MOLECULAR & CELLULAR TOXICOLOGY, Vol. 3, No. 4, 222-230, December 2007

Gene Expression Profiling of Human Bronchial Epithelial (BEAS-2B) Cells Treated with Nitrofurantoin, a Pulmonary Toxicant

Youn-Jung Kim[1], **Mee Song**[1] **& Jae-Chun Ryu**[1]

[1]Cellular and Molecular Toxicology Laboratory, Korea Institute of Science and Technology, PO Box 131, Cheongryang, Seoul 130-650, Korea
Correspondence and requests for materials should be addressed to J. C. Ryu (ryujc@kist.re.kr)

Accepted 8 November 2007

Abstract

Some drugs may be limited in their clinical application due to their propensity towards their adverse effects. Toxicogenomic technology represents a useful approach for evaluating the toxic properties of new drug candidates early in the drug discovery process. Nitrofurantoin (NF) is clinical chemotherapeutic agent and antimicrobial and used to treatment of urinary tract infections. However, NF has been shown to result in pulmonary toxic effects. In this research, we revealed the changing expression gene profiles in BEAS-2B, human bronchial epithelial cell line, exposed to NF by using human oligonucleotide chip. Through the clustering analysis of gene expression profiles, we identified 136 up-regulated genes and 379 down-regulated genes changed by more than 2-fold by NF. This study identifies several interesting targets and functions in relation to NF-induced toxicity through a gene ontology analysis method including biological process, cellular components, molecular function and KEGG pathway.

Keywords: Nitrofurantoin, Pulmonary, Gene ontology, Differentially Expressed Gene (DEG)

Nitrofurantoin (NF) is one of 5-nitrofuran derivatives, antimicrobial and commonly used in the treatment of urinary tract infections caused by gram-positive or gram-negative bacteria. But this clinical agent has been associated with acute, subacute and chronic pulmonary adverse reactions. The acute reaction induced by NF occurs in about 1/5,000 women after their first exposure to this agent[1]. Also, about 85 percent of patients prescribed NF present with related to pulmonary reaction. Common symptoms induced by NF are dry cough, chest pain, dyspnea and peripheral eosinophilia[2]. NF administered chronically for more than 6 months induces pulmonary fibrosis which is a typical side effect of NF treatment reported in 1973[3]. 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. 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[4].

The presence of NADPH and microsomes under aerobic condition 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^-. The toxicity of NF is mediated through superoxide and its secondary metabolites H_2O_2[5]. H_2O_2 induced by NF reacts with lysosomal Fe^{2+} to form Reactive Oxygen Species (ROS). ROS formation causes lysosomal lipid peroxidation, membrane disruption, protease release and cell death[6].

Toxicogenomics is a study on the response of a genome to drug or hazardous substance, using transcriptomics, proteomics and metabolomics in combination with bioinformatic methods and conventional toxicology. Classsical toxicology tools require hundreds of animals and provide little information with respects to mechanisms. But, recently, as the rapid progress in cellular and molecular biology, toxicology is now experiencing a renaissance fuelled by the application of 'omic' technologies to gain a better understanding of the biological basis of toxicology of drugs and other environmental factors[7]. Microarray technologies are comprised in toxicogenomics tools have been widely used for comprehensive gene expression analysis as well as mutation and single nucleotide polymorphism detection. In particular, large-scale microarray analysis of gene expression enables researchers to analyze changes in thousands of genes significant patterns. The Gene Ontology (GO) analysis of microarray data provides structured and controlled vocabularies and classifications for several domains of molecular and cellular biology[8]. GO is generally used to annotate the functions of genes and is composed of three domains, biological process,

cellular components and molecular functions. However, it is not deal with the relations between pathways and sub-pathways or between pathways and their related biological phenomena[9]. So, the association of Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway containing information on biological pathways and GO is useful for analysis of function of genes. Combination of microarray and pathway data may highlight the processes taking place in the cell providing information on the process-specific function of the genome. Biological process can be prospected according to combination of microarray results and biological pathway information[10].

The objective of this study is the identification of function of genes through analysis of GO and of potential gene-based markers induced by NF. We subjected a toxicogenomic approach to understand the mechanism and to identify gene expression profiles and potential novel markers of NF in human bronchial epithelial cell line.

Cytotoxicity of NF in BEAS-2B Cells

Relative survival of BEAS-2B cells following exposure to a range of concentrations of NF 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 hrs of exposure to NF in BEAS-2B cells as shown in Figure 1. The IC_{20} value for NF was 89.45 μM.

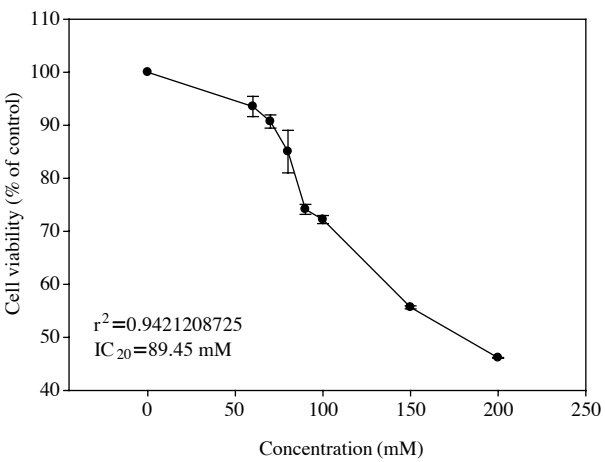

Figure 1. Cell viability of BEAS-2B cells treated to NF. Dose-response curve assessed after treatment. BEAS-2B cells were treated with NF at the indicated concentration in 24-well plate for 48 hrs. MTT (4 mg/mL in PBS) solution was added to each well and incubated for 3 hrs. DMSO solution was added to each well and transfer in 96 well plates. The optical density (O.D.) of the purple formazan product was measured at a wavelength of 540 nm. The IC_{20} value of NF was calculated to 89.45 μM after 48 hrs treatment. Values are expressed as percentage of corresponding controls and standard deviation of data from triplicate experiments.

Gene Expression Analysis

BEAS-2B cells were treated with 89.45 μM NF for 48 hrs, and the total RNA was subjected to microarray analysis. Gene expression changes were analyzed by comparing with treated group and control group using a statistical criteria of $\geq$2-fold changes with $P < 0.01$. In this analysis, 136 genes were up-regulated and 379 genes were down-regulated.

These genes were classified according to KEGG pathway to analyse molecular mechanism exposed to NF. The up- and down-regulated genes are listed in Table 1 and 2. Differentially changed genes involved in 1) MAPK signaling pathway, 2) cytokine-cytokine receptor interaction, 3) apoptosis, 4) ribosome, 5) glycolysis/glucogenesis, 6) axon guidance, 7) metabolism of xenobiotics cytochrome P450, 8) hematopoietic cell linage, 9) focal adhesion, 10) extracellular matrix (ECM)-receptor interaction, 11) cell adhesion molecules (CAMs), 12) Jak-STAT signaling pathway, 13) Wnt signaling pathway, 14) TGF-beta signaling pathway and 15) cell cycle (Figure 2). In KEGG pathway analysis, MAPK signaling pathway, cytokine-cytokine receptor interaction, apoptosis, ribosome, glycolysis/glucogenesis, axon guidance and metabolism of xenobiotics by cytochrome P450 are up-regulated. cytokine-cytokine receptor interaction, hematopoietic cell linage, focal adhesion, ECM-receptor interaction, CAMs, MAPK signaling pathway, Jak-STAT signaling pathway, apoptosis, Wnt signaling pathway, TGF-β signaling pathway and cell cycle are down-regulated.

We investigated an enrichment of GO annotations in the up-regulated and down-regulated genes related to the pulmonary toxic effects of NF. The categories of the function in differentially expressed genes are presented in Figure 3. The biological processes profile could be subdivided into angiogenesis, anti-apoptosis, apoptosis, biosynthesis, catabolism, cell cycle, cell migration, cell organization and biogenesis, chemotaxis, development, DNA metabolism, hemopoiesis, immune response, ion transport, lipid metabolism, NF-kappaB, phosphorylation, protein ubiquitination, response to stress, RNA splicing, signal transduction, transcription, transport and ubiquitin cycle. In this data, the common functions of up-regulated and down-regulated genes are apoptosis, ion transport, immune response and transcription. The

Table 1. Key pathway of up-regulated genes in NF-treated BEAS-2B cells.

Accession no.	Gene name	Gene symbol	Fold change
MAPK SIGNALING PATHWAY			
CA943742	Transcribed locus, strongly similar to XP_429266.1		2.699
AB209361	Fas (TNF receptor superfamily, member 6)	FAS	2.439
NM_006597	Heat shock 70 kDa protein 8	HSPA8	2.205
AK128769	calcium channel, voltage-dependent, beta 2 subunit	CACNLB2	2.200
NM_005345	Heat shock 70 kDa protein 1A	HSPA1A	2.121
CYTOKINE-CYTOKINE RECEPTOR INTERACTION			
AK090482	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	CXCL12	2.695
AB209361	Fas (TNF receptor superfamily, member 6)	FAS	2.439
NM_134470	Interleukin 1 receptor accessory protein	IL1RAP	2.084
NM_003840	Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	TNFRSF10D	2.027
APOPTOSIS			
AB209361	Fas (TNF receptor superfamily, member 6)	FAS	2.439
NM_134470	Interleukin 1 receptor accessory protein	IL1RAP	2.084
NM_003840	Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	TNFRSF10D	2.027
GAP JUNCTION			
NM_006087	Tubulin, beta 4	TUBB4	2.262
AK024002	Tubulin, alpha 4	TUBA4	2.127
RIBOSOME			
BF983391	Ribosomal protein S27-like	RPS27L	2.982
BU902070	Ribosomal protein L36a-like	RPL36AL	2.079
GLYCOLYSIS / GLUCONEOGENESIS			
S81916	Phosphoglycerate kinase 1	PGK1	2.128
AF153821	Alcohol dehydrogenase IB (class I), beta polypeptide	ADH1B	2.106
AXON GUIDANCE			
AK090482	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	CXCL12	2.695
AF055634	Unc-5 homolog C (*C. elegans*)	UNC5C	2.330
METABOLISM OF XENOBIOTICS BY CYTOCHROME P450			
AK123482	Microsomal glutathione S-transferase 1	MGST1	2.171
AF153821	Alcohol dehydrogenase IB (class I), beta polypeptide	ADH1B	2.106

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

main functions of categorized genes are listed up in Table 3. Analysis of cellular localization reveals enrichment for genes associated with membrane, nucleus, ribosome, chromosome and collagen. And the majority of profiles in molecular function are ion binding, nucleic acid binding and signal transducer activity, mostly membrane transport.

Discussion

The clinical use of NF is limited due to its pulmonary toxicity. Classifying the gene alterations, analysing the gene expression patterns and understanding mechanism associated with NF-induced toxicity should allow earlier identification of clinically relevant toxicological findings in compound screening, and aid in the development of therapeutics to reduce pulmonary toxicity. Using microarray in conjunction with statistical analyses, this study has identified a number of differentially expressed genes associated with NF-mediated pulmonary toxicity.

When NF is prescribed chronically, pulmonary fibrosis is mainly induced. As shown in Table 1 and 2, the differentially expressed genes in NF-treated BEAS-2B cells are related to functions such as MAPK signaling pathway, Jak-STAT signaling pathway, TGF -beta signaling pathway, ECM-receptor interaction, cytokine-cytokine receptor interaction, apoptosis, ribosome, CAMs and cell cycle. MAPK signaling pathway is modulated by ROS, and is associated with pulmonary fibrosis[11]. Jak-STAT signaling pathway is a major signaling pathway converting the cytokine signal into gene expression programs regulating the proliferation and differentiation of the immune cells[12]. Pulmonary fibrosis is followed by an abnormal repair

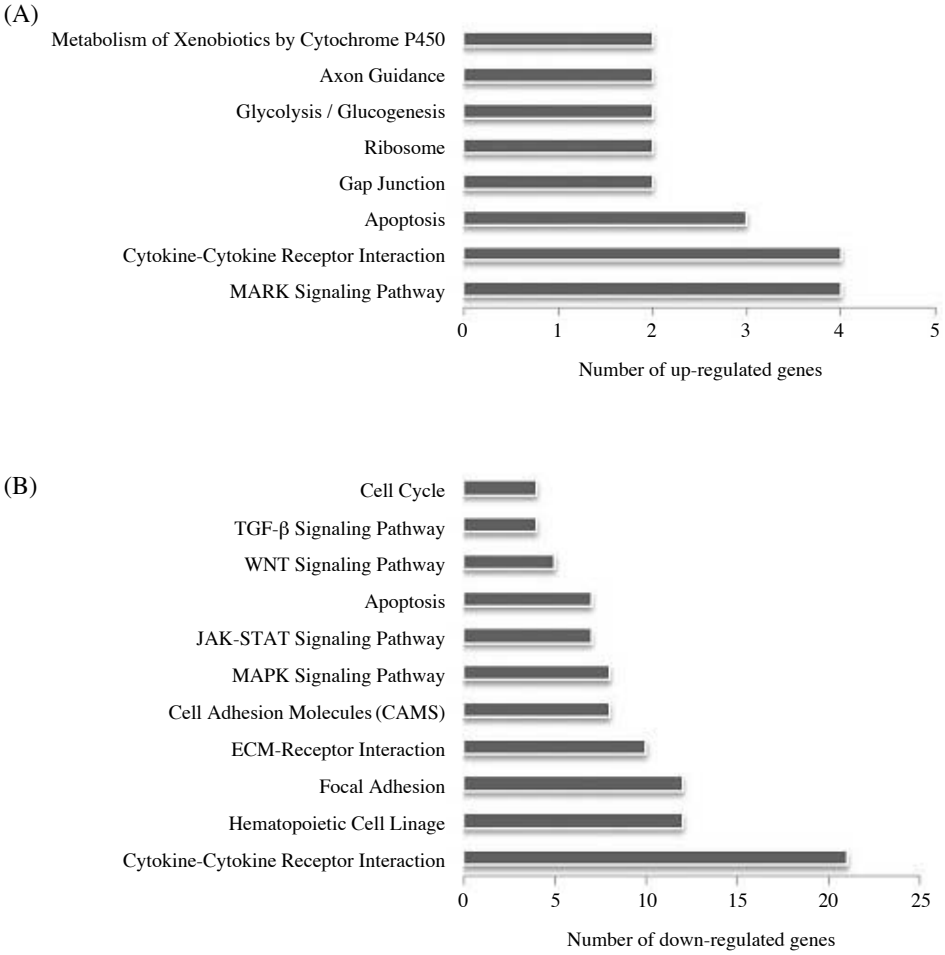

Figure 2. The pathway analysis of differentially expressed up-regulated (A) and down regulated (B) genes using KEGG pathway database.

process, aberrant cytokines and growth factor production. Various growth factors control the accumulation of connective tissue in lung tissue. TGF-β1 is a known regulator of collagen synthesis, and related to proliferation, cellular adhesion, angiogenesis and ECM synthesis[13]. TGF-β1 has been implicated as one of the mediators in the initiation and progression of fibrosis. TGF-β1 initiates the signal by binding to TGF-βRI and TGF-βRII and the binding activates serine/threonine kinases of TGF-βR complexes, which phosphorylate smad-2/3. After phosphorylation, the conformation of smad-2/3 changes thereby facilitates the binding with smad-4. The smad complex then translocates into the nucleus, where it acts to modulate the extracellular matrix (ECM) gene transcription[14]. Ribosomes catalyze mRNA-directed protein synthesis in all organisms. Ribosomal proteins play an important role in stabilization of mRNA and rRNA. Apoptosis is recognized as early features in pulmonary fibrosis[15]. TUBB4 and TUBA4 are involved in apoptosis classification. Tubulin is the major protein of microtubules. β-tubulin is widely distributed in the normal and diseased respiratory tract and is found in many lung tumors, particularly in small and large cell carcinomas[16].

In conclusion, our results showed that the changes of gene expression patterns were associated with pulmonary toxicity induced by NF. And it is suggested that toxicogenomic analysis using oligomicroarray is an efficient technology for evaluation the gene regulation and the possibility to identify the molecular markers. Also, GO analysis is good for a prediction of the mechanism associated with differentially expressed genes in cells or organisms influenced by chemical.

Methods

Materials

Nitrofurantoin, 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

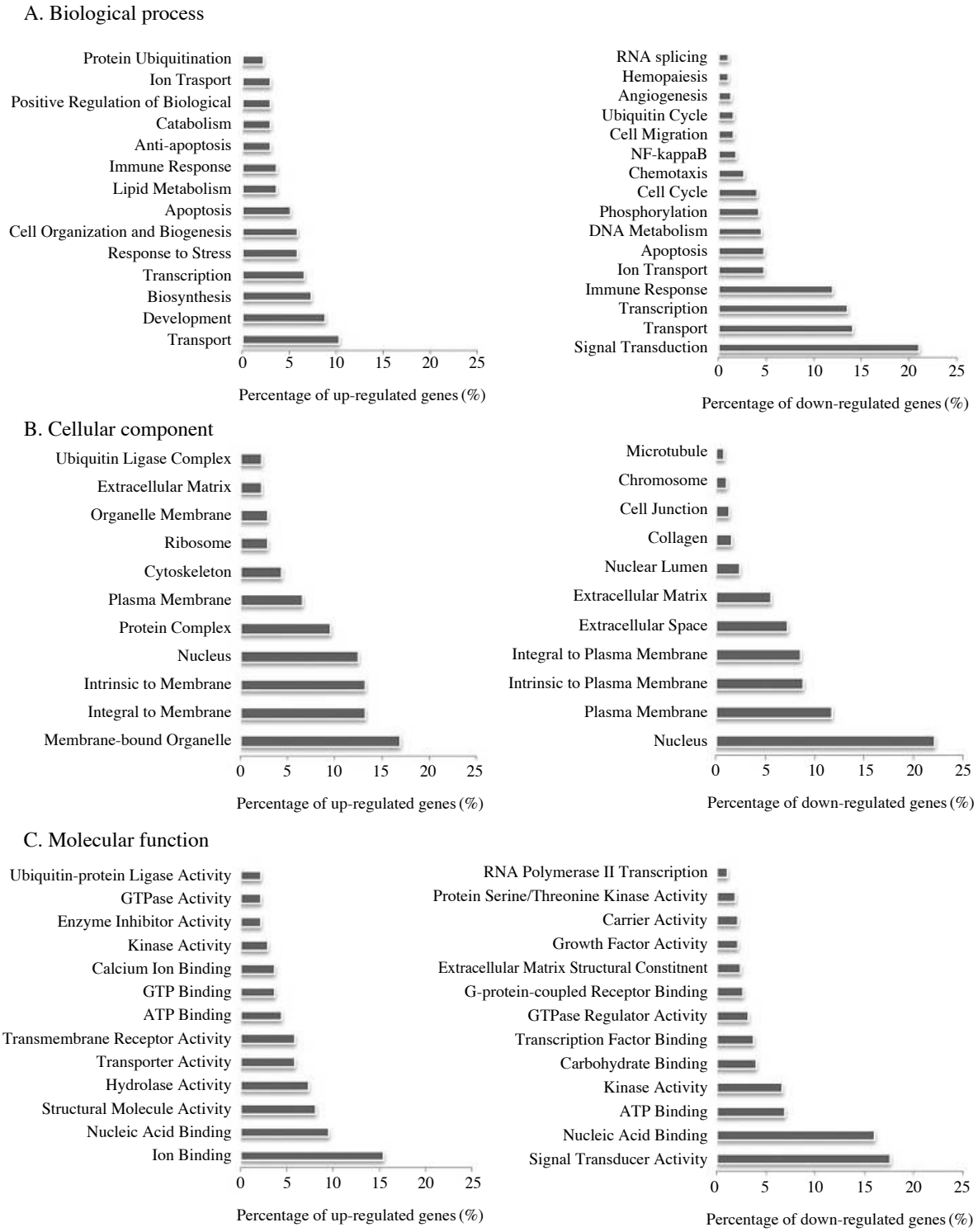

Figure 3. The distribution of up- and down-regulated genes according to gene ontology categories in NF-induced BEAS-2B cells.

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} (Carlsbad, CA,

Table 2. Key pathway of down-regulated genes in NF-treated BEAS-2B cells.

Accession no.	Gene name	Gene symbol	Fold change
CYTOKINE-CYTOKINE RECEPTOR INTERACTION			
BC071593	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT	0.498
NM_000638	Vitronectin (serum spreading factor, somatomedin B, complement S-protein)	VTN	0.494
X03663	Colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog	CSF1R	0.492
BC040531	Activin A receptor, type IB	ACVR1B	0.482
BC028148	Tumor necrosis factor (TNF superfamily, member 2)	TNF	0.474
BE873976	Colony stimulating factor 2 (granulocyte-macrophage)	CSF2	0.455
NM_002994	Chemokine (C-X-C motif) ligand 5	CXCL5	0.433
NM_000600	Interleukin 6 (interferon, beta 2)	IL6	0.425
BC053653	Chemokine (C-X-C motif) ligand 2	CXCL2	0.419
BC071718	Chemokine (C-C motif) ligand 4-like 1	CCL4L1	0.415
BC021117	Colony stimulating factor 1 (macrophage)	CSF1	0.405
BC008678	Interleukin 1, beta	IL1B	0.393
NM_000575	Interleukin 1, alpha	IL1A	0.362
NM_172220	Colony stimulating factor 3 (granulocyte)	CSF3	0.321
AA995107	Transcribed locus, moderately similar to XP_517807.1 PREDICTED: interleukin 7 receptor [Pan troglodytes]	IL7R	0.292
U81234	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	CXCL6	0.280
NM_002982	Chemokine (C-C motif) ligand 2	CCL2	0.211
BC020698	Chemokine (C-C motif) ligand 20	CCL20	0.107
FOCAL ADHESION			
NM_000638	Vitronectin (serum spreading factor, somatomedin B, complement S-protein)	VTN	0.494
NM_001845	Collagen, type IV, alpha 1	COL4A1	0.482
NM_003629	Phosphoinositide-3-kinase, regulatory subunit 3 (p55, gamma)	PIK3R3	0.473
NM_001165	Baculoviral IAP repeat-containing 3	BIRC3	0.452
NM_003371	Vav 2 oncogene	VAV2	0.441
NM_002160	Tenascin C (hexabrachion)	TNC	0.440
NM_212482	Fibronectin 1	FN1	0.359
AK128124	Calpain 5	CAPN5	0.352
NM_198679	Rap guanine nucleotide exchange factor (GEF) 1	RAPGEF1	0.345
NM_198129	Laminin, alpha 3	LAMA3	0.268
NM_000393	Collagen, type V, alpha 2	COL5A2	0.139
ECM-RECEPTOR INTERACTION			
NM_000638	Vitronectin (serum spreading factor, somatomedin B, complement S-protein)	VTN	0.494
AB007937	Syndecan 3 (N-syndecan)	SDC3	0.489
NM_004393	Dystroglycan 1 (dystrophin-associated glycoprotein 1)	DAG1	0.488
NM_001845	Collagen, type IV, alpha 1	COL4A1	0.482
Z74615	Collagen, type I, alpha 1	COL1A1	0.462
NM_002160	Tenascin C (hexabrachion)	TNC	0.440
NM_002999	Syndecan 4 (amphiglycan, ryudocan)	SDC4	0.368
NM_212482	Fibronectin 1	FN1	0.359
NM_198129	Laminin, alpha 3	LAMA3	0.268
NM_000393	Collagen, type V, alpha 2	COL5A2	0.139
CELL ADHESION MOLECULES (CAMS)			
AY358094	Claudin 17	CLDN17	0.500
AB007937	Syndecan 3 (N-syndecan)	SDC3	0.489
NM_000425	L1 cell adhesion molecule	L1CAM	0.483
NM_016369	Claudin 18	CLDN18	0.459
AK001872	Programmed cell death 1 ligand 2	PDCD1LG2	0.436
NM_002999	Syndecan 4 (amphiglycan, ryudocan)	SDC4	0.368
AY358815	Neural cell adhesion molecule 1	NCAM1	0.293
NM_015259	Inducible T-cell co-stimulator ligand	ICOSLG	0.180
MAPK SIGNALING PATHWAY			
CR614015	CD14 antigen	CD14	0.500
BC040531	Activin A receptor, type IB	ACVR1B	0.482

Table 2. Continued.

Accession no.	Gene name	Gene symbol	Fold change
BC028148	Tumor necrosis factor (TNF superfamily, member 2)	TNF	0.474
BC008678	Interleukin 1, beta	IL1B	0.393
NM_000575	Interleukin 1, alpha	IL1A	0.362
NM_014002	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	IKBKE	0.338
NM_002891	Ras protein-specific guanine nucleotide-releasing factor 1	RASGRF1	0.241
NM_000944	Protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform (calcineurin A alpha)	PPP3CA	0.229
JAK-STAT SIGNALING PATHWAY			
NM_003629	Phosphoinositide-3-kinase, regulatory subunit 3 (p55, gamma)	PIK3R3	0.473
NM_003955	Suppressor of cytokine signaling 3	SOCS3	0.472
BE873976	Colony stimulating factor 2 (granulocyte-macrophage)	CSF2	0.455
NM_000600	Interleukin 6 (interferon, beta 2)	IL6	0.425
NM_002309	Hypothetical protein MGC20647	LIF	0.415
NM_172220	Colony stimulating factor 3 (granulocyte)	CSF3	0.321
AA995107	Transcribed locus, moderately similar to XP_517807.1 PREDICTED: interleukin 7 receptor [Pan troglodytes]	IL7R	0.292
APOPTOSIS			
BC028148	Tumor necrosis factor (TNF superfamily, member 2)	TNF	0.474
NM_003629	Phosphoinositide-3-kinase, regulatory subunit 3 (p55, gamma)	PIK3R3	0.473
NM_001165	Baculoviral IAP repeat-containing 3	BIRC3	0.452
NM_000575	Interleukin 1, alpha	IL1A	0.362
AK128124	Calpain 5	CAPN5	0.352
NM_000944	Protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform (calcineurin A alpha)	PPP3CA	0.229
TGF-BETA SIGNALING PATHWAY			
U68019	SMAD, mothers against DPP homolog 3 (Drosophila)	SMAD3	0.500
BM906235	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	ID3	0.485
BC040531	Activin A receptor, type IB	ACVR1B	0.482
BC028148	Tumor necrosis factor (TNF superfamily, member 2)	TNF	0.474

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

Table 3. GO analysis on differential expressed genes in NF-treated BEAS-2B cells.

GO term	Expression	Genes contributing to the differential expression
Apoptosis	Up	RHOB, FAS, UNC5C, HSPA1A, NALP12, BAG1, TNFRSF10D
	Down	CD14, CTSB, TNF, SOCS3, P8, ESPL1, TP73, BIRC3, TNFAIP3, ANGPTL4, IL6, IL1B, IL1A, SULF1, TNFSF9, ABL1, CCL2
Transcription	Up	PIR, BHLHB2, ZNF469, TULP4, ATF6, LOC339344, ST18, HOXB7, ZFHX2
	Down	SMAD3, GLIS3, KIAA1718, KLF1, NCOR1, SOX4, NFATC1, GATA2, ID3, ETV6, IRF4, VGLL4, TNF, EN1, ARID5B, VSX1, ATF3, TP73, BCOR, TLE3, TCF1, ZNF326, SOX2, MLL2, TNFAIP3, IQGAP3, PARP1, OLIG3, NFE2L1, HDAC11, THRAP2, NAB2, BRF1, MAFK, KIAA1055, ZFM1, SQSTM1, SMARCC2, MAFB, POLR2A, NEUROD6, HES4, HES6, MEF2B, ABL1, ATOH8, HBXAP, ELF3, WBSCR14
Immune response	Up	CXCL12, FAS, IL1RAP, NALP12, FTH1
	Down	CD14, VTN, CSF1R, IRF4, DMBT1, TNF, PTX3, CXCL1, SECTM1, KIF13B, CSF2, CRHR1, C3, PDCD1LG2, CXCL5, APOL3, IL6, CXCL2, NFE2L1, CCL4L1, LIF, CSF1, C1R, IL1B, ITK, CXCL3, IL1A, FN1, TNFSF9, SQSTM1, SAA1, IKBKE, MBP, CSF3, SAA4, IL7R, SERPINA3, SAA2, CXCL6, BF, CCL2, KLRC3, ICOSLG, CCL20
Ion transport	Up	UCP2, GRIK4, CACNLB2, FTH1
	Down	SCN8A, COL4A1, SLC23A2, EN1, COL1A1, ATP2B4, TPCN1, COL4A5, COL12A1, SFXN5, SLC22A7, MFI2, C1QTNF1, SLC12A7, COL8A1, SCN5A, HTR3A, COL5A2

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 ana-

lytical 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[17]. 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 hrs. After treatment, cells were incubated for 3 hrs 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 NF 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 treated with 89.45 μM NF for 48 hrs using 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. The amount of each total RNA sample was measured by a spectrophotometer, and its quality was checked by Experion™ (Bio-Rad Laboratories, Hercules, CA, USA).

Oligonucleotide Microarray Hybridization

Gene expression analysis was conducted on the RNA samples using human whole 44 k microarray (Agilent Technologies, Palo Alto, CA, USA). Labeling and hybridization were performed by instruction of FairPlay® Microarray Labeling Kit (Stratagene, Glenville, VA, USA). This was 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 hrs. After washing ($2 \times$ SSC/0.1% SDS for 2 min at 58˚C, $1 \times$ SSC for 3 min at RT, $0.2 \times$ 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, Union City, CA, USA). Scanned images were analyzed with GenePix 4.1 software (Axon Instruments, Union City, CA, USA) 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, WA, USA). Significance Analysis of Microarray (SAM) was performed for the selection of the genes with significant gene expression changes[18]. 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 0.5 or less than -0.5, i.e., 2.0-fold difference in expression level, and when the q-values were < 1.

References

1. Williams, E. M. & Triller, D. M. Recurrent acute nitrofurantoin-induced pulmonary toxicity. *Pharmacotherapy* **26**:713-718 (2006).
2. Bobbak, V. & Bernadette, M. M. Nitrofurantoin pulmonary toxicity: A brief review. *Cur Respir Med Rev* **2**:439-442 (2006).
3. Adamson, I. Y. Drug-induced pulmonary fibrosis. *Environ Health Perspect* **55**:25-36 (1984).
4. Daba, M. H., El-Tahir, K. E., Al-Arifi, M. N. & Gubara, O. A. Drug-induced pulmonary fibrosis. *Saudi Med* **25**:700-706 (2004).
5. Gram, T. E. Chemically reactive intermediates and pulmonary xenobiotic toxicity. *Pharmacol Rev* **49**: 297-341 (1997).
6. Pourahmad, J., Khan, S. & O'Brien, P. J. Lysosomal oxidative stress cytotoxicity induced by nitrofurantoin redox cycling in hepatocytes. *Adv Exp Med Biol* **500**:261-265 (2001).
7. Ryu, J. C., Kim, M. K., Choi, M. H. & Chun, T. H. Promising next generation technology in toxicology-

toxicogenomics. *Mol Cell Toxicol* **1**:1-6 (2005)
8. Ashburne, M. *et al*. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* **25**:25-29 (2000).
9. Tatsuya, K. *et al*. Event Ontology: A Pathway-Centric Ontology for Biological Processes. *Pac Symp Biocomput* **11**:152-163 (2006).
10. Te Pas, M. F. *et al*. Biochemical pathways analysis of microarray results: regulation of myogenesis in pigs. *BMC Dev Biol* **7**:66-80 (2007).
11. Mossman, B. T., Lounsbury, K. M. & Reddy, S. P. Oxidants and signaling by mitogen-activated protein kinases in lung epithelium. *Am J Respir Cell Mol Biol* **34**:666-669 (2006).
12. Park, H. J. *et al*. Identification of Gene-based potential biomarkers for cephalexin-induced nephrotoxicity in mice. *Mol Cell Toxicol* **2**:193-201 (2006).
13. Chen, C. M. *et al*. Up-regulation of connective tissue growth factor in hyperoxia-induced lung fibrosis. *Pediatr Res* **62**:128-133 (2007).
14. Wang, X. M. *et al*. Caveolin-1: a critical regulator of lung fibrosis in idiopathic pulmonary fibrosis. *J Exp Med* **203**:2895-2906 (2006).
15. Fridlender, Z. G. *et al*. Telomerase activity in bleomycin-induced epithelial cell apoptosis and lung fibrosis. *Eur Respir J* **30**:205-213 (2007).
16. Sheppard, M. N. & Thurlow, N. P. Distribution of the cytoskeletal protein beta-tubulin in normal lung, cryptogenic fibrosing alveolitis and lung tumors. *Histopathology* **20**:421-425 (1992).
17. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**:55-63 (1983).
18. Tusher, V. G., Tibshirani, R. & Chu, G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* **98**:5116-5121 (2001).