ToxEHS

Identification of Genes Induced by Carbamazepine in Human Bronchial Epithelial BEAS-2B Cells

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Abstract

Some drugs are limited in their clinical application due to their propensity for inducing adverse side effects. We examined some clinical chemotherapeutic agents that have pulmonary toxic effects. Carbamazepine (CBZ) is an antiepileptic agent and its long-term use is associated with interstitial pneumonia, pulmonary fibrosis, and pulmonary infiltration with eosinophilia. CBZ is persistent in the environment and is frequently detected in water systems. A new technique in toxicity screening, "toxicogenomic technology", represents a useful approach for evaluating the toxic properties of new drug candidates early in the drug discovery process and their potential effects on the environment. To this end, we have examined gene expression profiles in BEAS-2B cells (a human bronchial epithelial cell line) following exposure to CBZ, which induced pulmonary toxicity, by using a human oligonucleotide chip. We identified 518 up- and 496 down-regulated genes whose expression had changed by more than 1.5-fold ($p < 0.01$) following CBZ exposure. Gene Ontology (GO) analysis showed elevation in the expression of genes involved in several key biological processes related to pulmonary toxicity, such as cholesterol metabolism, cell proliferation, and cell cycle regulation. In conclusion, the present study indicates that CBZ exerts its toxicity by modulating mRNA expression in BEAS-2B cells. We suggest that genes expressed by CBZ might serve as a molecular signature, which could be used more widely when implemented in combination with more traditional techniques, for the assessment and prediction of toxicity following CBZ-exposure.

Keywords: Carbamazepine (CBZ), Pulmonary toxicity, Microarray, Gene ontology (GO)

Carbamazepine (CBZ) is widely used as an antiepileptic agent in the treatment of epilepsy, neuropathic pain, and bipolar affective disorder. However, it has been associated with a wide variety of side effects, including hematological, hepatic, neurological, cardiac, dermatological, and renal disorders[1]. Furthermore, it is suspected that long-term use of CBZ elicits immune-mediated hypersensitivity[1]. In pulmonary toxicity, interstitial pneumonitis, bronchiolitis obliterans organizing pneumonia, bronchospasm, pulmonary edema, pulmonary nodules, and pulmonary fibrosis have all been reported[1-3].

CBZ is chronically administered to patients at a daily dosage of 100-2,000 mg[4]. The extensive use of CBZ requires a high worldwide production rate[4]. Production of generic CBZ in the United States was estimated to be approximately 43,000 kg in 2000, and 35,000 kg in 2003[5]. Approximately 50% of administrated CBZ and its metabolites are excreted in urine, and thus end up at municipal wastewater treatment plants (WWTPs)[6]. Since CBZ is highly resistant to degradation in WWTPs, a proportion of the CBZ survives the wastewater treatment processes and enters the local water systems[4]. As a result, CBZ has become a frequently detected pharmaceutical in WWTP effluents and in environmental water systems[7,8]. CBZ has been detected in wastewater effluents (up to 6.3 μg/L), surface waters (up to 2.1 μg/L), groundwater (up to 0.41 μg/L) and even in drinking water (up to 0.26 μg/L)[4]. In Korea, the total levels of target pharmaceuticals in the effluents are below 1 μg/L, of which CBZ showed the highest concentration (0.178 μg/L) in municipal WWTPs[9]. Furthermore, CBZ (21-56%) is the dominant pharmaceutical in municipal WWTP effluent[9]. CBZ has a relatively long dissipation half-life (~328 days) and is, therefore, classified as a highly persistent organic compound in the environment[10-12]. In natural ecosystems, CBZ residues in the environment could cause potential damage to the liver and immune systems[13].

Although many researchers are performing risk as-

sessment and toxicological studies of CBZ by physical and chemical measurements, this physicochemical analysis might not be sufficient to provide detailed information on pulmonary toxicity and on the cellular effects of CBZ at a molecular level. Therefore, a toxicological study to assess the in vitro effects of CBZ in human models at the molecular level is required, with gene expression analysis being an appropriate method.

The aim of this study was to identify potential gene-based markers for CBZ toxicity. 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 by using several methods of statistical analysis. The examination of differentially expressed genes (DEGs) may assist in the identification of potential biomarkers and may improve our understanding of the molecular toxicological mechanisms of CBZ in human bronchial epithelial BEAS-2B cells.

Results

Cytotoxicity of CBZ in BEAS-2B Cells

The MTT assay was used to determine the relative survival of BEAS-2B cells following exposure to a range of concentrations of CBZ, which was used to induce pulmonary toxicity. The survival percentage relative to the control (solvent alone, DMSO) was determined as the percentage optical density measured after treatment. Based on the results of the MTT assay, the 20% cell viability inhibitory concentration (IC_{20}) of CBZ was calculated. Dose-dependent cell viability curves were obtained after 48 h of exposure to CBZ in BEAS-2B cells as shown in Figure 1. The IC_{20} value for CBZ was determined to be 324 μM. The IC_{20} value as a minimum cytotoxic concentration was used for the selection of genes less affected by cell cytotoxicity and the identification for appropriate number of genes using microarray.

Gene Expression Profiles Altered by CBZ in BEAS-2B Cells

To study the gene expression response to CBZ-induced toxicity, BEAS-2B cells were treated with 324 μM of CBZ for 48 h, and total RNA was isolated for microarray analysis. Gene expression changes were analyzed by comparing the treated group against the control group by using statistical criteria ($\geq$1.5-fold change, $p < 0.01$). Three independent experimental samples were analyzed to determine RNA transcription levels. We identified 518 up-regulated and 496 down-regulated genes in response to CBZ exposure. To confirm the mRNA expression results of the microarray

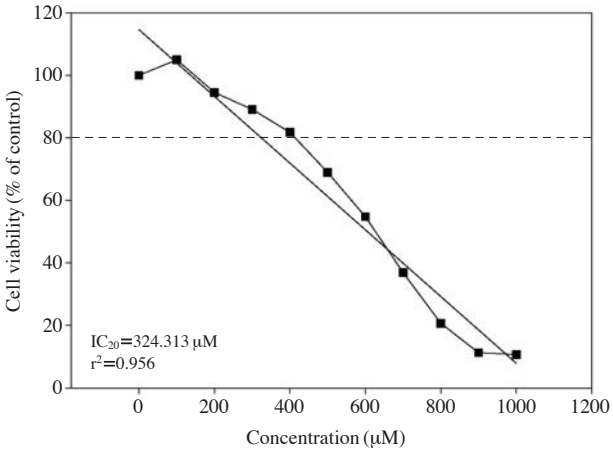

Figure 1. Cell viability measured by MTT assay. BEAS-2B cells were exposed to different concentrations of CBZ for 48 h. After exposure, cell viability for each treatment was determined based on spectrometry of formazan formation, and represented the viability percentage relative to control (DMSO) exposure. The MTT assay was carried out at least in triplicate.

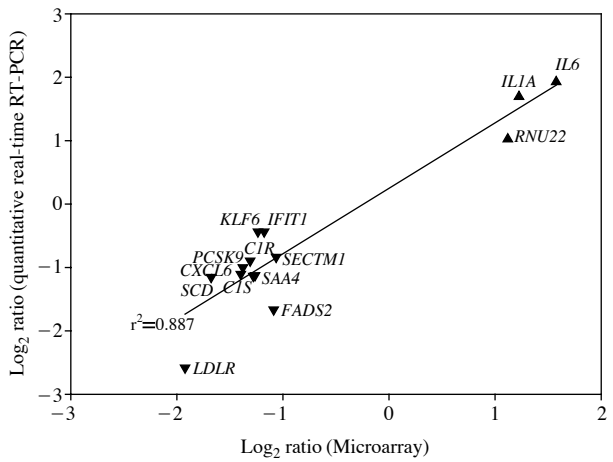

Figure 2. Comparison of DNA microarray and quantitative real-time RT-PCR data in CBZ-induced expressed genes. The fold change in the mean mRNA ratio for CBZ was $\log_2$ transformed and plotted with microarray versus real-time RT-PCR (r^2; correlation coefficient).

data for 3 up-regulated genes (*IL6*, *IL1A*, and *RNU22*) and 11 down-regulated genes (*SECTM1*, *IFIT1*, *KLF6*, *CXCL6*, *SAA4*, *C1R*, *C1S*, *LDLR*, *FADS2*, *PCSK9*, and *SCD*), total RNA from CBZ exposed BEAS-2B cells was analyzed using quantitative real-time RT-PCR with SYBR green fluorescent dye. Expression of these genes showed similar patterns in the quantitative real time RT-PCR as for the microarray analysis data. The correlation coefficient (r^2) of up-regulated and down-regulated genes between microarray and quantitative

Table 1. GO and KEGG annotations for carbamazepine-induced genes.

Up		Down	
GO-Biological Process	Genes[1]	GO-Biological Process	Genes[1]
Amine biosynthetic process	7	Cholesterol metabolic process	13
Positive regulation of cell proliferation	16	Steroid metabolic process	14
Inflammatory response	15	Negative regulation of cell proliferation	19
Biogenic amine metabolic process	7	Tissue development	27
Cellular amino acid metabolic process	14	Epidermis development	12
		Cell morphogenesis	17
		Ectoderm development	12
		Complement activation	6
		Nervous system development	34
		Cell morphogenesis involved in differentiation	13
		Complement activation, classical pathway	5
		Inflammatory response	15
		Humoral immune response mediated by circulating immunoglobulin	5
		Cell cycle arrest	8
		Mitosis	11
		Nuclear division	11
		M phase of mitotic cell cycle	11
		Lymphocyte mediated immunity	6
KEGG Pathway	Genes[1]	KEGG Pathway	Genes[1]
Aminoacyl-tRNA biosynthesis	8	Complement and coagulation cascades	9
Cytokine-cytokine receptor interaction	12	Viral myocarditis	8
Melanoma	5	Regulation of actin cytoskeleton	11
Selenoamino acid metabolism	3	Focal adhesion	10
		Antigen processing and presentation	6
		ECM-receptor interaction	6
		Gap junction	6
		Steroid biosynthesis	3
		Cell cycle	7
		Systemic lupus erythematosus	6
		Arrhythmogenic right ventricular cardiomyopathy (ARVC)	5

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

real-time RT-PCR was 0.887 (Figure 2).

Gene Ontology (GO) Analysis and KEGG Pathway of Genes Altered by CBZ in BEAS-2B Cells

We classified the 518 up-regulated and 496 down-regulated genes in BEAS-2B cells exposed to CBZ according to the GO terms of biological process, in order to analyze the molecular mechanisms related to the exposure of CBZ. EASE analysis (http://david.abcc.ncifcrf.gov/) was performed to find the biological processes that were significantly over-represented in these genes in order to identify any biological themes that arise in response to CBZ. The key biological processes that were significantly affected (Fisher exact test, $p < 0.01$) by CBZ are shown in Table 1. We identified 518 up-regulated genes and found 5 biological processes containing at least 2 gene hits in GO Biological Process 4 (BP4) categories. We found differentially down-regulated genes involved in amine biosynthesis, positive regulation of cell proliferation, inflammatory response, biogenic amine metabolism, and cellular amino acid metabolism (Table 1). Further, 496 down-regulated genes were identified, and 18 biological processes containing at least 2 gene hits in GO Biological Process 4 (BP4) categories were found. We found differentially up-regulated genes involved in cholesterol metabolism, the negative regulation of cell proliferation, tissue development, cell morphogenesis, inflammatory response, cell cycle arrest, and mitosis (Table 1).

Up- and down-regulated genes were also classified according to the KEGG pathway system to identify molecular mechanisms related CBZ exposure. In the KEGG pathway analysis, aminoacyl-tRNA biosynthesis, cytokine-cytokine receptor interaction, melanoma, and selenoamino acid metabolism were positively associated with the up-regulated genes (Table 1). Further, the regulation of actin cytoskeleton, focal adhesion, ECM-receptor interactions, gap junctions, steroid biosynthesis, and cell cycle were positively associated with down-regulated genes (Table 1).

Among these biological functions identified using

Table 2. Key functions of genes in carbamazepine-treated BEAS-2B cells.

GenBank Accession No.	Gene symbol	Gene name	Mean intensity (Cy5/Cy3)
Cholesterol metabolic process			
NM_198336	*INSIG1*	Insulin induced gene 1	0.26
BX648281	*LDLR*	Low density lipoprotein receptor (familial hypercholesterolemia)	0.26
AK124635	*PCSK9*	Proprotein convertase subtilisin/kexin type 9	0.38
BC051385	*SREBF2*	Sterol regulatory element binding transcription factor 2	0.49
AK096736	*CLN8*	Chromosome 8 open reading frame 61	0.51
BC000054	*DHCR7*	7-dehydrocholesterol reductase	0.54
NM_014762	*DHCR24*	24-dehydrocholesterol reductase	0.55
NM_033261	*IDI2*	Isopentenyl-diphosphate delta isomerase 2	0.58
NM_002130	*HMGCS1*	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	0.59
NM_145343	*APOL1*	Apolipoprotein L, 1	0.60
NM_002957	*RXRA*	Retinoid X receptor, alpha	0.61
BC035638	*LSS*	Lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	0.62
AF111801	*TRERF1*	Transcriptional regulating factor 1	0.64
Negative regulation of cell proliferation			
AK091170	*CDKN2C*	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	0.50
AK021874	*TGFB2*	Transforming growth factor, beta 2	0.51
NM_003238	*TGFB2*	Transforming growth factor, beta 2	0.51
NM_000165	*GJA1*	Gap junction protein, alpha 1, 43 kDa (connexin 43)	0.52
NM_001753	*CAV1*	Caveolin 1, caveolae protein, 22 kDa	0.54
NM_014762	*DHCR24*	24-dehydrocholesterol reductase	0.55
AY033611	*PLIF*	Homo sapiens placenta immunoregulatory factor PLIF mRNA, complete cds.	0.56
NM_078487	*CDKN2B*	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	0.58
AB208813	*RBM5*	RNA binding motif protein 5	0.60
BC003684	*CXADR*	Coxsackie virus and adenovirus receptor	0.61
NM_002957	*RXRA*	Retinoid X receptor, alpha	0.61
Z29093	*DDR1*	Discoidin domain receptor family, member 1	0.61
NM_058197	*CDKN2A*	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	0.62
L07594	*TGFBR3*	Transforming growth factor, beta receptor III (betaglycan, 300 kDa)	0.63
AB209416	*ILK*	Integrin-linked kinase-2	0.65
NM_003255	*TIMP2*	TIMP metallopeptidase inhibitor 2	0.65
BC067842	*CDKN1C*	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	0.65
NM_004585	*RARRES3*	Retinoic acid receptor responder (tazarotene induced) 3	0.65
NM_003641	*IFITM1*	Interferon induced transmembrane protein 1 (9-27)	0.65
NM_001005333	*MAGED1*	Melanoma antigen family D, 1	0.66
Positive regulation of cell proliferation			
CR593609	*PBEF1*	Pre-B-cell colony enhancing factor 1	1.51
M92424	*MDM2*	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)	1.52
BX647657	*PRKCQ*	Protein kinase C, theta	1.58
CR749236	*FOXP2*	Forkhead box P2	1.60
NM_002006	*FGF2*	Fibroblast growth factor 2 (basic)	1.61
NM_002253	*KDR*	Kinase insert domain receptor (a type III receptor tyrosine kinase)	1.63
AB209032	*ATF3*	Activating transcription factor 3	1.68
BC025765	*PLEKHK1*	Pleckstrin homology domain containing, family K member 1	1.70
NM_001058	*TACR1*	Tachykinin receptor 1	1.72
CR614398	*ODC1*	Ornithine decarboxylase 1	1.74
NM_006889	*CD86*	CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)	1.76
NM_000641	*IL11*	Interleukin 11	1.79
NM_002009	*FGF7*	Galactokinase 2	1.89
NM_206956	*PRAME*	Preferentially expressed antigen in melanoma	1.96
NM_000600	*IL6*	Interleukin 6 (interferon, beta 2)	2.98
NM_001759	*CCND2*	Cyclin D2	3.03

Table 2. Continued.

GenBank Accession No.	Gene symbol	Gene name	Mean intensity (Cy5/Cy3)
Cell cycle			
NM_006306	*SMC1L1*	SMC1 structural maintenance of chromosomes 1-like 1 (yeast)	0.49
AK091170	*CDKN2C*	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	0.50
NM_003238	*TGFB2*	Transforming growth factor, beta 2	0.51
NM_078487	*CDKN2B*	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	0.58
NM_058197	*CDKN2A*	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	0.62
NM_006739	*MCM5*	MCM5 minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)	0.64
BC067842	*CDKN1C*	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	0.65

KEGG pathway analysis, we found several key functions related to CBZ-responsive genes, such as cholesterol metabolism, cell proliferation, and cell cycle control. Table 2 shows the genes involved in biological processes related to the CBZ response in BEAS-2B cells.

Discussion

The clinical use of CBZ is limited due to its pulmonary toxicity. Classifying the gene alterations, analyzing the gene expression patterns, and understanding the mechanism associated with CBZ-induced toxicity should enable earlier identification of clinically relevant toxicological findings in compound screenings; it should also aid the development of therapeutics to reduce pulmonary toxicity. The aim of this study was to identify genes involved in the biological process and to elucidate the cellular response to CBZ in mammalian cells by using toxicogenomic technology[14]. By using microarrays in conjunction with statistical analyses, we identified a number of DEGs associated with CBZ-mediated pulmonary toxicity.

It has been reported that CBZ influences the expression of *CYP3A4*, *ABCB1*, *DRD1*, *CYP2C8*, *HLA-B*, *PLA2G4A*, *ABCC2*, *EPHX1*, *NR1I2*, *ESR1*, *CCND1*, *CYP2B6*, *GRIK2*, *NR3C1*, and *SLC5A5*[15-22]. The genetic variants in *ABCC2*, *EPHX1*, *TNF*, and *HSPA1L* that affect susceptibility to CBZ can be used to predict maintenance doses of CBZ[21,23-25]. CBZ exerts an influence on genes related to the Keap1-Nrf2-ARE signaling pathway, enzymes involved in oxidative-stress response, glutathione transferase, and heat shock proteins[26]. According to the Comparative Toxicogenomics Database (CTD), CBZ will have an effect on lung neoplasm induced by disruption of *CCND1*, *CYP2A6*, *ERBB2*, *ESR1*, *FOS*, *NOS2*, and *TNF* expression.

In this study, we have used this approach to identify the gene expression profiles induced by CBZ in BEAS-2B cells by using a 44K whole human genome microarray. Triplicate assays were performed for each chemical to minimize errors. From the microarray study and the subsequent statistical analysis, 518 genes were identified as up-regulated and 496 as down-regulated by CBZ exposure. We investigated the enrichment of GO annotations in these up- and down-regulated genes. Although we noted several changes in gene expression (Table 1), the focus of this discussion will be on gene expression changes that are associated with cholesterol metabolism, cell proliferation, and cell cycle.

Both *ABCA1* and *ABCG1* (related to cholesterol and phospholipid efflux) play a key role in severe pulmonary abnormalities such as asthma[27,28]. ABCA1-knockout mice exhibit massive lipid accumulation in alveolar macrophages, type II pneumocytes and lung parenchyma, abnormal lung morphology, and shallow breathing[27]. In our data, genes involved in cholesterol metabolism such as *LDLR*, *PCSK9*, and *SREBF2* were down-regulated by CBZ. In addition, CBZ down-regulated *FADS2* and *SCD* genes, which play important roles in lipid metabolism[29]. It is well known that amiodarone (a phospholipidosis-inducing agent) induces the up-regulation of lipid metabolism-related genes[30]. The up-regulation of lipid metabolism-related genes induced lipid accumulation in lysosome, however, the down-regulation of lipid metabolism-related genes didn't induce lipid accumulation[30]. Thus, the down-regulation of lipid metabolism-related genes may be lead to the disruption of lipid accumulation. CBZ and amiodarone are frequently used in combination to treat cardiomyopathies[30].

Pulmonary fibrosis is a neoproliferative disorder of the lung[31]. Genetic alterations, response to growth and inhibitory signals, resistance to apoptosis, myofibroblast origin and behavior, altered cellular communications, and intracellular signaling pathways are all fundamental pathogenic markers for both pulmonary

fibrosis and cancer[31]. Thus, the disruption of cell proliferation and cell cycle phase may contribute to pulmonary toxicity. From our data, mitosis-related genes (*SPAG5*, *CCNG2*, *TUBB*, *ASPM*, *CDCA3*, *MGC8685*, *CNAP1*, *HGF*, *SMC1L1*, and *CDCA2*) were down-regulated by CBZ. CBZ induces mitotic arrest and apoptosis arising from defects in spindle formation related to cell cycle arrest[32]. In our previous study, CBZ slightly increased early apoptosis and ROS generation[32]. CBZ causes oxidative stress by increasing ROS production and lipid peroxidation, which was induced by the production of CYP3A4 that resulted in mitochondrial toxicity[32]. From these data, we can infer that CBZ may induce pulmonary toxicity through the disruption of cholesterol metabolism, cell proliferation, and cell cycle.

It can be concluded that toxicogenomic analysis using an oligonucleotide microarray is an efficient technology for evaluating gene regulation that is affected by toxic exposure and that this approach also offers the possibility of identifying molecular markers. Furthermore, GO analysis has been shown to be efficient for predicting the mechanisms associated with DEGs in cells or organisms subjected to various environmental pollutants. Our results indicate that changes in gene expression are associated with CBZ-induced toxicity and that these genes could be promising biomarkers for detecting CBZ-induced toxicity.

Materials and Methods

Chemicals and Reagents

Carbamazepine, sodium bicarbonate and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Phosphate buffer saline (PBS), 0.5% trypsin-EDTA, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), sodium pyruvate, penicillin and streptomycin were the products of Gibco™ (Carlsbad, CA, USA). Trizol reagent was produced by Invitrogen (Carlsbad, CA, USA) and RNeasy mini kit and RNase-free DNase set were purchased from Qiagen (Valencia, CA, USA). All other chemicals used were of analytical grade or the highest grade available.

Cell Lines and Culture

A human bronchial cell line, BEAS-2B was purchased from Korean Cell Line Bank (KCLB, Seoul, Korea) was maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C. The culture medium was 90% DMEM supplemented with 10% FBS plus 0.044 M sodium bicarbonate, 10 mM sodium pyruvate, 1% penicillin and streptomycin. The medium was refreshed every two or three days.

Determination of Cell Viability

The MTT assay for measuring cytotoxicity and cell growth was performed following the modifications described by Mosmann. MTT is a tetrazolium salt which can be metabolized to formation salt by viable cells and can be quantified spectrophotometrically at 540 nm. For the cytotoxicity assay, BEAS-2B cells plated in 24-well cell culture plates at a seeding density of 3×10^4 cells/mL. After reaching to 80% confluency, cells were exposed to various concentrations of NF in culture medium for 48 h. After treatment, cells were incubated for 3 h with MTT (4 mg/mL, in PBS) at 37˚C. The reaction was stopped by removing the medium and adding DMSO. The absorbance of each sample was measured at 540 nm. The value of untreated sample was regarded as 100% and the 20% inhibitory concentration (IC_{20}) of cell proliferation by CBZ was defined as the concentration that causes 20% reduction in the cell viability versus the untreated control. The IC_{20} values were directly determined from the semi-logarithmic dose-response curves. The MTT assay was carried out having at least in triplicate experiments.

RNA Extraction

Total RNA was extracted from the BEAS-2B cells after 324 μM CBZ 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 44 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

Table 3. Primer sequences.

GenBank Accession No.	Gene	Primer Sequence (5′→3′)	
		F	R
NM_000600	*IL6*	ACTGGTGTTCAAACCCTCACCACT	ATGGGTCTGCGGCATATGGAAACA
NM_000575	*IL1A*	TCTCCACAAGCGCCTTCG	CTCAGGGCTGAGATGCCG
AK095849	*RNU22*	AGAGCTGCGTTGCACTTGTTTACG	AACTTGCTTCCCGTTCTTCAGGGA
NM_003004	*SECTM1*	ACTGGTGTTCAAACCCTCACCACT	ATGGGTCTGCGGCATATGGAAACA
NM_001733	*C1R*	TATTACCACATGGGCAGACTGGCA	GGCCTTGGCCCGTTCATAATTCTT
NM_001734	*C1S*	TGGGCAGAGCCTATTGGGACATAA	ACCCTGGAACGGCTGATGAGTTTA
U81234	*CXCL6*	AACCTGCCTAATGGTGACTTCCGT	TTCTTCCAAATGCCCTGTGCTGTG
BG564326	*SAA4*	ACACCTGAGAAACTGGAGAACGCA	GCCCTTTGAGCCAAGCAGGTTATT
NM_004265	*FADS2*	AGGACGGCTACAGCTACCC	CTCCAGGCAGATGTTCACG
AK124635	*PCSK9*	ACTCGGTGAGCTGTGAAAGGCTAT	ACCCAAGTCATCCTGCTCCTTCAT
NM_005063	*SCD*	AACTTGATACGTCCGTGTGTCCCA	CTGTATGTTTCCGTGGCAATGCGT
BX648281	*LDLR*	AGGACGGCTACAGCTACCC	CTCCAGGCAGATGTTCACG
NM_002046	*GAPDH*	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGA

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, lowless, print-tip, and scaled normalization methods. Obtained data were represented to volcano plot of genes that behaved similarly across the chlordane treatment using GeneSpring GX 7.3.1 software. We utilized an algorithm based on the Pearson's correlation to separate genes exhibiting similar patterns.

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.

Quantitative Real-time PCR

Messenger RNA expression levels for the genes of interest were analyzed via quantitative real time reverse transcription polymerase chain reaction (RT-PCR) using a Bio-Rad iCycler system (Bio-Rad, USA). Total RNA was reverse-transcribed into cDNA using an Omniscript RT kit (Qiagen). Primer specificity was tested by running a regular PCR for 40 cycles (95°C for 20 s and 60°C for 1 min). Real time RT-PCR was performed using a SYBR supermix kit (Bio-Rad). Samples were subjected to 45 cycles of 95°C for 20 s and 60°C for 1 min. PCR efficiency was determined by running serial dilutions of template cDNA and melting curve data were collected to assure PCR specificity. Each cDNA sample was analyzed in triplicate and the corresponding no-RT mRNA sample was included as a negative control. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer was included in every plate as an internal loading control. The mRNA level of each sample for each gene was normalized against that of GAPDH mRNA. The relative mRNA level was determined as $2^{[(Ct/GAPDH\text{-}Ct/gene\ of\ interest)]}$. All data were presented as the mean ± standard deviation (SD) of three separate experiments. The primers used for the quantitative real time RT-PCR are listed in Table 3.

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References

1. Archibald, N. *et al*. Carbamazepine-induced interstitial pneumonitis in a lung transplant patient. *Respir. Med.* **100**, 1660-1662 (2006).
2. Wilschut, F. A. *et al*. Recurrent respiratory distress associated with carbamazepine overdose. *Eur. Respir. J.* **10**, 2163-2165 (1997).

3. Ozkan, M., Dweik, R. A. & Ahmad, M. Drug-induced lung disease. *Cleve. Clin. J. Med.* **68**, 782-785, 789-795 (2001).
4. Zhang, W., Ding, Y., Boyd, S. A., Teppen, B. J. & Li, H. Sorption and desorption of carbamazepine from water by smectite clays. *Chemosphere.* **81**, 954-960 (2010).
5. Thacker, P. D. Pharmaceutical data elude researchers. *Environ. Sci. Technol.* **39**, 193A-194A (2005).
6. Lienert, J., Güdel, K. & Escher, B. I. Screening method for ecotoxicological hazard assessment of 42 pharmaceuticals considering human metabolism and excretory routes. *Environ. Sci. Technol.* **41**, 4471-4478 (2007).
7. Zhang, Y., Geissen, S. U. & Gal, C. Carbamazepine and diclofenac: removal in wastewater treatment plants and occurrence in water bodies. *Chemosphere.* **73**, 1151-1161 (2008).
8. Carballa, M., Omil, F. & Lema, J. M. Comparison of predicted and measured concentrations of selected pharmaceuticals, fragrances and hormones in Spanish sewage. *Chemosphere.* **72**, 1118-1123 (2008).
9. Sim, W. J., Lee, J. W. & Oh, J. E. Occurrence and fate of pharmaceuticals in wastewater treatment plants and rivers in Korea. *Environ. Pollut.* **158**, 1938-1947 (2010).
10. Tixier, C., Singer, H. P., Oellers, S. & Müller, S. R. Occurrence and fate of carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, and naproxen in surface waters. *Environ. Sci. Technol.* **37**, 1061-1068 (2003).
11. Löffler, D., Römbke, J., Meller, M. & Ternes, T. A. Environmental fate of pharmaceuticals in water/sediment systems. *Environ. Sci. Technol.* **39**, 5209-5218 (2005).
12. Chenxi, W., Spongberg, A. L. & Witter, J. D. Determination of the persistence of pharmaceuticals in biosolids using liquid-chromatography tandem mass spectrometry. *Chemosphere.* **73**, 511-518 (2008).
13. Ferrari, B., Paxéus, N., Lo Giudice, R., Pollio, A. & Garric, J. Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac. *Ecotoxicol. Environ. Saf.* **55**, 359-370 (2003).
14. Yeo, M. K. & Kim, H. E. Gene expression in zebrafish embryos following exposure to TiO_2 nanoparticles. *Mol. Cell. Toxicol.* **6**, 97-104 (2010).
15. Oscarson, M. *et al.* Transcriptional profiling of genes induced in the livers of patients treated with carbamazepine. *Clin. Pharmacol. Ther.* **80**, 440-456 (2006).
16. Seo, T. *et al.* ABCB1 polymorphisms influence the response to antiepileptic drugs in Japanese epilepsy patients. *Pharmacogenomics.* **7**, 551-561 (2006).
17. Mann, L. *et al.* Inhibition of specific adenylyl cyclase isoforms by lithium and carbamazepine, but not valproate, may be related to their antidepressant effect. *Bipolar Disord.* **11**, 885-896 (2009).
18. Oscarson, M. *et al.* Transcriptional profiling of genes induced in the livers of patients treated with carbamazepine. *Clin. Pharmacol. Ther.* **80**, 440-456 (2006).
19. Hung, S. I. *et al.* Genetic susceptibility to carbamazepine-induced cutaneous adverse drug reactions. *Pharmacogenet. Genomics.* **16**, 297-306 (2006).
20. Ghelardoni, S., Tomita, Y. A., Bell, J. M., Rapoport, S. I. & Bosetti, F. Chronic carbamazepine selectively downregulates cytosolic phospholipase A2 expression and cyclooxygenase activity in rat brain. *Biol. Psychiatry.* **56**, 248-254 (2004).
21. Kim, W. J. *et al.* A nonsynonymous variation in MRP2/ABCC2 is associated with neurological adverse drug reactions of carbamazepine in patients with epilepsy. *Pharmacogenet. Genomics.* **20**, 249-256 (2010).
22. Meng, Q. W. *et al.* Inhibitory effect of carbamazepine on proliferation of estrogen-dependent breast cancer cells. *Ai Zheng.* **25**, 967-973 (2006).
23. Makmor-Bakry, M. *et al.* Genetic variants in microsomal epoxide hydrolase influence carbamazepine dosing. *Clin. Neuropharmacol.* **32**, 205-212 (2009).
24. Pirmohamed, M., Lin, K., Chadwick, D. & Park, B. K. TNFalpha promoter region gene polymorphisms in carbamazepine-hypersensitive patients. *Neurology.* **56**, 890-896 (2001).
25. Alfirevic, A. *et al.* Serious carbamazepine-induced hypersensitivity reactions associated with the HSP70 gene cluster. *Pharmacogenet. Genomics.* **16**, 287-296 (2006).
26. Lu, W., Li, X. & Uetrecht, J. P. Changes in gene expression induced by carbamazepine and phenytoin: testing the danger hypothesis. *J. Immunotoxicol.* **5**, 107-113 (2008).
27. Delvecchio, C. J., Bilan, P., Nair, P. & Capone, J. P. LXR-induced reverse cholesterol transport in human airway smooth muscle is mediated exclusively by ABCA1. *Am. J. Physiol. Lung Cell Mol. Physiol.* **295**, L949-957 (2008).
28. Pie, J. E. *et al.* Correlation between nutrition intake and gene expression profiles in children with asthma. *Mol. Cell. Toxicol.* **6**, 313-319 (2010).
29. Song, M., Kim, Y. J. & Ryu, J. C. Phospholipidosis induced by PPARγ signaling in human bronchial epithelial (BEAS-2B) cells exposed to amiodarone. *Toxicol. Sci.* **120**, 98-108 (2011).
30. Leite, S. A., Leite, P. J., Rocha, G. A., Routledge, P. A. & Bittencourt, P. R. Carbamazepine kinetics in cardiac patients before and during amiodarone. *Arq. Neuropsiquiatr.* **52**, 210-215 (1994).
31. Vancheri, C., Failla, M., Crimi, N. & Raghu, G. Idiopathic pulmonary fibrosis: a disease with similarities and links to cancer biology. *Eur. Respir. J.* **35**, 496-504 (2010).
32. Song, M., Kim, Y. J. & Ryu, J. C. Induction of ROS, p38 MAP Kinase and apoptosis via pulmonary toxic drugs. *BioChip J.* **3**, 306-315 (2009).