Altered Gene Expression Patterns in MCF-7 Cells Induced by the Urban Dust Particulate Complex Mixture Standard Reference Material 1649a

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Abstract

Human exposures to polycyclic aromatic hydrocarbon (PAH) occur in complex mixtures. Here, gene expression patterns were investigated using standard reference material (SRM) 1649a (urban dust). MCF-7 cells were exposed to SRM 1649a alone or SRM 1649a with either benzo[a]pyrene (BP) or dibenzo[a,l]pyrene (DBP) for 24 hours. Global analyses of the gene expression data revealed alterations of 41 RNA transcripts with at least 2-fold change (signal log ratio ≤−1 or ≥1) in response to SRM 1649a exposure. Increase in expression of cytochrome P450 (CYP) genes was observed in response to BP exposure (CYP1A1 and CYP1B1; signal log ratio of 4.7 and 2.5, respectively). An additive induction of CYP1A1 and CYP1B1 was observed with cotreatment of SRM 1649a and BP. On the contrary, no change in gene expression of CYP1A1 and CYP1B1 was observed when the cells were exposed to DBP. Furthermore, to study the effect of complex PAH mixtures on the metabolic activation of carcinogenic PAH to DNA-binding derivatives and to relate this with gene expression studies, PAH-DNA adduct formation was determined. SRM 1649a decreased the total level of BP-DNA adducts in comparison to either DBP or SRM 1649a alone. No significant difference in adduct levels was observed in response to either DBP alone or in combination with SRM 1649a. These results provide a transcriptional signature for chemical carcinogen exposure; in addition, they suggest a major factor in carcinogenic activity of PAH within complex mixtures is their ability to promote or inhibit the activation of carcinogenic PAH by the induction of CYP enzymes. (Cancer Res 2005; 65(4); 1251-8)

Introduction

Ambient air contains dust particles that are complex, potentially genotoxic mixtures that pose danger to human health (1). Air pollution exposure resulting from the incomplete combustion of organic matter is suspected of contributing to cancer risk (2, 3). Recently, it has been shown that reduction of exposure to particulate air pollution lowers the risk of heritable mutations in mice with implications for health risks to future generations (4, 5). Because air pollutants are composed of 2,800 chemicals (6), their synergistic, antagonistic or additive effects may be important factors in human carcinogenesis (7). Studies have shown that the main contributors to genotoxicity are polycyclic aromatic hydrocarbon (PAH) and its derivatives (8–10).

Chemical analysis of complex mixtures in ambient air is difficult because several biologically active components occur at low concentrations. The National Institute of Standards and Technology have developed a number of natural matrix standard reference materials (SRM) since the early 1980s for the determination of organic contaminants in environmental matrices (11). The first particle-based, environmental natural matrix SRM developed by the National Institute of Standards and Technology for organic contaminants was SRM 1649 (urban dust/organics) which was issued in 1982 (12, 13). SRM 1649a is the same particulate material that was issued previously in 1982 as SRM 1649 (14); this material has been rebottled and reanalyzed to provide updated certified values as well as certified, reference, and information values for additional constituents. For our studies, we used SRM 1649a in addition to representative examples of carcinogenic PAHs, benzo[a]pyrene (BP) and dibenzo[a,l]pyrene (DBP) to understand the metabolic activation and DNA binding of these PAHs within complex mixtures.

It is understood that exposure to PAHs can target the expression of regulatory proteins involved in activation, detoxification and DNA repair, thus altering the ability of the cells to maintain genomic integrity. Jeffy et al. (15) have suggested that exposure to PAHs may be a predisposing factor in the etiology of sporadic breast cancer by disrupting the expression of BRCA-1, a tumor suppressor gene. Identification of genes whose expression is specifically modified by exposure to complex mixtures of PAH would provide a better understanding of their mechanisms of action and allow development of sensitive and specific biomarkers of exposure and susceptibility for use in both mechanistic laboratory and epidemiology studies. Therefore, in the current work we used high density oligonucleotide microarray to compare the effects of the complex environmental mixture SRM 1649a on gene expression, metabolic activation and PAH-DNA binding of BP and DBP in human mammary carcinoma derived MCF-7 cells in culture. Here we focused on cytochrome P450 (CYP) CYP1A1 and CYP1B1, which account for PAH metabolism, and also confirmed the expression of these relevant genes by real-time PCR. To study the relationship of gene expression to DNA damage, we also determined the DNA adduct formation and protein expression in MCF-7 cells exposed to SRM 1649a alone and in combination with BP or DBP.

Materials and Methods

Cell Culture and Treatment. The MCF-7 cells (obtained from the Karmanos Cancer Center, Detroit, MI) were cultured in a 75-cm² flask (Corning, Corning, NY) in a 1:1 mixture of F-12 Nutrient Mixture and DMEM (Life Technologies, Grand Island, NY). The medium was supplemented with
10% fetal bovine serum (Intergen, Purchase, NY), containing 15 mM/L HEPES buffer and antibiotics (200 units/mL penicillin, 200 μg/mL streptomycin, and 25 μg/mL ampicillin) at 37°C with 5% CO₂. Cell cultures were subcultured at a ratio of 1:4 when the cells covered the entire surface of the flask.

All cell culture flasks, in which the cells covered ~70% of the surface, were replenished with fresh medium (20 mL) 24 hours before treatment. SRM 1649a was obtained from the National Institute of Standards and Technology (Gaithersburg, MD) and had a unit of SRM in a bottle containing 2.5 g of atmospheric particulate material (14). A detailed description and chemical composition of SRM 1649a is available under http://patapsco.nist.gov/srmcatalog/certificates/1649a.pdf. SRM 1649a treatments in cell culture were done using a dose of 400 μg in 50 μL of DMSO. The cells were treated with either solvent alone as a control (DMSO, 75 μL), SRM 1649a (400 μg), BP (20.2 μg), SRM 1649a (400 μg) plus BP (20.2 μg), DBP (0.2 μg), or SRM 1649a (400 μg) plus DBP (0.2 μg). The concentrations were based on previous studies in our laboratory, which indicated that these doses gave detectable levels of DNA binding without causing excessive toxicity to the cells (16). The cells were harvested after 24 hours of continuous exposure.

High-Density Oligonucleotide Array Expression Analysis. Total RNA was isolated from treated and control cultures of MCF-7 cells using RNeasy Kit (Qiagen, Valencia, CA) and subsequent steps leading to hybridization and scanning of the human genome U133A arrays were done according to the DNA microarray manufacturer's instructions (Affymetrix, Santa Clara, CA). Briefly, total RNA (10 μg) was used for the preparation of cDNA using a T7-poly-T primer and the reverse transcriptase Superscript II (Life Technologies, Carlsbad, CA). Labeled cRNA transcript was generated from the cDNA sample by an in vitro transcription reaction using Enzo Bioarray high yield RNA transcription labeling kit (Enzo Diagnostic, Farmingdale, NY). The labeled cRNA transcript was purified (RNeasy, Qiagen) and each cRNA sample (20 μg) was fragmented by mild alkaline treatment (94°C, 35 min). Each fragmented cRNA sample (15 μg) was hybridized to U133A human genome arrays, representing ~22,000 genes (45°C, 16 hours; in rotisserie oven set at 60 rpm). Subsequent washing and staining of the probe arrays was done using the GeneChip fluids station protocol EukGE-WS2. Furthermore, probe arrays were scanned using the Affymetrix Gene Array 2500 Scanner. The differential gene expression data analysis was done using Microarray Suite 5.0 software. Two biological replicates were used for every treatment. A signal log ratio (SLR) of one was considered equivalent to 2-fold change in gene expression.

Quantitative Real-time PCR Analysis. Relative quantitation with real-time reverse-transcriptase PCR (RT-PCR) was done for both CYP1A1 and CYP1B1 using an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA), according to manufacturer’s instructions. The cDNA prepared for microarray analysis was diluted 1:50 and 1.5 μL was used as template to perform RT-PCR in a 50-μL reaction using 20× assays-on-demand gene expression primers and probes for both CYP1A1 and CYP1B1 (Hs00153120_m1/Hs0061_102612), CYP1B1 (Hs01643833_ml/U03688) and housekeeping gene GAPDH (Hs99999908v1; PE Applied Biosystems/Genbank). RT-PCR consisted of initial denaturing for 10 minutes at 95°C, 40 cycles of 95°C for 15 seconds, and 50°C for 1 minute. Each sample was assayed in quadruplicates and the cycle threshold (Ct) values were normalized to the house keeping gene GAPDH and the fold change was calculated using 2 -ΔΔCt method (17).

DNA Isolation. A standard DNA isolation protocol was used (18). Briefly, cell culture samples were homogenized in a glass homogenizer containing EDTA, SDS buffer [10 mM/L Tris, 1 mM/L NaEDTA, and 1% SDS (w/v)], pH 8). The homogenates were treated with RNase, DNase-free (50 units/mL; Boehringer-Mannheim Co., Indianapolis, IN) and RNase T1 (1,000 units/mL; Boehringer-Mannheim) at 37°C for 1 hour, followed by treatment with proteinase K (500 μg/mL; Sigma Chemical, St. Louis, MO) at 37°C for 1 hour. The DNA was extracted with equal volumes of Tris-equilibrated phenol and chloroform/isooamyl alcohol (24:1) and then with equal volumes of chloroform/isooamyl alcohol (24:1). The aqueous layer was treated with 1/10 volume of 5 mol/L NaCl and twice the volume of cold 100% ethanol to precipitate the DNA which was then dissolved in double-distilled water; its concentration was determined by UV absorbance at 260 nm.

³²P-Postlabeling of DNA Adducts. Post-labeling was carried out as described previously (19). Briefly, 10 μg DNA isolated from MCF-7 cells after treatment were digested with nucleases P1 and protamine acid phosphatase, post-labeled with [γ-³²P]ATP (3,000 Ci/mmole), cleaved to adducted mononucleotides with snake venom phosphodiesterase I, and prepurified with a Sep-Pak C18 cartridge (Waters, Milford, MA). Subsequent separation by analytic high performance liquid chromatography (HPLC-Varian system equipped with two pumps and an autosampler; Varian Systems, CA) was carried out using a C18 reverse-phase column (5 μm Ultrasound ODS, 4.6 × 250 mm). Adducts were resolved by elution at 1 mL/min with 0.1 mol/L ammonium phosphate (pH 5.5, solvent A) and 100% HPLC grade methanol (solvent B). The elution gradient was as follows: 44% to 60% solvent B over 40 minutes, 60% to 80% solvent B over 10 minutes, isocratic elution at 80% solvent B over 10 minutes, and 84% to 44% B over 5 minutes. The radiolabeled nucleotides were detected by an online radioisotope flow detector (Packard Instruments, Downers Grove, IL), and the level of DNA binding was calculated based on the labeling efficiency of a [³²P]JHβ[a]P-7,8-dihydroxy 9,10-epoxide standard (20). Three independent sets of the post-labeling reaction were carried out for every sample treated, to determine the total PAH-DNA adduct levels.

Microsomal Isocyanate. Microsomes were prepared as described previously (21) with minor modifications. Briefly, cell culture samples were homogenized with a steel homogenizer in microsomal homogenization buffer (0.25 mol/L K2HPO4, 0.15 mol/L KCl, 10 mmol/L EDTA, and 0.25 mol/L phenylmethylsulfonyl fluoride) and were centrifuged at 15,000 × g for 20 minutes at 4°C. The supernatant was centrifuged at 100,000 × g for 90 minutes at 4°C, and the pellet was resuspended in microsomal dilution buffer (0.1 mol/L KH2PO4, 20% glycerol, 10 mmol/L EDTA, 0.1 mmol/L DTT, and 0.25 mol/L phenylmethylsulfonyl fluoride). The protein concentration was spectrophotometrically determined at 562 nm using the Bicinchoninic acid colorimetric assay (Pierce, Rockford, IL).

Ethynoresorufin O-Deethylation Assay. Fifty micrograms of microsomal protein were added to 200 μmol/L 7-ethynoresorufin (Sigma Chemical) in 0.1 mol/L Tris-HCl (pH 7.8) buffer and added in a blank 96- well plate (E&K Scientific, Campbell, CA). NADPH (Sigma Chemical) was added to each well and the fluorescence was measured with a Spectra MAX Gemini plate reader (Molecular Devices, Sunnyvale, CA). The excitation wavelength was 335 nm, emission 585 nm and the assay was monitored over 10 minutes. Each sample was assayed in triplicate and the amount of resorufin produced was calculated from the fluorescence of a known concentration of resorufin.

Western Blots. Microsomal proteins were separated by SDS-PAGE on a 7.5% READY gel (Bio-Rad, Hercules, CA). Microsomal proteins (50 μg) were diluted with loading buffer [0.0625 mol/L Tris-base (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue]. Proteins were denatured by boiling for 3 minutes, followed by rapid cooling on ice. Following gel electrophoresis at 200 V for 1 hour, the microsomal proteins were transferred to nitrocellulose using a mini Trans-Blot electrophoretic transfer cell (Bio-Rad) at 100 V for 45 minutes. Uniformity of sample loading was confirmed by staining the nitrocellulose membrane with Ponceau S (Sigma Chemical). The membrane was washed thrice with PBS-T [PBS with 0.3% (w/v) Tween 20] for 5 minutes each and blocked with 1:3 Nap-Sure blocker (Geno Technology, St. Louis, MO): PBS-T for 1 hour on a shaker. After three further washes with Nap-Sure: PBS-T (1:7), the membrane was incubated with the primary antibody for 2 hours and was washed again with Nap-Sure blocker: PBS-T (1:7). Human CYP1A1 was detected by a rabbit polyclonal CYP1A1 antibody (1:1500) prepared against purified recombinant human CYP1A1 protein. The antibody against human CYP1A1 was a gift from Dr. F.P. Guengerich (Center for Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, TN). Dr. C. Marcus (Department of Pharmacology and Toxicology, University of New Mexico, Albuquerque, NM) prepared and provided the human CYP1B1 rabbit polyclonal antibody (1:1000). The immunoreactive proteins were detected by incubating the membrane with peroxidase-conjugated anti-rabbit IgG (1:30,000 for CYP1A1 and 1:20,000 for CYP1B1) for

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30 minutes. After three washes with Nap-Sure blocker: PBS-T (1:7), the immunoreactive proteins were observed using an enhanced chemiluminescence detection method, as described by the manufacturer (Amersham Life Science, Arlington Heights, IL). Microsomal protein (10 μg) from V79 cells expressing either human CYP1A1 or CYP1B1 (18) was used as a positive control in the immunoblots.

Statistical Analysis. Correlation coefficients were determined according to the method of Pearson using Excel (22).

Results

Altered Gene Expression in MCF-7 Cells in Response to Complex Mixture SRM 1649a

Exposure of MCF-7 cells to BP, DBP, SRM 1649a, BP plus SRM 1649a, and DBP plus SRM 1649a resulted in altered gene expression patterns in many RNA species. Global gene expression analysis (at least 2-fold change SLR ≤ −1 or ≥ 1) revealed 129, 41, and 39 genes that were altered in response to BP, SRM 1649a, and DBP, respectively. The data reported here with regard to microarray studies were based on 2-fold up-regulated or down-regulated genes as being significant (P ≤ 0.05).

Functional Classification of Differentially Expressed Genes

The differentially expressed genes were classified based on their function and selected genes that were altered on exposure to BP, SRM 1649a, and DBP are listed in Tables 1, 2 and 3 respectively (with a short functional description). The genes are arranged with their short name/gene symbol, so they can be easily looked up and linked to the text.

Genes involved in cell cycle, proliferation/apoptosis, DNA repair and tumor suppression. On exposure to BP, 8 of 10 genes were categorized as those involved in cell cycle regulation/apoptosis and DNA repair. Whereas SRM 1649a exposure induced three genes, DBP influenced only 2 of 10 genes categorized here. Most genes that were induced or repressed were either directly or indirectly involved in cell cycle regulation (CDKN1A, BAX, BCL2L11, PPARG, OKL38, CYR61, and DUSP1) and or apoptosis. Nuclear accumulation of p53 and the transcriptional induction of various target genes such as CDKN1A has been shown previously on human cells exposed to BP, DBP, or its ultimate metabolite (23, 24). Peroxisome proliferative activated receptor γ (PPARG) was up-regulated on exposure to DBP or SRM 1649a. Two important genes

Table 1. Selected genes that were induced at least SLR of ≥ 1 (2-fold) in response to BP exposure

<table>
<thead>
<tr>
<th>Genbank accession no.</th>
<th>Gene symbol</th>
<th>Gene title</th>
<th>Treatment</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>BP</td>
</tr>
<tr>
<td>NM_000499</td>
<td>CYP1A1</td>
<td>Cytochrome P450, family 1, subfamily A, polypeptide 1</td>
<td>4.7 ± 0.28</td>
</tr>
<tr>
<td>NM_001924</td>
<td>GADD45A</td>
<td>Growth arrest and DNA-damage-inducible, α</td>
<td>3.1 ± 0.00</td>
</tr>
<tr>
<td>NM_015516</td>
<td>E2IG4</td>
<td>Hypothetical protein, estradiol-induced</td>
<td>2.3 ± 0.35</td>
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<tr>
<td>NM_000104</td>
<td>CYP1B1</td>
<td>Cytochrome P450, family 1, subfamily B, polypeptide 1</td>
<td>2.5 ± 0.28</td>
</tr>
<tr>
<td>NM_002083</td>
<td>GPX2</td>
<td>Glutathione peroxidase 2</td>
<td>2.1 ± 0.14</td>
</tr>
<tr>
<td>NM_001353</td>
<td>AKR1C1</td>
<td>Aldo-keto reductase family 1, member C1</td>
<td>1.7 ± 0.07</td>
</tr>
<tr>
<td>U05598</td>
<td>AKR1C2</td>
<td>Aldo-keto reductase family 1, member C2</td>
<td>1.6 ± 0.07</td>
</tr>
<tr>
<td>NM_000463</td>
<td>UGT1A10</td>
<td>UDP glycosyltransferase 1 family, polypeptide A10</td>
<td>1.7 ± 0.14</td>
</tr>
<tr>
<td>NM_015869</td>
<td>PPARG</td>
<td>Peroxisome proliferative activated receptor, γ</td>
<td>1.7 ± 0.21</td>
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<tr>
<td>NM_000389</td>
<td>CDKN1A</td>
<td>Cyclin-dependent kinase inhibitor 1A (p21, Cip1)</td>
<td>1.5 ± 0.00</td>
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<tr>
<td>NM_000120</td>
<td>EPHX1</td>
<td>Epoxide hydrolase 1</td>
<td>1.4 ± 0.07</td>
</tr>
<tr>
<td>U19599</td>
<td>BAX</td>
<td>BCL2-associated X protein</td>
<td>1.3 ± 0.00</td>
</tr>
<tr>
<td>X06399</td>
<td>CYP2B6NS</td>
<td>Cytochrome P450, family 2, subfamily B, polypeptide 6</td>
<td>1.1 ± 0.14</td>
</tr>
<tr>
<td>AA629050</td>
<td>BCL2L11NS</td>
<td>BCL2-like 11 (apoptosis facilitator)</td>
<td>1.3 ± 0.21</td>
</tr>
<tr>
<td>NM_001065</td>
<td>TNFRSF1A</td>
<td>Tumor necrosis factor receptor superfamily, member 1A</td>
<td>1.1 ± 0.00</td>
</tr>
<tr>
<td>NM_000107</td>
<td>DDR2</td>
<td>Damage-specific DNA binding protein 2</td>
<td>1.0 ± 0.00</td>
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<tr>
<td>NM_013370</td>
<td>OKL38</td>
<td>Pregnancy-induced growth inhibitor</td>
<td>1.0 ± 0.00</td>
</tr>
<tr>
<td>NM_000903</td>
<td>NQO1</td>
<td>NADPH dehydrogenase, quinone</td>
<td>2.0 ± 0.07</td>
</tr>
</tbody>
</table>

NOTE: Selected genes that were induced by at least a SLR of ≥ 1 in response to BP treatment. The data represents mean ± SD where n = 2 arrays per treatment. All genes were significantly altered (P ≤ 0.05) on exposure to SRM 1649a except for those indicated as not significant (NS) following the gene symbol.
linked to DNA repair (GADD45A and DDB2) were observed to be up-regulated on exposure to BP and DBP, whereas an increase in the expression of the mitochondrial tumor suppressor gene MTS1 was observed on DBP treatment. DDB2 being a nucleotide excision repair protein (25) substantiates its increased expression in our study, on exposure to BP and DBP.

**Genes involved in immune response, cell structure/adhesion, transport, and receptor/inhibitor.** Two genes (TNFRSF6 and E21G4) were up-regulated on exposure to BP, whereas three unique genes involved in cell structure/adhesion and transport (CTGF, JAG1, and SLC2A1) respectively, increased in expression on exposure to SRM 1649a. DBP and SRM 1649a exposure indicated an increase in the expression of connective tissue growth factor (CTGF), whereas tissue factor pathway inhibitor (TFPI2) and tumor necrosis factor receptor (TNFRSF6) showed increased expression on exposure to DBP.

**Oncogenes.** Proto-oncogenes (RAβ6, FOS, JUN, and MCL1) were particularly up-regulated on exposure to SRM 1649a and DBP but expressed relatively low on exposure to BP.

**Genes involved in metabolism.** Several of the genes known to be involved in the metabolism of PAHs (CYP1A1, CYP1B1, AKR1C1, AKR1C2, NQO1, EPHX1, UGT1A1, CYP2B6, and GPX2) were found to be up-regulated on exposure to SRM 1649a.

### Table 2. Selected genes that were induced at least SLR of ≥1 (2-fold) in response to SRM 1649a exposure

<table>
<thead>
<tr>
<th>Genbank accession no.</th>
<th>Gene symbol</th>
<th>Gene title</th>
<th>Treatment</th>
<th>1649a</th>
<th>BP</th>
<th>DBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_000104</td>
<td>CYP1B1</td>
<td>Cytochrome P450, family 1, subfamily B, polypeptide 1</td>
<td>1.8 ± 0.07</td>
<td>2.5 ± 0.28</td>
<td>−0.2 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>M92934</td>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
<td>2.0 ± 0.70</td>
<td>0.6 ± 0.49</td>
<td>1.8 ± 0.91</td>
<td></td>
</tr>
<tr>
<td>BC00490</td>
<td>FOS</td>
<td>v-fos FBJ murine osteosarcoma viral oncogene homologue</td>
<td>1.7 ± 0.28</td>
<td>−0.3 ± 0.21</td>
<td>0.9 ± 1.20</td>
<td></td>
</tr>
<tr>
<td>NM_001554</td>
<td>CYB61</td>
<td>Cysteine-rich, angiogenic inducer, 61</td>
<td>1.3 ± 0.49</td>
<td>0.1 ± 0.28</td>
<td>0.1 ± 0.77</td>
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<tr>
<td>NM_004417</td>
<td>DUSP1</td>
<td>Dual specificity phosphatase 1</td>
<td>1.2 ± 0.42</td>
<td>−0.1 ± 0.07</td>
<td>0.9 ± 0.77</td>
<td></td>
</tr>
<tr>
<td>BF59446</td>
<td>MCL1</td>
<td>Myeloid cell leukemia sequence 1 (BCL2 related)</td>
<td>1.2 ± 0.28</td>
<td>0.5 ± 1.06</td>
<td>0.8 ± 0.14</td>
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<tr>
<td>U73936</td>
<td>JAGN</td>
<td>Jagged 1 (Alagille syndrome) involved in cell communication</td>
<td>2.0 ± 0.84</td>
<td>2.0 ± 0.99</td>
<td>2.6 ± 0.35</td>
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<tr>
<td>NM_015869</td>
<td>PPARG</td>
<td>Peroxisome proliferative activated receptor, γ</td>
<td>1.2 ± 0.14</td>
<td>1.7 ± 0.21</td>
<td>0.9 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>BG491844</td>
<td>JUN</td>
<td>v-jun sarcoma virus 17 oncogene homologue (avian)</td>
<td>1.0 ± 0.00</td>
<td>0.3 ± 0.14</td>
<td>0.4 ± 0.28</td>
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<tr>
<td>AI091047</td>
<td>SLC2A1</td>
<td>Solute carrier family 2 (facilitated glucose transporter), member 1</td>
<td>1.7 ± 0.00</td>
<td>0.3 ± 0.92</td>
<td>0.8 ± 0.00</td>
<td></td>
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</tbody>
</table>

**NOTE:** Selected genes that were induced by at least a SLR of ≥1 in response to SRM 1649a treatment. The data represents mean ± SD where n = 2 arrays per treatment. All genes were significantly altered (P ≤ 0.05) on exposure to SRM 1649a except for those indicated as not significant (NS) following the gene symbol.

### Table 3. Selected genes that were induced at least (2-fold) SLR of ≥1 in response to DBP exposure

<table>
<thead>
<tr>
<th>Genbank accession no.</th>
<th>Gene symbol</th>
<th>Gene title</th>
<th>Treatment</th>
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<tr>
<td>NM_001924</td>
<td>GADD45A</td>
<td>Growth arrest and DNA damage-inducible, α</td>
<td>3.0 ± 0.21</td>
<td>1.1 ± 0.92</td>
<td>3.1 ± 0.00</td>
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<tr>
<td>M92934</td>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
<td>1.8 ± 0.92</td>
<td>2.0 ± 0.71</td>
<td>0.6 ± 0.49</td>
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<tr>
<td>NM_000839</td>
<td>CDKN1A</td>
<td>Cyclin-dependent kinase inhibitor 1A (p21, Cip1)</td>
<td>1.5 ± 0.28</td>
<td>0.2 ± 0.14</td>
<td>1.5 ± 0.00</td>
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<tr>
<td>AA164751</td>
<td>TNFRSF6</td>
<td>Tumor necrosis factor receptor superfamily, member 6</td>
<td>1.3 ± 0.14</td>
<td>−0.7 ± 0.71</td>
<td>1.2 ± 0.35</td>
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<tr>
<td>L27624</td>
<td>TFF2</td>
<td>Tissue factor pathway inhibitor 2</td>
<td>1.2 ± 0.14</td>
<td>0.7 ± 0.07</td>
<td>−1.5 ± 0.21</td>
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<tr>
<td>BE552421</td>
<td>MTS1</td>
<td>Mitochondrial tumor suppressor gene 1</td>
<td>1.2 ± 0.21</td>
<td>1.1 ± 0.49</td>
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</tr>
<tr>
<td>NM_000107</td>
<td>DB2</td>
<td>Damage-specific DNA binding protein 2</td>
<td>1.2 ± 0.21</td>
<td>0.0 ± 0.14</td>
<td>1.0 ± 0.00</td>
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<tr>
<td>NM_002869</td>
<td>RAB6A</td>
<td>RAB6A, member RAS oncogene family</td>
<td>1.1 ± 0.07</td>
<td>1.0 ± 0.28</td>
<td>0.3 ± 0.78</td>
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</tr>
<tr>
<td>L08599</td>
<td>CDHI</td>
<td>Cadherin 1, type 1, E-cadherin (epithelial)</td>
<td>1.0 ± 0.07</td>
<td>0.8 ± 0.07</td>
<td>−0.3 ± 0.92</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** Selected genes that were induced by a SLR of ≥1 in response to DBP treatment. The data represents mean ± SD where n = 2 arrays per treatment. All genes were significantly altered (P ≤ 0.05) on exposure to DBP.
to be up-regulated especially on exposure to BP, whereas CYP1B1 levels increased on exposure to SRM 1649a. It has been shown (26) that CYP1A1 and CYP1B1 are key enzymes involved in the metabolism of the two carcinogenic PAH (BP and DBP) used in this study. AKR1CI and AKR1C2 have also been shown to be involved in the diversion of PAH trans-dihydrodiols from diol-epoxides to o-quinones (27, 28) that may thus provide an alternative pathway of PAH activation.

Quinone oxidoreductase (NQO1) is known to catalyze the activation of some environmental procarcinogens present in tobacco smoke, and the genotypes of NQO1 was recently shown to play an important role in the development of smoking related bladder cancer (29). BP being a carcinogenic PAH present in environmental tobacco smoke it was no surprise to see NQO1 up-regulated in our study on cells exposed to BP. Another enzyme epoxide hydrolase (EPHX) is known to play a dual role in the metabolism of the two carcinogenic PAH (BP and DBP) used in this study on exposure to BP which provides confirmation of the ability of EPHX to hydrolate PAH epoxy metabolites to the carcinogenic PAH diol-epoxides (32). Here, we have further analyzed and focused on the altered transcripts of CYP1A1 and CYP1B1.

A significant increase in the expression of CYP1A1 was observed in MCF-7 cells exposed to BP plus SRM 1649a in comparison to SRM 1649a alone (Fig. 1A). On the contrary, DBP plus SRM 1649a did not show significant increase in CYP1A1 expression when compared with 1649a. The data array was validated with RT-PCR, and correlated with microarray data with regard to the transcript detection for CYP1A1 (r = 0.95). A similar pattern in the expression of CYP1B1 was also observed where there was an increase of CYP1B1 expression when treated with BP plus SRM 1649a in comparison to SRM 1649a alone (Fig. 1B). DBP plus SRM 1649a did not show a significant increase in CYP1B1 expression in comparison to SRM 1649a. This expression analysis of CYP1B1 was also validated by RT-PCR that correlated well with the transcript detection for CYP1B1 (r = 0.97).

**DNA Adduct Formation in MCF-7 Cells in Culture**

The effect of the complex mixture SRM 1649a on DNA adduct formation in human mammary carcinoma MCF-7 cells was compared with BP and DBP both individually and in combination with SRM 1649a. The cells were treated for 24 hours, and the DNA adducts were 32P-postlabeled and analyzed by HPLC. MCF-7 cells treated with DMSO (vehicle control) or SRM 1649a alone did not exhibit any DNA binding. Quantitative analysis of the total average levels of DNA binding is illustrated in Fig. 3. Treatment with a combination of BP and SRM 1649a indicated a 2-fold decrease in DNA binding in comparison to BP alone. On the other hand, treatment with a combination of DBP and SRM 1649a did not show any significant difference in the formation of DNA adducts (Fig. 2).

**Cytochrome P450 Expression**

The ethoxyresorufin O-deethylation assay was done using microsomes from MCF-7 cells exposed to PAs and SRM 1649a. The effect of SRM 1649a on CYP induction as measured by the ethoxyresorufin O-deethylation assay is shown in Table 4. Both DMSO- and DBP-treated samples exhibited background levels of CYP activity. Treatment with SRM 1649a, although revealed CYP induction, a combination treatment of BP plus SRM 1649a and DBP did not show any additive increase in CYP activity, in comparison to BP or DBP alone (Table 4).

Western analyses were done to examine the ability of the complex mixture SRM 1649a to induce the expression of human...
CYP 1A1 and CYP 1B1 proteins in MCF-7 cells (Fig. 3). Treatment with DMSO or DBP alone did not express either CYP 1A1 or CYP 1B1 proteins (Fig. 3A and B). Whereas BP and SRM 1649a expressed both proteins CYP 1A1 and CYP 1B1, an additive increase in the expression of both proteins was evident in the treatment with BP plus 1649a (Fig. 3A and B).

Discussion

Testing complex mixtures presents a formidable scientific problem, because most recently available toxicologic data have been obtained from single substance studies and are not simply applicable to mixtures of chemicals. Although there are no special strategies and standard protocols available for determining toxic and genotoxic effects of complex mixtures, the fundamental concepts of evaluation are the same as those for single substances (33). Furthermore, toxicologic as well as genotoxic evaluations of complex mixtures are complicated by the potential for the additive, antagonistic or synergistic component interactions. Therefore, in our study, we have documented the effects of two different carcinogenic PAHs (BP, moderately potent; DBP, extremely potent), the complex mixture SRM 1649a and the results of their interactions relative to the individual compound or mixture alone particularly with regard to two PAH metabolism genes CYP1A1 and CYPIB1.

Several research organizations have conducted animal carcinogenicity bioassays on specific chemicals and has identified many of them as mammary carcinogens (ref. 34; including PAHs and nitro-PAHs) that show evidence of genotoxicity. Gammon et al. (35) have assessed PAH exposure by measuring PAH-DNA adducts and although they observed a lack of a relationship between PAH-DNA adducts and exposures to grilled food and tobacco smoke as primary sources of PAHs, other sources such as air pollution may be more important (36). Given the modest relative risks of environmental factors, associated with breast cancer, discovery of a risk would be valuable if that could save many thousands of lives (36). Recent studies have shown evidence that biologically effective amounts of PAH compounds associated with particulate matter were transferred to the intracellular environment and elicited the activation of various genes (37, 38).

The selection of a human mammary carcinoma derived cell line in this study reflects our interest in breast cancer and or pulmonary health effects that are potentially attributable to exposure to urban dust. We discuss here two genes (CTGF and CYR61) that indicated increased expression on exposure to SRM 1649a (Table 2 in our study and their possible link to breast cancer. Connective tissue growth factor (CTGF) is known to be a potent angiogenic factor. The results obtained by Frazier and Grotendorst (39) suggest that cancer stroma formation involves induction of fibroproliferative growth factors such as CTGF. Recent studies in samples from primary breast tumors suggest that CTGF and CYR61 may play a role in the progression of breast cancer and might serve as a valuable tool for monitoring tumor status in breast cancer patients (40). Tsai et al. (41) have further shown that CYR61 is a tumor-promoting factor and a key regulator of breast cancer progression. SRM 1649a in our study increased the expression of CTGF and CYR61, which is the first report connecting urban dust particle and genes involved in breast cancer progression. Overall, although there is some overlap in the genes modified between different PAHs and SRM 1649a, these data suggest that each PAH modifies expression of a largely unique set of genes that may be characteristic of each treatment.

PAHs can induce tumor promotion through several, likely combinatorial, mechanisms including negative effects on transcription of tumor-suppressor genes (42), transcriptional activation of proto-oncogenes (43), and activation of enzymes that oxidize procarcinogenic PAHs to reactive metabolites (44). To date, no mutations in the BRCA-1 gene have been identified in sporadic breast cancers, whereas the expression levels of BRCA-1 in breast tumors are lower than those observed in normal mammary tissue (45). Recent work by Jeffy et al. (46) suggest that BP, a carcinogenic PAH found in tobacco smoke, environmental pollution, and foods may contribute to breast carcinogenesis through the inhibition of the tumor suppressor gene BRCA-1. Under the experimental conditions used in our study, the global gene expression analysis revealed no alterations in the tumor suppressor gene BRCA-1;
however, Jeffy et al. (15) in a study of BRCA-1 expression in MCF-7 cells, did see a decrease after 72 hours of BP treatment.

Here, we have mainly focused on the alteration of two important genes CYP1A1 and CYP1B1 known to be involved in the metabolism of PAHs (26) in MCF-7 cells treated with BP, SRM 1649a, DBP and combination treatments of BP plus SRM 1649a and DBP plus 1649a. The data obtained on several other genes that were altered by >2-fold on exposure to combination treatments (BP plus SRM 1649a and DBP plus SRM 1649a) will be reported in a separate article in view of the specific nature of this present discussion. Bartosiewicz et al. (47) have shown that administration of BP to mice up-regulated CYP1A1 and CYP1A2 genes. The gene expression data in our study using the human U133A microarray representing >22,000 genes indicated expression (>2-fold) of CYP1A1 and CYP1B1 in cells treated with BP, DBP, and SRM 1649a (Fig. 1A and B). An additive increase in expression of CYP1A1 and CYP1B1 was noted only in cells treated with BP plus SRM 1649a (Fig. 1A and B). Shimada and Fujii-Kuriyama (26) have shown that BP and other carcinogenic PAHs, such as dibenz[a]anthracene, benzo[b]fluoranthene and DBP induce CYP1B1 as well as CYP1A1 in liver and lung of AhR (+/+) mice, but not in those of AhR (−/−) mice. Also, CYP1A1 and CYP1B1 enzymes having similar, but not identical, substrate specificity towards various PAHs (26) which would explain the significant increase in CYP1A1 and CYP1B1 genes in cells treated with BP and SRM 1649a. The gene expression data correlates directly with the CYP1A1 activity as measured by ethoxyresorufin O-deethylation assay and the protein expression levels of both CYP1A1 and CYP1B1 as determined by Western blot (Table 4; Fig. 3).

In our study, relating CYP1A1 or CYP1B1 gene and protein induction levels (Figs. 1A and B and 3A and B) to metabolism of the PAH forming DNA adducts (Fig. 2) in cells treated with SRM 1649a in comparison to SRM 1649a plus BP did not account for the observed decrease in PAH-DNA adduct level. A similar outcome was observed in an in vitro study with SRM 1597 derived from coal tar (16). Although SRM 1649a did induce the CYP1A1 and CYP1B1 gene and protein, the induction levels were not higher than those calculated in cells treated with SRM 1649a plus BP or BP alone. These results suggest that the CYP enzymes metabolized many of the various PAH in SRM 1649a to non-DNA-binding metabolites.

However, the decrease in DNA adducts formed in cells treated with SRM 1649a plus BP may indicate the ability of PAH within mixtures to competitively inhibit the activation of carcinogenic PAH. Recently, Binkova and Sram (23) showed that, in human diploid lung fibroblasts, BP-diol epoxide adduct levels were up to 5-fold lower in an artificial carcinogenic PAH mixture and up to 10-fold lower in an environmental mixture and they attribute this decrease in adduct formation to competition for the metabolic activation enzymes, their saturation and/or inactivation by PAH metabolites. It has also been shown previously that the covalent binding of different dibenzopyrenes to DNA as binary mixtures formed relatively lower amounts of DNA adducts when compared with DBP in skin and lungs (48). Similar inhibition or reduction in PAH mixture induced tumor formation has also been reported (16, 48). Competitive inhibition of the activation of PAH could occur in several ways. One is simply competitive inhibition of the CYP1A1 metabolism of the PAH, another is that the metabolism of the PAH could be shifted to produce metabolites not capable of being activated to DNA binding intermediates such as phenols. Conducting biochemical experiments with SRM 1649a to understand classic Michaelis-Menten kinetics would clearly elucidate the competitive inhibition of the CYP enzymes that may be operating.

Another mechanism by which SRM 1649a may be affecting the formation of DNA adducts when cotreated with BP is by altering the global gene expression pattern (Table 2). Cells treated with SRM 1649a in comparison to BP exhibited an increase in the expression of oncogenes (FOS and JUN) and a decrease in tumor suppressor gene (CDKN1A; Tables 2 and 3). These responses would make it possible for increased tumor formation allowing for a decrease in adduct formation in the cotreated SRM 1649a plus BP group compared with BP alone. Although our studies with SRM 1649a were done using cells in culture, the results are in good agreement with a published animal study using SRM 1597 (16) where a decrease in adduct formation in the cotreated SRM 1597 plus BP group compared with BP alone was observed in spite of a similar tumorigenic response between the two treatment groups.

In conclusion, the microarray results from our study shows a broad spectrum of PAH-specific patterns of expression among the genes examined. Future work will focus on using the broad spectrum genes expressed to explore basic mechanisms of PAH and/or urban dust particulate toxicity and to generate new hypotheses. Also, the gene response patterns may shed new light on the mechanisms of toxic chemical mixture-induced human diseases and may also be useful for development of molecular biomarkers of exposure and/or effect in mechanistic, epidemiologic, and risk assessment studies.

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Altered Gene Expression Patterns in MCF-7 Cells Induced by the Urban Dust Particulate Complex Mixture Standard Reference Material 1649a

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