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The Autoxidation of the Reduced Forms of EO9

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The properties of the semiquinone radical from [3-hydroxy-5-aziridinyl-1-methyl-2-(1H-indole-4,7-indi one)-prop-β-en-α-ol], EO9, have been studied using pulse-radiolysis techniques. The reduction potential of the semiquinone of EO9 at pH 7.4, E(EO9/EO9$^{\cdot-}$), is −253 ± 6 mV and hence this quinone can be readily reduced by one-electron reducing enzymes such as cytochrome P450 reductase and xanthine oxidase. However, the radical is unstable in the presence of oxygen ($k = 1.3 \pm 0.15 \times 10^8$ M^{-1} s^{-1}). The semiquinone radicals and the hydroquinone are in equilibrium, although the formation of the hydroquinone is favoured at physiologically relevant pH. The hydroquinone of EO9 is also unstable in the presence of oxygen and it is predicted that in fully aerated solutions, its half life is 1.5 ± 0.3 seconds. These results are discussed in view of the selective cytoxicity of EO9 and its ability to undergo bioreductive activation by one-electron reducing enzymes and DT-diaphorase.

Key words: EO9, semiquinone, hydroquinone, DT-diaphorase, autoxidation, pulse radiolysis

INTRODUCTION

EO9 [3-hydroxy-5-aziridinyl-1-methyl-2-(1H-indole-4,7-indione)-prop-β-en-α-ol] is a bioreductive alkylating indoloquinone (Figure 1) with a distinct activity towards solid tumours and is currently undergoing phase I/II clinical trials.[1] Several *in vitro* studies have shown that this compound is up to 1000 times more toxic towards hypoxic cells compared to cells which are fully oxygenated.[2,3]

It has been proposed that EO9 undergoes bioreductive activation and alkylates DNA. The reduction can be produced by several one-electron reducing enzymes including cytochrome P450 reductase, xanthine oxidase and cytochrome b_5 reductase.[4,5] However, the quinone is an excellent substrate for the obligatory two-electron reducing enzyme, DT-diaphorase [NAD(P)H quinone oxireductase, EC 1.6.99.2] and there is a good correlation between the activity of DT-diaphorase present in a tumour cell and the cytotoxicity of the drug.[6] However, recent work has

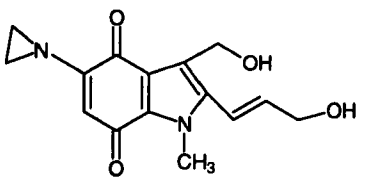

FIGURE 1 The structure of EO9

shown that this correlation is only true under oxic conditions and indeed, it appears that under hypoxic conditions, where EO9 is most toxic, DT-diaphorase may be protecting the cells against damage by the drug.[5]

In view of the conflicting mechanisms proposed for the cytotoxicity of EO9, we have investigated the chemical properties of the semiquinone radical and the reactivities of the radical and the hydroquinone with oxygen.

MATERIALS AND METHODS

EO9 was obtained from the EORTC under the auspices of Kyowa Hakko, UK and was found to be>95% pure, based on determination by HPLC. Bovine xanthine oxidase, allopurinol and hypoxanthine were from Sigma (St Louis, MO, USA). All of the pulse radiolysis solutions contained 0.1 M sodium formate and were buffered using 10mM phosphate (pH 4.5–9.0) or 10 mM borate/NaOH (pH 8.5–10.0). All other reagents were of the highest grade commercially available.

The pulse radiolysis experiments were conducted at the Paterson Institute linear accelerator facility.[7] The pulse lengths were less than 0.1 μs. The optical detection system consisted of a xenon or tungsten lamp, a Kratos monochromator and an EMI 9558QA photomultiplier. The optical cells were capillary cells (3 mm internal diameter) with path lengths of 0.4 and 2.5 cm. The signals from the photomultiplier were recorded on a Tektronix 7612AD programmable digitizer.

The stopped flow measurements used an Applied Photophysics RX1000 stopped-flow apparatus coupled to HP8452A diode array spectrophotometer. The kinetic analysis were carried out using the Fig P (registered trademark, Biosoft, Cambridge, UK) program assuming second order kinetics. All experiments were performed at 21 ± 2°C.

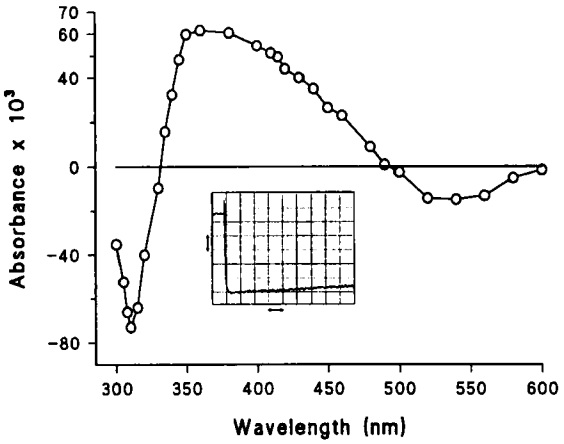

FIGURE 2 The difference spectrum of the EO9 semiquinone recorded at 20μs after the pulse. All absorbances were normalised to a radical concentration of 6.5μM in a 2.5 cm optical cell. Insert: A typical trace produced by pulsing an argon saturated solution containing 150μM EO9 in 0.1M formate, 10mM phosphate (pH 7.4) at 400nm. Radical concentration = 5.7μM, 20μs/div, 1.8% transmission/div.

RESULTS

When an argon saturated buffered solution of sodium formate (100 mM, pH 4.5–9) and quinone (20μM–2mM) is irradiated, the following reactions are initiated:

$$H_2O \rightarrow {}^{\bullet}OH, {}^{\bullet}H, e_{aq}^{-}, H_2O_2, H_2 \quad (1)$$

$${}^{\bullet}OH({}^{\bullet}H) + HCO_2^{-} \rightarrow H_2O\,(H_2) + CO_2^{\bullet -} \quad (2)$$

$$CO_2{}^{\bullet -}\,(e_{aq}^{-}) + Q \rightarrow Q^{\bullet -} + CO_2 \quad (3)$$

Hence within a few microseconds, all of the primary radicals from water produce semiquinone radicals. The difference spectrum (absorbance of semiquinone minus absorbance of parent quinone) produced at 20 μs after the pulse for the solutions at pH 7.4 is shown in Figure 2. The spectrum of the radical was the same at pH 7.4 and 10 although at pH values below about 6, the absorbances were found to decrease in the range 350–450 nm. The pH dependence of the absorbances at 400 nm are shown in Figure 3. Unfortunately, due to the instability of EO9 in acid and

alkaline pH,[4,8,9] it was not possible to do the experiments outside the pH range 4.5–10.0.

In the absence of oxygen, the semiquinone radicals were found to decay at pH 7.4 over a period of milliseconds in a dose dependent manner. A typical trace is included in Figure 3.

This is consistent with the well established reaction for semiquinone radicals:

$$Q^{\bullet-} + Q^{\bullet-} + 2H^+ \rightarrow QH_2 + Q \qquad (4)$$

Where QH_2 is the hydroquinone of EO9.

This reaction was investigated at 400 nm where the rate constant was calculated to be $5.2 \pm 1.6 \times 10^7\ M^{-1}\ s^{-1}$ at pH 7.4. This value was independent of the radical concentration in the range 1.0–5.2 μM and quinone concentrations up to 2.0 mM. However, within the pH range *ca.* 8–10 the apparent rate constant for reaction 4 was found to significantly increase and the absorptions due to the radicals did not disappear completely, but relaxed to a value which remained stable for at least several tens of milliseconds. The apparent rates of the reaction and the absorbance values at the end of the reaction were found to be dependent on the initial quinone concentration. A typical trace at pH 9.0 is included in Figure 3. This is consistent with reaction 4 being an equilibrium in alkaline pH:

$$Q^{\bullet-} + Q^{\bullet-} + 2H^+ \rightleftarrows QH_2 + Q \qquad (4')$$

Measurements of the final absorbance at 400 nm, where only the semiquinone have appreciable absorptions, enabled the concentration, R_e, of the semiquinone at equilibrium to be calculated. The equilibrium constant K_4 was then calculated from the formula:

$$K_4 = \frac{\left(A_o - \frac{(R_o + R_e)}{2}\right)\left(\frac{(R_o - R_e)}{2}\right)}{R_e^2} \qquad (5)$$

where A_o is the initial quinone concentration (50–200 μM) and R_o is the concentration of semiquinone radicals produced immediately after the pulse (1.7–5.6 μM). From these calculations it was found that K_4 was 8 ± 2 at pH 10.0 and 84 ± 12 at pH 9.0. By using high concentrations of quinone (100–500 μM) and lower concentrations of radicals (0.4–1 7 μM), it was observed that the equilibrium occurred at pH 8. However, due to the relatively small absorbance values at equilibrium, only a lower limit of $K_4 > 700$ could be determined. Similarly, from using a much higher concentration of quinone (2.0 mM in a 0.4 cm optical cell), the limit on the value at pH 7.4 was found to be >4000.

The semiquinone radicals formed at pH 7.4, were unstable in the presence of oxygen. This is consistent with the reaction.[27]

$$Q^{\bullet-} + O_2 \rightarrow Q + O_2^{\bullet-} \qquad (6)$$

The rate of this reaction was determined by adding small concentrations of oxygen to the argon saturated solutions and observing the decay of the semiquinone radicals at 400nm. The variation of rate with oxygen concentration is shown in Figure 4. From the slope of the line, the rate constant for reaction 6 was calculated as $1.3 \pm 0.15 \times 10^8\ M^{-1}\ s^{-1}$

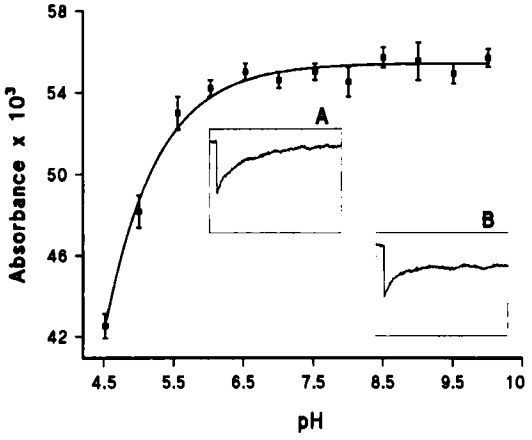

FIGURE 3 The variation of the absorbance of EO9 semiquinone radicals as a function of pH. All values were normalised to a radical concentration of 6.5 μM in a 2.5 cm optical cell. Inset A: The decay of EO9 semiquinone radicals at pH 7.4 (λ = 400 nm: radical concentration = 2.65 μM, 10 ms/division and 1.2% transmission/division). Inset B: The formation of an equilibrium with 100 μM, EO9 at pH 9.0 (λ = 400 nm: radical concentration = 2.6 μM, 4 ms/div and 1.2% transmission/division).

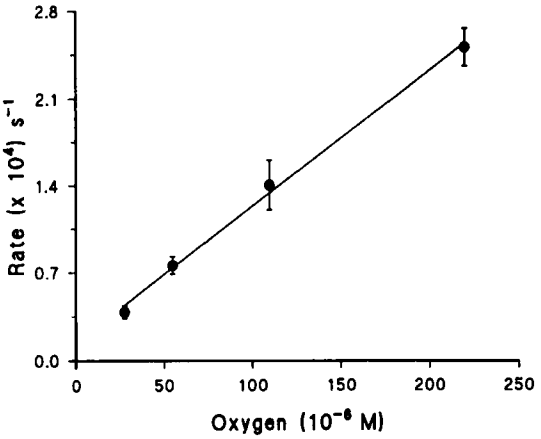

FIGURE 4 The dependence of the rate of decay of semiquinone radicals on oxygen concentration.

The reduction potential of the quinone/semiquinone couple at pH 7.4 was determined by setting up an equilibrium between the semiquinone radicals of EO9 and mitomycin C (MMC):

$$MMC^{\bullet-} + EO9 \rightleftarrows MMC + EO9^{\bullet-} \qquad (7)$$

The equilibria were investigated at 415nm, where the $EO9^{\bullet-}$ radicals have a much higher absorbance than that of $MMC^{\bullet-}$ radicals and at 490nm, where the $MMC^{\bullet-}$ radicals have much higher absorbance than $EO9^{\bullet-}$ radicals[10]. The concentration of mitomycin C was kept at 100 μM while the EO9 concentration was varied between 10–40 μM.

It can easily be shown[11] that at equilibrium:

$$K = (A_e - A_{MMC})/(A_{EO9} - A_e) \times [MMC]/[EO9] \qquad (8)$$

where, A_e is the absorbance value at equilibrium and A_{MMC} is the absorbance of Mitomycin C radicals in the absence of EO9 and A_{EO9} is the absorbance of the EO9 radicals in the absence of Mitomycin C. The equilibrium constant, K is related to the reduction potentials[11,12]:

$$E(MMC/MMC^{\bullet-}) = E(EO9/EO9^{\bullet-}) - 59\log K \qquad (9)$$

TABLE 1

Concentration of EO9 (μM)	λ(nm)	Absorbance at equilibrium (A_e) × 10^{-3}	K
10	490	37.4	9.0
	415	24.2	9.6
20	490	25	9.3
	415	31.8	9.0
40	490	15.1	9.6
	415	39.2	9.5

The absorptions of EO9 radicals at 415 and 490 nm are 49.5 and 0.7×10^{-3}/6.5 μM radicals in the absence of MMC. The absorption of MMC radicals are 0 and 70.4×10^{-3}/6.5 μM radicals at the same wavelengths respectively.

The absorbance values, normalised to a radical concentration of 6.5μM, are given in Table 1. From using the derived average value of K in Table 1 and assuming $E(MMC/MMC^{\bullet-}) = -310 \pm 6$ mV[10], it can be shown that $E(EO9/EO9^{\bullet-}) = -253 \pm 6$ mV.

It has recently been shown that the reduction of EO9 by the obligatory two electron reducing enzyme, DT-diaphorase is influenced by the presence of oxygen.[13] Several preliminary experiments involving rapidly mixing the hydroquinone produced in the pulse radiolysis experiments with air indicated that the hydroquinone is unstable with respect to autoxidation:

$$QH_2 + O_2 \rightarrow Q + H_2O_2 \qquad (10)$$

This reaction was studied by initially reducing the quinone (20–50μM) in one half of a stopped flow mixing chamber with xanthine oxidase/hypoxanthine (100 μM hypoxanthine, 0.1 M phosphate buffer (pH 7.4), 0.6U xanthine oxidase, argon saturated) until the solution became colourless. This was then rapidly mixed with an air saturated solution (200 μM allopurinol and 2 μM EDTA in 0.1 M phosphate buffer, pH 7.4). The total time for the initial reduction of the quinone and the subsequent mixing with air was less than 10 min and it was determined by HPLC analysis, consistent with recent studies,[4] that the reactions did not produce any changes in the

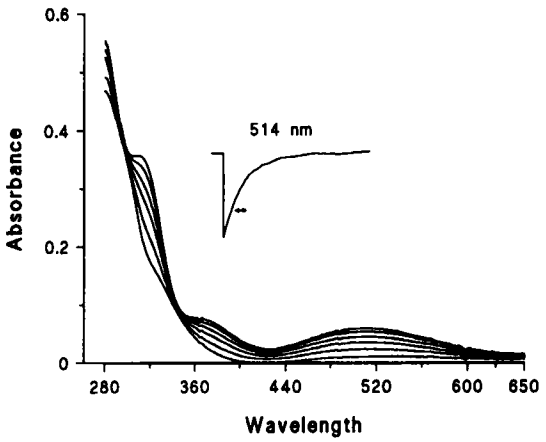

FIGURE 5 The autoxidation of the hydroquinone of EO9. Spectra measured at 2 sec intervals. Insert: Decay of EO9 hydroquinone at 514nm in the presence of 110μM oxygen. The arrow refers to a time period of 2 sec.

structure of the quinone during this period. A typical stopped-flow trace is shown in Figure 5 and the insert shows the kinetics at 514 nM. If it is assumed that the oxygen concentration in a fully air-saturated solution is 220 μM (ie. 110 μM in the stopped flow mix), then the rate constant for reaction 10 is $2.1 \pm 0.4 \times 10^3$ M^{-1} s^{-1}. Thus the half-life of a low concentration of the hydroquinone of EO9 in air is 1.5 ± 0.3 seconds.

DISCUSSION

The semiquinone radicals of EO9 have a reduction potential, $E(Q/Q^{\bullet-})$, of − 253 ± 6 mV at pH 7.4. Hence, according to previous studies on the dependence of enzyme rates and reduction potentials,[14,15,16] EO9 should be readily reduced by the one electron reducing enzymes such as cytochrome P450 reductase and xanthine oxidase. This has recently been demonstrated and indeed EO9 can be reduced by xanthine oxidase in the presence of DNA to form crosslinks.[4] Therefore, although EO9 may be an excellent substrate for diaphorase, the selectivity cytotoxicity of the quinone should also be mediated by the levels of the one-electron reducing enzymes within the cells.

The spectrum of the radical did not change between pH 6.5 and 10. (Figure 3). This implies that the aziridine on the semiquinone radical does not have a pK in this region. However, the absorbances at 400 nm were found to decrease in the pH range of 4.5–6.5. This decrease is probably due to both the protonation the aziridine and the $-O^{\bullet-}$ on the semiquinone radical (ie. $Q^{\bullet-}$ in neutral protonating to $QH^{\bullet}$ in acid). Most semiquinone radicals have a pK of around 4.0 to 6.0.[11] Unfortunately, due to the instability of EO9 in acid solutions,[4,8] it was not possible to accurately determine either the pK of the aziridine or of the semiquinone.

The semiquinone radicals were found to be in equilibrium with the hydroquinone and parent quinone. The equilibrium constant, K_4 was 84 ± 12 at pH 9.0 and 8 ± 2 at pH 10.0. Similar equilibria have been observed for several quinones including simple benzoquinones, naphthoquinones and adriamycin.[17,18,19] For simple quinones, the decrease in the equilibrium constant in going from neutral to alkaline pH is normally due to the involvement of protons in the equilibrium reaction and the deprotonation of the resulting hydroquinone in alkaline pH.[17] Hence, although the equilibrium constant could not be accurately measured at pH 7.4, there is no reason to suppose that an equilibrium does not occur at this pH and indeed it has been reported that when EO9 is reduced by DT-diaphorase, semiquinone e.s.r signals can be detected.[26]

The semiquinone of EO9 reacts with oxygen (reaction 6) with a rate constant of $1.3 \pm 0.15 \times 10^8$ M^{-1} s^{-1} and thus is expected to be removed in well oxygenated cells. Similarly, the hydroquinone of EO9 also reacts relatively rapidly with oxygen to form hydrogen peroxide (reaction 10).

The autoxidation of hydroquinones has been extensively studied[17,24] and is believed to be mediated by the semiquinone radicals involving reactions of the type:

$$Q^{\bullet-} + Q^{\bullet-} + 2H^+ \rightleftarrows QH_2 + Q \qquad (4')$$

$$Q^{\bullet-} + O_2 \rightarrow Q + O_2^{\bullet-} \qquad (6)$$

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (11)$$

$$O_2^{\bullet-} + Q^{\bullet-} + 2H^+ \rightarrow Q + H_2O_2 \qquad (12)$$

Interestingly, the one-electron potentials for duroquinone and EO9 are similar[12] and yet the rate of autoxidation of durohydroquinone is several orders of magnitude slower.[17] However, in the case of duroquinone, the equilibrium is strongly in favour of hydroquinone formation[11] and hence the amount of available semiquinone that can react with oxygen at any one time, is expected to be much less than that of EO9.

In a previous study, it was shown that DNA strand breaks are formed when DNA is incubated with EO9 and DT-diaphorase in the presence of air.[20] The extent of strand breaks was unaffected by the presence of superoxide dismutase and so it was proposed that the strand breaks were as a consequence of DNA alkylation by EO9. Unfortunately, the effects of catalase or metal chelators were not reported in this study. Hence, an alternative explanation could be that the strand breaks were produced simply as a consequence of the formation of hydrogen peroxide. It is significant that a recent study on the formation of EO9-DNA cross links produced by DT-diaphorase under nitrogen reported that strand breaks were not detected.[4] These simple reactions could, at least in part, explain the enhanced cytotoxicity of EO9 in cells under hypoxia.[2,3] If it is assumed that the toxicity in air is primarily due to hydrogen peroxide and superoxide anions, then these products are expected to be much less damaging than the formation of DNA cross links and strand breaks formed from the direct interactions of the reduced form(s) of EO9 with DNA.

However, it has recently been shown that although DT-diaphorase contributes to the cytotoxicity under aerobic conditions, it appears to protect cells under hypoxia.[5] The conclusion from this study would be that, under hypoxia, the hydroquinone reactions are not damaging. It was suggested that as a protonated aziridine is necessary for reactions with DNA bases, the pK of the aziridine on the semiquinone may be much higher than that of the hydroquinone and hence at physiologically relevant pH the semiquinones could be more reactive. However, the present studies show (Figure 3) that the pK of the aziridine on the semiquinone must be less than about 5.5. Surprisingly, the pK of the aziridine on EO9 has not been reported but it is expected to be similar to that of other aziridinylquinones which are 2.8 for mitomycin C[21] and typically between 2.5 and 4.0 for diaziridinylbenzoquinones (Butler *et al.*, unpublished). As the predicted order in the pK's of the aziridines should be quinone<hydroquinone< semiquinone, it is unlikely that the pK's of the semiquinone and the hydroquinone will be sufficiently different to account for the difference in reactivities. However, the semiquinones could be more reactive than the hydroquinones towards DNA alkylation due to other factors such as different hydrogen bonding between the DNA bases (which does, in some instances, favour the formation of crosslinks as opposed to single alkylations),[22,23] or selective activation of the vinyl group. Unfortunately, the structures of the toxic DNA crosslinks produced from reduced EO9 are not known.

The present results clearly show that the semiquinones and hydroquinones can be in equilibrium although it is extensively in the direction of hydroquinone formation at physiologically relevant pH. Nonetheless, the DNA crosslinks formed by the reduction of relatively high concentrations of EO9 with purified DT-diaphorase[4] could be explained by a direct reaction of a more reactive semiquinone which is produced from the equilibrium in a similar manner as the hydroquinone apparently reacts with oxygen. It is also significant that in this study, the DNA crosslinking from both the DT-diaphorase and the

xanthine oxidase reductions was found to increase as the pH was changed from 5.5 to 7.0. This is contrary to what would be expected from the activation of the aziridines[10,22] but it is consistent with the equilibrium being more in favour of semiquinone formation.

The question arises as to whether this equilibrium will be of significance in cellular systems under hypoxic conditions. The concentrations of EO9 necessary to kill cells are typically in the nanomolar range.[2,3,5] Hence although it may be possible that the semiquinones could, in principle, react together and form hydroquinones it is unlikely that the back reaction of the equilibrium will be important at these very low concentrations of drug. Thus, within cellular systems, the reduction of EO9 by DT-diaphorase should lead simply to the formation of the hydroquinone. Within the cell, the hydroquinone may be less reactive than the semiquinone, as discussed above, or the hydroquinone could be detoxified and excreted following the formation of glucoronide-, sulphate- or glutathione-adducts. Consequently, hypoxic cells which have high levels of DT-diaphorase could be protected against damage by EO9[5] by these mechanisms.

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