Biochemical Pharmacology, Vol. 39, No. 6, pp. 1133–1135, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
© 1990. Pergamon Press plc

SHORT COMMUNICATIONS

Reaction kinetics of 4-methoxy ortho benzoquinone in relation to its mechanism of cytotoxicity: a pulse radiolysis study

(*Received* 7 *September* 1989; *accepted* 2 *November* 1989)

Studies of the phenolic depigmenting agent 4-hydroxyanisole (4-HA) have demonstrated that it is able to act as a substrate for the melanogenic enzyme tyrosinase [1], and that it is oxidized to give rise both to the corresponding orthoquinone, 4-methoxy ortho benzoquinone (anisyl-3,4-quinone) [2, 3] and, under appropriate conditions, to the orthosemiquinone, 4-methoxy ortho benzosemiquinone (anisyl-3,4-semiquinone) [4]. There is evidence that in model systems, in which the oxidation takes place exterior to the cell, cytotoxicity is associated with the formation of the quinone species [5] which is able to react with nucleophiles such as amino or thiol groups [6]. However, cytotoxicity is exacerbated by hypoxia [7], and since the yield of semiquinone radicals is increased in conditions of low oxygen tension [8], this may also implicate the related semiquinone as a damaging species. As, in the model system, the relatively hydrophilic oxidation products of 4-HA are generated exterior to the cells, it is probable that the cytotoxic action is exerted at the plasma membrane [5]. Possible cytotoxic mechanisms include inactivation of plasma membrane ion pumps by the quinone and initiation of lipid peroxidation by the semiquinone by hydrogen abstraction from unsaturated membrane lipids [9].

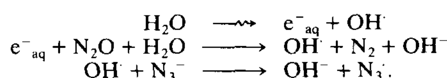
Starting with the quinone the alternative toxic mechanisms may be classed as either (a) *direct* involving: (i) covalent reaction with —SH or —NH_2 groups; or (ii) depletion of cellular antioxidants by redox reactions (e.g. with ascorbate) or (b) *indirect* involving: (iii) disproportionation of the quinone and hydroquinone to give the semiquinone, followed by either (iv) single electron donation to oxygen to give $O_2^{\cdot -}$; or (v) hydrogen abstraction from unsaturated fatty acid to initiate lipid peroxidation.

We have examined the reaction kinetics of these types of reaction by pulse radiolysis in an attempt to assign the relative likelihood that the reactions are involved in the cytotoxic pathway.

Materials and methods

3,4-Dihydroxyanisole was prepared by oxidizing 4-hydroxyanisole to 4-methoxy ortho benzoquinone with potassium nitrosodisulphonate and the quinone immediately reduced to 3,4-dihydroxyanisole with sodium dithionite [10]. Cysteine, glutathione, dithiothreitol, arginine, glutamine and *trans*-2-butenoic acid were used as supplied by the Sigma Chemical Co. (Poole, U.K.). NaN_3, KSCN, KH_2PO_4 and Na_2HPO_4 were AnalaR grade from BDH Chemicals (Poole, U.K.). Medicinal grade nitrous oxide was supplied by BOC (Guildford, U.K.). Water was redistilled from alkaline permanganate. In order to prevent oxidation by dissolved air, 3,4-dihydroxyanisole was added subsequent to bubbling of the solutions with N_2O.

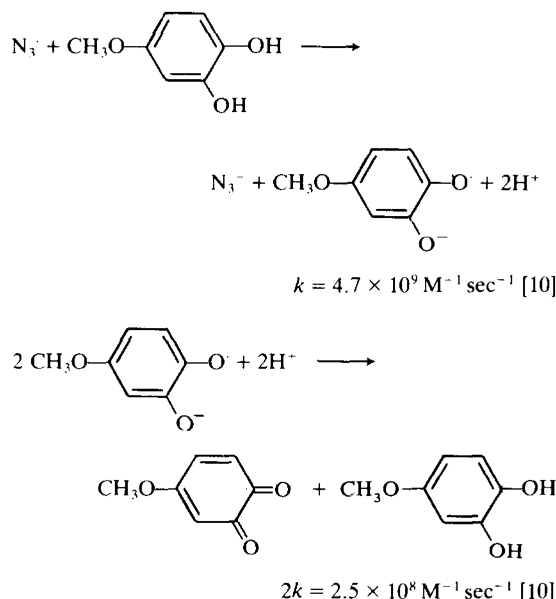
The pulse radiolysis experiments were performed [11] with a 9–12 MeV Vickers linear accelerator using 10–500 ns pulses with doses up to 60 Gy and quartz capillary cells of optical path 2.5 cm. Absorbed doses were determined from the transient $(SCN)_2^{\cdot -}$ yield of 0.30 μM/Gy and $\varepsilon_{500\ nm} = 7100\ M^{-1}\ cm^{-1}$ [12]. Generation of the one-electron oxidizing species $N_3^{\cdot}$ was achieved by irradiating N_2O-saturated aqueous solutions of 5×10^{-2} M NaN_3. Under such conditions $N_3^{\cdot}$ radicals are formed within 0.1 μsec after the radiation pulse via the reactions:



$$H_2O \rightsquigarrow e^-_{aq} + OH^{\cdot}$$
$$e^-_{aq} + N_2O + H_2O \longrightarrow OH^{\cdot} + N_2 + OH^-$$
$$OH^{\cdot} + N_3^- \longrightarrow OH^- + N_3^{\cdot}.$$

Results

4-Methoxy ortho benzoquinone was prepared by pulse radiolysis of N_2O-saturated $\sim 10^{-3}$ M solutions of 3,4-dihydroxyanisole containing 5×10^{-2} M NaN_3 buffered to pH 7.0 with 10^{-1} M phosphate [10]. This method of preparation, via disproportionation of 4-methoxy ortho benzosemiquinone following one-electron oxidation of 3,4-dihydroxyanisole:



$k = 4.7 \times 10^9\ M^{-1}\ sec^{-1}$ [10]

$2k = 2.5 \times 10^8\ M^{-1}\ sec^{-1}$ [10]

allows the almost instantaneous (within 20 msec) introduction of quinone into the solution. The quinone, monitored at 420 nm, was seen to be stable over several seconds, although at longer times (minutes to hours) it progressively polymerizes.

Addition of 2.0×10^{-4} M cysteine to the above solution hardly reduced the initial amount of quinone formed, consistent with the low rate constant ($2 \times 10^8\ M^{-1}\ sec^{-1}$ [13]) for the reaction of $N_3^{\cdot}$ with cysteine, but did result in the subsequent almost complete loss of absorption at 420 nm over a period of several tens of milliseconds. Under the pulse dose conditions employed, ~55 Gy, corresponding to an initial quinone yield of $\sim 1.7 \times 10^{-5}$ M, the decay at 420 nm was unimolecular, corresponding to a second order rate constant of $3.5 \times 10^5\ M^{-1}\ sec^{-1}$ for the reaction of the quinone with cysteine.

Addition of 2.0×10^{-4} M glutathione instead of cysteine to the same solution led to a similar shortening of the lifetime of 4-methoxy ortho benzoquinone, corresponding to a second order rate constant of 3.1×10^{5} M^{-1} sec^{-1} for the reaction of the quinone + glutathione. Likewise, addition of 1.14×10^{-4} M dithiothreitol led to a rate constant of 3.5×10^{5} M^{-1} sec^{-1} for the reaction of the dithiol with 4-methoxy ortho benzoquinone.

Because amines, like thiols, can undergo nucleophilic reductive addition to quinones, the reactions of arginine and glutamine with 4-methoxy ortho benzoquinone were investigated. Addition of 10^{-2} M arginine to $\sim 10^{-3}$ M 3,4 dihydroxyanisole containing 5×10^{-2} M NaN_3, buffered to pH 7.0 with 10^{-1} M phosphate, led to no significant increase in the rate of decay of quinone over the time scale studied (5 sec), leading to a rate constant limit of ≤ 1 M^{-1} sec^{-1}. Higher concentrations were precluded as they seriously interfered with the initial radiation chemical formation of 4-methoxy ortho benzoquinone. Arginine does react very slowly with 4-methoxy ortho benzoquinone since an earlier report [14] showed that addition of arginine to a mixture of tyrosinase and 4-hydroxyanisole in PBS buffer leads to an increase in the product quinone decay rate, monitored over time scales of minutes to hours.

Addition of 10^{-2} M glutamine also failed to affect the decay of pulse radiolytically produced 4-methoxy ortho benzoquinone, leading to a similar limit of ≤ 1 M^{-1} sec^{-1} for the reaction of the quinone with glutamine.

A clear reaction of the redox active agent ascorbic acid with 4-methoxy ortho benzoquinone was detected in the present experiments. Addition of 1.1×10^{-3} M ascorbic acid to 2×10^{-3} M 3,4-dihydroxyanisole + 5×10^{-2} M NaN_3 in phosphate buffer pH 7.0, although leading to ~30% reduction in the initial amount of quinone formed as expected from the comparatively high rate constant for the reaction of $N_3^{\cdot}$ with ascorbic acid (2.9×10^{9} M^{-1} sec^{-1} [15]), did result in loss of the quinone over a period of several hundreds of milliseconds. The corresponding rate constant found for the reaction of 4-methoxy ortho benzoquinone with ascorbic acid was 1.0×10^{4} M^{-1} sec^{-1}.

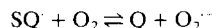
In an earlier study we described the pulse radiolytic production of 4-methoxy ortho benzosemiquinone via $N_3^{\cdot}$-induced one-electron oxidation of 3,4-dihydroxyanisole [10]. Amongst the properties of the radical recorded were its absorption spectrum and its low reactivity with oxygen $k \leq 10^{5}$ M^{-1} sec^{-1}. The propensity of the radical to abstract H atoms from unsaturated fatty acids has now been investigated by measuring the effect of *trans*-2-butenoic acid upon the decay of the 320 nm absorption peak of 4-methoxy ortho benzosemiquinone. Addition of up to 2×10^{-3} M *trans*-2-butenoic acid to 2×10^{-3} M 3,4 dihydroxyanisole containing 5×10^{-2} M NaN_3 and 10^{-1} M phosphate buffer, pH 7.0, had no effect upon the initial amount of 4-methoxy ortho benzosemiquinone formed nor its second order decay which proceeded with a first half-life of ~2.5 msec under the pulse dose conditions employed (4.4 Gy). The resultant limit obtained for reaction of the semiquinone with *trans*-2-butenoic acid was $\leq 2 \times 10^{5}$ M^{-1} sec^{-1}.

Discussion

The rate constants for the reaction of 4-methoxy ortho benzoquinone (Q) with —SH compounds indicate that these are relatively rapid reactions ($k = 3$–4×10^{5} M^{-1} sec^{-1}) and are consistent with the proposal that one of the major cytotoxic mechanisms could be quinone addition to thiol-containing proteins. These addition reactions are more than an order of magnitude faster than redox exchange with ascorbate ($k = 1.0 \times 10^{4}$ M^{-1} sec^{-1}).

The present 4-methoxy ortho benzoquinone-thiol rate constants are of a similar order of magnitude to some para naphthoquinone-thiol rate constants measured previously [16], although in the latter case the variation in rate constants between the thiols was larger. In view of the proximity of the pK values for the equilibria $RSH \rightleftharpoons RS^- + H^+$ to the pH value at which the present measurements were carried out (7.0), there is every expectation that the thiol rate constants would be pH sensitive since only RS^- may be reactive.

The rapid disproportionation to completion of the 4-methoxy ortho benzosemiquinone ($SQ^{\cdot}$) generated by pulse radiolysis [10] does not seem to favour the generation of $SQ^{\cdot}$ by the reverse reaction, but the equilibrium could be affected by subsequent $SQ^{\cdot}$ reactions. Our data [10] show that the equilibrium constant of the reaction:



$$SQ^{\cdot} + O_2 \rightleftharpoons Q + O_2^{\cdot -}$$

is over three orders of magnitude in favour of electron donation by superoxide to form the semiquinone. Thus, significant single electron reduction of oxygen by the semiquinone is very unlikely.

The alternative possibility that the semiquinone radical initiates lipid peroxidation by hydrogen abstraction was also examined. Previous attempts to demonstrate lipid peroxidation in a similar system by estimating lipid peroxidation products, such as malondialdehyde, by the thiobarbituric acid assay were hampered by melanoid pigment interference with the TBA spectrum [17]. In this study, we examined an aqueous system containing butenoic acid as a model of unsaturated lipid.

The low reactivity of 4-methoxy ortho benzosemiquinone with *trans*-2-butenoic acid obtained ($\leq 2 \times 10^{5}$ M^{-1} sec^{-1}) implies that in aqueous conditions hydrogen abstraction from unsaturated acyl chains is very inefficient. Since the pulse radiolysis system used for generating the semiquinone is an aqueous system, due to low solubilities we have not examined the effect of increasing the chain length of the fatty acid. Increasing the hydrophobicity of the potential electron donor may also introduce problems since phase changes in the system would be expected to interfere with reactivity. Had the reaction proceeded rapidly, it could still have been argued that this factor could militate against the initiation of lipid peroxidation by a predominantly hydrophilic species such as the 4-methoxy ortho benzosemiquinone. We conclude that, if under these relatively optimal conditions, the hydrogen abstraction reaction is not favoured, it is unlikely that the initiation of lipid peroxidation by the semiquinone radical is a major pathway of cytotoxicity, although we cannot exclude other possible reactions of the semiquinone. Our data lead us to conclude that semiquinone radical-mediated reactions affecting membrane integrity do not feature prominently among the mechanisms of toxicity of 4-hydroxyanisole oxidation products. This observation may be significant in relation to the frequently observed membrane defects in melanosomes in malignant melanomas [18]. One possible source of such defects could be the degradation of the melanosomal membrane by the initiation of lipid peroxidation by radical products generated during melanogenesis. Since the semiquinone radical produced by tyrosinase oxidation of 4-hydroxyanisole serves as a model of radical intermediates of the melanogenic pathway, our present data would seem to exclude this mode of intrinsic damage to melanosomes.

We conclude, therefore, that the combination of the low yield of the $SQ^{\cdot}$ species and the unfavourable kinetics of its reactions with either molecular oxygen or unsaturated fatty acids makes it more probable that the major toxic reaction of 4-methoxy ortho benzoquinone involves covalent addition reactions with cellular nucleophiles, especially thiols. There is evidence that certain crucial protein components of the plasma membrane, such as the calcium pumps, are potential targets for reactions with quinones [19] and the cytotoxic sequaelae of raised intracellular calcium levels and other ionic disturbances are well documented [20].

In summary, rate constants quantifying the reactivity of 4-methoxy ortho benzoquinone, formed in the metabolic

activation of 4-hydroxyanisole, a possible melanocytotoxic drug under current assessment as a treatment for malignant melanoma, have been determined by pulse radiolysis. The quinone is reactive towards the thiols cysteine ($k = 3.5 \times 10^5\ M^{-1}\ sec^{-1}$), glutathione ($k = 3.1 \times 10^5\ M^{-1}\ sec^{-1}$) and dithiothreitol ($k = 3.5 \times 10^5\ M^{-1}\ sec^{-1}$), but relatively unreactive towards other nucleophiles such as arginine ($k \leq 1\ M^{-1}\ sec^{-1}$) and glutamine ($k \leq 1\ M^{-1}\ sec^{-1}$). Redox exchange with ascorbate also occurs ($k = 1.0 \times 10^4\ M^{-1}\ sec^{-1}$). In view of the low reactivity of 4-methoxy ortho benzosemiquinone towards oxygen ($k \leq 10^5\ M^{-1}\ sec^{-1}$) and the model lipid *trans*-2-butenoic acid ($k \leq 2 \times 10^5\ M^{-1}\ sec^{-1}$), it is unlikely that initiation of lipid peroxidation by the semiquinone is a major source of cytotoxicity. A more likely toxicity pathway appears to be covalent addition reactions of 4-methoxy ortho benzoquinone with cellular nucleophiles, especially thiols, and/or redox exchange reactions of the quinone leading to antioxidant depletion.

Acknowledgements—This work was supported by the Cancer Research Campaign and the Association for International Cancer Research.

E. J. LAND*†
C. J. COOKSEY‡
P. A. RILEY§

* *Paterson Institute for Cancer Research*
Christie Hospital and Holt Radium Institute
Manchester M20 9BX, U.K.
‡ *Department of Chemistry*
University College
Gordon St.
London WC1H 0AJ, U.K.
§ *Department of Chemical Pathology*
University College and Middlesex School of Medicine
Windeyer Building
Cleveland St.
London W1P 6DB, U.K.

REFERENCES

1. Riley PA, Radicals and melanoma. *Phil Trans R Soc Lond B* **311**: 679–689, 1985.
2. Passi S and Nazzaro-Porro M, Molecular basis of substrate and inhibitory specificity of tyrosinase: phenolic compounds. *Brit J Derm* **104**: 659–665, 1981.
3. Naish S, Cooksey CJ and Riley PA, Initial mushroom tyrosinase-catalysed oxidation product of 4-hydroxyanisole is 4-methoxy-ortho-benzoquinone. *Pigment Cell Res* **1**: 379–381, 1988.
4. Nilges MJ, Swartz HM and Riley PA, Identification by electron spin resonance of free radicals formed during the oxidation of 4-hydroxyanisole catalysed by tyrosinase. *J Biol Chem* **259**: 2446–2451, 1985.
5. Naish S, Holden JL, Cooksey CJ and Riley PA, Major primary cytotoxic product of 4-hydroxyanisole oxidation by mushroom tyrosinase is 4-methoxy ortho benzoquinone. *Pigment Cell Res* **1**: 382–385, 1988.
6. Naish S and Riley PA, Studies on the kinetics of oxidation of 4-hydroxyanisole by tyrosinase. *Biochem Pharmacol* **38**: 1103–1107, 1989.
7. Naish S, Cytotoxicity of 4HA oxidation products in a model system. In: *Hydroxyanisole: Recent Advances in Anti-melanoma Therapy* (Ed. Riley PA), pp. 83–100. IRL Press, Oxford, 1984.
8. Nilges MJ and Swartz HM, Quinone and semiquinone intermediates in the reaction of 4-hydroxyanisole and tyrosinase. In: *Hydroxyanisole: Recent Advances in Anti-melanoma Therapy* (Ed. Riley PA), pp. 25–34. IRL Press, Oxford, 1984.
9. Dahle LK, Hill EG and Holman RT, The thiobarbituric acid reaction and the autoxidations of polyunsaturated fatty acid methyl esters. *Arch Biochem Biophys* **98**: 253–261, 1962.
10. Cooksey CJ, Land EJ, Riley PA, Sarna T and Truscott TG, On the interaction of anisyl-3,4-semiquinone with oxygen. *Free Rad Res Commun* **4**: 131–138, 1987.
11. Butler J, Hodgson BW, Hoey BM, Land EJ, Lea JS, Lindley EJ, Rushton FAP and Swallow AJ, Experimental studies of some moderately fast processes initiated by radiation. *Radiat Phys Chem* **34**: 633–646, 1989.
12. Adams GE, Boag JW, Currant J and Michael BD, The pulse radiolysis of aqueous solutions of thiocyanate ion. In: *Pulse Radiolysis* (Ed. Ebert M, Keene JP, Swallow AJ and Baxendale JH), pp. 117–129. Academic Press, London, 1965.
13. Land EJ and Prutz WA, Reaction of azide radicals with amino acids and proteins. *Int J Radiat Biol* **36**: 75–83, 1979.
14. Holden JL, Dewey DL and Galpine AR, Cytotoxicity produced by the oxidation of 4-hydroxyanisole by tyrosinase. In: *Hydroxyanisole: Recent Advances in Anti-melanoma Therapy* (Ed. Riley PA) pp. 101–110. IRL Press, Oxford, 1984.
15. Hoey BM and Butler J, The repair of oxidized amino acids by antioxidants. *Biochim Biophys Acta* **791**: 212–218, 1984.
16. Wilson I, Wardman P, Lin T-S and Sartorelli AC, Reactivity of thiols towards derivatives of 2- and 6-methyl-1,4-naphthoquinone bioreductive alkylating agents. *Chem Biol Interact* **61**: 229–240, 1987.
17. Riley PA, Sawyer B and Wolff MA, The melanocytotoxic action of 4-hydroxyanisole. *J Invest Derm* **64**: 86–89, 1975.
18. Borovansky J, Hach P, Mirejovsky P and Riley PA, The possible relationship between abnormal melanosome structure and cytotoxic phenomena, in preparation.
19. Orrenius S, Oxidative stress studies in intact mammalian cells. *Phil Trans R Soc Lond B* **311**: 673–677, 1985.
20. Trump BF, Berezesky IK and Osornio-Vargas AR, Cell death and the disease process: the role of calcium. In: *Cell Death in Biology and Pathology* (Ed. Bowen ID and Lockshin RA), pp. 209–224. Chapman and Hall, London, 1981.

† To whom correspondence should be addressed.