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Involvement of NADPH: cytochrome P450 reductase in the activation of indoloquinone EO9 to free radical and DNA damaging species

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Abstract

Evidence suggests that DT-diaphorase is involved in the activation and mechanism of cytotoxicity of the investigational indoloquinone anticancer drug EO9 under aerobic conditions. Data also implicate a role for other enzymes including NADPH: cytochrome P450 reductase, especially in low DT-diaphorase tumour cells and under hypoxic conditions. Here, we used purified rat NADPH: cytochrome P450 reductase to provide additional evidence in support of a role for this enzyme in activation of EO9 to generate free radical and DNA-damaging species. Electron spin resonance spectrometry studies showed that NADPH: cytochrome P450 reductase reduced EO9 to a free radical species, including a drug radical (most likely the semiquinone) and reactive oxygen species. Plasmid DNA experiments showed that reduction of EO9 catalysed by NADPH: cytochrome P450 reductase results in single-strand breaks in DNA. The information obtained may contribute to the understanding of the molecular mechanism of DNA damage and cytotoxicity exerted by EO9 and may be useful in the design of future bioreductive drugs.

Keywords: EO9; NADPH: cytochrome P450 reductase; Bioreductive agents; Electron spin resonance spectroscopy; Reduction; DNA damage

1. Introduction

The bioreductive alkylating agent mitomycin C has been used clinically for the treatment of several tumour types. Despite significant antitumour activity, its widespread use is limited as a result of its extensive and unpredictable dose-limiting myelosuppression [1]. An indoloquinone analogue of mitomycin C, EO9 (Fig. 1; [2]), has undergone preclinical and clinical development under the auspices of the EORTC. EO9 was found to have a different spectrum of preclinical antitumour activity to mitomycin C, showed preference for solid tumours over leukaemias, and most importantly was non-myelopsuppressive [3,4]. As a result of these features and information regarding its mechanism of enzymatic activation (see below), EO9 entered Phase I and II clinical trial [5–7]. Although responses were seen in Phase I trials [6], activity was not confirmed in Phase II [7]. Nevertheless, clinical development of EO9 continues, potentially to define a clinical role for this agent in a setting where its short plasma half-life is not so critical, e.g. intravescicular therapy for bladder cancer. In addition, studies continue with the aim of identifying bioreductive agents that will be clinically more effective than EO9 [8].

A characteristic of bioreductive alkylating agents such as EO9 is that they are designed to require reduction for activation to a cytotoxic species. Several enzymes are

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Abbreviations: EORTC, European Organisation for the Research and Treatment of Cancer; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; and AN and AH, hyperfine-splitting constants for nitrogen and hydrogen, respectively.

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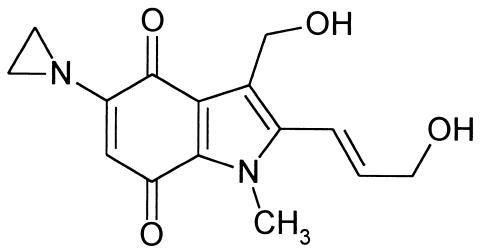

Fig. 1. Chemical structure of EO9.

known to be involved in the reduction of quinone compounds [8]. These include those catalysing one-electron reduction, e.g. NADPH: cytochrome P450 reductase and cytochrome *b*5 reductase as well as DT-diaphorase, which carries out two-electron reduction. As part of an 'enzyme-directed approach' to bioreductive drug development [8–10], we were interested in elucidating the molecular enzymology and mechanism of action of EO9. We had previously focused on the involvement of the two-electron reducing flavoenzyme DT-diaphorase and provided data to support a role for this enzyme in the activation of EO9 to a cytotoxic species [11–15].

Initially, extracts of the high DT-diaphorase expressing rat Walker tumour and human HT29 colon carcinoma cells were shown to catalyse reduction of EO9 [12,15]. The rate of reduction decreased in the presence of the potent DT-diaphorase inhibitor dicoumarol. Dicoumarol is, however, also known to affect other enzyme systems. Proof of the ability of DT-diaphorase to reduce EO9 was provided by use of purified rat, mouse, and human recombinant DT-diaphorase [11,16]. Activation of EO9 catalysed by DT-diaphorase resulted in generation of a DNA-damaging metabolite(s) able to induce strand breaks and interstrand cross-links in DNA [13]. Studies correlating cellular sensitivity with enzyme expression provided further evidence for the importance of DT-diaphorase in activation of EO9 under aerobic conditions [17–21] but not in hypoxia [22]. Interestingly, cells expressing low levels of DT-diaphorase were greatly sensitised to EO9 under hypoxic compared with aerobic conditions [19,23]. Also, inclusion of dicoumarol in cytotoxicity assays did not provide complete protection against the cytotoxic effects. These data suggested that other enzymes may also be important in reductive activation of EO9, especially under hypoxia.

Malipaard *et al*. [24] have shown that xanthine oxidase is able to reduce EO9 to generate a DNA-damaging species. NADPH: cytochrome P450 reductase is known to reduce other bioreductive quinone compounds such as AZQ and mitomycin C [25–27]. Furthermore, recent experiments by Saunders and co-workers [28], involving transfection and overexpression of the NADPH: cytochrome P450 reductase gene, have strongly implicated a role for this enzyme in the cellular reduction of EO9. Here, we provide additional evidence in support of a role for this enzyme in activation of EO9 to free radical and DNA-damaging species. The experiments were carried out under predominantly aerobic conditions and are therefore more directly relevant to cytotoxic effects that EO9 may generate in well-oxygenated normal cells (contributing to toxicity) and in the aerobic population of tumour cells (contributing to therapeutic response). Understanding the enzymology of EO9 metabolism could be useful in the future clinical development of the drug, as well in the development of analogues of EO9 and related indoloquinone bioreductive agents, which continues to be an area of significant activity [8,28].

2. Materials and methods

2.1. Materials

EO9 was a generous gift from the EORTC New Drugs Development Office, Amsterdam. Rat NADPH: cytochrome P450 reductase was kindly supplied by Professor C. R. Wolf, Dundee, Scotland and plasmid pBR322 DNA was obtained from Boehringer Manheim UK Ltd., Lewis, East Sussex, UK.

2.2. Electron spin resonance spectroscopy

Electron spin resonance spectroscopy was carried out using a Varian E109 Century X-band (9.3 GHz) spectrometer. ESR parameters were in general: scan range $\pm$ 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 cps and microwave frequency 9.51 G. All reactions were carried out at room temperature.

Standard reaction constituents included: NADPH: cytochrome P450 reductase (1.3 μg), EO9 (800 μM dissolved in DMSO and diluted in buffer), and NADPH (1 mM) made up to a total volume of 400 μL Tris-Cl buffer (50 mM) pH 7.4. Concentrations given are the final concentrations in the reaction. Spin trapping studies were carried out using *N*, *N* dimethyl pyrolline *N*-oxide (DMPO, 100 mM) in the presence and absence of superoxide dismutase (400 U) and catalase (200 U). Reactions were initiated by addition of cofactor to the reaction mixture and rapidly mixing. Constituents were then transferred directly to the flat cell for analysis by ESR.

2.3. DNA strand break assays

The ability of EO9 to induce strand breaks in DNA following activation by NADPH: cytochrome P450 reductase was determined using a cell-free agarose gel method similar to that described by Walton and co-workers [15]. Supercoiled pBR 322 (1 μg) was incubated at 37° for 30 min in the presence of EO9 (100 μM), purified rat NADPH: cytochrome P450 reductase (0.1 μg), and NADPH (2 mM) made up to a total volume of 60 μL in 100 mM sodium phosphate buffer pH 7.4. Control incubations involved omission of each reaction component. The effect of varying

the concentrations of NADPH: cytochrome P450 reductase (0–1 μg), EO9 (1–400 μM), and incubation time (0–90 min) was also investigated. The reaction was terminated by addition of 20-μL aliquots of reaction mixture to 5 μL of stop buffer (SDS [0.5%], EDTA [5 mM], glycerol [60% v/v], and bromophenol blue [0.001%]). Samples were then electrophoresed on a 1% agarose gel and bands visualised by staining with ethidium bromide. DNA damage was quantified using a laser densitometer. In order to account for the differential binding capacity of ethidium bromide with supercoiled plasmid compared with open circular and linear forms, a correction factor of 1.22 was applied to the supercoiled form [26].

3. Results

3.1. Reduction of EO9 and free radical formation catalysed by NADPH: cytochrome P450 reductase

Reproducible ESR spectra were obtained following aerobic incubation of EO9 with NADPH and NADPH: cytochrome P450 reductase. Fig. 2a shows a typical 7-line hyperfine spectrum superimposed over a broad single spectrum (see Fig. 2a). This free radical, attributable to the EO9 drug radical, persisted for 30 min and was dependent on the presence of NADPH, EO9, and enzyme (results not shown). ESR spectrometry in combination with DMPO, a spin trap for short-lived radicals [29], was used to investigate the formation of reactive oxygen as a result of EO9 reduction by NADPH: cytochrome P450 reductase under aerobic conditions. Fig. 2b shows that reduction of EO9 by NADPH: cytochrome P450 reductase in the presence of 0.01% DMSO (as a drug diluent) resulted in a spectrum with hyperfine splitting 1:1:1:1:1:1 (A_N = 16.0G, A_H = 22.0G), superimposed above the 7-line spectrum of the EO9 radical. This is consistent with formation of DMPO-CH_3, the presence of which indicates that EO9 stimulated hydroxyl radical attack on DMSO to generate the methyl radical that is subsequently trapped by DMPO. Addition of both superoxide dismutase and catalase (Fig. 2c), scavengers of superoxide anions and hydrogen peroxide respectively, resulted in no reactive oxygen-derived spin trap over the same time scale. The DMPO-CH_3 six-line spectrum diminished during the scan time of the 5-min incubation, as indicated by the asymmetry of the spectrum observed. After 10 min incubation, the spin-trapped spectrum was no longer observed (Fig. 2d), although a 7-line spectrum persisted that was identical to that observed for EO9 incubations in the absence of DMPO (compare Fig. 2, b and d).

3.2. DNA strand break induction

Following aerobic incubation of plasmid DNA with EO9, NADPH: cytochrome P450 reductase, and NADPH cofactor, DNA strand breaks were detected reproducibly by agarose gel electrophoresis. Typical results are shown in Figs. 3, 4, and 5. Plasmid DNA in the supercoiled conformation (form I) is converted to open circular (form II) DNA following single-strand break induction. As a result of their differing conformations, these migrate differentially in the gel. The extent of DNA strand breaks was found to increase with increasing drug (Fig. 3) or enzyme concentration (Fig. 4) as well as with increasing incubation time up to 90 min (Fig. 5). Control incubations where either drug, enzyme, or cofactor were omitted showed little or no evidence of DNA damage (Figs. 3–5).

4. Discussion

We and others (see Introduction) have provided considerable evidence to suggest that the obligate two-electron reducing enzyme DT-diaphorase is able to catalyse reduction of EO9 and play a major role in the mechanism of the drug cytotoxicity under aerobic conditions. However, as also outlined in the Introduction, the results of these studies indicated that other enzymes are also likely to be involved, particularly under hypoxic conditions [22]. In the present studies, we show that purified rat NADPH: cytochrome P450 reductase was able to catalyse the reduction of EO9 to generate free radical and DNA-damaging species. Such aerobic metabolism may contribute to the cytotoxicity of EO9 in well-oxygenated normal tissues and also to the antitumour activity of EO9 in the well-vascularized population. In addition, the drug radical observed following cytochrome P450 metabolism of EO9 could contribute to the cytotoxicity of EO9 in the hypoxic tumour population.

ESR spectrometry was used to show that purified NADPH: cytochrome P450 reductase can reduce EO9 to generate free radical species. The ESR spectrum observed for EO9 free radical is likely to be an oxygen-centered semiquinone species with nitrogen involvement. Semiquinones often generate singlet spectra as a result of line broadening due to their slow rotation on the ESR time scale in biological media. The multiple-line hyperfine spectrum superimposed on a broad singlet observed for EO9 semiquinone suggests spin coupling of the free radical electron to the aziridinyl nitrogen and associated protons. An identical spectrum was previously observed for DT-diaphorase-mediated formation of EO9 free radicals [11]. The DT-diaphorase-mediated EO9 free radical was likely to be a consequence of EO9 obligate 2-electron reduction and concomitant quinone/hydroquinone comproportionation to form the semiquinone observed. NADPH: cytochrome P450 reductase is a one-electron reductase and hence the semiquinone observed is likely to be directly generated, although comproportionation probably accounts for the apparent longevity of the free radical species observed. Previously, it was shown that EO9 semiquinones are oxygen-sensitive [30] and will generate reactive oxygen [11]. The present study also shows that when EO9 is reduced by NADPH:

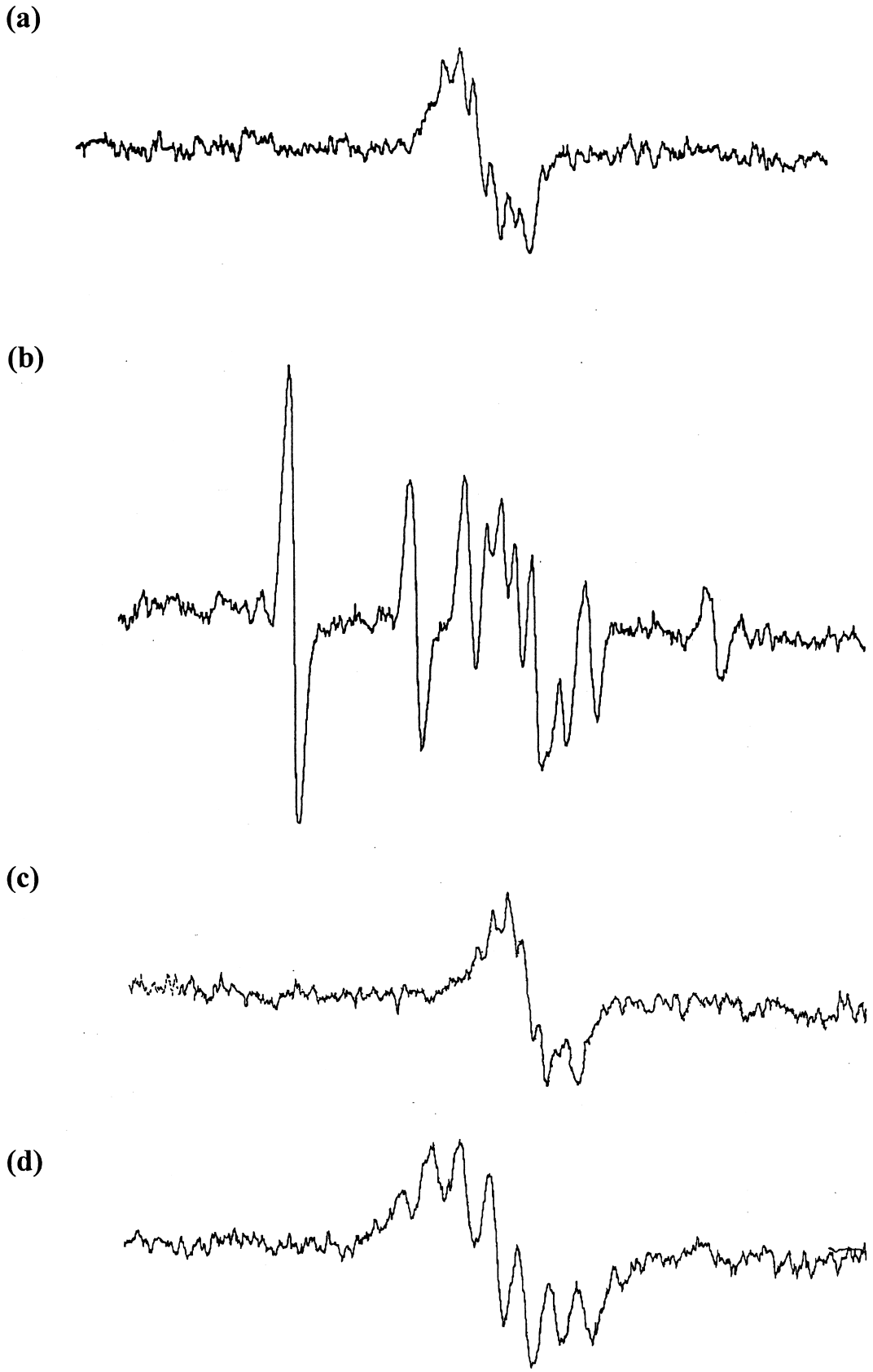

FIG. 2. ESR spectra obtained during the reduction of EO9 catalysed by NADPH: cytochrome P450 reductase. Unless otherwise stated, standard reaction conditions included NADPH: cytochrome P450 reductase (1.3 μg), EO9 (800 μM), and NADPH (1 mM) in a total volume of 400 μL. (a) Complete system recorded 4 min after initiation of reaction; (b) complete system in the presence of spin trap DMPO (100 mM) recorded at 5 min.; (c) complete system plus superoxide dismutase (400 U) and catalase (200 U) recorded at 8 min; (d) complete system in the presence of spin trap DMPO (100 mM) recorded at 10 min.

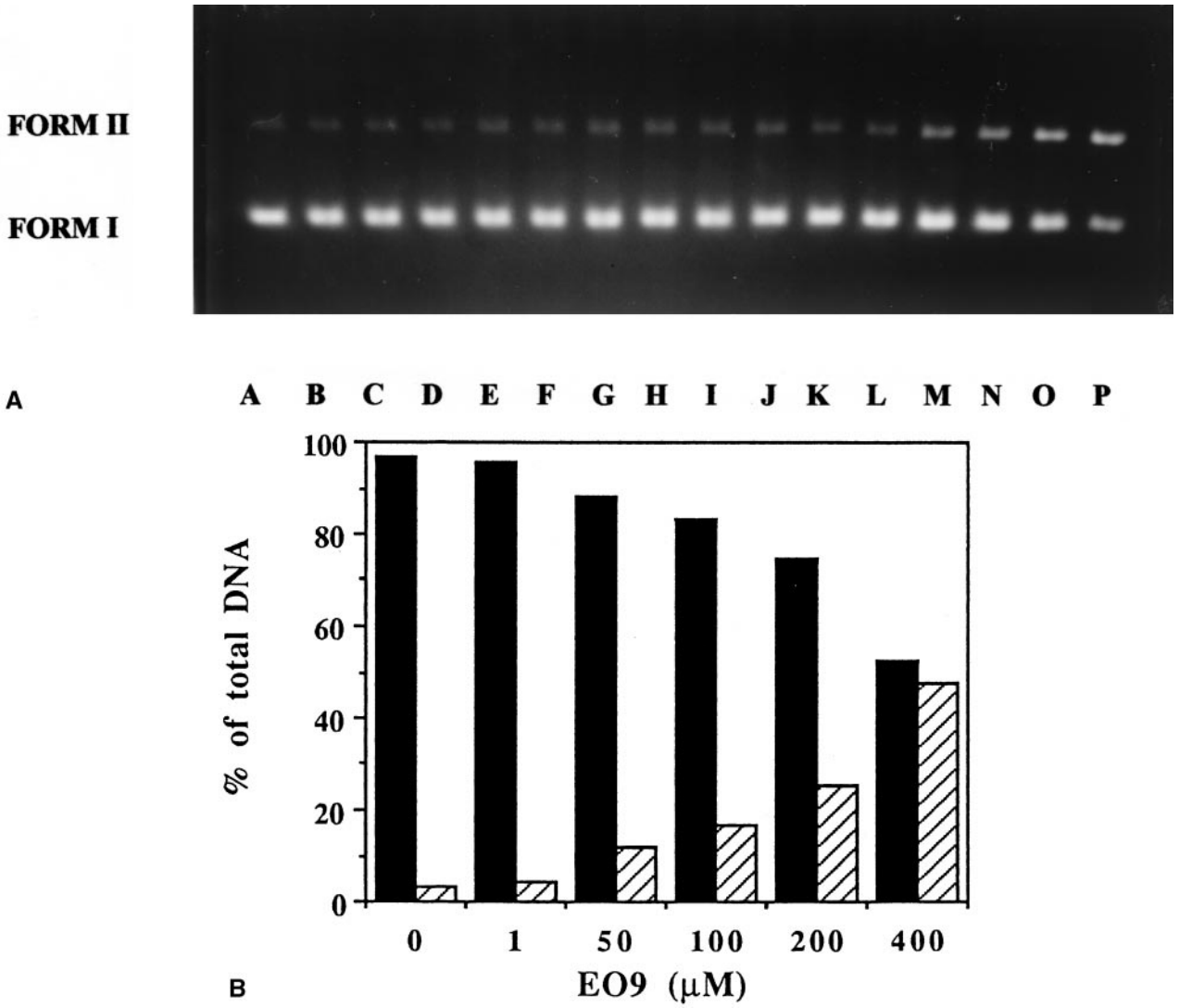

Fig. 3. The effect of altering drug concentration on EO9 induced plasmid strand break frequency following activation by NADPH: cytochrome P450 reductase in the presence of cofactor. Unless otherwise stated, incubation mixtures contained: pBR 322 (1.0 μg), NADPH: cytochrome P450 reductase (0.1 μg), and NADPH (2 mM) with varying concentrations of EO9 (0–400 μM). Solid bars represent % supercoiled DNA and hatched bars % open circular DNA. Panel A: Agarose gel. Lanes: (A) plasmid-alone control; (B) plasmid plus NADPH: cytochrome P450 reductase only; (C) plasmid plus NADPH only; (D) control omitting EO9; (E) control omitting EO9 but including drug vehicle; (F) as E but omitting NADPH; (G–K) increasing EO9 concentrations (1, 50, 100, 200, and 400 μM EO9) omitting NADPH: (L–P) as (G–K) but including NADPH. Panel B: Quantification of DNA damage by densitometry. Data correspond to lanes E and L–P in the upper panel.

cytochrome P450 reductase in the presence of DMSO, the formation of a spin-trapped methyl radical was observed. The radical was dependent on aerobic conditions and was abolished in the presence of reactive oxygen scavenging enzymes. This demonstrates that EO9 is directly generating reactive oxygen, which in turn is responsible for radical attack on DMSO. The absence of a spin-trapped methyl radical under anaerobic conditions indicates that EO9 does not directly react with hydrogen atom donors. EO9 appears to be generating anaerobic conditions in about 10 min, presumably by autocatalytic oxidation of EO9 semiquinone which completely consumes the dioxygen available in the sealed flat cell employed in these ESR spectrometry experiments. This leads to loss of the spin trap ESR signal but not the semiquinone spectrum which persists under anaerobic conditions. In support of this, the semiquinone of EO9 has been shown to react rapidly with oxygen with a rate constant of $1.31 +/- 0.15 \times 10^8\ M^{-1}\ s^{-1}$ [30].

The plasmid DNA experiments that we carried out show that the reduction of EO9 by purified NADPH: cytochrome P450 reductase results in the production of single-strand breaks in DNA, as also seen with DT-diaphorase [15]. The presence of the aziridine ring and hydroxyl-leaving groups confer the potential of EO9 to act as a bioreductive alkylating agent and/or cross-linking agent. However, the precise contribution of these moieties remains unclear. As is normally the case, the drug concentrations used in the ESR spectroscopy, and DNA strandbreak experiments, are higher

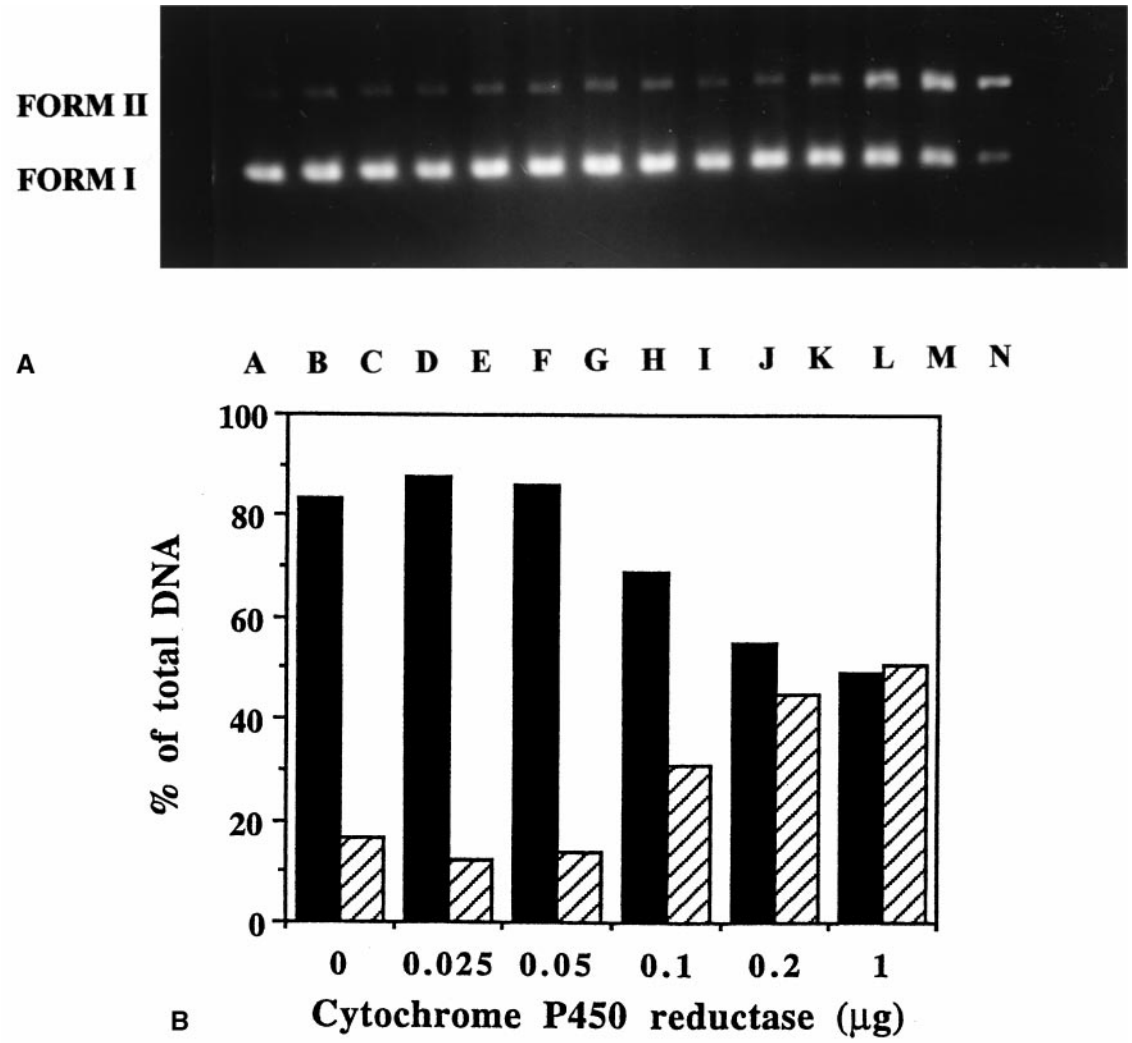

Fig. 4. The effect of altering enzyme concentration on EO9 induced plasmid strand break frequency following activation by NADPH: cytochrome P450 reductase in the presence of cofactor. Unless otherwise stated, standard reaction conditions contained pBR 322 (1.0 μg), EO9 (100 μM), NADPH (2 mM) with varying concentrations of NADPH: cytochrome P450 reductase (0–1 μg). Solid bars represent % supercoiled DNA and hatched bars % open circular DNA. Panel A: Agarose gel. Lanes: (A) plasmid control; (B) vehicle control; (C–H) no NADPH control for varying enzyme concentrations (0, 0.025, 0.5, 0.1, 0.2, 1 μg); (I–N) increasing enzyme concentration (0, 0.025, 0.5, 0.1, 0.2, 1 μg) including NADPH. Panel B: Quantification of DNA damage by densitometry. Data correspond to lanes I–N in the upper panel.

than those causing cytotoxicity. However, alkaline elution experiments showed EO9 to be capable of causing both DNA strand breaks and interstrand cross-links in the DNA of intact tumour cells at cytotoxic concentrations [13]. The plasmid assay used here is unlikely to detect any cross-links in the presence of strand breaks.

Cytotoxicity arising from EO9 treatment is likely to be determined by: 1) the reactive drug species or metabolites produced; 2) the resulting DNA damage that occurs; and 3) the particular cell's response to that damage. The present paper deals with aspects of points 1) and 2). Further studies are required to elucidate factors downstream of DNA damage that may influence cellular response to EO9.

Previous studies measuring the *in vitro* cytotoxicity of EO9 in cells with low and high DT-diaphorase activities [17–23] have suggested that this enzyme plays a major role in the cytotoxicity of EO9 under aerobic conditions. On the other hand, for cell lines with little or no DT-diaphorase activity under aerobic conditions, or in all cell lines under hypoxic conditions, the results suggested that other enzymes might be predominant. The present studies show directly that NADPH: cytochrome P450 reductase is also capable of mediating EO9 bioreductive activation to free radical and DNA-damaging species. Production of free radicals was obtained with similar amounts of NADPH: cytochrome P450 reductase to DT-diaphorase [11]. These data support the view that cytochrome P450 reductase is likely to contribute to the antitumour cytotoxicity of EO9. Additional support at the cellular level is provided by results of gene transfer studies [28]. Our results would suggest that the production of oxygen radicals by cytochrome P450 reductase may contribute unwanted toxic effects in well-oxygen-

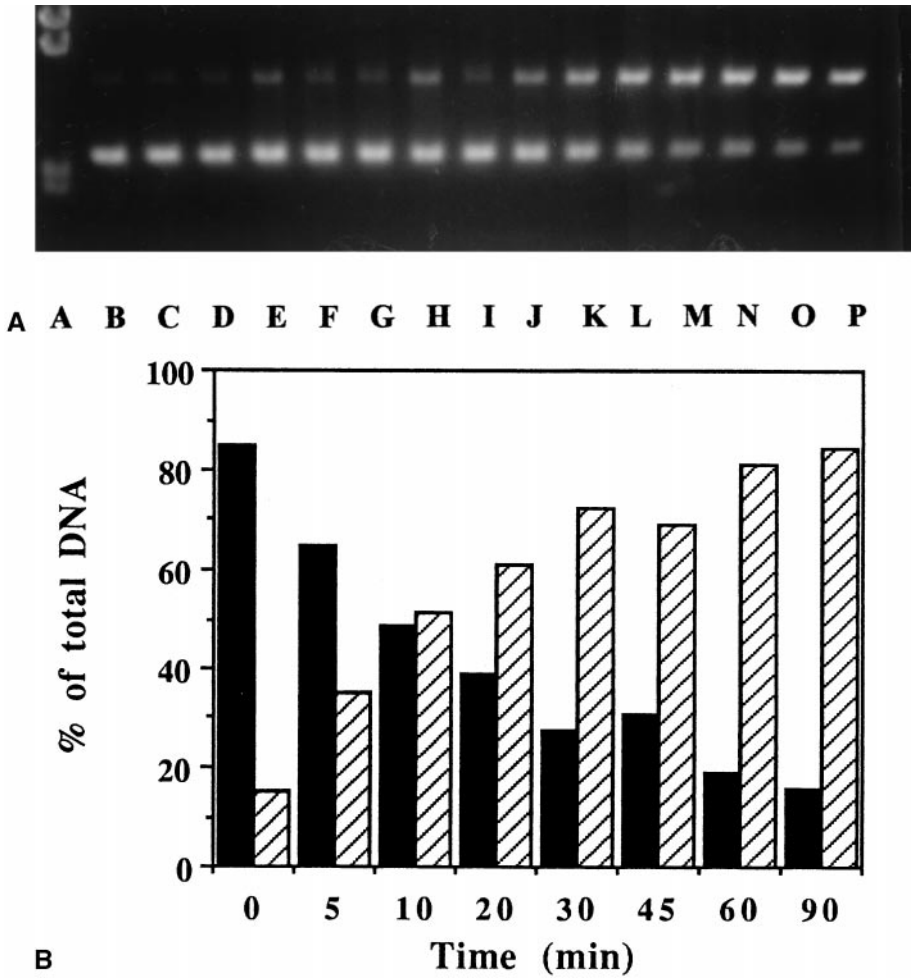

Fig. 5. The effect of altering incubation time on EO9 induced plasmid strand break frequency following activation by NADPH: cytochrome P450 reductase. Unless otherwise stated, standard reaction conditions contained pBR 322 (1.0 μg), EO9 (100 μM), NADPH (2 mM) and NADPH: cytochrome P450 reductase (0.1 μg). Solid bars represent % supercoiled DNA and hatched bars % open circular DNA. Panel A: Agarose gel. Lanes: (A) λ hind III markers; (B) plasmid alone; (C) plasmid and EO9; (D) plasmid and NADPH: cytochrome P450 reductase; (E) plasmid and NADPH; (F) no NADPH control; (G) no enzyme control; (H) no EO9 control; (I–P) increasing incubation time (0, 5, 10, 20, 30, 45, 60, 90 min). Panel B: Quantification of DNA damage by densitometry. Data correspond to lanes I–P in the upper panel.

ated normal tissues and also to beneficial effects in the more aerobic tumour cell population. In hypoxic tumour cells that are relatively low in DT-diaphorase, reduction by cytochrome P450 reductase is likely to contribute in a more significant way, as a result of activated drug species leading directly to DNA strand breaks and cross-links.

As discussed in detail previously [8,9], there is a complex relationship between chemosensitivity, enzymology (particularly high and low DT-diaphorase levels in relation to cytochrome P450 reductase), and oxygenation status. EO9 can clearly be activated by both DT-diaphorase and cytochrome P450 reductase. Our results may be useful in interpreting the results of the ongoing clinical studies with EO9. In addition, based on the present work and other studies, we suggest that it would be useful to aim to design analogues that are activated predominantly by either DT-diaphorase or cytochrome P450 reductase and to explore their use in the treatment of patients with tumours that have high levels of either DT-diaphorase or hypoxia, respectively.

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