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Comparative Cytotoxic Effects of *N*-Acetyl-*p*-benzoquinone Imine and Two Dimethylated Analogues

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SUMMARY

N-acetyl-*p*-benzoquinone imine (NAPQI), a reactive metabolite of acetaminophen, has previously been shown to be toxic to hepatocytes freshly isolated from rat liver [*Mol. Pharmacol.* **28**:306–311 (1985)] NAPQI arylates and oxidizes cellular thiols, and either one or both reactions may be important in the pathogenesis of cytotoxicity. Two dimethylated analogues of NAPQI, *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine (3,5-diMeNAPQI) and *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine (2,6-diMeNAPQI), were prepared to determine whether one reaction might be more damaging to cells than the other. Of the three quinone imines, the least potent cytotoxin to rat hepatocytes was 3,5-diMeNAPQI. However, the cytotoxicity of 3,5-diMeNAPQI was markedly enhanced by pretreatment of cells with 1,3-bis-(2-chloroethyl)-*N*-nitrosourea, which inhibits glutathione reductase. Reactions of 3,5-diMeNAPQI with GSH, both chemically and in hepatocytes, indicated that this quinone imine primarily oxidized thiols. These findings were corroborated by results of covalent binding experiments, which showed that radiolabeled 3,5-diMeNAPQI bound only to a small extent to hepatocyte proteins. On the other hand, 2,6-diMeNAPQI, the most potent cytotoxin of the three quinone imines that was investigated, bound extensively to hepatocyte proteins. In addition, 2,6-diMeNAPQI reacted with GSH, both chemically and in hepatocytes, to form significant amounts of GSSG. Reduction products of NAPQI and its dimethylated analogues were not important contributors to cytotoxicity or GSSG formation based on the following results: 1) the quinone imines did not increase oxygen consumption by hepatocytes nor did they lead to oxygen uptake in solution; 2) dicoumarol, an inhibitor of the reductase, DT-diaphorase, had no effect on cytotoxicity caused by the quinone imines. Evidence for the involvement of *ipso*-adducts of the quinone imines in their reactions with cellular thiols is provided by results of investigations on the effects of DTT on the metabolism, covalent protein binding, and cytotoxic effects of the quinone imines.

NAPQI is an oxidation product of the widely used analgesic and antipyretic drug acetaminophen and it has been implicated in hepatotoxicity caused by acetaminophen (1, 2). In fact, NAPQI is a metabolite of acetaminophen (3–5) that has both chemical and biochemical properties that are consistent with its role as the major cytotoxin formed by cytochrome P-450 oxidation of acetaminophen (6–11).

Although NAPQI is a potent cytotoxin, it is not clear how it causes cell death. It is highly reactive both as an electrophile and as an oxidant (4, 6, 10), and either one or both properties may be important in the pathogenesis of toxicity. Previous studies with isolated rat hepatocytes indicated that NAPQI caused dose-dependent cytotoxicity (9), which was sensitive to the presence of a variety of protective agents. However, some of the best protective agents (e.g., GSH) could act by one or more mechanism. Later work with the rat hepatocyte model system revealed that cytotoxicity caused by NAPQI is also dependent on the integrity of the cytoprotective enzyme GSSG reductase (10). This finding provided further evidence that oxidative mechanisms could participate in the overall process of cytotoxicity caused by NAPQI.

In order to better delineate the mechanism(s) of NAPQI-mediated cytotoxicity, we have compared, in isolated rat hepatocytes, the toxic effects of NAPQI with those of two of its dimethylated analogues. Chosen for study were 2,6- and 3,5-diMeNAPQI. Previous studies with these compounds indicated that they react with GSH in different ways (12) and that a relatively stable semiquinone imine is generated only from the 3,5-dimethylated analogue (13, 14). Of the three compounds, 3,5-diMeNAPQI reacted with GSH to form only GSSG, whereas both NAPQI and 2,6-diMeNAPQI formed stable GSH conjugates, and NAPQI formed GSSG as well.

Therefore, the major aim of this work was to establish

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ABBREVIATIONS: NAPQI, *N*-acetyl-*p*-benzoquinone imine; BCNU, 1,3-bis-(2-chloroethyl)-*N*-nitrosourea; 3,5-diMeNAPQI,*N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine; 2,6-diMeNAPQI,*N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine; DEM, diethylmaleate; DTT, dithiothreitol; MDA, malondialdehyde; HEPES, *N*-2-hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid; HPLC, high performance liquid chromatography.

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whether the dimethylated quinone imines caused cytotoxicity at levels similar to those found for NAPQI and to determine the importance of the oxidative capacity of the quinone imines in this cytotoxicity. Results of these studies indicate that oxidative stress, independent of lipid peroxidation, probably contributes to the cytotoxic effects of all three quinone imines, although more so with 3,5-diMeNAPQI than either 2,6-diMeNAPQI or NAPQI.

A second aim of the work was to determine whether the quinone imines caused cytotoxicity under conditions that paralleled the reactivities of their respective hydroxyacetanilides as cytotoxins (15). It is not possible to directly relate the results from investigations with the quinone imines to results from investigations with their respective hydroxyacetanilides, because it is not known whether the quinone imines are the only toxic metabolites formed from the hydroxyacetanilides and because mechanisms of toxicity probably differ when the metabolites are generated *in situ* compared with when they are added exogenously in a bolus dose. However, results of the studies reported herein demonstrated that the toxicity of the quinone imines could be modulated by treatments that often produce effects parallel to those on cytotoxicity caused by the hydroxyacetanilides. Thus, we are more confident that the quinone imines, and/or metabolites derived from the quinone imines, are important contributors to the cytotoxic effects observed with their respective hydroxyacetanilides.

Materials and Methods

Collagenase (grade II), DTT, and HEPES were obtained from Boehringer (Mannheim, Federal Republic of Germany). Desferrioxamine [(*N*-[5-3-amino-pentyl)-hydroxycarbonyl]-3-[-5-(*N*-hydroxyacetamido) pentyl]-carbamoyl)-propionyl-hydroxamine-methansulfonic acid) was from Ciba-Geigy, Ltd. (Basle, Switzerland). Acetaminophen (4'-hydroxyacetanilide), DEM, GSH, GSSG, and β-glucuronidase/sulfatase (type H-2) were purchased from Sigma (St. Louis, MO) whereas 2,6-dimethylphenol, 3,5-dimethylphenol, and acetic anhydride were purchased from Aldrich (Milwaukee, WI). BCNU was a generous gift from Bristol Laboratories (Stockholm, Sweden) and [1-^{14}C]acetic anhydride (10 mCi/mmol) was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA). All other reagents were obtained from local commercial sources and were of the highest available quality.

NAPQI was synthesized and purified as previously described (7). [*acetyl*-^{14}C]NAPQI was prepared by the oxidation of [*acetyl*-^{14}C] acetaminophen as reported elsewhere (4). Both 3,5- and 2,6-diMeNAPQI were synthesized as described by Fernando *et al.* (16). [*acetyl*-^{14}C]3,5-diMeNAPQI and [*acetyl*-^{14}C]2,6-diMeNAPQI were prepared by the same methods starting with their respectively labeled hydroxyacetanilides, the synthesis and purification of which have been described elsewhere (15). The quinone imines and their radiolabeled analogues were all 98% pure as determined by comparison of melting points with literature values (16) and by HPLC analysis as previously described (12). The labeled quinone imines were diluted with their respective unlabeled compounds to achieve specific activity of approximately 0.1 mCi/mmol and were dissolved immediately before use in distilled and dried (Linde molecular sieves, type 4A) dimethyl sulfoxide.

Male Sprague-Dawley rats (200–250 g) were used in all experiments and were allowed free access to food and water up to the time of hepatocyte preparation. Hepatocytes were isolated by collagenase perfusion of rat livers as previously described (17), and cell incubations were performed at 37° in rotating, round-bottom flasks at a concentration of 2×10^6 cells/ml under an atmosphere of 95% O_2 and 5% CO_2.

Inhibition of glutathione reductase in hepatocytes was performed as previously described (10) in a modified Krebs-Henseleit buffer, supplemented with 25 mM HEPES and an amino acid mixture. After 25 min of treatment with BCNU, the cells were washed and resuspended in the same medium, without BCNU, and incubated for an additional hour in order to restore the GSH level. The incubation medium was then changed to Krebs-Henseleit buffer without amino acids, pH 7.4, supplemented with 25 mM HEPES. Under these conditions, glutathione reductase activity was inhibited by about 90% and GSH was restored to nearly normal concentrations.

Depletion of GSH in hepatocytes was performed by the administration of DEM (0.5 ml/kg intraperitoneally in corn oil) to rats 1 hr before the isolation of hepatocytes. This treatment routinely depleted soluble thiols in hepatocytes to 10–15% of control levels. DT-diaphorase was inhibited almost completely (>95%) by preincubation of cells for 5 min with dicoumarol (1%, w/v, in dimethylsulfoxide to achieve a final concentration in cells of 30 μM).

Assays were performed as follows. Concentrations of GSH were determined with the sulfhydryl reagent monobromobimane (Thiolyte; Calbiochem-Behring, La Jolla, CA) both with and without treatment of cells with DTT (18). In some experiments, GSH and GSSG were determined by HPLC (19) with the following modification: HPO_3 was used in place of perchloric acid to precipitate proteins. Covalent binding of radiolabeled compounds was determined as previously described for NAPQI (10). Protein was measured by a modified method of Lowry (20). The formation of malondialdehyde, as a measure of lipid peroxidation, was determined by the method of Smith *et al.* (21). Hepatocyte integrity was monitored as previously described (22), by the exclusion of trypan blue.

Oxygen consumption was assayed polarographically with a Clark oxygen electrode (model 53; Yellow Springs Instrument Co., Yellow Springs, OH) in a 3.0-ml chamber maintained at 37°. Hepatocytes (2×10^6/ml) were maintained in HEPES-supplemented Krebs-Henseleit buffer, pH 7.4. KCN (1 mM) was added to inhibit mitochondrial respiration.

Products of the reactions of the quinone imines with GSH and with hepatocytes were analyzed by reverse phase thin layer chromatography on RP-18 F_{254} precoated thin layer chromatography plates (5 × 20 cm, 0.25-mm thickness; E, Merck, Darmstadt, Federal Republic of Germany). Aliquots (50 ml) of either reaction mixtures or cell supernatants, after treatment with β-glucuronidase/sulfatase (2780 units/ml), were applied to the plates, which were then developed in a system of H_2O/methanol/acetic acid/ethyl acetate (90:15:1:0.5, v/v) for 15 cm. Synthetic standards had the following mobilities: acetaminophen, 2–3 cm; 3-(*S*-glutathionyl)acetaminophen, 7–10 cm; 3,5-dimethylacetaminophen, 1–2 cm; 2,6-dimethylacetaminophen, 1–3 cm; and 3-(*S*-glutathionyl)-2,6-di-methylacetaminophen, 7–10 cm. After development, the plates were divided into 1-cm sections, which were scraped into scintillation vials and vortexed with 1 ml of 50% methanol/H_2O. To this was added 5 ml of Instagel Scintillation cocktail (Packard B. V., Groningen, The Netherlands) and radioactivity was determined on an LKB 1216 liquid scintillation counter. Representative samples from incubations of each substrate were also analyzed by an HPLC method described previously (12). Both assays revealed that the GSH conjugates and hydroxyacetanilide reduction products were the major (>90%) metabolites formed.

Results

NAPQI and its dimethylated analogues caused substrate concentration-dependent cytotoxicity to hepatocytes isolated from untreated rats as measured by trypan blue uptake (Fig. 1). Of the three quinone imines, 2,6-diMeNAPQI was the most potent cytotoxin, followed in order by NAPQI and 3,5-diMeNAPQI. Both BCNU and DEM pretreatment of hepatocytes enhanced rates of cytotoxicity after the administration of all three quinone imines. BCNU most markedly enhanced the initial rate of cytotoxicity (0–1 hr) caused by 3,5-diMeNAPQI, and DEM most markedly enhanced the initial rate of cytotoxicity caused by 2,6-diMeNAPQI (Fig. 2). After 4 hr, overall

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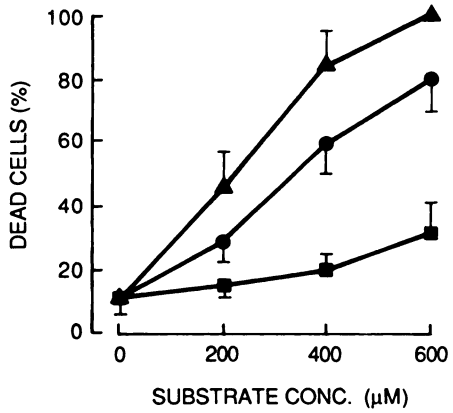

Fig. 1. Dose-response of NAPQI and its dimethylated analogues on the viability of isolated hepatocytes. The cytotoxic effects, as monitored by trypan blue uptake, 1 hr after the addition of various concentrations of NAPQI (●), 3,5-diMeNAPQI (■), and 2,6-diMeNAPQI (▲) to hepatocytes isolated from untreated rats. Values are means ± standard errors from four different experiments.

enhancement of cytotoxicity was greatest for 3,5-diMeNAPQI, which was essentially nontoxic at 300 μM if the hepatocytes were not subjected to either BCNU or DEM treatments.

All three quinone imines were found to rapidly deplete cellular GSH, part of which was recoverable by DTT treatment (Fig. 3). The extent of recovery was dependent on which quinone imine was used as the substrate. The least amount of GSH that was recoverable after DTT treatment occurred after treatment of cells with NAPQI. Cytotoxic concentrations of NAPQI (400 μM) caused approximately an 80% loss of the cellular GSH within 15 sec, and only about one third of that was recoverable by treatment of cells with DTT. In contrast, the same concentration of 3,5-diMeNAPQI caused a 60% loss of GSH, most of which was recoverable by treatment with DTT. Like NAPQI, 2,6-diMeNAPQI caused about 80% loss of cellular GSH, but unlike NAPQI, approximately two thirds of the GSH were recoverable after DTT treatment.

Previously, we found that NAPQI primarily reacted with GSH both chemically and in hepatocytes to form the conjugate 3-(*S*-glutathionyl)-acetaminophen; lesser amounts of GSSG were formed (9). This result was confirmed in the present studies (Table 1) and shows that the GSH not recovered by DTT treatment is primarily conjugated with acetaminophen. In contrast, no GSH conjugate of 3,5-diMeNAPQI was detected either in chemical reactions of the quinone imine with GSH or in hepatocytes. In the chemical reaction, GSSG and 3,5-dimethylacetaminophen accounted for essentially all of the products and in hepatocytes they accounted for most of the products (Table 1).

Interestingly, the most toxic quinone imine, 2,6-diMeNAPQI, also formed significantly more GSSG than did NAPQI, both chemically and in hepatocytes (Table 1), a finding that is consistent with the results of DTT-recoverable GSH (Fig. 3). This result was surprising because some of our earlier studies reported a lack of formation of GSSG in the chemical reaction between 2,6-diMeNAPQI and GSH (12). However, the assay that was used to monitor GSSG formation was indirect, whereas a direct HPLC assay was used in this investigation. Small amounts of 3-(*S*-glutathionyl)-2,6-dimethylacetaminophen were formed in the reactions of 2,6-diMeNAPQI with GSH in both the chemical and hepatocyte systems (Table 1).

Radiolabel from both [*acetyl*-^{14}C] NAPQI and [*acetyl*-^{14}C] 2,6-diMeNAPQI covalently bound to hepatocyte proteins, whereas even at concentrations that caused cytotoxicity, [^{14}C-[*acetyl*-^{14}C]3,5-diMeNAPQI bound only to a minor extent (Fig. 4). The time course of binding for all of the quinone imines is unusual, in that significantly more radiolabel is bound at earlier time points. The addition of DTT to the hepatocytes 4 min after the addition of substrates significantly decreased the extent of covalent binding to proteins of all quinone imines, particularly 2,6-diMeNAPQI.

A possible role for lipid peroxidation in cytotoxicity caused by the quinone imines was investigated. Of the quinone imines, both NAPQI and its 2,6-dimethylated analogue enhanced the rate of MDA formation in hepatocytes, whereas the 3,5-dimethylated analogue had no effect (Fig. 5). However, the time course of MDA formation did not precede the time course of cell death. MDA formation could be blocked by the addition of desferrioxamine to the hepatocytes without an effect on the time course of cytotoxicity (Fig. 5). Furthermore, pretreatment of cells with BCNU had no significant effect on MDA formation and, although DEM pretreatment almost doubled the rate of MDA formation, this effect could completely be blocked by desferrioxamine without an effect on the time course of cytotoxicity of the quinone imines (data not shown).

Redox cycling also does not appear to play a major role in cytotoxicity caused by the three quinone imines. They neither increased oxygen consumption by hepatocytes, nor did they lead to oxygen consumption in solution (Table 2). Furthermore, dicoumarol had no effect on cytotoxicity caused by NAPQI or its dimethylated analogues (Table 2). Dicoumarol is known to inhibit cellular DT-diaphorase [NAD(P)H:(quinone acceptor)oxidoreductase], which catalyzes the two electron reduction of quinones (23). This allows for an increased fraction of some quinones, such as menadione, to be reduced by enzymes such as NADPH-cytochrome P-450 reductase to the semiquinone one-electron-reduced products (24).

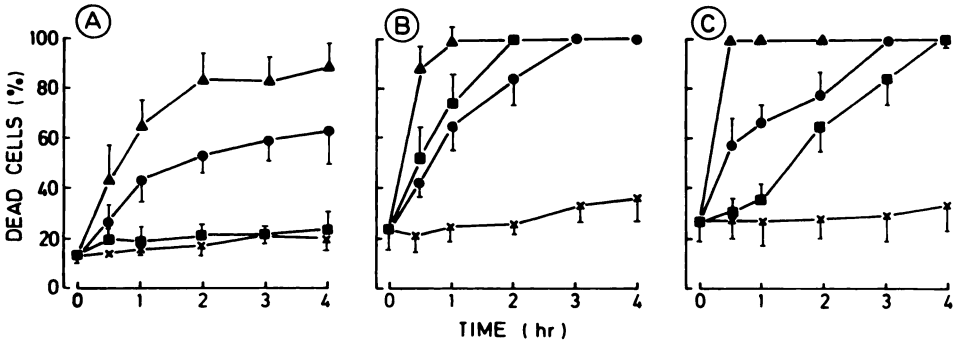

Fig. 2. Effects of BCNU and DEM on the time course of cytotoxicity of NAPQI and its dimethylated analogues. NAPQI and its dimethylated analogues (300 μM) were incubated with rat hepatocytes that were untreated (A), treated with BCNU (B), or treated with DEM (C). Treatments are described in Materials and Methods. *Symbols* are the same as those in Fig. 1. Values for hepatocytes treated with dimethylsulfoxide only are designatd by the X.

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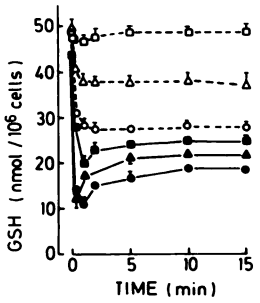

Fig. 3. Effects of DTT on GSH depletion caused by NAPQI and its dimethylated analogues. NAPQI (*circles*), 3,5-diMeNAPQI (*squares*), and 2,6-diMeNAPQI (*triangles*) were incubated (400 μM) with hepatocytes (2 × 10^6/ml) and GSH was determined by the monobromobimane method described in Materials and Methods. *Closed symbols* are assays carried out in the absence of DTT, and *open symbols*, after the addition of DTT. Values are means ± standard error from three different experiments.

Discussion

In a previous study (15), we found that acetaminophen and two of its dimethylated analogues, 2,6-dimethyl- and 3,5-dimethylacetaminophen, were toxic to rat hepatocytes. The study demonstrated that the rate at which cytotoxicity developed was markedly different for each analogue and was dependent on the non-protein thiol status of the cells. Because the quinone imine NAPQI is a known toxic metabolite of acetaminophen (3–11) and because indirect evidence supports a role for quinone imines in the cytotoxic reactions of the dimethylated analogues (15, 25), we investigated the mechanisms of cell injury caused by the quinone imines in rat hepatocytes.

Of the three quinone imines, 2,6-diMeNAPQI was the most potent cytotoxin and caused 50% loss of cell viability after 1 hr at a concentration of 200 μM (Fig. 1). A similar cytotoxic effect required 300 μM NAPQI and approximately 800 μM 3,5-diMeNAPQI (by extrapolation of the results in Fig. 1). These results deserve comment in regards to the results of our previous studies with the respective hydroxyacetanilides for two reasons. First, the quinone imines are considerably more potent cytotoxins than the corresponding hydroxyacetanilides, which required concentrations of 5 mM to cause cytotoxicity (15). Moreover, the quinone imines were cytotoxic to hepatocytes from uninduced rats, whereas the hydroxyacetanilides were only cytotoxic to hepatocytes of rats pretreated with an inducer of cytochrome P-450. Secondly, NAPQI and acetaminophen are more potent cytotoxins than their respective 3,5-dimethylated analogues. Therefore, these results are consistent with the hypothesis that the quinone imines are reactive metabolites of the hydroxyacetanilides and contribute significantly to their cytotoxic effects.

However, whereas 2,6-diMeNAPQI was the most cytotoxic of the quinone imines examined, 2,6-dimethylacetaminophen was the least cytotoxic of the hydroxyacetanilides (15). We believe that the explanation for this apparent contradiction resides in chemical considerations of the redox potentials of the hydroxyacetanilide/quinone imine couples. The most difficult hydroxyacetanilide to chemically oxidize, presumably because of steric constraints, is 2,6-dimethylacetaminophen (15). Analogously, we believe that 2,6-dimethylacetaminophen is enzymatically oxidized more slowly than acetaminophen or 3,5-dimethylacetaminophen. Biochemically, this phenomenon would be expected to slow the rate of covalent binding of radiolabeled 2,6-dimethylacetaminophen to hepatocyte proteins, which is indeed the case (15).

The effects of BCNU and DEM on the initial rates (first 1–2 hr) of developing cytotoxicity caused by the quinone imines paralleled their effects on cytotoxicity caused by the respective hydroxyacetanilides (Ref. 15 and Fig. 2). Treatment of hepatocytes with BCNU most markedly increased the initial rate of cell death caused by 3,5-diMeNAPQI (Fig. 2B), whereas DEM most markedly increased the initial rate of cell death caused by 2,6-diMeNAPQI (Fig. 2C). Although overall enhancement of cytotoxicity (4 hr) was greatest for 3,5-diMeNAPQI after both treatments, this effect is most likely a reflection of progression from a control condition of essentially no toxicity to one in which cytotoxicity is initiated.

Remarkably, all three quinone imines reacted very rapidly (within 1–2 min) to deplete cellular GSH (Fig. 3) and bind to cellular proteins (Fig. 4). None of the free quinone imines were detectable after 1 min of incubation with the hepatocytes (data not shown). Despite this fact, the time course and extent of cytotoxicity is clearly different for each quinone imine. The very interesting implication that can be drawn from these observations is that the differences in toxicity are primarily related to differences in the rates of progression of injury.

There are two distinct differences in the reactions of NAPQI and 3,5-diMeNAPQI with cellular constituents that may lead to the significant differences observed in their expression of

TABLE 1

Reactions of quinone imines with GSH chemically and in rat hepatocytes treated with BCNU

The quinone imines (400 μM) were incubated (37°) either with GSH (4 mM) in Krebs-Henseleit buffer (pH 7.4) or with rat hepatocytes (2 × 10^6/ml) for 15 min, after which time aliquots were removed for the analysis of GSSG by HPLC (23). DTT (10 mM) was added to incubations of cells 4 min after the addition of quinone imines. Final concentration of DMSO in all incubations was 30 μM and had no antioxidant effects of its own. The two GSH conjugates and reduced quinone imines (the respective hydroxyacetanilides) were analyzed as described in Materials and Methods. Cytotoxicity was determined after 2 hr by trypan blue uptake. The results are expressed as means ± standard error of three to four separate experiments.

Quinone imine	Chemical reaction products			Treatment	Cellular reaction products			Cytotoxicity
	GSH conjugate	GSSG	Hydroxy-acetanilide		GSH conjugate	GSSG	Hydroxy-acetanilide	
	nmol/ml				*nmol/ml*			*% dead cells*
NAPQI	236 ± 33	125 ± 20	142 ± 25	−DTT	44 ± 7	11 ± 4	215 ± 19	97 ± 2
				+DTT	55 ± 8	0	240 ± 22	84 ± 3[a]
3,5-diMeNAPQI	0	355 ± 26	381 ± 21	−DTT	0	30 ± 7	346 ± 25	100 ± 0
				+DTT	0	0	352 ± 30	67 ± 8[a]
2,6-diMeNAPQI	37 ± 8	332 ± 18	344 ± 20	−DTT	12 ± 5	24 ± 3	308 ± 15	100 ± 0
				+DTT	15 ± 5	0	361 ± 23	62 ± 10[a]

[a] $p < 0.05$ by *t* test for paired comparisons.

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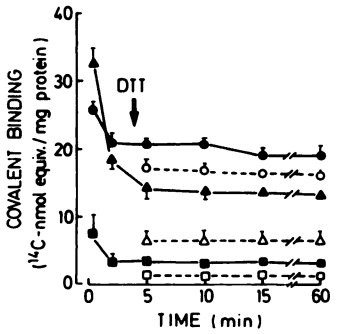

Fig. 4. Effect of DTT on the covalent binding of radiolabel from NAPQI and its dimethylated analogues to hepatocyte proteins. [*acetyl*-^{14}C] NAPQI (*circles*), [*acetyl*-^{14}C]3,5-diMeNAPQI (*squares*) and [*acetyl*-^{14}C] 2,6-diMeNAPQI (*triangles*) were incubated (400 μM) with hepatocytes (2 × 10^6/ml) and aliquots of the incubations were assayed for covalent protein binding as described in Materials and Methods. *Closed symbols* are determinations from incubations without the addition of DTT and *open symbols*, from incubations with the addition of DTT (10 mM) 4 min after the addition of substrate. For each substrate it was determined that binding had reached a plateau value by 3 min. Values are means ± standard errors from three different experiments.

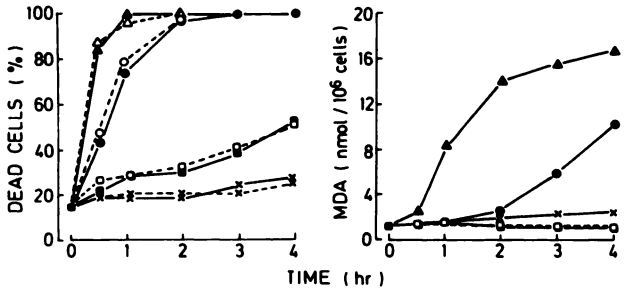

Fig. 5. Effect of desferrioxamine on cytotoxicity and lipid peroxidation caused by NAPQI and its dimethylated analogues. NAPQI (*circles*), 3,5-diMeNAPQI (*squares*), and 2,6-diMeNAPQI (*triangles*) were incubated (600 μM) with hepatocytes (2 × 10^6/ml) and aliquots of the incubations were assayed for cytotoxicity by trypan blue uptake and lipid peroxidation by MDA formation as described in Materials and Methods. *Closed symbols* are determinations from incubations without the addition of desferrioxamine and *open symbols*, from incubations with the addition of desferrioxamine (250 μM) 2 min after the addition of substrates. Values are the averages of duplicate experiments and were within 11% for cytotoxicity and 8% for MDA. Addition of desferrioxamine essentially blocked the formation of MDA with all substrates.

TABLE 2

Effects of quinone imines on some parameters related to oxygen activation

Oxygen consumption was determined as described in Materials and Methods. Cytotoxicity was determined by trypan blue uptake. Dicoumarol was added to the cells in 10 μl of DMSO to achieve a final concentration of 30 μM. Cells were incubated for 5 min with either dicoumarol or 10 μl of DMSO (without dicoumarol) before the addition of quinone imines. The cytotoxicity is reported at 2 hr. Prior time points showed no significant differences.

Compound	O_2 consumption		Cytotoxicity	
	Chemical	Hepatocytes	−Dicoumarol	+Dicoumarol
	nmol/min	*nmol/min/ 10^6 cells*	*% dead cells*	
None	0	3.1	22 ± 5	39 ± 6
NAPQI (400 μM)	0	−1.8	75 ± 11	80 ± 9
3,5-diMeNAPQI (600 μM)	0	2.3	42 ± 13	47 ± 7
2,6-diMeNAPQI (400 μM)	0	3.0	92 ± 7	93 ± 6

lethal cell injury. The first is that the GSH depletion caused by NAPQI is primarily a result of GSH conjugate formation, whereas GSH depletion caused by 3,5-diMeNAPQI is primarily a result of the oxidation of GSH to GSSG (Table 1). Correspondingly, much more GSH is recoverable after the addition of DTT to cells treated with 3,5-diMeNAPQI than with NAPQI (Fig. 3).

The second major difference in the reactions of NAPQI and 3,5-diMeNAPQI with hepatocytes is that NAPQI binds covalently to cellular proteins to a greater extent than does 3,5-diMeNAPQI (Fig. 4). Because the quinone imines are known to undergo some hydrolysis by microsomal enzymes (14) the interpretation of the covalent binding data is somewhat confounded by the position of the radiolabel (^{14}C-acetyl). Although the extent of hydrolysis of all the quinone imines is probably small, as inferred by recovery of >90% of the label in each case (GSH conjugate plus hydroxyacetanilide plus covalently bound; Table 1), only a small amount of binding to critical target molecules may be required for toxicity. Therefore, additional work is needed to assess the significance of the observed differences in covalent binding.

The rate of development of cytotoxicity caused by 3,5-diMeNAPQI was decreased to a significantly greater extent by DTT treatment of hepatocytes than was that caused by NAPQI (Table 1). This result, coupled with the finding that GSSG formation is significantly greater in 3,5-diMeNAPQI treated cells, suggests that oxidative stress contributes more to the development of cytotoxicity caused by this analogue than by NAPQI. These results are consistent with the results shown in Fig. 2, which indicate that, unless cellular mechanisms that are protective against oxidative stress are compromised (e.g., BCNU or DEM treatments), 3,5-diMeNAPQI is a less potent

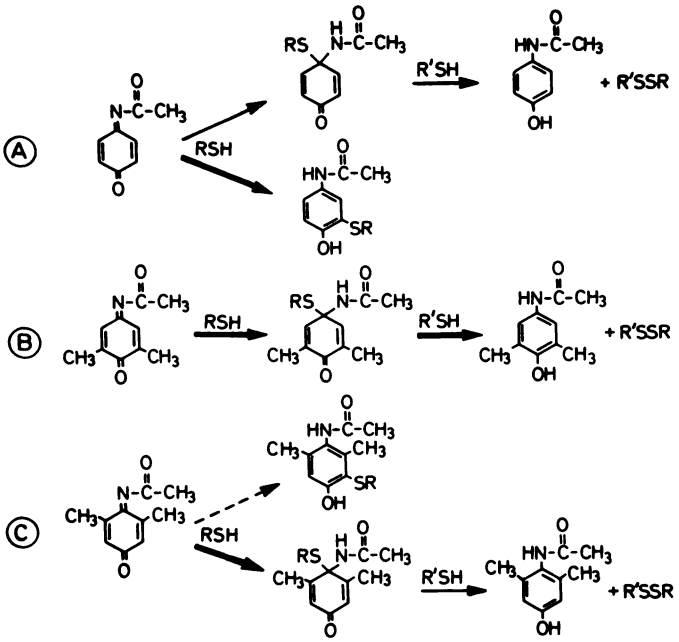

Fig. 6. Possible reactions of NAPQI (A), 3,5-diMeNAPQI (B), and 2,6-diMeNAPQI (C) with thiols. If both RSH and R'SH are glutathione, then GSH *ipso*-adducts or 3-adducts will be formed and R'SSR will be glutathione disulfide (GSSG). RSH and R'SH may also be protein thiols and result in protein *ipso*-adducts and 3-adducts as well as protein disulfides or mixed disulfides with GSH. *Bold arrows* indicate major routes, *normal arrows* indicate less major routes, and *dashed arrows* indicate minor routes of reaction of the quinone imines with thiols.

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cytotoxin than NAPQI. Similar results have been reported by others (26).

Interestingly, the oxidative stress associated with 3,5-diMeNAPQI and the other quinone imines is not a result of either redox cycling (Table 2) or lipid peroxidation (Fig. 5). Semiquinone reduction products of some quinones can reduce molecular oxygen and thereby redox cycle (24, 27). However, results of previous studies with NAPQI (14, 28) and its dimethyl analogues (13, 14) suggest that their semiquinone imines are not good reductants of molecular oxygen. Furthermore, inhibition of DT-diaphorase, an enzyme that decreases redox cycling of quinones via semiquinones (23), had no significant effect on cytotoxicity caused by the quinone imines (Table 2). This is not surprising inasmuch as NAPQI has been found to be a poor substrate for DT-diaphorase (29). Finally, lipid peroxidation observed in our system occurs late, after initial cell injury has occurred, and apparently does not significantly contribute to the progression of toxicity because MDA formation can be blocked by desferrioxamine without altering the time course of cytotoxicity (Fig. 5).

The question then arises as to how oxidative stress occurs. Smith and Mitchell (30) previously suggested the formation of thiol *ipso*-adducts of the quinone imines, which might react with other thiols, in what is essentially an inner-sphere two-electron redox reaction, to produce the corresponding hydroxyacetanilide and oxidized thiols (Fig. 6). Whereas NAPQI also can react as a Michael acceptor to form a thioether adduct at C-3 of the aromatic ring, the C-3 and C-5 positions are blocked in 3,5-diMeNAPQI and GSSG is the major thiol product detected.

The reactions of 2,6-diMeNAPQI deserve special comment. This analogue forms significant amounts of GSSG in hepatocytes (Table 1; Fig. 3) and lesser amounts of a stable GSH conjugate (Table 1) and displays an unusual time course of covalent binding to hepatocyte proteins (Fig. 4). Moreover, DTT treatment of cells releases about 50% of the bound hydroxyacetanilide (Fig. 4). Inasmuch as 2,6-diMeNAPQI is the only quinone imine known to form relatively stable *ipso*-adducts (16), we speculate that 2,6-diMeNAPQI reacts most efficiently with thiols in hepatocytes to form an *ipso*-adduct. A protein thiol *ipso*-adduct of 2,6-diMeNAPQI, if not sequestered, can undergo reaction with other thiols (e.g., GSH) to produce a disulfide (viz., GSSG) and the hydroxyacetanilide. However, this quinone imine also can react at the C-3 or C-5 positions of the aromatic ring to yield the stable arylated thiol adduct (Fig. 6). Thus, 2,6-diMeNAPQI may be particularly cytotoxic because of its ability to both arylate and cause oxidative stress.

In conclusion, two isomeric dimethylated quinone imine analogues of the known cytotoxin NAPQI have been found to be cytotoxic to isolated hepatocytes. The results of the present study cannot be directly correlated with the results of a previous study concerning the hydroxyacetanilides because the quinone imines were added extracellularly as a bolus dose at relatively high concentrations. Despite these limitations, the results are consistent with a role for quinone imines in hydroxyacetanilide-mediated cytotoxicity and provide, within their own framework, information on the contribution of oxidative stress to cytotoxicity caused by quinone imines. Thus, the analogues have been found to react with hepatocytes, like NAPQI, to cause oxidative stress that is independent of lipid peroxidation and to bind covalently to hepatocyte proteins. Differences in toxic potencies of the quinone imines primarily seem to be related to their abilities to oxidize and arylate cellular thiols. Thus, 3,5-diMeNAPQI, the least potent analogue, primarily acts to oxidize, an insult that is apparently readily repaired by the hepatocyte unless protective mechanisms in the repair process are compromised. The unusual time course and reactivity of protein-bound adducts of 2,6-diMeNAPQI, the most potent analogue, suggest the formation of relatively stable *ipso*-adducts in a reaction that merits further investigation.

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