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# Pharmacological and Biochemical Determinants of the Antitumour Activity of the Indoloquinone EO9

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**ABSTRACT.** EO9 is a novel bioreductive drug which has recently undergone extensive clinical evaluation. Its mechanism of action remains to be clearly defined. Antitumour activity of EO9 has been determined in 2 human colon cancer xenografts (HT-29 and BE) and 2 murine colon adenocarcinomas (MAC 16 and 26) after intratumoural injection of 250 μg of drug. Levels of the major bioreductive enzymes (DT-diaphorase, cytochrome P-450 reductase and cytochrome b5 reductase) were measured in tumours using cytochrome c reduction and menadione as the intermediate electron acceptor. There was no correlation between chemosensitivity (T/C: HT-29, 15%; BE, 27%; MAC 16, 33% and MAC 26, 60%) and enzyme activity ($r^2 = 0.47$ for DT-diaphorase, $r^2 = 0.1$ for cytochrome P-450 reductase and $r^2 = 0.52$ for cytochrome b5 reductase). Drug metabolism was followed in vitro using tumour homogenates incubated under aerobic and anaerobic conditions. Four metabolites were identified by HPLC and characterised by UV-visible spectroscopy. With the exception of the hydrolysis product EO5A, all other metabolites appeared to be drug adducts. No correlation was observed between the kinetics of metabolite formation and antitumour activity. A good correlation ($r^2 = 0.86$) was found with the rate of disappearance of parent drug and antitumour activity. These data show that the overall capacity of a tumour to metabolise EO9 is the most important determinant of antitumour activity rather than the expression of the major bioreductive enzymes and that the parent drug rather than a metabolite leads to the active form of the drug. BIOCHEM PHARMACOL **55**;3:253–260, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** Indoloquinone EO9; antitumour activity; xenografts; bioreductive enzymes; DT-diaphorase; drug metabolites

Indoloquinone EO9 (5-(1-aziridinyl)-3-(hydroxymethyl)-2 [(E)-3-hydroxyprop-1-enyl]-1-methyl-1H-indole-4,7-dione; NSC-382459; E-85/053)‡ was originally synthesised as an analogue of the clinically active, naturally occurring, bioreductive drug mitomycin C (MMC) (for structures see Fig. 1) [1]. The mechanism of action of EO9 has generally been assumed to be similar to MMC, involving enzyme catalyzed one or two electron reduction followed by the generation of either drug derived DNA alkylating moieties or DNA damaging reactive oxygen species (ROS) [2]. Nonetheless, several important details remain to be clarified in this reaction scheme such as the enzymology of metabolic activation in tumour tissue, the identity of the reactive intermediates formed after EO9 quinone reduction and the nature of the DNA covalent modifications produced by drug activation [3, 4]. Fundamental mechanistic differences between EO9 and MMC are beginning to emerge [5].

EO9 displays potent *in vitro* activity in the NCI disease orientated panel of human tumour cell lines [6] and broad spectrum *in vivo* antitumour activity against both refractory murine colon tumours [7] and a number of human xenografts including non-small cell lung (NSCLC), ovarian and breast cancer [6]. However, it is inactive against leukaemic cells both *in vitro* and *in vivo* and produces an unusual pattern of toxicity where a lack of myelotoxicity was recorded in rats, mice [6] and man [8]. In contrast to MMC, the drug can display markedly enhanced cytotoxicity to hypoxic cells by up to a factor of 49-fold [9, 10]. EO9 has recently completed phase I and II clinical evaluation [8, 11].

The aim of the present study has been to attempt to identify the major biochemical and pharmacological determinants of the *in vivo* antitumour activity of EO9. These investigations included studying the role of bioreductive enzymes such as the obligate two electron reductase DT-diaphorase (NAD(P)H: Quinone Oxidoreductase; E.C. 1.6.99.2; NQO1) and drug metabolism, the latter being important in view of the fact that EO9 has a very short half

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‡ *Abbreviations:* EO9, 5-(1-aziridinyl)-3-(hydroxymethyl)-2 [(E)-3-hydroxyprop-1-enyl]-1-methyl-1H-indole-4,7-dione; MeDZQ, 2,5-dimethyl-3,6-diaziridinyl 1,4-benzoquinone; MMC, mitomycin C; NCI, National Cancer Institute of America; NSCLC, non-small cell lung cancer; ROS, reactive oxygen species; T/C, mean drug treated tumour volume/mean control tumour volume.

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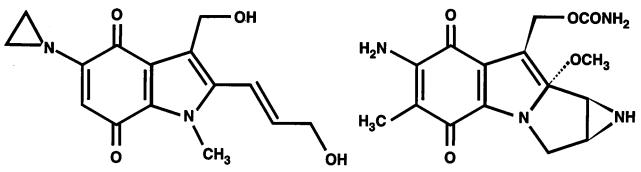


**FIG. 1. Molecular structures of EO9 and mitomycin C.**

life [8, 12] and is extensively metabolised [13]. Finally, in conjunction with a recently published study on the properties of EO9 reactive intermediates [5], a further aim was to seek to define the active form(s) of the drug in tumour tissue.

## MATERIALS AND METHODS

### *Materials*

All EO9 used throughout was kindly supplied by Dr. Hans Hendriks of the New Drug Development Office (NDDO) of the European Organization for Research and Treatment of Cancer (EORTC) under the auspices of Mr. John Kelly and Dr. Hiroshi Furukawa of Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan.

### *Antitumour Activity of EO9*

The antitumour activity of EO9 was determined in a panel of 4 solid tumours: two human colon cancer xenografts (HT-29 and BE) and two murine adenocarcinomas of the colon (MAC 16 and MAC 26).

The MAC 16 and 26 tumours and breeding pairs of NMRI mice were kindly supplied by Professor J. A. Double and Dr. M. C. Bibby, Clinical Oncology Unit, University of Bradford, U.K. NMRI mice were maintained under standard laboratory conditions of heating and lighting, fed a standard mouse diet (RM3(E): SDS, Witham, Essex, U.K.) and given access to water *ad lib*. The MAC tumours were maintained by serial subcutaneous passage of 1–3 mg of viable tumour via a trochar needle to animals weighing between 25 and 30 g. When tumours reached 0.02–0.1 $cm^3$ (after 2–3 weeks), animals were either randomised into groups of 5–8 for antitumour studies or eventually sacrificed as a source of tumour material. All tumours were stored at −80° prior to use.

HT-29 and BE xenografts were established in house in nude mice (nu/nu) from their respective cell lines. The BE cell line was kindly supplied by Dr. Neil W. Gibson, Pfizer Inc., Groton, U.S.A. Nude mice were housed in special isolators (Moredun Isolators, Edinburgh, U.K.), fed RM3(E) mouse diet treated by irradiation and given free access to water. Xenografts were maintained by serial subcutaneous passage of 1–3 mg of viable tumour via a trochar needle to animals weighing between 20 and 25 g. When xenografts reached 0.02–0.1 $cm^3$ (after 4 weeks for HT-29 and 6 weeks for BE) animals were randomised in groups of 5–8 for antitumour studies or eventually sacrificed for collection of tumour material as above. Different passages of all four tumours were subjected to histological analysis to confirm their pathology.

All animals were dosed with 250 μg of EO9 made up fresh in 200 μL PBS/0.5% Tween 80 immediately prior to direct intratumoural (i.t.) injection. Control animals received an i.t. injection of drug vehicle only. Tumour volumes were determined by caliper measurement and calculated using the formula: $0.5 \times \text{length} \times \text{width}^2$. The antitumour activity of EO9 was expressed as a percentage of the mean drug treated tumour volume/mean control tumour volume (T/C) on day 14 for the MAC tumours and day 21 for the xenografts after approximately 3–4 tumour doublings.

### *Tumour Quinone Reductase Activity*

Isolation of subcellular fractions (microsomes and cytosols) from all four tumours was carried out using a standard differential ultracentrifugation method [14]. The protein content of subcellular fractions was determined by the Biuret method [15] standardised with BSA (Cohn Fraction V). After fixation, microsomal fractions were examined using a Jeol 100CXII transmission electron microscope to confirm the presence of microsomal vesicles and the absence of mitochondria [16].

Quinone reductase activity in subcellular fractions was measured spectrophotometrically by following the reduction of cytochrome c at 550 nm (using the molar extinction coefficient, $e = 21.1 \times 10^3$ M/cm) with menadione as the intermediate electron acceptor and either NADH or NADPH as cofactor [14]. DT-diaphorase activity was calculated from the dicoumarol inhibitable fraction in cytosolic and microsomal incubations utilizing both NADH and NADPH. Cytochrome P-450 reductase activity was calculated from the non-dicoumarol inhibitable fraction in microsomes utilizing NADPH and cytochrome b5 reductase as the non-dicoumarol inhibitable fraction in microsomes utilizing NADH. A combined pool of xanthine dehydrogenase/oxidase and carbonyl reductase activity was calculated as the non-dicoumarol inhibitable fraction in cytosols. Quinone reductase assays were performed in triplicate.

### In Vitro *Metabolism of EO9 by Tumour Homogenates*

Whole tumours (0.5–1.5 g) were allowed to partially thaw, then washed in saline prior to homogenisation in 0.25 M sucrose, 5 mM Tris-buffer, 0.5 mM EDTA, pH 7.4 (33% w/v). Drug incubations were carried out at 37° in a total volume of 1 mL which consisted of 0.9 mL of tumour homogenate and 0.1 mL of 1 mg/mL EO9 made up in 0.1 M sodium phosphate buffer, pH 7.4 (final concentration of EO9 was 100 μg/mL). The amount of each tumour homogenate added to drug incubations was adjusted to yield a linear rate of EO9 disappearance over the incubation period studied. At 45 min intervals, 100 μL samples were with-

**TABLE 1. Tumour quinone reductase activity* (menadione as substrate) in isolated cytosolic and microsomal subcellular fractions**

| | Cytosol | | | Microsomes | | | |
|---|---|---|---|---|---|---|---|
| | DT-diaphorase# | | | DT-diaphorase | | Cytochrome P-450 | Cytochrome |
| Tumour | NADH | NADPH | Others§ | NADH | NADPH | reductase | b5 reductase |
| MAC 16 | 477 ± 19.2 | 328 ± 20.5 | 379 ± 37.3 | 5.1 ± 0.9 | 9.7 ± 3.1 | 17.2 ± 2.1 | 18.4 ± 0.9 |
| MAC 26 | 19.3 ± 5.9 | 15.7 ± 3.6 | 49.8 ± 3.7 | ND | ND | 14.8 ± 1.4 | 16.9 ± 4.9 |
| BE | ND | ND | 16.2 ± 1.5 | ND | ND | 15.6 ± 1.6 | 29.6 ± 3.1 |
| HT-29 | 2776 ± 205 | 2103 ± 371 | 99.5 ± 36 | 98.0 ± 7.1 | 50.6 ± 10 | 115 ± 9.4 | 100 ± 14.1 |
| Correlation to | $r^2 = 0.47$ | 0.46 | 0.01 | $r^2 = 0.44$ | 0.48 | 0.09 | 0.52 |
| antitumour activity | $P = 0.31$ | 0.31 | 0.89 | $P = 0.33$ | 0.31 | 0.68 | 0.27 |

* All enzyme activities are expressed as nmol cytochrome c reduced/min/mg protein using the extinction coefficient, $e = 21.1 \times 10^3$ M/cm. Each value represents the mean ± SE from three separate determinations. ND = no detectable activity.

# DT-diaphorase activity was measured using both NADH or NADPH as co-factors.

§ Others refers to the non-dicoumarol inhibitable fraction of quinone reductase activity in cytosols.

drawn, mixed with 100 μL of methanol and centrifuged for 2 min at 15,000 *g* in a microcentrifuge to remove protein. Thereafter, 20 μL was analyzed by HPLC to determine EO9 and its metabolites (see below). Control incubations included all the above components minus EO9. In aerobic incubations, test tubes (50 mL, tapered) were open to the atmosphere. In hypoxic incubations, tumour homogenates were sparged with helium for 5 min prior to drug addition. An hypoxic environment was maintained throughout the incubation with a flow of helium gas delivered to test tubes via a rubber stopper and glass pipette assembly.

### *Drug Analysis Techniques*

EO9 and its aziridine ring opened hydrolysis EO5A were determined in drug incubations by HPLC using a Hewlett Packard 1090 liquid chromatograph with a diode array detector (set to monitor chromatograms at 280 nm and UV-visible spectra from 200–600 nm). The stationary phase was LiChrosorb RP-18 (7 μm particle size), which consisted of a 250 mm × 4 mm diameter analytical column and a 10 mm long × 4 mm diameter pre-column (5 μm particle size, Merck Ltd., Poole, Dorset, UK). The mobile phase consisted of 10 mM sodium phosphate buffer, pH 7.5 and methanol, 74:26. Elution was isocratic at a flow rate of 1 mL/min and the column was maintained at 40°C.

## RESULTS

### *Relationship Between EO9 Antitumour Activity and Bioreductive Enzyme Activity*

EO9 produced a significant effect on the growth of all four tumours. T/C values were 60% for MAC 26 and 33% for MAC 16. Comparable activity has been obtained in these two tumours when EO9 was administered systemically at a dose of 3 mg/kg i.p. (T/C 66% in MAC 26 and 45% in MAC 16) [7]. A T/C value of 27% was recorded with BE and the HT-29 xenograft appeared to be the most responsive of all four tumours with a T/C of 15%. Interestingly, HT-29 has been reported to be resistant to EO9 when the drug was administered at its maximum tolerated dose of 6 mg/kg i.v. on four consecutive days [17]. The chemosensitivity of the BE xenograft to EO9 has, to the best of our knowledge, not been described previously.

Quinone reductase activity (menadione as substrate) of the major bioreductive enzymes are presented in Table 1. DT-diaphorase levels were 25-fold higher in MAC 16 compared to MAC 26, in keeping with the 16-fold elevation already described [7]. The HT-29 xenograft expressed the highest level of DT-diaphorase (2776 nmol/min/mg), very similar to that published for the HT-29 cell line (2760 nmol/min/mg) [18] but much higher than that published for the HT-29 xenograft when 2,6-dichlorphenol-indophenol (DCPIP) was utilised as a substrate (359 nmol/min/mg) [17]. However, the activity of DT-diaphorase in HT-29 cells is highly proliferation dependent [19], and this factor together with different experimental techniques may account for the above difference. The BE xenograft was confirmed to lack DT-diaphorase activity which is due to a point mutation in the NQO1 gene [20, 21].

Low levels of cytochrome P-450 reductase activity were confirmed in all tumours with the exception of HT-29 (Table 1) [7, 22]. The activity of cytochrome b5 reductase was similar to that of cytochrome P-450 reductase but much lower than cytosolic DT-diaphorase in the case of MAC 16 and the HT-29 xenograft (Table 1) mirroring a trend previously observed in cell lines and human tumour biopsies [23, 24]. While cytochrome P-450 reductase is capable of accepting EO9 as a substrate for reduction [25], little is known about the ability of cytochrome b5 reductase to metabolise the drug, although this enzyme can perform metabolic activation of MMC [26].

There was no correlation between the activity of any one of three major bioreductive enzymes and antitumour activity to EO9: DT-diaphorase, $r^2 = 0.47$ ($P = 0.31$); cytochrome P-450 reductase, $r^2 = 0.09$ ($P = 0.68$) and cytochrome b5 reductase, $r^2 = 0.52$ ($P = 0.27$). Species differences exist in the ability of mouse and human DT-diaphorase to metabolise EO9 and this may partly account for the poor correlation [27]. Nevertheless, the difference in activity between the two enzymes is small (3-fold difference

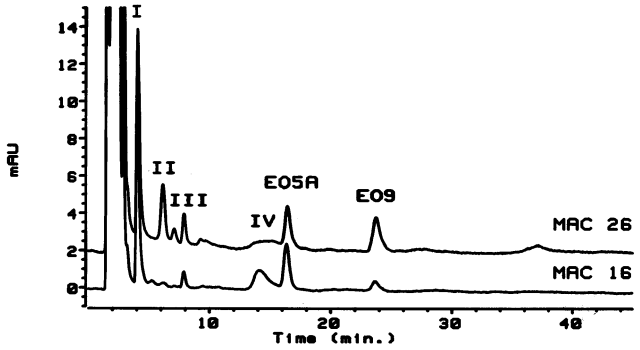


**FIG. 2. Aerobic 45 min incubation of EO9 (100 μg/mL) with MAC 16 and 26 tumour homogenates followed by HPLC. In MAC 16 peaks are: EO9 (0.4 μg/mL); EO5A (2.1 μg/mL); I (6.1 μg/mL) and III (0.2 μg/mL). In MAC 26 peaks are: EO9 (1.8 μg/mL); EO5A (2.0 μg/mL); I (5.6 μg/mL); II (1.8 μg/mL) and III (0.8 μg/mL). Peaks I–III are expressed as EO9 equivalents.**

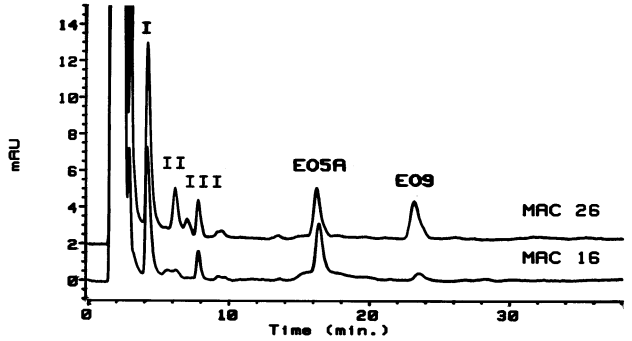


**FIG. 4. Hypoxic 45 min incubation of EO9 (100 μg/mL) with MAC 16 and 26 tumour homogenates followed by HPLC. In MAC 16 peaks are: EO9 (0.5 μg/mL); EO5A (2.7 μg/mL); I (5.8 μg/mL) and III (1.4 μg/mL). In MAC 26 peaks are: EO9 (1.7 μg/mL); EO5A (2.6 μg/mL); I (7.1 μg/mL); II (1.9 μg/mL) and III (1.7 μg/mL). Peaks I–III are expressed as EO9 equivalents.**

in $K_m$; no difference in $V_{max}$) [27] and would not manifest itself at the saturating substrate concentration used in the present study (100 μg/mL, 347 μM). Less of a correlation to antitumour activity was observed with the non-dicoumarol inhibitable fraction of quinone reductase activity in tumour cytosols, which includes xanthine oxidase/ dehydrogenase and carbonyl reductase ($r^2 = 0.01$, $P = 0.89$). HT-29 and BE cell lines are reported not to contain xanthine oxidase [22] and only a limited role for xanthine dehydrogenase in EO9 bioreduction is suggested [24]. The involvement of carbonyl reductase in the metabolism of EO9 remains unclear [4].

### In Vitro *Formation of EO9 Metabolites by Tumour Homogenates and Relationship to Antitumour Activity*

In addition to EO9 and EO5A, three other metabolites (I–III) were detected routinely in drug incubations (see Figs. 2–5). The characteristics of these metabolites are summarised in Table 2 and work in progress is aimed at establishing their chemical structure by HPLC-mass spectrometry. Preliminary evidence from the fragmentation pattern produced from electron impact mass spectra indicated the presence of ions corresponding to EO9/EO5A as well as higher molecular weight species. This is indicative of peaks I–III being adducts of either EO9 or EO5A and is consistent with their chromatographic properties where they exhibited shorter retention times than the parent compounds (Table 2). Based on their spectral properties (see Fig. 6), it is possible to tentatively ascribe a probable identity to the three metabolites. Peak I would appear to be a protein/peptide adduct due to the strong UV-absorption maximum at 280 nm, but is not a glutathione (GSH) conjugate since reaction of EO9 with GSH produced chromatographic peaks with different retention times and absorption spectra from metabolites I–III [16]. Neither was it the product of alkaline catalyzed degradation of EO9 which produced a single product that eluted close to the solvent front with a retention time of 3 min [16]. Peak II would appear to be an adduct of EO5A since opening of the aziridine ring is associated with a spectral shift in absorption

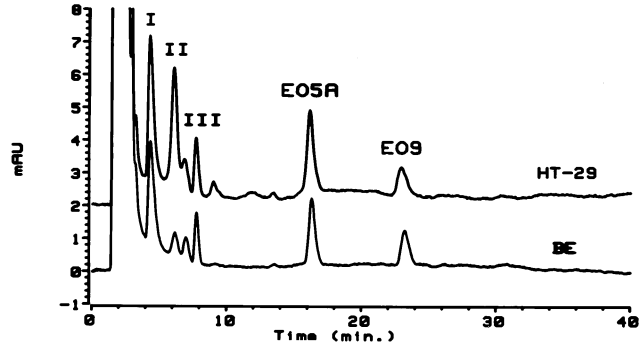


**FIG. 3. Aerobic 45 min incubation of EO9 (100 μg/mL) with BE and HT-29 tumour homogenates followed by HPLC. In BE peaks are: EO9 (1.2 μg/mL); EO5A (1.9 μg/mL); I (3.2 μg/mL); II (0.3 μg/mL) and III (1.0 μg/mL). In HT-29 peaks are: EO9 (1.1 μg/mL); EO5A (2.4 μg/mL); I (4.6 μg/mL); II (3.2 μg/mL) and III (1.1 μg/mL). Peaks I–III are expressed as EO9 equivalents.**

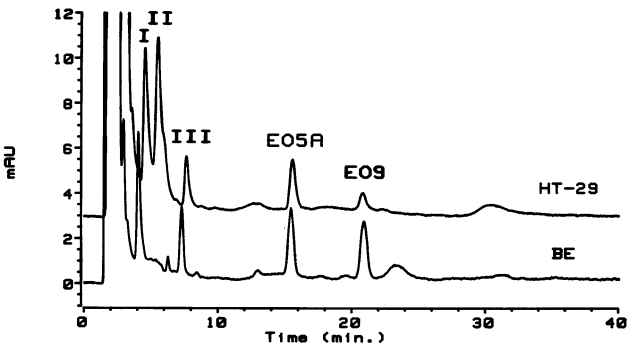


**FIG. 5. Hypoxic 45 min incubation of EO9 (100 μg/mL) with BE and HT-29 tumour homogenates followed by HPLC. In BE peaks are: EO9 (2.4 μg/mL); EO5A (2.4 μg/mL); I (2.8 μg/mL) and III (2.3 μg/mL). In HT-29 peaks are: EO9 (1.1 μg/mL); EO5A (2.2 μg/mL); I (3.7 μg/mL); II (4.1 μg/mL) and III (2.2 μg/mL).**

**TABLE 2. Chromatographic characterization of the major tumour metabolites of EO9**

| Metabolite | Retention time (min) | Absorption maxima (nm) | Effect of hypoxia | Proposed identity |
|---|---|---|---|---|
| EO9 | 21–23 | 268, 313, 505 | | |
| EO5A | 14–16 | 276, 321, 550 | | aziridine ring opened hydrolysis product |
| I | 4.2–4.4 | 280 | nil | drug protein/peptide adduct |
| II | 5.4–6.2 | 276, 321, 550 | increase | aziridinyl adduct |
| III | 7.5–8.0 | 240, 340 | increase | hydroquinone adduct |

maximum from 505 to 550, 268 to 276 and 313 to 321 respectively (Table 2) [13, 28]. Peak III had an unusual spectrum and is different for any EO9 metabolite previously described [13]. The absence of a visible absorption maximum may suggest that this product is related in structure to the fully reduced form of EO9, the hydroquinone. It is unlikely to be the native hydroquinone since this has a half life of only 1.5 sec [5] but probably represents a more stable adduct. Three water soluble metabolites of EO9 were also identified by HPLC after incubation of the drug with purified rat DT-diaphorase [29] and at least 5 water soluble metabolites with spectral properties similar to EO9 have been identified in mouse plasma after i.p. drug administration. To the best of our knowledge, the data of Table 2 represent the first observations of EO9 metabolism by solid tumours.

The main thrust of the present report has been to address the question of possible relationships between *in vitro* metabolite formation in tumour homogenates and antitumour activity. Incubations were carried out over a 90-min period under both aerobic and hypoxic conditions and repeated in triplicate. For the sake of clarity, these data are presented as a series of HPLC chromatograms taken at the 45 min time point (Fig. 2, MAC 16 and MAC 26 in air; Fig. 3, HT-29 and BE in air; Fig. 4, MAC 16 and 26 hypoxic and Fig. 5, HT-29 and BE hypoxic).

Levels of metabolite I produced by all 4 tumours were very similar and did not increase or decrease under hypoxia. Significantly, metabolite I was present at approximately

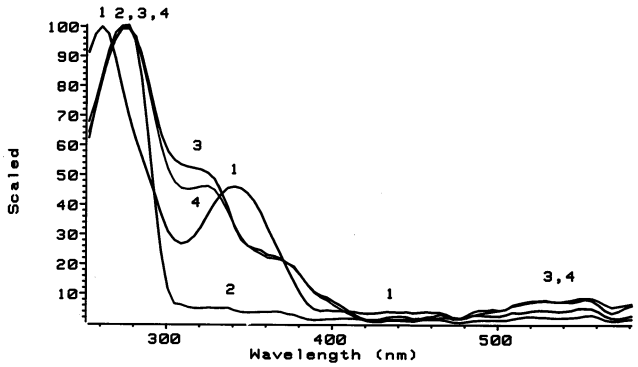


**FIG. 6. UV-visible absorption spectra of metabolite peaks generated during hypoxic incubation of HT-29 tumour homogenates with EO9 (100 μg/mL) (see Fig. 5). Spectrum 1 is metabolite peak III; spectrum 2 is metabolite peak I; spectrum 3 is EO5A and spectrum 4 is metabolite peak II.**

50% its maximum value at time zero and then increased linearly with time. These data strongly suggest that metabolite I is not generated as a consequence of EO9 bioreduction but forms spontaneously through chemical alkylation. Chemical alkylation or modification of naked DNA to form EO9 monoadducts has been demonstrated to occur in the absence of enzyme activation [30]. Production of metabolite II did vary significantly from tumour to tumour whereby high levels were detected with HT-29, intermediate levels with MAC 26 and low levels with BE and MAC 16 (see Figs. 2–5). This pattern of formation did not follow the level of expression of any of the enzymes measured in Table 1, nor did it correlate with antitumour activity. Metabolite II was not present at time zero. Concentration time curves for this product revealed complex kinetics: peak levels were achieved by 45 min and thereafter declined. Several primary metabolites of mitomycin C also exhibit short half lives both *in vitro* [31] and *in vivo* [32] due to the fact that they act as substrates for further metabolism [31].

Production of metabolite III was similar in MAC 26, HT-29 and BE but lower in MAC 16. As with metabolite II, metabolite III was not present at time zero, it did increase under hypoxia, it displayed complex kinetics and did not correlate with either bioreductive enzyme expression or antitumour activity.

There was no correspondence between formation of EO5A and antitumour activity. This is in line with several studies which have shown very poor *in vitro* cytotoxicity for this species [28, 33]. EO5A is not formed by enzyme catalyzed bioreductive metabolism but probably represents a chemical degradation product [29]. However, EO5A can act as a substrate for the generation of subsequent metabolites [13] and this is reflected in its kinetics where peak levels were achieved after 45 min but in most incubations declined thereafter.

### In Vitro *Metabolism of EO9 by Tumour Homogenates and the Relationship to Antitumour Activity*

The overall capacity of each of the four tumour homogenates to catabolize EO9 under aerobic and hypoxic conditions is presented in Table 3. While these rates of reaction did not relate to the expression of a single bioreductive enzyme, there was a much stronger correlation to antitumour activity, particularly under hypoxic conditions ($r^2$ =

**TABLE 3. Relationship between rates of EO9 metabolism by tumour homogenates and *in vivo* antitumour activity**

| Tumour homogenate | EO9 disappearance (nmol/45 min/mg protein) | | Antitumour activity# (T/C %) |
|---|---|---|---|
| | Aerobic | Hypoxic | |
| MAC 16 | 4.1 ± 0.2* | 3.9 ± 1.0 | 33 |
| MAC 26 | 2.4 ± 0.8 | 2.1 ± 0.8 | 60 |
| BE | 5.7 ± 2.9 | 9.0 ± 0.1 | 27 |
| HT-29 | 13.8 ± 3.2 | 7.1 ± 2.2 | 15 |
| Correlation to antitumour activity | $r^2 = 0.82$ $P = 0.17$ | $r^2 = 0.86$ $P = 0.07$ | |

* Each value represents the mean ± SE from three separate experiments.

# Antitumour activity was expressed as a percentage of the mean drug treated tumour volume/mean control tumor volume (T/C) on day 14 for the MAC tumours and day 21 for the xenografts after approximately 3–4 tumour doublings.

0.86, $P = 0.07$ and $r^2 = 0.82$, $P = 0.17$ for aerobic conditions).

The rate of EO9 disappearance in tumour homogenate incubations will include bioreductive metabolic activation to DNA and protein binding species, biotransformation to metabolites, chemical hydrolysis to EO5A and non-enzymatic alkylation of biomolecules. EO9 is relatively stable at neutral pH (pH 7.4) [28] and this was observed in the present study, where the generation of chemical degradation products such as EO5A and metabolite I (see above) accounted for only approximately 8% of the starting material and other metabolites such as II and III accounted for an even smaller percentage [16]. Thus, the rate of EO9 metabolism probably represents principally bioreductive metabolic activation of the drug, and hence the closer correlation to antitumour activity.

## DISCUSSION

The main finding of the present study was that the antitumour efficacy of EO9 in a panel of 4 solid tumours did not correlate with the level of activity of a single major bioreductive enzyme, or the formation of a metabolite, but did correlate with the overall ability of tumour homogenates to metabolise the parent drug. By contrast, a large number of studies have demonstrated in cell lines that the activity of EO9 in air correlates very closely to the level of expression of DT-diaphorase [7, 10, 17, 19, 34, 35]. EO9 is a good substrate for purified preparations of DT-diaphorase and the enzyme converts the drug into both DNA damaging species [36] and DNA crosslinking agents [29, 30]. In addition, when human DT-diaphorase (NQO1 cDNA) was transfected into CHO cells, clones expressing high levels of enzyme were 3-fold more sensitive to the drug, providing strong evidence that DT-diaphorase can bioactivate EO9 into cytotoxic species in whole cells [37]. Nonetheless, a complex role is proposed for DT-diaphorase in metabolic activation of EO9, since overexpression of the enzyme is believed to protect hypoxic cells from cytotoxicity rather than promote it and marked overexpression of the enzyme does not necessarily result in an increase in drug efficacy [19, 24, 37].

In cell lines low in DT-diaphorase, EO9 exhibits a high hypoxic cell kill differential clearly implicating the participation of one electron reductases in metabolic activation [10, 19, 24]. At least two one electron reductases can activate EO9 into DNA reactive species: xanthine oxidase [29] and cytochrome P-450 reductase [25]. Transfection of MDA231 cells with the cDNA for human cytochrome P-450 reductase enhances the cytotoxicity of EO9 10–30 fold under hypoxic conditions [38] and in the A549 non-small cell lung cancer (NSCLC) cell line at least 50% of EO9 bioreduction was attributed to cytochrome b5 reductase [39].

Thus, it is evident that metabolic activation of EO9 in tumour cells is not the domain of one enzyme, several enzymes can participate. The enzyme(s) which will predominate will depend on their comparative level of expression, the prevailing physiological conditions present in the tumour such as oxygen tension and pH, the proliferative state of cells and a number of poorly understood factors including DNA repair, availability of antioxidant defenses and induction of apoptosis [4]. Therefore, it is perhaps less surprising that in a panel of 4 solid tumours with vastly different enzyme contents and markedly different histology and biology, no association was evident between the level of expression of a single major bioreductive enzyme and antitumour activity. A lack of a correlation between DT-diaphorase expression and antitumour activity has recently been reported for EO9 in a large panel of human tumour xenografts [17].

A similar lack of a correlation between *in vitro* and *in vivo* studies has emerged with the related bioreductive drug MeDZQ (2,5-dimethyl-3,6-diaziridinyl 1,4-benzoquinone). MeDZQ is one of the best drug substrates available for recombinant human DT-diaphorase [40] and is more readily metabolised than EO9 [4]. Furthermore, cytotoxicity to MeDZQ is increased 4-fold in CHO cells transfected with human NQO1 cDNA [37]. However, in a panel of human NSCLC xenografts, antitumour activity did not

correspond to the level of expression of a single bioreductive enzyme [41].

Another significant finding of the present study is the observation that native EO9 (and subsequent reactive intermediates) rather than a metabolite is likely to be the active form(s) of the drug. Recently, our laboratory has reported on the properties of the two main EO9 reactive intermediates generated after quinone reduction, namely: the semiquinone free radical, the product of one electron reduction and the fully reduced hydroquinone intermediate, the product of two electron reduction [5]. Unusually, the hydroquinone of EO9 was unstable in the presence of air (half life 1.5 sec) and underwent autoxidation via the generation of ROS. Therefore, in previous studies where a correlation was observed between the cytotoxicity of EO9 in air and DT-diaphorase expression [7, 10, 17, 19, 34, 35], this effect was probably mediated via ROS. DT-diaphorase catalyzed metabolism of EO9 results primarily in DNA damage (single and double stranded breaks) consistent with ROS generation [36] rather than DNA crosslinking which only occurs under hypoxic conditions [29]. In this respect, the action of DT-diaphorase is essentially analogous to a one electron reductase and may be seen as a complimentary pathway, as opposed to a competing one. Under hypoxic conditions, at neutral pH, after one or two electron reduction, the hydroquinone and semiquinone exist in equilibrium, with the equilibrium greatly favouring the hydroquinone ($K_{eq} > 3000$). However, the equilibrium shifts towards the semiquinone at more alkaline pH ($K_{eq} = 84$, pH 9.0). The observation that DNA crosslinking increases under hypoxia as the pH is shifted from 5.5 to 7.5 would suggest that the semiquinone form of the drug is the more active crosslinking agent [29].

Since intact EO9 is required to produce the antitumour effect of this drug, then chemical stability, pharmacokinetics and tumour drug penetration become equally important issues to the enzymology of metabolic activation. Several preclinical and clinical pharmacokinetic studies have demonstrated that EO9 has a very short half life and is extensively metabolised: $t_{1/2} = 1.9$ min in mice, 3.0 min in rats [12], and 0.8–19 min in man [8]. Throughout extensive phase II trials conducted in Europe, EO9 failed to produce any responses [11]. It is equally the case that the absence of clinical activity may be due to poor chemical stability and rapid metabolism resulting in poor tumour drug penetration, rather than a failure of the drug to undergo metabolic activation in tumour cells.

In conclusion, the present studies have revealed that the *in vivo* antitumour activity of EO9 cannot be predicted for by measuring tumour levels of bioreductive enzymes such as DT-diaphorase and cytochrome P-450 reductase. Tumour drug metabolism of EO9 appears to produce a number of covalent drug adducts in addition to the chemical hydrolysis product EO5A but appearance of these metabolites does not correspond to antitumour activity. Finally, a good correlation was observed between the *in vitro* rate of EO9 metabolism by tumour homogenates, which possibly reflects metabolic activation, and antitumour activity. Therefore, *in vitro* drug incubations with tumour biopsies are now recommended as a more reliable predictive indicator of the antitumour activity of EO9.

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