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Potentiation of EO9 Anti-tumour Activity by Hydralazine

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EO9[3-hydroxy-5-aziridinyl-1-methyl-2(1H-indole-4,7-dione)prop-β-en-α-ol] has been selected for phase I evaluation in Europe. Activity has been seen previously in a highly refractory, necrotic mouse adenocarcinoma (MAC 16) but EO9 is shown here to be inactive against early tumours (MAC 15A and MAC 13) and a well vascularised, well-differentiated established adenocarcinoma (MAC 26). EO9 becomes active against MAC 26 tumours when hydralazine (10 mg/kg) is administered 1 min after EO9. Co-administration of hydralazine decreases EO9 plasma clearance and increases plasma area under the curve values (0.053 to 0.115 μg h/ml). These pharmacokinetic changes are accompanied by anti-tumour activity but no increase in bone marrow toxicity so this therapeutic gain may be due, at least in part, to microenvironmental changes resulting from hydralazine induced tumour vascular shutdown.
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INTRODUCTION

THE INDOLOQUINONE EO9 [3-hydroxy-5-aziridinyl-1-methyl-2-(1H-indole-4,7-dione)-prop-β-en-α-ol] has been selected for phase I evaluation in Europe under the auspices of the EORTC New Drug Development Coordinating Committee and Office. EO9 was chosen from a series of indoloquinones synthesised by Oostveen and Speckamp [1] because of good activity against human solid tumour cell lines *in vitro* and promising activity against solid murine and human tumour xenografts *in vivo* [2].

Bioreductive alkylation is thought to play a major role in the mechanism of action of EO9 with the activation being catalysed by the two-electron donating flavoenzyme DT-diaphorase [NAD(P)H: (quinone-acceptor) oxidoreductase, EC1.6. 99.2]. The two-electron reduction of EO9 via DT-diaphorase generates DNA damaging species *in vitro* [3].

Hypoxic cells within a solid tumour mass exist in an environment that is more conducive to reductive reactions than their well-oxygenated counterparts [4]. It is likely then that EO9 might be more active against established solid tumours than against ascitic tumours or early tumour deposits, assuming that drug delivery to the hypoxic fraction of solid tumours is not impaired. Previous studies in this laboratory have shown activity against a normally refractory tumour (MAC 16) [5] which becomes highly necrotic [6, 7]. Another way to test the "bioreductive potential" of EO9 *in vivo* might be to utilise an initially well-vascularised tumour which can be rendered hypoxic by the use of a vasoactive agent. Hydralazine has been shown to enhance the effectiveness of other bioreductive agents [8, 9]. The present study examines the *in vivo* activity of EO9 against three members of a panel of murine colon adenocarcinomas (MAC tumours) comprising an ascitic tumour and two solid tumours of varying growth characteristics and morphology. The investigation aims to compare activities against ascites, recently implanted and advanced tumours, and also to examine the influence of co-administration of hydralazine on the activity of EO9 against a well vascularised tumour in which the vasculature has already been shown to respond to hydralazine [10]. Since in a previous study we have demonstrated that hydralazine causes significant changes to the pharmacokinetics of the anti-cancer agent tauromustine [11] this investigation will also examine the influence of hydralazine on mouse plasma pharmacokinetics of EO9 and on mouse bone marrow toxicity.

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MATERIALS AND METHODS

Test compounds

EO9 used in the initial studies was obtained via the Screening and Pharmacology Group of the EORTC. The combination studies used formulated EO9 [12]. Hydralazine was purchased from the Sigma Chemical Co. (Poole, U.K.).

Chemotherapy

The tumours grown in NMRI mice have been previously well characterised. MAC 15A is an ascitic tumour grown in the peritoneal cavity. MAC 13 and MAC 26 are grown subcutaneously (s.c.) in the flank.

Chemotherapy began 18 days after implantation for the slow growing MAC 26 and after 2 days for MAC 13 and MAC 15A. EO9 was administered as a single intraperitoneal (i.p.) injection.

For hydralazine combination experiments MAC 26 tumours of approximately 1 g were used as they have a well established vascular supply which has been extensively studied [10]. Both compounds were given as a single intravenous (i.v.) bolus in saline, EO9 at a dose range of 3–12 mg/kg body weight and hydralazine at 10 mg/kg [10] 1 min after EO9. Groups of eight mice were used and experiments duplicated.

Pharmacokinetic studies

EO9 (6 mg/kg) and hydralazine (10 mg/kg) were injected (i.v.) to MAC 26 bearing mice as in the chemotherapy experiments. Mice were treated with drug alone or drug with hydralazine within the same experiment and the experiment was repeated three times.

Blood samples from one mouse per time point were taken by cardiac puncture under ether anaesthesia into heparinised tubes and plasma separated at 4°C.

One hundred microlitres of internal standard [12] [(0.30 μg/ml in phosphate buffer (0.1 mol/l, pH 7.0)] was added to 300 μl of plasma and made up to 500 μl with buffer. EO9 was extracted from plasma and quantified using high pressure liquid chromatography [12]. No interfering compounds were detected and calibration curves were linear.

The drug concentration versus time curve for EO9 + hydralazine was fitted to a bi-exponential equation whereas a mono-exponential equation described the EO9 only data. Calculations have been described previously [11].

Bone marrow toxicity

The effects of EO9 (i.v.) with or without hydralazine (i.v.) on the bone marrow were assayed by the spleen colony forming unit method of Till and McCulloch [13]. This assay is described in detail elsewhere [14].

RESULTS

EO9 was initially screened as one of a series of analogues and results against the MAC 15A ascites tumour and the MAC 13 solid tumour are presented in Table 1. EO9 showed no anti-tumour activity against either of these tumours when treated 2 days after implantation. Similar results were seen using saline as the solvent.

Activity of EO9 (i.v.) with or without hydralazine against the established MAC 26 tumour is shown in Fig. 1. There is significant ($P < 0.005$) anti-tumour activity when EO9 (6 mg/kg) is given in combination with hydralazine (10 mg/kg i.v.) whereas hydralazine alone has no effect. EO9 shows no activity at the other dose levels or by the i.p. route (Table 1).

The pharmacokinetic parameters in the mice administered with EO9 (6 mg/kg) only and EO9 (6 mg/kg) with hydralazine (10 mg/kg) are shown in Table 2 with the plasma levels represented graphically in Fig. 2. Points from all three experiments have been included to demonstrate reproducibility. C_0 values for the hydralazine treated and non-treated mice were the same (approx. 2 μg/ml Table 2). This would be expected as hydralazine was given after the EO9. The plasma concentration in EO9-alone mice decreased rapidly ($t_{1/2\alpha}$ of only 1.8 min). The clearance was immediately decreased following hydralazine administration and after a short initial $t_{1/2\alpha}$ of 1.4 min a terminal half-life of 40 min was calculated. The effect of this decrease in clearance was to double the area under the curve (AUC) from 0.053 (non-treated) to 0.115 μg h/ml (treated).

The spleen colony forming assay indicated that the administration of hydralazine (i.v.) 1 min after EO9 (6 mg/kg i.v.) gave no significant increase ($P > 0.05$) in bone marrow toxicity as compared with the EO9 only treated mice with survival fractions (± 1 SD) being 0.54 (± 0.14) for EO9 and 0.51 (± 0.08) for EO9 plus hydralazine.

Table 1. Evaluation of EO9 against MAC 15A, MAC 13 and MAC 26 tumours

Tumour	Dose (mg/kg)	Solvent	Route	Treatment day	Drug Deaths	T/C%
MAC 15A	6	Ethanol/oil*	i.p.	2	5/5	Toxic
	4				5/5	Toxic
	2.6				0/5	71†
	1.7				0/5	93
MAC 13	9	Ethanol/oil	i.p.	2	5/5	Toxic
	4				5/5	Toxic
	2.6				0/5	109‡
MAC 26	4.5	Saline	i.p.	18	10/10	Toxic
	3				0/10	68^{NS}§
	2				0/10	69^{NS}

* Similar results were seen for MAC 15A and MAC 13 when saline was used as solvent.
† Assessed from median life span of treated/control.
‡ Assessed from tumour weights when control tumour weighed 1 g.
§ Assessed from tumour volume at regrowth.
NS = Not significantly different from control.

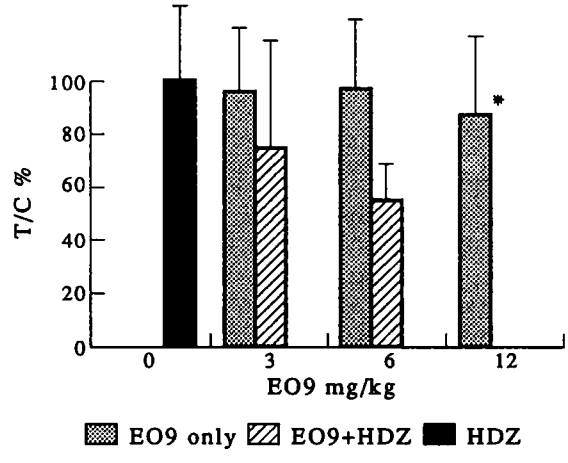

Fig. 1. Activity of formulated EO9 ± hydralazine against MAC 26 tumours in NMRI mice (errors shown are ± 1 S.D.) * 1 death in eight mice in the experiment shown.

Table 2. Summary of pharmacokinetic parameters in mice after treatment with formulated EO9 (6 mg/kg, i.v.) both with and without the administration of hydralazine (10 mg/kg). Range in brackets

	EO9 only	EO9 + HDZ
Dose mg/kg	6	6
A^* μg/ml	1.87 (1.34–2.87)	2.04 (1.51–3.13)
B^* μg/ml	—	0.104 (0.069–0.144)
$t_{1/2}\alpha$ min	1.78 (1.34–2.31)	1.40 (1.20–1.80)
$t_{1/2}\beta$ min	—	40.3 (24.6–52.2)
AUC μg h/ml	0.053 (0.040–0.070)	0.115 (0.081–0.153)
Clearance ml/min	49.7 (35.7–62.5)	23.2 (16.3–30.8)

* A and B are the ordinate intercepts of the α and β phases, respectively. $C_0 = A + B$.

DISCUSSION

The hydralazine dose used here has been shown previously to cause an 80% vascular shutdown in MAC 26 tumours [10] and to increase significantly the plasma and tissue AUC of tauromustine in mice [11]. The present data show similar changes in plasma EO9 AUC values following hydralazine treatment with the EO9 only data giving similar kinetic parameters to those of Workman *et al.* [15]. As hydralazine is known to affect blood flow in both tumour and normal tissue in mice [16], including kidneys and liver, this increased AUC was thought to be due to a combination of decreased renal clearance and drug metabolism. Since the anti-tumour effects of EO9 produced by co-administration of hydralazine are not accompanied by an increase in bone marrow toxicity these data suggest a real therapeutic gain for the combination.

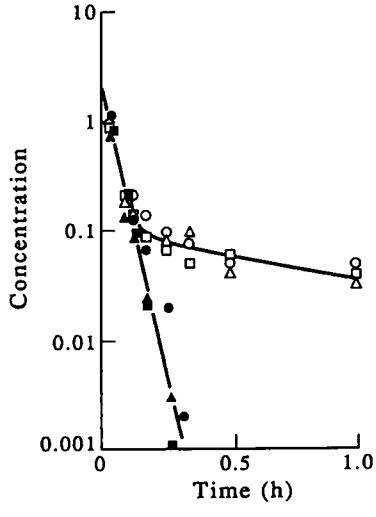

Fig. 2. Plasma concentrations (μg/ml) of EO9 (6 mg/kg) both with (open symbols) and without (closed symbols) the administration of hydralazine (10 mg/kg).

Other factors such as the role of DT-diaphorase activity, transient tumour hypoxia or microenvironmental changes resulting from reduced tumour blood perfusion, for bioactivation of the quinone moiety, cannot be ruled out. Further studies to identify the importance of these factors are ongoing.

The relevance of vaso-manipulation such as the one described here for clinical cancer is open to debate and further work is clearly required however this study has confirmed the observations of Brown [9] by demonstrating manipulation of an experimental tumour to potentiate the activity of a bioreductive agent.

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