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Molecular Mechanism For Prevention of *N*-Acetyl-*p*-benzoquinoneimine Cytotoxicity By the Permeable Thiol Drugs Diethyldithiocarbamate and Dithiothreitol

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SUMMARY

The present study was carried out to elucidate the mechanism by which the permeable thiol drug diethyldithiocarbamate (DEDC) exhibited an antidotal effect against acetaminophen-induced hepatotoxicity *in vivo*. DEDC was found to act as an antidote against acetaminophen-induced cytotoxicity in hepatocytes isolated from a pyrazole-pretreated rat without affecting cytochrome P-450 levels. The mechanism of protection exhibited against reactive intermediate *N*-acetyl-*p*-benzoquinoneimine (NAPQI)-induced cytotoxicity by DEDC was then investigated and compared with that exhibited by the permeable thiol-reductant dithiothreitol (DTT). Cytotoxicity induced by the dimethylated analogue 2,6-dimethyl-*N*-acetyl-*p*-benzoquinoneimine (2,6-diMeNAPQI) was prevented if the hepatocytes were preincubated with DEDC for 5 min and removed before addition of 2,6-diMeNAPQI. Both DEDC and DTT were also found to act as antidotes against NAPQI- and 2,6-diMeNAPQI-induced cytotoxicity in isolated rat hepatocytes if added within 2 min of the addition of the quinoneimines. However, the addition of DEDC or DTT 10 min after either quinoneimine did not prevent subsequent cytotoxicity or restore GSH levels, indicating that the alkylation of GSH and of protein thiols was irreversible at that time. Fast atom bombardment mass spectrometry was used to show that DEDC formed conjugates with both NAPQI and 2,6-diMeNAPQI. Furthermore, these conjugates were found to be nontoxic. This suggests that DEDC acts as a trap for the toxic quinoneimines, thus preventing alkylation of essential macromolecules. In contrast, DTT reduced the quinoneimines to their respective nontoxic parent compounds and presumably also reduced mixed-protein disulfides and GSSG, thereby regenerating protein thiols and GSH. Therefore, this study suggests that DEDC and DTT act as antidotes by two different mechanisms.

Acetaminophen (paracetamol, 4-acetamidophenol) is a widely used analgesic and antipyretic drug that, when administered in high concentrations, produces centrilobular liver necrosis in humans and other susceptible species (1–6). Metabolic activation of acetaminophen by the microsomal cytochrome P-450 mixed function oxidase system (6, 7) results in the formation of NAPQI, which is the putative reactive metabolite implicated in acetaminophen hepatotoxicity (6, 8–11). It is a highly reactive electrophile as well as an oxidant (6, 9). NAPQI-induced cytotoxicity in isolated hepatocytes follows the oxidation of GSH, GSH conjugate formation, and protein alkylation, as well as alterations in mitochondrial function (6, 9, 10). The dimethylated NAPQI analogue 2,6-diMeNAPQI also forms a GSH conjugate with GSH *in vitro* (12) and in isolated hepatocytes (9).

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DEDC (see Fig. 1) is a thiol drug that has been reported to protect mice *in vivo* against such toxicants as carbon tetrachloride, allyl alcohol, bromobenzene, and thioacetamide (13–15). Younes *et al.* (16) later found that DEDC protected phenobarbital-treated rats *in vivo* against acetaminophen-induced hepatotoxicity. DEDC was also found to be a potent antidote against hepatic damage caused by acetaminophen in mice (17). Younes *et al.* (18) also showed that DEDC not only was capable of suppressing acetaminophen-induced hepatotoxicity and nephrotoxicity but also inhibited the acetaminophen-induced GSH depletion in the liver and kidney. Furthermore, the oxidized DEDC metabolite disulfiram (see Fig. 1) was also found to protect rats against acetaminophen-induced hepatotoxicity when injected chronically for 1 week before administration of acetaminophen (19). Earlier studies have shown that a single intraperitoneal injection of DEDC or disulfiram caused a significant decrease in cytochrome P-450 but only after 6–24 hr in rats (20–23). From these studies it was suggested that DEDC

ABBREVIATIONS: NAPQI, *N*-acetyl-*p*-benzoquinoneimine; 2,6-diMeNAPQI, 2,6-dimethyl-*N*-acetyl-*p*-benzoquinoneimine; DEDC, diethyldithiocarbamate; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; FAB, fast atom bombardment; DMSO, dimethyl sulfoxide.

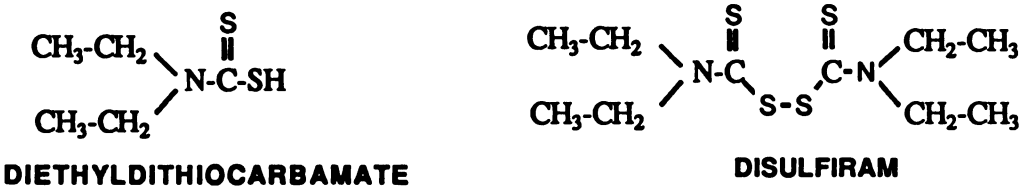
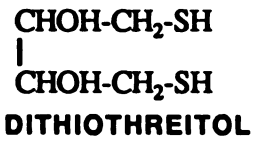


Fig. 1. Molecular structure of the thiol drug DEDC, its toxic metabolite disulfiram, and the sulfhydryl-reducing agent DTT.



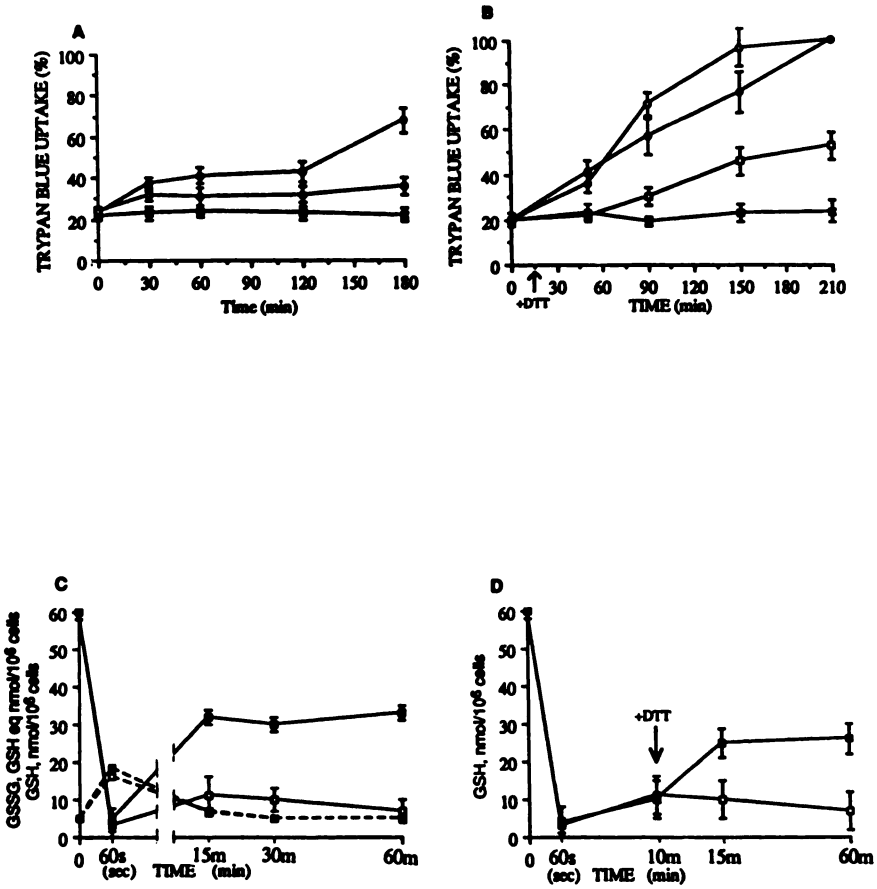


Fig. 2. A, Protection against acetaminophen-induced cytotoxicity by DEDC in isolated rat hepatocytes (10^6 cells/ml) from a pyrazole-pretreated rat. Cell viability was determined by trypan blue exclusion at various time points. Hepatocytes were incubated with acetaminophen (10 mM) alone (●), with DEDC (1 mM) added at 2 min after acetaminophen (○), or with no addition (control cells) (×). Values represent averages of three separate experiments, with *error bars* representing the standard deviations. B, Protection against NAPQI-induced cytotoxicity by DEDC in isolated rat hepatocytes (10^6 cells/ml). Cell viability was determined by trypan blue exclusion at various time points. Hepatocytes were incubated with NAPQI (300 μM) alone (○), with DEDC (1 mM) added at 2 min (□) after quinoneimine, with DTT (10 mM) added at 10 min after quinoneimine (●), or with no addition (control cells) (×). Values represent averages of three separate experiments, with *error bars* representing the standard deviations. C, Effect of NAPQI, with or without DEDC, on hepatocyte GSH and GSSG (– – –) levels. Hepatocytes (10^6 cells/ml) were incubated with NAPQI (300 μM) alone (□) or with DEDC (1 mM) (■). GSH and GSSG levels were determined by the method of Reed *et al.* (34), as described in Experimental Procedures. Values represent averages of three separate experiments, with *error bars* representing the standard deviations. D, Effect of DTT on hepatocyte GSH levels in cells treated with NAPQI. Hepatocytes (10^6 cells/ml) were incubated with NAPQI (300 μM) alone (□) or with DTT (10 mM) added at 10 min after the quinoneimine (■). GSH levels were determined by the method of Reed *et al.* (34), as described in Experimental Procedures. Values represent averages of three experiments, with *error bars* representing the standard deviations.

or disulfiram (injected before acetaminophen) protects against acetaminophen-induced liver damage by inhibiting the cytochrome P-450 mixed function oxidase system believed to catalyze the oxidation of acetaminophen to NAPQI (13, 16–19). However, inactivation of cytochrome P-450 does not explain the protective effect of DEDC *in vivo* when administered 1 hr after acetaminophen. The purpose of the present study was to use isolated hepatocytes to elucidate the possible mechanism of protection from NAPQI toxicity observed with DEDC, in order to understand its antidotal activity *in vivo*. Its dimethylated analogue 2,6-diMeNAPQI was used in order to better elucidate the antidotal effect of DEDC and DTT on the quinoneimines.

DTT (see Fig. 1) is a thiol reductant used to protect protein sulfhydryls and restore enzyme activity lost by the oxidation of sulfhydryl groups *in vitro* (24–26). Because DTT was also found to delay NAPQI-induced cytotoxicity in isolated hepatocytes (6, 9), the antidotal effect of the thiol drugs DEDC and DTT against NAPQI-induced cytotoxicity will be compared in this study, in order to better understand the protective action of DEDC in isolated hepatocytes.

In this study, isolated hepatocytes, which have been shown to be a useful and flexible model cell system for studying molecular cytotoxic mechanisms leading to liver necrosis (9), were used. Isolated hepatocytes have the advantage of an *in vitro* system as well as most of the properties of an *in vivo* system, without its obvious limitations due to the difficulties of controlling various parameters.

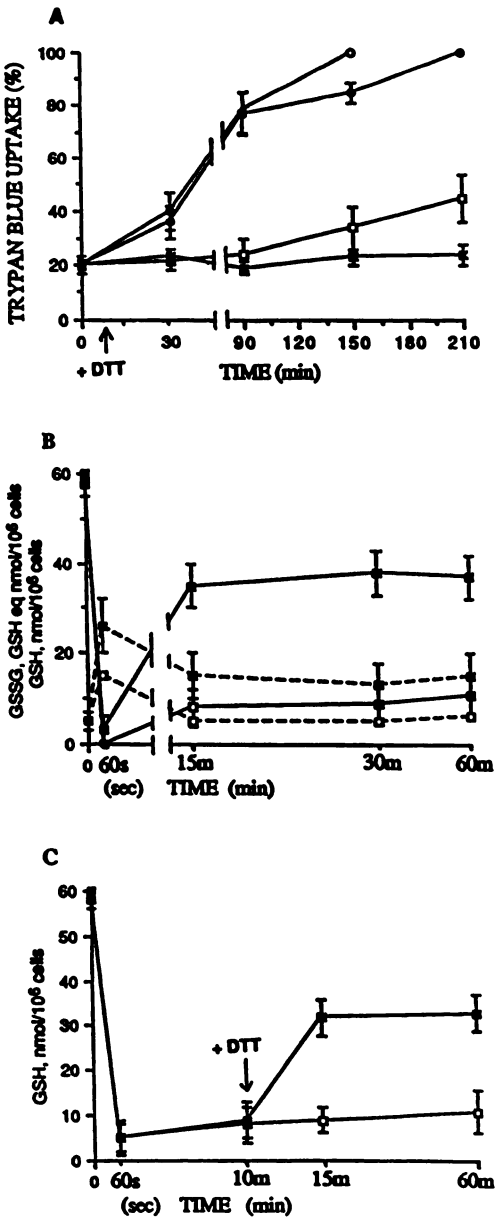


Fig. 3. A, Protection against 2,6-diMeNAPQI-induced cytotoxicity by DEDC in isolated hepatocytes (10^6 cells/ml). Cell viability was determined by trypan blue exclusion at various time points. Hepatocytes were incubated with 2,6-diMeNAPQI (250 μM) alone (○), with DEDC (1 mM) added at 2 min (□) after quinoneimine, with DTT (10 mM) added at 10 min after quinoneimine (●), or with no addition (control cells (×). B, Effect of 2,6-diMeNAPQI, with or without DEDC, on hepatocyte GSH and GSSG (– – –) levels. Hepatocytes (10^6 cells/ml) were incubated with 2,6-diMeNAPQI (150 μM) alone (□) or with DEDC (1 mM) (■). GSH and GSSG levels were determined by the method of Reed *et al.* (34), as described in Experimental Procedures. Values represent averages of three experiments, with *error bars* representing the standard deviations. C, Effect of DTT on hepatocyte GSH levels in cells treated with 2,6-diMeNAPQI. Hepatocytes (10^6 cells/ml) were incubated with 2,6-diMeNAPQI (150 μM) alone (□) or with DTT (10 mM) added at 10 min after the quinoneimine (■). GSH levels were determined by the method of Reed *et al.* (34), as described in Experimental Procedures. Values represent averages of three experiments, with *error bars* representing the standard deviations.

The present study shows that DEDC formed a nontoxic stable conjugate with NAPQI and 2,6-diMeNAPQI. The formation of this conjugate prevented the complete alkylation of GSH and, presumably, protein thiols critical for cell viability.

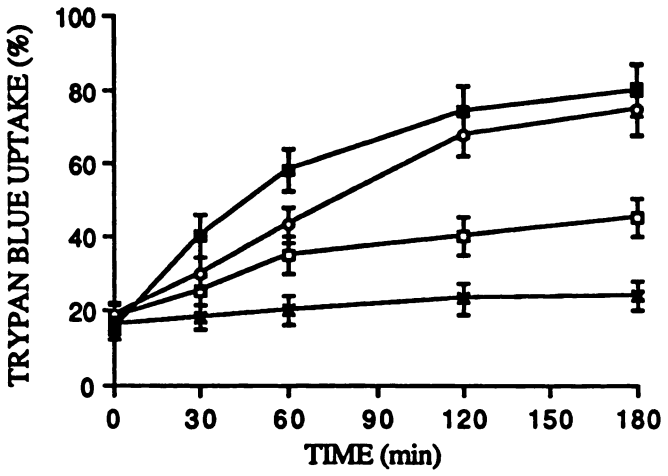


Fig. 4. Effect of preincubating isolated hepatocytes with DTT (1 mM) (○) or DEDC (1 mM) (□) for 5 min, then centrifuging the cells, and discarding the extracellular medium. The pellet was washed and resuspended in incubation buffer before the addition of 2,6-diMeNAPQI (200 μM) (■). 2,6-DiMeNAPQI (200 μM) was added to all the flasks after preincubation, apart from the control cells (×), which were also washed and resuspended in incubation buffer. Values represent averages of three separate experiments, with *error bars* representing the standard deviations.

In contrast, DTT was found to prevent cytotoxicity induced by NAPQI or 2,6-diMeNAPQI, by reducing the quinoneimines to their respective nontoxic parent compounds as well as reducing nonprotein and protein mixed disulfides.

Experimental Procedures

Materials. DEDC, disulfiram, acetaminophen, trypan blue, fluoro-2,4-dinitrobenzene, iodoacetic acid, GSH, GSSG and pyrazole were obtained from Sigma Chemical Co. (St. Louis, MO). DTT was obtained from Aldrich (Milwaukee, WI). Collagenase (from *Clostridium histoliticum*), HEPES and bovine serum albumin were purchased from Boehringer-Mannheim (Montréal, Canada). HPLC solvents were purchased from Caledon Laboratories Ltd (Georgetown, Ontario, Canada). NAPQI and 2,6-diMeNAPQI were purchased from Dalton Chemical Co. (Toronto, Canada). 2,6-Dimethylacetaminophen was synthesized as described by Fernando *et al.* (27). All other chemicals used were of the highest grade purity that was commercially available.

Isolation of rat hepatocytes and cytotoxicity study. Adult male Sprague-Dawley rats (190–210 g) fed *ad libitum* were used to prepare hepatocytes. In order to induce the cytochrome P-450 IIE1 isozyme system, adult male Sprague-Dawley rats were pretreated intraperitoneally with pyrazole, at a daily dose of 200 mg/kg, for 2 days. Pyrazole has been found to efficiently induce P-450 IIE1 or P-450j in rats (28). This specific isozyme, P-450 IIE1, is also known to catalyze the metabolism of acetaminophen to NAPQI (29–32). Isolated hepatocytes were prepared by collagenase perfusion of the liver, as described by Moldéus *et al.* (33). Cell viability was measured by trypan blue exclusion (final concentration, 0.16%, w/v), and the initial viability of hepatocytes was routinely 85%. Cells (15 ml of 1 × 10^6 cells/ml) were suspended in Krebs-Henseleit buffer, pH 7.4, supplemented with only 12.5 mM HEPES, under 95% O_2/5% CO_2, in round-bottomed flasks continuously rotating in a water bath at 37°. DEDC and DTT were dissolved in incubation buffer. Acetaminophen, NAPQI, and 2,6-diMeNAPQI, were dissolved in DMSO, to a final concentration of 0.1–0.5% DMSO (0.1% DMSO for acetaminophen). These solutions were prepared immediately before use and were added to hepatocytes after 30 min of preincubation. DEDC or DTT was added 2 or 10 min after the quinoneimine or acetaminophen was added to the hepatocyte incubate.

To determine whether the thiol drugs permeated into the cells,

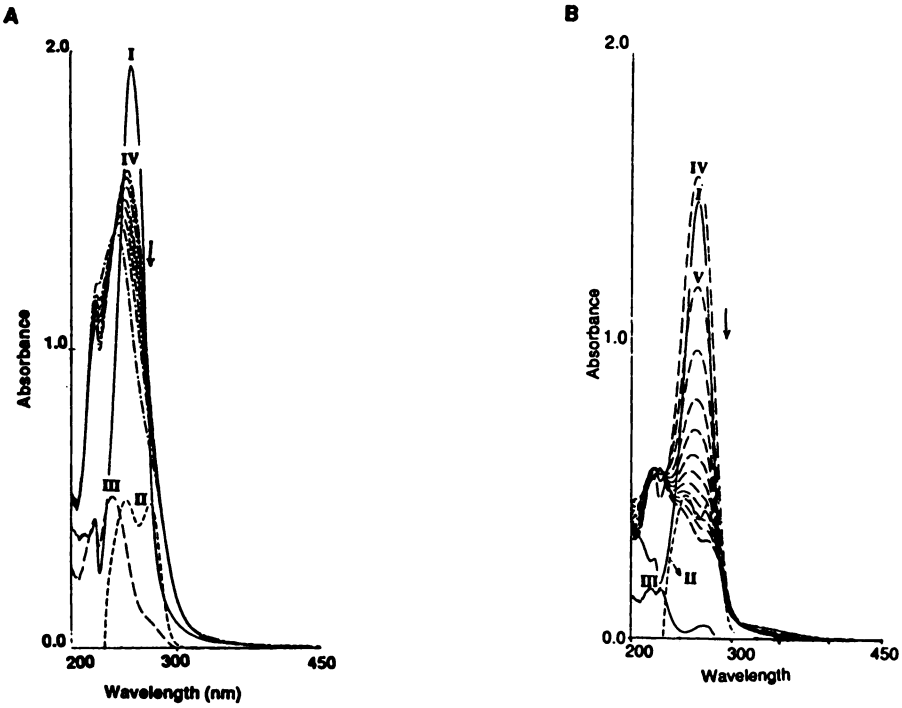


Fig. 5. Spectrophometrical measurement of the reaction of quinoneimines with DEDC. A, NAPQI (50 μM) (I) was reacted with DEDC (100 μM) (II) in 3.0 ml of Tris·HCl buffer, pH 7.4, at 20°. The reaction product (IV) was consecutively scanned over time. Control scans of acetaminophen (50 μM) (III) and DEDC (50 μM) (II) were also measured. B, 2,6-DiMeNAPQI (50 μM) (I) was reacted with DEDC (50 μM) (II) as in A. The reaction product (IV) was consecutively scanned (V) over time. Control scans of 2,6-dimethylacetaminophen (50 μM) (III) and DEDC (50 μm) (II) were also measured.

isolated hepatocytes were pretreated with DEDC, DTT, or GSH for 5 min and then centrifuged (50 × *g*), and the extracellular medium was discarded. The pellet was washed and resuspended in incubation buffer before the addition of 2,6-diMeNAPQI. Cell viability was assayed as described above.

Glutathione assay. The total amounts of GSH and GSSG in isolated hepatocytes were measured on deproteinized samples (5% metaphosphoric acid) after derivatization with iodoacetic acid and fluoro-2,4-dinitrobenzene, as described by Reed *et al.* (34). Quantitation was carried out on a μBondapak NH_2 column, using a Waters 6000A solvent delivery system equipped with a model 660 solvent programmer, a WISP 710A automatic injector, and a data module (Waters Associates, Milford, MA). GSH and GSSG were used as external standards.

Spectral determination of cytochrome P-450. The postmitochondrial supernatant was obtained from normal and DEDC (2 mM)-treated (for 30 min) isolated rat hepatocytes (35). Protein content was readily determined by the colorimetric method of Lowry *et al.* (36), with reference to a standard curve of bovine serum albumin. Cytochrome P-450 was determined by difference spectroscopy, as described by Omura and Sato (37), using an extinction coefficient of 91 mM^{-1} cm^{-1}.

UV spectral analysis of reaction products of quinoneimines and DEDC. The formation of the quinoneimine-DEDC conjugate was detected spectrophotometrically using a Beckman DU-7 spectrophotometer. Incubations were carried out in 3.0 ml of Tris·HCl buffer solution (0.1 M, pH 7.4) containing 50 μM NAPQI or 2,6-diMeNAPQI, dissolved in DMSO (final concentration, 0.15%), and 100 or 50 μM DEDC, respectively.

Quantitative analysis of reaction products of quinoneimines and DEDC or DTT. A quantitative analysis of the reaction of the quinoneimines and DEDC was carried out by HPLC (Shimadzu LC 6A with a Shimadzu SPD-6AV UV-visible spectrophotometer and a Shimadzu Chromatopac C-R3A recorder). DEDC (50, 100, or 200 μM) or DTT (200 μM) and each quinoneimine (100 μM) were combined in Tris·HCl buffer solution (0.1 M, pH 7.4) for various periods of time and analyzed using a C18 μBondapak reverse phase column (0.3 mm × 30 cm) (Waters Associates, Milford, MA) eluted at a flow rate of 1 ml/min with a linear gradient system of methanol/water (10:90 to 100:0 over 25 min) (UV monitoring at 260 nm). The NAPQI-DEDC conjugate was eluted with a retention time of 17.5 min, and the 2,6-diMeNAPQI-DEDC conjugate was eluted with a retention time of 17.8 min.

Large-scale isolation of the conjugates was carried out using HPLC (Beckman 421 controller, 110B solvent delivery module) with a Waters Millipore (Milford, MA) C18 μBondapak reverse phase column (0.3 mm × 30 cm). NAPQI (200 μM) or 2,6-diMeNAPQI (200 μM) and DEDC (200 μM) were reacted in 0.1 M Tris·HCl buffer, pH 7.4, and analyzed as described above. Methanol was removed from pooled samples with compressed nitrogen gas, and the samples were lyophilized and stored at −20° under nitrogen for mass spectrometry analysis.

Isolation of *N*-acetyl-2,6-dimethyl-3-(diethyldithiocarbamate-*S*-yl)-4-hydroxyaniline from hepatocytes. Hepatocytes (2×10^7 cells/ml) were incubated with 2,6-diMeNAPQI (5 mM) and DEDC (20 mM) for 15 min at 37°, under 95% O_2/5% CO_2. Stock solutions (1 M) of 2,6-diMeNAPQI were made up in DMSO, and an equivalent amount of DMSO was added to the control. DEDC stock solution (5 M) was dissolved in incubation buffer. Incubations were terminated by the addition of metaphosphoric acid (final concentration, 5%). The precipitated protein was removed by centrifugation, and the pH of the supernatant was adjusted to pH 5.5. Samples were stored at −20° (38). Aliquots were examined by HPLC (260 nm), using a C18 μBondapak reverse phase column (0.3 mm × 30 cm) (Waters Associates) eluted at a flow rate of 1 ml/min with a shallow linear gradient system of methanol/water (10:90 to 100:0 over 30 min). *N*-Acetyl-2,6-dimethyl-3-(diethyldithiocarbamate-*S*-yl)-4-hydroxyaniline was eluted with a retention time of 17.8 min. 2,6-DiMeNAPQI was used instead of NAPQI for this study because of the high cost of NAPQI.

Mass spectrometry of conjugate. Mass spectra were recorded using a VG Analytical ZAB-1F instrument equipped for FAB for the 2,6-diMeNAPQI-DEDC conjugate and the NAPQI-DEDC conjugate. For FAB analysis, compounds were dissolved in thioglycerol and placed directly on the probe.

Results

Effect of DEDC and DTT on acetaminophen- or NAPQI-induced cytotoxicity and GSH levels. Acetaminophen-induced hepatotoxicity has been found to be increased after chronic ethanol consumption (29–31). In addition, pyra-

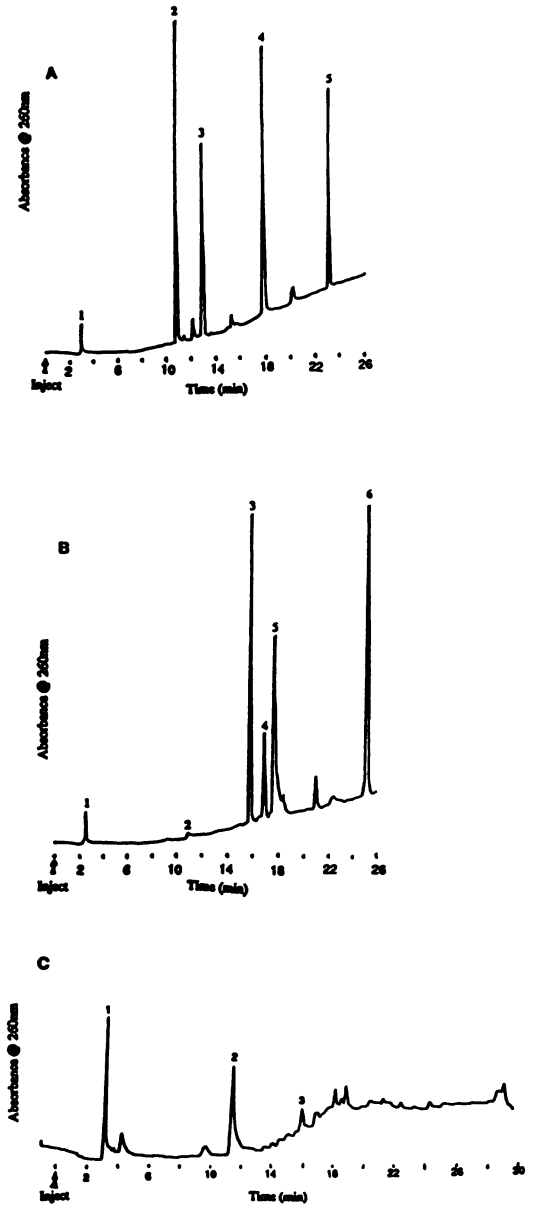


Fig. 6. HPLC elution profiles of the reaction products of quinoneimines and DEDC or DTT. Chromatographic conditions consisted of a linear gradient system of methanol/water (10:90 to 100:0), at a flow rate of 1 ml/min, over 25 min. A, Reaction of DEDC (100 μM) with NAPQI (100 μM) in 1 ml of 0.1 M Tris·HCl buffer, pH 7.4, after 1.5 min. *Peak 1*, solvent front, 2.8 min; *peak 2*, acetaminophen, 10.6 min; *peak 3*, NAPQI, 12.7 min; *peak 4*, *N*-acetyl-3-(diethyldithiocarbamate-*S*-yl)-4-hydroxyaniline, 17.5 min; *peak 5*, disulfiram, 22.8 min. B, Reaction of DEDC (100 μM) with 2,6-diMeNAPQI (100 μM) in 1.0 ml of 0.1 M Tris·HCl buffer, pH 7.4, after 1.5 min. *Peak 1*, solvent front, 2.9 min; *peak 2*, 2,6-dimethylacetaminophen, 11.1 min; *peaks 3* and *4*, 2,6-diMeNAPQI, 15.9 and 17 min; *peak 5*, *N*-acetyl-2,6-dimethyl-3-(diethyldithiocarbamate-*S*-yl)-4-hydroxyaniline, 17.8 min; *peak 6*, DEDC, 25.2 min. C, Reaction of DTT (200 μM) with 2,6-diMeNAPQI (100 μM) in 1.0 ml of 0.1 M Tris·HCl buffer, pH 7.4, after 1.5 min. *Peak 1*, solvent front and DTT, 3.4 min; *peak 2*, 2,6-dimethyl-acetaminophen, 12.1 min; *peak 3*, 2,6-diMeNAPQI, 16.0 mins.

zole and ethanol both induce cytochrome P-450 IIE1 (28), which catalyzes the oxidation of acetaminophen to its reactive metabolite, NAPQI (32). As shown in Fig. 2A, DEDC (1 mM) protected pyrazole-induced isolated rat hepatocytes against acetaminophen-induced cytotoxicity when added 2 min after acetaminophen (10 mM). DEDC (5 mM) is not cytotoxic to isolated hepatocytes (39). Because DEDC has been shown to inactivate hepatic cytochrome P-450 *in vivo* (20–23), the specific content of cytochrome P-450 was measured in the post-mitochondrial supernatant of the hepatocytes. It was found that the cytochrome P-450 content of isolated rat hepatocytes pretreated with DEDC (2 mM) for 30 min was unchanged (0.05 ± 0.01 nmol/mg of protein) from the cytochrome P-450 content in control isolated hepatocytes.

The reactive metabolite of acetaminophen, NAPQI, caused up to 60% cytotoxicity in isolated hepatocytes after 90 min, as determined by trypan blue uptake. The addition of DEDC (1 mM) or the sulfhydryl-reducing agent DTT (10 mM) (data not shown) protected hepatocytes against NAPQI (300 μM)-induced cytotoxicity when added 2 min after NAPQI (Fig. 2B). However, neither DEDC (1 mM) (data not shown) nor DTT (10 mM) prevented subsequent NAPQI (300 μM)-induced cytotoxicity when added 10 min after NAPQI.

Hepatocyte GSH levels were rapidly depleted in the presence of 300 μM NAPQI (Fig. 2C). The GSH levels partially recovered after 15 min of incubation but then gradually decreased throughout the course of the experiment. Presumably, the early GSH recovery reflects the reduction of GSSG formed by the oxidation of approximately 30% of the GSH. In the presence of DEDC (1 mM), added 60 sec after NAPQI (300 μM), GSH levels recovered 50% and remained sustained throughout the course of the experiment. In order to determine whether NAPQI-induced cytotoxicity is the result of alkylation or oxidative stress caused by the formation of mixed protein disulfides, a large excess of DTT (10 mM) was added 10 min after NAPQI (300 μM). This resulted in the recovery of only 30% of the GSH, indicating that the alkylation of GSH and of protein thiols was mostly irreversible (Fig. 2D).

Effect of DEDC and DTT on 2,6-diMeNAPQI-induced cytotoxicity and GSH levels. The dimethylated NAPQI analogue 2,6-diMeNAPQI (250 μM) caused 70% cell death, as determined by trypan blue uptake, after 90 min, as shown in Fig. 3A. DEDC (1 mM) added 2 min after the quinoneimine protected hepatocytes from 2,6-diMeNAPQI-induced cytotoxicity (Fig. 3A). However, as with NAPQI, neither DEDC (1 mM) nor DTT (10 mM), added 10 min after 2,6-diMeNAPQI, prevented subsequent cytotoxicity induced by 2,6-diMeNAPQI. As shown in Fig. 3B, hepatocyte GSH levels were rapidly depleted when 2,6-diMeNAPQI (150 μM) was added to hepatocytes, but the GSH levels partly recovered after 1–2 min, reflecting the reduction of some of the GSSG formed initially. GSH levels remained low during the course of the experiment. In the presence of DEDC (1 mM), added 2 min after the quinoneimine, GSH levels recovered 60% and remained sustained throughout the course of the experiment (Fig. 3B). DTT (10 mM), added 10 min after 2,6-diMeNAPQI, did not prevent cytotoxicity, although approximately 50% of the GSH was restored, indicating that part of the GSH depletion was due to the formation of mixed protein disulfides.

The effectiveness of the antidotal action of DEDC and DTT against 2,6-diMeNAPQI-induced cytotoxicity was further investigated by determining whether these thiol drugs permeated the cell membrane and were retained by the hepatocyte. To avoid the direct reaction of 2,6-diMeNAPQI and DEDC in the medium and to determine whether DEDC can carry out its antidotal activity intracellularly, hepatocytes were preincubated with DEDC for 5 min and resuspended in fresh medium

TABLE 1

Reaction products from separate *in vitro* reaction of DEDC with the two quinoneimines

Values represent DEDC equivalent nmol of reactant or products detected. Values for the conjugate are expressed as the equivalent amounts of quinoneimine not accounted for by phenol and disulfiram formation. NAPQI and 2,6-diMeNAPQI (100 nmol) were incubated with increasing concentrations of DEDC for 1.5 min, and the reaction products were determined as described in Experimental Procedures.

Reactants	Products formed				
	Remaining quinone, 12.7 min[a]	Remaining DEDC, 25.2 min	Phenol, 10.6 min	Disulfiram, 22.8 min	Conjugate, 17.5 min
	nmol		*nmol*		
DEDC (0.05 M) + NAPQI (0.1 M)	49		15	12	38
DEDC (0.1 M) + NAPQI (0.1 M)	6		31	30	63
DEDC (0.2 M) + NAPQI (0.1 M)	0	90	45	50	62
	Products formed				
Reactants	Remaining quinone, 15.9 and 17.0 min	Remaining DEDC, 25.2 min	Phenol, 11.1 min	Disulfiram, 22.8 min	Conjugate, 17.8 min
	nmol		*nmol*		
DEDC (0.05 M) + 2,6-diMeNAPQI (0.1 M)	45				56
DEDC (0.1 M) + 2,6-diMeNAPQI (0.1 M)	20	23	11	10	72
DEDC (0.2 M) + 2,6-diMeNAPQI (0.1 M)	3	101	12	10	84

[a] Retention time.

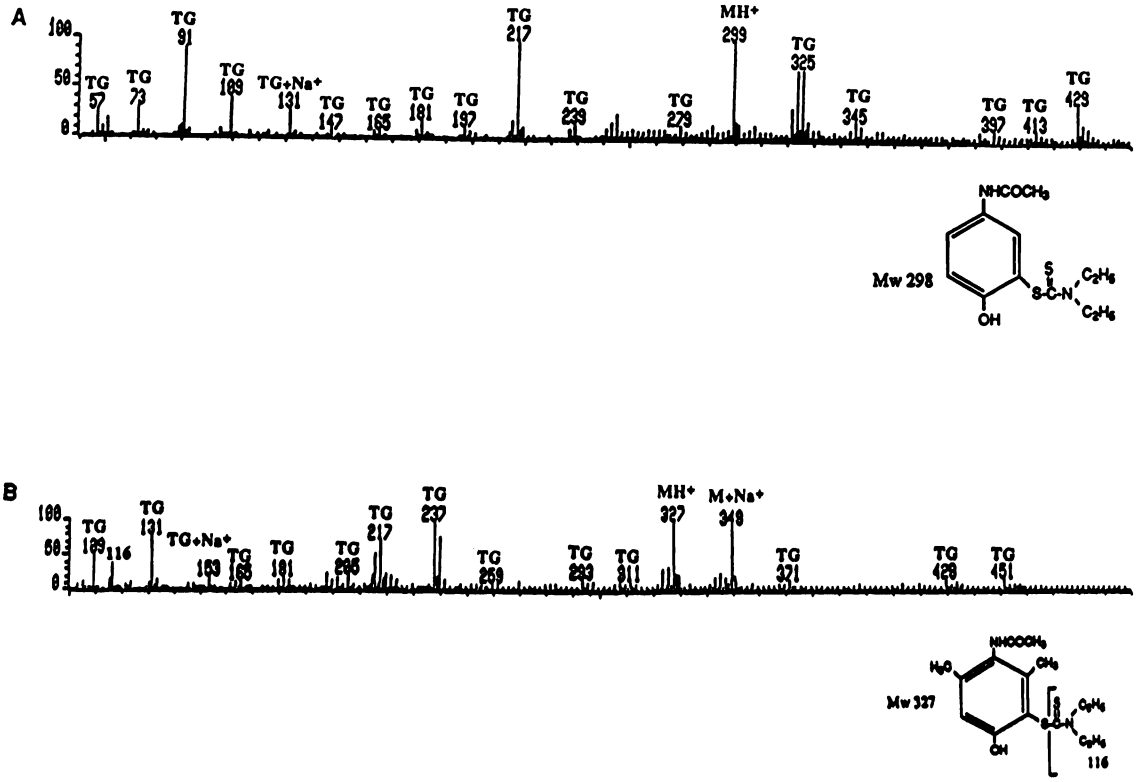


Fig. 7. A, FAB mass spectrum of DEDC/NAPQI conjugate identified as *N*-acetyl-3-(diethyldithiocarbamate-*S*-yl)-4-hydroxyaniline. *TG*, thioglycerol. B, FAB mass spectrum of DEDC-2,6-diMeNAPQI conjugate identified as *N*-acetyl-2,6-dimethyl-3-(diethyldithiocarbamate-*S*-yl)-4-hydroxyaniline.

before the addition of 2,6-diMeNAPQI. As shown in Fig. 4, substantial protection was found, indicating that DEDC readily permeated the cell membrane and was retained by the hepatocyte. In contrast, DTT was much less effective, indicating that DTT was not retained by the hepatocyte. GSH did not protect hepatocytes against 2,6-diMeNAPQI-induced cytotoxicity, which strongly suggests that it did not permeate the cell membrane (data not shown).

Quantitation and identification of the reaction products of DEDC or DTT with NAPQI or 2,6-diMeNAPQI. The UV spectrophotometrical analysis of 100 μM DEDC (II) reacted with NAPQI (I) in 0.1 M Tris·HCl buffer, pH 7.4, formed the product IV (Fig. 5A). In Fig. 5B, the UV spectrophotometrical analysis of an equimolar concentration of 2,6-diMeNAPQI (I) reacted with DEDC (II) resulted in the formation of the product IV, which decreased with time (V). The HPLC elution profile of the reaction products of DEDC (100 μM) with NAPQI (100 μM) in 0.1 M Tris·HCl buffer, pH 7.4, after 90 sec (Fig. 6A) showed the formation of a single new product, with a retention time of 17.5 min, which comprised 70% of the reaction products. The remaining 30% of the products comprised the oxidation-reduction reaction products,

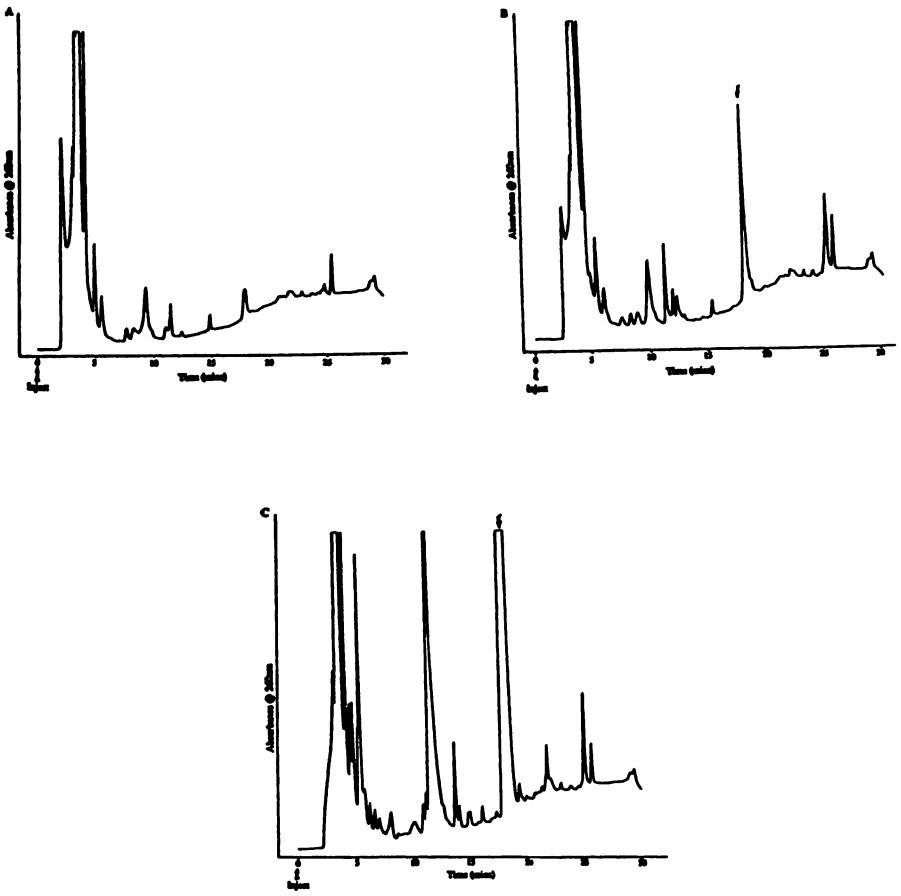


Fig. 8. HPLC tracings of hepatocyte extracts from cells treated with 2,6-diMeNAPQI and DEDC. A, Control cells. B, Control cells spiked with standard *N*-acetyl-2,6-dimethyl-3-(diethyldithiocarbamate-*S*-yl)-4-hydroxyaniline, as shown by the *arrow* (retention time, 17.8 min). C, Cells treated with 5 mM 2,6-diMeNAPQI and 20 mM DEDC. *Arrow*, *N*-acetyl-2,6-dimethyl-3-(diethyldithiocarbamate-*S*-yl)-4-hydroxyaniline (retention time, 17.8 min). In each case, hepatocytes (2×10^7 cells/ml) were incubated at 37° for 10 min with substrate, and the reaction was terminated by the addition of metaphosphoric acid (final concentration, 5%).

namely, acetaminophen and disulfiram, as shown in Fig. 6A and Table 1. The new product was quantitated according to the amount of DEDC and NAPQI missing and the amount of disulfiram and acetaminophen formed after the reaction. As shown in Fig. 7A, FAB mass spectral analysis of the isolated product yielded a base peak with *m/z* 299, corresponding to the (MH^+) ion. This was consistent with the structure *N*-acetyl-3-(diethyldithiocarbamate-*S*-yl)-4-hydroxyaniline. The HPLC elution profile of the reaction of 2,6-diMeNAPQI (100 μM) and DEDC (100 μM) in 0.1 M Tris·HCl buffer, pH 7.4, at 90 sec (Fig. 6B) revealed a single new product, with a retention time of 17.8 min, which accounted for approximately 80% of the reaction products formed, as shown in Table 1. Quinoneimines are very reactive compounds to work with and, therefore, are difficult to analyze by HPLC, which explains why 2,6-diMeNAPQI eluted as two peaks. The 300 MHz ^1H NMR and ^{13}C NMR spectras of 2,6-diMeNAPQI, which were provided by Dalton Chemical Co. (Toronto, Canada) showed only the expected signals, which certified that 2,6-diMeNAPQI was 100% pure. As shown by Fernando *et al.* (27), 2,6-diMeNAPQI has been found to react with various compounds, such as ethyl alcohol. However, when the amount of conjugate formed by the reaction of 2,6-diMeNAPQI with DEDC was quantitated, both peaks of 2,6-diMeNAPQI were taken into account. As shown in Table 1, only 20% of the reaction was accounted for by oxidation-reduction to disulfiram and 2,6-dimethylacetaminophen. Incubation of hepatocytes (2×10^7 cells/ml) with 5 mM 2,6-diMeNAPQI (equivalent to 250 μM substrate with 1×10^6 cells/ml) led to the formation of approximately 80% *N*-acetyl-2,6-dimethyl-3-(diethyldithiocarbamate-*S*-yl)-4-hydroxyaniline, as determined by HPLC (Fig. 8C). As shown in Fig. 7B, FAB mass spectral analysis of the isolated product yielded a base peak at *m/z* 327, corresponding to the (MH^+) ion and a peak at *m/z* 349 ($M + Na^+$). This was consistent with the structure of the conjugate *N*-acetyl-2,6-dimethyl-3-(diethyldithiocarbamate)-4-hydroxyaniline.

As shown in Fig. 6C, the HPLC elution profile of the reaction products of DTT (200 μM) with 2,6-diMeNAPQI (100 μM) in 0.1 M Tris·HCl buffer, pH 7.4, demonstrated the complete reduction of the quinoneimine to its nontoxic parent compound, 2,6-dimethylacetaminophen. No evidence of conjugate formation was found.

Discussion

In order to explain the antidotal properties of DEDC against acetaminophen hepatotoxicity *in vivo*, the present study investigated the molecular mechanisms by which DEDC protects isolated hepatocytes against the toxic acetaminophen metabolite NAPQI. The dimethylated quinoneimine analogue 2,6-diMeNAPQI, which is representative of alkylation-induced cytotoxicity (12), was also used in order to better delineate the possible mechanism of protection by DEDC. Furthermore, the mechanism of protection by DEDC against NAPQI-induced cytotoxicity was compared with that exhibited by the sulfhydryl reducing agent DTT, in order to better understand the protective action of DEDC in isolated hepatocytes.

As was found *in vivo* (13–17), DEDC was found to protect against acetaminophen-induced cytotoxicity in isolated rat hepatocytes *in vitro*. Other investigators have concluded that

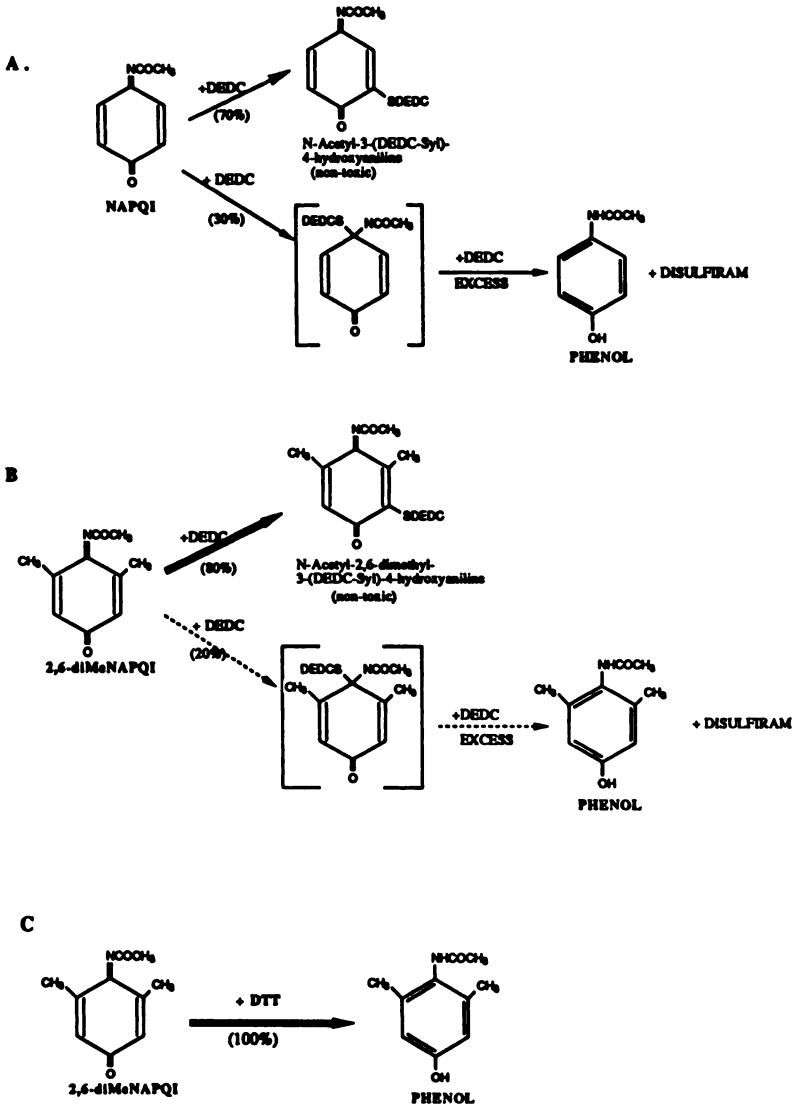


Fig. 9. Hypothetical scheme describing the reaction pathway and subsequent toxicity of DEDC with NAPQI (A), DEDC with 2,6-diMeNAPQI (B), and DTT with 2,6-diMeNAPQI (C).

DEDC protected against acetaminophen-induced hepatotoxicity *in vivo* by inactivating the mixed function oxidase believed to catalyze the oxidation of acetaminophen to NAPQI (11–13, 16, 17). However, isolated hepatocytes pretreated for 30 min with DEDC at the concentration used in this study did not exhibit changes in cytochrome P-450 levels. DEDC may require a longer period of incubation or a higher concentration to inactivate this enzyme (14). For this reason, another mechanism explaining the antidotal activity of DEDC against acetaminophen-induced cytotoxicity was worth investigating.

Acetaminophen was found to rapidly deplete GSH levels over the course of 2 hr in isolated hepatocytes (2, 40, 41). However, as shown by Younes *et al.* (18), DEDC inhibited the acetaminophen-induced GSH depletion in the liver and kidney. DEDC was also found to readily protect isolated rat hepatocytes against NAPQI-induced cytotoxicity. The quinoneimine also reacted very rapidly to deplete cellular GSH, with or without DEDC. However, in the presence of DEDC, the cellular GSH levels recovered more, while GSSG levels remained low, suggesting that DEDC prevented the complete alkylation of GSH and protein thiols. The sulfhydryl reducing agent DTT, which has been found to delay acetaminophen-induced cytotoxicity in isolated hepatocytes (41), also prevented NAPQI-induced cytotoxicity when added 2 min after the quinoneimine. DEDC or DTT did not, however, prevent subsequent cytotoxicity or restore total GSH levels when added 10 min after NAPQI addition, suggesting that the alkylation of GSH and protein thiols is irreversible at that time. Cytotoxicity induced by NAPQI may, therefore, be due mainly to the alkylation of GSH and protein thiols, rather than oxidative stress.

Recently, Rundgren *et al.* (9) have shown that 66% of the GSH depleted in hepatocytes by NAPQI was the result of GSH conjugate formation, whereas the remaining GSH was oxidized to GSSG. GSH oxidation was attributed to GSH reacting with an *ipso*-conjugate. In the present study, 70% of the DEDC depletion was attributed to a NAPQI-DEDC conjugate, which was identified by FAB mass spectrometry as *N*-acetyl-3-(diethyldithiocarbamate-*S*-yl)-4-hydroxyaniline. Only 30% of the DEDC was oxidized to disulfiram, presumably via an *ipso*-adduct. GSH and DEDC, therefore, appear to react at similar positions on the NAPQI molecule, that is, at the C-3 position to form a stable conjugate and at the C-1 position to form an unstable postulated *ipso*-conjugate, which reacts with the thiol compound to form the oxidized thiol compound. Because the

oxidation-reduction reaction accounts for only 30% of the reaction, the amount of disulfiram formed is not enough to enhance cytotoxicity (42), even when excess DEDC is used to protect hepatocytes from NAPQI. The DEDC-NAPQI conjugate formed explains why GSH levels were not completely depleted, because DEDC, GSH, and protein thiols presumably compete for NAPQI. Therefore, DEDC protects isolated hepatocytes from NAPQI-induced cytotoxicity by conjugating NAPQI and preventing the extensive GSH depletion and cytotoxic alkylation of protein thiols (Fig. 9A).

DEDC also protected isolated hepatocytes from 2,6-diMeNAPQI-induced cytotoxicity. This quinoneimine also readily depleted GSH levels, with some GSSG formation, both in the presence and in the absence of DEDC. However, in the presence of DEDC, GSH recovered more, which further suggests that DEDC prevented the alkylation of GSH and protein thiols by 2,6-diMeNAPQI. Rosen *et al.* (12) postulated that 2,6-diMeNAPQI-induced cytotoxicity was attributed to the alkylation of GSH and protein thiols. However, Rundgren *et al.* (9) found that 2,6-diMeNAPQI formed significant amounts of GSSG in hepatocytes and lesser amounts of a stable GSH conjugate. Thus, they concluded that 2,6-diMeNAPQI may be especially cytotoxic because of its ability to both alkylate and cause oxidative stress.

However, GSH and DEDC differ in their reactivity with the 2,6-diMeNAPQI molecule. Rundgren *et al.* (9) reported that GSH reacts chemically with 2,6-diMeNAPQI to form 10% GSH conjugate, with 90% being oxidized to GSSG. In the experiments reported here, analysis of the products formed by the reaction of 2,6-diMeNAPQI with DEDC showed that approximately 80% of the 2,6-diMeNAPQI underwent conjugation with DEDC to the nontoxic *N*-acetyl-2,6-dimethyl-3-(diethyldithiocarbamate-*S*-yl)-4-hydroxyaniline, thereby explaining the protective mechanism of DEDC for 2,6-diMeNAPQI. This result was further verified when the HPLC analysis of the reaction of DEDC and 2,6-diMeNAPQI in isolated hepatocytes demonstrated the production of approximately 80% 2,6-diMeNAPQI-DEDC conjugate. Furthermore, only 20% of the products were formed by an oxidation-reduction reaction, yielding 2,6-dimethylacetaminophen and disulfiram, as depicted in Fig. 9B. Rundgren *et al.* (9) have speculated that 2,6-diMeNAPQI oxidizes GSH by forming an *ipso*-adduct. Steric factors may prevent DEDC from reacting with 2,6-diMeNAPQI at the C-1 position on the quinoneimine molecule to form an *ipso*-adduct or reacting with the *ipso*-adduct to form disulfiram.

Therefore, DEDC protected isolated hepatocytes from 2,6-diMeNAPQI-induced cytotoxicity by reacting with the quinoneimine at the C-3 position of the aromatic ring and forming a nontoxic conjugate, thus preventing alkylation of GSH and protein thiols. We have also found that DEDC protected against benzoquinone-induced cytotoxicity by forming a nontoxic conjugate, thus preventing the alkylation of critical macromolecules by benzoquinone (39).

Addition of the sulfhydryl reducing agent DTT at 4 min to hepatocytes treated with 2,6-diMeNAPQI restored approximately 66% of the GSH and significantly decreased the extent of covalent binding to proteins (9). Because 2,6-diMeNAPQI was the only quinoneimine to form relatively stable *ipso*-adducts (27), it was speculated that 2,6-diMeNAPQI must react very efficiently with GSH and protein thiols in hepatocytes to form *ipso*-adducts. DTT only delayed the cytotoxicity when added at 4 min (6, 9), which suggests that DTT could restore GSH by reducing GSSG or mixed protein disulfides (26), as well as by reacting with the *ipso*-adducts of 2,6-diMeNAPQI formed with GSH or protein thiols. As analyzed by HPLC, DTT did reduce the quinoneimine to its nontoxic parent compound, 2,6-dimethylacetaminophen. However, addition of DTT 10 min after addition of the quinoneimine did not prevent cytotoxicity, even though 50% of the GSH was restored. This suggests that, at 10 min, irreversible alkylation of protein thiols by 2,6-diMeNAPQI occurred and caused hepatocyte cytotoxicity (Fig. 9C).

The antidotal activity of the thiol drugs DEDC and DTT was further investigated by determining whether these thiols could react with the quinoneimines intracellularly. Preincubating the hepatocytes with DEDC for 5 min and resuspending them in fresh medium before addition of 2,6-diMeNAPQI prevented cytotoxicity, showing that DEDC readily permeates the cell membrane and is retained, presumably because of its lipophilicity. The short preincubation time and lack of inhibition by 25 mM serine, 25 mM alanine, or 25 mM glutamic acid (results not shown) suggest that an amino acid-transporting system is not involved. Research in our laboratory has demonstrated that DTT readily reduces intracellular GSSG, indicating that DTT readily permeates the cell membrane.[1] However, DTT only delayed the cytotoxicity induced by 2,6-diMeNAPQI, which suggests that DTT, being more water soluble than DEDC, is not retained by the cell, thus explaining the lack of complete protection. GSH did not protect against 2,6-diMeNAPQI-induced cytotoxicity, thus suggesting that it does not permeate the cell membrane.

The theory that DEDC protects against *in vivo* acetaminophen hepatotoxicity by inactivating the mixed function oxidase believed to catalyze the oxidation of acetaminophen to NAPQI (13–19) could still apply, particularly when a high enough dose of DEDC is administered before acetaminophen. A single intraperitoneal injection of DEDC or disulfiram caused a significant decrease in cytochrome P-450 in rats, but only after 6–24 hr (20–23). However, DEDC also prevented hepatic damage caused by acetaminophen in mice when added 1 hr after acetaminophen, and DEDC has been recommended as a valuable antidote against acetaminophen overdosage (17). Because acetaminophen-induced liver injury occurs only after GSH depletion of 70% or more (43), the repletion of liver GSH levels when DEDC is present could explain its antidotal activity. Because DEDC acts as an antidote against NAPQI-induced cytotoxicity in isolated hepatocytes by forming a nontoxic DEDC-NAPQI conjugate, it is possible that DEDC may act in a similar way *in vivo*, which could explain the antidotal effect exhibited *in vivo*.

This study demonstrated the antidotal activity of two thiol drugs against NAPQI-induced cytotoxicity in isolated hepatocytes. DTT was found to completely reduce the quinoneimines to their nontoxic parent compound as well as regenerate GSH from GSSG and mixed protein disulfides. The disadvantage of this mechanism of protection is that DTT has no effect on alkylation-induced cytotoxicity, and the parent compound could reoxidize to its reactive intermediate, thus subsequently resulting in hepatotoxicity. On the other hand, DEDC conju-

[1] J. M. Silva, and P. J. O'Brien. Protection against nitrofurantoin-induced cytotoxicity by dithiothreitol. Manuscript in preparation.

gates the reactive intermediate, thus preventing the complete alkylation of GSH and presumably protein thiols and macromolecules that are critical for cell viability.

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