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Induction of ROS, p38 MAP Kinase and Apoptosis via Pulmonary Toxic Drugs

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Abstract

Some drugs may be limited in their clinical application due to their propensity towards their adverse effects. Among these drugs, some clinical chemotherapeutic agents with pulmonary toxic effects were subjected in this study. Moreover, a new paradigm in toxicity screening, toxicogenomic technology represents a useful approach for evaluating the toxic properties of new drug candidates early in the drug discovery process. In this respect, we identified functional mechanisms through analysis of biological process for gene alteration in BEAS-2B cells, human bronchial epithelial cell line, exposed to four drugs-methotrexate (MTX), nitrofurantoin (NF), amiodarone (AM), and carbamazepine (CBZ)-induced pulmonary toxicity, by using human oligonucleotide chip. In this study, we confirmed that pulmonary toxicity-related common mechanisms were apoptosis, cell cycle process, cell development and cell differentiation. Out of common functions, we showed that the treatment with MTX, NF, AM and CBZ resulted in the induction of apoptosis, the increase of ROS generation and the activation of p38 MAPK. Thus, we provide a clue for pulmonary toxic mechanism of these chemotherapeutic agents.

Keywords: Pulmonary toxicity, ROS, Apoptosis, p38 MAPK, Drug

Introduction

Only 19 drugs as having the potential to cause pulmonary disease were identified in 1972, but more than 150 agents were recognized in 2001 and the list of drug-induced pulmonary toxicity will undoubtedly continue to grow as new drugs are developed[1]. So, to decrease the potential adverse effects from drug-induced pulmonary diseases, it is important understanding of mechanisms for drug-induced pulmonary toxicity.

Drug-induced pulmonary toxicity may be due to several mechanisms. Oxidant molecules such as oxygen and hydrogen peroxide which may induce redox reactions resulting in the oxidation of fatty acid in cell membrane play a key role in drug-induced pulmonary toxicity[2]. The pulmonary vascular damage is induced by the increase of permeability resulting from the activation of inflammatory and immune responses in vascular endothelium[2]. Amphiphilic compounds such as amiodarone (AM) may cause deposition of phospholipids because of the inhibition of phospholipase A within lysosomes in cells[1]. Drugs can play a role in antigens or haptens causing immune responses which can lead to pulmonary edema, interstinal lung disease and lupus erythematosus[2]. DNA strand breakage causing chromosomal injury is a direct toxic effect inducing lung diseases such as pulmonary fibrosis[2].

Bleomycin-induced lung diseases appear 6 to 12 weeks after beginning therapy and occur in 3-5%[3]. Mitomycin may cause pulmonary toxicity in 3-7% of patients[3]. Long-term use of busulfan causes pulmonary fibrosis in approximately 5% of patients[3]. Cyclophosphamide-induced pulmonary fibrosis occurs in less than 1% after a dose of as little as 150 mg in patients[3]. The incidence of methotrexate (MTX)-induced pulmonary hypersensitivity reactions is approximately 2-5% for low-dose prescription as patients with rheumatoid arthritis[2]. The rate of death from AM-induced pulmonary disease is 10% of cases[2]. The respiratory damage by AM generally occurs months after initiation of therapy in patients taking dosages greater than 400 mg/d[3]. Angiotensin-converting enzyme (ACE) inhibitors develop a chronic non-productive cough in up to 44% of patients after months of therapy[3]. Nitrofurantoin (NF) administered chronically for more than 6 months induces pulmonary fibrosis which is a typical side effect of NF treatment reported in 1973[4].

In this study, we used MTX, NF, AM and carbamazepine (CBZ) as representative drugs inducing pulmonary toxicity. These drugs have been related to the induction of apoptosis with intracellular oxidation or oxidative stress[5-9]. MTX treatment is associated with a number of adverse reactions, including pneumonitis and pulmonary fibrosis[10]. MTX-induced pneumonitis stimulates the release of IL-8, MCP-1, G-CSF and GM-CSF in airway epithelial (A549) cells[11]. Lung

provides an enormous surface area of about 140 m^2 to air is vulnerable to oxidative damage by a lot of toxicants. The toxicity of NF is mediated through superoxide and its secondary metabolites H_2O_2 that reacts with lysosomal Fe^{2+} to form reactive oxygen species (ROS)[6]. The presence of NADPH and microsomes catalyzes a one-electron reduction of the nitro group of NF to produce a nitro free radical (R-NO_2-) that spontaneously reacts with oxygen to regenerate the original nitro compound and reduces oxygen to O_2^{-}[6]. ROS formation in turn causes lysosomal lipid peroxidation, membrane disruption and cell death[6]. AM induces steatosis and non-alcoholic steatohepatitis due to mitochondrial dysfunction involving in the synthesis of mitochondrial β-oxidation of fatty acids in liver[7]. The generation of ROS by the damaged respiratory chain reaction induces lipid peroxidation. ROS and lipid peroxidation can lead to apoptosis or necorsis[7]. Long-term use of CBZ has been suspected to be an immune-mediated hypersensitivity. CBZ induces interstitial pneumonia, pulmonary fibrosis and pulmonary infiltration with eosinophilia[12,13]. CBZ induces mitotic arrest and apoptosis from defects of spindle related with cell cycle arrest[8]. And CBZ causes oxidative stress by the increase of ROS production and lipid peroxidation which was induced by the production of CYP3A4 resulted in mitochondrial toxicity[9].

The generation of ROS leads to production of proinflammatory cytokines and chemokines and interacts with DNA causing DNA damage such as point mutation and rearrangements[14]. Drug-induced pulmonary fibrosis may involve release of free oxygen radicals and various cytokines, for example, IL-Iβ, TNF-α, TNF-β and TGF-β[6]. Also, drug-induced pulmonary fibrosis includes inhibition of the phospholipases of macrophages and lymphocytes with the resultant accumulation of phospholipids and reduction of the immune system[15].

The objective of this study is the identification of functional mechanisms through analysis of biological process out of gene ontology (GO) in gene alteration by four drugs (MTX, NF, AM and CBZ). We subjected a toxicogenomic approach to identify potential mechanisms for pulmonary toxicity in human bronchial epithelial cell line.

Results and Discussion

Cytotoxicity in BEAS-2B Cells

Relative survival of BEAS-2B cells following exposure to a range of concentrations of pulmonary toxicity induced-drugs (MTX, AM, NF and CBZ) was determined by MTT assay. The survival percentage relative to solvent control (DMSO) was determined as a percentage of optical density value measured after treatment. Based on the results of MTT assay, the 20% cell viability inhibitory concentration (IC_{20}) of each compound was calculated. Dose dependent cell viability curves were obtained after 48 h of exposure to pulmonary toxicants in BEAS-2B cells as shown in Figure 1. The IC_{20} value determined as 0.144 μM, 29.388 μM, 89.450 μM, and 324.313 μM for MTX, AM, NF and CBZ, respectively.

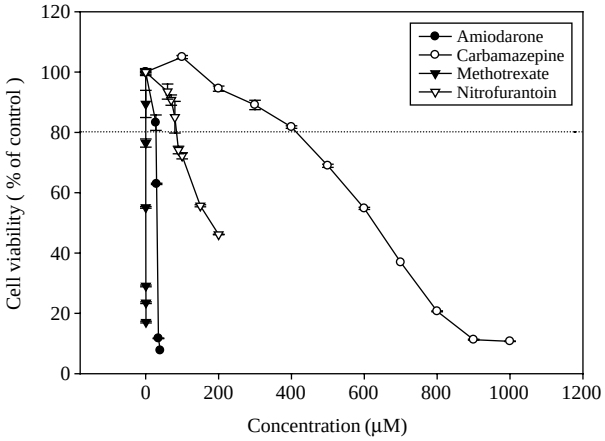

Figure 1. Cell viability by MTT assay. BEAS-2B cells were treated for 48 h with methotrexate, amiodarone, carbamazepine and nitrofurantoin, and the dose-response curve was plotted.

The Comparison of Functional Classification of MTX, NF, AM and CBZ

Understanding of lung injury-related mechanisms using gene alteration induced by MTX, NF, AM and CBZ will aid in the development of therapeutics to reduce pulmonary toxicity. As shown in Table 1, there were enriched GO categories of biological process in MTX, NF, AM and CBZ, respectively. The most prominent annotated terms from biological process by MTX included cellular metabolic process, biological regulation, nucleic acid metabolic process, cell differentiation, cell development or cell cycle process. The biological process terms such as biological regulation, cell communication, signal transduction, cell differentiation, cell development or apoptosis appeared in NF. The biological process terms like localization, transport, cell differentiation, cell cycle, lipid metabolic process, apoptosis or inflammation response represented in AM. And the prominent biological process terms by CBZ involved in cell differentiation, cell development, response to stress, apoptosis, cell cycle or organic acid metabolic process.

There were 4 common biological processes in MTX,

Table 1. GO annotations for methotrexate, nitrofurantoin, amiodarone and carbamazepine-induced genes.

	UP		DOWN	
	GO-Biological Process	Genes[1]	GO-Biological Process	Genes[1]
Methotrexate	Cellular metabolic process	507	Cellular metabolic process	386
	Biological regulation	381	Gene expression	196
	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	293	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	179
	Gene expression	257	Biosynthetic process	99
	Developmental process	224	Translation	67
	RNA metabolic process	223	Cellular component assembly	49
	Transcription	206	DNA metabolic process	48
	Cell differentiation	132	Cell cycle process	42
	Protein modification process	122	RNA processing	34
	Cell development	96	Ribonucleoprotein complex biogenesis and assembly	26
Nitrofurantoin	Biological regulation	243	Biological regulation	585
	Developmental process	167	Cell communication	441
	Cell differentiation	108	Developmental process	414
	System development	94	Multicellular organismal process	410
	Cell development	75	Signal transduction	403
	Response to stress	73	Localization	310
	Organ development	71	System development	254
	Apoptosis	54	Transcription, DNA-dependent	247
	Cellular localization	52	RNA biosynthetic process	247
	Response to chemical stimulus	46	Cell differentiation	235
Amiodarone	Developmental process	72	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	61
	Localization	69	Organelle organization and biogenesis	50
	Transport	56	Cell cycle	40
	Cell differentiation	47	Mitotic cell cycle	29
	Lipid metabolic process	34	Chromosome organization and biogenesis	28
	Cell development	30	M phase	27
	Response to stress	27	Cellular component assembly	23
	Apoptosis	24	Cellular localization	23
	Regulation of cell proliferation	16	Cell division	21
	Inflammatory response	12	DNA packaging	20
Carbamazepine	Multicellular organismal development	62	Developmental process	96
	System development	48	Multicellular organismal development	74
	Biosynthetic process	43	Anatomical structure development	72
	Organ development	38	System development	60
	Cellular biosynthetic process	35	Cell differentiation	58
	Response to external stimulus	31	Cell development	44
	Response to stress	31	Organ development	43
	Nitrogen compound metabolic process	26	Response to stress	37
	Carboxylic acid metabolic process	25	Apoptosis	28
	Organic acid metabolic process	25	Cell cycle	28

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

NF, AM and CBZ, which were apoptosis, cell cycle process, cell development and cell differentiation (Figure 2). The impairment of the barrier function of pulmonary endothelium and epithelium leading to pulmonary edema induces apoptosis of endothelial and epithelial cells[16]. Bleomycin causes apoptosis associated with p53-dependent response in the lung[17]. AM induces apoptosis through the activation of specific intracellular death-related pathways including the bax-dependent caspase-3 activation in human lung epithelial cells[18]. In this study, we focused on apoptosis as a mechanism related with drug-induced pulmonary toxicity. In microarray data, four drugs changed the expression of apoptosis-related genes. Although there were the same matching biological processes among four groups, the involved genes were different (Figure 3).

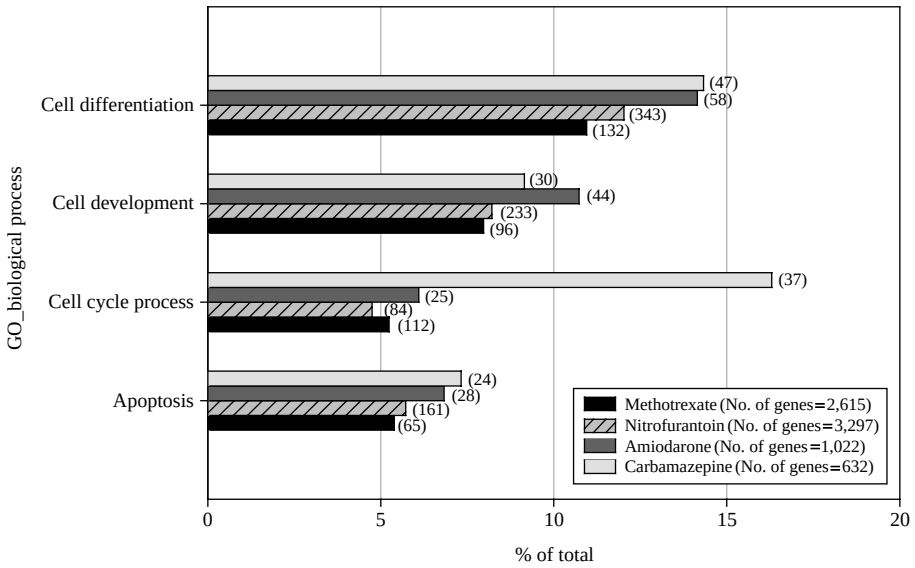

Figure 2. Common gene ontology (GO) mapping of differentially expressed gene by four drugs (methotrexate, nitrofuratoin, amiodarone and carbamazepine).

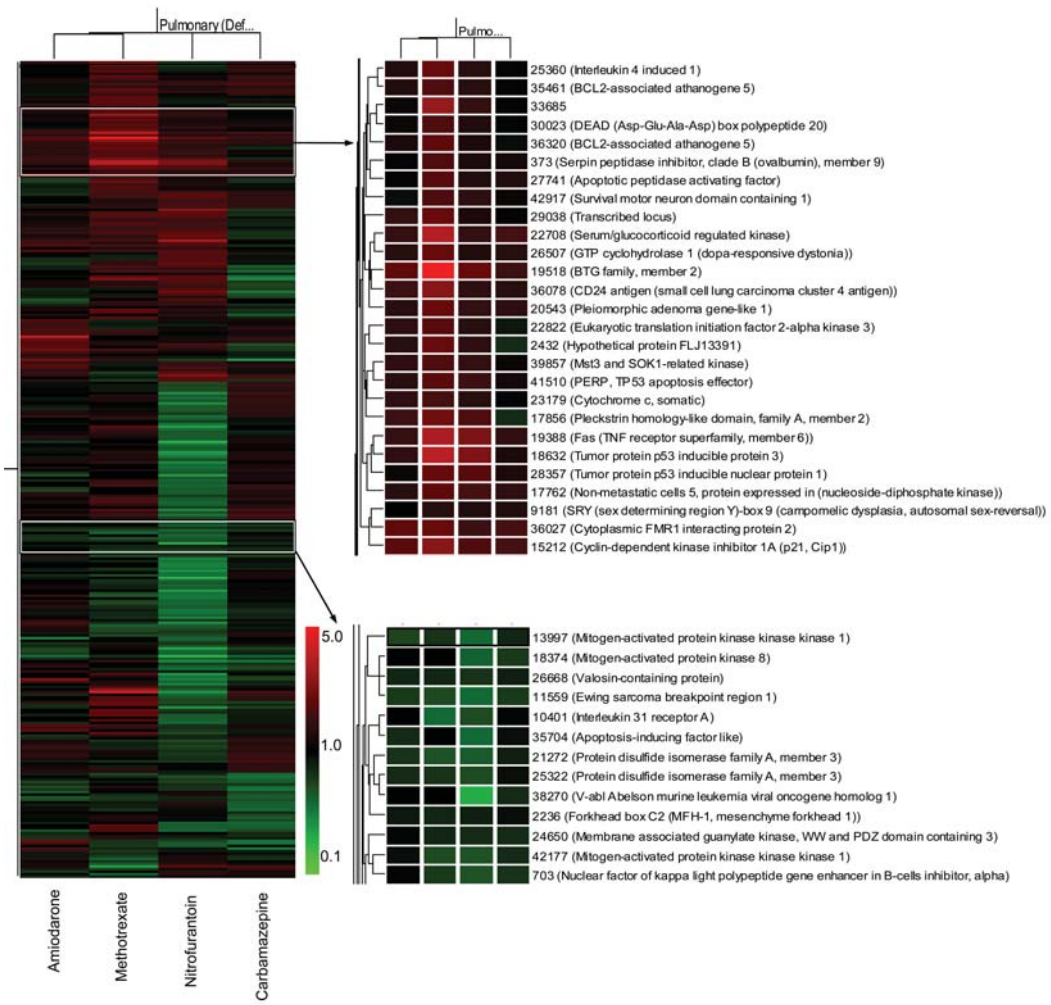

Figure 3. Apoptosis-related gene expression patterns in methotrexate, nitrofuranotin, amiodarone and carbamazepine. Hierarchical clustering was performed using a smooth correlation coefficient.

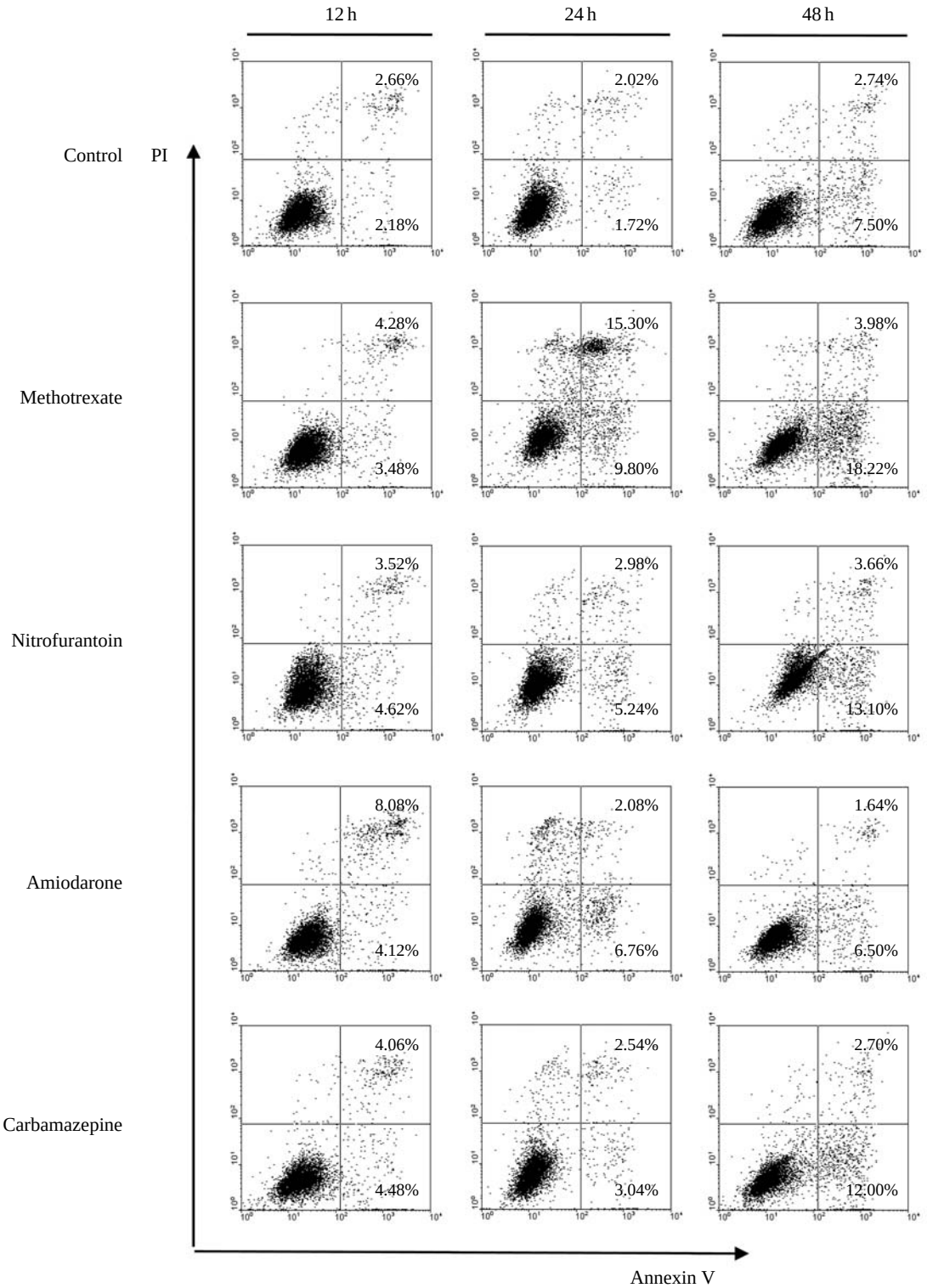

Figure 4. The effect of methotrexate, nitrofurantoin, amiodarone and carbamazepine on either early or late stage of apoptosis in BEAS-2B as detected by flow cytometry. After 12 h, 24 h and 48 h, the cells were harvested, stained with Annexin V-FITC and propidium iodide, and analysed by flow cytometry. Data are expressed as % of Annexin V-FITC and PI-negative cells (early state of apoptosis) and as % of Annexin V-FITC and PI-positive cells (late stage of apoptosis). Diagrams of Annexin V/PI flow cytometry in a representative experiment are presented the graphs. The lower right quadrants represent the cells in the early stage of apoptosis. The upper right quadrants contain the cells in the late stage of apoptosis.

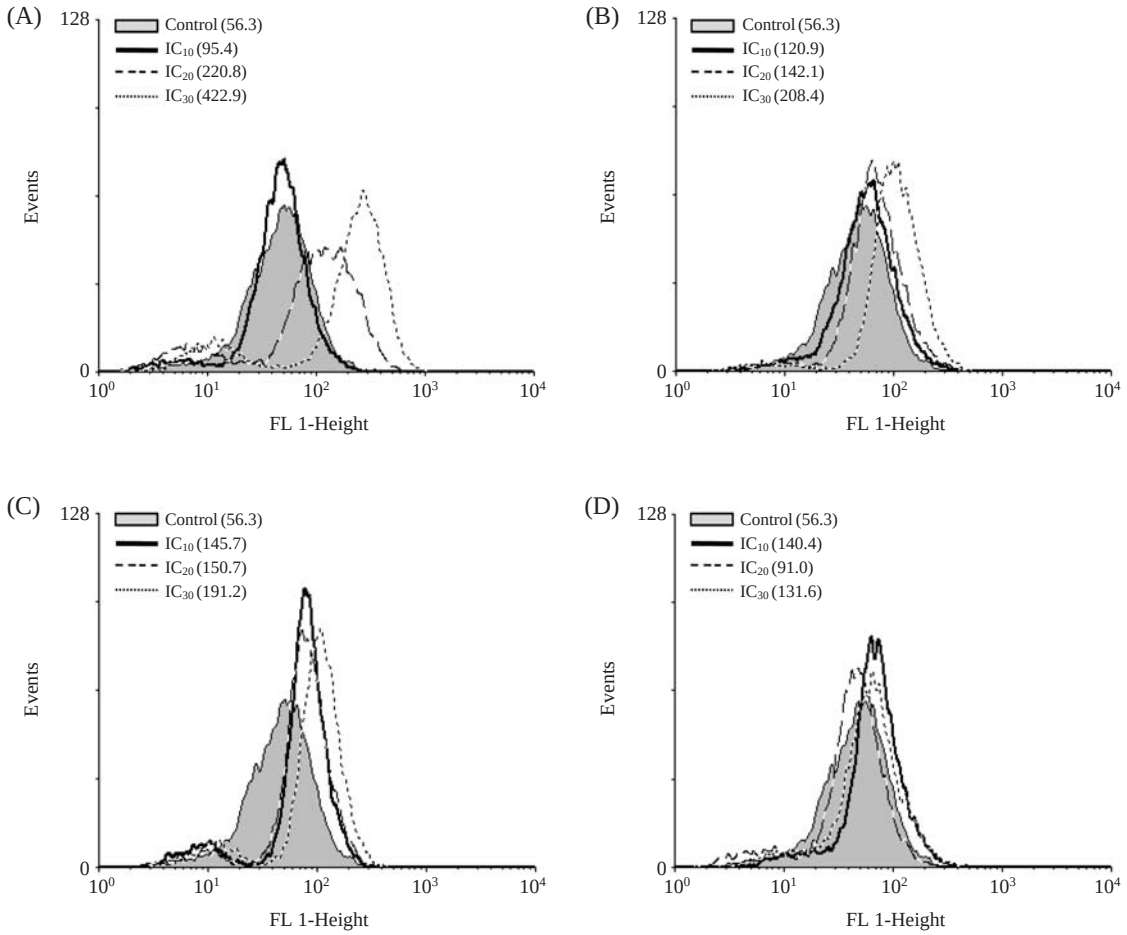

Figure 5. Four drugs-induced ROS generation in BEAS-2B cells. Cells (1×10^6 cells/mL) were treated with methotrexate (A), nitrofurantoin (B), amiodarone (C) and carbamazepine (D) at various dose points (IC_{10}, IC_{20} and IC_{30}) for 48 h. FL 1-Height is fluorescence intensity in log scale. Numbers within parenthesis indicate representative of mean fluorescent intensity.

The Induction of ROS Generation, p38 MAPK and Apoptosis by 4 Drugs

As shown in Figure 4, MTX significantly influenced the proportions of early apoptotic cells which represent 3.48%, 9.80% and 18.22% in 12 h, 24 h, and 48 h respectively. MTX dramatically induced late apoptosis at 24 h, from 2.02% to 15.30%. When expose to NF, the number of early apoptotic cells increased 4.62%, 5.24% and 13.10% at 12 h, 24 h and 48 h. The treatment of AM increased the number of early apoptotic cells which was 4.12%, 6.76% and 6.50% at 12 h to 48 h. CBZ had a minor influence on apoptosis at 12 h and increased the number of early apoptotic cells which was 12.00% at 48 h. Therefore, MTX significantly influenced early and late apoptosis and NF, AM and CBZ slightly increased early apoptosis.

Apoptosis is presented as early features in pulmonary fibrosis[19]. Apoptosis in bronchial and alveolar epithelial cells may be regulated by the Fas-FasL pathway, TGF-β, p53 and p21. TGF-β induces idiopathic pulmonary fibrosis[20]. Oxidative stress and mitochondrial damage may also play a key role in pulmonary fibrosis affecting apoptosis[20]. Oxidative stress appears in damaged lung epithelial cells representing idiopathic interstitial pneumonia[20]. NADPH oxidase-produced ROS contributes to apoptosis in human dermal microvascular and umbilical vein endothelial cells[16]. So, we identified whether four drugs induce ROS generation or not in a dose-dependent manner. Figure 5 showed that the treatment of BEAS-2B cells with MTX, NF and AM in IC_{10} to IC_{30} induced the increase of ROS production in a dose-dependent manner. And CBZ slightly affected the generation of ROS in IC_{10} and IC_{30}, not dose dependent.

The generation of ROS induces membrane lipid peroxidation[21]. The lipid peroxidation of cell membrane may lead to necrosis through the increase of membrane permeability[22]. The depletion of glutathione

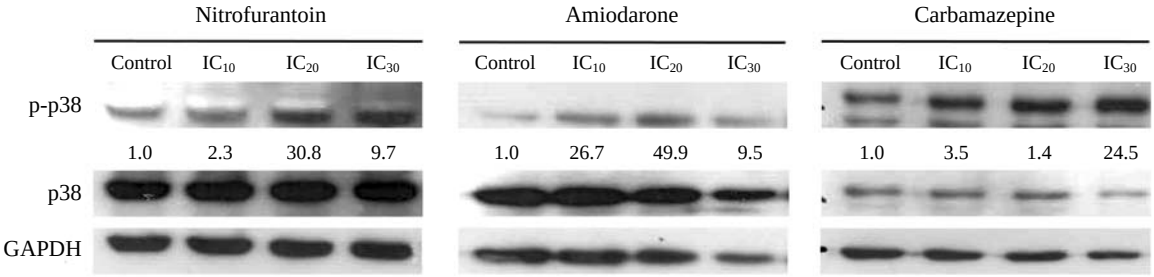

Figure 6. Effect of nitrofurantoin, amiodarone and carbamazepine on protein phosphorylation in the p38 MAPK signaling cascade. Cells were exposed to nitrofurantoin, amiodarone and carbamazepine for 48 h and then subjected to western blotting using the following primary antibodies: anti-phospho-p38 MAPK (Thr180/Tyr18), anti-p38 MAPK. phosphorylated proteins were quantified using GelScope 1.5.

(GSH) levels which control intracellular ROS levels induces apoptosis[21,23]. It was reported that MTX, NF, AM and CBZ caused a significant decrease in GSH levels[24-27]. Thus, the intensity of ROS production and GSH levels have an influence on the induction of apoptosis and the type of cell death[21].

The generation of ROS also has an influence on apoptosis by activation of caspase and MAPKs cascades and disrupting the mitochondrial membrane potential[22]. The activation of caspase 3 by oxidative stress such as SO in both the intrinsic and extrinsic pathways plays a pivotal role in the occurrence of apoptosis[23]. In MAPKs signaling, ERK is involved in cellular responses such as cell proliferation and survival, whereas JNK and p38 are related to proapoptotic events[23]. So, in this study, we examined whether four drugs induce p38 MAPK in a dose-dependent manner. It was reported that MTX induced the phosphorylation of p38 MAPK[28]. And we confirmed that p38 MAPK was activated by NF, AM and CBZ (Figure 6). Pulmonary fibrosis is caused by the modulation of ROS through MAPKs signaling pathway[19].

From this study, we can propose a mechanism that apoptosis for drug-induced pulmonary toxicity is associated with induction of ROS generation and the activation of p38 MAPK.

Conclusions

The clinical use of MTX, AM, CBZ and NF is limited due to its pulmonary toxicity. In this study, we identified that pulmonary toxicity-related common mechanisms were apoptosis, cell cycle process, cell development and cell differentiation through microarray analysis. Out of common functions, we showed that the treatment with MTX, NF, AM and CBZ resulted in the induction of apoptosis, the increase of ROS generation and the activation of p38 MAPK. Thus, we provide a clue for pulmonary toxic mechanism of these chemotherapeutic agents.

Materials and Methods

Materials

MTX, NF, AM, CBZ, sodium bicarbonate and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), DMSO and dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma (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^{TM} (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

The human bronchial epithelial cell line, BEAS-2B cells, was purchased from Korean Cell Line Bank (KCLB, Korea) and was maintained under a humidified atmosphere of 5% CO_2 and 95% air at 37˚C. The culture medium was 90% DMEM (GIBCO^{TM}) supplemented with 10% FBS (GIBCO^{TM}), sodium bicarbonate (Sigma), sodium pyruvate (GIBCO^{TM}) and penicillin and streptomycin (GIBCO^{TM}). The medium was refreshed every 2 to 3 days.

Determination of Cell Viability

To determine the cytotoxicity and effects on cell growth, the MTT cell proliferation assay was performed using the modifications described by Mosmann[29]. MTT is a tetrazolium salt that is metabolized by living cells to form an insoluble purple salt, which can be quantified spectrophotometrically at 540 nm. In the cytotoxicity assay, BEAS-2B cells were seeded in 24-

well cell culture plates (BD Falcon™, USA) at a density of 3×10^4 cells/mL. After reaching 80% confluence, the cells were exposed to various concentrations of MTX, AM, CBZ and NF (Sigma) for 48 h. After exposure, the cells were incubated for 3 h with 4 mg/mL MTT in PBS at 37˚C. To quench the reaction, the medium was removed and DMSO (Sigma) was added. The absorbance of each sample was measured at 540 nm. Untreated samples were used as the negative control (100% viable). The 20% inhibitory concentration (IC_{20}) for cell proliferation was defined as the MTX, AM, CBZ and NF concentration that reduced cell viability by 20% compared with the untreated control. The IC_{20} values were determined directly from semilogarithmic dose-response curves. The MTT assay was performed in triplicate for each sample.

RNA Extraction

BEAS-2B cells were seeded in a 100 mm dish at a density of 1.24×10^6 cells/mL. After incubation for 24 h at 37˚C, the cells were treated with 0.144 μM MTX, 29.388 μM AM, 324.313 μM, CBZ and 89.450 μM NF for 48 h.

Oligonucleotide Microarray Hybridization

Gene expression analysis was conducted on the RNA samples using human whole 44 k microarray (Agilent Technologies, USA). Labelling and hybridization were followed by the coupling of the Cy3 dye for the controls or 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 3 min at RT, 0.2 × SSC for 2 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) to obtain gene expression ratios.

Data Analysis

The fluorescent intensity of each spot was calculated by local median background subtraction. We used the robust scatter-plot smoother LOWESS function to perform intensity dependent normalization for the gene expression. Scatter plot analysis was made by Microsoft Excel 2000 (Microsoft, USA). Significance Analysis of Microarray (SAM) was performed for the selection of the genes with significant gene expression changes[30]. The statistical significance of the differentially expressed of genes was assessed by computing a q-value for each gene. To determine the q-value, a permutation procedure was used, and for each permutation, two-sample *t* statistics were computed for each gene. Genes were differentially expressed when logarithmic gene expression ratios in three independent hybridizations were more than 0.66 or less than −0.66, i.e., 1.5-fold difference in expression level, and when the q-values were < 5.

Functional 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.

Measurement of ROS Production by Flow Cytometry

BEAS-2B cells (1×10^6 cells/mL) suspended in PBS were incubated with 20 μM DCFH-DA for 15 min at 37˚C. Then cells were washed twice or three times and resuspended in PBS at 4˚C. Intracellular ROS generation was analyzed on a FACS Calibur flow cytometer using CellQuest software (Becton-Dickinson, USA).

Analysis of Apoptosis by Flow Cytometry

The *ApoScan*™ Annexin V-FITC apoptosis detection Kit (BioBud, Korea) was used to detect apoptosis by flow cytometry. BEAS-2B cells (1×10^6 cells/mL) were plated onto 60 mm dishes and incubated with MTX, NF, AM and CBZ for 12 h, 24 h or 48 h. BEAS-2B cells were trypsinized, harvested and resuspended (1×10^6 cells/mL) in DMEM. BEAS-2B cells were washed in cold PBS and pelleted by centrifugation at 1,000 × g for 5 min. They were then resuspended in a 1 × binding buffer (500 μL) and incubated with 1.25 μL of Annexin V-FITC (200 μg/mL) at room temperature for 15 min. The cells were resuspended in 500 μL of a 1 × binding buffer and then cell suspensions were stained with 10 μL of PI (30 μg/mL) at 4˚C in the dark. The fluorescence was determined using a FACScan flow cytometer (Becton-Dickinson). A computer system (CellQuest Pro.) was used for data acquisition and analysis. Data for 5,000 events were stored.

Cell Lysates and Western Blotting

BEAS-2B cells of each treatment group were washed in PBS and lysed via gentle agitation in ice-cold lysis buffer [20 mM Tris buffer (pH 8.0), 137 mM NaCl, 2 mM EDTA, 10% (v/v) NP-40] containing freshly added protease inhibitor tablets (Roche Applied Science, Germany) and phosphatase inhibitor (Sigma). Lysates were clarified by centrifugation at 12,000 rpm for 10 min at 4˚C. Total protein in the supernatant was quan-

tified using the BCA assay (Pierce, Germany), which was performed in triplicate for each sample.

Proteins were separated via SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 5% bovine albumin serum (Sigma) and 0.5% Tween 20, followed by overnight incubation at 4˚C. Phosphorylated proteins were detected using the following primary antibodies: anti-phospho-p38 MAPK$^{Thr180/Tyr182}$, anti-p38 MAPK (Cell Signaling Technology, USA) and anti-GAPDH (Chemicon, USA). Secondary antibodies included horseradish peroxidase (HRP)-conjugated horse anti-rabbit IgG (Cell Signaling Technology) and HRP-conjugated horse anti-mouse IgG (Calbiochem, USA). Immunoreactivity was detected using an enhanced chemiluminescence kit (Amersham Biosciences, UK), according to the manufacturer's protocol. All results were standardized against the signal obtained for GAPDH and band intensity was quantified using GelScope 1.5.

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