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Toxicogenomic Effect of Liver-toxic Environmental Chemicals in Human Hepatoma Cell Line

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Abstract

Some environmental chemicals have been shown to cause liver-toxicity as the result of bioaccumulation. Particularly, fungicides have been shown to cause varying degrees of hepatictoxicity and to disrupt steroid hormone homeostasis in *in vivo* models. The principal objective of this study was to evaluate the liver-toxic responses of environmental chemicals-in this case selected fungicides and parasiticides-in order to determine whether or not this agent differentially affected its toxicogenomic activities in hepatic tumor cell lines. To determine the gene expression profiles of 3 fungicides (triadimefon, myclobutanil, vinclozolin) and 1 parasiticide (dibutyl phthalate), we utilized a modified HazChem human array V2. Additionally, in order to observe the differential alterations in its time-dependent activities, we conducted two time (3 hr, 48 hr) exposures to the respective IC20 values of four chemicals. As a result, we analyzed the expression profiles of a total of 1638 genes, and we identified 70 positive significant genes and 144 negative significant genes using four fungicidic and parasiticidic chemicals, using SAM (Significant Analysis of Microarray) methods (q-value < 0.5%). These genes were analyzed and identified as being related to apoptosis, stress responses, germ cell development, cofactor metabolism, and lipid metabolism in GO functions and pathways. Additionally, we found 120 genes among those time-dependently differentially expressed genes, using 1-way ANOVA (*P*-value < 0.05). These genes were related to protein metabolism, stress responses, and positive regulation of apoptosis. These data support the conclusion that the four tested chemicals have common toxicogenomic effects and evidence respectively differential expression profiles according to exposure time.

Keywords: Fungicide, Toxicogenomics, HazChem, Liver-toxicity, HepG2

Fungi can cause serious damage to agricultural crops, resulting in critical losses in yield, quality, and profits. Fungicides are chemicals that are utilized to inhibit fungi or fungal spores and are used both agriculturally and to fight fungal infections in animals. However, some fungicides have been shown to cause varying degrees of hepatic toxicity and to disrupt steroid hormone homeostasis in *in vivo* models[1]. Some triazole fungicides evidence some degree of toxicity in mammals, and the effects reported from fungicide exposure in rodents include male infertility for myclobutanil and triadimefon in rats[2]. Some fungicides are known to be dangerous to human health, including vinclozolin, which is no longer used[3]. In this study, we utilized four fungicides and parasiticides-myclobutanil, triadimefon, vinclozolin, and dibutyl phthalate (Figure 1). Myclobutanil and triadimefon are agrichemical fungicides that block ergosterol biosynthesis, and thus control several species and strains of fungi[4,5]. Dibutyl phthalate can affect you when breathed in, and may be absorbed through the skin; high-level exposure can cause headache, nausea, drowsiness, and seizures. Thus, it may cause mutations, cause reproductive damage, or affect the nervous system. Vinclozolin is a fungicide that is commonly used in vineyards and is known as an endocrine disruptor chemical that interferes with the normal functioning of reproductive hormones[6]. In order to evaluate the toxicogenomic effects of these environmental toxicants, we utilized a modified HazChem human array V2[7]. It was manufactured using VOCs- and PAHs-specific genes and

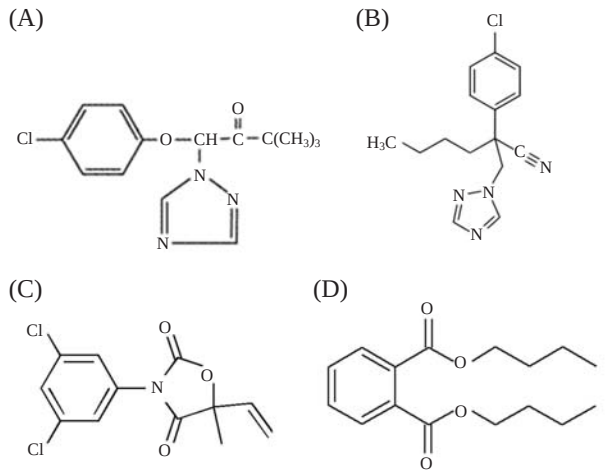

Figure 1. Chemical structure of 4 fungicidal and parasiticidal chemicals. (A) Triadimefon, (B) Myclobutanil, (C) Vinclozolin, (D) Dibutyl phthalate.

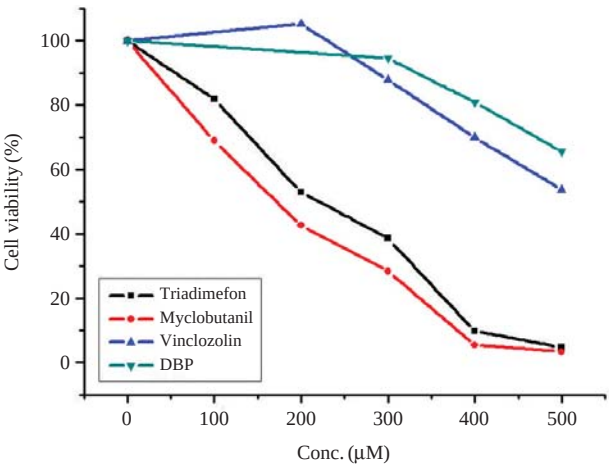

Figure 2. Cell viabilities of 4 chemicals using MTT assay. IC20 values (20% inhibition concentration of control) are respectively 96 μM (triadimefon, box), 72 μM (myclobutanil, circle), 320 μM (vinclozolin, triangle), and 340 μM (dibutyl phthalate (DBP), inverse triangle).

commonly expressed genes. We chose to modify and add certain liver-toxicity-related genes, as the four chemicals utilized herein have been shown to evidence varying degrees of hepatic toxicity[8]. In this study, genomic data from *in vitro* studies of four fungicidal and parasiticidal chemicals were analyzed, demonstrating that there are regulated hepatic biological functions in active human hepatocytes that are commonly disrupted by these chemicals. These differentially expressed genes and affected functions contributed to our understanding of the liver-toxicity induced by fungicidal and parasiticidal chemicals.

Cell Viability of Four Chemicals

The respectively cytotoxicity of four fungicides and parasiticides were assessed via MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assays in a HepG2 cell line. The absorbance of this colored solution was quantified via measurement at a wavelength of 540 nm by a spectrophotometer, and the IC20 values were determined from the linear concentration-response curve (Figure 2). The cultured HepG2 cells were exposed to various doses of 4 chemicals over a range of 0-1000 μM. The observed IC20 values of the four chemicals were calculated, respectively, as 96 μM (triadimefon), 72 μM (myclobutanil), 320 μM (vinclozolin), and 340 μM (dibutyl phthalate).

Modified HazChem Human Array V2

The HazChem human array V2 is composed of 600 genes that selected from VOCs and PAHs shown in previous studies to be expressed specifically on each type of chemicals or commonly expressed on the two types. In order to assess the toxicogenomic effects of these environmental toxicants, we utilized a modified HazChem human array V2. We modified and added 1038 liver-toxicity-related genes, because the four chemicals have been reported to evidence varying degrees of hepatic toxicity. 1038 liver toxicity-related genes were selected that were previously shown in rat *in vivo* studies to be homologous with human genes. The modified HazChem human array V2 is comprised of a total of 1638 genes.

Gene Expression Profile

Extracted data from Agilent Feature Extraction v10.7 software log-transformed on the background-subtracted signals were analyzed using GeneSpring GX 10 software. LOWESS normalization was conducted to eliminate intensity-dependent dye bias from each array. Total gene expression profiles of eight samples were analyzed using the Euclidean distance and the average linkage algorithm (Figure 3). The results of hierarchical clustering showed that the similarity between gene expression induced by the four fungicidal and parasiticidal chemicals. Red colors indicate upregulation, green indicates downregulation, and black indicates no expressional change. The expression profiles of four chemical-exposure samples exhibited very similar patterns.

Significant Common Expressionally Changed Genes

Significant expression changes were analyzed via comparison with the exposed and control groups of all chemicals using the Significant Analysis of Microar-

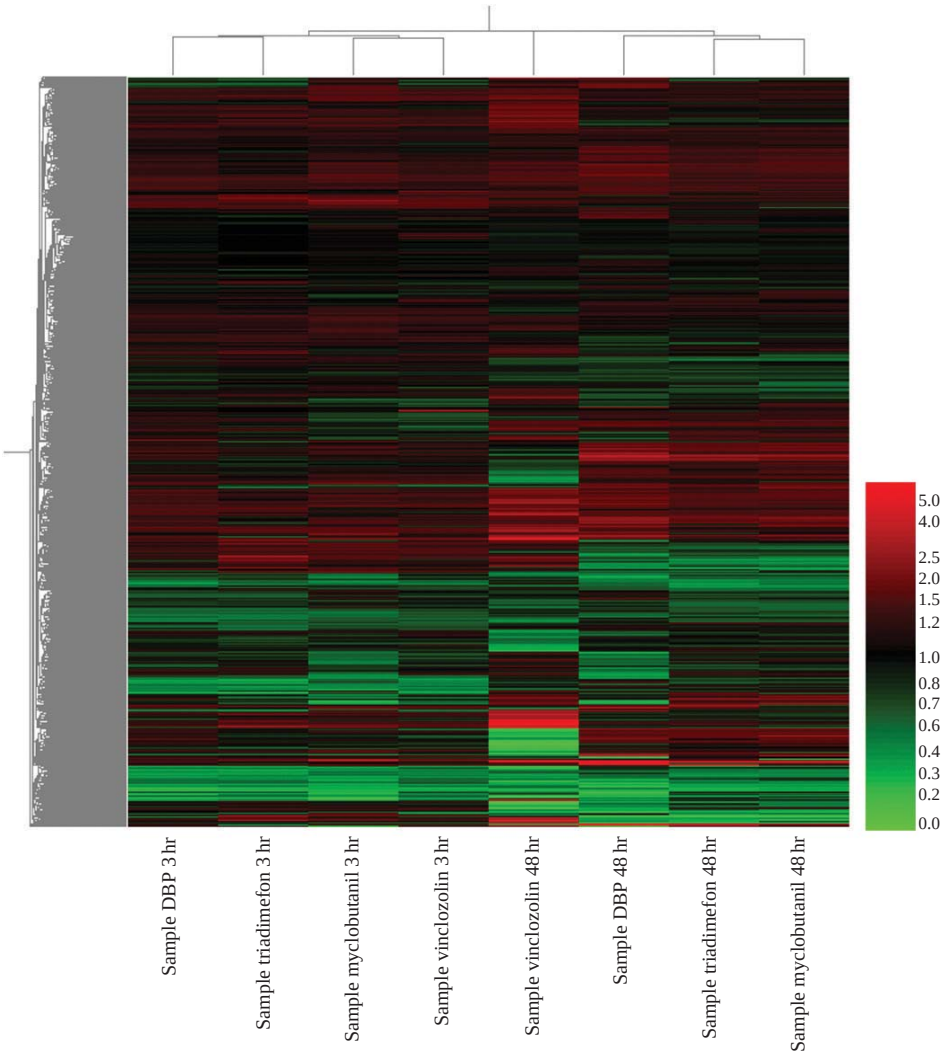

Figure 3. Total gene expression profiles. A total of 1638 genes with similar expression patterns are clustered. Similarity Measure is a Euclidean distance, the clustering algorithm is an average linkage. Red color indicates overexpression, green color indicates downregulation, and black color is normal.

ray (SAM) method (score $> \pm 1$, q-value < 0.05). In all chemical groups, a total of 214 genes were selected (Figure 4), 70 genes were significantly positively regulated, and 144 genes were significantly negatively regulated. These genes were identified as being related with apoptosis, stress responses, germ cell development, cofactor metabolism, and lipid metabolism in GO functions and pathways (Table 1). In particular, 26 genes (12%) related with apoptosis were identified as significantly expressionally altered by treatment with 4 chemicals. This data supports the notion that the 4 chemicals were barely toxic.

Time-dependent Varied Gene

In order to determine whether the differential response between early (3 hr) and late (48 hr) times after the exposure of 4 chemicals, we compared them with the expression profiles at early and late times, and then observed the differences in responses. We conducted a statistical analysis using a time-parametric Welch's t-test, and the Benjamini-Hochberg false discovery rate (FDR-adjusted P-value < 0.05). As a result, we were able to identify 120 genes that were differentially expressed in a time-dependent manner between early and late phases (Figure 5). Among those, 47% (56/120) were relatively upregulated at a late time as compared to an early time, whereas 53% (64/120) were relatively downregulated. These genes were found to be associated with protein metabolism, stress responses, and positive regulation of apoptosis.

Discussion

Fungicides are known chemicals that are used to inhibit fungi. They are utilized both in agriculture and

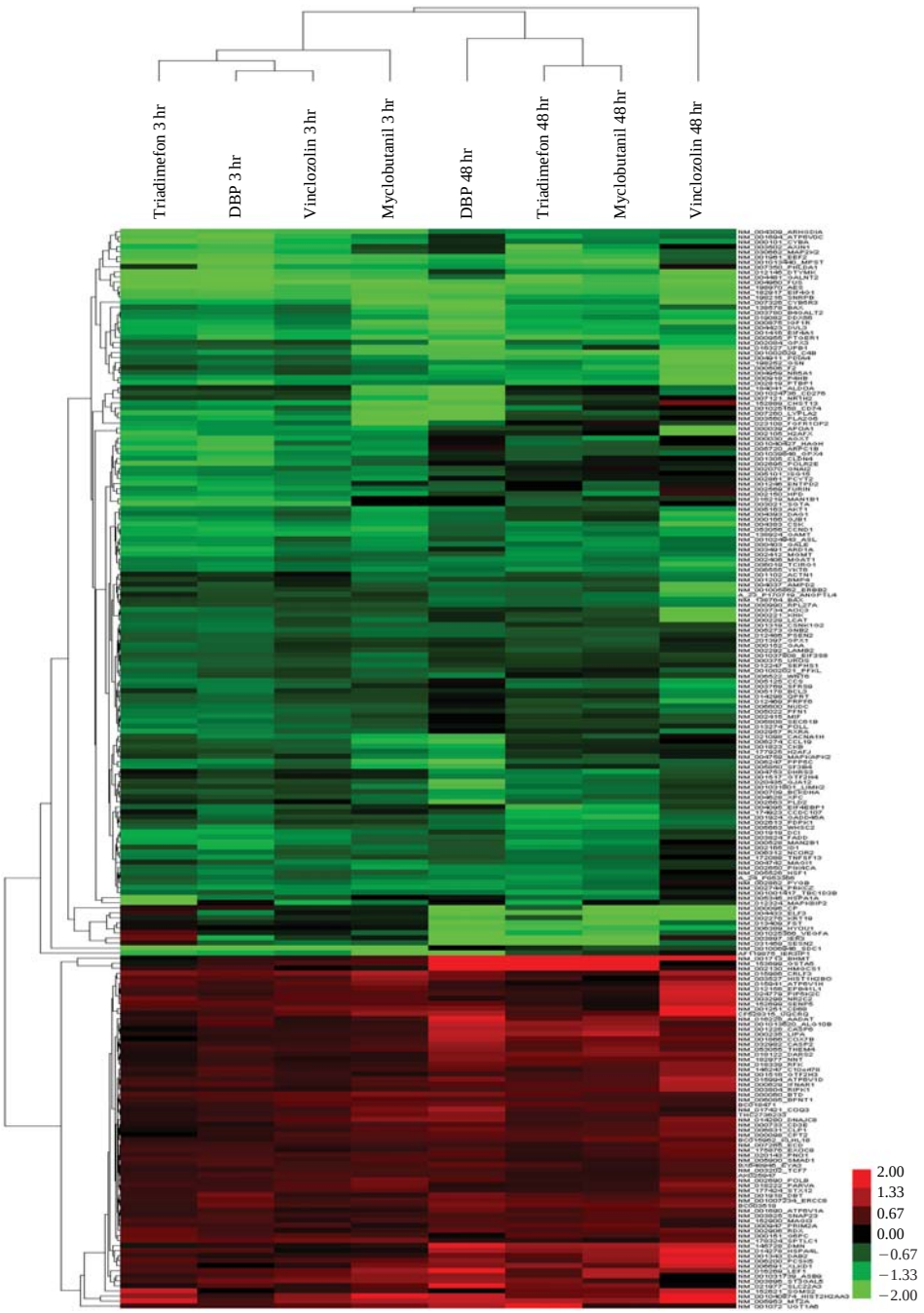

Figure 4. Genes commonly expression-altered by four chemicals. To select common expression-changed genes, we used significant analysis of microarray (SAM) methods. Cut-off values were q-values of less than 0.5% and scores in excess of ± 1.214 common expressionally altered genes with similar expression patterns are clustered.

to fight fungal infections in animals. However, some fungicides have been demonstrated to induce varying degrees of hepatic toxicity. The principal objective of this study was to compare the gene expression profiles of four fungicide and parasiticide chemicals, and to identify common expression changes that better characterized toxicities by exposure. The differences in time-dependent responses in human hepatoma cells were also evaluated via this toxicogenomic approach[9,10]. Observing changes in gene expression across different chemicals and exposure times allowed us to confirm the differential gene expression patterns common to the four fungicidal and parasiticidal chemicals, and also that they differed significantly across exposure times. As shown in previous studies, we assessed the toxicogenomic effects associated with apoptosis, stressresponses and lipid metabolism resulting from exposure to fungicides and parasiticides.

Table 1. GO ontology classification of common changed 214 genes.

Category	Common genes	% of Genes in category	*P*-value
Apoptosis	26	13.61	3.80E-06
Germ cell development	5	2.618	1.40E-05
Lipid metabolism	23	12.04	8.88E-05
Response to stress	31	16.23	9.65E-05
Cofactor metabolism	10	5.236	0.000497
Hydrogen transport	6	3.141	0.000506
Catabolism	20	10.47	0.00103
Alcohol metabolism	11	5.759	0.00222
Energy reserve metabolism	4	2.094	0.0023
Response to endogenous stimulus	12	6.283	0.00233
Insulin-like growth factor receptor signaling pathway	2	1.047	0.00282
BMP signaling pathway	3	1.571	0.00299

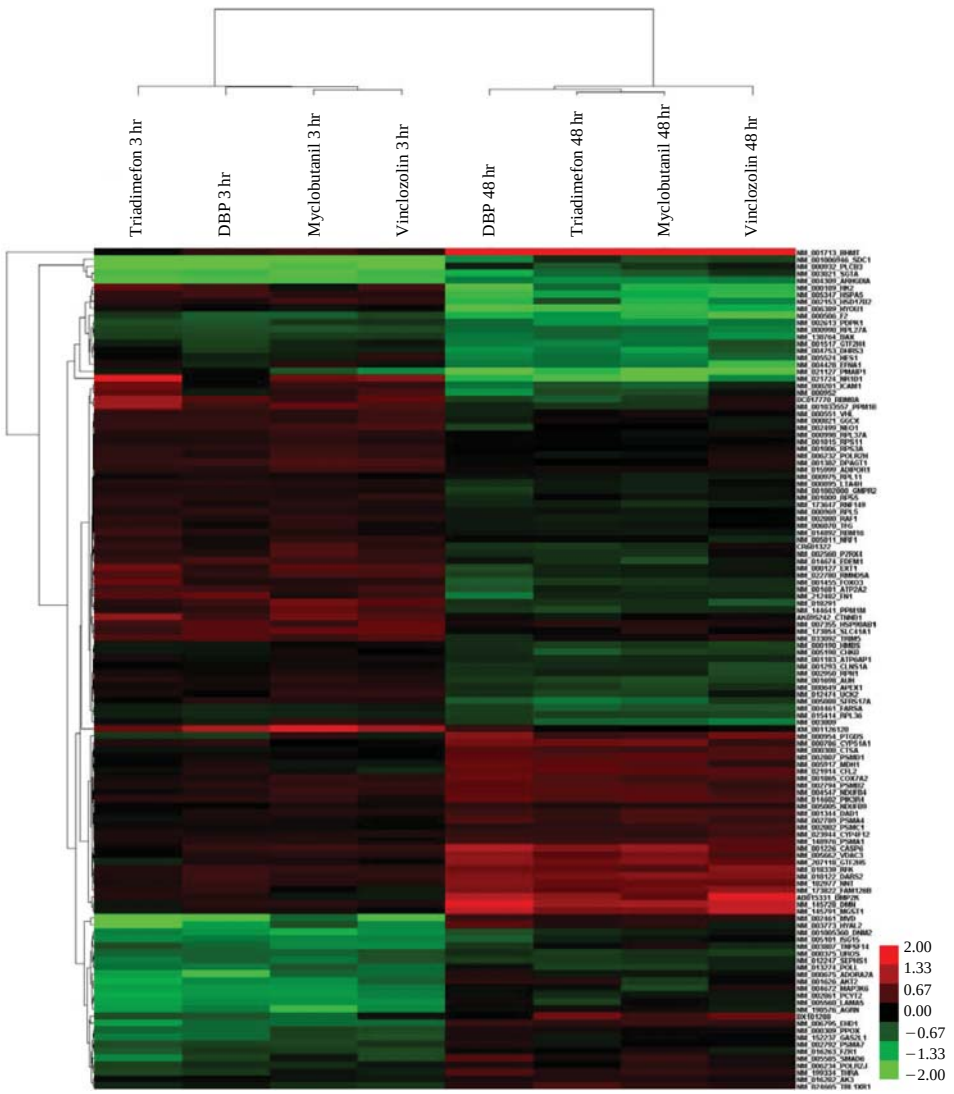

Figure 5. Time-dependent distinct genes. Benjamini-Hochberg correction for false discovery rate (FDR) was used for examine time-dependent differences. We selected expressed genes that a FDR-adjusted *P* value of less than 0.05 according to the ANOVA (test type is Welch's t-test). 120 distinct genes with similar expression patterns are clustered.

Some of the genes are related to germ cell development. Our results support the conclusions of previous studies, in which it was asserted that bioaccumulations of fungicides and parasiticides can induce abnormali-

ties of reproducibility[11]. Responses to toxicant treatment at early and late times differ greatly[12]. In the present study, we confirmed the findings of these previous studies showing an obvious and distinct gene expression pattern. These patterns were clearly related to stress responses and positive regulation of apoptosis, as shown by GO ontology analyses. As time progressed, acute responses were reduced and cell death increased in the gene expression profiles.

In this study, we utilized toxicogenomic analysis, identified common changes in four chemicals, and assessed distinct changes over exposure time. These toxicogenomic approaches were applied to delineate the molecular mode of actions and to assess the risks of environmental toxicants[13]. This approach should prove quite useful and may also be effective as a routine basic tool in the field of toxicology.

Materials & Methods

Chemicals and Reagents

Dibutyl phthalate (CAS No, 84-74-2), triadimefon (CAS No. 43121-43-3), vinclozolin (CAS No, 50471-44-8), myclobutanil (CAS No. 88671-89-0), Dimethylsulphoxide (DMSO) and 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from the Sigma-Aldrich Chemical Company (USA). RPMI-1640 Culture Medium, Dulbecco's Phosphate Buffered Saline (PBS) and Fetal Bovine Serum (FBS) were obtained from GIBCO^{TM} (USA). All other chemicals used in this study were of analytical grade or the highest available grade.

Cell Line and Culture

A human hepatocellular carcinoma cell line (HepG2), purchased from the Korean Cell Line Bank (Korea), was used throughout the study. HepG2 cells were grown in DMEM medium supplemented with 10% inactivated FBS plus 0.044 M sodium bicarbonate, 10 mM sodium pyruvate, and 1% penicillin at 37˚C in an atmosphere of 5% CO_2. For cell growth, the medium was renewed every two or three days. The HepG2 cells were brought to approximately 80% confluence by plating 2×10^6 cells/mL in 100 mm culture dishes[14].

Determination of Cell Viability

MTT assays[15] were conducted to determine cell viability. 24-well plates were utilized for cytotoxicity assays. Cells were seeded at a seeding density of 80 $\times 10^4$ cells/mL on a well in 500 μL of media. Cells were exposed for 48 h to various concentrations of triadimefon, myclobutanil, vinclozolin, dibutyl phthalate in culture medium at 37˚C. 75 μL of MTT (4 mg/mL in PBS) solution was added to each well and incubated for 3 hrs. DMSO solution was added to each well and transferred to 96-well plates. The optimal density (OD) of the purple formazan product was measured at a wavelength of 540 nm. The 20% inhibitory concentration (IC_{20}) of cell proliferation in a particular chemical was defined as the concentration required to cause a 20% reduction in the cell viability versus the solvent-treated control. The IC_{20} values were determined directly from the linear dose-response curves.

Preparation of HazChem Array

The HazChem human array V2 was composed of 600 genes selected from those shown in previous studies to be specifically expressed in response to specific VOCs and PAHs or commonly expressed in responses to the two types of chemicals. In order to assess the toxicogenomic effects of these environmental toxicants, we used a modified HazChem human array V2. We have modified this by the addition of 1038 liver-toxicity related genes, because the four chemicals used herein were reported to evidence varying degrees of hepatic toxicity. The 1038 liver toxicity-related genes were all shown in previous rat *in vivo* studies to be homologous to human genes. Thus, our modified HazChem human array V2 included a total of 1638 genes.

RNA Extract & Hybridization

Total RNA was extracted from the cells with Trizol reagent (Invitrogen Life Technologies) and purified with an RNeasy Mini Kit (Qiagen, USA) in accordance with the manufacturer's instructions. Genomic DNA was removed using an RNase-free DNase set (Qiagen, USA) during RNA purification. The quantity of each RNA concentration was quantified using Nanodrop, and the RNA quality was evaluated using an Agilent Bioanalyzer 2100 (Agilent Technologies, CA).

Each extracted total RNA sample (30 μg) was labeled with Cyanine (Cy3) or Cyanine (Cy5) conjugated dCTP (Amersharm, Piscataway, NJ) via a reverse transcription reaction using reverse transcriptase, PrimeScrip Reverse Transcriptase (TaKaRa, Japan). The labeled cDNA mixture was then concentrated via ethanol precipitation. The concentrated Cy3 and Cy5-labeled cDNAs were resuspended in 10 μL of hybridization solution (GenoCheck, Korea). The two labeled cDNAs were then mixed, placed on a modified HazChem human array V2 (GenoCheck, Korea), and covered with an Agilent 8-plex Gasket chamber (Agilent Technologies, CA). The slides were then hybridized for 12 hr at 62˚C with an Agilent hybridization system (Agilent Technologies, CA). The hybridized slides were washed in 2 X SSC, 0.1% SDS for 2 min, 1 X SSC for

3 min, and 0.2 X SSC for 2 min at room temperature. The slides were then centrifuged for 20 seconds at 3000 rpm to dry.

Microarray Data Analysis

The hybridized slides were scanned with an Agilent scanner and the scanned images were analyzed with the Feature Extraction v10.7 (Agilent Technologies, CA) and GeneSpring GX 10 software (Agilent Technologies, CA). Spots that were adjudged as substandard via the visual examination of each slide were flagged and excluded from further analysis. Spots that harbored dust artifacts or spatial defects were manually flagged and excluded. In an effort to filter out the unreliable data, spots with signal-to-noise ratios (signal-background-background SD) below 10 were excluded from our data. The data were normalized by intensity LOWESS normalization for data reliability. The expression profile data were clustered into groups of genes that behaved similarly across the drug treatment experiments using GeneSpring GX 10 (Agilent Technologies, CA). We utilized an algorithm based on Euclidean distance and average linkage to separate genes evidencing similar patterns. In order to select commonly expressionally-altered genes, we used significant analysis of microarray (SAM) methods. Q-values of less than 0.5% and scoresin excess of ± 1 were used as cut-off values. Thus, the Benjamini-Hochberg correction for false discovery rate (FDR) was used to evaluate time-dependent differences. We selected expressed genes with FDR-adjusted *P* values of less than 0.05 according to ANOVA results (test type is Welch's t-test) using GeneSpring GX 10, ArraytoKegg software (GenoCheck, Ansan, Korea) was utilized for the functional analysis of genes within differentially expressed genes[16].

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