Gene Expression Analysis of Methotrexate-induced Hepatotoxicity between *in vitro* and *in vivo*

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Abstract

The recent DNA microarray technology enables us to understand gene expression profiling in cell line and animal models. The technology has potential possibility to comprehend mechanism of multiple genes were related to compounds which have toxicity in biological system. So, microarray system has been used for the prediction of toxicity through gene expression induced by toxicants. It has been shown that compounds with similar toxic mechanisms produce similar changes in gene expression *in vivo* system. Here we focus on the use of toxicogenomics for the determination of gene expression analysis associated with hepatotoxicity in rat liver and cell line (WB-F344). Methotrexate (MTX) is a chemotherapy agent that has been used for many years in the treatment of cancer because it affects cells that are rapidly dividing. Also it has been known the toxicity of MTX, in a MTX abortion, it stops embryonic cells from dividing and multiplying and is a non-surgical method of ending pregnancy in its early stages. We have shown DNA microarray analyses to assess MTX-specific expression profiles *in vivo* and *in vitro*. Male Sprague-Dawely VAF+ albino rats of 5-6 weeks old and WB-F344 cell line have been treated with MTX. Total RNA was isolated from Rat liver and cell line that has treated with MTX. 4.8 K cDNA microarray in house has been used for gene expression profiling of MTX treatment. We have found quite distinct gene expression patterns induced by MTX in a cell line and *in vivo* system.

Keywords: Methotrexate, toxicogenomics, gene expression, rat liver, epithelial cells

Recently, many pharmaceutical companies and research groups are making databases of gene expression related to toxic mechanism induced by compounds that were well characterized. These collected databases of microarray associated with toxicity will shorten the toxicity evaluation steps that are often the rate-limiting step in the discovery and development of new pharmaceuticals. Therefore, the toxicogenomics through this technology may be very powerful for understanding the effect of unknown toxic mechanisms in biological system.

We are gaining information of numerous candidate genes that have been known and unknown their function in biological system through many projects has been done and are processing. Many techniques that are able to analyze many genes and proteins simultaneously in once are used to interpret the information. Microarray technology, one of them, permits the comparison of thousands of genes in different biological systems. Lately, microarray system has been used for the prediction of toxicity through gene expression induced toxicant[1,2] and has shown that compounds with similar toxic mechanisms produce similar changes in gene expression *in vivo*[3] and *in vitro* system[4].

Methotrexate (MTX) is a chemotherapy agent that has been used for many years in the treatment of cancer because it affects cells that are rapidly dividing. Also it has been known the toxicity of MTX, in a MTX abortion, it stops embryonic cells from dividing and multiplying and is a non-surgical method of ending pregnancy in its early stages. Methotrexate treatment for psoriasis is known to cause hepatic fibrosis in some patients, which might progress to cirrhosis[5,6].

To compare gene expression patterns of *in vivo* and

in vitro system, we have been used cell line which was treated with MTX for 12 hours and animal model which was treated with MTX for 1, 7, and 14 days. We have treated high dose MTX and low dose MTX to *in vivo* and *in vitro* system.

Methods

Cell Line Treated

WB-F344 (WB) cell was cultured in D-media (Formula No. 78-5470EF, Gibco BRL, Grand Island, NY, USA) containing 3 mL/L PSN mixture in the presence of 5% fetal bovine serum. Cells were incubated in a 37°C humidified incubator containing 5% CO_2 and 95% air. Cells were grown in 175 cm^2 flask and the culture medium was changed every other day.

The cytotoxic effects of MTX on WB cells were measured by MTT assay, based on the ability of live cells to convert tetrazolium salt into purple formazan. In brief, the cells were seeded at a density of 2×10^5 cells/mL in 24-well microplates and incubated overnight at 37°C, 5% CO_2. Then the cells were treated with different concentrations of chemicals or its vehicle, deionized water for 12 h. Following treatment, the cells were rinsed three times with PBS and then incubated with addition of MTX stock for the last 4 hr of incubation. After media was removed, added 200 μL of DMSO to each well and then pipetted up and down to dissolve the crystals.

WB cells were seed at a density of 2×10^5 cells/mL in 10 cm dishes and incubated overnight at 37°C, 5% CO_2. On reaching confluency, cells were then dosed for 12 h.

Animals and Samples Treated

Sixty Sprague-Dawley VAF+ albino rats at 5-6 weeks age were purchased from Biogenomics Inc. (Seoul, Korea). Animals were weighed, weight-ranked, and assigned randomly to each of the treatment (5 rats/group) and control groups (5 rats/group) before administration and then housed in one in polycarbonate, wire-mesh cages (Myung-Jin Machinery, Korea) during the study. Methotrexate (MTX) were obtained from Sigma (St. Louis, MO). Dosing suspensions of all compounds were prepared using a high speed homogenizer, and all dose suspensions were continuously stirred until completion of dosing. The MTX were prepared using vehicle. Body weights and food consumption were measured weekly. Based on the most recently recorded body weights, the volume of drug administered was adjusted. Animals were observed 2-3 times daily for signs of overt toxicity.

RNA Isolation

Cell line

Following the appropriate incubation period, WB cells were washed three times in PBS and the cells scraped into 1 mL TRIzol Reagent™ (Invitrogen, Carlsbad, CA) per dish. RNA was extracted from four individual culture incubations and equal amount of RNA from each conditions were pooled. The extracted RNA was dissolved in RNase-free water, and its concentration and purity was determined from absorbance measurements at 260 and 280 nm using a spectrophotometer.

Rat liver

Total RNA was extracted from liver tissues using Micro-to-Midi Total RNA Purification System (Life Technologies. Inc.). Liver sections of 40-50 mg were used for midiprep. Lysis buffer was added to frozen liver sections, and the tissue was immediately homogenized using a Turrax Homogenizer. Final product yielded 260 nm/280 nm ratios of 1.8-2.0, purity was confirmed via gels, and concentration was determined based on 260 nm absorbance using a spectrophotometer. Quality of the RNA was checked by visualizaion of the 28S : 18S ribosomal RNA ratio on a 1% agarose gel.

Hybridization in Microarray

A rat 4.8 K cDNA microarray, developed in-house at GenoCheck Co. Ltd., was used for gene expression profiling studies. A complete listing of the genes on this microarray is available at the following Web site: http://www.genocheck.com. cDNA microarray chips were prepared according to DeRisi *et al.*, 1996.[7] cDNAs were amplified in 100 μL polymerase chain reaction (PCR) mixture. A sample of PCR products (5 μL) was separated on 1% agarose gels to ensure quality of the amplification. The remaining PCR product were purified by ethanol precipitation and resuspended in spotting buffer kit (Genocheck Co. Ltd, Ansan) and spotted onto CMT-GAPS II slides (Corning Inc., Corning NY). For microarray hybridization, total RNA of control liver RNA was pooled and used for hybridization and each total RNA sample (50 μg) was labeled with Cyanine (CY3) or Cyanine (Cy5)-conjugated dCTP (Amersharm, Piscataway, NJ) by a reverse transcription reaction using reverse transcriptase, SuperScript (Invitrogen, Carlsbad, California). The fluorescently labeled cDNAs were mixed and hybridized simultaneously to the cDNA microarray chip. The cDNA chips were scanned with an Axon 4000B Scanner (Axon Instruments, Foster City CA) using laser excitation of the 2 fluors at 532 and 635 nm wave length for the Cy3 and Cy5 labels. The raw

intensity data was global normalized by intensity dependent normalization on lowess method, then was normalized by with-print-tip group normalization method for each print-tip, 8 tips was used for making 4.8 K cDNA microarray. We gathered fold changes from M, log_2R-log_2G (R is Cy5 signal and G is Cy3 signal). We have chose gene expression of two fold changed, over 2 and down 0.5 ($M \geq 1$ and $M \leq -1$). The fold change of gene expression was selected on the bias that two individuals have over fold changed among three individuals treated with chemical simultaneously.

Data Management

Gene expression values for each chip were normalized to the intensity-dependent (LOWESS) normalization, then was normalized by with-print-tip group normalization method for each print-tip, 8 tips were used for making 4.8 K cDNA microarray. We gathered fold changes from M, log_2R-log_2G (R is Cy5 signal and G is Cy3 signal). We have chose gene expression of two fold changed, over 2 and down 0.5 ($M \geq 1$ and $M \leq -1$). Experiment normalized, cluster analysis and ontology were performed with GeneSpring software 5.0 (Agilent Technologies).

In this study, a rat 4.8 K cDNA microarray which contains approximately 4,800 toxicity-related genes has designed. Sprague-Dawely VAF+ albino rats of 5-6 weeks old were treated with each compound for 1, 7 and 14 days and WB-F344 (WB) cell was treated for 12 hours. Toxicity in livers of all of the treated animals with histological examinations were performed. In order to determine gene expression profiles associated with chemical exposure, liver total RNA was extracted after 1, 7 and 14 days of daily exposures to the compound. To reduce variability in the level of gene expression of rats treated with vehicle, control total RNA has pooled and used for hybridizations on the 4.8 K cDNA microarray[8-10].

To compare gene expression patterns of *in vivo* and *in vitro* system, we selected 2 fold up- and down-regulated genes in condition of high and low concentrations of MTX *in vitro* (Fig. 1). The selected genes have been compared with liver tissue treated with high concentration of MTX for 1, 7 and 14 days. 174 genes have been shown 2 fold changes in a high concentration of MTX *in vitro* for 12 hr treatment. Although under condition of 2 different concentrations of MTX *in vitro*, the gene expression pattern was shown similar expression patterns (Fig. 1). The expression profiles of selected genes were quite different from gene expression patterns in other conditions; high and low concentration treatments for 1, 7 and 14 days *in vivo*. 29 genes have been shown up-regulated and 14 genes have been shown down-regulated *in vitro* by MTX (Table 1, 2). Most of genes treated with MTX *in vitro* showed the different gene expression profiles *in vivo* condition.

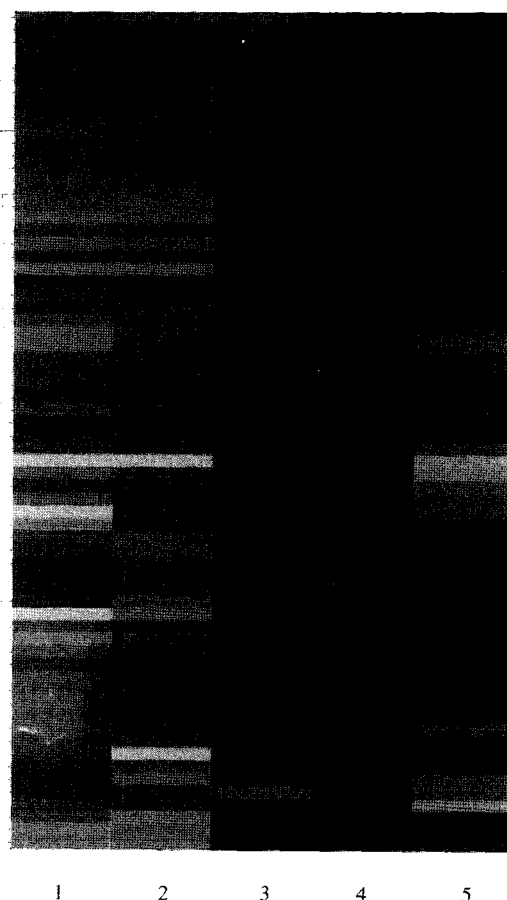

Fig. 1. Gene expression profiles of MTX treatment *in vitro* and *in vivo*. Genes were exchanged simultaneously *in vitro* treated high and low dose condition. High does in cell line: 1, low dose in cell line: 2, high dose treatment *in vivo* for 1 day: 3, for 7 days: 4 and for 14 days: 5.

Table 3 shows the genes that regulated over 2 fold induction by high dose MTX treatment. The upregulated genes were characterized into 6 groups; Metabolism, Human disease, Genetic Information Processing, Environmental Information Processing, Environmental Information Processing and Cellular processes. Most of genes were cataloged into metabolism, environmental Information processing and cellular processes. In metabolism, most genes were characterized into lipid, carbohydrate, and amino acid metabolism. In environmental Information processing, most genes were involved in signal transduction and in cellular processes.

Table 1. Expression profiles of up-regulated genes induced by MTX

Gene name	*In vitro* (12 hr)		*In vivo* (1 day)		*In vivo* (7 day)		*In vivo* (14 day)	
	High	Low	High	Low	High	Low	High	Low
B-cell translocation gene 2	4.37	0.53	0.41	0.64	−0.43	−0.14	1.33	0.29
Serum-inducible kinase	3.29	0.44	−0.33	−0.27	−0.34	−0.13	−0.56	−0.08
Regulator of G-protein signaling protein 2	2.81	−0.16	−0.21	−0.06	−0.38	0.13	0.34	0.06
Dynactin 1	2.67	0.20	−0.16	−0.10	−0.11	−0.27	0.02	0.01
Cyclin L	2.28	0.04	−0.17	−0.18	−0.01	−0.38	0.32	0.10
Serine proteinase inhibitor, member 1	2.21	0.11	0.03	0.15	−0.19	0.02	0.58	−0.19
Neuritin	2.12	0.33	−0.17	−0.29	0.18	0.05	0.13	0.02
Myeloid differentiation primary response gene 116	2.02	−0.12	0.02	0.36	0.16	0.28	−0.08	−0.08
Mercaptopyruvate sulfurtransferase	1.84	−0.08	−0.14	−0.03	0.00	−0.02	0.29	0.18
Diacylglycerol kinase, alpha (80 kDa)	1.78	0.29	0.10	0.06	−0.06	−0.22	−0.26	0.09
Selenoprotein P, plasma, 1	1.67	2.55	0.13	−0.05	0.25	0.17	−0.22	0.05
Surfactant, pulmonary-associated protein B	1.66	−0.07	0.27	0.03	0.11	0.75	0.46	0.09
Diphtheria toxin receptor	1.62	0.21	−0.12	−0.06	−0.18	−0.43	0.04	0.09
Protein tyrosine phosphatase 4a1	1.57	0.05	0.05	−0.05	0.23	0.16	−0.01	0.01
Peroxiredoxin 5	1.57	1.51	0.24	0.25	0.20	−0.14	−0.38	−0.19
Neurexophilin 4	1.56	0.37	0.09	0.06	0.33	0.30	0.40	0.19
Activated leukocyte cell adhesion molecule	1.55	−0.14	0.27	0.29	−0.20	−0.20	−0.17	−0.42
Interferon-related developmental regulator 1	1.52	0.10	0.29	0.12	−0.14	−0.04	0.36	−0.25
Translation initiation factor eIF-2B alpha-subunit	1.50	0.31	−0.01	−0.04	−0.22	−0.18	−0.12	−0.08
Cannabinoid receptor 1	1.49	−0.15	−0.39	−0.35	−0.06	−0.28	0.20	0.11
HNF-3/forkhead homolog-1	1.47	0.56	0.14	0.20	−0.04	−0.09	0.19	0.09
Protein tyrosine kinase	1.39	0.33	0.19	0.24	0.15	0.10	0.10	0.09
Ribosomal protein L18	1.33	0.11	0.08	0.02	0.01	−0.19	−0.23	−0.17
Epoxide hydrolase 1	1.32	0.27	0.54	0.72	−0.66	−0.29	−0.89	0.06
Hsp70-interacting protein	1.30	−0.03	−0.18	0.17	0.00	0.17	0.12	0.06
Peptidoglycan recognition protein	1.29	−0.16	0.02	−0.13	−0.04	0.21	0.15	0.03
EGF-like-domain, multiple 4	1.29	0.94	−0.13	−0.15	−0.06	−0.09	−0.15	−0.02
Hairy and enhancer of split 1	1.26	−0.25	0.44	−0.45	−0.40	0.17	−0.11	−0.08
Glucose-6-phosphatase	1.21	−0.01	−0.02	0.33	−0.40	−0.20	−1.28	−0.30

Table 2. Expression profiles of down-regulated genes induced by MTX

Gene name	*In vitro*		*In vivo* (1 day)		*In vivo* (7 day)		*In vivo* (14 day)	
	High	Low	High	Low	High	Low	High	Low
Solute carrier family 27, member 32	−1.02	0.04	−0.02	−0.14	0.22	0.17	−0.97	−0.19
Ribosomal protein S26	−1.02	−0.27	0.06	0.07	−0.14	−0.05	−0.18	−0.08
Microsomal glutathione S-transferase 1	−1.14	−0.12	−0.19	−0.06	−0.08	−0.15	0.57	0.24
Aldehyde reductase 1	−1.14	−0.08	0.07	0.01	−0.01	0.04	−0.19	−0.10
Branched chain aminotransferase 2, mitochondrial	−1.16	−0.38	−0.04	0.09	0.06	0.01	0.01	0.14
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	−1.17	0.24	−0.54	−0.35	−0.56	0.17	0.34	−0.23
Solute carrier family 10, member 1	−1.22	0.04	−0.21	0.11	0.02	−0.09	−0.03	−0.11
Serine proteinase inhibitor, clade H, member 1	−1.22	0.15	−0.15	−0.16	−0.06	0.08	0.53	−0.06
Interleukin 6 signal transducer	−1.32	0.02	0.37	0.43	0.00	−0.20	0.00	−0.58
Nuclear pore glycoprotein 62	−1.34	−0.18	0.03	−0.01	−0.13	−0.06	−0.43	0.18
Stearoyl-Coenzyme A desaturase 2	−1.52	0.55	0.02	−0.03	0.03	−0.04	−0.10	0.00
Ras-related small GTP binding protein 4	−1.54	−0.12	0.20	0.06	−0.09	0.25	0.18	−0.19
Isocitrate dehydrogenase 1, soluble	−1.60	−0.02	−0.17	−0.15	−0.04	0.03	−0.05	0.09
Vascular cell adhesion molecule 1	−1.70	−0.35	−0.12	0.14	−0.01	0.04	0.34	0.02

Discussion

The MTX is an anticancer drug also used as an antimalarial inhibitor. The interferon-related developmental regulator increased only *in vitro* treated with high dose MTX. The 1-Cys peroxiredoxin have been shown the relation to MTX. In the result of high dose *in vitro*, peroxiredoxin was up-regulated, but was not shown in other cases. The effect of alpha-

Table 3. The pathway analysis of 2 fold changed genes *in vitro*

	Pathway	Gene
Metabolism	Nucleotide	8
	Other amino acids	9
	Cofactors and vitamins	7
	Lipid	25
	Glycan biosynthesis	4
	Energy	8
	Carbohydrate	32
	Biosynthesis of secondary	9
	Biodegradation of xenobiotics	6
	Amino acid	37
Human disease	Neurodegenerative disorders	3
Genetic information processing	Translation	2
	Transcription	1
	Replication and repair	2
	Folding, sorting and degradation	1
Environmental information processing	Signal transduction	49
Environmental information processing	Ligand-Receptor interaction	16
Cellular processes	Immune system	5
	Development	1
	Cell motility	7
	Cell growth and death	8
	Cell communication	16

and gamma-interferon on methotrexate cytotoxicity against human promyelocytic cell line HL-60 has been evaluated[11-13]. The EGF-like domain gene increased in both vitro treated with high and low dose. MTX can be increased EGF. Only the accumulation of MTX, raltitrexed or topotecan, was significantly enhanced after hormone pre-treatment both in intact renal tissue and in human renal cell carcinoma[14-16].

We found glutathione S-transferase 1 gene was decreased by MTX *in vitro* treated with high for 12 hours. W-H75 cells were generally more resistant than the parental cells to most of the agents that were tested, they were collaterally sensitive to the antimetabolites methotrexate and glutathione S-transferase[17]. We examined the expression by microarray serial time *in vivo* and high and low dose MTX *in vivo* and *vitro*. The Interleukin 6 signal transducer gene was only decrease *in vitro* treated with high dose. MTX significantly inhibited the enhancement by IL-17 of TNF-alpha-stimulated IL-6 synthesis. MTX reduced the levels of IL-6 induced by 12-O-tetradecanoylphorbol 13-acetate, a direct activator of protein kinase C (PKC), suggesting that MTX inhibits PKC signals for IL-6 synthesis[18-20].

Our study has shown the comparison between *in vivo* and *in vitro* expression profilings of methotrexate-induced hepatotoxicity using microarray method. Most of the changes in gene expression induced by MTX were different *in vitro* and *in vivo*. This demonstrates that the employed *in vitro* model treated by high dose of MTX is not able to reflect most of the expression changes due to MTX toxicity were not observed *in vivo*. If we have optimized toxic concentrations of MTX *in vivo*, we would be able to make more related expression patterns. The results obtained from the microarray analysis revealed significant toxicity related gene expression changes and elucidated interesting expression changes and biological function relationships. However, we also found many interesting ESTs which have no functional informations.

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References

1. Thai, S.F., Allen, J.W., DeAngelo, A.B. & George, M.H. Altered gene expression in mouse livers after dichloroacetic acid exposure. *Mutat. Res.* **543**, 167-180 (2003).
2. Wilbert, F.M. & Kemmelmeier, C. Identification of deoxynivalenol, 3-acetyldeoxynivalenol, and zearalenone in galactose oxidase-producing isolates of Fusarium graminearum. *J. Basic Microbiol.* **43**, 148-157 (2003).
3. Hamadeh, H.K. *et al.* Gene expression analysis reveals chemical-specific profiles. *Toxicol. Sci.* **67**, 219-231 (2002).
4. Burczynski, M.E. *et al.* Toxicogenomics-based discrimination of toxic mechanism in HepG2 human hepatoma cells. *Toxicol. Sci.* **58**, 399-415 (2000).
5. Affleck, J.G., Neumann, K., Wong, L. & Walker, V.K. The Effects of Methotrexate on Drosophila Development, Female Fecundity, and Gene Expression. *Toxicol. Sci.* Nov. **9** (2005).
6. Hytiroglou, P., Tobias, H., Saxena, R., Abramidou, M., Papadimitriou, C.S. & Theise, N.D. The canals of hering might represent a target of methotrexate hepatic toxicity. *Am. J. Clin. Pathol.* **121**, 324-329 (2004).
7. DeRisi J. *et al.* Trent J.M. Use of a cDNA microarray to analyse gene expression patterns in human cancer. *Nat Genet.* **14**, 457-460 (1996).
8. Jin, W.J. *et al.* Gene Expression Analysis of Peroxi-

some Proliferators- and Phenytoin-Induced Hepatotoxicity Using cDNA Microarray. *J. Vet. Med. Sci.* **66**, 1329-1333 (2004).

9. Wan, S.L. *et al.* The intelligent data management system for Toxicogenomics. *J. Vet. Med. Sci.* **66**, 1334-1338 (2004).
10. Gyoung, J.L. *et al.* cDNA microarray gene expression analysis and toxicological phenotype for anticancer drug. *J. Vet. Med. Sci.* **66**, 1339-1343 (2004).
11. Sur, P., Matsuo, Y., Otani, T. & Minowada, J. Modulation of methotrexate cytotoxicity with natural interferon upon human leukemia cell line HL-60. *Oncology.* **48**, 469-473 (1991).
12. Couret, M., Combe, B., Reme, T. & Sany, J. Production of interleukin 2 and interferon-gamma in rheumatoid polyarthritis during a longitudinal study conducted on patients treated with methotrexate. *Rev Rhum. Mal. Osteoartic.* **57**, 641-645 (1990).
13. Machy, P., Arnold, B., Alino, S. & Leserman, L.D. Interferon sensitive and insensitive MHC variants of a murine thymoma differentially resistant to methotrexate-containing antibody-directed liposomes and immunotoxin. *J. Immunol.* **136**, 3110-3115 (1986).
14. Fleck, C., Wennek-Klose, J., Wange, J. & Oelschlager, H. Effects and toxicity of new fomocaine derivatives and of 2-hydroxypropyl-beta-cyclodextrin inclusion compounds in rats. *Arzneimittelforschung.* **54**, 265-274 (2004).
15. Chauhan, S.S. *et al.* Reduced endocytosis and altered lysosome function in cisplatin-resistant cell lines. *Br. J. Cancer.* **88**, 1327-1334 (2003).
16. Xian, C.J., Cool, J.C., Howarth, G.S. & Read, L.C. Effects of TGF-alpha gene knockout on epithelial cell kinetics and repair of methotrexate-induced damage in mouse small intestine. *J. Cell Physiol.* **191**, 105-115 (2002).
17. Kroll, D.J., Borgert, C.J., Wiedmann, T.W. & Rowe T.C. Drug sensitivity of heat-resistant mouse B16 melanoma variants. *Radiat Res.* **124**, 15-21 (1990).
18. Yoshida, M. *et al.* Methotrexate suppresses inflammatory agonist induced interleukin 6 synthesis in osteoblasts. *J. Rheumatol.* **32**, 787-795 (2005).
19. Notenboom, S. *et al.* Short-term exposure of renal proximal tubules to gentamicin increases long-term multidrug resistance protein 2 (abcc2) transport function and reduces nephrotoxicant sensitivity. *J. Pharmacol Exp. Ther.* **315**, 912-920 (2005).
20. Guo, J., Chu, M., Abbeyquaye, T. & Chen, C.Y. Persistent nicotine treatment potentiates amplification of the dihydrofolate reductase gene in rat lung epithelial cells as a consequence of Ras activation. *J. Biol. Chem.* **280**, 30422-30431 (2005).
20. Caetano, N.N., Campello, A.P., Carnieri, E.G., Kluppel, M.L. & Oliveira M.B. Effect of methotrexate (MTX) on NAD (P)+ dehydrogenases of HeLa cells: malic enzyme, 2-oxoglutarate and isocitrate dehydrogenases. *Cell Biochem. Funct.* **15**, 259-264 (1997).