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Papers

DT-diaphorase Activity Correlates with Sensitivity to the Indoloquinone EO9 in Mouse and Human Colon Carcinomas

Michael I. Walton, Michael C. Bibby, John A. Double, Jane A. Plumb and Paul Workman

The indoloquinone EO9 exhibits promising *in vitro* and *in vivo* antitumour activity. EO9 is metabolised to DNA damaging species by DT-diaphorase *in vitro*. In the present study DT-diaphorase specific activity was 16 fold higher in the mouse adenocarcinoma MAC 16, a tumour which is quite responsive to EO9 *in vivo*, compared with levels in the more resistant mouse adenocarcinoma MAC 26. This order of responsiveness is the reverse of that seen with the most active of the clinically used agents in these tumours [chloroethylnitrosoureas and 5-fluorouracil (5-FU)]. In addition, when the *in vitro* sensitivity of two human colon carcinoma cell lines was compared, EO9 was 15–30 fold more active in the DT-diaphorase rich HT29 line than in the enzyme-deficient BE cell line counterpart. These results are consistent with the hypothesis that DT-diaphorase expression may be a major determinant of the sensitivity of tumours to EO9. This should be considered in the clinical development of the drug.

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INTRODUCTION

THE INDOLOQUINONE EO9 [3-hydroxymethyl-5-aziridinyl-1-methyl-2(*H*-indole-4,7-indione)-propenol] is about to enter phase I clinical trial under the auspices of the EORTC New Drug Development Coordinating Committee and Office. It exhibits potent cytotoxic activity *in vitro*, particularly against hypoxic cells, displays a unique profile in the US NCI *in vitro* human tumour cell line panel, shows promising *in vivo* solid tumour activity against both syngeneic mouse neoplasms and human xenografts, and is non-myelosuppressive [1–3].

The structure of EO9 suggests that it would be likely to act as a bioreductive alkylating agent [4] following reduction of the quinone moiety by appropriate cellular enzymes. In the case of the related bioreductive prodrugs mitomycin C and diaziquone, this metabolic activation can be catalysed by the obligate two-electron-donating flavoenzyme DT-diaphorase [NAD(P)H: (quinone-acceptor) oxidoreductase, EC1.6.99.2] [5, 6]. We have shown recently that EO9 is a much more efficient substrate than is mitomycin C for the DT-diaphorase present in both HT29 human colon carcinoma and Walker 256 rat mammary carcinoma cells [7]. Moreover, metabolism of EO9 by the purified enzyme from Walker tumour cells results in the production of DNA-damaging species [7]. It is therefore possible that the expression of DT-diaphorase will be a major determinant of the sensitivity of cells to EO9. Since DT-diaphorase is commonly overexpressed in experimental carcinogenesis models and in rodent and human tumours [8–12], any dependence of sensitivity to EO9 on DT-diaphorase activity should contribute to a favourable therapeutic index for the drug. Two important consequences would follow: patients could be selected to receive EO9 on the basis of their tumour DT-diaphorase activity ('enzyme profiling'), and second generation analogues might be developed to optimise specific activation by DT-diaphorase ('enzyme-directed bioreductive drug development') [13].

Although the biochemical and molecular data strongly support a role for DT-diaphorase, until now there has been no evidence that enzyme activity predicts for solid tumour activity *in vivo*. In this report we demonstrate such a correlation in a pair of transplantable mouse adenocarcinoma of the colon (MAC) tumour lines [14, 15]. The MAC tumours are an excellent model for this purpose because they exhibit biological properties and relative chemoresistance closely similar to human colon cancer [14–18], and colon tumours have been reported to express high levels of DT-diaphorase [8–11]. We also show here that the *in vitro* EO9 sensitivity of the DT-diaphorase rich human colon carcinoma line HT29 is about 15–30-fold greater than that of the DT-diaphorase-deficient BE cell line counterpart.

MATERIALS AND METHODS

MAC 16 and 26 solid tumours were produced by subcutaneous implantation in the flanks of NMRI female mice weighing 20 g [14–18]. When tumours reached 40 mm^3, mice were either entered into a chemotherapy experiment or killed and the

Correspondence to P. Workman. CRC Department of Medical Oncology, Cancer Research Campaign Beatson Laboratories, University of Glasgow, Switchback Road, Bearsden, Glasgow G61 1BD, U.K.; M.I. Walton was with P. Workman at MRC Clinical Oncology and Radiotherapeutics Unit, Medical Research Council Centre, University of Cambridge, Hills Road, Cambridge CB2 2QH, U.K. and are members of the EORTC Pharmacology and Molecular Mechanisms Group; M.I. Walton is now at Laboratory of Molecular Pharmacology, National Cancer Institute, Building 37, Room 5C19, Bethesda, Maryland 20892, U.S.A.; M.C. Bibby and J.A. Double are at Clinical Oncology Unit, University of Bradford, Bradford, West Yorkshire BD7 1DP, U.K. and are members of the EORTC Screening and Pharmacology Group; J.A. Plumb is at CRC Department of Medical Oncology, Cancer Research Campaign Beatson Laboratories, University of Glasgow, Switchback Road, Bearsden, Glasgow G61 1BD, U.K.
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tumours removed and stored at −70°C or lower prior to enzyme analysis. Chemotherapy experiments were conducted according to standard protocols for the MAC solid colon carcinomas [14–18]. Briefly, mice were randomised at 8–10 per group to receive either the previously established maximum tolerated dose of EO9 (MTD, < 15% weight loss and < 10% lethality), two-thirds of this dose or control vehicle. All injections were given intraperitoneally in saline (10 ml/kg). Tumour growth was followed by serial calliper measurements and tumour volumes calculated. Antitumour activity was determined from the ratio of tumour weights in treated and control groups (T/C%) and by regrowth delay.

For enzyme assay, tumours were homogenised in all-glass homogenisers and cell debris pelleted at 600 *g*. The enzyme activity in the supernatant was determined by a standard spectrophometric method using menadione (20 μmol/l) as quinone substrate, NADH (2 mmol/l) as reduced cofactor, KCN (1 mmol/l) and cytochrome *c* (77 μmol/l) as terminal electron acceptor all in 0.1 mol/l) phosphate buffer pH 7.4 containing 0.14% w/v bovine albumin [7, 19]. Reaction curves were linear with time and the initial reaction velocity was linear with protein concentration. DT-diaphorase activity was calculated as that component which was inhibited by 100 μmol/l of the DT-diaphorase inhibitor dicoumarol. Cytochrome P450 reductase activity was measured by the standard assay involving the direct reduction of cytochrome *c* with NADPH as reduced cofactor [20]. Results are reported as nmol cytochrome *c* reduced per min per mg tissue protein.

The sensitivity of the HT29 and BE human colon carcinoma cell lines was determined by the MTT tetrazolium dye-based microtitration, optimised for these lines [21, 22]. Cells in exponential growth phase were exposed to EO9 for 24 h. Eight concentrations of drug were used (10^{-6}–10^{-11} mol/l) with four wells per concentration and plates were set up in triplicate. The cells were then allowed to grow in drug-free medium for 3 days. MTT dye reduction was used to assess final cell numbers. Drug sensitivity is expressed as the concentration of drug required to kill 50% of the cells, i.e. to reduce the absorbance value to half that of the control untreated cells. Results were calculated for individual plates and are presented as the mean (S.E.) of three plates. The corresponding results for MAC 16 and MAC 26 as *in vitro* cell lines are for nominal 96 h exposures [23].

RESULTS AND DISCUSSION

The results of a typical chemotherapy experiment are summarised in Table 1. It can be seen that MAC 16 is more responsive to EO9 than is MAC 26. A 2+ T/C score was obtained in MAC 16 compared to a 1+ score for MAC 26. The same T/C scores were obtained in a repeat experiment. With respect to the tumour growth delays, it should be pointed out that the MAC 16 and MAC 26 tumours both have volume doubling times of around 4 days. Thus the results are equivalent to almost one volume doubling for MAC 16, but less than half a volume doubling for MAC 26. This is particularly significant as MAC 16 is generally unresponsive to the major classes of cytotoxic therapy. Whilst MAC 26 is generally insensitive compared to most rodent tumour models, greater responses can be achieved than in MAC 16 [14–17]. For example, with 5-FU and chloroethylnitrosourea-like agents no response is seen in MAC 16 whereas in MAC 26 2+ scores can be obtained with 5-fluorouracil, mitozolomide and TCNU. Similarly, the nitrogen mustards chlorambucil and cyclophosphamide have no effect in MAC 16, but give 2+ T/C scores in MAC 26; however, melphalan produces a 2+ response in both tumours. Interestingly, the only conventional drugs which score better in MAC 16 than in MAC 26 are mitomycin C and doxorubicin (2+). Like EO9, these are quinone-containing drugs which can undergo metabolic reduction, and mitomycin C is also thought to be activated by DT-diaphorase [6].

Table 1. Response of MAC 16 and MAC 26 tumours to indoloquinone EO9

	MAC 16			MAC 26		
Dose mg/kg	T/C* (%)	Score†	Growth‡ delay (days)	T/C* (%)	Score†	Growth‡ delay (days)
2	51	+	2.5	89	0	0.5
3	45	++	3.0	66	+	1.5

Tumour response was determined by standard protocols for MAC 16 tumours [14–17].
* T/C = treated/control tumour volume at 10 days, expressed as a percentage.
† Scores correspond to the following T/C values: ++ = 26–50%; + 51–75%; 0 = > 75%.
‡ Growth delay = difference in time to reach 4 × treatment volume for treated compared with control tumours.
Results are typical of those obtained in various repeat experiments. No toxic deaths were seen. Note that MAC 16 is normally less responsive to standard drugs than MAC 26 (see text).

Table 2 summarises the specific activities of the bioreductive enzymes DT-diaphorase and cytochrome P450 reductase in the two MAC tumours. Activities of cytochrome P450 reductase were similar in the two different tumours ($P > 0.05$). What is striking, however, is the 15-fold higher level of DT-diaphorase activity in the more EO9-sensitive MAC 16 tumour compared with the less responsive MAC 26 tumour ($P < 0.05$).

The above results are consistent with (but do not necessarily prove), an important role for DT-diaphorase in the antitumour response to indoloquinone EO9. In particular, they provide the first evidence of the involvement of DT-diaphorase in solid tumours *in vivo*. Taken together with our previous biochemical and molecular data demonstrating that EO9 acts as a good substrate for the enzyme and that the reaction generates DNA damaging species (most likely via the activation of electrophilic centres in the hydroquinone) [7] the new evidence points to a potentially crucial role in human cancers. There is clearly a need to corroborate the apparent predictive value of DT-diaphorase in a greater number of tumours and further studies are planned using other rodent tumours and also human tumour xenografts.

Table 2. Specific activities of DT-diaphorase and cytochrome P450 reductase in MAC 16 and 26 solid tumours

	DT-diaphorase	P450 reductase
MAC 16	98.3 (9.8)	10.7 (1.5)
MAC 26	6.2 (1.9)	13.5 (2.9)

Results are mean (2 S.E.) ($n = 4$ independent assays).
Units are nmol cytochrome *c* reduced/min/mg protein.

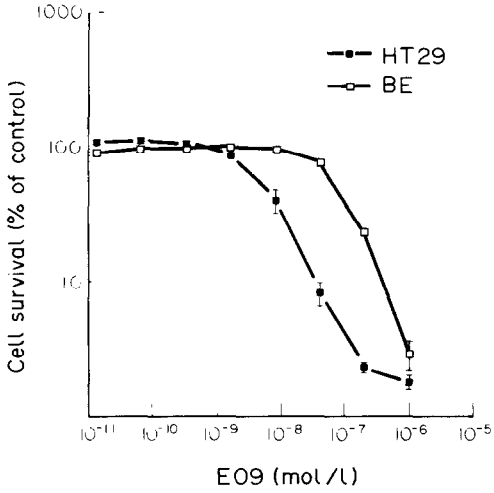

Fig. 1. Dose–response curve for the cytotoxicity of indoloquinone EO9 against DT-diaphorase rich HT29 human colon carcinoma cells and the enzyme-deficient BE counterparts. Results shown are for one of three independent experiments (see text for IC50 values). Data points are the mean (S.E.) for three replicate dose–response curves performed as individual plates, with four wells included per plate for each concentration.

Preliminary chemosensitivity experiments with MAC 16 and 26 cell lines have also confirmed the higher sensitivity of the former *in vitro*. Thus IC50 values (S.E.) for a nominal 96 h exposure were 73.2 (31.4) nmol/l for MAC 16 and 2056 (174) nmol/l for MAC 26, a difference of 28 fold. To further test the hypothesis of a key function for the bioactivation of EO9 by DT-diaphorase in colon cancer we also compared the *in vitro* drug sensitivity of the BE human colon carcinoma cell line which essentially lacks DT-diaphorase activity with that of the HT29 human colon carcinoma which has high levels [5, 6]. Data from a typical experiment using a 24 h exposure are shown in the Fig. 1. The IC50 values (S.E.) for this experiment were 110.0 (11.6) nmol/l for BE cells and 6.7 (1.2) nmol/l for HT29 cells. Thus the DT-diaphorase rich human colon tumour line was 16.4-fold more sensitive than the enzyme-deficient BE cell counterpart. The corresponding differences in two repeat experiments were 15.7 and 31.0-fold, respectively.

It should be pointed out that whereas several other relevant parameters are not markedly different between the two human colon carcinoma lines [5, 6] the HT29 line has high levels of the O^6-alkyl guanine DNA alkyl transferase repair protein whereas the BE line is again deficient [24, 25]. This protein removes damaging lower alkyl and chloroethyl adducts from the O^6 position of guanine in DNA, and hence the BE cells are much more sensitive to methylating and chloroethylating agents than are HT29 cells. We can however discount the involvement of the O^6-alkyl transferase in the response to EO9 for two reasons. Firstly, the repair protein does not recognise/remove bulky DNA adducts such as those which are formed by mitomycin C and which would be likely to be induced by the structurally related EO9 [24, 26]. Secondly, if the alkyl transferase did play a role in removing EO9 DNA adducts then the BE repair protein-deficient cell line would be more sensitive to EO9 than the repair proficient HT29 whereas the reverse is true. Although other factors, including alternative DNA repair pathways, will no doubt be found to influence cellular sensitivity to EO9, the present results are clearly consistent with a major involvement of DT-diaphorase.

Other experiments which may be carried out to further explore the role of DT-diaphorase in EO9 sensitivity include the use of dicoumarol and related agents to inhibit the enzyme. On the other hand there are a number of concerns over the lack of specificity of these inhibitors using intact cell assays [27] and these would be exacerbated by pharmacokinetic interactions *in vivo*. We have however shown that dicoumarol inhibits the DNA damage induced by EO9 in the presence of purified DT-diaphorase [7].

EO9 will soon enter phase I trial within EORTC. The increased expression of DT-diaphorase activity in tumour compared to surrounding normal tissue reported for human colon, lung, breast and liver tumours [8–12] makes these especially attractive targets for EO9 therapy. We suggest that the clinical protocols for the drug should take advantage of the opportunity to correlate any response to EO9 with tumour DT-diaphorase level. In addition, we are continuing to investigate the relationship between drug structure and ability to act as a substrate for DT-diaphorase. As with the dinitrophenylaziridine CB 1954 [28, 29], preliminary results indicate that quite small changes in structure can result in marked differences in both the enzymatic reduction and cytotoxicity of indoloquinones [30] and analogues with improved specificity may therefore be identified. We suggest that a detailed understanding of the molecular enzymology of DT-diaphorase-mediated bioreductive activation is vital to the rational design and clinical development of indoloquinones and related agents [13].

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c-*myc* Gene Amplification in Selected Node-negative Breast Cancer Patients Correlates with High Rate of Early Relapse

M.Roux-Dosseto, S. Romain, N. Dussault, C. Desideri, L. Piana, P. Bonnier, N. Tubiana and P.M. Martin

In breast cancers with histologically negative axillary nodes selected for high frequency of recurrence, the amplification of c-*myc*, *erb*B-2 and *int*-2 genes was found to concern, respectively 25% (16/65), 31% (25/81) and 14% (10/70) of tumours. Their relation with tumour progression expressed by relapse-free survival is reported. Using univariate analyses, c-*myc* amplified tumours showed significant association with early (30-month period after diagnosis) ($P = 0.0013$) and intermediate (50-month period after diagnosis) ($P = 0.0398$) risks of recurrence. In contrast, only a trend towards higher relapse was observed in *erb*B-2 amplified breast cancers with respect to later events (occurring over the first 30-month period). Multivariate analyses indicated that c-*myc* amplification is an independent prognostic factor stronger than oestrogen receptor status and tumour size to define a high risk subset in node-negative patients selected for high frequency of recurrence.
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INTRODUCTION

To date, involvement of axillary lymph nodes is the best prognostic indicator in breast cancer. It has been related to a considerable increase in early mortality. Nevertheless, high recurrence and early death rate are continuously observed among node-negative patients (30% relapse during the first 5 years after local-regional therapy). Several prognostic factors are used to describe high risk node negative subsets, mainly tumour size, oestrogen receptor level, tumour ploidy and mitotic index [1]. Nevertheless, these parameters do not match perfectly the disease evolution; this has resulted in the search for new prognostic parameters capable of distinguishing high risk patients in