Biochemical Pharmacology, Vol. 56, pp. 613–621, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S006-2952(97)00661-8

Reduction of the Indoloquinone Anticancer Drug EO9 by Purified DT-Diaphorase: A Detailed Kinetic Study and Analysis of Metabolites

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ABSTRACT. DT-diaphorase has been implicated in the activation and mechanism of cytotoxicity of the investigational indoloquinone anticancer drug EO9. Here, we have used a highly purified DT-diaphorase isolated from rat Walker tumour cells to provide unambiguous evidence for the ability of this enzyme to catalyze reduction of EO9 and to provide a more detailed characterization of the reaction. Under the conditions used hypoxia had no effect on the initial rate of this reduction but did effect the nature and stability of metabolites formed. Electron spin resonance (ESR) spectrometry studies showed that DT-diaphorase reduced EO9 to a highly oxygen-sensitive metabolite that is probably the hydroquinone. In the presence of air, this metabolite is auto-oxidized to generate both drug- and oxygen-based radicals. Comproportionation:disproportionation reactions may also be involved in the generation of these radical species. The identification of these metabolites may contribute to the understanding of the molecular mechanism of DNA damage and cytotoxicity exerted by EO9. BIOCHEM PHARMACOL **56**;5:613–621, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. EO9; hypoxia; bioreductive agents; electron spin resonance spectroscopy; reduction

The indoloquinone anticancer drug EO9 [(3-hydroxy-5-aziridinyl-1-methyl-2-(1H-indole-4,7-dione)prop-β-en-α-ol); Fig. 1] is related to the bioreductive alkylating agent mitomycin C [1] but exhibits a different antitumour profile and is non-myelosuppressive [2, 3]. Unfortunately, despite showing promising activity in Phase I clinical trial [4], results of Phase II studies were less favourable with no partial or complete responses observed [5, 6]. Possible reasons for the lack of activity of EO9 in the chemical trials are the rapid clearance of EO9 from the body and the lack of monitoring for tumour DT-diaphorase activity. In addition, an understanding of the enzymology of this compound could help elucidate the reason for this somewhat disappointing result and may provide valuable information for future bioreductive drug development and optimization of clinical usage.

Quinones can undergo one-electron reduction catalyzed by a variety of enzymes such as NADPH:cytochrome P450 reductase (e.g. [7, 8, 9]) and NADH:cytochrome b_5 reductase [10]. This process produces semiquinones, which are usually oxygen sensitive and under aerobic conditions participate in redox-cycling to generate potentially cell damaging reactive oxygen- and drug-based radicals. In contrast, DT-diaphorase [NAD(P)H acceptor oxidoreductase (quinone); E.C. 1.6.99.2] can be an obligate two-electron transferring flavoenzyme [11]. As a result of this property it is often considered to be a detoxifying enzyme as it catalyses two-electron reduction of quinones directly to hydroquinones, thus bypassing the toxic radical-producing intermediates [12]. However, in some cases hydroquinones are also oxygen-sensitive and may participate in redox cycling (e.g. diaziquone, [13]). In addition, certain bioreductive agents are activated to alkylating species following reduction [12]. Thus, DT-diaphorase may either activate or detoxify depending on the specific chemical nature of the drug [11].

DT-diaphorase has a broad substrate specificity, although within related groups of compounds a slight chemical modification can profoundly alter the ability of a particular compound to be reduced [14, 15]. A range of quinones have

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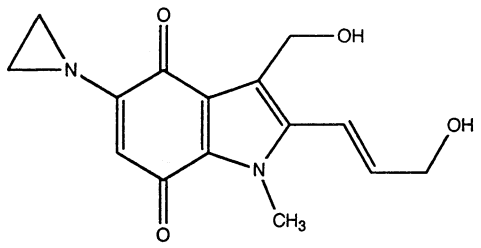
Received 22 May 1997; accepted 17 November 1997.



EO9

FIG. 1. Structure of indoloquinone EO9

been found to be metabolized by DT-diaphorase including mitomycin C [16] and AZQ [8, 13]. Expression and activity of DT-diaphorase is increased in some tumours compared with normal tissue of the same origin [11, 17, 18, 19, 20]. Thus DT-diaphorase may be a "target" for anticancer prodrugs that are activated by this enzyme.

It has been shown that EO9 may undergo reduction catalyzed by sonicates of UK rat Walker 256 tumour cells and human HT29 colon carcinoma cells [15, 21, 22] that express high levels of DT-diaphorase [13, 23]. This reductase activity was largely negated by inclusion of dicoumarol, a potent DT-diaphorase inhibitor, thus suggesting the involvement of this enzyme. The purified rat Walker DT-diaphorase also activated EO9 to a species capable of inducing strand-breaks in plasmid DNA [21]. To date, kinetic analyses of DT-diaphorase-mediated EO9 reduction have been reported only for the enzyme present in sonicates of rat Walker tumour cells and human HT29 cells, and these have relied on the use of dicoumarol as a diagnostic inhibitor. More recent reports have shown that dicoumarol is not specific and is able to inhibit other enzymes such as NADH:cytochrome b_5 reductase [10] and xanthine dehydrogenase [24]. As a result of these pleiotropic effects caution should be employed when interpreting results obtained using dicoumarol [25]. Studies reported in this paper employed a highly purified form of the rat Walker DT-diaphorase to confirm unequivocally that EO9 could be reduced by this enzyme. Detailed kinetics are presented and HPLC and ESR* spectroscopy studies were used to characterize the nature and identity of metabolites formed during DT-diaphorase-catalyzed reduction of EO9, with emphasis on radical species.

MATERIALS AND METHODS

Materials

EO9 was a generous gift from the European Organization for Research and Treatment of Cancer (EORTC) New Drug Development Office, Amsterdam, The Netherlands. Nitrogen and helium were purchased from British Oxygen Co. All other chemicals were obtained from Sigma. Rabbit anti-rat Walker tumour cell DT-diaphorase polyclonal antiserum was obtained as described previously [23].

Purification of DT-Diaphorase from Rat Walker 256 Tumour Cells

DT-diaphorase was isolated from rat Walker tumour cells and purified to homogeneity by gel filtration and anion exchange chromatography as previously described [26].

Spectrophotometric Determination of EO9 Reduction by DT-Diaphorase

CYTOCHROME C REDUCTION ASSAY. DT-diaphorase reduction of EO9 was monitored spectrophotometrically using a method similar to that described by Ernster [27]. The reaction was followed by detecting a decrease in absorbance of the signal molecule cytochrome *c* at 550 nm. Reaction constituents were: cytochrome *c* (77 μM), BSA (0.14%), purified rat Walker tumour cell DT-diaphorase, quinone dissolved in a mixture of DMSO (final concentration of 2% or less) and Tris-HCl buffer (pH 7.5) with BSA (0.14%) in a final volume of 1 mL of Tris-HCl buffer pH 7.5. The reaction was initiated by addition of NADH (2 mM) and was monitored at 37° for 1–2 min. DT-diaphorase activity was calculated as the fraction inhibited by dicoumarol (100 μM). Menadione, a known substrate for DT-diaphorase, was included as a standard and as a positive control. Little variation was observed between repeat assays.

The effect of hypoxia on EO9 reduction catalyzed by purified rat Walker DT-diaphorase was determined by the cytochrome *c* reduction assay described above. These studies utilized a Thunberg type quartz cuvette (Belmont Instruments, Glasgow, Scotland, U.K.) and degassing was carried out with nitrogen gas (black spot grade < 6 parts per million). The reaction was initiated by rapid inversion of the cuvette, thus mixing the cofactor (NADH) present in the bulb with the remaining reaction constituents.

Activity was calculated in μmol of cytochrome *c* reduced/min/mg of protein using an extinction coefficient of 21.1 mM cm^{-1}. Kinetic constants were determined from Hanes Woolf plots where substrate concentration (S)/initial velocity (v) is plotted against substrate concentration. Values were obtained using the computer programme Enzyme Kinetics (Trinity Software) and were confirmed by comparison with other graphical methods.

Direct Spectrophotometry

Reduction of EO9 was monitored directly in the absence of cytochrome *c* by following the change in absorbance of the reaction mixture over the range 200–700 nm at 1 min

* *Abbreviations:* DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; and ESR, electron spin resonance.

intervals for a 10-min period. In these experiments the NADH concentration was 200 μM, EO9 was 50 μM and purified rat Walker DT-diaphorase 0.25 μg in a total volume of 1 mL of Tris-HCl buffer (50 mM, pH 7.5).

Additional experiments monitored loss of EO9 absorbance, under aerobic conditions, at 500 nm. Although 500 nm was not the λ max of EO9 it was selected because it was a principal wavelength at which EO9 continued to absorb light but that avoided overlap with the absorption peak of the nicotinamide cofactor, NADH. The reaction constituents were EO9 (100 μM), NADH (500 μM) and purified rat Walker DT-diaphorase (5 μg) in a total volume of 3 mL of Tris-HCl buffer (50 mM; pH 7.5) containing BSA (0.14%). Due to the oxygen sensitivity of EO9 metabolites, a small magnetic flea was included in the cuvette (3-mL volume) where possible to ensure complete mixing of the reaction components.

HPLC

EO9 (100 μM), NADH (500 μM) and either 5, 50, 250 ng or 25 μg of purified rat Walker DT-diaphorase were incubated at 37° in 1 mL of Tris-HCl buffer (50 mM, pH 7.5) containing BSA (0.14%). Every 6 min, aliquots (10 μL) were injected on to the HPLC (Gilson, Anachem) with a Partisphere C18 column and a mobile phase of 40% methanol 60% water. Reduction was followed at 260- and 340-nm wavelength and also by monitoring fluorescence.

ESR Spectrometry

ESR spectrometry was carried out at room temperature using a Varian E109 Century Series X-band (9.3 GHz) spectrometer (Varian Instruments). ESR parameters were typically: scan range ± 50 G; time constant 1 sec; modulation amplitude 1.25 G; receiver gain 1.25×10^5 and 1.25×10^6; microwave power 5 mW; field set 3394 G; scan time 4 min; modulation frequency 100 mHz; and microwave frequency 9.51 G.

Standard reaction constituents were: DT-diaphorase (0.25 μg), EO9 (800 μM dissolved in DMSO), NADPH (1 mM) and BSA (0.14%) made up to a total volume of 400 μL of Tris-HCl buffer (50 mM), pH 7.4. The effect of altering DT-diaphorase concentration was also investigated. Spin-trapping studies were carried out using DMPO (100 mM) in the presence and absence of superoxide dismutase (400 U) and catalase (200 U). For inhibition studies dicoumarol (100 μM) or rabbit anti-rat Walker DT-diaphorase antiserum (43 μL) were included. The antiserum was preincubated for 15 min at room temperature with purified DT-diaphorase before addition of the other components. Reactions were initiated by addition of cofactor. In some of the DT-diaphorase assays, NADH (1–2 mM) was substituted with NADPH.

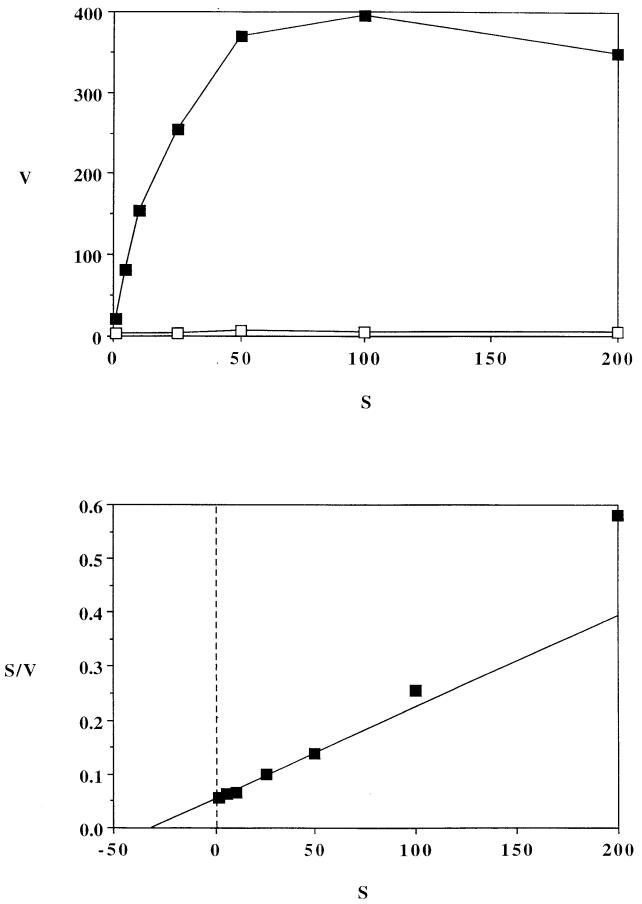


FIG. 2. Reduction of EO9 catalysed by purified rat Walker tumour cell DT-diaphorase displayed as (A) a plot of initial velocity (v) against substrate concentration (S) in the presence (□) and absence (■) of the inhibitor dicoumarol (100 μM). (B) S/V versus S plot. Units of V are in μmol cytochrome c reduced/min/mg of enzyme. S is given in μM. Data shown are from a typical experiment.

RESULTS

Evidence for EO9 Reduction Using the Cytochrome c Reduction Assay

EO9 underwent efficient reduction catalyzed by purified rat Walker DT-diaphorase when incubated in the presence of cofactor NADH (Fig. 2A and B; Table 1). Controls where one of the reaction constituents was omitted showed only background levels of reaction indicating that enzyme, EO9 and cofactor were all required for reduction (results not shown). Kinetics were consistent with Michaelis-Menten

TABLE 1.

Drug		Km (μM)	Kcat (min^{-1})	Kcat/Km
Menadione	Air (a)	1.87	3.13×10^4	1.67×10^4
	Air (b)	1.65	3.74×10^4	2.27×10^4
EO9	Air (a)	31.36	1.58×10^4	5.02×10^4
	Air (a)	31.41	1.58×10^4	5.02×10^4
	Hypoxia (a)	18.68	1.56×10^4	8.36×10^4

Final concentration of DMSO used as a solvent is (a) 2% and (b) 1% in the reaction

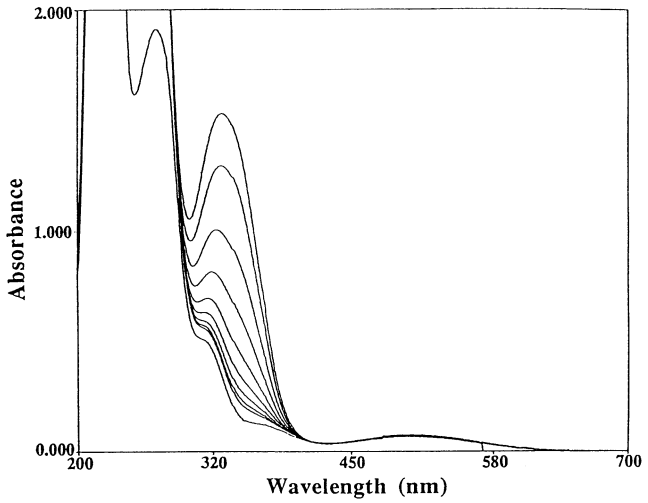


FIG. 3. Sequential UV-visible absorbance scans associated with the reduction of EO9 (50 μM) catalysed by DT-diaphorase (0.25 μgα) in the presence of cofactor NADH (200 μM/mL). The lower curve represents EO9 in buffer in the presence and absence of cofactor and enzyme and the uppermost EO9 plus NADH in the absence of enzyme. Intermediate scans were taken sequentially each minute following addition of DT-diaphorase. Data shown are from an individual experiment and were confirmed in an independent repeat assay.

behaviour. The rate of reduction increased with increasing EO9 concentration (Fig. 2A) up to 50 μM, above which saturation occurred and even some degree of inhibition at 200 μM EO9. Activity also increased in a linear fashion with increasing enzyme concentration over the range examined (results not shown). Dicoumarol inhibited EO9 reduction by >97% (Fig. 2A). Compared with menadione, EO9 was only about two-fold less efficiently metabolized in terms of K_{cat} values (Table 1). However, because of the much higher K_m value for EO9 the K_{cat}/K_m ratio was about 30-fold lower.

Direct Measurement of NADH-dependent EO9 Reduction

Change in absorbance was monitored over the wavelengths 200–700 nm. For sequential spectrophotometric scans at intervals of 1 min a decrease in peak height at 340 nm was observed (Fig. 3). This was consistent with oxidation of the NADH cofactor. Little absorbance change was noted at 450–550 nm, the absorption region of EO9. Control incubations where drug was substituted by vehicle or where enzyme was omitted resulted in no detectable NADH oxidation. However, loss of absorbance occurred at 500 nm (not shown) using approximately seven-fold higher concentrations of DT-diaphorase. This decrease persisted over 3 min before again increasing to near original levels at 6–7 min. Visual observance of the reaction mixture showed a concomitant loss and reappearance of EO9 (purple) colour consistent with the formation and subsequent reoxidation of a colourless EO9 reduction product.

HPLC

HPLC analyses provided further evidence for the ability of EO9 to undergo reduction catalyzed by purified rat Walker DT-diaphorase. This was indicated by a decrease in peak height of NADH that eluted at 0.55–0.6 min (Fig. 4). However, under these conditions, only a slight decrease in that attributed to EO9 was observed. Increasing enzyme concentration increased EO9 reduction. In control experiments where either drug, enzyme or cofactor were omitted no decrease in NADH was noted.

The Effect of Hypoxia on DT-Diaphorase-catalyzed Reduction of EO9

The effect of hypoxia on DT-diaphorase catalyzed reduction of EO9 was determined both spectrophotometrically and by HPLC. Results of the cytochrome *c* reduction assays

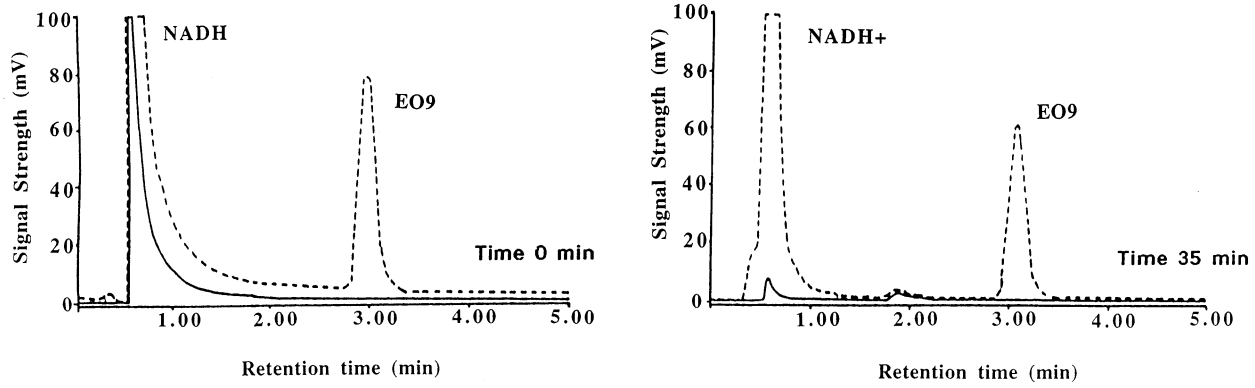


FIG. 4. HPLC chromatograms (taken at Time 0 min and Time 35 min) showing change in peak height of EO9 and NADH absorbance at 260 nm (broken line) and by fluorescence absorbance (solid line) during DT-diaphorase catalysed metabolism. Reaction constituents included: 50 μL of 1:100 dilution of purified Walker tumour cell DT-diaphorase, 500 μM NADH and 100 μM EO9 in 1 mL of Tris-HCl buffer pH 7.5. Data are taken from an individual experiment. The same trends were seen in similar experiments where only enzyme concentration was altered.

showed the initial velocity of DT-diaphorase metabolism of EO9 to be almost identical in terms of the K_{cat} value under aerobic and hypoxic conditions although a slight difference was observed in the K_m value (Table 1). During HPLC experiments, anaerobic reduction of EO9 was detected visually by conversion of the purple coloured parent quinone to form a colourless metabolite. This product remained stable over the time course of the assay. However, during HPLC analysis no apparent loss of EO9 was evident as judged by the lack of change in EO9 peak height (not shown). These results indicated the auto-oxidation of EO9 metabolite(s) during analyses, as also suggested by the spectrophotometric studies.

Radical Formation Following DT-Diaphorase Reduction of EO9

ESR analyses showed that aerobic incubation of EO9 in the presence of either NADPH or NADH supplemented purified Walker tumour cell DT-diaphorase resulted in the formation of a seven-line hyperfine spectrum superimposed over a broad singlet spectrum. In the presence of either cofactor this spectrum was clearly visible after 8 min (Fig. 5A) and persisted for 30 min (data not shown). Formation of this hyperfine spectrum was not evident in the absence of either NAD(P)H, drug or enzyme, and was inhibited by DT-diaphorase antibody and dicoumarol (results not shown).

ESR spectrometry in combination with DMPO, a spin trap for short-lived free radicals [28] was used to investigate directly the formation of reactive oxygen as a result of EO9 reduction by DT-diaphorase under aerobic conditions. The results (Fig. 5B) show that EO9, in the presence of DMSO as a drug diluent, generates an ESR spectrum with hyperfine splitting 1:1:1:1:1:1 [AN (hyperfine splitting constant for nitrogen) = 16.2; AH (hyperfine splitting constant for hydrogen) = 23.0]. This is consistent with formation of the DMPO-CH_3 adduct, the presence of which indicates that EO9 stimulates hydroxyl radical attack on DMSO to generate the methyl radical that is subsequently trapped by DMPO as the adduct observed [29]. Addition of both superoxide dismutase and catalase (Fig. 5E and F), scavengers of superoxide anions and hydrogen peroxide, respectively, resulted in no reactive-oxygen-derived spin trap over the same time scale. Interestingly, addition of superoxide dismutase alone (not shown) resulted in an intense spectrum of the DMPO-CH_3 adduct. This indicates that superoxide dismutase alone can facilitate production of H_2O_2, which in the absence of catalase would mediate hydroxyl radical formation via Fenton chemistry and/or Haber-Weiss driven process. Addition of catalase alone resulted in abolition of the oxygen radical (Fig. 5G). No spin-trapped ESR spectrum was observed in the absence of EO9 or DT-diaphorase (results not shown).

The DMPO-CH_3 six-line spectrum generated as a result of EO9 aerobic incubation with DT-diaphorase diminished during the scan time of the 3-min (Fig. 5B) incubation as judged by the asymmetry of the spectrum observed. After 8-min incubation the spin-trapped spectrum was no longer observed but was replaced with a seven-line spectrum superimposed over a broad ESR singlet (Fig. 5C). This

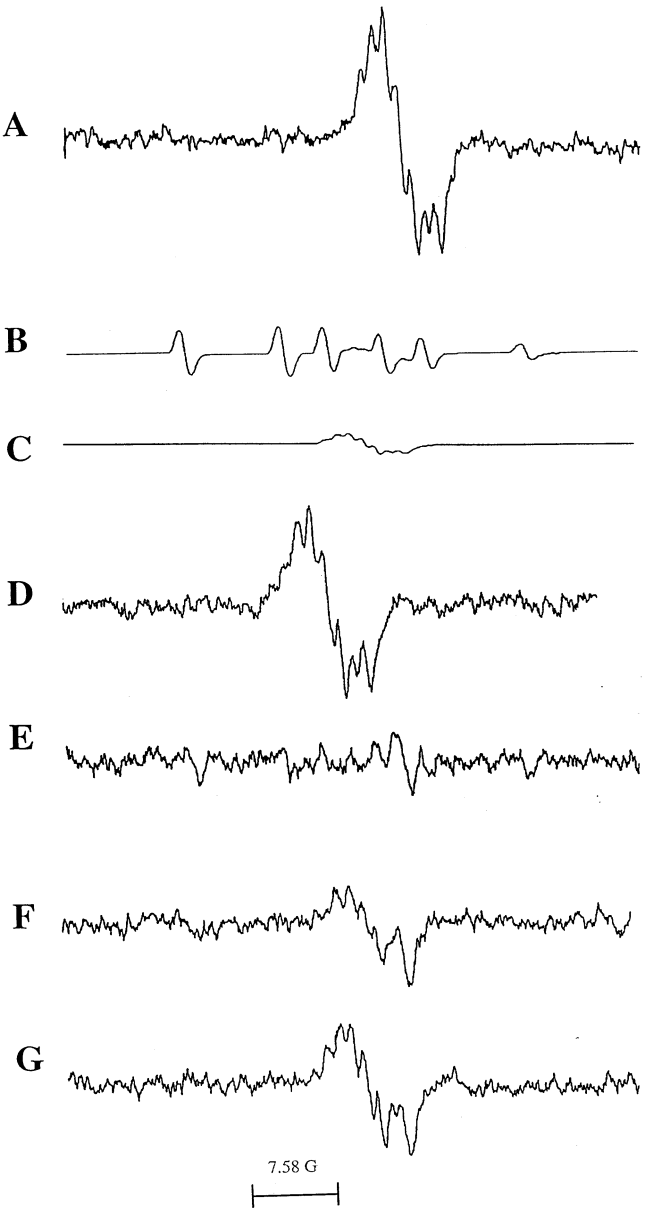


FIG. 5. ESR spectra of (A) EO9 free radical generated by purified rat Walker tumour cell DT-diaphorase and (B-G) the same incubation mixture in the presence of DMPO, a reactive oxygen spin-trapping agent. A typical incubation mixture was: DT-diaphorase (0.25 μg), EO9 (800 μM) and NADPH (1 mM) in a volume of 400 μL. Spectra are as follows: (A) typical incubate after 3 min; (B, C and D) typical incubate plus 100 mM DMPO after (B) 3 min (1.25×10^5 gain); (C) 8 min (1.25×10^5 gain); and (D) 8 min (1.25×10^6 gain); (E, F) typical incubate plus DMPO (100 mM), superoxide dismutase (400 U) and catalase (200 U) taken (E) 3 and (F) 8 min after the initiation of the reaction; (G) complete system plus DMPO (100 mM) and catalase (200 U) after 8-min incubation. Results are from a typical experiment. ESR spectrometer operating conditions were as described in the methods section using an instrument gain of 1.25×10^6 unless otherwise stated.

spectrum was not as a result of free radical oxygen formation because it was observed after 8 min in the presence or absence of superoxide dismutase and/or catalase. It was identical to the EO9 free radical spectrum observed in the absence of DMPO (Fig. 5A). Addition of DT-diaphorase antibody to EO9 incubations partially inhibited, whilst dicoumarol almost totally inhibited, the formation of all ESR spectra (results not shown).

In experiments where either the enzyme concentration was lowered to 0.007 μg (in 400 μL reaction volume) or increased to 2 or 5 μg (in 400 μL) the drug-based radical was no longer observed. At lower enzyme concentrations it is likely that the hydroquinone is produced more slowly and therefore auto-oxidation occurs to generate the semiquinone and quinone without permitting sufficient amounts of semiquinone to build up to form a detectable signal. At higher enzyme concentrations, reduction of EO9 would be very rapid and thus may quickly consume the oxygen present in the system, causing the sealed flat cell to become hypoxic. Under these conditions the hydroquinone may be more stable because, although comproportionation:disproportionation reactions could still occur, auto-oxidation may not. Hence, diminished levels of semiquinone may result. Visible evidence existed for the occurrence of EO9 metabolism at these higher enzyme concentrations in that loss of colour was seen.

DISCUSSION

The two-electron donating flavoenzyme DT-diaphorase may provide an attractive target for selective activation of bioreductive antitumour agents due to its increased activity and expression in some tumours compared with normal tissues of the same origin [11, 17, 18, 19, 20]. DT-diaphorase is able to catalyze metabolism of a range of substrates, including quinone compounds that bear a structural similarity to EO9. However, whether DT-diaphorase participates as an activation or a detoxification enzyme is dependent on the chemical nature of the individual substrate. An "enzyme-directed" approach to bioreductive drug development that aims to tailor drug treatment to suit the catalytic preferences of target enzymes has been described [30, 31]. As part of this strategy we have examined the role of DT-diaphorase in the activation of EO9.

Cellular studies have previously shown a direct correlation between DT-diaphorase activity and EO9 cytotoxicity under aerobic conditions, whereas an inverse correlation has been observed under hypoxia [32, 33, 34, 35, 36]. In addition, sonicates of rat Walker tumour cells and human HT29 colon carcinoma cells are able to catalyze dicoumarol-inhibitable reduction of EO9, suggesting the involvement of DT-diaphorase in the drug's mode of action [15, 21]. Due to the lack of complete specificity of dicoumarol care should be taken when interpreting data obtained using this inhibitor [25]. For this reason, a highly purified DT-diaphorase has been used in the present study and a variety of techniques have unambiguously shown that this enzyme is able to catalyze reduction of EO9.

The results illustrate that EO9 is an excellent substrate for DT-diaphorase, being only two-fold poorer in terms of Kcat than the benchmark substrate menadione although taking into account the higher K_m value, the K_{cat}/K_m ratio suggests that it is 30-fold less efficiently reduced. EO9 reduction was greatly diminished in the presence of dicoumarol, consistent with the role of the latter as an inhibitor of DT-diaphorase. Interestingly, this novel anticancer drug is shown to be more efficiently metabolized by DT-diaphorase at pH 7.4 than is mitomycin C, the prototypical bioreductive alkylating agent. The difference in enzymological activation of EO9 and mitomycin C has been suggested to explain the lack of myelosuppression observed with EO9 as bone marrow contains low levels of the activating enzyme DT-diaphorase [3]. Conversely, Plumb and coworkers [32] proposed that high levels of this enzyme in the kidney may account for the dose limiting toxicity in Phase I clinical trials of EO9, namely proteinurea.

The ability of EO9 to undergo DT-diaphorase-catalyzed reduction was confirmed by direct spectrophotometry. Reduction of EO9 was indicated by oxidation of NADH, although no corresponding decrease in the absorbance ascribed to EO9 was detectable. This may be due to either the formation of a metabolite that has a similar absorption spectrum to EO9 or more likely the metabolite formed is oxygen sensitive and undergoes extensive auto-oxidation back to the parent compound, thus resulting in no apparent change in the amount of EO9 observed. At higher DT-diaphorase protein and NADH concentrations a decrease in the absorption at 500 nm was observed coincidentally with a visible loss of the purple color of EO9. With time a return to the purple colouration was evident at the reaction buffer/air interface, suggesting that auto-oxidation of the reduced metabolite to regenerate the EO9 was occurring.

HPLC analyses further confirmed the oxidation in NADH with little alteration in the concentration of EO9. Rapid auto-oxidation following metabolism by DT-diaphorase have also been reported for the aziridinylbenzoquinone AZQ [13].

Hypoxia has often been associated with resistance to conventional chemotherapy and radiotherapy [37]. It may however, provide a selective target for bioreductive anticancer agents. Tumour cell lines have been shown to have increased sensitivity to EO9 under hypoxia [32, 38, 39] with differentials as large as 1000-fold being observed [38]. Interestingly, an inverse correlation appears to exist between DT-diaphorase expression and hypoxic differential with large differential values being reported for cell lines expressing low levels of DT-diaphorase [32, 33]. For this reason, the effect of hypoxia on the rate of DT-diaphorase catalyzed reduction was examined. Results (Table 1) showed an identical rate of reduction of EO9 under aerobic and hypoxic conditions in terms of K_{cat} values. HPLC studies also gave similar results although, somewhat unexpectedly, no decrease in the level of EO9 was detected

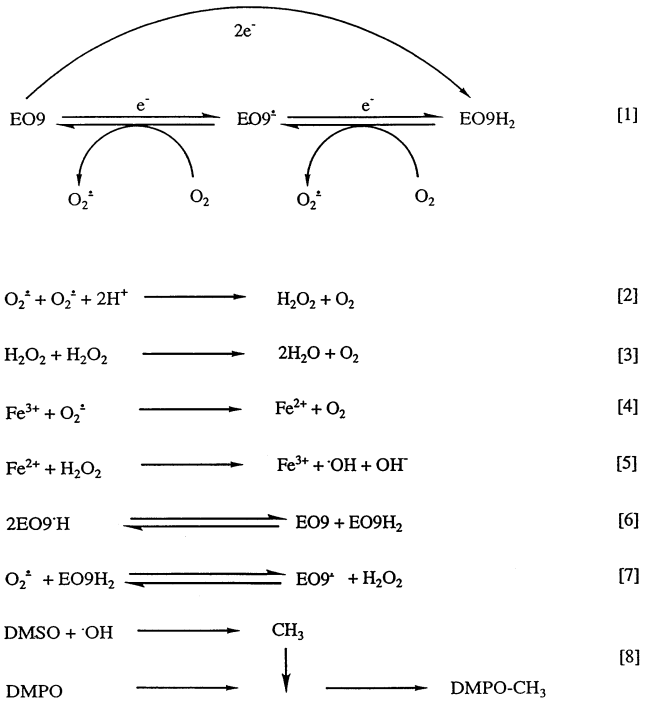


FIG. 6. Schematic illustrating the proposed chemical reactions that occur during reduction of EO9 catalysed by DT-diaphorase. For detailed comments see text.

under hypoxia. This suggested that the metabolites were so oxygen sensitive that auto-oxidation occurred during the HPLC analyses. However, using different HPLC conditions Maliepaard and coworkers [40] did observe a decrease in EO9 concomitant with the formation of three unidentified metabolites following DT-diaphorase catalyzed reduction under anoxic conditions. A lack of differential between aerobic and hypoxic metabolism catalyzed by DT-diaphorase has also been reported for other oxygen sensitive bioreductive compounds including mitomycin C [16], AZQ [13] and CB 1954 [14, 26]. Nevertheless hypoxia may affect the nature and levels of EO9 metabolites formed by affecting their stability, particularly as it appears that the metabolites resulting from DT-diaphorase-catalyzed reduction of EO9 are oxygen sensitive.

In order to characterize the radical products of DT-diaphorase-catalyzed reduction of EO9 ESR spectrometry studies were carried out. These revealed that the DT-diaphorase-catalyzed reduction of EO9 generated a drug-based radical. The ESR spectrum observed for EO9 free radical is likely to be due to an oxygen-centered semiquinone species with nitrogen involvement. Semiquinones often generate singlet spectra as a result of broadening due to their slow rotation on the ESR time scale in biologic milieu [41]. The multiple-line hyperfine spectrum thus observed for EO9 semiquinone suggests spin coupling to the aziridinyl nitrogen and associated protons.

DT-diaphorase is an obligate two-electron reducing enzyme [11, 42] and as such would be expected to catalyze reduction of EO9 to generate hydroquinone (Fig. 6, Eqn 1) that is ESR silent. However, comproportionation of EO9 hydroquinone (Fig. 6, Eqn 6) with EO9 could explain the EO9 semiquinone ESR spectrum observed in this study. Previously, it has been shown using pulse radiolysis mediated formation of EO9 semiquinone that this free radical is in equilibrium with EO9 and EO9 hydroquinone at physiological pH [43]. The time delay in appearance of the EO9 semiquinone is consistent with the oxygen sensitivity of the DT-diaphorase-generated EO9 reduction products. Specifically, the EO9 hydroquinone is unstable with respect to autoxidation (Fig. 6, Eqn 1) and has a half-life of 1.5 + 0.3 sec in air [43]. The two-electron oxidation of EO9 hydroquinone by dioxygen will result in H_2O_2 formation (Fig. 6, Eqn 7). Furthermore, EO9 hydroquinone-catalyzed formation of H_2O_2 will react with adventitious iron to generate hydroxyl radicals (Fig. 6, Eqn 5) which, via H-atom abstraction from DMSO, would explain the formation of the methyl radical spin adduct observed by ESR spectrometry (Fig. 6, Eqn 8). The autocatalytic oxidation of EO9 hydroquinone consumes completely the dioxygen in the stoppered flat cell employed in these ESR spectrometry experiments. This results eventually in an anaerobic incubation condition—a process that appears to take about 3 min under the conditions used. In the absence of dioxygen, an increase in the concentration of the EO9 hydroquinone will result and, as indicated previously, EO9 can comproportionate with EO9 hydroquinone to generate the EO9 semiquinone observed (Fig. 6, Eqn 6). Hence, although the EO9 semiquinone is not an obligate intermediate in DT-diaphorase mediated EO9 reduction, one-electron autoxidation of this free radical formed as a result of EO9/EO9 hydroquinone comproportionation will, under aerobic conditions, contribute to the formation of the reactive oxygen observed in this study. In support of this, the semiquinone of EO9 has been shown to react rapidly with oxygen with a rate constant of $1.31 \pm 0.15 \times 10^8\ M^{-1}\ s^{-1}$ [43].

Consistent with the idea that the drug-based radical results from reduction of EO9 catalyzed by DT-diaphorase is the finding that replacing the cofactor NADPH with NADH gave almost identical results. A characteristic of this enzyme is its ability to utilize NADH and NADPH with equal efficiency. Increasing NADH concentration to 2 mM resulted in a similar initial peak intensity to that obtained with 1 mM; however, in contrast to the result with 1 mM cofactor the signal began to decrease after 9 min giving no detectable peak after 24 min. Based on the presumption that EO9 is being reduced to the hydroquinone by DT-diaphorase and that auto-oxidation subsequently occurs, the additional amount of reducing equivalent may continue to drive the forward reaction until hypoxia prevails. This could stabilize the hydroquinone and result in a diminished level of the radical species.

DNA damage has been suggested as a mechanism of EO9 induced cytotoxicity. Both DNA strand breaks and DNA interstrand cross-links have been detected in cells treated with EO9 [15]. Previous studies have shown that reduction of EO9 by purified rat Walker tumour cell DT-diaphorase

results in the breakage of plasmid DNA in a cell-free system [15, 21]. Data from ESR experiments presented in this paper have provided evidence for the formation of reactive oxygen species presumed to be involved in these strand breaks. Catalase inhibited the formation of oxygen radicals as detected by ESR and also inhibited DNA damage [44].

In conclusion, data presented in this paper have confirmed that EO9 can undergo reduction catalyzed by purified rat Walker tumour cell DT-diaphorase and provided a more detailed characterization of the process. Results indicated that this reduction generates a highly oxygen-sensitive metabolite that, under aerobic conditions, is autooxidized to yield its semiquinone radical and ultimately the parent quinone. The latter is then available for further reduction. This process of redox cycling produces oxygen-based radicals. It is likely that these contribute toward DNA strand breaks that may be involved in the mechanism of EO9 induced aerobic cytotoxicity. Hypoxia did not affect the initial rate of reduction of EO9 by DT-diaphorase but it is likely to affect the stability of metabolites so generated, as well as of those produced following reduction by other enzymes such as NADPH:cytochrome P450 reductase. Differences in the hypoxic versus oxic tumour cells *in vivo* may have consequences for the degree of cytotoxicity seen in the different environments. Other reducing enzymes have also been shown to catalyze reduction of EO9 including NADPH:cytochrome P450 reductase [44, 45] and xanthine oxidase [40]. Thus cytotoxicity may also be influenced by the levels and affinities of these other reducing enzymes within the cell [31]. Careful *in vivo* studies will be required to clarify the role of various parameters in animal models and human tumours.

This work was supported by the U.K. Cancer Research Campaign. Prof. Paul Workman acknowledges a CRC Life Fellowship.

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