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Identification of Hepatotoxicity Related Genes Induced by Hexachlorobenzne (HCB) in Human Hepatocellular Carcinoma (HepG2) Cells

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Abstract

Hexachlorobenzene (HCB) is a bioaccumulative, persistent, and toxic pollutant. HCB is one of the 12 priority of Persistent Organic Pollutants (POPs) intended for global action by the United Nations Environment Program (UNEP) Governing Council. POPs are organic compounds that are resistant to environmental degradation through chemical, biological, and photolytic processes. Some of HCB is ubiquitous in air, water, soil, and biological matrices, as well as in major environmental compartments. HCB has effects on various organs such as thyroid, bone, skin, kidneys and blood cells and especially, revealed strong toxicity to liver. In this study, we identified genes related to hepatotoxiciy induced by HCB in human hepatocellular carcinoma (HepG2) cells using microarray and gene ontology (GO) analysis. Through microarray analysis, we identified 96 up- and 617 down-regulated genes changed by more than 1.5-fold by HCB. And after GO analysis, we determined several key pathways which known as related to hepatotoxicity such as metabolism of xenobiotics by cytochrome P450, complement and coagulation cascades, and tight junction. Thus, our present study suggests that genes expressed by HCB may provide a clue for hepatotoxic mechanism of HCB and gene expression profiling by toxicogenomic analysis also affords promising opportunities to reveal potential new mechanistic markers of toxicity.

Keywords: Persistent Organic Pollutants (POPs), Hexachlorobenzene (HCB), Microarray, Gene ontology (GO)

The major research goals developing biomarkers are the development and validation of biomarkers that permit the prediction of the risk of disease. Toxicogenomic study has been widely used to characterize toxicological properties of disease. In this paper, we approached to Hexachlorobenzene (HCB) evaluated and their effects on genes with toxicogenomic study.

There are thousands of Persistent Organic Pollutants (POPs) chemicals, often coming from certain series or 'families' of chemicals (e.g there are theoretically 209 different polychlorinated biphenyls, differing from each other by level of chlorination and substitution position). POPs are persistent in the environment, having long half-lives in soils, sediments, air, or biota. Several POPs have been used as pesticides and some others are used in industrial processes and in the production of a range of goods such as solvents, polyvinyl chloride, and pharmaceuticals. Though there are a few natural sources of POPs, most POPs are created by humans in industrial processes, either intentionally or as byproducts[1]. HCB is one of the 12 priority of POPs and it is a chlorinated monocyclic aromatic white crystalline solid compound in which the benzene ring is fully substituted by chlorine (chemical formula C_6Cl_6). HCB does not occur naturally but can be synthesizes or formed as a byproduct during the manufacture of chemicals. Small amounts of HCB can also be produced during combustion processes like burning city wastes[2]. Using and intentionally making HCB is no longer allowed in the US but until 1965, the compound was widely used as a pesticide[3]. Commercial uses were essentially discontinued in the 1970s and becoming virtually complete by the early 1990s, because of concern about adverse effects on the environmental and human health. HCB is listed by the US EPA as a probable human carcinogen. Other reported health effects include impairment of thyroid, liver, bone, skin, kidneys, and blood cells, as well as damage to the immune, endocrine, developmental, and nervous system[4-7]. Laboratory animal studies revealed that chronic exposure to HCB could induce liver cell tumors in rat, mice, hamsters renal adenomas in rats, and tumors, haemangioendotheliomas and thyroid adenomas in

hamsters[7-10]. And HCB caused a massive outbreak of porphyria cutanea tarda (PCT) in Turkey and reported to induce porphyria in several animal species[10]. PCT, the most common form of porphyria in humans, is clinically characterized by photosensitivity with vesicular eruption on sun-exposed areas, hirsutism, impaired hepatic function, and dark red urine[11]. Uroporphyrinogen decarboxylase is the key enzyme of the heme metabolic pathway that is blocked in this porphyria, not only in humans, but also in experimental HCB-induced models. This block impairs the regulation of the heme pathway with the concomitant accumulation in the liver of highly carboxylated porphyrins[12]. The mechanism of uroporphyrinogen decarboxylase inactivation is not known; however, the most favored hypothesis is that of an inhibitor generated during the cytochrome CYP1A2-catalyzed uroporphyrinogen oxidation[13]. Like these, many researchers are performing risk assessments and toxicological studies of HCB with various animals and organs by means of physical and chemical measurements, but such physic-chemical analysis may not be sufficient to provide detailed information on how HCB affects the cells on a molecular level. Therefore, a toxicological study looking at the effects of HCB on the molecular level is required, with gene expression analysis being an appropriate method.

The aim of this study is the identification of potential gene-based markers on hepatotoxicity of HCB. We examined global gene expression in a small number of well-matched exposed-control subject pairs. Genes with differential expression were then ranked and selected for further examination using several forms of statistical analysis. The identification of differentially expressed genes (DEGs) may assist in the identification of potential biomarker and may understand molecular toxicological mechanisms of HCB in human hepatocytes.

Cytotoxicity of HCB in HepG2 Cells

To determine the optimal concentration, cytotoxicity of HCB was assessed by MTT [3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The survival percentage relative to solvent control (DMSO) was determined as a percentage of optical density values measured after treatment. Based on results of cytotoxicity assay, 20% cell viability inhibitory concentration (IC_{20}) of HCB was calculated. But HCB had a low solubility in DMSO. So, IC_{20} was about 16% inhibitory concentration which revealed maximam solubility in DMSO. Dose-dependent cell viability curve as obtained after 48 h exposure of HCB in HepG2 cells as shown in Figure 1, The IC_{20} value for HCB was 80 μM.

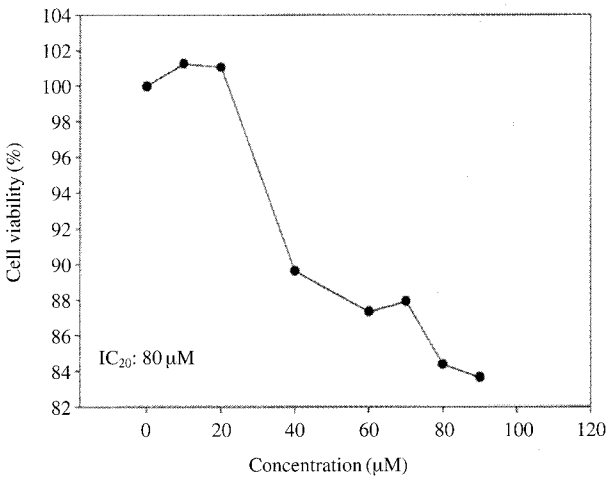

Figure 1. Cell viability measured by MTT assay. HepG2 cells were exposed to different concentrations of HCB for 48 hrs. After exposure, cell viability for each treatment was determined based on spectrometry of formazan formation, and represented the viability percentage relative to control (DMSO) exposure.

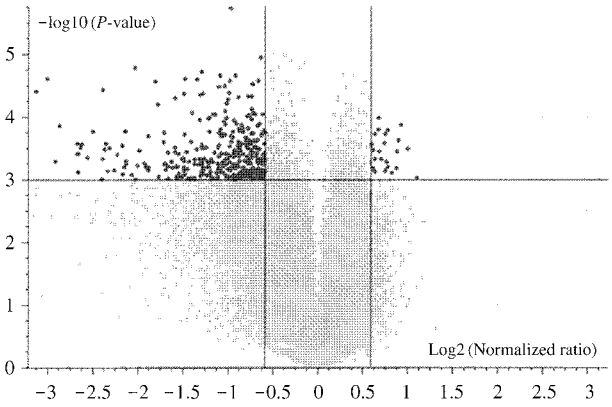

Figure. 2. Volcano plot representation of microarray data. Each dot represents one gene that had detectable expression in HCB. Treated HepG2 cells. The horizontal line marks the threshold ($P<0.01$) for defining a gene as up-regulated in right dots and down-regulated in left dots , with a combined change >1.5-fold. The vertical lines represent 1.5-fold change in expression.

Gene Expression Analysis

HepG2 cells were treated with 80 μM HCB for 48 h, and the total RNA was subjected to microarray analysis. Gene expression changes analyzed by comparing with treated group and control group using a statistical criteria of ≥ 1.5-fold changes with $P<0.001$. Ninty-six up-regulated genes and 617 down-regulated genes were selected from the experimental group using approximately 45,220 oligonucleotide probes (Figure 2).

These genes were classified according to KEGG

Table 1. Key pathways of up-regulated genes in HCB-treated HepG2 cells.

Accession no.	Gene name	Gene symbol	Fold change
Metabolism of xenobiotics by cytochrome P450			
NM_153699	glutathione S-transferase A5	GSTA5	2.251
NM_000846	glutathione S-transferase A2	GSTA2	1.855
NM_001072	UDP glucuronosyltransferase 1 family, polypeptide A6	UGT1A6	1.775
NM_000761	cytochrome P450, family 1, subfamily A, polypeptide 2	CYP1A2	1.873
NM_000847	glutathione S-transferase A3	GSTA3	1.984
NM_145740	glutathione S-transferase A1	GSTA1	1.951
Complement and coagulation cascades			
NM_000301	plasminogen	PLG	1.524
NM_000014	alpha-2-macroglobulin	A2M	1.776
NM_001002236	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	SERPINA1	2.100
NM_000242	mannose-binding lectin (protein C) 2, soluble (opsonic defect)	MBL2	2.000
NM_000509	fibrinogen gamma chain	FGG	1.529
NM_000130	coagulation factor V (proaccelerin, labile factor)	F5	1.505
Ribosome			
XM_931362	similar to ribosomal protein S3A		1.582
NM_001014	ribosomal protein S10	RPS10	1.529
NM_001017	ribosomal protein S13	RPS13	1.690
NM_021104	ribosomal protein L41	RPL41	1.607
NM_000997	ribosomal protein L37	RPL37	1.561

All genes identified in the present study had been submitted to GeneBank and assigned accession numbers.

pathway to analyse molecular mechanisms. Up- and down-regulted genes are listed in Table 1 and 2. In KEGG pathway analysis, metabolism of xenobiotics by cytochrome P450, complement and coagulation cascades, and ribosome are prominently annotated with up-regulated genes. Also insulin signaling pathway, tight junction, glioma, chronic myeloid leukemia, ErbB signaling pathway, fructose and mannose metabolism, colorectal cancer, non-small cell lung cancer, citrate cycle (TCA cycle), glycine, serine and threonine metabolism, cholera-Infection, carbon fixation, and cyanoamino acid metabolism are prominently annotated with down-regulated genes.

We investigated an enrichment of GO annotations in the up-regulated and down-regulated genes. These genes were classified according to GO biological, cellular, and molecular process to analyze molecular mechanism of HCB. EASE analysis was performed on genes to find biological, cellular, and molecular process significantly, represented and to identify any biological terms that response to HCB using program in http://david.abcc.ncifcrf.gov/. And then, the biological, cellular and molecular process terms were condensed to the most common parent term without going higher than the fourth GO level below biological, cellular, and molecular process. The categories of the function in expressed genes are presented in Table 3. The biological processes profile could be subdivided to each top 10, organ development, translation, tissue development, hemostasis, inflammatory response, sterol biosynthetic process, isoprenoid metabolic process, regulation of coagulation, actin filament capping, fibrinolysis, apoptosis, cellular lipid metabolic process, lipid biosynthetic process, MAPKKK cascade, cell growth, lipoprotein metabolic process, glycolysis, caspase activation, cofactor catabolic process, and protein destabilization. Analysis of cellular localization reveals enrichment for genes associated with cytoplasm, intracellular, extracellular, and ribosome. And the majority of profiles in molecular function are protein binding, structural molecule activity, catalytic activity, and endopeptidase inhibitor activity.

Discussion

In mammals, metabolism HCB of is very slow and occurs primarily in the liver, with most of the compound being excreted unchanged. Reductive dechlorination of HCB catalyzed by enzymes located in the microsomal fraction of liver, lung, kidney, and intestine appears to be an important metabolic pathway[14]. Following the accidental human poisoning in Turkey, the toxicity of HCB has been extensively studied. Porphyria is regarded as the major potential toxic manifestation of HCB in experimental animals and human. HCB-induced hepatic porphyria is characterized by a deficiency of the enzyme uroporphyrinogen decarbo-

Table 2. Key pathways of down-regulated genes in HCB-treated HepG2 cells.

Accession no.	Gene name	Gene symbol	Fold change
Insulin signaling pathway			
NM_002840	protein tyrosine phosphatase, receptor type, F	PTPRF	0.347
AK054771	v-akt murine thymoma viral oncogene homolog 2	AKT2	0.602
NM_001654	v-raf murine sarcoma 3611 viral oncogene homolog	ARAF	0.487
NM_001013839	exocyst complex component 7	EXOC7	0.561
NM_005163	v-akt murine thymoma viral oncogene homolog 1	AKT1	0.327
NM_002086	growth factor receptor-bound protein 2	GRB2	0.599
NM_004475	flotillin 2	FLOT2	0.327
NM_182470	pyruvate kinase, muscle	PKM2	0.415
NM_002591	phosphoenolpyruvate carboxykinase 1 (soluble)	PCK1	0.345
NM_003029	SHC (Src homology 2 domain containing) transforming protein 1	SHC1	0.442
NM_005184	calmodulin 3 (phosphorylase kinase, delta)	CALM3	0.537
NM_004563	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	PCK2	0.496
NM_002862	phosphorylase, glycogen; brain	PYGB	0.328
Tight junction			
NM_201629	tight junction protein 2 (zona occludens 2)	TJP2	0.549
AK054771	v-akt murine thymoma viral oncogene homolog 2	AKT2	0.602
NM_014225	protein phosphatase 2 (formerly 2A), regulatory subunit A, alpha isoform	PPP2R1A	0.577
NM_005964	myosin, heavy chain 10, non-muscle	MYH10	0.461
NM_003277	claudin 5 (transmembrane protein deleted in velocardiofacial syndrome)	CLDN5	0.566
NM_005417	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	SRC	0.362
NM_021807	exocyst complex component 4	EXOC4	0.647
NM_001102	actinin, alpha 1	ACTN1	0.457
NM_005163	v-akt murine thymoma viral oncogene homolog 1	AKT1	0.327
NM_001614	actin, gamma 1	ACTG1	0.572
Glioma			
AK054771	v-akt murine thymoma viral oncogene homolog 2	AKT2	0.602
NM_001654	v-raf murine sarcoma 3611 viral oncogene homolog	ARAF	0.487
NM_002660	phospholipase C, gamma 1	PLCG1	0.489
NM_172171	calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma	CAMK2G	0.507
NM_005163	v-akt murine thymoma viral oncogene homolog 1	AKT1	0.327
NM_003029	SHC (Src homology 2 domain containing) transforming protein 1	SHC1	0.442
NM_005184	calmodulin 3 (phosphorylase kinase, delta)	CALM3	0.537
NM_002086	growth factor receptor-bound protein 2	GRB2	0.599
Chronic myeloid leukemia			
AK054771	v-akt murine thymoma viral oncogene homolog 2	AKT2	0.602
NM_001654	v-raf murine sarcoma 3611 viral oncogene homolog	ARAF	0.487
NM_138578	BCL2-like 1	BCL2L1	0.469
NM_005163	v-akt murine thymoma viral oncogene homolog 1	AKT1	0.327
NM_003029	SHC (Src homology 2 domain containing) transforming protein 1	SHC1	0.442
NM_002086	growth factor receptor-bound protein 2	GRB2	0.599
NM_021574	breakpoint cluster region	BCR	0.262
ErbB signaling pathway			
AK054771	v-akt murine thymoma viral oncogene homolog 2	AKT2	0.602
NM_001654	v-raf murine sarcoma 3611 viral oncogene homolog	ARAF	0.487
NM_002660	phospholipase C, gamma 1	PLCG1	0.489
NM_005417	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	SRC	0.362
NM_172171	calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma	CAMK2G	0.507
NM_005163	v-akt murine thymoma viral oncogene homolog 1	AKT1	0.327
NM_003029	SHC (Src homology 2 domain containing) transforming protein 1	SHC1	0.442
NM_002086	growth factor receptor-bound protein 2	GRB2	0.599
Fructose and mannose metabolism			
NM_184041	aldolase A, fructose-bisphosphate	ALDOA	0.513
NM_000303	phosphomannomutase 2	PMM2	0.531
NM_004567	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	PFKFB4	0.409
NM_004566	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	PFKFB3	0.564
NM_138412	retinol dehydrogenase 13 (all-trans/9-cis)	RDH13	0.607
NM_000189	hexokinase 2	HK2	0.641

Table 2. Continued.

Accession no.	Gene name	Gene symbol	Fold change
NM_000221	ketohexokinase (fructokinase)	KHK	0.661
Colorectal cancer			
NM_006266	ral guanine nucleotide dissociation stimulator	RALGDS	0.414
AK054771	v-akt murine thymoma viral oncogene homolog 2	AKT2	0.602
NM_001654	v-raf murine sarcoma 3611 viral oncogene homolog	ARAF	0.487
U68019	SMAD family member 3	SMAD3	0.387
NM_005163	v-akt murine thymoma viral oncogene homolog 1	AKT1	0.327
NM_138763	BCL2-associated X protein	BAX	0.460
NM_002086	growth factor receptor-bound protein 2	GRB2	0.599
Non-small cell lung cancer			
AK054771	v-akt murine thymoma viral oncogene homolog 2	AKT2	0.602
NM_001654	v-raf murine sarcoma 3611 viral oncogene homolog	ARAF	0.487
NM_002660	phospholipase C, gamma 1	PLCG1	0.489
NM_001455	forkhead box O3	FOXO3	0.665
NM_005163	v-akt murine thymoma viral oncogene homolog 1	AKT1	0.327
NM_002086	growth factor receptor-bound protein 2	GRB2	0.599
Citrate cycle (TCA cycle)			
NM_004168	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	SDHA	0.615
NM_002591	phosphoenolpyruvate carboxykinase 1 (soluble)	PCK1	0.345
AK128072	CDNA FLJ46193 fis, clone TESTI4006234		0.628
NM_004563	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	PCK2	0.496
NM_001098	aconitase 2, mitochondrial	ACO2	0.556
Glycine, serine and threonine metabolism			
NM_004169	serine hydroxymethyltransferase 1 (soluble)	SHMT1	0.584
NM_005412	serine hydroxymethyltransferase 2 (mitochondrial)	SHMT2	0.629
NM_138412	retinol dehydrogenase 13 (all-trans/9-cis)	RDH13	0.607
NM_006623	phosphoglycerate dehydrogenase	PHGDH	0.483
NM_000481	aminomethyltransferase	AMT	0.621
Cholera-Infection			
NM_002660	phospholipase C, gamma 1	PLCG1	0.489
NM_001659	ADP-ribosylation factor 3	ARF3	0.650
NM_001661	ADP-ribosylation factor-like 4D	ARL4D	0.443
NM_001662	ADP-ribosylation factor 5	ARF5	0.637
NM_001614	actin, gamma 1	ACTG1	0.572
Carbon fixation			
NM_184041	aldolase A, fructose-bisphosphate	ALDOA	0.513
NM_182470	pyruvate kinase, muscle	PKM2	0.415
AK128072	CDNA FLJ46193 fis, clone TESTI4006234		0.628
NM_001064	transketolase (Wernicke-Korsakoff syndrome)	TKT	0.604
Cyanoamino acid metabolism			
NM_004169	serine hydroxymethyltransferase 1 (soluble)	SHMT1	0.584
NM_005412	serine hydroxymethyltransferase 2 (mitochondrial)	SHMT2	0.629
NM_013430	gamma-glutamyltransferase 1	GGT1	0.631

All genes identified in the present study had been submitted to GeneBank and assigned accession numbers.

xylase, which is essential for the synthesis of heme, an iron containing pigment[15,16].

As shown in Table 1 and 2, the expressed genes in HCB-treated HepG2 cells are related to pathways such as metabolism of xenobiotics by cytochrome P450, complement and coagulation cascades, tight junction, fructose and mannose metabolism, and citrate cycle (TCA cycle). The importance of xenobiotics and the P450 isoenzyme complex in the pathogenesis of porphyria cutanea tarda (PCT) was first proven in rat liver tissue[17]. In human, the cytochrome P450 (CYP) enzyme complex also comprises important enzymes, in particular CYP1A1 and CYP1A2, for the metabolism of xenobiotics such as heterocyclic amines, polycyclic hydrocarbons, and various endogenous substrates[17-19]. The liver plays a predominant role in the regulation

Table 3. GO annotations for HCB-induced genes.

UP		DOWN	
GO-Biological Process (top 10 out of 43 total)	Genes[1]	GO-Biological Process (top 10 out of 176 total)	Genes[1]
organ development	12	apoptosis	40
translation	8	cellular lipid metabolic process	28
tissue development	6	lipid biosynthetic process	16
hemostasis	6	MAPKKK cascade	11
inflammatory response	5	cell growth	10
sterol biosynthetic process	3	lipoprotein metabolic process	6
isoprenoid metabolic process	3	glycolysis	5
regulation of coagulation	3	caspase activation	4
actin filament capping	2	cofactor catabolic process	4
fibrinolysis	2	protein destabilization	2
GO-Cellular Component (top 8 out of 11 total)	Genes[1]	GO-Cellular Component (top 10 out of 62 total)	Genes[1]
cytoplasm	40	intracellular part	331
cytosol	8	cytoplasm	240
ribosome	6	nucleus	145
cytosolic ribosome (sensu Eukaryota)	5	nuclear lumen	28
cytosolic part	5	organelle envelope	25
ribosomal subunit	5	microtubule	12
cytosolic large ribosomal subunit (sensu Eukaryota)	3	vacuole	11
large ribosomal subunit	3	spindle	7
		pore complex	6
		polar microtubule	2
GO-Moleculer Function (top 7 out of 17 total)	Genes[1]	GO-Moleculer Function (top 10 out of 54 total)	Genes[1]
calcium ion binding	10	adenyl nucleotide binding	58
serine-type endopeptidase inhibitor activity	5	kinase activity	44
endopeptidase inhibitor activity	5	transcription cofactor activity	19
glutathione transferase activity	4	GTPase activator activity	12
protein heterodimerization activity	3	symporter activity	10
retinal binding	2	FAD binding	6
retinoid binding	2	carbohydrate transmembrane transporter activity	5
		oxidoreductase activity, acting on the CH-NH group of donors, NAD or NADP as acceptor	4
		insulin receptor binding	4
		RNA polymerase II transcription mediator activity	3

[1]Some genes are counted in more than one annotation category.

of haemostasis. By producing most clotting factors (except tissue factor TF) and inhibitors (antithrombin III, protein C, protein S, C1 inhibitor), as well as a number of the proteins involved in fibrinolysis (plasminogen, a2-antiplasmin), and by clearing from the bloodstream activated enzymes involved in clotting or fibrinolysis, the liver protects against both bleeding and under activation of coagulation. There ia s common bleeding profile emerging in the overwhelming majority of liver diseases[20-22]. Tight junctions hepatocyte of play key roles in characteristic liver functions, including bile formation and secretion. Tight junctions are major components of cell-cell adhesion complexes that separate apical from basolateral membrane domains and maintain cell polarity by forming an intramembrane fence that restricts diffusion of lipids in the exoplasmic leaflet of the plasma membrane[23].

In conclusion, HCB is has been implicated in a broad range of adverse human environmental health effects, including impaired reproduction and neurological dysfunction, immunologic effects, hepatotoxic, skin effects, and cancer. As long as HCB-containing products are being produced, and convincing sub stantiveevidence exists for the actual and potential toxic impact of this both to human health and to the environment, international cooperation in addressing the presence of HCB in the global environment must be achieved. Our results showed that the gene expression

patterns were associated with hepatotoxicity induced by HCB. And classifying the gene alterations, analyzing the gene expression patterns, and understanding mechanism associated with HCB-induced toxicity should allow earlier identification of clinically relevant toxicological findings in compound screening and aid in the development of new chemical to reduce hepatotoxicity.

Materials & Methods

Chemicals and Reagents

Hexachlorobenzene (HCB), dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (USA). Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Phosphate Buffered Saline (PBS), 0.5% trypsin-EDTA and Fetal Bovine Serum (FBS) were the products of GIBCO™ (USA). Trizol reagent was produced by Invitrogen (USA) and RNeasy mini kit and RNase-free DNase set were purchased from Qiagen (USA). All other chemicals used were of analytical grade or the highest grade available.

Cell Lines and Culture

Human hepatocellular carcinoma cell line (HepG2) used throughout the study was purchased from Korean Cell Line Bank (Korea). HepG2 cells were grown in DMEM medium supplemented with 10% inactivated FBS, 0.044 M sodium bicarbonate, 10 mM sodium pyruvate and 1% penicillin at 37°C in 5% CO_2 atmosphere. For cell growth, the medium was renewed every two or three days. HepG2 cells were approximately 80% confluence achieved by plating 6×10^6 cells/mL in 100 mm culture dish.

Determination of Cell Viability

To determine the cyotoxicity and effects on cell growth, MTT assay was performed[24]. In case of HepG2 cells, 24-well plates were used and cells were seeded at a seeding density of 80×10^4 cells/mL on a well in 500 μL of media. And cells were exposed to various concentrations of HCB in culture medium at 37°C for 48 h exposure times. After exposure, the cells were incubated for 3 h with 4 mg/mL MTT in PBS. To quench the reaction, the medium was removed and DMSO was added and transferred to 96 well plate. The optimal density (O.D) 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 that causes a 20% reduction in the cell viability versus the solvent treated control. The IC_{20} values were directly determined from the linear dose-response curves.

RNA Extraction

Total RNA was extracted from the HepG2 cells after 80 μM HCB exposure for 48 h, using the Trizol reagent and purified using RNeasy mini kit according to the manufacturer's instructions. Genomic DNA was removed using RNase-free DNase set during RNA purification procedure. The amount of each total RNA was quantified using NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies Inc., USA). Only samples with an A260/A280 ratio between 1.9 and 2.2 were considered for suitable use and its quality was checked by Experion™ (Bio-Rad, USA).

Oligonucleotide Microarray Hybridization

Gene expression analysis was conducted on the RNA samples using 4X44 K whole human genome microarray (Agilent Technologies, USA). Triplicate analysis was simultaneously performed. Labeling and hybridization were performed by instruction of Platinum Biochip Reagent Kit (GenoCheck Co. Ltd, Korea). This was followed by the coupling of the Cy3 dye for the controls (DMSO) and Cy5 dye for the treated samples. Hybridization was performed in a hybridization oven at 62°C for 12 h. After washing (2 × SSC/0.1% SDS for 2 min at 58°C, 1 × SSC for 2 min at RT and 0.2 × SSC for 3 min at RT), the slide was dried by centrifugation at 800 rpm for 3 min at RT. Hybridization images on the slides were scanned by GenePix 4000B (Axon Instruments, USA). Scanned images were analyzed with GenePix 4.1 software (Axon Instruments, USA) to obtain gene expression ratios.

Data Analysis

After analyzing of scanned images, spots that adjudged as substandard via the visual examination of each slide were flagged and excluded from further analysis. Spots that harboured dust artifacts or spatial defects were manually flagged and excluded. In an attempt to filter out the unreliable data, spots with signal-to-noise (signal-background-background SD) ratios below 10 were not included in the data. Data were normalized via global, lowess, print-tip, and scaled normalization methods. Obtained data were represented to volcano plot of genes that behaved similarly across the HCB treatment using GeneSpring Gx 7.3.1 software. We utilized an algorithm based on the Pearson's correlation to separate genes exhibiting similar patterns[25].

Functional Grouping and Clustering Analysis

In order to classify the selected genes into groups

with a similar pattern of expression, each gene was assigned to an appropriate category according to its main cellular function. The necessary information to categorize each gene was obtained from several databases particularly the database located at http://david.abcc.ncifcrf.gov/home.jsp

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