Development of Polyclonal Antibodies for Detection of Protein Modification by 1,2-Naphthoquinone

Jiang Zheng and Bruce D. Hammock*

Departments of Entomology and Environmental Toxicology, University of California, Davis, California 95616

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Naphthoquinones have been reported to be toxic to liver cells *in vitro.* Protein modification is associated with naphthoquinone-induced cytotoxicity. In addition, 1,2-naphthoquinone was found to bind covalently to cysteine residues of proteins of lung Clara cells incubated with naphthalene. To further identify the target proteins of the naphthoquinone, we raised polyclonal antibodies by immunizing rabbits with 1,2-naphthoquinone protein adducts. A high titer of polyclonal antibodies was obtained by antiserum dilution tests. Competitive ELISA showed that the antibodies specifically recognize the 1,2-naphthoquinone *N*-acetylcysteine adduct. Very weak cross reactivity toward *N*-acetylcysteine and its 1,4-naphthoquinone as well as naphthalene oxide adducts was observed. For covalent binding studies, we incubated mouse liver homogenates with 1,2-naphthoquinone at concentrations of 1.0 and 10 μM at 37 °C for 1 h. The resulting protein samples were developed by SDS−PAGE, followed by Western blotting and immunostaining using the polyclonal antibodies. Chemiluminescent bands developed with ECL chemiluminescence kit were observed on the poly(vinylidene difluoride) microporous membrane blotted with the mouse liver homogenates exposed to 1.0 and 10 μM 1,2-naphthoquinone. One chemiluminescent band at a molecular weight of 22 kDa was observed in the lane loaded with the protein sample incubated with 1.0 μM 1,2-naphthoquinone, and many chemiluminescent bands at a wide range of molecular weights were observed in the lane loaded with the protein sample incubated with 10 μM quinone. As expected, no chemiluminescent bands were detected on the membrane blotted with the proteins exposed to vehicle. We have successfully raised polyclonal antibodies to recognize 1,2-naphthoquinone cysteine adducts and developed immunostaining to detect protein modification by 1,2-naphthoquinone.

Introduction

Naphthoquinones, which are known as electrophilic species, have been reported to be toxic to hepatocytes *in vitro.* Blebbing on cellular surface along with a dose-dependent decrease in intracellular glutathione is observed after isolated hepatocytes are exposed to naphthoquinones (*1*). Arylation of nucleophilic amino acid residues such as cysteine of cellular proteins by naphthoquinones has been suggested to be an important mechanism of quinone-induced cytotoxicity (*2*−*4*). Pessah and co-workers demonstrated that naphthoquinones caused activation of Ca^{2+}-induced Ca^{2+} release channel and suggested that the cytotoxicity of quinones may be related to alternation of normal cellular Ca^{2+} regulation (*5, 6*). Naphthoquinones have been suggested to be toxic reactive metabolites of naphthalene. Naphthalene is an important environmental pollutant (*7*) and has been reported to produce selective necrosis of lung Clara cells in the mouse (*8, 9*). Covalent binding of cellular proteins by reactive metabolites of naphthalene is associated with naphthalene-induced cytotoxicity (*10*). The pathway of the formation of reactive metabolites of naphthalene is proposed as shown in Scheme 1. Naphthalene metabolism is initiated by the formation of naphthalene oxide mediated by cytochrome P-450. The epoxide metabolite is proposed to bind covalently to nucleophilic amino acid residues of cellular proteins. In addition, the resulting epoxide metabolite is spontaneously and enzymatically hydrolyzed by epoxide hydrolase to dihydrodiol and further oxidized to 1,2-dihydroxynaphthalene (1,2-naphthohydroquinone) by dihydrodiol dehydrogenase. Alternatively, naphthalene oxide is chemically rearranged to 1-naphthol which can then be hydroxylated to 1,2- and 1,4-dihydroxynaphthalenes (naphthohydroquinones). These hydroquinone metabolites of naphthalene are readily oxidized to the corresponding naphthoquinones which possess high chemical reactivity with nucleophiles of intracellular proteins forming protein adducts. Recently we identified 1,2-naphthoquinone as the major quinone metabolite of naphthalene which covalently conjugates to cysteine residues of intracellular proteins after Clara cells are incubated with naphthalene. In addition to 1,2-naphthoquinone, epoxide metabolite of naphthalene was found to bind covalently to cysteine residues of Clara cell proteins (*11*).

Immunoblotting of protein adducts of reactive metabolites has been successfully developed to identify target cellular proteins modified by reactive metabolites and to investigate the toxicological importance of protein modification in experimental animals and in humans pre-exposed to acetaminophen (*12*) and halothane (*13*). In addition, the antibodies prepared for immunoblotting can be used for the development of enzyme-linked immunosorbent assays (ELISAs)[1] to monitor urinary mercapturic acid metabolites (*14*) and plasma protein adducts (*15*) derived from the reactive metabolite as biomarkers of

* Address correspondence to this author at: Departments of Entomology and Environmental Toxicology, University of California, Davis, CA 95616. Tel: (916) 752-7519; FAX: (916) 752-1537; E-mail: bdhammock@ucdavis.edu.

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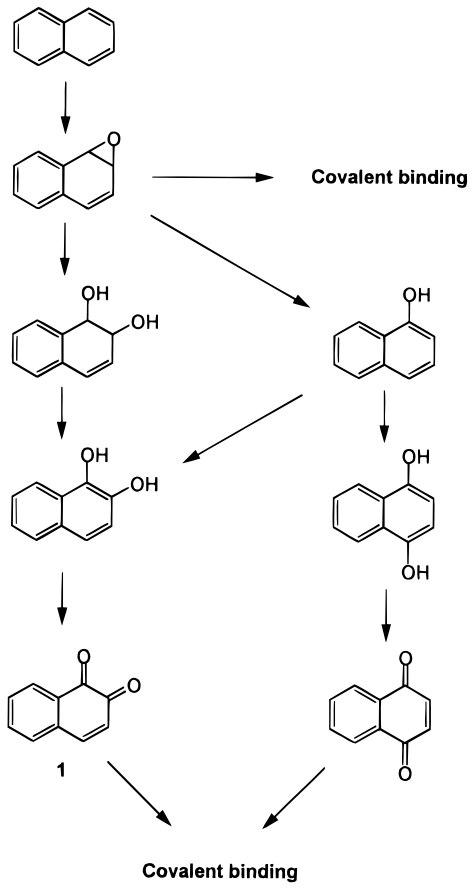
Scheme 1



human exposure to potential toxins. The objective of this study was to prepare polyclonal antibodies and to develop immunoblotting used as a probe to detect proteins modified by 1,2-naphthoquinone. This antibody-based technique will facilitate our investigation on the mechanism of naphthalene-induced cytotoxicity.

Experimental Section

Chemicals and Instruments. Chemical reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Mouse albumin, conalbumin, *N*-acetylcysteine, and immunochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Water-soluble keyhole limpet hemocyanin (KLH) and *N*-succinimidyl (3(2)-pyridyldithio)propionate (SPDP) were purchased from Pierce (Rockford, IL). HPLC analysis and purification of synthetic chemicals were performed using a Varian 9010 solvent delivery system equipped with a Varian 9050 UV−vis detector. A reverse phase C_{18} chromatography column (250 × 4.6 mm) purchased from Vydac (Hesperia, CA) was used for HPLC analysis and purification of synthetic compounds. ^{1}H-NMR spectra were obtained from a QE-300 spectrometer (General Electric, 300 MHz). Chemicals were analyzed on a VG/Fisons Quattro-BQ triple quadruple mass spectrometer (VG Biotech, Altrincham, U.K.) using 1:1 CH_3CN/H_2O together with 1.0% formic acid as the mobile phase. Spectra were obtained in positive mode. Immunoassays were performed with polystyrene 96-well microtiter plates purchased from Nunc (Maxisorb, Roskilde, Denmark). The absorbances were read with a V_{max} microplate reader (Molecular Devices, Menlo Park, CA). The curve fitting was performed using SigmaPlot.

[1] Abbreviations: ELISAs, enzyme-linked immunosorbent assays; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; SPDP, *N*-succinimidyl (3(2)-pyridyldithio)propionate.

Synthesis. 1,2-Naphthoquinone (1). 1,2-Naphthoquinone was prepared by the method Fieser (*16*). Briefly, aminonaphthol (1.5 g) was dissolved in 100 mL of 0.5 N HCl, and the insoluble solid was discarded by filtration. To the solution 15 mL of 10% (w/v) $FeCl_3$ dissolved in 3 N HCl was dropwise added over 10 min, with vigorous stirring at room temperature. The mixture was stirred at room temperature for 30 min, and the resulting brownish crystals were recrystallized in hexane and ethyl acetate, giving bright orange crystals.

***N*-Acetyl-*S*-(1,2-dihydroxy-4-naphthyl)cysteine (2).** *N*-Acetylcysteine (50 mg, 0.3 mmol) was dissolved in 2.0 mL of water which had been purged with nitrogen gas. 1,2-Naphthoquinone (31 mg, 0.2 mmol) was dissolved in 1.0 mL of nitrogen-purged acetone and added dropwise to the cysteine solution under nitrogen. After 30 min stirring at room temperature, the acetone was removed by rotary evaporation. The resulting mixture was injected into HPLC for purification. The chromatography was performed using a gradient of 20−80% acetonitrile in water with 0.1% of trifluoroacetic acid. The desired fractions were collected and pooled. The acetonitrile was removed by evaporation with a stream of nitrogen, and the remaining aqueous fraction was evaporated by lyophylization, giving a brownish solid. ^{1}H-NMR (DMSO) δ 2.00 (s, 3H, CH_3CONH), 3.46 (dd, J = 8.7, 13.8 Hz, 1H, SCH_aH$_b$CH, diastereomers), 3.74 (dd, J = 4.8, 13.8 Hz, 1H, SCH$_a$$H_b$CH, diastereomers), 4.4 (m, 1H, SCH$_2$CH), 4.9 (b, 2H, H hydroxyl), 6.62 (s, H aromatic), 7.63 (t, J = 7.5 Hz, 1 H, H aromatic), 7.75 (t, J = 7.5 Hz, 1H, H aromatic), 7.93 (t, J = 7.8 Hz, 1H, H aromatic), 8.12 (d, J = 7.5 Hz, 1H, H aromatic). ESIMS: 322 $(M + 1)^+$.

***N*-Acetyl-*S*-(1,4-dihydroxy-2-naphthyl)cysteine (3).** The procedure was similar to the synthesis of **2** as described above. *N*-Acetylcysteine (50 mg, 0.3 mmol) was reacted with 1,4-naphthoquinone (31 mg, 0.2 mmol). The product was purified by reverse phase HPLC. ^{1}H-NMR (DMSO) δ 1.74 (s, 3H, CH_3-CONH), 2.92 (dd, J = 8.7, 13.2 Hz, 1H, SCH_aH$_b$CH, diastereomers), 3.10 (dd, J = 4.5, 13.2 Hz, 1H, SCH$_a$$H_b$CH, diastereomers), 3.7 (b, 2H, H hydroxyl), 4.2 (m, 1H, SCH$_2$CH), 6.70 (s, 1H, H aromatic), 7.4 (m, 2H, H aromatic), 7.9 (m, 2H, H aromatic). ESIMS: 322 $(M + 1)^+$.

***N*-Acetyl-*S*-(1,2-dihydro-1-hydroxy-1-naphthyl)cysteine (4).** Compound **4** was synthesized by the reaction of naphthalene oxide with *N*-acetylcysteine as described by Marco and co-workers (*14*).

Keyhole Limpet Hemocyanin−1,2-Naphthoquinone Adduct (Immunogen I). Water-soluble keyhole limpet hemocyanin (KLH, 20 mg) was dissolved in 1.0 mL of 4.0 M guanidinum chloride, followed by addition of 20 mg of dithiothreitol. The mixture was stirred under nitrogen at room temperature in the dark overnight. The reduced proteins were loaded to a G-25 gel filtration column (1.0 × 10 cm), and the column was eluted with deionized water. The fractions containing protein were identified by a UV spectrometer at 254 nm and pooled as desired. To the protein solution, 1,2-naphthoquinone (3.0 mg) dissolved in 0.5 mL of acetone was added under nitrogen. After stirring for 20 min, the mixture was washed with ethyl acetate and dried by lyophilization.

Thiolated KLH−1,2-Naphthoquinone Adduct (Immunogen II). Water-soluble KLH (20 mg) was dissolved in 1.0 mL of water, followed by addition of 2.0 mg of *N*-succinimidyl (3(2)-pyridyldithio)propionate (SPDP) dissolved in 50 μL of dimethyl sulfoxide. After 1.5 h stirring at room temperature, 25 mg of dithiothreitol dissolved in 50 μL of water was added to the mixture. The reduction was performed under nitrogen, with stirring at room temperature for 30 min. The mixture was loaded onto a G-25 gel filtration column (1.0 × 10 cm), and the column was eluted with deionized water. The fractions containing protein were collected and pooled. The reduced protein was immediately reacted with 3.0 mg of 1,2-naphthoquinone dissolved in 0.5 mL of acetone as described above in the preparation of immunogen **I**. The resulting protein adducts were washed with ethyl acetate and lyophilized. The lyophilization of the protein adducts gave a brownish powder.

Mouse Albumin−1,2-Naphthoquinone (Coating Antigen). Mouse serum albumin (20 mg) was reduced by dithiothreitol as described above to reduce disulfides of the protein. The reduced mouse albumin was separated from the reducing agent by gel filtration and immediately reacted with 3.0 mg of 1,2-naphthoquinone using a similar procedure for the arylation of proteins as described above. The protein adducts were purified by gel filtration and dried by lyophilization.

Immunization of the Rabbits. Female New Zealand White rabbits (Herbert's Rabbitry, Plymouth, CA) weighing 2.5−3.0 kg were immunized with either immunogen **I** or **II**. The immunogen (100 μg) was dissolved in 0.5 mL of PBS buffer (pH = 7.4), followed by emulsification with 0.5 mL of Freund's complete adjuvant. The rabbits were injected subcutaneously with the emulsion (1.0 mL/rabbit) at multiple sites in the back. After 4 weeks these animals were boosted several times with a interval of four weeks by the same procedure except that Freund's incomplete adjuvant was used in place of Freund's complete adjuvant. These rabbits were boosted until no further increase in antibody titer was observed.

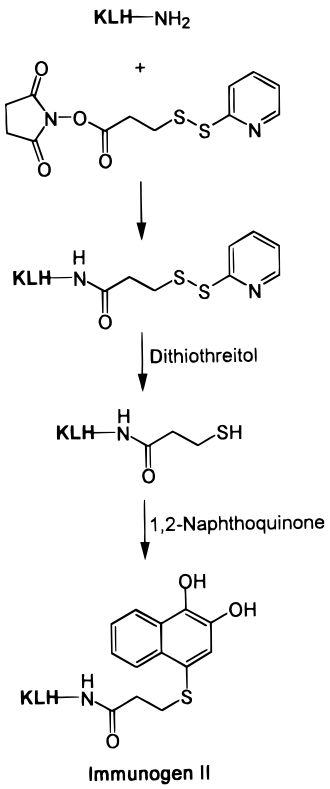
Analysis of Titer. The titer of the serum obtained from the rabbits was determined by measuring the binding of serial dilutions (1/100 to 1/128 000) to microtiter plates coated with several concentrations of mouse albumin−1,2-naphthoquinone adducts. Optimal concentration for the coating antigen and antisera dilution were determined (*17*).

Competitive Enzyme Immunosorbent Assay. To each well of 96-well microtiter plates, 100 μL of coating antigen mouse albumin−1,2-naphthoquinone at 2.0 μg/mL (200 ng/well) in PBS buffer (pH 7.4) was added and incubated at 4 °C overnight covered with adhesive plate seals. Serial dilutions of the analytes were prepared in PBST buffer (0.2 M phosphate-buffered saline solution containing 0.05% Tween-20, pH 7.4) with 1% ethanol. The resulting analyte solution was mixed (1:1 v/v) with primary antisera (1/1000 dilution) in 2% nonfat milk dissolved in PBST buffer, followed by incubation at 4 °C overnight. The following day the plates were washed 5 times with PBST buffer and blocked by addition of 1.0% nonfat milk in PBST buffer (120 μL/well) and incubation for 1.0 h at room temperature. The plates were washed as described before. The preincubated antiserum with analytes at various concentrations was added to the plates (100 μL/well) and incubated at room temperature for 2.0 h. After the same process of washing, 100 μL of a 1/12 000 diluted goat anti-rabbit IgG−horseradish peroxidase (HRP) solution was added to each well. The mixture was incubated for an additional 1 h, and after another wash step, 100 μL of a substrate solution containing 0.3 mM tetramethylbenzidine and 0.1 mM H_2O_2 in 0.1 M acetate buffer (pH = 5.5) was added and incubated at room temperature for 30 min, followed by addition of 50 μL of a 4 M sulfuric acid solution to quench colorimetric development. The absorbance at dual wavelength (450 − 650 nm) was read.

***In Vitro* Study.** Freshly harvested mouse liver which had been perfused with diluted heparin saline solution to remove blood was homogenated using a tissue blender. The resulting homogenates were centrifuged at 1000*g* for 30 min. The pellet was discarded, and the supernatants (5.0 mg of protein/mL) were incubated with 1,2-naphthoquinone at 37 °C at concentrations of 1.0 or 10 μM for 1.0 h, then frozen and stored at −80 °C for gel electrophoretic separation and immunoblotting.

Electrophoresis and Immunoblotting. The protein bands resolved by SDS−polyacrylamide gel electrophoresis (Mini-Protean II, Bio-Rad) according to Laemmli (*18*) using 3.3% stacking and 12% resolving gels were transferred to poly(vinylidene difluoride) microporous membranes (Immobilon-P transfer membranes, Millipore) by an electroblotter (LKB Novablot electrophoretic transfer kit). After 3.0 h transferring (43 mA), blots were blocked by shaking overnight in 5% nonfat dry milk in PBST buffer. The blotted poly(vinylidene difluoride) membranes were incubated for 1.0 h with a 1/4000 dilution of primary rabbit antiserum in PBST buffer with 3% nonfat milk in the absence or presence of *N*-acetyl-*S*-(1,2-dihydroxy-4-naphthyl)cysteine. The immunoblots were incubated for 1.0 h with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (1/8000 in PBST buffer, Sigma). The blotted protein bands were detected by chemiluminescence using ECL Western blotting kits (Amersham International plc, England).

Scheme 2



Results and Discussion

Previous studies (*10*) have shown that cysteine residues of cellular proteins were modified by the 1,2-naphthoquinone metabolite of naphthalene after isolated mouse Clara cells were exposed to naphthalene (0.5 mM) at 37 °C for 1.0 h. This finding led to identification of the cellular proteins chemically modified with the naphthoquinone metabolite of naphthalene as a step in elucidation of the mechanism of naphthalene-induced cytotoxicity. Western blotting and immunostaining techniques (*12*) have been used as powerful tools to identify protein adducts by detecting the hapten moiety of protein adducts formed during the exposure of cellular proteins to electrophilic species (haptens). In an attempt to identify target proteins of the 1,2-quinone metabolite of naphthalene, we took advantage of immunoblotting as a detection tool by using polyclonal antibodies which can recognize the 1,2-naphthohydroquinone moiety of proteins modified with 1,2-naphthoquinone. As the first step of antibody preparation, immunogens (**I** and **II**) were designed and synthesized. For the preparation of immunogen **I**, free sulfhydryl groups of carrier protein KLH were generated by reduction with dithiothreitol and immediately reacted with 1,2-naphthoquinone. Immunogen **II** was synthesized as shown in Scheme 2. Water-soluble KLH was thiolated by reacting with SPDP, and the resulting disulfide products were reduced by dithiothreitol to thiols, which immediately reacted with 1,2-naphthoquinone. As shown in Figure 1, immunogen **II** is a lysine derivative which mimics the structure of 1,2-naphthoquinone cysteine residue adducts (immunogen **I**) except for the lack of acylamido substitution. The side

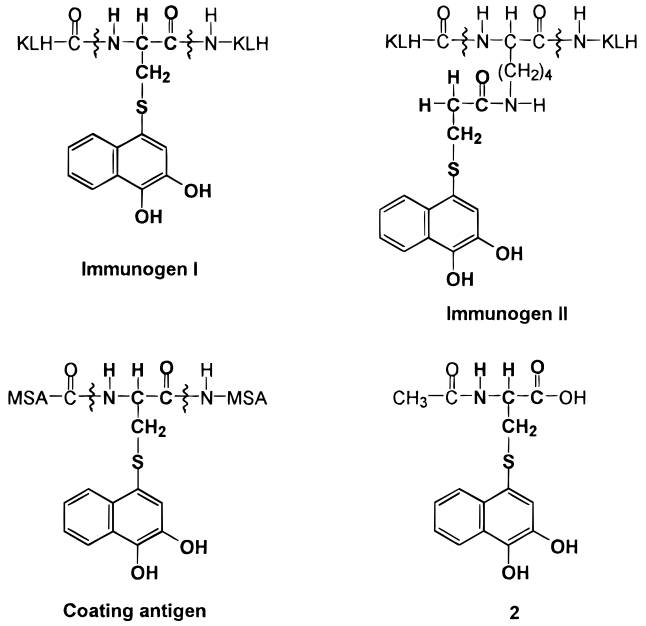


Figure 1. Chemical structures of immunogens, coating antigen, and compound **2** used for immunization and ELISAs. KLH: keyhole limpet hemocyanin; MSA: mouse serum albumin.

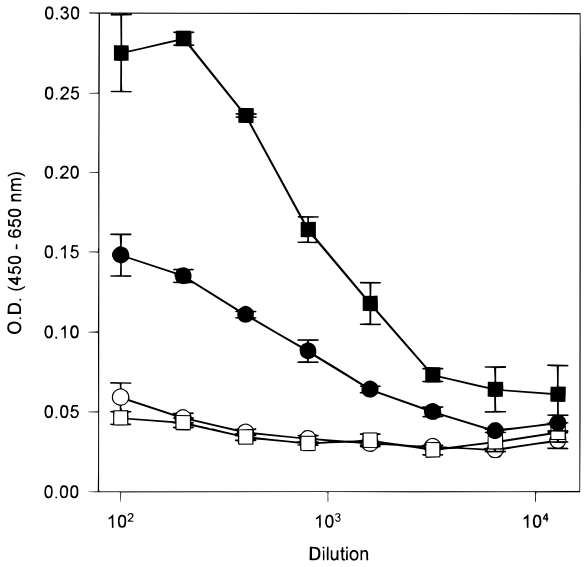


Figure 2. Titration tests of antisera raised against immunogen **I** (□ and ■) and immunogen **II** (○ and ●) in a microtiter plate coated with 1,2-naphthoquinone mouse albumin adduct (■ and ●) and native mouse albumin (□ and ○). The values are mean ± SD for triplicate assays.

chain of lysine residues supplies a spacer between the carrier protein KLH and 1,2-naphthohydroquinone. Mouse albumin 1,2-naphthoquinone adduct used as the coating antigen was prepared by conjugating 1,2-naphthoquinone to mouse albumin which had been treated with dithiothreitol similarly as described in the preparation of immunogen **I**.

Immunizations of rabbits were carried out with immunogens **I** and **II**. Using a checkerboard titration, the antisera of each animal were tested against mouse albumin 1,2-naphthoquinone adduct after every bleeding until no increase in the titer was observed. As shown in Figure 2, the antiserum raised against immunogen **I** showed a higher titer than the one prepared by the immunization with immunogen **II**. The low titer of antiserum raised against immunogen **II** may result from

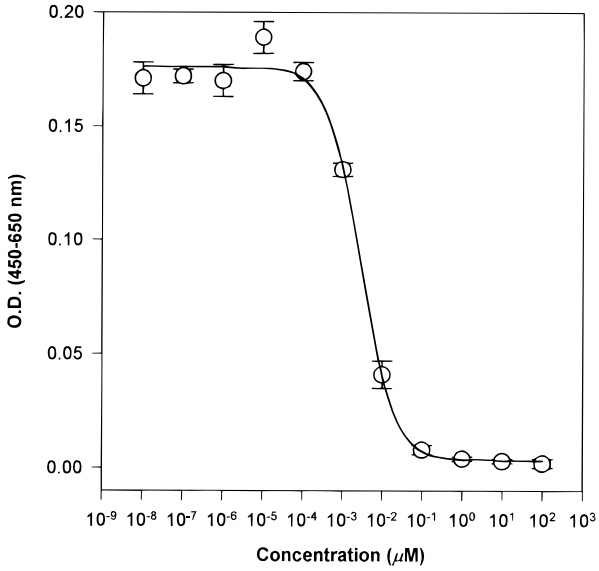
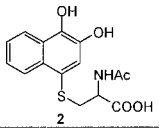
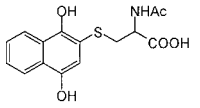
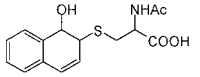
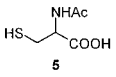


Figure 3. Analysis of *N*-acetyl-*S*-(1,2-dihydroxy-4-naphthyl)-cysteine in a competitive ELISA using an antiserum raised against immunogen **I** in a microtiter plate coated with the 1,2-naphthoquinone adduct on mouse serum albumin. Error bars represent standard deviation of separate runs on different days. The values are mean ± SD for triplicate assays.

using heterologous coating antigen for the titration test. In addition, both antisera showed very low affinity toward native mouse serum albumin, the carrier protein of the coating antigen.

Competitive ELISA experiments were conducted to characterize the antibodies. Both antisera raised against immunogen **I** and **II** were diluted to 1/4000 and used for the ELISA in plates coated with the same coating antigen as the one for the titration tests, and *N*-acetyl-*S*-(1,2-dihydroxy-4-naphthyl)cysteine, **2**, employed as an analyte. Optical density measured at 450 nm minus 650 nm was plotted against the logarithm of the various concentrations of the analyte. As demonstrated in Figure 3, a sigmoid curve was obtained from the competitive ELISA using the antiserum raised against immunogen **I**. The resulting curve was fitted by a four-parameter logistic equation. An I_{50} of 2.82 nM was observed with A/D = 61.5, slope = 0.98, and r = 0.987 (A: maximal absorbance; D: noise absorbance; r: regression coefficient). In addition, the competition test showed that the presence of 1.0 μM analyte **2** inhibits 98% of the binding of the antiserum to the coating antigen. The remaining 2.0% of the binding unable to be reversed by the analyte may result from nonspecific binding. This may indicate that 1,2-naphthoquinone reacted with cysteine residues rather than other nucleophilic amino acid residues of proteins in the synthesis of the immunogen and the coating antigen. Interestingly, no competition by *N*-acetyl-*S*-(1,2-dihydroxy-4-naphthyl)cysteine, analyte **2**, was observed using the antiserum generated against immunogen **II**, although the antiserum was found to recognize the coating antigen mouse albumin 1,2-naphthoquinone adduct in the titration tests (Figure 2). It is unknown why analyte **2** did not reverse the recognition of the coating antigen by the antiserum raised against immunogen **II**. The explanation is likely that the antibodies raised against immunogen **II** recognize the thionaphthohydroquinone moiety of the coating antigen. However, the cavity size of the antibody binding sites is not big enough to hold analyte **2**. It thus is unable to displace the coating antigen from the antibodies.

Table 1. I_{50} Values, Cross Reactivities, and Slopes for Analytes

Structures of analytes	I_{50}	Cross reactivity	Slope
 2	2.8 nM	100%	0.98
 3	1.0 μM	0.27%	1.06
 4	10.2 μM	0.02%	0.91
 5	22.6 μM	0.01%	0.71

As shown in Scheme 1, naphthalene oxide, another reactive metabolite of naphthalene, has been found to modify cysteine residues of cellular proteins of Clara cells after exposure to naphthalene (*11*). Additionally, 1,4-naphthoquinone has been suggested to be another quinone metabolite of naphthalene and likely to react with cysteine residues to form protein adducts (*1*, *2*). To determine the cross reactivity of the antibodies toward naphthalene oxide and 1,4-naphthoquinone modified proteins, we conducted competitive ELISA of *N*-acetyl-*S*-(1,4-dihydroxy-2-naphthyl)cysteine (**3**) and *N*-acetyl-*S*-(1,2-dihydro-1-hydroxy-1-naphthyl)cysteine (**4**) as competitive analytes, using the antiserum raised against immunogen **I**. As expected, very weak cross reactivity (less than 0.3%) was observed to these two *N*-acetylcysteine adducts relative to the hapten (Table 1). Additionally, we ran competitive ELISA of *N*-acetylcysteine, **5**, as a competitive analyte using the same antiserum, and only 0.01% of cross reactivity was observed as shown in Table 1. This suggests that an intact structure of 1,2-naphthoquinone cysteine adduct is required for hapten recognition by the antibodies.

In an effort to develop immunostaining for the detection of 1,2-naphthoquinone protein adducts using the antiserum described above, protein samples of mouse liver homogenate were exposed to vehicle or 1,2-naphthoquinone at concentrations of 10 μM (20 nmol of quinone/mg of protein) and 1.0 μM (2.0 nmol of quinone/mg of protein), and the resulting protein samples were separated by SDS−PAGE, followed by blotting to an Immobilon-P transfer membrane. The blotted membrane was incubated with the antiserum raised against immunogen **I** and consecutively incubated with goat anti-rabbit IgG−horseradish peroxidase. The resulting antibody-incubated membranes were treated with ECL chemiluminescence kit. As shown in Figure 4, many chemiluminescent bands at a wide range of molecular weights were observed in the lane loaded with the protein sample incubated with 10 μM 1,2-naphthoquinone. However, only one chemiluminescent band at a molecular weight of 22 kDa was observed in the lane loaded with the protein sample incubated with 1.0 μM quinone. As expected, no chemiluminescent bands were observed in the lane loaded with control protein sample which was

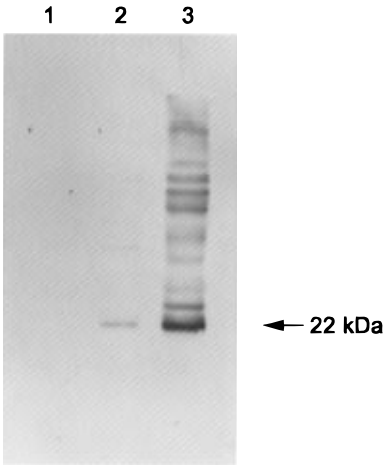


Figure 4. Immunochemical detection of 1,2-naphthoquinone−proteins in mouse liver homogenates incubated with vehicle (lane 1), 1.0 μM 1,2-naphthoquinone (lane 2), or 10 μM 1,2-naphthoquinone (lane 3). Each gel lane was loaded with 2.0 μg of protein sample.

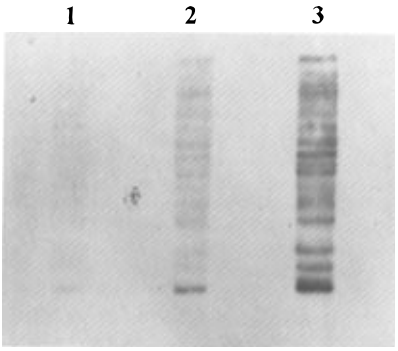


Figure 5. Competitive immunostaining of 1,2-naphthoquinone−proteins in mouse liver homogenates incubated with 10 μM 1,2-naphthoquinone in the presence of vehicle (lane 3) or 1.0 μM (lane 2) or 10 μM (lane 1) *N*-acetyl-*S*-(1,2-dihydroxy-4-naphthyl)-cysteine, analyte **2**. Each lane was loaded with 2.0 μg of protein sample.

processed in the same manner in the absence of 1,2-naphthoquinone. This suggests that the antibodies are able to detect cellular proteins modified by 1,2-naphthoquinone and show no cross reaction toward unmodified native cellular proteins.

Competitive immunostaining was conducted to determine the specificity of antibody recognition of the modification at cysteine residues of proteins by 1,2-naphthoquinone. The Immobilon-P transfer membranes blotted with mouse liver proteins which had been exposed to 10 μM 1,2-naphthoquinone were incubated with a 1/4000 dilution of the antiserum raised against immunogen **I** in the absence or presence of *N*-acetyl-*S*-(1,2-dihydroxy-4-naphthyl)cysteine (**2**) at various concentrations. As Figure 5 shows, the presence of 1.0 μM analyte **2** in the incubation of the blotted membrane with the antiserum partially inhibited the immunostaining of the protein adducts, and the presence of 10 μM analyte **2** completely prevented the antibodies from binding to the protein adducts. This indicates that the binding of the antibodies to 1,2-naphthoquinone protein adducts attributes the immunorecognition of the cysteine residue modification by the naphthoquinone. This antiserum would be a useful tool to detect protein modification by 1,2-naphthoquinone and facilitate the investigation of the mechanism of naphthoquinone- and naphthalene-induced cytotoxicity.

In summary, we have successfully raised polyclonal antibodies to detect 1,2-naphthoquinone cysteine adduct. These antibodies are able to detect 1,2-naphthoquinone-modified cellular proteins with no cross reactivity toward native cellular proteins, providing a powerful tool for the investigation of the mechanism of cytotoxicity induced by 1,2-naphthoquinone and naphthalene. In addition, the antibodies could be used for ELISAs to detect urinary 1,2-naphthoquinone-derived mercapturic acid as a biomarker of human exposure to naphthalene.

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Supporting Information Available: Supporting information of the analysis of *N*-acetyl-*S*-(1,4-dihydroxy-2-naphthyl)-cyteine (**3**), *N*-acetyl-*S*-(1,2-dihydro-1-hydroxy-1-naphthyl)-cysteine (**4**), and *N*-acetylcysteine (**5**) in a competitive ELISA using an antiserum raised against immunogen **I** in a microtiter plate coated with the 1,2-naphthoquinone adduct on mouse serum albumin (3 pages) is available. Ordering information can be found on any current masthead.

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