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INHIBITION OF MITOCHONDRIAL RESPIRATION BY A PARA-QUINONE METHIDE

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SUMMARY: A relatively stable *para* quinone methide was prepared from 4-allyl-2,6-dimethoxyphenol. In aqueous solution the quinone methide had a half-life of 52 min, yet reacted rapidly with thiols such as glutathione or cysteine. The unusual stability of this quinone methide allowed us to directly test its effects on mitochondrial respiration. The quinone methide was a potent inhibitor of succinate-dependent mitochondrial respiration (IC_{50} = 47 μM). The inhibition appeared to be due to the depletion of protein thiols, since its effects were comparable to 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent). This quinone methide may prove a useful tool in the investigation of the specific effects of quinone methides on cells which lead to cytotoxicity.

Several *para*-alkylphenols are known to form quinone methides during oxidative biotransformation by cytochrome P450 (1). These reactive intermediates are thought to mediate cytotoxicity of the parent phenols through depletion of intracellular glutathione and subsequent covalent binding to cellular macromolecules. Evidence to support this general sequence of events includes the detection of specific alkylphenol-glutathione adducts, covalent binding to protein, the protective effects of exogenous thiols and metabolism inhibitors, and enhanced toxicity when glutathione is depleted (2-5).

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Abbreviations used: ADMP, 4-allyl-2,6-dimethoxyphenol; ADMP-QM, 4-allylidene-2,6-dimethoxy-2,5-cyclohexadien-1-one (quinone methide of ADMP); DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); ACR, acceptor control ratio.

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Although substantial evidence exists to support the involvement of quinone methides in the cytotoxicity of alkylphenols, the direct effects of quinone methides on cellular processes has not been measured. This is because most quinone methides are too reactive to study directly. They can often be synthesized in dilute solutions of organic solvents, but are unstable and polymerize rapidly upon standing or when concentrated. They are also usually incompatible with biological buffers since they undergo rapid hydrolysis.

During a study on the toxicity of several 4-alkyl-2-methoxyphenols we came across a compound (4-allyl-2,6-dimethoxyphenol, ADMP) which forms a quinone methide that is remarkably stable in aqueous solution (6). This compound can be synthesized in acetonitrile in relatively concentrated solutions (50 mM) and subsequently used for experiments in biological systems. We report here on the reactivity profile of this quinone methide and its effects on mitochondrial respiration.

MATERIALS AND METHODS

4-Allyl-2,6-dimethoxyphenol and silver(I) oxide were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI) while Hepes (4[2-hydroxyethyl]piperazine-1-ethanesulfonic acid) was from Fluka Chemical Corp. (Ronkonkoma, NY) and mannitol was from Spectrum Chemical Mfg. Corp. (Gardena, CA). 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), glutathione, thioctic acid, coenzyme A and all buffer chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

The quinone methide (4-allylidene-2,6-dimethoxy-2,5-cyclohexadien-1-one, ADMP-QM) was synthesized following the general procedure described by Zanarotti (7), with the exception that acetonitrile was used as solvent. To 5 mL of acetonitrile was added 44.4 μL of parent phenol (48.5 mg; 0.25 mmol) and 175 mg silver oxide (0.75 mmol). The mixture was vigorously stirred at room temperature for 20 minutes, then centrifuged at 2000 x g for 10 minutes. The supernatant (50 mM quinone methide) was decanted into a clean test tube and used directly for all subsequent experiments.

Kinetic experiments were carried out at room temperature using an HP 8452 diode array spectrophotometer equipped with a temperature controlled, stirred cuvette. Quinone methide was added to 2 mL of phosphate buffer (0.1 mM, pH 7.4), with or without nucleophiles present, and disappearance over time was measured at 368 nm. The half-life of ADMP-QM was calculated according to the following formula: $k=(2.303/t)\log(A_0-A_\infty/A_t-A_\infty)$. A_∞ was determined at the end of each experiment following the addition of excess glutathione.

Mitochondria were prepared from male Sprague-Dawley rat livers (Harlan Laboratories, Indianapolis, IN) following the procedure described by Pedersen *et al.* (8) using H-medium. Respiration was measured using a YSI 5300 oxygen monitor and a Clark-type oxygen electrode. Incubations were carried out at room temperature and contained 1 mg/mL mitochondrial protein and 5 mM succinate in a total volume of 1.5 mL of respiration buffer (70 mM sucrose, 210 mM mannitol, 3 mM Hepes, 2.5 mM potassium phosphate, 0.5 mM EDTA, 2.5 mM magnesium chloride and 1 mg/mL fatty acid-free bovine serum albumin, pH 7.4). Test compounds were preincubated with mitochondria for 1 minute prior to the addition of succinate. ADP (150 μM) was added after a stable baseline respiratory rate was obtained with succinate. Acceptor control ratios (ACR) were measured as the rate of state 3 (in the presence of ADP)/state 4 (in the absence of ADP) respiration.

Mitochondrial thiols were measured using Ellman's reagent (DTNB) following the procedure described by Sedlak and Lindsay (9).

RESULTS AND DISCUSSION

The quinone methide of 4-allyl-2,6-dimethoxyphenol was prepared by oxidation with sliver(I) oxide (6). The synthetic quinone methide (ADMP-QM) had an absorbance maximum at 368 nm and a half-life of 3143 seconds (52 minutes) in aqueous solution (0.1 M phosphate buffer, pH 7.4). In Table 1 the half-life of ADMP-QM in phosphate buffer is contrasted with its half-life in the presence of several nucleophiles, including glutathione, lysine and histidine. In these experiments, 25 μM ADMP-QM was added to cuvettes containing 10 mM solutions of nucleophile in phosphate buffer, and the disappearance of ADMP-QM was followed at 368 nm. In the presence of glutathione, the half-life was too fast to accurately measure with this technique ($<$ 5 seconds). Lysine and histidine also significantly decreased the half-life of the quinone methide (by 40 and 60%, respectively), however, not nearly so much as glutathione. The quinone methide appeared to be quite specific for thiol nucleophiles.

Upon the addition of an equimolar amount of glutathione to an aqueous solution of 25 μM ADMP-QM, the quinone methide immediately disappeared (Figure 1). When a 100-fold excess of glutathione was added (2.5 mM), the ADMP-QM disappeared in less than 2 seconds. The rate of reaction was probably limited by the mixing time required after addition of the thiol. With glutathione, subsequent HPLC analysis of the products revealed that three isomeric monoglutathione adducts were formed with ADMP-QM. The *trans*-1,8-glutathione adduct of ADMP-QM has recently been characterized (6). Other thiol nucleophiles also reacted rapidly with the quinone methide, including cysteine, lipoic acid and coenzyme A (data not shown). The amine nucleophiles (histidine and lysine) also formed adducts with ADMP-QM as detected by HPLC (data not shown).

The molar extinction coefficient of ADMP-QM in phosphate buffer was determined to be 52,103 cm^{-1} (log ϵ =4.72). Figure 2 shows the UV/VIS spectrum of the quinone methide (25 μM) in phosphate buffer, followed by the addition of aliquots of glutathione (6.25 μM). Addition of an equivalent amount of glutathione completely reacted with the quinone methide.

Alkylphenol toxicity is thought to be mediated, at least in part, by alkylation of vital cellular macromolecules via reactive quinone methide intermediates. However, the critical

TABLE 1

Half-life of ADMP-QM in the presence of various nucleophiles

Nucleophile	t-1/2 (seconds)
Phosphate buffer (0.1 M, pH 7.4)	3143
Glutathione (10 mM)	< 5
Lysine (10 mM)	1794
Histidine (10 mM)	1226

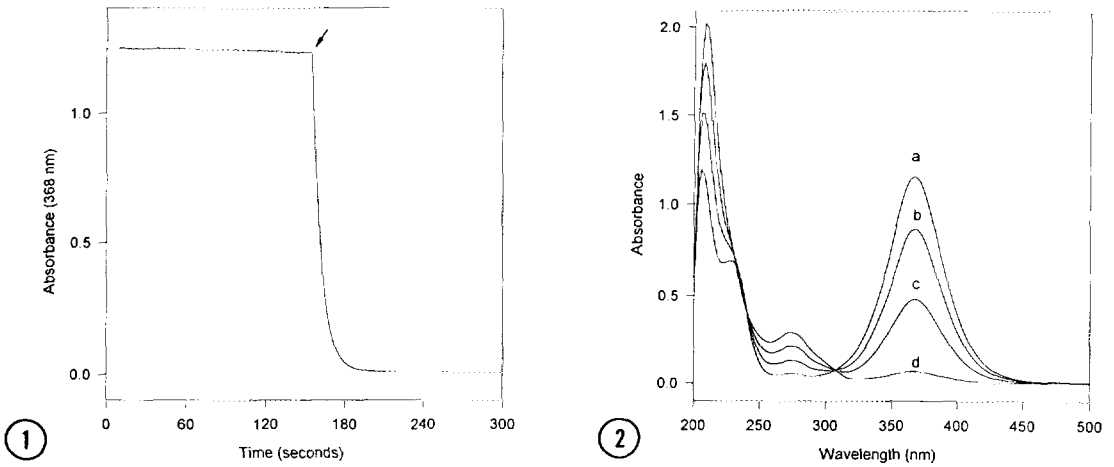


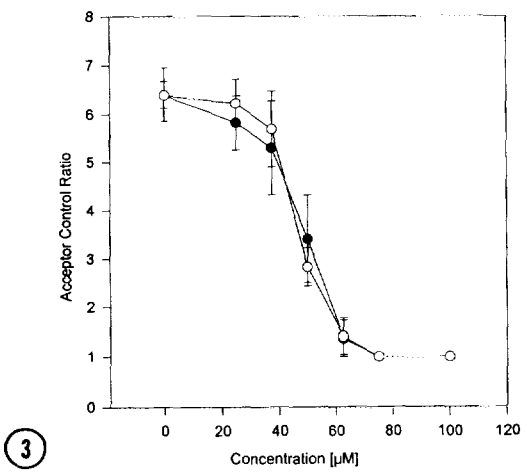
Figure 1. Reaction of ADMP-QM with glutathione. ADMP-QM (25 μM) was incubated in 2 mL phosphate buffer (0.1 M, pH 7.4) for a total of 5 minutes. At the arrow, 25 μM glutathione was added.

Figure 2. UV/VIS spectrum of ADMP-QM and titration with glutathione. ADMP-QM (25 μM) was added to 2 mL phosphate buffer (0.1 M, pH 7.4). Scans were taken one minute after each addition. KEY: Scan (a) ADMP-QM alone; Scan (b) + 6.25 μM glutathione; Scan (c) + 12.5 μM glutathione; Scan (d) + 25 μM glutathione.

intracellular targets of quinone methide alkylation are not known. One possible target is mitochondria. Impairment of mitochondrial function is suspected to play a role in the mechanism of toxicity of a number of environmental and medicinal toxicants (10). In addition, several mitochondrial enzymes are known to be dependent on sulfhydryl groups for enzymatic activity, and respiration is markedly influenced by redox status (11-14). Therefore, ADMP-QM was tested for its effects on mitochondrial respiration.

As shown in Figure 3, ADMP-QM was an effective inhibitor of succinate-dependent respiration. Acceptor control ratio (state 3 respiratory rate/state 4) was used as an indicator of mitochondrial function. In control experiments, the acceptor control ratio was approximately 6.5. Respiratory control was completely inhibited (i.e. ACR equal to 1.0) at a concentration of 75 μM ADMP-QM. The quinone methide had a sharp concentration-response curve, with no significant inhibition being seen until approximately 37.5 μM, and an IC_{50} of 47 μM. We compared the effects of ADMP-QM with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), a compound known to react with both protein and nonprotein sulfhydryls. Both compounds inhibited respiration at comparable concentrations (DTNB IC_{50} = 46 μM).

Although thiols are known to play an important role in the regulation of mitochondrial function, nonprotein-sulfhydryls can be depleted without affecting mitochondrial respiration. For example, chlorodinitrobenzene, a compound which depletes glutathione through conjugation via glutathione S-transferase activity, does not inhibit respiration (15). Chlorodinitrobenzene has



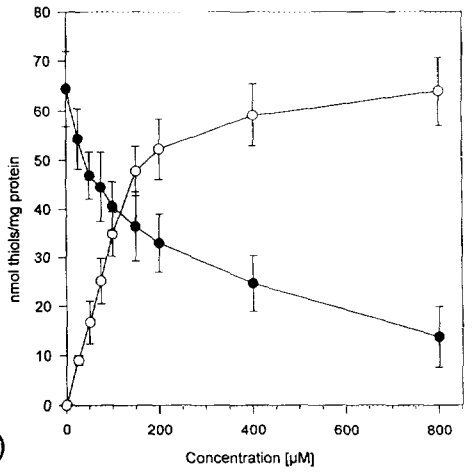


Figure 3. Inhibition of mitochondrial acceptor control ratio by ADMP-QM and DTNB. Test compounds were preincubated for one minute with mitochondria prior to measuring respiration rates. Data points represent mean ± SE. KEY: (●) ADMP-QM; (○): DTNB.

Figure 4. Reaction of ADMP-QM with mitochondrial thiols. Mitochondria were incubated either alone or with ADMP-QM and then total thiols were measured with DTNB. Data points represent mean ± SE. KEY: (●) ADMP-QM; (○): DTNB.

no effect on protein thiols. Similarly, (R,S)-3-hydroxy-4-pentenoate, which is oxidized to a metabolite which selectively depletes mitochondrial glutathione (but not protein thiols), does not impair mitochondrial function in intact hepatocytes (16). Conversely, N-ethylmaleimide binds to both soluble and protein thiols and is known to inhibit mitochondrial respiration (17). In addition, we tested the effects of diethyl maleate, a compound which depletes nonprotein sulfhydryls via glutathione S-transferase-dependent conjugation reactions, on respiration and observed little or no effect at concentrations up to 10 mM (data not shown). These results suggested that ADMP-QM was depleting protein thiols in addition to nonprotein sulfhydryls in mitochondria.

Total mitochondrial thiols were titrated with DTNB and observed to be approximately 65 nmol/mg protein (Figure 4). Of this amount, only about 2 nmol/mg protein (3% of total thiols) was measured as nonprotein sulfhydryls. Mitochondria were also exposed to various concentrations of ADMP-QM for 10 minutes, and the remaining thiols measured with DTNB. With ADMP-QM, a concentration-dependent depletion of thiols was observed (Figure 4). These data demonstrate that ADMP-QM is a potent agent for interacting with mitochondrial thiols, including protein thiols. Similar to DTNB, the inhibition of respiration by ADMP-QM appears to be due to protein thiol depletion. In the respiration experiments, a total of about 100 nmol thiols were present per incubation. With both DTNB and ADMP-QM, inhibition of respiration occurred with an IC_{50} of approximately 50 μM. At these concentrations, data from Figure 4

indicate that approximately 25% of the available thiols were depleted. Once this level of thiol depletion was reached, mitochondrial function was markedly inhibited.

In summary, we have described the synthesis and reactivity profile of a remarkably stable *para*-quinone methide. It is highly reactive toward thiol nucleophiles, including protein thiols, yet is also somewhat reactive toward amine nucleophiles as well. When added directly to intact mitochondria, the quinone methide potently inhibits respiration, probably through alkylation of protein thiol residues. This quinone methide may prove a useful tool in the further investigation of the specific effects of quinone methides on cells which lead to cytotoxicity.

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REFERENCES

1. Thompson, D.C., Thompson, J.A., Sugumaran, M. and Moldéus, P. (1993) *Chem.-Biol. Interactions* 86:190-162.
2. Thompson, D.C., Perera, K. and London, R. (1995) *Chem. Res. Toxicol.* 8:55-60.
3. Bolton, J.L., Valerio, Jr., L.G. and Thompson, J.A. (1992) *Chem. Res. Toxicol.* 5:816-822.
4. Bolton, J.L., Comeau, E. and Vukomanovic, V. (1995) *Chem.-Biol. Interactions* (in press).
5. Thompson, D., Constantin-Teodosiu, D., Egestad, B., Mickos, H. and Moldéus, P. (1990) *Biochem. Pharmacol.* 39:1587-1595.
6. Thompson, D.C., Perera, K., Krol, E.S. and Bolton, J.L. (1995) *Chem. Res. Toxicol.* (in press).
7. Zanarotti, A. (1985) *J. Org. Chem.* 50:941-945.
8. Pedersen, P.L., Greenawalt, J.W., Reynafarje, B., Hullihen, J., Decker, G.L., Soper, J.W. and Bustamente, E. *Methods Cell. Biol.* 20:411-481.
9. Sedlak, J. and Lindsay, R.H. (1968) *Anal. Biochem.* 25:192-205.
10. Jones, D.P. and Lash, L.H. (1993) In: *Mitochondrial Dysfunction* (L.H. Lash and D.P. Jones, eds.), *Methods in Toxicology, Vol. 2,* pp. 1-7, Academic Press, San Diego.
11. LeQuoc, K., LeQuoc, D. and Gaudemer, Y.(1981) *Biochemistry* 20:1705-1710.
12. LeQuoc, K. and LeQuoc, D. (1982) *Arch. Biochem. Biophys.* 216:639-651.
13. LeQuoc, K. and LeQuoc, D. (1985) *J. Biol. Chem.* 260:7422-7428.
14. Moore, G.A., Weis, M., Orrenius, S. and O'Brien, P.J. (1988) *Arch. Biochem. Biophys.* 267:539-550.
15. Jocelyn, P.C. and Cronshaw, A. (1985) *Biochem. Pharmacol.* 34:1588-1590.
16. Shan, X., Jones, D.P., Hasmi, M. and Anders, M.W. (1993) *Chem. Res. Toxicol.* 6:75-81.
17. LeQuoc, K., LeQuoc, D. and Gaudemer, Y. (1979) *Biochim. Biophys. Acta* 546:356-364.