

New Isocyanate-Specific Albumin Adducts of 4,4'-Methylenediphenyl Diisocyanate (MDI) in Rats

Anoop Kumar, Nagaraju Dongari, and Gabriele Sabbioni*

Department of Environmental Health Sciences, School of Public Health and Tropical Medicine,
Tulane University, 1440 Canal Street, Suite 2100, New Orleans, Louisiana 70112

Received August 5, 2009

4,4'-Methylenediphenyl diisocyanate (MDI) is the most important of the isocyanates used as intermediates in the chemical industry. Among the main types of damage after exposure to low levels of MDI are lung sensitization and asthma. Albumin adducts of MDI might be involved in the etiology of sensitization reactions. It is, therefore, necessary to have sensitive and specific methods for monitoring the isocyanate exposure of workers. To date, urinary metabolites or protein adducts have been used as biomarkers in workers exposed to MDI. However, with these methods it is not possible to determine whether the biomarkers result from exposure to MDI or to the parent aromatic amine 4,4'-methylenedianiline (MDA). This work presents a procedure for the determination of isocyanate-specific albumin adducts. In a long-term experiment, designed to determine the carcinogenic and toxic effects of MDI, rats were exposed chronically for 3 months, to 0.0 (control), 0.26, 0.70, and 2.06 mg MDI/m³ as aerosols. Albumin was isolated from plasma, digested with Pronase E, and analyzed by LC-MS/MS. MDI formed adducts with lysine: N⁶-[({4-[4-aminobenzyl]phenyl}amino)carbonyl]lysine (MDI-Lys) and N⁶-[({4-[4-(acetylaminobenzyl)phenyl]amino)carbonyl]lysine (AcMDI-Lys). For the quantitation of the adducts in vivo, isotope dilution mass spectrometry was used to measure the adducts in 2 mg of albumin. The adducts found in vivo (MDI-Lys and AcMDI-Lys) and the corresponding isotope labeled compounds (MDI-[¹³C₆¹⁵N₂]Lys and Ac[²H₄]MDI-Lys) were synthesized and used for quantitation. The MDI-Lys levels increased from 0–24.8 pmol/mg albumin, and the AcMDI-Lys levels increased from 0–1.85 pmol/mg albumin. The mean ratio of MDI-Lys/AcMDI-Lys for each dose level was greater than >20. The albumin adducts correlate with other biomarkers measured in the same rats in the past: urinary metabolites and hemoglobin adducts released after mild base hydrolysis. This method will enable one to measure isocyanate-specific albumin adducts in workers. This new biomonitoring procedure will allow for the assessment of suspected exposure sources and may contribute to the identification of individuals who are particularly vulnerable for developing bronchial asthma and other respiratory diseases after exposure to isocyanates. In addition, it will help to improve the production of antigens for the analysis of antibodies in exposed workers.

Introduction

Isocyanates are highly reactive compounds that have a variety of commercial applications. Diisocyanates such as 4,4'-methylenediphenyl diisocyanate (MDI¹), are increasingly used for manufacturing polyurethane foam, elastomers, paints, adhesives, coatings, and insecticides, and consolidation of loose rock zones in coal mining or tunneling, and many other products (1). The worldwide annual production of diisocyanates is estimated to be more than 6 million tons. The high chemical reactivity of diisocyanates makes them toxic. A number of adverse effects at the cellular and subcellular level have been reported, such as

irritative and immunological reactions. Inhalation of diisocyanate vapors is associated with various pulmonary ailments, such as eosinophilic airway inflammation, airway hyper-reactivity, early and late-onset asthma, exogenous allergic alveolitis, and direct toxic responses (2–6). Diisocyanates are of great concern with regard to environmental and occupational health, being considered one of the main causes of occupational asthma (2, 7, 8). The steady rise in asthma over the past decades points strongly to the potential relationship between isocyanates in consumer products and increasing prevalence of asthma in the general population, especially children (9). The prevalence and incidence of diisocyanate-induced disorders depend on the degree of exposure. Occupational exposure to diisocyanates may take place during their production and application in the production of polyurethane foam and other products containing monomeric or polymeric diisocyanates. The predominant route of occupational exposure is through inhalation.

Arylisocyanates react directly with biomolecules and/or hydrolyze to arylamines. Arylisocyanates (10, 11) and arylamines (12, 13) can bind with proteins and/or DNA (Figure 1) and lead to cytotoxic and genotoxic effects. Protein adducts are believed to be involved in the etiology of sensitization reactions (14, 15). Arylamines are metabolized to highly reactive *N*-

* Corresponding author. Tel/Fax: (504) 9882771. E-mail: gabriele.sabbioni@bluewin.ch.

¹ Abbreviations: AcMDA, N¹-[4-(4-aminobenzyl)phenyl]-acetamide; Ac[²H₄]-MDA, N¹-[4-(4-amino-3,5-dideuteriobenzyl)-2,6-dideuteriophenyl]acetamide; AcMDI-Lys, N⁶-[({4-[4-(acetylaminobenzyl)phenyl]amino)carbonyl]lysine; Ac[²H₄]MDI-Lys, N⁶-[({4-[4-(acetylaminobenzyl)-2,6-dideuteriophenyl]amino)carbonyl]lysine; BocMDA, *tert*-butyl [4-(4-aminobenzyl)phenyl]carbamate; BocMDI, *tert*-butyl [4-(4-isocyanatobenzyl)phenyl]carbamate; MDA, 4,4'-methylenedianiline; MDI, 4,4'-methylenediphenyl diisocyanate; MDI-Lys, N⁶-[({4-[4-aminobenzyl]phenyl}amino)carbonyl]lysine; MDI-[¹³C₆¹⁵N₂]Lys, N⁶-[({4-[4-aminobenzyl]phenyl}amino)carbonyl] [¹³C₆¹⁵N₂]lysine; MDI-Val-Hyd, 3-[4-(4-aminobenzyl)phenyl]-5-isopropyl-1,3-imidazolidine-2,4-dione; N_α-Boc-Lys, N_α-(*tert*-butoxycarbonyl)-L-lysine.

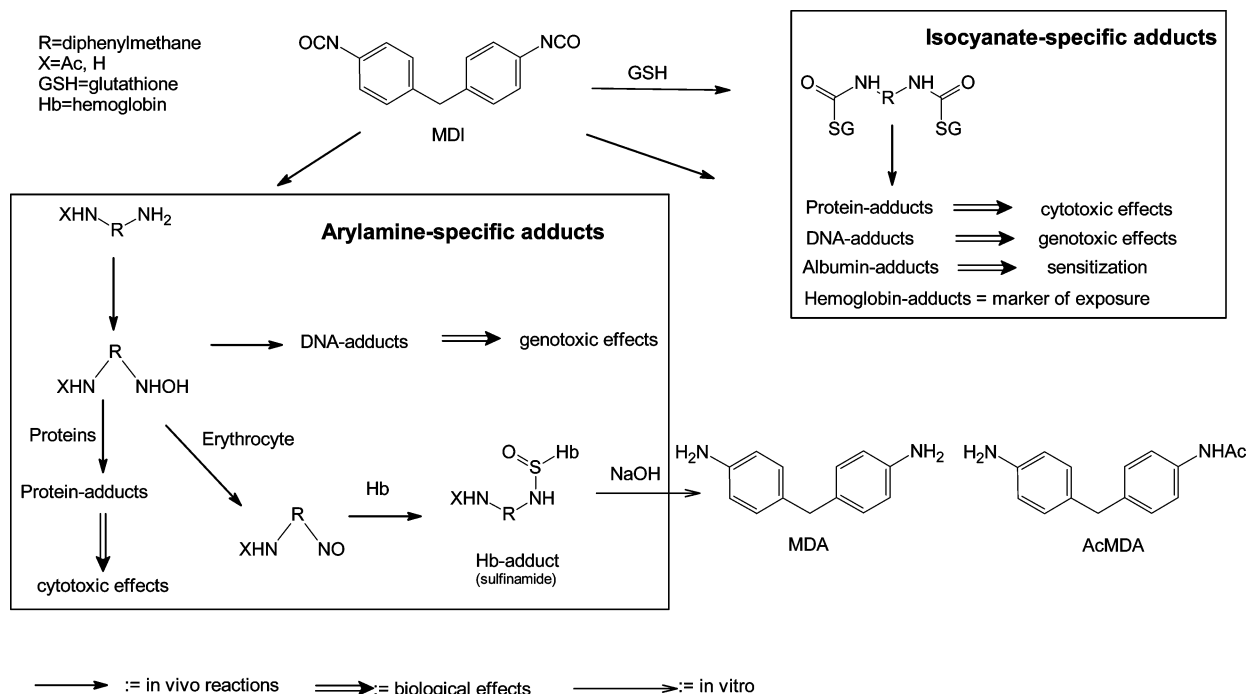


Figure 1. Metabolism and possible reaction products of MDI.

hydroxy arylamines (12) by mixed function monooxygenases. *N*-Hydroxy arylamines can be further metabolized to reactive conjugates, which are responsible for the genotoxic and cytotoxic effects of this class of compounds. In exposed animals, arylamines such as 4-aminobiphenyl (16), a human bladder carcinogen, are known to form adducts with DNA, with tissue proteins, and with the blood proteins albumin and hemoglobin in a dose-dependent manner. Arylamine-specific adducts are of the sulfonamide type (17, 18), which can be cleaved with mild base hydrolysis. The methods of identification of such adducts are well established (13, 19–21).

In contrast, isocyanates do not need any further activation to react with biomolecules (Figure 1). Important vehicles for isocyanates are their reaction products with glutathione (22, 23). The glutathione adducts release the isocyanate moiety to react with other nucleophiles, e.g., proteins. Therefore, glutathione adducts are thought to be responsible for the transport of isocyanate to reactive sites away from the site of isocyanate uptake. Isocyanates can react with the following amino acids under physiological conditions (4, 24): the α -amino group of the *N*-terminal amino acids, the sulfhydryl group of cysteine, the hydroxyl groups of tyrosine and especially serine, the ϵ -amino group of lysine, and the imidazole ring of histidine. 4-Methylphenyl isocyanate adducts with the α -amino group of valine and aspartic acid, with the ϵ -amino group of lysine, with the hydroxyl of serine and tyrosine, and with the sulfur of cysteine have been synthesized and characterized by NMR and MS (25). The chemical stability was tested under different conditions. Carbamylation products with cysteine and tyrosine can be hydrolyzed with base under mild conditions (25). For the products with the other amino acids boiling with acid or base is needed to cleave the products.

Urinary metabolites of MDA have been found in rats exposed chronically to MDI (26). Arylamine-specific adducts with hemoglobin were found in rats exposed chronically to MDI (26). Isocyanate-specific adducts with the *N*-terminal valine of hemoglobin with MDI (27) and 2,4-toluenediisocyanate (28) have been quantified in rats and in humans, respectively. The DNA adducts (29) of the nasal epithelial cell and the hemoglobin

adducts (26) correlate with the dose (11) in rats chronically exposed to MDI. In rats, hemoglobin adducts have been found to correlate with the administered dose of either MDI (26) or the corresponding arylamine MDA (30). MDA binds to hemoglobin (30) and to DNA (31). In globin of rats exposed to MDI, hydantoin (MDI-Val-Hyd) has been found in a dose-dependent manner (27).

Isocyanate-specific adducts of MDI with albumin have not been identified *in vivo* up to date. For the present project, we developed a method to find isocyanate-specific adducts with albumin, which can be applied to human samples. Albumin is one of the potential targets involved in the etiology of sensitization reactions (14–15). Therefore, albumin adducts would be a marker of exposure and a marker which is related to the mechanism of sensitization caused by isocyanates.

Material and Methods

Caution: The aromatic amines used in this work are potentially carcinogenic. Isocyanates are strong irritants. Triphosgene is severely irritating to the eyes and skin. Avoid contact and inhalation. All these compounds should be handled with protective clothing in a well-ventilated fume hood.

Animal Experiment. The animal experiment was published previously. The inhalation studies were performed at the Fraunhofer Institute in Hannover (26, 27, 29, 32). Female Wistar rats (CrI:[WI]BR, Charles River WIGA GmbH, Sulzfeld) were used for all experiments.

Chronic Exposure. The rats were exposed for 17 h per day, 5 days per week over 3 months. The concentrations of MDI in the inhalation chambers measured were 0.26, 0.70, and 2.06 mg/m³. Ten rats from each dose group and from the control group were sacrificed after 3 months of exposure.

Single Exposure. A single high dose experiment with 20 mg/m³ for 6 h was carried out with the same strain of rats and exposure protocol (whole body exposure) used in the chronic experiment. Groups of five animals were sacrificed immediately after exposure, 1 day and 7 days after exposure. Urine was collected for 24 h prior to sacrifice, from the animals sacrificed 1 and 7 days after exposure.

Chemicals. Amicon ultra centrifugal filter tube (30K MWC; 4 mL) (#UFC803096), methanol (A454-4) for sample preparation,

and methanol (Optima, A456-4) for LC-MS/MS were obtained from Fisher Scientific (New Jersey, USA). 4,4'-Methylenedianiline (#32950), triphosgene (#15217), Pronase E from *Streptomyces griseus* (#81748), ammonium formate (#17843), sodium sulfide hydrate puriss. p.a. (32–38%) (#71975), and sodium hydroxide (#71687) were purchased from Fluka (Buchs, Switzerland). The reagent grade tris(hydroxymethyl)amino-methane (#252859), sodium phosphate monobasic monohydrate (#P0662), sodium thiocyanate (#251410), $^2\text{H}_6$]DMSO, dry 1,4-dioxane (#296309), N_α -(*tert*-butoxycarbonyl)-L-lysine (#15456), L- $^{13}\text{C}_6^{15}\text{N}_2$]lysine hydrochloride (#608041), copper(II) carbonate basic (#207896), trifluoroacetic acid (#T62200), di-*tert*-butyl dicarbonate (Reagent plus 99%) (#205249), human serum albumin (#A1653), and water for LC-MS/MS (Chromasolv; #39253) were purchased from Sigma-Aldrich (St. Louis, MO). Silicagel 60 (70–230 mesh ASTM) was obtained from Merck (Darmstadt, Germany). Coomassie plus protein assay reagent (#23236) for protein determination was acquired from Thermo Scientific (Rockford, IL). Strata-X-33u (8B-S100-FBJ) polymeric reversed phase columns (200 mg/3 mL) was purchased from Phenomenex Inc. (Torrance, CA). N^1 -[4-(4-aminobenzyl)phenyl]-acetamide (AcMDA) (31) and N^1 -[4-(4-amino-3,5-dideuteriobenzyl)-2,6-dideuteriophenyl]acetamide (Ac $^{13}\text{C}_6^{15}\text{N}_2$ -MDA) (30) were synthesized as described previously.

Instrumentation. A API 4000 Q Trap (Applied Biosystems, Foster City, CA) mass spectrometer interfaced to a HPLC (Shimadzu Prominence 20AD) was used for LC-MS/MS analyses. HPLC analyses to determine the purity of the compounds were performed on a Hewlett-Packard 1100 system with a quaternary HPLC pump and a photodiode array detector: Lichrosphere RP18 (125 × 4.6 mm, 5 μm) column, with a 20 min 30–80% methanol gradient in ammonium acetate (10 mM), flow rate of 1.0 mL/min, and λ = 250 nm. A Beckman Coulter DU 800 spectrophotometer was used for protein determination. Centrifugations were performed on a Beckman Coulter Allegra X-22R centrifuge equipped with a SX4250 swing out bucket rotor. NMR spectra were recorded on a Bruker AC 500 instrument with $^2\text{H}_6$]DMSO as the solvent and as the internal standard. The degree of substitution of the C atoms was determined using the distortionless enhancement by polarization transfer (DEPT) method. The raw NMR data were processed with the program MestRe-C (Cobas, J. C., Cruces, J., and Sordina, F. J., Magnetic Resonance Companion, Departamento de Química Orgánica, Universidad de Santiago de Compostela, 15706 Santiago de Compostela, Spain).

***tert*-Butyl [4-(4-aminobenzyl)phenyl]carbamate (BocMDA).** 4,4'-Methylenedianiline (MDA) (1.0 g, 5.05 mmol) was dissolved in methanol (5 mL). Di-*tert*-butyl dicarbonate (1.102 g, 5.05 mmol) in methanol (5 mL) was added slowly every 5 min over the period of 1 h. The reaction was stopped after 3 h at 30 °C. BocMDA (70%) and diBocMDA (30%) were present according to HPLC analysis (conditions described above). MDA (t_R = 8.1 min), BocMDA (t_R = 16.3 min), and diBocMDA (t_R = 20.5 min) were obtained. BocMDA (R_f = 0.40) was obtained with 93% purity after silicagel column chromatography with *n*-hexane/ethylacetate (60:40 v/v).

***tert*-Butyl [4-(4-isocyanatobenzyl)phenyl]carbamate (BocMDI).** BocMDA (100 mg, 0.335 mmol) was added to a solution of triphosgene (99.3 mg, 0.335 mmol) and a triethylamine (33.8 mg, 0.335 mmol) mixture in anhydrous 1,4-dioxane (5 mL), and the mixture was stirred for 3 h at 80 °C under nitrogen. This reaction mixture was used without further cleanup for the next steps. According to HPLC analyses, most of the BocMDA (t_R = 16.3 min) was converted into BocMDI (t_R = 18.0 min).

N^6 -[([4-(4-aminobenzyl)phenyl]amino)carbonyl]lysine (MDI-Lys). N_α -(*tert*-Butoxycarbonyl)-L-lysine (N_α -Boc-Lys) (82.4 mg, 0.335 mmol) was dissolved in 0.25 M sodium bicarbonate (7.0 mL, pH 8.3). Hot BocMDI in 1,4-dioxane (5 mL) was added dropwise at 80 °C. After 3 h, the reaction was cooled with ice, filtered, and concentrated in vacuo to approximately 10 mL. The reaction mixture was washed successively with *n*-hexane (3 × 15 mL), dichloromethane (2 × 15 mL), and ethyl acetate (3 × 15 mL). The pooled ethyl acetate extracts were washed with water (2 × 10 mL), dried

on anhydrous sodium sulfate, filtered, and evaporated in vacuo. The residue (39.5 mg, % yield, 97% pure (t_R = 7.6 min) was treated with TFA (400 μL) for 25 min at room temperature. After evaporation of TFA in vacuo, the residue was recrystallized from ethyl acetate. MDI-Lys was isolated as a flax-colored solid with a purity of 98%; (25.1 mg, 97.9%).

^1H NMR ($^2\text{H}_6$]DMSO) δ ppm 8.24 (s, 1H, $\text{NC}_6\text{H}_5\text{NHCO}$), 8.00 (broad s, 3H), 7.26 (d, J = 8.36 Hz, 2H, aromatic H, meta position to NHCONH group), 7.01 (d, J = 8.36 Hz, 2H, aromatic H, ortho position to NHCONH group), 6.88 (d, J = 8.11 Hz, 2H, aromatic H, meta position to NH_2), 6.56 (d, J = 8.11 Hz, 2H, aromatic H, ortho position to NH_2), 6.09–5.98 (m, 1H, $\text{NH}-\text{CH}_2$), 3.94–3.81 (m, 1H, CHCOOH), 3.68 (s, 2H, CH_2), 3.14–3.00 (m, 2H, CH_2N), 2.50 (d, J = 1.61 Hz, 1H), 1.80–1.71 (m, 2H, CH_2 , beta), 1.51–1.37 (m, 4H, CH_2 delta and CH_2 gamma).

^{13}C NMR ($^2\text{H}_6$]DMSO) δ ppm 170.7 (COOH), 155.2 (NH-CONH), 138.2 (C), 134.5 (C), 128.9 (CH), 128.4 (CH), 117.7 (CH), 115.0 (CH), 51.9 (CHCOOH), 39.8 (CH $_2$), 38.6 (CH $_2$), 29.6 (CH $_2$), 29.3 (CH $_2$), 21.6 (CH $_2$), 2 signals missing for the aromatic ring carbons.

ESI-MS (positive) m/z : 785.4 [$3\text{M} + 2\text{Na}$] $^+$, 763.3 [$2\text{M} + \text{Na}$] $^+$, 741.4 [$2\text{M} + \text{H}$] $^+$, 393.3 [$\text{M} + \text{Na}$] $^+$, 371.1 [$\text{M} + \text{H}$] $^+$. MS/MS of m/z 371.1 = 199.0 (61%) [$\text{M}-\text{C}_7\text{H}_{12}\text{N}_2\text{O}_3$] $^+$, 173.1 (100%) [$\text{M}-\text{C}_{13}\text{H}_{15}\text{N}_2$] $^+$, 147.1 (5%) [$\text{M}-\text{C}_{14}\text{H}_{14}\text{N}_2\text{O}$] $^+$.

ESI-MS (negative) m/z : 761.3 [$2\text{M} + \text{Na}$] $^-$, 739.5 [$2\text{M} + \text{H}$] $^-$, 391.3 [$\text{M} + \text{Na}$] $^-$, 369.1 [$\text{M} - \text{H}$] $^-$. MS/MS of 369.1 = 171 (5%) [$\text{M}-\text{C}_{13}\text{H}_{14}\text{N}_2$] $^-$, 145.1 (100%) [$\text{M}-\text{C}_{14}\text{H}_{13}\text{N}_2\text{O}$] $^-$.

UV: λ (nm) = 246 max, 285 shoulder.

N^6 -[([4-(4-aminobenzyl)phenyl]amino)carbonyl] $^{13}\text{C}_6^{15}\text{N}_2$] lysine (MDI- $^{13}\text{C}_6^{15}\text{N}_2$]Lys). A solution of L- $^{13}\text{C}_6^{15}\text{N}_2$] lysine hydrochloride (10 mg, 0.052 mmol) in water (2 mL), 100 μL of NaOH (20 mg/mL), and copper(II) carbonate (11.5 mg, 0.052 mmol) was refluxed for 30 min. The reaction mixture was cooled to room temperature and used for further reaction. Hot BocMDI (15.5 mg, 0.052 mmol) in 1,4-dioxane (1 mL) was added slowly to the copper complex of L- $^{13}\text{C}_6^{15}\text{N}_2$]Lys. After 4 h at room temperature, Na_2S (20 mg) (33) was added and stirred for 10 min. The precipitated cuprous sulfide was eliminated after centrifugation and filtration. The filtrate was washed with ethyl acetate (3 × 3 mL), acidified to pH 4 with 2 M HCl, and extracted again with ethyl acetate (3 × 4 mL). The latter organic phases were dried on anhydrous Na_2SO_4 , filtered, and evaporated under vacuum. BocMDI- $^{13}\text{C}_6^{15}\text{N}_2$]Lys (4.1 mg) was obtained as a white solid and treated with 100 μL of TFA for 25 min. After evaporation of TFA, MDI- $^{13}\text{C}_6^{15}\text{N}_2$]Lys was recrystallized from ethyl acetate to yield (59%) MDI- $^{13}\text{C}_6^{15}\text{N}_2$]Lys of 88% purity.

ESI-MS (positive) m/z : 401.2 [$\text{M} + \text{Na}$] $^+$, 379.1 [$\text{M} + \text{H}$] $^+$. MS/MS of m/z 379.1 = 199.0 (61%) [$\text{M}-^{13}\text{C}_6^{15}\text{N}_2\text{CH}_2^{15}\text{N}_2\text{O}_3$] $^+$, 181.1 (100%) [$\text{M}-\text{C}_{13}\text{H}_{14}\text{N}_2$] $^+$.

ESI-MS (negative) m/z : 377.1 [$\text{M} - \text{H}$] $^-$. MS/MS of 377.1 = 179.0 (4%) [$\text{M}-\text{C}_{13}\text{H}_{14}\text{N}_2$] $^-$, 153.0 (100%) [$\text{M}-\text{C}_{14}\text{H}_{13}\text{N}_2\text{O}$] $^-$.

UV: λ (nm) = 246 max, 285 shoulder.

N^6 -[([4-(4-(acetylamino)benzyl)phenyl]amino)carbonyl]lysine (AcMDI-Lys). N_α -Boc-L-Lys (143 mg; 0.5 mmol) was dissolved in 0.25 M sodium bicarbonate (8.0 mL, pH 8.3). AcMDI (0.5 mmol) synthesized as described in ref 27 in 1,4-dioxane (10 mL) was added slowly at 80 °C. After 3 h at 80 °C, the reaction mixture was cooled to room temperature, filtered, concentrated to approximately 6 mL, and washed with ethyl acetate (3 × 15 mL). The pH of the aqueous phase was adjusted to pH 2. The white precipitate was filtered and dried in a desiccator: (AcMDI- N_α -Boc-Lys, 90 mg, purity 99%, t_R = 13.0 min). AcMDI- N_α -Boc-Lys was treated with TFA (250 μL) for 15 min. After evaporation in vacuo, the solid was recrystallized from ethyl acetate. AcMDI-Lys (49.2 mg, 68.2%) was obtained as a cream-colored solid with a purity of 97% (t_R = 9.0 min).

^1H NMR ($^2\text{H}_6$]DMSO) δ ppm 9.87 (s, 1H), 9.04 (s, 1H), 8.00 (broad s, 2H), 7.46 (d, J = 8.13 Hz, 2H), 7.32 (d, J = 7.97 Hz, 1H), 7.09 (d, J = 8.13 Hz, 2H), 7.01 (d, J = 7.97 Hz, 2H), 6.77 (broad s, 1H), 3.76 (s, 2H), 3.43 (m, 1H), 3.11–2.99 (m, 2 H), 2.01 (s, 3H), 1.84–1.62 (m, 2H), 1.46–1.22 (m, 4H).

ESI-MS (positive) m/z : 847.5[2M + Na]⁺, 825.4 [2M + H]⁺, 435.3[M + Na]⁺, 413.2 [M + H]⁺. MS/MS of m/z 413.2 = 241.2 (100%) [M-C₇H₁₃N₂O₃]⁺, 173.1 (46%) [M-C₁₅H₁₅N₂O]⁺, 147.1 (4%) [M-C₁₆H₁₄N₂O₂]⁺.

ESI-MS (negative) m/z : 823.1 [2M - H]⁻, 411.2[M - H]⁻. MS/MS of 411.2 = 145.1 (100%) [M-C₁₆H₁₅N₂O₂]⁻.

UV: λ (nm) = 250 max.

N^ε-[4-[4-(Acetyl-amino-3,5-dideuteriobenzyl)-2,6-dideuteriophenyl]carbonyl]lysine (Ac^[2H₄]MDI-Lys). (Ac^[2H₄]MDA) (7.6 mg, purity 93%) was synthesized from *N*-1-[4-(4-amino-3,5-dideuteriobenzyl)-2,6-dideuteriophenyl]acetamide (Ac^[2H₄]MDA) (10 mg, 0.041 mmol), triphosgene, and *N_α*-Boc-Lys using the same procedure as that described for the synthesis of AcMDI-Lys.

ESI-MS (positive) m/z : 439.3[M + Na]⁺, 417.1 [M + H]⁺. MS/MS of m/z 417.1 = 245.1 (100%) [M-C₇H₁₃N₂O₃]⁺, 172.9 (50%) [M-C₁₅ [^{2H₄}] H₁₅N₂O]⁺, 147.2 (4%) [M-C₁₆ [^{2H₄}] H₁₀N₂O₂]⁺.

ESI-MS (negative) m/z : 415.1[M - H]⁻. MS/MS of 415.1 = 145.1 (100%) [M-C₁₆ [^{2H₄}] H₁₀N₂O₂]⁻.

UV: λ (nm) = 250 max.

Isolation of Albumin. Albumin was isolated from plasma (0.15 mL) with a HiTrap Blue (1.0 mL) affinity column using the solvents described in ref 34. The column were first equilibrated with 3.0 mL of binding buffer (10 mM Tris-HCl, pH 7.4), and the plasma sample (0.15 mL) was diluted in 0.85 mL of binding buffer, applied on column, and washed with 3 mL of binding buffer. The retained albumin was eluted with 4 mL of elution buffer (50 mM Tris-HCl, pH 7.4, + 0.2 M NaSCN). The HiTrap columns were regenerated by washing with 3.0 mL of binding buffer. Purified fractions were concentrated in an Amicon ultra centrifugal filter tube (30K MWC; 4 mL) by centrifuging with 4000 rpm at 4 °C for 10–15 min and washed with water (3 × 4 mL). Samples were redissolved in 10 mM sodium phosphate buffer (pH 7.0). The concentrations of the isolated albumin solutions were determined with a Coomassie protein assay kit for total protein quantitation from Pierce.

Digestion of Albumin. Albumin (2 mg) in 50 mM ammonium bicarbonate, pH 8.9 (0.9 mL), was spiked with MDI-[¹³C₆¹⁵N₂]₂Lys (13.23 pmol) and Ac^[2H₄]MDI-Lys (12.02 pmol). Samples were digested and incubated with 133.4 μ L of freshly prepared Pronase E solution (5 mg/mL; 50 mM ammonium bicarbonate, pH 8.9) for 15 h at pH 8.9 and 37 °C. The digest was acidified to pH 4.0 with 2 M hydrochloric acid and purified with solid phase extraction (Strata-X-33u, polymeric reversed phase columns, 200 mg). The column was first activated with 3 mL of methanol and then equilibrated with 3 mL of 0.1% formic acid (pH, 4.0). The sample was applied on the column and subsequently washed with a 3 mL fraction of 0, 10, and 20% methanol in 0.1% formic acid. MDI-Lys and AcMDI-Lys were eluted with 6 mL of 80% methanol in 0.1% formic acid. The eluate was concentrated to approximately 1 mL in a speed evaporator.

Quantification of Albumin Adducts, MDI-Lys and AcMDI-Lys, Using LC-MS/MS. Shimadzu Prominence 20AD interfaced to a API 4000 Q Trap LC-MS/MS (Applied Biosystems, Foster City, CA) mass spectrometer system was used for all of the quantitative analyses. The MS parameter was optimized in the electrospray ionization mode (ESI). Parameter optimization was carried out with a 100 pg/ μ L solution of analyte with a flow rate of 10 μ L/min in the negative ionization mode. MDI-Lys and AcMDI-Lys were showing a corresponding peak at m/z 369.1 and 411.1 [M - H]⁻. Quantitative optimization mode was used to maximize the signal and set the maximum suitable compound parameters for the compounds. For better resolution and sensitivity of the analyte, quadrupole mass analyzers (Q1 and Q3) were set on a 0.7 $\pm$ 0.1 amu resolution window.

The mass spectrometer was operated in negative ionization mode with an electrospray voltage of -4500 V and a source temperature of 500 °C. Nitrogen was used as ion spray (GS1), drying (GS2), and curtain gas at 40, 45, and 10 arbitrary units, respectively. The declustering potential (DP) and collision energy (CE) for MDI-Lys and AcMDI-Lys were -75 and -80 V, and -28 and -30 V, respectively. The entrance potential (EP) for both compounds was

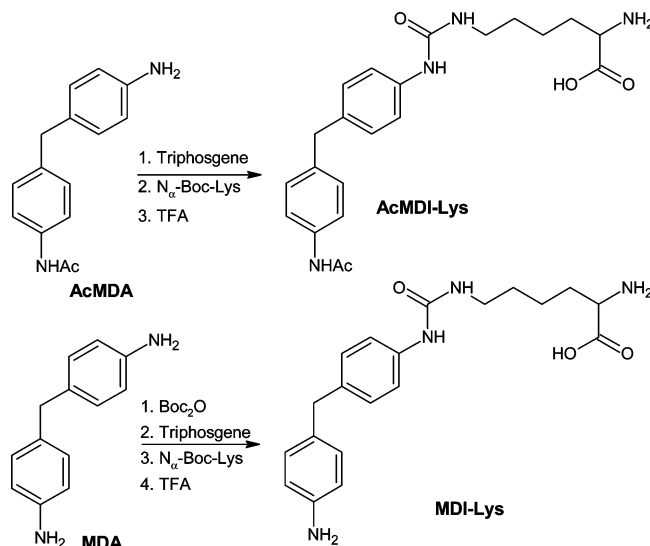


Figure 2. Procedure for the synthesis of the albumin adducts MDI-Lys and AcMDI-Lys.

-10 V. All data were processed using Analyst software 1.4.2 (Applied Biosystems/MDS Sciex).

The MDI-Lys and AcMDI-Lys were detected with multiple reaction monitoring (MRM) m/z 369.1/145.1 and 411.1/145.1, respectively. The corresponding internal standards MDI-[¹³C₆¹⁵N₂]₂Lys and Ac^[2H₄]MDI-Lys were monitored with the transitions m/z 377.1/153.0 and 415.1/145.1, respectively. Chromatographic separation was achieved on a Luna C18(2) (100 Å, 150 × 2.0 mm, 3 μ m) (Phenomenex Inc., Torrance CA) protected by a C18 guard column (AJO-4287; 4 mm L × 3.0 mm ID), using a gradient system with solvent A (10 mM ammonium formate) and solvent B (methanol) at a flow rate of 0.2 mL/min: 0 min (B 20%), 3 min (B 20%), 16 min (B 90%), and 20 min (B 90%). The retention times (t_R) of MDI-Lys and AcMDI-Lys were 14.7 and 15.3 min, respectively. The column flow was diverted away from the ESI ion source except for the time period from 5 to 18 min.

To generate the calibration line, 2 mg of human serum albumin (Sigma) was spiked with different amounts of MDI-Lys (0.00, 0.54, 2.7, 8.11, 27.03, 54.05, and 135.1 pmol) and AcMDI-Lys (0.00, 0.49, 2.43, and 7.28 pmol) along with MDI-[¹³C₆¹⁵N₂]₂Lys (13.23 pmol) and Ac^[2H₄]MDI-Lys (12.02 pmol) and worked up as described for the rat samples. The calibration lines for MDI-Lys and AcMDI-Lys were generated over the range of 0–135.1 and 0–7.28 pmol/2 mg albumin, respectively. The concentration levels are plotted against the peak area ratio of the analyte against the peak area of the internal standard (e.g., peak area ratio MDI-Lys/MDI-[¹³C₆¹⁵N₂]₂Lys). The regression coefficient values r^2 = 0.998 (MDI-Lys) and 0.990 (AcMDI-Lys) were found using the method of regression option linear and 1/x weighting factor.

Results

Synthesis of Isocyanate Adducts. The urea derivatives with the ϵ -amino group of lysine were synthesized according to the scheme depicted in Figure 2. AcMDI was generated from AcMDA and triphosgene in dioxane, and used without prior purification for the reaction with *N_α*-Boc protected lysine. After elimination of the Boc with TFA at room temperature, AcMDI-Lys was obtained. In order to obtain MDI-Lys, mono Boc-protected MDA was synthesized. BocMDI was synthesized from triphosgene and reacted with *N_α*-Boc-Lys. After elimination of the Boc groups with TFA, MDI-Lys was obtained. These products were characterized by NMR, MS, and UV. The signals were assigned according to the data of similar compounds synthesized in our laboratory (27). The internal standards and MDI-[¹³C₆¹⁵N₂]₂Lys and Ac^[2H₄]MDI-Lys were synthesized

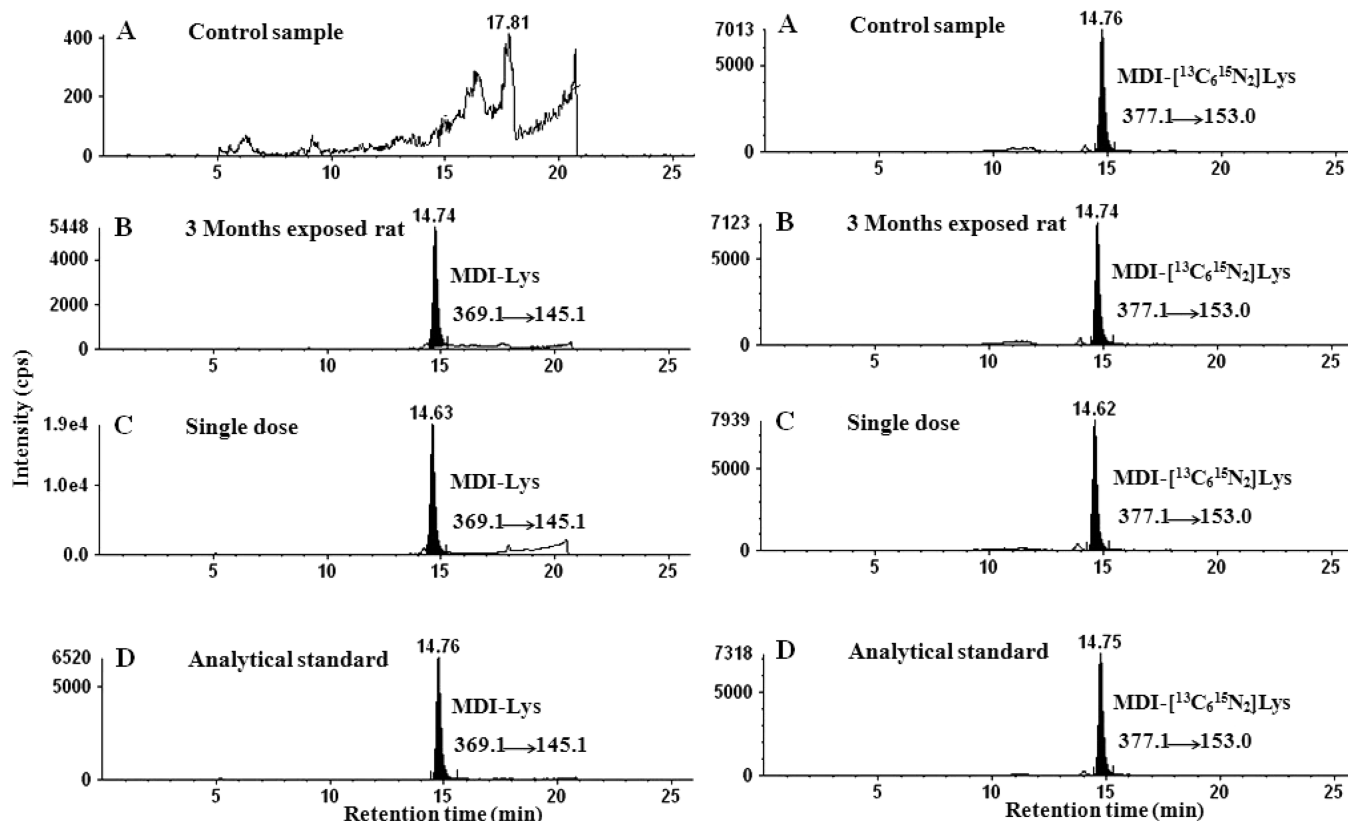


Figure 3. LC-MS/MS analysis of MDI-Lys found in vivo: (A) control rats, (B) rats exposed chronically to 0.7 mg/m³ MDI for 3 months, (C) rats exposed to a single dose 20 mg/m³ MDI and sacrificed after 6 h of exposure. (D) Standard compound MDI-Lys (3 ng) and internal standard MDI-[¹³C₆¹⁵N₂]Lys (5 ng).

accordingly with slight modifications and characterized by MS, HPLC, and UV. All adducts were synthesized from L-amino acids. The optical purity of the final products was not investigated.

Identification of Isocyanate Adducts with Albumin. In a long-term experiment designed to determine the carcinogenic and toxic effects of MDI, rats were exposed chronically for 3 months to 0.0 (control), 0.26, 0.70, and 2.06 mg of MDI/m³ as aerosols (35). Serum albumin was purified with affinity chromatography (with Cibacron Blue F3G-A as the ligand) on a HiTrap Blue column (35, 36). For the detection of low levels of MDI-Lys and AcMDI-Lys, a method was developed using LC-MS/MS. The best results were obtained using electrospray ionization in the negative ion mode. On column, up to 0.675 and 0.606 fmol of MDI-Lys and AcMDI-Lys, respectively, could be detected. For the quantitation of the adducts in vivo, isotope dilution mass spectrometry was used. Albumin was digested with Pronase E in the presence of the internal standards, MDI-[¹³C₆¹⁵N₂]Lys and Ac[²H₄]MDA-Lys. The digests were purified using solid phase extraction. The adducts MDI-Lys and AcMDI-Lys and the internal standards were eluted with 80% methanol in 0.1% formic acid. The eluates were concentrated to approximately 1 mL, and an aliquot was analyzed by LC-MS/MS. The chromatograms are presented in Figures 3 and 4. The adducts found in vivo were quantified against a calibration line for MDI-Lys and AcMDI-Lys generated over the range of 0–135.1 and 0–7.28 pmol/2 mg albumin, respectively. The adducts were found in a dose-dependent manner. The increase in the amount of adduct was linear for the two lowest doses. The limit of quantitation was tested by spiking 2 mg of albumin with standards. The samples were worked up as the in vivo samples. The limit of the quantification for was 67.5 fmol/2 mg and 60.6 fmol/2 mg albumin for MDI-Lys and AcMDI-Lys, respectively. Five aliquots of a rat sample were digested, worked-up, and

analyzed with LC-MS/MS. The adduct levels were 6.62 ± 0.53 pmol of MDI-Lys/mg albumin and 0.51 ± 0.022 pmol of AcMDI/mg albumin, which corresponds to a coefficient of variation of 8.0% and 4.3%, respectively.

The pronase digestion was tried under different conditions. Hydrolysis experiments were performed at pH 7.4 with molar ratios of albumin to Pronase E of 10:1 and 3:1, and at pH 8.9 with molar ratios of albumin to Pronase E of 3:1 and 1:1. The best yields of MDI-Lys and AcMDI-Lys were obtained with a ratio of albumin to Pronase E of 3:1 at pH 8.9. The results obtained with albumin to Pronase E 10:1 (pH 7.4) and albumin to Pronase E 3:1 (pH 8.9) were compared with regression analysis (Figure 5). At least 2.5 times higher yields of MDI-Lys were obtained at pH 8.9 than at pH 7.4. The ratio of MDI-Lys/AcMDI-Lys at pH 8.9 and pH 7.4 was 23.7 ± 3.0 and 11.5 ± 2.1 , respectively. These results suggest that more MDI-Lys is released from albumin than AcMDI-Lys at pH 8.9 or that some AcMDI-Lys is hydrolyzed at the higher pH. Therefore, the stability of AcMDI-Lys was tested under such conditions. A synthetic standard of AcMDI-Lys was incubated with albumin and Pronase E at pH 8.9 for 48 h. No MDI-Lys was formed under such conditions. This was confirmed by the fact that no [²H₄]MDA-Lys was formed from the internal standard Ac[²H₄]MDA-Lys added to the in vivo samples.

The results of the adduct levels present in the rats after 3 months of exposure are presented in Figure 6. The adduct levels increased linearly with the dose, although for AcMDI-Lys and MDI-Lys the increase could be described with a nonlinear curve. The adduct levels MDI-Lys and AcMDI-Lys correlated with $r^2 > 0.96$. The MDI-Lys/AcMDI-Lys ratio was 22.1, 23.9, and 25.2 for doses 0.26, 0.7, and 2.06 mg/m³, respectively. The difference was statistically different for the highest dose compared to the

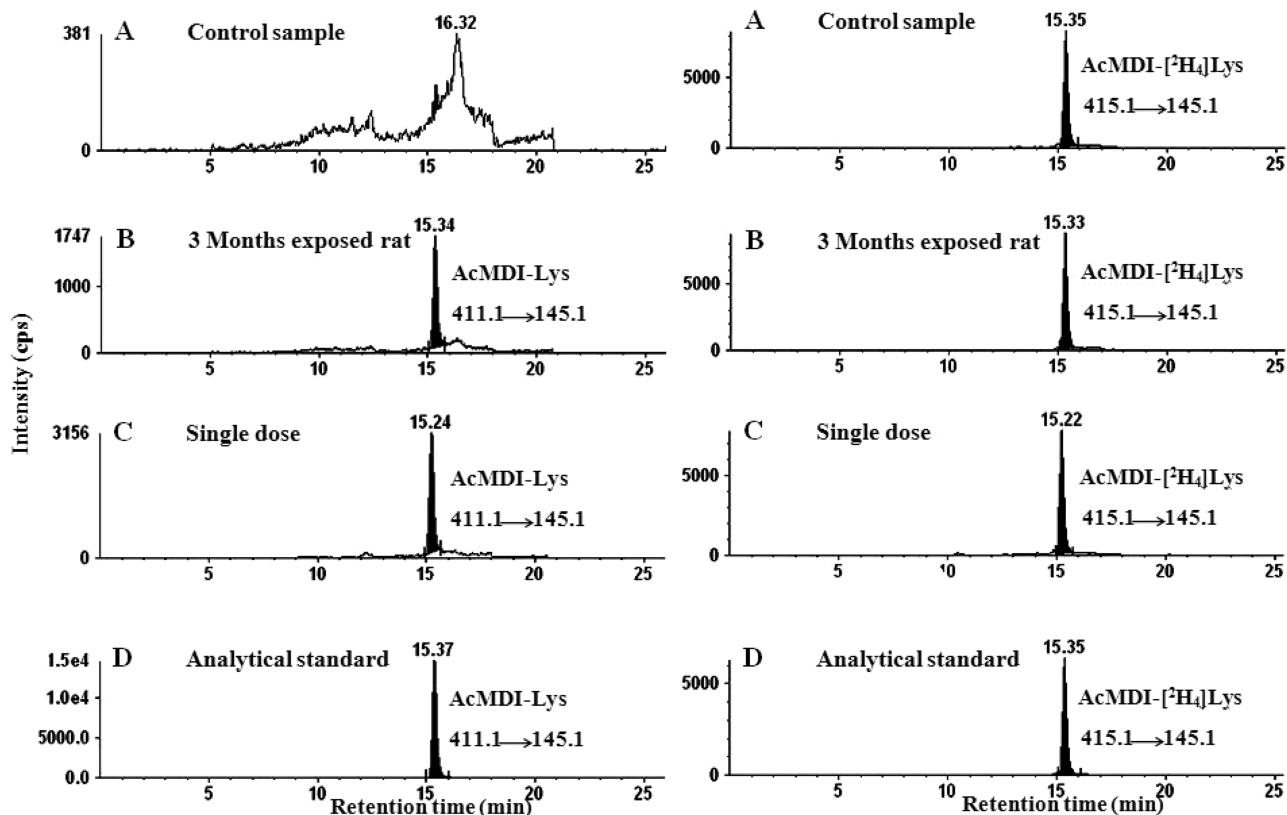


Figure 4. LC-MS/MS analysis of AcMDI-Lys found in vivo: (A) control rats, (B) rats exposed chronically to 0.7 mg/m³ MDI for 3 months, (C) rats exposed to a single dose of 20 mg/m³ MDI and sacrificed after 6 h exposure. (D) Standard compound AcMDI-Lys (3 ng) and internal standard Ac[²H₄]MDI-Lys (5 ng).

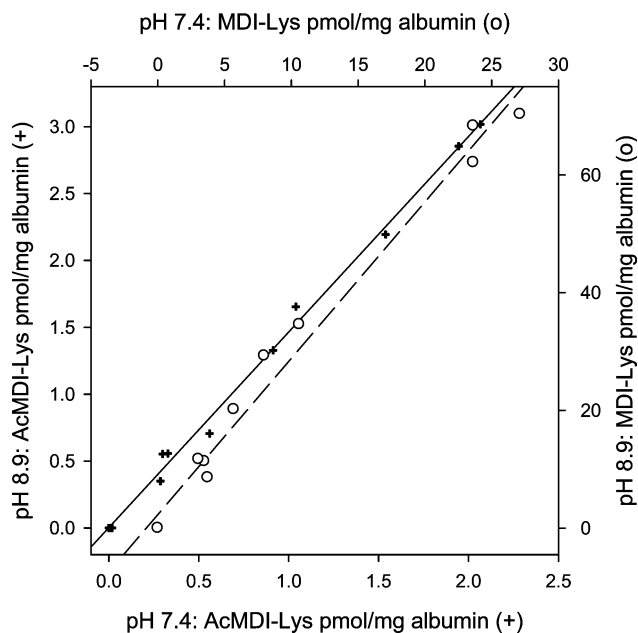


Figure 5. Comparison of the results obtained after digestion of albumin of chronically exposed rats following two protocols: (a) pH 7.4, albumin/Pronase E = 10:1 (w/w); (b) pH 8.9, albumin/Pronase E = 3:1 (w/w). The following regression curves were obtained: (AcMDI-Lys, pH 8.9) = 0.003 + 1.462(AcMDI-Lys, pH 7.4), $r^2 > 0.99$; and (MDI-Lys, pH 8.9) = 2.27 + 2.656(MDI-Lys, pH 7.4), $r^2 > 0.98$.

lower doses (independent *t*-test). Therefore, adduct formation for AcMDI-Lys increased less than MDI-Lys.

A group of rats was exposed to a single dose by whole body exposure as for the chronic exposure experiment. The rats were exposed for 6 h to 20 mg/m³ MDI and sacrificed immediately after exposure, 1 day and 7 days after exposure. Albumin was

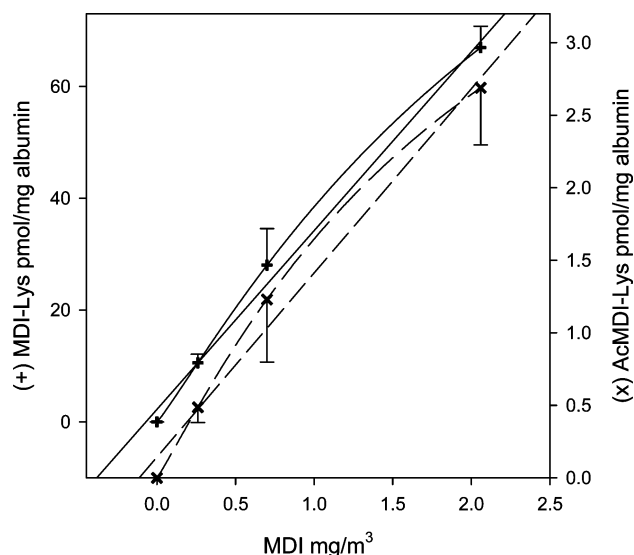


Figure 6. Isocyanate-specific adducts of MDI with albumin of rats chronically exposed for 3 months at three different doses.

isolated and analyzed for the detection of isocyanate-specific adducts. MDI-Lys and AcMDI-Lys could be found after all time points. The adduct levels were the highest just after exposure. The adduct levels (MDI-Lys) were 44.5 ± 5.0 pmol/mg albumin in the rats sacrificed immediately after exposure. These levels were compared to the values found in rats chronically exposed to MDI. The increase of MDI-Lys in chronically exposed rats can be described with the equation: $[\text{MDI-Lys}] = 2.25 + 31.97 \times \text{dose}$. Taking this equation, the adduct level after a single dose (6 h) corresponds to the adduct levels in rats dosed chronically with 1.32 mg MDI/m³ for 17 h per day and 3 months. Further, animals exposed to a single dose of MDI were

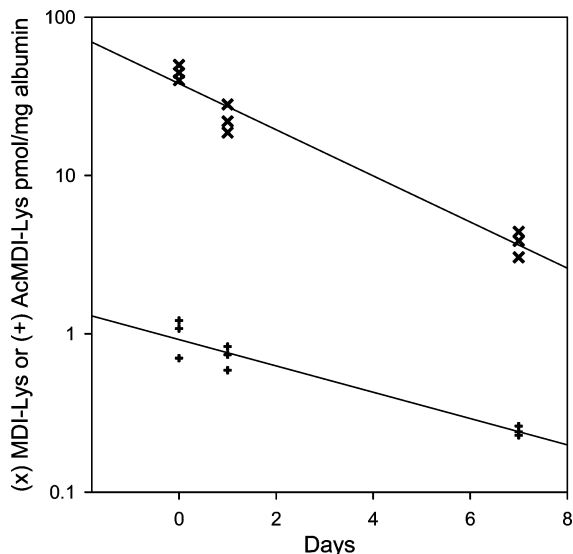


Figure 7. Decrease of the MDI-albumin adduct levels in rats exposed one time for 6 h with 20 mg/m³ MDI.

sacrificed after 1 and 7 days of exposure. The adduct levels were plotted on a logarithmic scale against the days after exposure on a linear scale (Figure 7). The decrease of the MDI-Lys levels could be described well with a linear equation: $\log[\text{MDI-Lys}] = 1.58 - 0.146 \times \text{days}$, $r^2 = 0.96$. This equation yields a half-life of 2.06 days for the adduct, which is a little shorter than the half-life (2.5–3 days) of unadducted rat albumin (37). The decrease of AcMDI-Lys adducts yields a half-life of 3.6 days using the equation $\log[\text{AcMDI-Lys}] = -0.0363 - 0.0831 \times \text{days}$, $r^2 = 0.92$. The half-life is longer than that for unadducted albumin. The ratio of MDI-Lys/AcMDI-Lys decreases from day 0–day 7: MDI-Lys/AcMDI-Lys = 46.5 ± 9.1 , 31.7 ± 2.1 , and 15.6 ± 3.5 . The ratio is significantly different (*t*-test) at day 7 compared to that at day 1 and/or day 0. Therefore, it appears that albumin adducts of AcMDI are formed later than MDI, as seen for the adducts with hemoglobin (26).

Discussion

This is the first method reported to quantify isocyanate-specific adducts with albumin. The levels of these albumin adducts were compared to other biomarkers measured in the same rats in the past (26, 27). Hemoglobin of the same rats was hydrolyzed under mild basic conditions, and the released AcMDA and MDA were measured using GC-MS or HPLC with electrochemical detection (26). MDA and AcMDA were also found as urinary metabolites (26). In the same rats, the isocyanate-specific adduct of MDI with the *N*-terminal valine of hemoglobin, *N*-([4-(4-aminobenzyl)phenyl]amino)carbonyl-valine, was determined (27). The *N*-terminal adduct with valine could be released from hemoglobin after acid hydrolysis, which yielded the corresponding hydantoin of valine, 3-[4-(4-aminobenzyl)phenyl]-5-isopropyl-1,3-imidazoline-2,4-dione (MDI-Val-Hyd). The increase of all biomarker levels independent of the dose was plotted in Figure 8. Except for the adducts with albumin, the adducts with hemoglobin and the urinary metabolites did not increase linearly for the highest dose. This might be due to saturation effects of metabolic pathways. The adduct levels were the highest in albumin. The albumin adducts levels found, for example, at a dose of 0.7 mg/m³ were ca. 38 and 280 times higher in comparison to the MDI-Val-Hyd levels and with

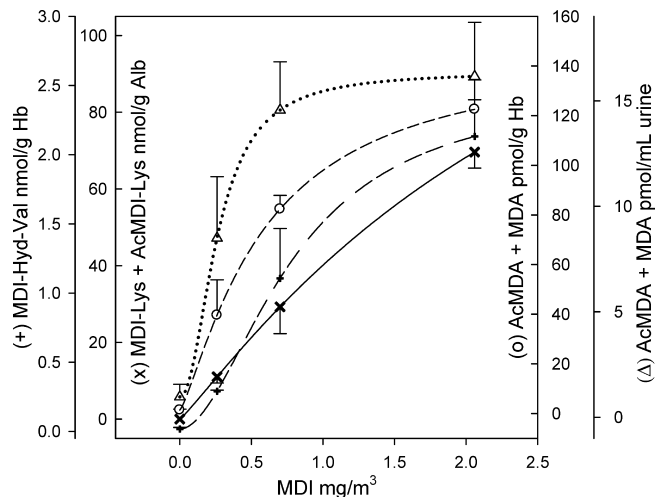


Figure 8. Comparison of the different biomarkers found in rats exposed to MDI: (a) the isocyanate-specific adduct levels with albumin (Alb); (b) the isocyanate-specific adduct (MDI-Val-Hyd) with hemoglobin (Hb) (27); (c) the total of MDA and AcMDA released from Hb after mild base hydrolysis (26); and (d) the total of AcMDA and MDA found in urine (26).

the sum of AcMDA and MDA obtained from mild base hydrolysis of hemoglobin (arylamine-specific adducts), respectively.

The ratio of the acetylated MDA varies greatly among the different biomarkers. The mean ratio (of the 3 dose groups 0.26, 0.70, and 2.06 mg MDI/m³) for the urinary metabolites AcMDA/MDA, the hemoglobin adducts (Hb-AcMDA/Hb-MDA), and the albumin adducts AcMDI-Lys/MDI-Lys was 34.2 ± 12.6 , 1.54 ± 0.23 , and 0.043 ± 0.005 , respectively. This is a further indication that the hemoglobin adducts and the urinary metabolites are the consequence of biologically available MDA which is then acetylated to a large degree. From these data, the following pathways can be postulated. The isocyanate-specific adducts result via the direct reaction of the isocyanate groups with albumin and hemoglobin or indirectly via the reaction of MDI with glutathione (Figure 1), as postulated for methyl isocyanate, the resulting thiocarbamate, which releases the isocyanate in the plasma and erythrocytes to yield the adducts with lysine of albumin and the adducts with the *N*-terminal adducts of hemoglobin. The arylamine-specific adducts result after MDI hydrolyses to MDA, which is further oxidized in the liver to its *N*-hydroxy arylamine and subsequently to the nitroso compound in the erythrocytes (Figure 1). The nitroso derivative of MDA and/or AcMDA yields the sulfinamide adducts, which are acid and base labile. Such mild conditions will release arylamines from the typical sulfinamide adducts formed by arylamines with the cysteine of hemoglobin. The presence of large amounts of AcMDA after mild base hydrolysis of hemoglobin indicates that these adducts were generated from MDA. However, mild base hydrolysis can also release isocyanate adducts with cysteine and partially adducts with tyrosine (30%, yield) (25). Serine adducts cannot be hydrolyzed under such conditions. Cysteine adducts of isocyanates are not stable in vivo (22, 23), and the formation of tyrosine adducts is not likely. The phenylhydroxy group in tyrosine appears not to be as reactive as the hydroxyl group in serine. Without protection of the tyrosine, only the serine alcohol group reacted with *p*-maleimidophenyl isocyanate (38). Furthermore, the isocyanate with the α -amino group of tyrosine could be transformed to an isocyanate without protecting the phenolic hydroxyl group (39). The isocyanate-specific adducts with albumin show that only for a small fraction one isocyanate group of MDI was

hydrolyzed to the amine and then acetylated. This is in contrast to the high AcMDA/MDA ratio found after mild hydrolysis of hemoglobin and to the large AcMDA/MDA ratio found as urinary metabolites (see above) (40). This indicates that the hydrolyzable hemoglobin adducts and the determined urinary metabolites do not originate from the same precursor (MDA vs MDI) as the albumin adducts MDI-Lys/AcMDI-Lys.

The MDI-Lys levels correlate with the MDA levels found after base extraction of urine and with the MDA levels released from hemoglobin (Hb-MDA) after base hydrolysis with $r^2 = 0.9$ and 0.85 . Accordingly, AcMDI-Lys correlates with the levels of AcMDA found after base extraction of urine and with the AcMDA levels released from hemoglobin (Hb-AcMDA) after base hydrolysis with $r^2 = 0.64$ and 0.84 . Therefore, urinary metabolites or hemoglobin adducts measured in the past might be a good indicator for the presence of albumin adducts in these workers.

The discovery and quantitation of isocyanate-specific adducts with albumin will enable one to monitor MDI exposed workers. New preventive measures can then be implemented at the workplace to identify presently unknown and unexpected exposures and to reduce them. The elucidation of these new adducts with albumin will contribute to the synthesis of antigens for immunological tests in exposed people. Several studies have been performed to monitor workers using immunological methods (reviewed in refs 41 and 42). In a review of 29 studies of occupational exposure to diisocyanates, Ott et al. (42) showed a considerable variability in assay methodology and heterogeneity in the prevalence of positive antibody responses across laboratories. Since the adduct structures found in vivo are unknown, this makes it impossible to produce antigens which correspond to the in vivo situation. Therefore, various methodologies have been used to generate diisocyanate adducts and to assess antibody binding to these adducts. Our method will enable one to determine the levels of isocyanate-specific adducts with lysine. Lysine adducts present in albumin modified with hexane 1,6-diisocyanate (HDI) appear to be crucial for the immunological response of antibodies present in exposed workers (40). HDI conjugated to albumin from cows is not recognized by human antibodies (40). Lys414 is present only in human albumin. Thus, the adduct of HDI with Lys414 of human albumin appears to be crucial for immunological responses (40, 41). In future studies, it will be important to produce antigens according to the adducts found in vivo. Then, the immunological tests, the disease status, and the albumin adduct levels can be compared to confirm the relationship between the two biomarkers and the disease.

Acknowledgment. This research was supported by the Tulane Cancer Center. NMR was run by Dr. Qi Zhao at the Coordinated Instrumentation Facility of Tulane University.

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