



Profiling of the dichloromethane-induced proteome expression changes

Seul-Ki Park and Mi-Young Lee*

Department of Medical Biotechnology, SoonChunHyang University, Asan, Chungnam 336 600, Korea

*Corresponding Author E-mail: miyoung@sch.ac.kr

Publication Info

Paper received:
25 May 2012

Revised received:
08 November 2012

Accepted:
24 December 2012

Abstract

A colorless volatile liquid dichloromethane (DCM) is used as solvents in chemical manufacturing processes. The major route of exposure is via inhalation and to a lesser extent through the skin and digestive tract. We investigated the effects of DCM on rats and analyzed their liver proteome expression changes. Approximately 1,100 protein spots that were detected by 2-dimensional gel electrophoresis showed reproducible abundance. Mass spectrometry based proteomics was used to characterize the changes in the liver proteome in response to DCM exposure. Consequently, 7 of these spots showed significant changes in expression level after DCM treatment. These proteins were 3 paralogues of glutathione S-transferase, beta 1 globin, 2 hemoglobin beta-2 and alpha-2 globulin. Of these, the expression of alpha-2 globulin was also confirmed by western blot. The differential expression of these proteins might be caused by DCM exposure.

Key words

Dichloromethane, Liver protein, Proteomics, Rat

Introduction

Dichloromethane, also called methylene chloride (CH_2Cl_2), is a volatile and colorless liquid having high diluting capacity. DCM is widely used in various industrial processes, such as paint stripping, metal cleaning and degreasing, pharmaceutical manufacturing, and solvent distribution and formulation (Kim *et al.*, 2007). The volatility has led to its use as an aerosol spray propellant and as a blowing agent for polyurethane foams. The International Agency for Research on Cancer reported that DCM is a possible carcinogen (group 2B) (IARC, 1987). DCM has also been used as a solvent in the production of hops, flavorings and caffeine in coffee. However, due to health concerns, DCM's use as an extraction solvent in food products and coffee has declined greatly over the years.

Occupational exposure to DCM has been associated with numerous adverse health effects on the central nervous system and the reproductive system, with liver and kidney toxicity, and

with carcinogenicity (Chellman *et al.*, 1986; Starr *et al.*, 2006). However, the carcinogenic risk posed to human health is uncertain due to lack of evidence from human studies because the carcinogenicity of DCM has been based only on studies in mammalian systems (Preston and William 2005; Watanabe *et al.*, 2007). For example, exposure to high doses of DCM increased the incidence of pulmonary and hepatic neoplasm in female and male mice; however, some reports have indicated that this mouse model is not appropriate for human health assessment (Green, 1997).

There are two major metabolic pathways for DCM bio transformation. The first pathway is detoxification process that begins with the oxidation of DCM by cytochrome P450 2E1 (CYP2E1). The second pathway is glutathione S-transferase theta 1 (GSTT1) catalysis, which includes the nucleophilic substitution of the thiol group of glutathione (GSH) by the halogen chloride of DCM. CYP2E1 activity on DCM produces mainly oxidized compounds, such as CO. Although CO has not been

proved to be genotoxic by itself, it induces cellular toxicity. At high concentrations, DCM is mostly metabolized by GSTT1, and this reaction produces S-haloalkyl glutathione and formaldehyde, which are conjugates that are more reactive than the parent compound (Olvera-Bello *et al.*, 2010).

The transformation of dichloromethane to carbon monoxide with subsequent formation of carboxyhemoglobin has already been reported (Ehler *et al.*, 2011). Limited information on the molecular and cellular level investigation about the toxicity of DCM is available, although a variety of studies have been performed detailing the monitoring and occupational studies. In the present study, differentially expressed proteins in rat livers following exposure to DCM were identified by proteomic tools. The differentially expressed proteins might be useful to discover biomarker candidate for DCM exposure

Materials and Methods

Reagents and treatment of animals : Dichloromethane (DCM) [CAS No. 75-09-2] ultrapure reagent was obtained from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were reagent grade and used without further purification. Healthy adult male Sprague-Dawley rats were housed at 7 rats/cage in a temperature- and light-controlled environment at $23\pm 2^{\circ}\text{C}$ with a 12 h light/dark cycle, and allowed to acclimate for 7 days before dosing. They were fed with commercial rat diet and drinking water ad libitum. For dosing, a single dose of DCM equivalent to 10% (160 mg kg^{-1}) and 50% (800 mg kg^{-1}) of the LD_{50} was dissolved in corn oil and administered orally (Berman *et al.*, 1995; Wirkner *et al.*, 1997). The vehicle treated rats received the same volume of corn oil. The rats were sacrificed 72 hr after treatment, and their livers were flushed to eliminate excess blood content by passage of a cold NaCl solution through the hepatic vein. Animal usage as part of this study was reviewed and approved by the laboratory Institutional Animal Care and Use Committee. To get total protein, the liver tissue was removed through a midline abdominal incision, frozen in liquid nitrogen and stored until analysis.

Preparation of liver samples: Livers were solubilized using tip-probe sonication in modified lysis buffer [7 M urea, 2 M thiourea, 4.5% (w/v) CHAPS, 40 mM Tris, 100 mM DTE] containing a protease inhibitor cocktail as reported previously (Park *et al.*, 2010). The solubilized tissue was centrifuged at $12,000 \times g$ for 50 min at 15°C . The supernatant fractions were transferred to new tubes, assayed for protein concentration, aliquoted, and stored at -70°C until analyzed. Pooled samples were created by combining equal amounts of total protein from each sample within a given treatment group.

Two dimensional gel electrophoresis and image analysis: Proteins were separated by charge in the first dimension using isoelectric focusing (IEF). Rat liver proteins were dissolved in a rehydration solution [7 M urea, 2 M thiourea, 4.5% (w/v) CHAPS,

40 mM Tris, 100 mM DTE, 0.25% IPG buffer (pH 3-10 nonlinear)] and aliquots of pooled protein (1 mg) in a total volume of 450 μl were loaded, and then incubated overnight in a reswelling tray. Focusing was performed following steps: 200 V/200 Vh, 500 V/500 Vh, 1,000 V/1,000 Vh, 8,000 V/13,500 Vh, 8,000 V/100,000 Vh at 20°C . After isoelectric focusing, the individual strips were soaked twice for 20 min in equilibration buffer [6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 5 mM tributylphosphine, 2.5% acrylamide, 0.01% bromophenol blue, 0.375 M Tris-HCl (pH 8.8)]. The second-dimensional SDS-PAGE was performed using 8-16% gradient gel, and was run at 10°C for 2 hr at 10 mA/gel, and then at 30 mA/gel; the current was stopped when the dye front began to exit the gel (Jeon *et al.*, 2011).

The gels were stained with colloidal coomassie brilliant blue by incubation of the gels in staining solution [34% methanol, 0.1% CBB G-250, 17% ammonium sulfate, 3% phosphoric acid] and the destained in 5% glacial acetic acid. The gels were scanned using a PowerLook 1100 flatbed UMAX scanner, and data were analyzed Image Master 2D Platinum 6.0 software (GE Healthcare Bio-Science, Piscataway, NJ, USA) (Park *et al.*, 2012).

Enzymatic in-gel digestion and mass spectrometry: Protein analysis was performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry on an Ettan MALDI-TOF instrument (Amersham Biosciences, Piscataway, NJ, USA). Proteins were subjected to in-gel trypsin digestion. Differentially expressed protein spots were excised from the gel. Gel spots were destained with 100 μl of destaining solution (50% methanol in 10% acetic acid) by shaking for 5 min. After removal of the solution, gel pieces were washed twice with 25 mM ammonium bicarbonate (pH 8.2) and 50% (v/v) acetonitrile (ACN), and then dehydrated by the addition of 100% ACN. 50 mM ammonium bicarbonate containing 0.2 mg modified trypsin was added to each gel piece and the digestion was performed overnight at 30°C . The peptide solution was automatically desalted, concentrated, and spotted onto Axima MALDI-TOF target plates (Kratos, Manchester, UK).

Tryptic peptides derived from protein spots were analyzed and amino acid sequences were deduced using a de novo sequencing program, PepSeq (Gippsland, Victoria, Australia). NCBI nr and EST databases using the PROFOUND search program (http://139.85.19.192/profound_bin/web_ProFound.exe) and BLAST were used in order to identify the proteins (Park *et al.*, 2011).

Immunoblot analysis: Alpha-2 globulin was selected for immunoblot analysis to verify the MALDI-TOF results. Rat liver proteins were denatured and resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, incubated overnight with mouse anti-rat alpha-2

globulin antibody at 4°C. The membrane was washed three times with TBS/0.05% Tween-20, incubated with horseradish peroxidase-conjugated goat-anti mouse IgG for 1 hr at room temperature, and washed three times for 30 min. The membranes were visualized using the ECL plus Western blotting detection system (Amersham Biosciences, Piscataway, NJ, USA) (Kim *et al.*, 2009).

Results and Discussion

Liver plays a major role in xenobiotic metabolism, chemical toxicity and bioavailability of metabolism (Ding and Kaminsky, 2003). 2-dimensional gel electrophoresis (DE) and mass spectrometry (MS) are powerful proteomic tools that may be used to examine the effects of chemical toxicants on protein expression profiles since they allow simultaneous measurement and comparison of the expression levels of various proteins. An important issue in the mechanisms of xenobiotic metabolism and chemical toxicity is whether cytochrome P450 enzymes expressed in various extrahepatic target tissues are critical for the tissue-selective toxicity of chemical compounds (Gu *et al.*, 2005).

A lethal dose (LD) is an indication of the lethality of given chemicals. The most commonly used LD₅₀ is a standard measurement of acute toxicity that represents the individual dose required to kill 50 % of a population of test animals. Lower the LD₅₀ dose, more toxic is the chemical. Liver proteins treated with (at 10% (160 mg kg⁻¹) and 50% (800 mg kg⁻¹) of its LD₅₀) or without (control) DCM were separated by two-dimensional gel electrophoresis (Fig. 1). Protein abundance is indicated by the relative spot volume. More than 1,100 proteins with pIs between 3 and 10 and relative molecular weights between 6.5 and 206 kDa were detected in triplicate gels of rat liver tissue. Among them, 7 proteins showed notable differences in protein expression compared to the control as shown in Table 1. Following DCM treatment at 160 mg kg⁻¹ and 800 mg kg⁻¹, all of these proteins, named 3 paralogues of glutathione S-transferase, beta 1 globin, 2 hemoglobin beta-2 subunits and alpha-2 globulin, were down-regulated.

Those protein as shown in Fig. 2 are from spot 1 to 7. Spot 1, 2 and 3, identified as glutathione S-transferases (GSTs) were down-regulated by exposure to DCM. Glutathione S-transferase is ubiquitous enzyme in animals and plants and protects tissues against oxidative damage or from toxic products produced during xenobiotic metabolism (Marrs, 1996). GSTs typically function by catalyzing the conjugation of glutathione to a variety of electrophilic and hydrophobic substrates to render the substrate less toxic or to produce a more water-soluble conjugate (Thatcher *et al.*, 2007). This detoxification process is part of a larger sequential three-phase detoxification process with cytochrome P450s and ATP-binding cassette transporters. The phytotoxic compounds detoxified by GSTs may be direct products of microbial or animal attack, or they may be endogenous byproducts of a defense or stress response (Thatcher *et al.*, 2007). The reduced GST level might be the result from liver tissue damage by DCM.

The hemoglobin molecule consists of four globular protein subunits. Each subunit is composed of a protein chain tightly associated with a non-protein heme group. Each protein chain arranges into a set of alpha-helix structural segments connected together in a globin fold arrangement. This folding pattern contains a pocket which strongly binds the heme group. A heme group consists of an iron ion held in a heterocyclic ring, known as a porphyrin (Keel *et al.*, 2008). Iron ion, which forms the site of oxygen binding, bonds with four nitrogens at the center of the ring, which all lie in one plane (Bassem *et al.*, 2007). Hemoglobin's oxygen-binding capacity is decreased in the presence of carbon monoxide because both gases compete for the same binding sites on hemoglobin, carbon monoxide binding preferentially in place of oxygen. Carbon dioxide occupies a different binding site on the hemoglobin (Park *et al.*, 2011). Spot 4, which was down-regulated by DCM, was identified as beta-1-globin and spot 5 and 6 were identified as hemoglobin beta-2 subunit. The expression changes of the globin and hemoglobin subunit by DCM might be related with subsequent formation of carboxyhemoglobin through carbon monoxide generated from DCM metabolism binding to hemoglobin (Ehler *et al.*, 2011).

Table 1 : List of differentially expressed proteins from rat liver following dichloromethane treatment

Spot No.	Identified protein	Accession No.	Cov %	pI	Molecular weight	Change	
						160 mg kg ⁻¹	800 mg kg ⁻¹
1	Chain a, glutathione transferase mutant Y115f	29726512	47	8.6	25920	–	–
2	Glutathione S-transferase, mitochondrial	31077128	69	9.13	25493	–	–
3	Glutathione S-transferase alpha-2 (Glutathione S-transferase Ya-2) (GST Ya2) (GST 1b-1b) (GSTA2-2)	121713	50	8.89	25559	–	–
4	Beta-1 globin	546056	82	7.98	15834	–	–
5	Similar to hemoglobin beta-2 subunit (Hemoglobin beta-2 chain) (Beta-2-globin) (Hemoglobin beta chain, minor-form)	109459168	87	8.91	15982	–	–
6	Similar to hemoglobin beta-2 subunit (Hemoglobin beta-2 chain) (Beta-2-globin) (Hemoglobin beta chain, minor-form)	109459168	82	8.91	15982	–	–
7	Alpha-2 globulin	204264	46	5.4	17340	–	–

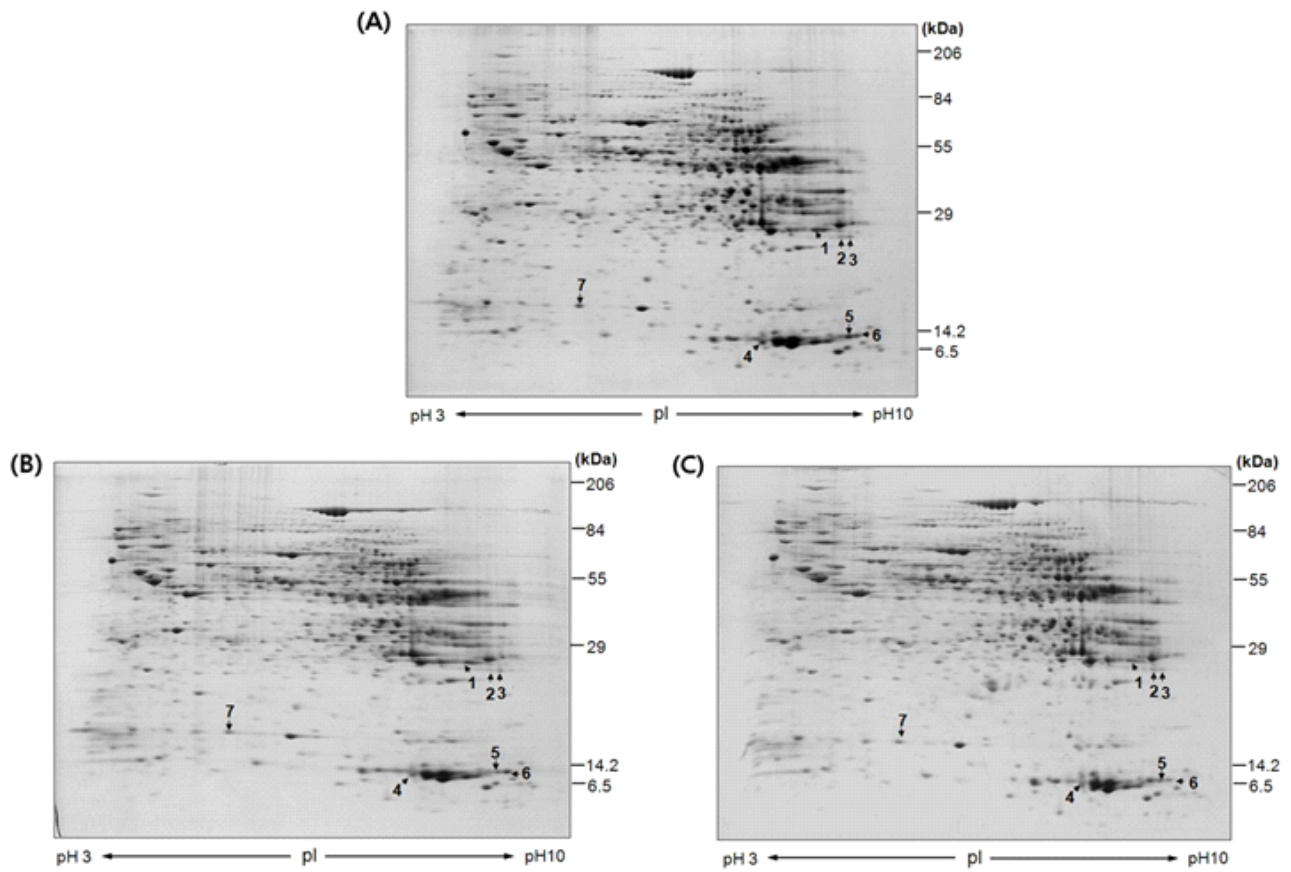


Fig. 1 : 2-dimensional gel electrophoresis representative images of differentially expressed rat liver proteins by dichloromethane. (A) control, (B) 160 mg/kg dichloromethane (C) 800 mg/kg dichloromethane

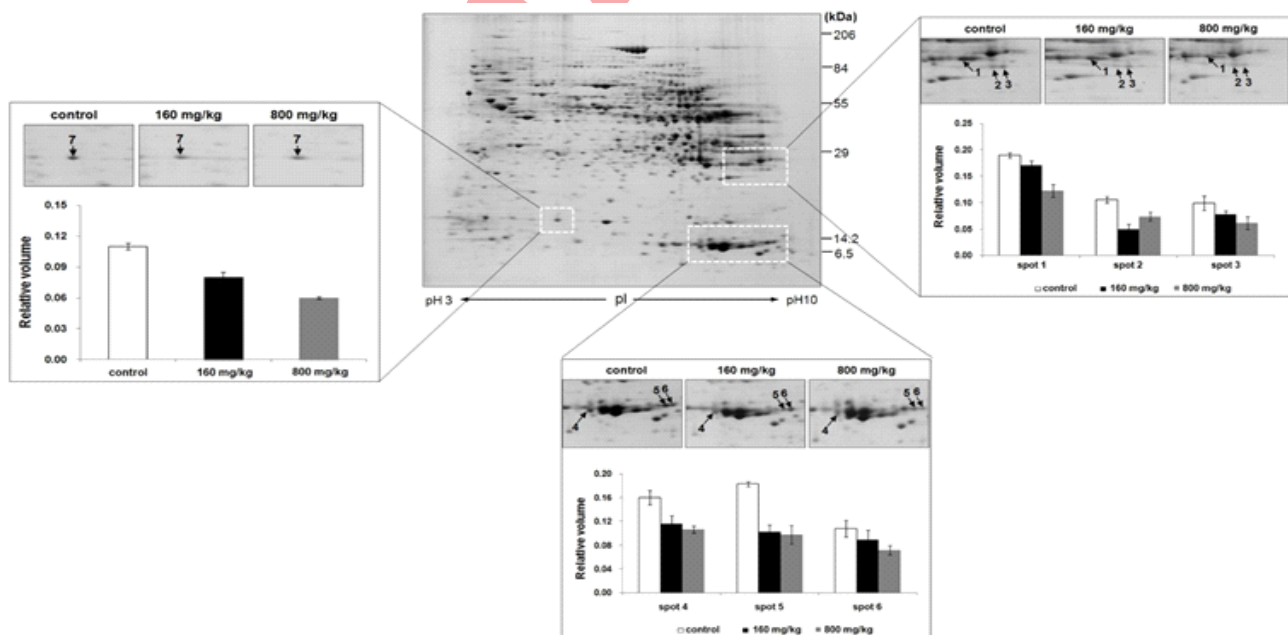


Fig. 2 : An enlarged portion of the map showing differentially expressed proteins by dichloromethane treatment in rat liver. Protein expression levels were determined by relative intensity using image analysis. Normalized spot intensities of the dichloromethane versus control group were compared. Mean spot intensities on individual gels are shown. $P < 0.05$ by Student's t-test

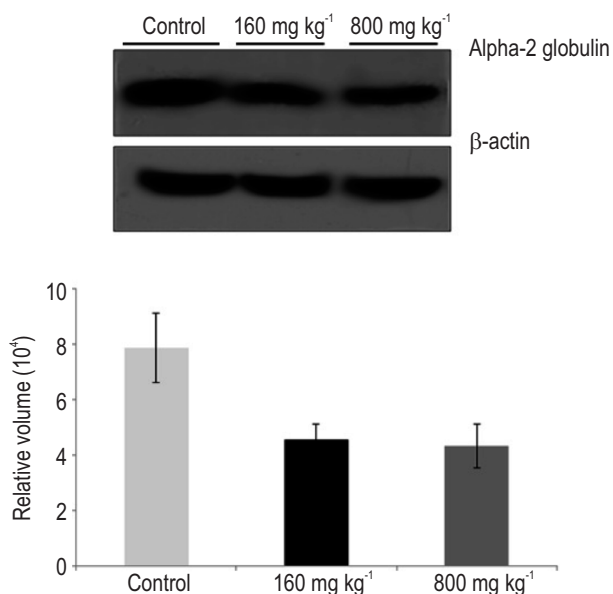


Fig. 3 : Validation of alpha-2 globulin expression by western blot analysis. Protein expression levels were determined by relative intensity against β -actin using image analysis

The expression of alpha-2 globulin, which has been known as a tumor risk factor, was down-regulated by DCM. This protein is a major urinary protein in adult male rats. The secreted urinary protein is synthesized mainly in the male rat liver. It is also synthesized in other tissues such as the salivary gland, lachrymal gland and preputial gland of both male and female rats (Soares *et al.*, 1987). Alpha-2u globulin is synthesized in liver of male but not in female rats, secreted into the bloodstream and excreted in urine. It binds pheromones that are released from drying urine and affect the sexual behavior of females. There are number of chemicals that induce toxic syndrome in male rats referred to as α 2u-globulin nephropathy (Wang *et al.*, 1997). This organ-specific toxicity is characterized by accumulation of protein droplets in the proximal tubules. It was suggested that these droplets might be formed due to association between the chemical and α 2u protein. High doses of lovastatin strongly decreased the abundance of alpha-2 globulin in liver (Steiner *et al.*, 2000).

To examine whether down-regulation of alpha-2 globulin expression can also be detected by immunological method, western blot analysis using alpha-2 globulin-specific antibody was performed (Fig. 3). The result indicated that alpha-2 globulin was present at considerably high level in vehicle-treated liver, while its expression was at low level in DCM-exposed liver. The result substantiates the down-regulation of alpha-2 globulin expression in rat liver in response to DCM exposure.

In conclusion, the present study revealed that 7 spots were differentially expressed after DCM treatment in rat liver. The investigation might be helpful to understand DCM-induced

dynamic changes in protein expression, and to unravel the underlying mechanism associated with hepatotoxicity in rats. The identified protein signatures may act as a candidate of biomarker panel for DCM exposure.

Acknowledgment

This work was supported in part by the SoonChunHyang University research grant

References

- Bassem, M. R., W. A. Samir and S. Nahed: Hemoglobin oxygen affinity of Egyptian obese smokers suffering from elevated non-functional hemoglobin derivatives. *World J. Zool.*, **2**, 9-14 (2007).
- Berman, E., M. Schlicht, V. C. Moser and R. C. MacPhail: A multidisciplinary approach to toxicological screening: I. systemic toxicity. *J. Toxicol. Environ. Hlth.*, **45**, 127-143 (1995).
- Chellman, G. J., R. D. White, R. M. Norton and J. S. Bus: Inhibition of the acute toxicity of methyl chloride in male B6C3F1 mice by glutathione depletion. *Toxicol. Appl. Pharmacol.*, **86**, 93-104 (1986).
- Ding, X. and L. S. Kaminsky: Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annu. Rev. Pharmacol. Toxicol.*, **43**, 149-173 (2003).
- Ehler, E., J. Latta, A. Eichlerová, M. Mrklovský and P. Urban: Exposure to iodomethane and dichloromethane associated with a confusional state. *Neurotoxicology*, **32**, 307-311 (2011).
- Green, T.: Methylene chloride induced mouse liver and lung tumors: an overview of the role of mechanistic studies in human safety assessment. *Hum. Exp. Toxicol.*, **16**, 3-13 (1997).
- Gu, J., H. Cui, M. Behr, L. Zhang, Q. Y. Zhang, W. Yang, J. A. Hinson and X. Ding: *In vivo* mechanisms of tissue-selective drug toxicity: Effects of liver-specific knockout of the NADPH-cytochrome P450 reductase gene on acetaminophen toxicity in kidney, lung, and nasal mucosa. *Mol. Pharmacol.*, **67**, 623-630 (2005).
- International Agency for Research on Cancer. Overall evaluations of carcinogenicity: an updating of IARC monographs volumes 1-42 (IARC Monographs on the Evaluation of Carcinogenic Risks to Human, Suppl. 7). Lyon, 194-195 (1987).
- Jeon, Y. M., S. K. Park, W. J. Kim, J. H. Ham and M. Y. Lee: The effects of TiO₂ nanoparticles on the protein expression in mouse lung. *Mol. Cell. Toxicol.*, **7**, 283-289 (2011).
- Keel, S. B., R. T. Doty, Z. Yang, J. G. Quigley, J. Chen, S. Knoblauch, P. D. Kingsley, I. De Domenico, M. B. Vaughn, J. Kaplan, J. Palis and J. L. Abkowitz: A heme export protein is required for red blood cell differentiation and iron homeostasis. *Sci.*, **319**, 825-828 (2008).
- Kim, J. J., Y. H. Kim and M. Y. Lee: Proteomic characterization of differentially expressed proteins associated with no stress in retinal ganglion cells. *BMB Rep.*, **42**, 456-461 (2009).
- Kim, S. N., J. Y. Seo, Da W. Jung, M. Y. Lee, Y. S. Jung and Y. C. Kim: Induction of hepatic CYP2E1 by a subtoxic dose of acetaminophen in rats: increase in dichloromethane metabolism and carboxyhemoglobin elevation. *Drug Metab. Dispos.*, **35**, 1754-1758 (2007).
- Marrs, K. A.: The functions and regulation of glutathione S-transferases in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **47**, 127-158 (1996).

- Olvera-Bello, A. E., E. Estrada-Muñiz, G. Elizondo and L. Vega: Susceptibility to the cytogenetic effects of dichloromethane is related to the glutathione S-transferase theta phenotype. *Toxicol. Lett.*, **199**, 218-224 (2010).
- Park, S. K., J. B. Seo and M. Y. Lee: Proteomic profiling of hempseed proteins from Cheungsam. *Biochem. Biophys. Acta*, **1824**, 374-382 (2012).
- Park, S. K., S. W. Nam and M. Y. Lee: Ethylbenzene-induced differential protein profiles in rat liver. *BioChip J.*, **4**, 272-278 (2010).
- Park, S. K., Y. M. Jeon, B. S. Son, H. S. Youn and M. Y. Lee: Proteomic analysis of the differentially expressed proteins by airborne nanoparticles. *J. Appl. Toxicol.*, **31**, 463-470 (2011).
- Park, S. K., Y. N. Seo, S. W. Nam and M. Y. Lee: Hepatic proteome responses against benz[a]anthracene in the rat. *BioChip J.*, **5**, 309-316 (2011).
- Preston, R. J. and G. M. Williams: DNA-reactive carcinogens: mode of action and human cancer hazard. *Crit. Rev. Toxicol.*, **35**, 673-683 (2005).
- Soares Vda, C., R. M. Gubits, P. Feigelson and F. Costantini: Tissue-specific and hormonally regulated expression of a rat alpha 2u globulin gene in transgenic mice. *Mol. Cell. Biol.*, **7**, 3749-3758 (1987).
- Starr, T. B., G. Matanoski, M. W. Anders and M. E. Andersen: Workshop overview: reassessment of the cancer risk of dichloromethane in humans. *Toxicol. Sci.*, **91**, 20-28 (2006).
- Steiner, S., C. L. Gatlin, J. J. Lennon, A. M. McGrath, A. M. Aponte, A. J. Makusky, M. C. Rohrs and N. L. Anderson: Proteomics to display lovastatin-induced protein and pathway regulation in rat liver. *Electrophoresis*, **21**, 2129-2137 (2000).
- Thatcher, L. F., C. Carrie, C. R. Andersson, K. Sivasithamparam, J. Whelan and K. B. Singh: Differential gene expression and subcellular targeting of *Arabidopsis* glutathione S-transferase F8 is achieved through alternative transcription start sites. *J. Biol. Chem.*, **282**, 28915-28928 (2007).
- Wang, K. S., D. A. McFadyen, J. Locke and R. B. Hodgetts: Three subsets of genes whose tissue specific expression is sex and age-dependent can be identified within the rat a2u-globulin family. *Dev. Genet.*, **21**, 234-244 (1997).
- Watanabe, K., R. G. Liberman, P. L. Skipper, S. R. Tannenbaum and F. P. Guengerich: Analysis of DNA adducts formed *in vivo* in rats and mice from 1,2-dibromomethane, 1,2-dichloromethane, dibromomethane, and dichloromethane using HPLC/accelerator mass spectrometry and relevance to risk estimates. *Chem. Res. Toxicol.*, **20**, 1594-1600 (2007).
- Wirkner, K., B. Damme, W. Poelchen and D. Pankow: Effect of long-term ethanol pretreatment on the metabolism of dichloromethane to carbon monoxide in rats. *Toxicol. Appl. Pharmacol.*, **143**, 83-88 (1997).

Online