numbers of mice in both treatment groups (35 vs. 10) and a possible cage effect in the group treated with SRM_{1975} plus BP. However, the alternative non-parametric Wilcoxon test that gives greater weight to earlier deaths (relative hazard decreasing with time) provides evidence of a treatment difference ($P = 0.04$). At the end of the observation period, the overall percentage of TBA was higher in animals exposed to the combination of BP and SRM_{1975} (84.6%) compared to those treated with BP alone (71.4%). Nevertheless the overall tumor burden was only slightly different and not statistically different (BP: 2.4 tumors/TBA vs. SRM_{1975} plus BP: 2.9 tumors/TBA; FET, $P = 0.58$) (Table 1).

No significant difference in time-until-tumor formation was observed for DBP when compared to SRM_{1975} plus DBP treated animals (Fig. 1*B*; LR and Cox regression, $P = 0.2$). However, the presence of SRM_{1975} significantly reduced the incidence of malignant tumors (i.e., carcinoma) and the overall tumor burden induced by DBP, compared with DBP exposure alone (FET; $P = 0.002$ and $P = 0.04$, respectively) (Table 1). Conversely, no significant difference in the incidence of benign tumors (i.e., papilloma) was observed between mice treated with DBP alone and those co-treated with SRM_{1975} plus DBP, followed by 25 weeks of promotion (FET, $P = 0.44$) (Table 1).

3.2. Influence of SRM_{1975} on PAH-DNA adduct levels

The effects of diesel exhaust on PAH-DNA adduct formation in the epidermis of Sencar mice upon treatment with carcinogens in the presence or absence of SRM_{1975} was investigated. Representative TLC autoradiographs are shown in Fig. 2*A*, and total PAH-DNA adduct levels using the nuclease P1 and butanol enrichment procedure are illustrated in Fig. 2B.

Epidermis isolated from mice 24 hours after topical treatment with 50 mg SRM_{1975} exhibited virtually no detectable DNA adducts and was therefore found comparable to the epidermis from mice of the solvent (toluene) control group (Fig. 2*A*). BP showed its typical DNA adduct pattern consisting of one major DNA adduct which was identified as 10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydro-BP [39]. One major and multiple minor DNA adducts were observed after treatment with DBP. No difference in adduct pattern was observed between nuclease P1 and butanol enrichment. Generally, enrichment with nuclease P1 digestion yielded higher DNA adduct levels than with butanol extraction, but the differences in adduct levels observed were small and not statistically significant (Fig. 2*B*, $P > 0.05$). Using nuclease P1 digestion the PAH-DNA adduct levels observed were higher in epidermis co-treated with SRM_{1975} plus BP (110 ± 44 RAL/10^8 nucleotides) when compared with BP alone (65 ± 31 RAL/10^8). However, this difference was not statistically significant ($P = 0.23$), and was even more diminished when using butanol extraction ($P = 0.71$). Similarly, no significant difference in the DNA adduct levels of epidermis treated with SRM_{1975} plus DBP was observed when compared with DBP alone, for both the nuclease P1 digestion (35 ± 27 vs. 24 ± 5 RAL/10^8 nucleotides; $P = 0.52$) and butanol extraction methods (29 ± 35 vs. 13 ± 2 RAL/10^8 nucleotides; $P = 0.49$).

3.3. Influence of SRM_{1975} on EROD activity

The EROD assay was conducted to determine the effect of diesel exhaust on CYP-mediated activity in epidermal cells from mice 24 hours after treatment with SRM_{1975} and PAHs (Table 2). A significant decrease in EROD activity was detected in the epidermis from animals after co-treatment with SRM_{1975} and BP (0.48 ± 0.41 pmol/min/mg) when compared with BP

treatment alone (1.33 ± 0.21 pmol/min/mg) (ANOVA; $P < 0.05$). However, no such difference in EROD activity was determined in epidermal microsomes retrieved from animals treated with SRM_{1975} plus DBP (0.66 ± 0.34 pmol/min/mg) when compared to DBP exposure (0.33 ± 0.27 pmol/min/mg) ($P = 0.49$). SRM_{1975} alone (0.69 ± 0.08 pmol/min/mg) was found to be without any detectable effect on CYP expression compared to baseline expression in animals exposed to solvent (toluene) only (0.65 ± 0.21 pmol/min/mg).

3.4. Changes in the expression of candidate genes

In order to determine the influence of diesel exhaust particulate matter on PAH-induced gene expression in vivo, several candidate genes were chosen based on the results obtained by global gene expression analysis of human breast carcinoma MCF-7 cells treated with diesel exhaust and the same two carcinogens (unpublished data). The selected genes were *CYP1A1*, *CYP1B1*, *AKR1C21*, and *NQO1*, all of which are involved in PAH metabolism. Expression levels of genes previously found to be over-expressed in breast and pulmonary tumors were also investigated, i.e., *TNFRSF21*, *CYR61*, *MCL1*, *LGALS8*, and *FOS* [42,43]. As indicated in Fig. 3 and Table 3, a slight but not significant ($P > 0.05$) increase in the expression levels of *AKR1C21* and *TNFRSF21* were observed in tumors harvested from mice after treatment with SRM_{1975} plus BP (1.8- and 1.2-fold, respectively) when compared with BP alone (1.0- and 0.8-fold, respectively). For *LGALS8*, the change of expression levels from 1.2-fold (BP) up to 3.9-fold (SRM_{1975} plus BP) was found to be significant ($P = 0.047$) (Fig. 3). Tumors derived from mice co-exposed to SRM_{1975} plus DBP exhibited a tremendous increase in the expression levels of all three genes, *TNFRSF21*, *LGALS8* and *AKR1C21*, when compared with vehicle control (toluene), BP, and SRM_{1975} plus BP (Fig. 3, Table 3; $P < 0.001$).

4. Discussion

Together with hundreds of different chemical compounds, PAHs are present in a variety of environmental complex mixtures such as urban air pollution, diesel exhaust, and tobacco smoke. Humans are primarily exposed to these complex mixtures rather than to single compounds. Therefore the effects and mechanisms exerted by such mixtures must be established in order to define the true health risks to those exposed. However, studying and characterizing complex mixtures is difficult and the results are often hard to interpret due to possible antagonistic or synergistic chemical interactions [24,38,44]. Most studies have thus been conducted in a simplified manner by applying single but extremely potent PAHs [44, 45]. The complex reference diesel exhaust material SRM_{1975} applied in this study consists of dozens of different unsubstituted PAHs such as naphthalene (0.67 mg/kg), phenanthrene (8 mg/kg), pyrene (0.42 mg/kg), chrysene (1.95 mg/kg), benzo[*b*]fluoranthene (BF, 3.2 mg/kg), benzo[*e*]pyrene (BeP, 0.268 mg/kg), benz[*a*]anthracene (BA, 0.092 mg/kg), dibenzanthracenes (DBAs, 0.079 mg/kg), and of their methylated and nitro-substituted derivatives [46]. However, BP and DBP, both of which have been well characterized according to their strong genotoxic and carcinogenic activity in rodent tumor models [31,32] could not be detected in this complex matrix. To exhibit carcinogenic activity these compounds require CYP-catalyzed metabolic conversion into their ultimate DNA-damaging forms, the BP-7,8-diol 9,10-epoxide (BPDE) and DBP-11,12-diol 13,14-epoxide (DBPDE) [47,48]. In order to better appreciate the influence of chemical complexity in environmental sources containing carcinogens of varying potency we were investigating and characterizing the effects of the complex matter SRM_{1975} on the genotoxicity and gene expression pattern induced by BP or DBP in mice in vivo.

Bioactivation of PAHs adsorbed to the surface of diesel exhaust particulates was shown to result in the formation of bulky PAH-DNA adducts and elevated mutation rates in lungs of animals exposed [49–51]. Hence, the carcinogenic activity of diesel exhaust demonstrated in rats [52] and also presumed for humans based on weak but evident epidemiological findings

[53] is mechanistically explainable at the molecular level. However, the effects found in rats were only small and not reproducible at low doses of diesel exhaust (< 2000 μg/m^3), or shorter than life-time exposures and in other species such as mice or hamsters [52]. Consistent to this weak potency, the diesel exhaust matter SRM_{1975} applied here topically onto mice skin proved to be virtually non-tumorigenic: only one papilloma could be observed at the end of the observation period of 25 weeks (Table 1, Fig. 1). On co-exposure with BP and DBP, SRM_{1975} reduced the percentage of TBA and the numbers of carcinomas and overall tumor burden initiated by DBP compared with DBP alone. By contrast, co-exposure to SRM_{1975} had slightly opposite effects on the number of BP-induced papillomas or carcinomas that emerged at the end of the observation period: the percentage of TBA increased from 71.4 up to 84.6% and the number of tumors per TBA was counted at 2.9 compared to 2.4 with BP alone (Table 1). In addition, during the observation time the tumors developed much earlier in animals co-treated with BP and SRM_{1975} compared to animals treated with BP alone (Fig. 1*A*). This is consistent with higher overall DNA adduct levels observed at early time points in those animals co-treated with diesel exhaust material and BP compared to animals treated with BP alone (110 ± 44 vs. 65 ± 31 RAL/10^8 24 h after treatment; Fig. 2*B*).

Decreases in carcinogenicity of PAHs have been described for binary, tertiary or complex mixtures [54]. Antagonistic interactions can be attributed to the disturbance of metabolic activation or a shift toward detoxification [24,26]. After decades of research on the Ah receptor-mediated CYP-inducing effects of planar carcinogenic PAHs such as BP (cf. below), inhibition of human CYP1A1 and CYP1B1 by PAHs such as BA, BF, BeP, and others, has been described just recently [55,56]. Compared to human CYP1A1, inhibition of human CYP1B1 by individual compounds was found always much stronger and usually IC_{50} values were determined < 150 nM. For instance, BF, BA and BeP inhibited CYP1B1- dependent EROD activity with IC_{50} values of 4.9, 9.1 and 17 nM, while corresponding values for inhibition of CYP1A1 were 250, 170 and > 1,000 nM, respectively [55]. Since most of these compounds were also detected in diesel exhaust SRM_{1975} particulate matter in considerable amounts (vide supra), preferential inhibition of murine CYP1B1 in mouse skin would provide a rationale for the reduced numbers of tumors observed in animals co-treated with SRM_{1975} plus DBP. Although the antagonistic effects of PAHs on murine CYP enzymes have as yet not been investigated, it is well established that murine CYP1B1-catalyzed activation plays a predominant role in DBP-induced DNA damage and carcinogenesis [57,58], while murine CYP1A1 is central in the bioactivation of BP and other planar carcinogenic PAHs such as BA or DBAs [58]. The presence of BA and DBAs as constituents of SRM_{1975} [46] on the back of mice along with unaffected activity of cutaneous CYP1A1 may therefore explain the slightly higher DNA adduct levels and earlier onset of skin tumorigenesis in mice co-treated with the mixture plus BP compared to BP alone (Fig. 1*A* and Fig. 2*B*). At early time points, the co-exposure seems to have resulted in additive effects of carcinogens that are predominantly activated toward DNA-damaging metabolites by CYP1A1. On the other side, both CYP1A1-dependent toxification of BA, DBAs, and others, and concurrent mixture-dependent inhibition of DBP activation may have contributed to an unchanged overall DNA adduct level (Fig. 2*B*) and time-course of tumorigenesis (Fig. 1B) in animals cotreated with SRM_{1975} plus DBP compared to DBP alone.

Besides direct inhibition of CYP enzymes involved in metabolic activation, PAH mixtures have been shown of being able to target the expression of regulatory proteins involved in metabolism, DNA repair, cell cycle regulation, cellular migration and adhesion, thus altering the cell's ability to maintain its genomic integrity and growth control [27,59–63]. In the present study we determined gene expression changes in tumor tissue developed in mice upon treatment with BP or DBP in the absence or presence of SRM_{1975} (Fig. 3,Table 3). Among those genes investigated, significant changes in expression levels were observed in papilloma tissue retrieved from mice for the following genes: *CYP1A1* (BP exposure group), *AKR1C21*,

TNFRSF21, or *LGALS8* (DBP & SRM_{1975} exposure group), and *LGALS8* (BP & SRM_{1975} exposure group). The 12-fold increase in *CYP1A1* gene expression within tumor tissue compared to control at the end of the observation period confirms the CYP1A1-inducing capability of planar carcinogenic PAHs such as BP in mouse skin as previously judged for early time points (24 h) via protein detection [26] or in the present study via activity assays (Table 2). Conversely, the presence of diesel exhaust SRM_{1975} particulates completely inhibited the CYP1A1-inducing effects of BP in tumor tissue at the end of the observation period (Table 3).

Among the other genes investigated in this study, *AKR1C21*, *TNFRSF21*, and *LGALS8* were found highly induced in tumor tissue that developed in mice upon co-treatment with SRM_{1975} and DBP (Table 3). Since aldo-keto reductases (AKR) catalyze reductive conversion of PAH dihydrodiols into catechols and *o*-quinones [64], up-regulation of certain forms of AKR1C2 most likely compensates for low monooxygenation rates under conditions when CYP enzymes are inhibited. Up-regulation of *AKR1C2* expression became evident in SRM_{1975}-treated mice upon co-exposure to DBP, but not BP (Table 3). This difference again may be due to the preferential and strong inhibition of CYP1B1 by constituents of SRM_{1975} and their only weak activity against CYP1A1 (vide supra). Since elevated *AKR1C2* expression levels have also been observed at CYP-suppressing conditions in human cells treated with SRM_{1975} [65], urban dust particulate matter [66] or cigarette smoke condensate [67], this gene may have the potential to serve as a biomarker for human cancers associated with environmental exposures to complex PAH mixtures [64,68].

TNFRSF21 belongs to the tumor necrosis factor receptor superfamily, responsible for inducing apoptosis through caspase activation and cytochrome c release from mitochondria [69]. We observed a dramatic increase in *TNFRSF21* expression in mice exposed to SRM_{1975} plus DBP (~ 5000-fold), compared to SRM_{1975} plus BP (~ 1.2-fold) or vehicle control (Fig. 3). These results compare with a significant increase of *TNFRSF1B* expression in human alveolar macrophages in response to diesel exhaust exposure [70]. Although the dose of 2 nmol DBP proved to be more tumorigenic than 200 nmol of BP (Table 1), upon addition of SRM_{1975} the total number of papillomas and number of tumors per TBA was found much smaller in the DBP co-treatment group (40 and 2.1, respectively) compared to the BP co-treatment group (64 and 2.9, respectively). One reason for this effect might have been the overexpression of *TNFRSF21* and subsequent promotion of receptor-dependent apoptosis, leading to decreased tumor burden at the end of the observation period. Unfortunately, due to only low-quality RNA preparations we were unable to conduct qRT-PCR analysis on tumors derived from the DBP-treated group of mice. However, analysis of *TNFRSF21* in human MCF-7 cells revealed that DBP alone is less effective in induction of this gene than BP alone or BP in combination with diesel exhaust particulates (unpublished data; see also [71,72]).

LGALS8 encodes for a β-galactoside-binding soluble lectin, galectin-8, and various studies showed that it is widely expressed in tumor tissues as well as in normal tissues [73]. The level of expression may correlate with the malignancy of human colon cancers and the degree of differentiation of lung squamous cell carcinomas and neuroendocrine tumors [74]. We observed a significant increase in the expression of this gene in tumor tissue of mice following co-treatment with SRM_{1975} plus either BP or DBP compared to BP alone (Fig. 3, Table 3), and a similar effect has been verified in human cells in culture (unpublished data). The association of increased galectin-8 expression with increased cell migration and malignancy may provide an answer to the observation that mice developed much earlier visible and spreading tumors on co-treatment with SRM_{1975} plus DBP or BP compared to BP alone (after 2 and 4 weeks vs. 10 weeks; Fig. 1).

In conclusion, our results demonstrate that diesel exhaust SRM_{1975} matter influences PAH-induced carcinogenesis in an antagonistic manner when bioactivation through CYP1B1 is required. By contrast, CYP1A1-mediated carcinogenesis seems rather slightly being accelerated and increased by this mixture. The explanation most likely lies in the much stronger inhibitory effects of certain PAHs present in these mixtures on CYP1B1 compared to CYP1A1 [55,56,75]. Since bioactivation of DBP, but not BP, depends to great extents on CYP1B1 enzyme activity, the inhibitory effects of diesel exhaust particulates on tumorigenesis were found significant in animals initiated with DBP. Molecular markers such as elevated *AKR1C21* or *TNFRSF21* gene expression levels were detected in tumor tissue derived from animals cotreated with SRM_{1975} plus DBP. Therefore we validate microarray data as a source to uncover potential molecular pathways involved in PAH-mediated cancer progression in vivo, and suggest that mining of these data will provide a focus for future studies for elucidating mechanisms involved in environmental chemical carcinogenesis. It is important to note that the results reported herein were obtained following a single application of the chemical mixture. Investigations into extended exposures to diesel exhaust in vivo are warranted to better compare with long-term human exposures to such environmental mixtures.

Acknowledgments

This work was supported by NCI grant CA28825, DHHS, Cancer Research UK and NIEHS Pre-Doctoral Training Grant T32 ES007060 (L.A.C.). V.M.A. and D.H.P. are members of ECNIS (Environmental Cancer Risk, Nutrition and Individual Susceptibility), a network of excellence operating with the European Union 6th Framework Program, Priority 5: Food Quality and Safety (Contract No. 513943). The authors thank Wan-Mohaiza Dashwood for assistance with the animals, Eric Brooks for technical help, and Yonghai Li for statistical assistance. We further acknowledge the Integrative Health Sciences Facilities and Services Core of the Environmental Health Sciences Center at OSU, supported by the NIEHS Center, grant P30 ES00210. We also wish to express appreciation to Brinda Mahadevan and Channa Keshava for critical review of this manuscript.

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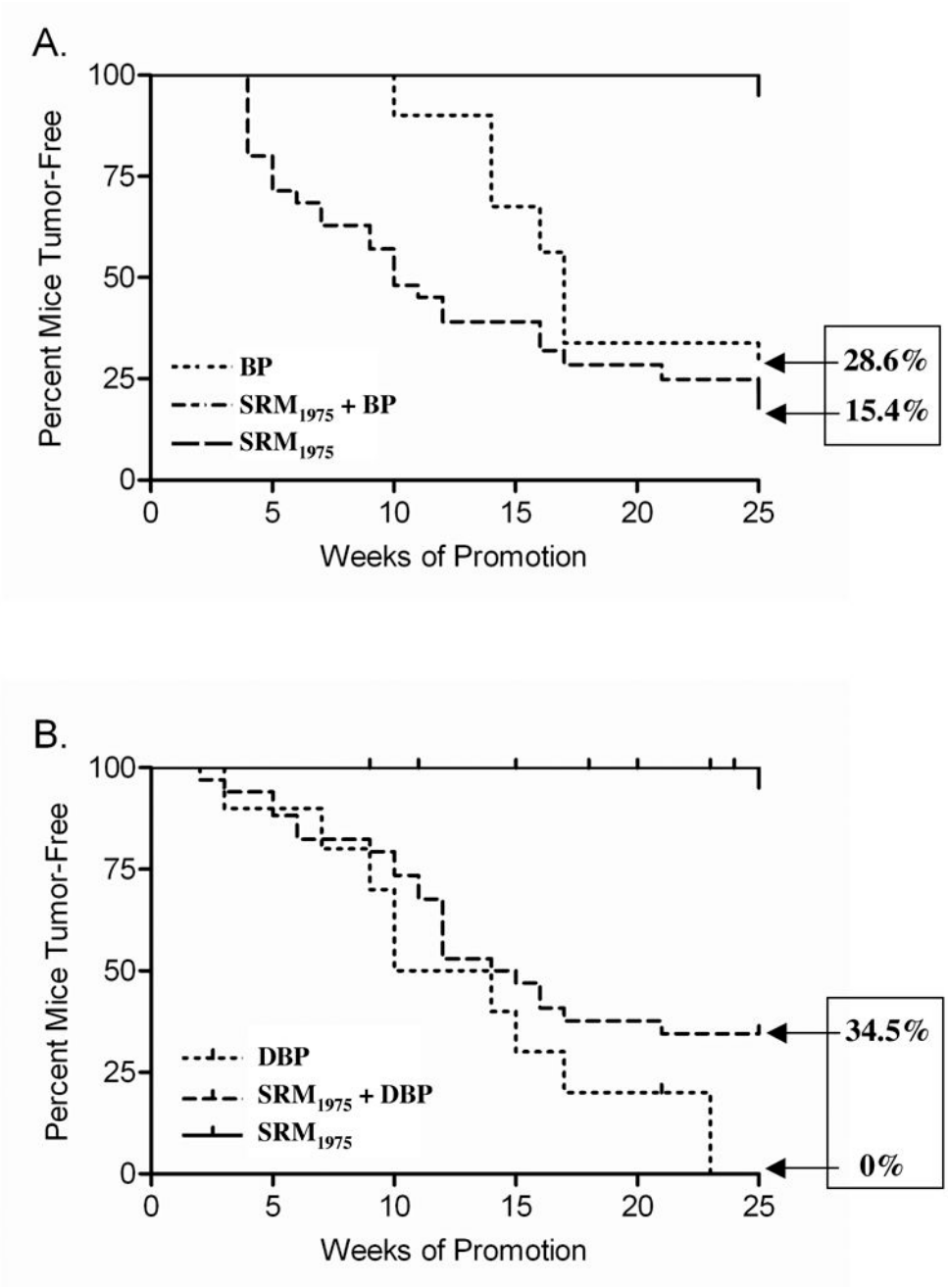

Fig. 1.

Time-until-tumor curves showing the effect of SRM_{1975} on the tumor initiating activity of BP and DBP, expressed as percent of tumor-free mice. Mice were treated with SRM_{1975} (n = 35), BP (n = 10), DBP (n = 10), SRM_{1975} plus BP or DBP (n = 35 each), or toluene (vehicle control) (n = 10) followed by twice-weekly promotion treatment with TPA for 25 weeks (as described in Materials and methods). Formation of skin tumors (includes both papillomas and carcinomas) were checked weekly. (A) Time-until-tumor curves showing the effect of SRM_{1975} on the tumor initiating activity of BP. (B) Time-until-tumor curves showing the effect of SRM_{1975} on the tumor initiating activity of DBP.

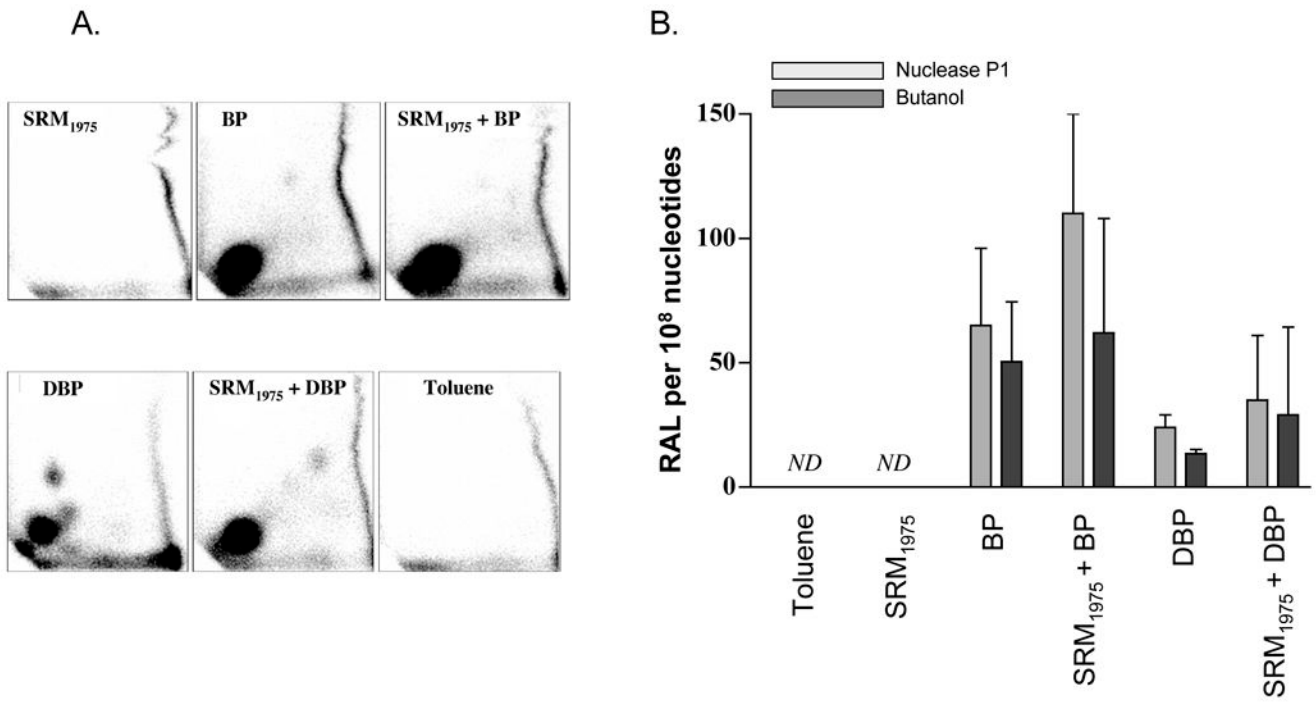

Fig. 2.

Relative adduct labeling (RAL) of total PAH-DNA adducts in Sencar epidermal DNA following 24 h topical exposure to SRM_{1975}, BP, SRM_{1975} plus BP, DBP, SRM_{1975} plus DBP, or toluene (vehicle control). (A) Representative TLC autoradiographs using the nuclease P1 digestion version of the ^{32}P-postlabeling assay [the profiles shown are representative of adduct profiles obtained using the butanol extraction version of the assay]. (B) Total PAH-DNA adducts using nuclease P1 digestion or butanol extraction. Values represent the mean ± SD of three animal experiments, each DNA sample determined by two separate postlabeling analyses. Statistical comparisons between PAH and SRM_{1975} plus PAH treatment groups were performed by ANOVA (no statistically significant difference observed between groups, $P > 0.05$). *ND* = not detected.

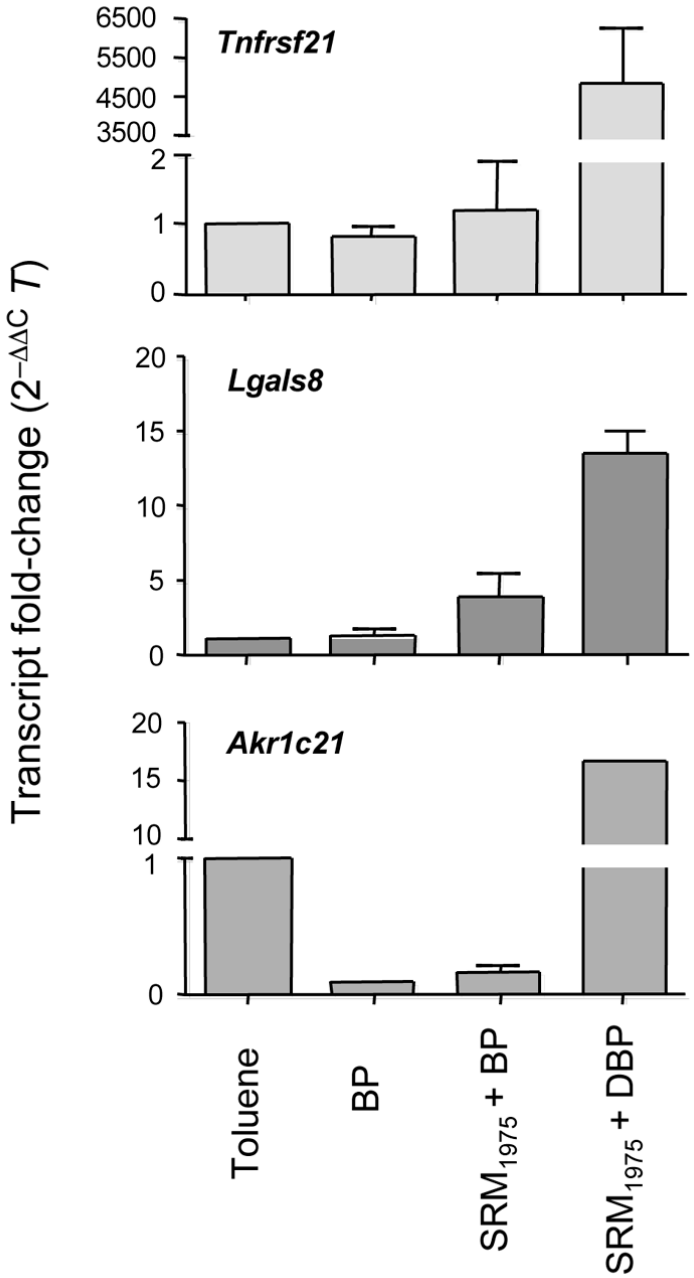

Fig. 3.

Changes in the expression of selected candidate genes, *TNFRSF21, LGALS8* and *AKR1C21*, in tumor tissue isolated from Sencar mice after treatment with SRM_{1975} plus PAH or PAH alone and promoted twice-weekly with TPA (as measured by qRT-PCR). The fold-change compared to control tissue isolated from toluene treated skin was calculated using the $2^{-\Delta\Delta C}T$ method [41]. Data represent the mean ± SD (n = 3 replicates per exposure group).

Table 1

Tumor initiating activity of SRM_{1975}, BP, SRM_{1975} plus BP, DBP, and SRM_{1975} plus DBP

Compound	Dose	# Mice/Treatment Group (Survivors[a])	# TBA (%)	Papillomas	Carcinomas	Total # Tumors[b]	# Tumors[b] per TBA[c]
Toluene (solvent)	200 µl	10 (6)	0 (0)	0	0	0	0
SRM_{1975}	50 mg	35 (26)	1 (3.8)	1	0	1	1.0
BP	200 nmol	10 (7)	5 (71.4)	11	1	12	2.4
SRM_{1975} + BP	50 mg + 200 nmol	35 (26)	22 (84.6)	58	6	64	2.9
DBP	2 nmol	10 (9)	9 (100)	14	9	23	2.6
SRM_{1975} + DBP	50 mg + 2 nmol	35 (29)	19 (65.5)	37	3*	40	2.1*

[a] Survivors, number of mice still living at the end of the observation period of 25 weeks.

[b] Skin papillomas and carcinomas together were considered as “tumors”.

[c] TBA, tumor-bearing animals (percent of surviving mice suffering from tumors).

* Statistically significant decrease in carcinoma incidence ($P = 0.002$) and overall tumor incidence ($P = 0.04$), compared with DBP exposure alone; Fisher’s Exact Test (FET).

Cancer Lett. Author manuscript; available in PMC 2009 June 28.

Table 2

Effect of SRM_{1975} on CYP enzyme activity in vivo following 24 h of topical exposure

Exposure Group	EROD Activity (pmol/min/mg)[a]
Toluene	0.65 ± 0.21
SRM_{1975}	0.69 ± 0.08
BP	1.33 ± 0.21
SRM_{1975} + BP	0.48 ± 0.41
DBP	0.33 ± 0.27
SRM_{1975} + DBP	0.66 ± 0.34

[a]EROD activities were determined in microsomes isolated from Sencar mice epidermis. Microsomes isolated from Chinese hamster ovary (CHO) cells over-expressing human recombinant CYP1A1 were used as the positive control (data not shown; 0.910 ± 0.20 pmol/min/mg). Microsomes from six mice per exposure group were pooled, and the average of three individual experiments is presented as mean ± SEM.

Table 3

Changes in the expression of candidate genes analyzed by qRT-PCR

	Toluene	BP	BP + SRM_{1975}	DBP + SRM_{1975}
Cyp1b1	1.00	0.01 ± 0.18	0.01 ± 0.51	1.37 ± 2.98
Cyp1a1	1.00	12.1 ± 20.8	0.01 ± 0.60	0.62 ± 7.11
Aldh3a1	1.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.23
Mcl-1	1.00	0.01 ± 0.02	0.03 ± 0.01	0.04 ± 0.20
Fos	1.00	0.11 ± 0.10	0.09 ± 0.05	0.28 ± 0.20
Nqo1	1.00	0.39 ± 0.36	0.11 ± 0.14	0.64 ± 0.23
Cyr61	1.00	0.71 ± 0.33	2.36 ± 2.02	0.81 ± 0.72
Tnfrsf21	1.00	0.82 ± 0.24	1.23 ± 0.95	4796 ± 2021
Lgals8	1.00	1.22 ± 0.73	3.89 ± 2.30	13.6 ± 2.3
Akr1c21	1.00	0.10 ± 0.00	0.18 ± 0.07	16.5 ± 0.0

[a] Each individual sample was assayed in triplicate and the cycle threshold values were normalized to the house-keeping gene *GAPDH* and the fold-change compared to solvent (toluene) control was calculated using the $2^{-\Delta\Delta C}T$ method [41]. Data represent the mean ± SD ($n = 3$ replicates per exposure group).