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## EO9: A Novel Bioreductive Alkylating Indoloquinone With Preferential Solid Tumour Activity and Lack of Bone Marrow Toxicity in Preclinical Models

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EO9 is a novel and fully synthetic bioreductive alkylating indoloquinone. Although structurally-related to mitomycin C, EO9 exhibits a distinct preclinical antitumour profile and there are also differences in its biochemical activation. In this study, EO9 was found to demonstrate preferential cytotoxicity against solid tumours *in vitro* as compared to leukaemia cell lines both in the Corbett two-tumour assay and in the disease-oriented human tumour cell line panel of the U.S. National Cancer Institute. In the latter system activity was particularly apparent in colon, melanoma and central nervous system lines, together with some renal and non-small cell lung lines. Preferential cytotoxicity towards hypoxic versus aerobic EMT6 mouse mammary tumour cells was observed. *In vivo*, EO9 was inactive against the P388 murine leukaemia, while exerting significant antiproliferative effects against several murine and human solid tumours, including the generally resistant MAC mouse colon tumours and gastric, ovarian and breast xenografts. These results confirmed *in vitro* observations of preferential solid tumour activity. In animal toxicology studies, EO9 induced vascular congestion in the gastrointestinal tract, but no significant bone marrow toxicity. The LD<sub>10</sub> value of EO9 after a single intravenous injection into mice was 9 mg/kg (27 mg/m<sup>2</sup>). A dose of one-tenth of the mouse equivalent LD<sub>10</sub> (2.7 mg/m<sup>2</sup>), the recommended starting dose for clinical phase I studies, was found to be safe in rats. Considering its distinct mechanism of bioactivation as compared to mitomycin C, its preferential solid tumour activity, its excellent activity against hypoxic cells, and lack of significant bone marrow toxicity in animal studies, EO9 has been selected for clinical evaluation within the framework of the EORTC.

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### INTRODUCTION

EO9 [3-HYDROXY-5-AZIRIDINYL-1-methyl-2-(1H-indole-4,7-dione)-propenol] (E85/053, NSC 382459) is the lead compound in a series of novel and fully synthetic bioreductive alkylating indoloquinones, originally synthesised by Oostveen and Spek-kamp [1]. Although structurally related to mitomycin C (MMC) (Fig 1), preliminary *in vitro* evaluation of EO9 has suggested that

this compound differs from MMC in its antitumour profile [2]. There are also differences in the mechanism of biochemical activation. In contrast to MMC, EO9 was shown to function *in vitro* as an excellent substrate for reduction by human and murine DT-diaphorase (DTD) at physiological pH [3, 4]. Experiments performed with purified rodent DTD have shown that EO9 reduction by this enzyme prompted the formation of bioactivated

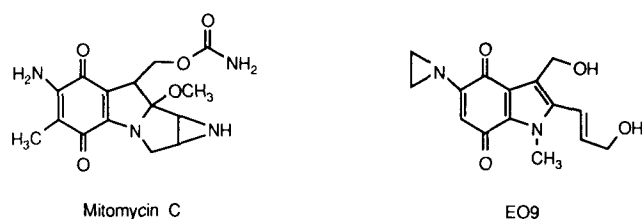


Fig. 1. Chemical structures of mitomycin C and EO9.

species which caused single strand breaks in plasmid DNA [4]. More recent studies have also demonstrated the formation of DNA crosslinks in DTD-rich rat tumour cells [5].

In this paper, we describe a series of *in vitro* and *in vivo* antitumour activity studies of EO9 in both murine and human tumour models, as well as animal toxicology studies, which led to the selection of this interesting new compound for phase I clinical evaluation within the framework of the EORTC.

## MATERIALS AND METHODS

### Drugs

EO9 (molecular weight: 288.1) was supplied by the EORTC New Drug Development Office. The bulk was stored at 4°C in the dark. For *in vitro* studies, EO9 was initially dissolved in dimethylsulphoxide and further diluted in culture medium so that the final concentration of solvent did not exceed 1%. For administration in animals, EO9 was dissolved in 0.9% sodium chloride. The stability of EO9 depends upon the pH of the medium and the temperature; the main breakdown product is EO5A [6, J.H. Beijnen, personal communication]. Under cell culture conditions, the half life of the drug is 6.3 h [6].

For animal toxicology studies, formulated EO9 was used. The vials contained 5 mg of freeze-dried drug with 50 mg lactose as excipient. After dilution with 0.9% sodium chloride (final EO9 concentration: 0.5 mg/ml), reconstituted vials were stable for 24 h at room temperature: 99.7 ± 0.7% EO9 remained after 4 h and 99.6 ± 2.9% after 24 h as measured by high performance liquid chromatography (HPLC) (J.H. Beijnen, personal communication).

MMC was obtained either from the US National Cancer Institute or Medac GmbH (Hamburg, Germany). The compound was solubilised in water, if necessary, further diluted in saline, and subsequently used for *in vitro* and *in vivo* experiments.

### In vitro cytotoxicity studies

The cytotoxicity profile of EO9 was studied in the human tumour cell line panel which constitutes the disease-oriented, *in vitro* drug-screening system of the National Cancer Institute (NCI). Detailed descriptions of the standard technical pro-

cedures and data analyses performed by the NCI have been published elsewhere [7–9]. Briefly, the assay involves plating the cells, preincubation for 24 h, followed by a 48 h continuous drug exposure over a broad concentration range (at least five 10-fold dilutions) against a panel of 56 cell lines comprising eight tumour types. The cytotoxicity was assessed with the sulphorhodamine B (SRB) protein assay. Three evaluation parameters were established, namely: (a) the drug concentration which inhibits growth by 50% ( $GI_{50}$ ); (b) the drug concentration which inhibits growth by 100% (total growth inhibition or TGI); and (c) the drug concentration which yields a 50% reduction in the amount of proteins at the end of the experiment in comparison with that amount at the start of the test ( $LC_{50}$ ), indicating a net loss of cells after drug treatment. The results were presented in the form of mean graphs [9] in which the drug effects are calculated as positive or negative deviations from the average sensitivity of the entire cell line panel. This generates a characteristic cell line “fingerprint” for each individual drug. Several standard chemotherapeutic agents (including MMC) have also been tested in this system, generating a reference database to allow comparisons of cytotoxicity profiles among novel and standard drugs.

The cytotoxicity of EO9 against EMT6 mouse mammary tumour cells was determined under both oxic and hypoxic conditions according to the method of Stratford and Stephens [10] using either the MTT assay or the clonogenic assay, as indicated in the Results. Incubations were carried out in glass to avoid problems of oxygen leakage from tissue culture plastic. The hypoxic cytotoxicity ratio (HCR) was given by:

$$HCR = \frac{GI_{50} \text{ for aerobic cells}}{GI_{50} \text{ for hypoxic cells}}$$

EO9 was also tested in the Corbett two-tumour assay previously described elsewhere [11] and designed to identify agents showing preferential cytotoxicity against carcinomas as opposed to leukaemias. Briefly, a measured concentration of EO9 was applied to a disc placed in an agar plate containing both murine leukaemia (L1210 or P388) and colon or pancreatic carcinoma (C38 or P03, respectively) cells. After incubation for several days, zones of growth inhibition of each cell type were measured.

Further characterisation of the *in vitro* cytotoxicity profile of EO9 was carried out using cell suspensions of human solid tumour xenografts in a two-layer soft agar culture system after continuous exposure to drugs [12, 13]. Tumour characteristics, including histology, growth rate and chemosensitivity to standard anticancer drugs have been described previously [14]. Drug effects were expressed as percentage survival obtained by comparing the mean number of colonies in the treated plates with the mean number of colonies of control plates (T/C%), according to the formula:

$$T/C\% = \frac{\text{Colony count (drug treated group)}}{\text{Colony count (control group)}} \times 100.$$

In accordance with the standard operating procedure, antitumour activity was considered to be significant if the compound reduced colony formation up to 30% or less of the control value ( $T/C \leq 30\%$ ).

### In vivo antitumour activity studies

**Murine tumours.** EO9 was tested in four different murine adenocarcinomas of the colon (MAC tumours) growing as solid

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tumours in inbred strain NMRI mice. The characteristics of these tumour models and their chemosensitivity to standard cytotoxic agents have been described previously [15–18]. Accordingly, MAC13 tumour fragments were implanted subcutaneously (s.c.) into the right flank of female mice by means of a trocar. Two days after transplantation when tumours were approximately 2 mm<sup>3</sup> (day 0), the mice were allocated into groups of 10 by randomisation prior to treatment. Chemosensitivity was assessed by mean tumour weight at day 21 and weight of treated tumours was expressed as a percentage of untreated weight (T/C%) [15]. MAC15A tumour cells were implanted into male mice by intraperitoneal (i.p.) injection of  $1 \times 10^6$  cells suspended in 0.2 ml of 0.9% saline (day 0). The animals were allocated into groups of 10 and treated on day 2 as previously mentioned. Antitumour activity was determined by comparing the lifespan of treated and control groups. Deaths were recorded and the median survival time determined [16]. MAC16 and MAC26 tumour fragments were implanted s.c. into the flank of female mice by means of a trocar. Positive takes could only be identified 2–3 weeks after implantation. Tumour bearing animals were allocated by randomisation into groups of 10. Treatment commenced when tumours could be reliably measured, i.e. when they had reached minimum dimensions of 4–5 mm. The effect of EO9 on MAC16 was assessed by tumour weight as above 14 days after treatment. The therapeutic effects on MAC26 were assessed by twice-weekly 2-dimensional caliper measurements of the tumour. The tumour volume was calculated from the formula  $(a^2 \times b)/2$  where  $a$  is the smaller diameter and  $b$  the larger. Tumour volumes were normalised with respect to starting volumes and graphs of the relative tumour volumes against time were plotted on semi-log graph paper. The percentage tumour inhibition or T/C% was then calculated. Criteria of activity for all four MAC tumour types can be found in Table 1.

The antitumour activity of EO9 was also tested against the

Table 1. Activity of EO9 against refractory murine colon adenocarcinomas in vivo (single intraperitoneal injection)

Tumour	Dose mg/kg	Schedule	T/C%	Survivors	Activity rating
MAC 15A	9.0	Day 2	14*	0/10	0
	4.0		50		0
	2.6		71		0
MAC 13	9.0	Day 2	Toxic	0/10	0
	4.0		Toxic	0/10	
	2.6		104†	10/10	
MAC 16	4.5	Day 20	Toxic	0/10	++
	3.0		45‡	10/10	
	2.0		51	10/10	
	1.3		88	3/10‡	
MAC 26	4.5	Day 15	Toxic	0/10	+
	3.0		66§	10/10	
	2.0		89	10/10	

\*Based on median survival time; activity rating in % increase in median survival time: <25%=0, 26–50%=±, 51–100%=+, 101–200%=++, 201–300%=+++, >300%=++++.

†Based on mean tumour weight; activity rating in % inhibition <24%=0, 25–49%=±, 50–74%=+, 75–89%=++, 90–95%=+++, 96–100%=++++.

‡Tumour associated deaths.

§Based on tumour volume; activity rating the same as for tumour weight.

Table 2. Antitumour activity of EO9 against murine leukaemia P388 in vivo

Tumour type	Route	Schedule	Dose (mg/kg)	Toxic death	MST* (days)	T/C%
P388	i.p.–i.p.	Once daily × 5	6.0	6/6	Toxic	—
			3.0	0/6	10.0	94
			1.5	0/6	11.3	106
		Control			10.6	100

\*MST=median survival time.

Activity rating in % MST: T/C% ≤120 = inactive.

P388 murine leukaemia model. Tumour origin, mouse host strain, route of administration, schedule, and activity criteria for drug testing have been described in detail elsewhere [19].

**Human tumour xenografts.** EO9 was evaluated in nude mice against a panel of human tumour xenografts (Table 6), which were initially selected on the basis of *in vitro* sensitivity testing [20, 21]. However, because of the low take rate of MAXF401, another breast cancer xenograft (MAXF449) was chosen instead for the *in vivo* evaluation. In addition, the relatively drug-sensitive human ovarian cancer xenograft MRI-H-207 was added to the panel.

Tumours were transplanted as fragments s.c. in both flanks of the mice. Tumour growth was recorded weekly by measuring two perpendicular diameters. The median diameter of all tumours at the start of the treatment was 7 mm [22]. EO9 was given intravenously (i.v.). Two injections at the maximum tolerated dose (MTD) plus a lower dose were given at a 1-week interval in all experiments, in accordance with the standard procedure.

Tumour size (TS) was calculated as the product of length × width ( $TS = a \times b$ ). Relative tumour size (RTS) values were calculated for each individual tumour by dividing the tumour size on day  $X$  by the tumour size at the start of treatment, day 0:

$$RTS = \frac{\text{Tumour size day } X}{\text{Tumour size day 0}} \times 100.$$

Median RTS values were used to construct semilogarithmic growth curves and to calculate treatment efficacy. Tumour doubling time (TD) of test and control groups was defined as the period (days) required to reach a median RTS value of 200%.

Treatment efficacy was assessed by three evaluation criteria used in parallel: specific growth delay (SGD), optimal growth inhibition (T/C%) and tumour growth curve. The SGD was calculated from the TD values of test and control groups as follows:

$$SGD = \frac{TD \text{ treated (days)} - TD \text{ control (days)}}{TD \text{ control (days)}}.$$

Optimal growth inhibition at a particular day within 4 weeks after the last drug injection was calculated from the RTS values of treated versus control groups, as follows:

$$T/C\% = RTS \text{ treated} / RTS \text{ control} \times 100\%.$$

Based on past experience with the models used, a T/C value

of less than 50% and a SGD of more than 1.0 were defined as standard criteria for antitumour activity.

#### Animal toxicology

Animal toxicological studies of EO9 were performed according to the EORTC guidelines as previously reported [23] at TNO-CIVO Institutes (Zeist, The Netherlands). The work was carried out in conformity with Good Laboratory Practice.

**Single dose intravenous lethality study in mice.** The aim of these studies was to determine the LD<sub>10</sub> and LD<sub>50</sub> values with a single intravenous (i.v.) injection of the test compound. Injections were given via the tail vein. After preliminary dose-finding tests, EO9 was administered at five appropriately spaced dose levels ranging 7.0–14.5 mg test substance/kg body weight to groups of 10 male and 10 female Swiss CD1 mice. The EO9 concentration was 1 mg/ml. After dosing, the animals were observed at least daily and morbidity and mortality were recorded. Extensive macroscopic examination of the cranial, thoracic and visceral cavities was performed on mice dying during the observation period of 35 days and those killed at the end of the study. Samples of the main organs were preserved for histology.

From the mortality incidence the LD<sub>50</sub> and LD<sub>10</sub> values were calculated by probit analysis according to Finney [24].

**Single dose i.v. toxicity study in mice.** A supplementary assessment of the acute toxicity of EO9 was carried out using 25 male mice that received a single i.v. dose at the LD<sub>10</sub> value (9 mg/kg body weight in a concentration of 0.5 mg EO9/ml). A control group of 25 males received i.v. saline in the same volume. Mice from both groups were killed at regular intervals during an observation period of 44 days after dosing. Body weights were registered on days 0, 7, 16, 21, 28 and 44. Haematological examinations were carried out on days 7, 16, 28 and 44; histopathological examinations and bone marrow counts were performed on days 16 and 28.

**Multiple dose intraperitoneal (i.p.) study in mice.** The toxicity of EO9 was investigated during multiple dosing and a subsequent recovery period of 28 days. In a preliminary multiple dose-finding study, EO9 could not be given daily i.v. due to severe local irritation at the injection site from the second day of treatment onwards.

After preliminary i.p. dose-finding tests, the main study was performed with a dose of 1.5 mg/kg (0.5 mg/ml) given i.p. daily to 15 male mice from days 0–4, 7–11, 14–18 and 21–25, followed by a recovery period until day 53. A control group of 15 male mice received the vehicle in the same volume as the drug-treated mice. At regular intervals, mice were killed during the dosing

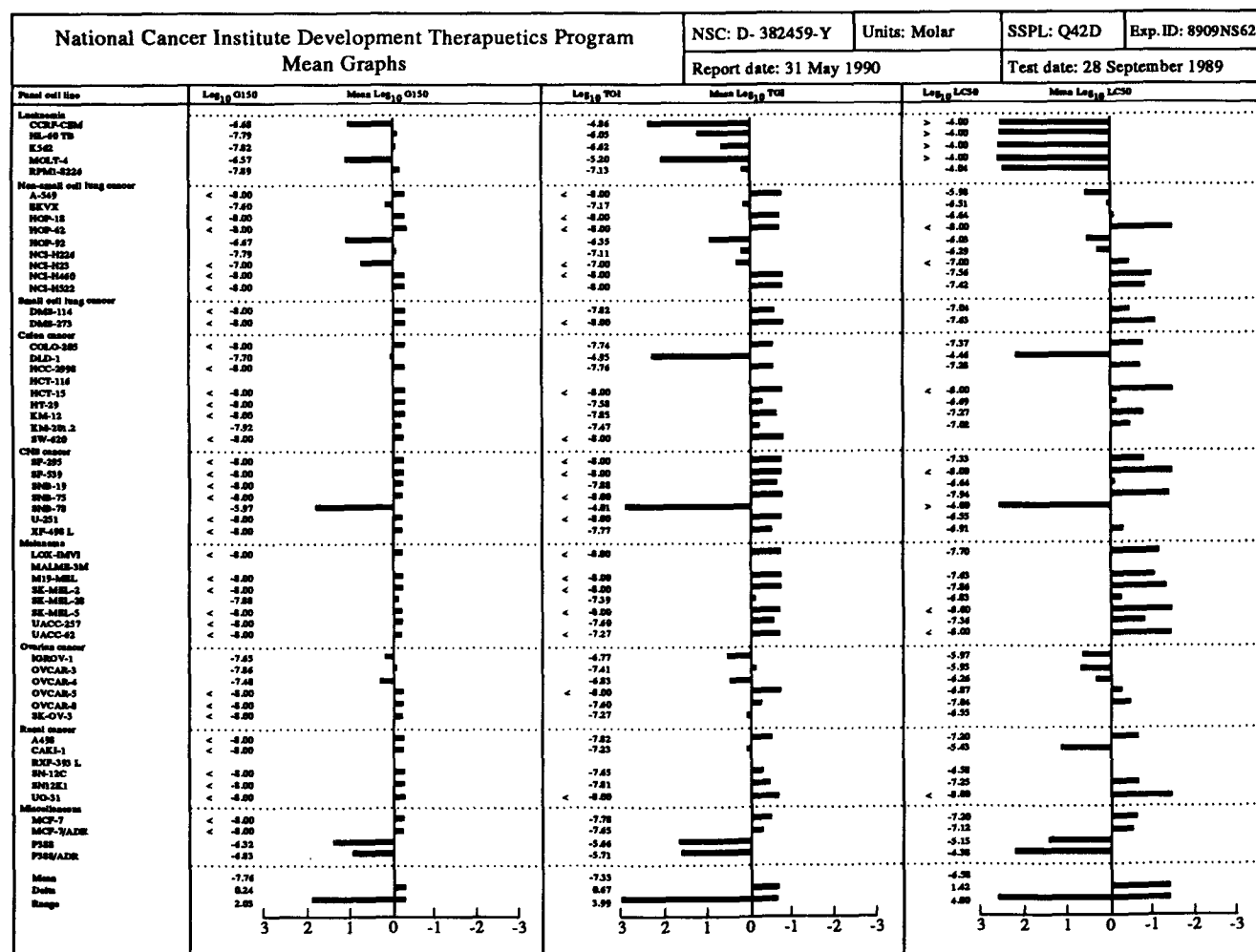


Fig. 2. Profiles of EO9 (a) and mitomycin C (b) in NCI *in vitro* disease-oriented human tumour cell line panel. GI<sub>50</sub>, TGI and LC<sub>50</sub> are interpolated log<sub>10</sub> values representing the concentrations at which the percentage growth is +50, 0, and -50%, respectively (see Materials and Methods).

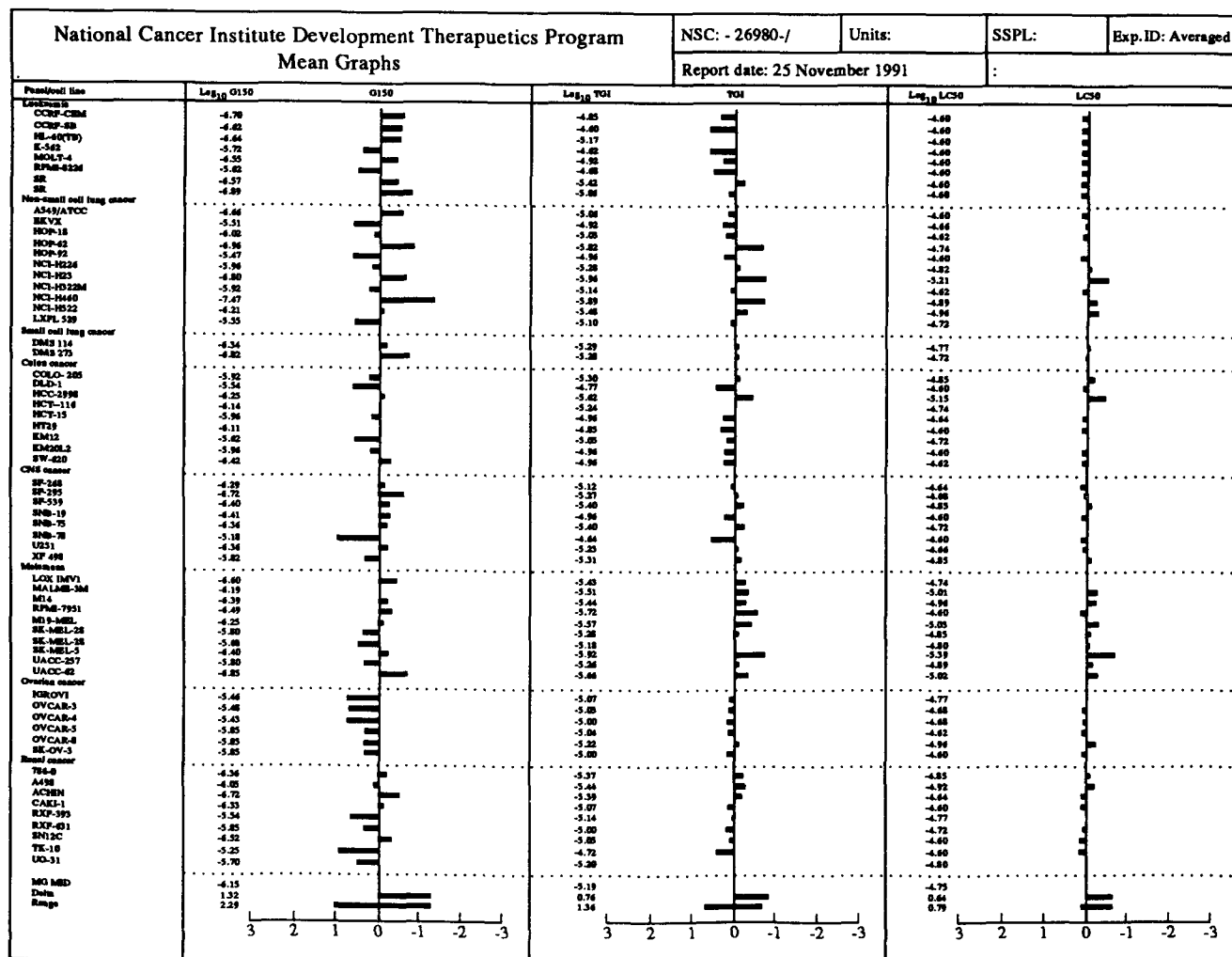


Fig. 2 Continued

and recovery periods. Clinical signs were noted and body weights were determined weekly and at the scheduled killing dates. Haematological examinations, organ weights and histopathology were performed on days 9, 28, 53; bone marrow counts were carried out on day 28.

**Single dose i.v. study in rats.** After the completion of single and multiple dose lethality and toxicity studies in mice, the safety of the one-tenth mouse equivalent LD<sub>10</sub>, the starting dose for clinical phase I studies, was studied in rats as a second species.

A single dose of EO9 (representing one-tenth of the mouse equivalent LD<sub>10</sub>) was administered i.v. in a volume of 1 ml/kg to a group of 10 rats. A control group of 10 males received saline in the same volume. The animals were observed daily for clinical signs. Body weights were registered on day 0, 7 and 14; histopathological examinations and bone marrow counts were performed on day 14.

**Multiple dose i.v. study in rats.** In this test, EO9 was administered i.v. at the one-tenth of the mouse equivalent LD<sub>10</sub> on days 0, 7, 14 and 21 to a group of 10 rats. The control group (10 males) received saline only. Thereafter, there was a recovery period until day 50. Clinical signs were noted daily. Body weights were registered weekly and on the scheduled killing

days. Autopsy, histopathological and haematological examinations were performed on day 23 and 50.

## RESULTS

### In vitro antitumour activity

Figure 2(a) shows the mean graph presentation of GI<sub>50</sub>, TGI and LC<sub>50</sub> results from a test with the indoloquinone EO9 in the NCI human tumour cell line panel. These computer-constructed graphs are centered at the arithmetic mean of the logarithm of the GI<sub>50</sub>, TGI or LC<sub>50</sub> values for the entire panel. Each bar indicates whether the sensitivity of a particular cell line is greater (bar to the right) or less (bar to the left) than the average response. EO9 displayed high potency, yielding GI<sub>50</sub> values <10<sup>-8</sup> mol/l (<3 ng/ml) in the majority of the sensitive lines. The log<sub>10</sub> of the mean GI<sub>50</sub>, TGI and LC<sub>50</sub> values of EO9 were -7.76 mol/l (1.7 × 10<sup>-8</sup> mol/l), -7.33 mol/l (4.7 × 10<sup>-8</sup> mol/l), and -6.58 mol/l (2.6 × 10<sup>-7</sup> mol/l), respectively.

Focusing on the TGI and LC<sub>50</sub> mean graphs, clusters of sensitive tumour cell lines corresponding to colon, melanoma, and central nervous system were identified. A similar degree of sensitivity was observed in some renal and non-small cell lung cancer lines. EO9 demonstrated preferential cytotoxicity against cell lines derived from human solid tumours (49/56) as opposed to the leukaemia lines included in the panel. Notably, the murine P388 leukaemia was among the least sensitive lines tested (GI<sub>50</sub>

$= 4.8 \times 10^{-7}$  mol/l). EO9 was equally cytotoxic to the MCF-7 breast adenocarcinoma cell line and its derivative expressing the multidrug resistance phenotype, MCF-7/ADR; both lines showed a  $GI_{50}$  value of  $<10^{-8}$  mol/l.

Considering the similarities of chemical structure between EO9 and MMC, the mean graph "fingerprint" of the latter is also presented for comparison (Fig. 2b). MMC is one of the standard agents used in the NCI *in vitro* screen and, therefore, has been tested many times. As a result, the mean graph provided is the average value for each of the test parameters for all of the tests carried out in the screen until November 1991. The pattern is strikingly different. MMC was in general less potent than EO9: mean  $\log_{10} GI_{50} = -6.15$  mol/l ( $7.1 \times 10^{-7}$  mol/l),  $\log_{10} TGI = -5.19$  mol/l ( $6.5 \times 10^{-6}$  mol/l),  $\log_{10} LC_{50} = -4.75$  mol/l ( $1.8 \times 10^{-5}$  mol/l). Moreover, except for melanoma, no clusters of MMC-sensitive cell lines could be recognised. Furthermore, in contrast to EO9, no preferential solid tumour activity was observed with MMC. In an earlier version of the mean graph of MMC (August 1989), the average  $GI_{50}$  values of murine leukaemia P388, human breast cell line MCF-7, and their ADR resistant sublines, were determined. P388, P388/ADR and MCF-7 were very sensitive to MMC ( $GI_{50}$  values  $1.6 \times 10^{-6}$ ,  $5 \times 10^{-6}$ ,  $2 \times 10^{-6}$  mol/l, respectively) as compared to the mean  $GI_{50}$  at that time ( $7.1 \times 10^{-6}$  mol/l), whereas MCF-7/ADR was rather resistant ( $GI_{50}$   $25.1 \times 10^{-6}$  mol/l) which is in contrast with the results for EO9.

Using the average  $GI_{50}$ , TGI and  $LC_{50}$  mean graphs for EO9 in comparison with the standard agent database of the NCI in the COMPARE programme [8], no correlation was found with MMC. At the  $LC_{50}$  level a moderate correlation was found with porfiromycin (Pearson correlation coefficient of 0.679). No significant correlation was seen with any other agent in the data base, all correlation coefficient values being  $<0.720$ .

Figure 3 shows the aerobic cytotoxicity of EO9 against EMT6 mouse mammary tumour cells using either a 1 h or a nominal continuous drug exposure, with analysis by MTT dye reduction. Data for MMC, obtained in the same experiment, are presented for comparison. It can be seen in Table 3 that EO9 is 10–20-fold more potent than MMC against this cell line. The comparative cytotoxicity of EO9 was compared towards EMT6 mouse mammary tumour cells under hypoxic versus oxic conditions using a 3 h exposure and analysis by clonogenic assay. Concentrations required to reduce cell survival to 10% of control were 3 and 10 ng/ml for hypoxic and oxic cells, respectively. Thus the hypoxic cytotoxicity ratio was 3.3.

In the Corbett two-tumour assay (Table 4), EO9 again exhib-

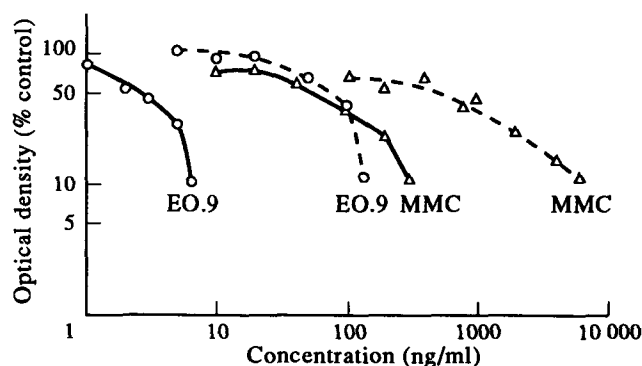


Fig. 3. *In vitro* cytotoxicity of EO9 and mitomycin C against EMT6 mouse mammary tumour cells for 1 h (broken lines) and continuous drug exposure (solid lines) under oxic conditions.

Table 3. *In vitro* cytotoxicity of EO9 and MMC against EMT6 mouse mammary tumour cells under normal oxic conditions (1h vs. continuous drug exposure, MTT assay)

	$IC_{50}$ ng/ml	
	1h	Continuous
EO9	75	2.5
MMC	500	40

Table 4. *In vitro* cytotoxicity of EO9 in the Corbett two-tumour assay

Tumours	$\mu$ g/disk	Tumour zone*		Difference
		Solid	Leukaemia	
Pancreatic 03/L1210	60	950	470	480
Pancreatic 03/P388	60	800	320	480
Colon 38/L1210	50	750	950	-200
	16	710	300	410

\*Data expressed in zone units. The larger the zone, the greater the cytotoxicity; if absolute cytotoxicity  $\leq 75$  zone units than compound considered to be inactive. If the differential between the solid tumours and the leukaemia lines is  $\geq 250$  zone units, the test agent is classified as either solid tumour- or leukaemia-selective.

ited more pronounced growth inhibitory effects in the cell lines derived from solid tumours (Colon 38 and Pancreatic 03) than in the leukaemia representatives (L1210 and P388). Furthermore, in the experiments using a colony forming assay with human tumour xenograft lines and continuous drug exposure (Table 5), EO9 showed significant activity ( $T/C \leq 30\%$ ) against the non-small cell lung cancer, breast, renal and colon tumour cell lines at drug concentrations ranging from 1 to 1000 ng/ml ( $3.5 \times 10^{-9}$ – $3.5 \times 10^{-6}$  mol/l).

Table 5. *In vitro* cytotoxicity of EO9 in the human tumour colony forming assay (continuous drug exposure for at least 7 days) in cells from human tumour xenografts

Tumour type	Line	EO9 concentration ng/ml			
		1	10	100	1000
Breast	MAXF 401	—	++	+++	+++
Colon	CXF 243	—	—	+	+++
Colon	CXF 676	—	—	++	+++
NSCLC	LXFL 529	+++	+++	+++	+++
Renal	RXF 423	—	—	+++	+++
Active/total		1/5	2/5	4/5	5/5

Inhibition of colony formation % T/C:

- $\geq 50\%$
- + 30–50%
- ++ 10–30%
- +++  $<10\%$ .

*In vivo antitumour activity*

EO9 was inactive against the P388 murine leukaemia (Table 2). The results obtained with the MAC tumours are summarised in Table 1. EO9 was inactive against the ascitic murine tumour MAC15A and the subcutaneous tumour MAC13. However, moderate activity was observed in MAC16 and, to a lesser extent, in MAC26 tumours. Notably, these tumours are relatively slow-growing and refractory to most standard agents. In addition, MAC16 tends to display significant central necrosis.

Turning to the human tumour xenografts (Table 6), EO9 induced tumour regression in the gastric cancer GXF97 and in the ovarian cancer MRI-H-207. Furthermore, a growth delay was observed in breast cancer MAXF449, and marginal activity was seen in the non-small cell lung cancer LXFL529. No antitumour activity was found in the renal cancer RXF243. When a third injection of EO9 was administered to MRI-H-207 bearing nude mice, the compound induced almost complete remissions.

In the experiments above, MMC was tested simultaneously against GXF97 and MRI-H-207. A single i.v. injection of 2 mg/kg MMC caused tumour regression in GXF97 (T/C 5.3%), and in MRI-H-207 two weekly i.v. injections of 5 mg/kg induced complete remission.

Considering that preliminary pharmacokinetic data indicated an extremely short plasma elimination half-life of EO9 in mice [25, 26], experiments were conducted in the MRI-H-207 tumour to evaluate whether frequent dosing on days 0 and 7 would improve efficacy as compared to single administration on the same days. Similar growth inhibition was observed in both schedules (Table 7). It should be noted however that although the frequent dosing would increase the overall exposure, a steady state would not be achieved.

*Animal toxicology*

*Single dose i.v. lethality study in mice.* Transient sluggishness, piloerection and pallor were seen in the animals 48 h following drug administration (at doses  $\geq$  8.4 mg/kg and 14.5 mg/kg in male and female animals, respectively). Transient irritation at the site of injection was observed in most animals. Mortality occurred between day 3 and day 25 after dosing. No specific drug-related macroscopic changes were observed at autopsy. The LD<sub>50</sub> values were 12.99 mg/kg (95% confidence limits:

Table 6. Antitumor activity of EO9 against human tumour xenografts in vivo (intravenously at day 0 and 7)

Tumour type	Line	Dose (mg/kg)	Toxic death	Optimal (%)	T/C%* (day)	SGD 1-2
Breast	MAXF 449	4.0	2/5	67.4	21	0.7
		6.0	0/5	49.2	28	1.6
	GXF 97	6.0	2/5	9.0	28	2.6
		4.0	0/6	67.4	21	0.3
Non-small cell lung cancer	LXFL 529	6.0	0/6	52.0	14	1.0
		5.0	0/7	3.8 <sup>†</sup>	24	NA
	MRI-H-207	6.0	1/7	2.4 <sup>†</sup>	24	NA
		5.0 <sup>‡</sup>	0/5	0.2 <sup>†</sup>	23	NA
Renal	RXF 423	4.0	0/4	98.6	21	0.1
		6.0	1/5	90.6	14	-0.03

\*Based on relative tumour size. NA = not applicable.

<sup>†</sup>Based on relative tumour volume. SGD = specific growth delay.

<sup>‡</sup>Schedule: i.v. administration at day 0, 7, and 14.

Table 7. Antitumour activity of EO9 (intraperitoneally) in various dose schedules against human ovarian xenograft MRI-H-207 in female NMRI nude mice

Dose (mg/kg)	Schedule (day)	Toxic death	Optimal (%)	T/C%* (day)	SGD 1-2
7.2	0,7	1/6	4	17	8.7
5.0	0,7	0/5	16	10	0.8
1.2	every h $\times$ 6; 0,7	0/5	4	17	7.6
0.8	every h $\times$ 6; 0,7	0/5	29	23	0.3

\*Based on relative tumour volume.

11.6–15.4 mg/kg) and 13.95 mg/kg (12.3–17.0 mg/kg) in male and female mice, respectively. The LD<sub>10</sub> values were 9.23 mg/kg (7.4–10.5 mg/kg) and 9.91 mg/kg (7.9–10.5 mg/kg) in male and female animals, respectively.

*Single dose i.v. toxicity study in mice.* On the basis of the LD<sub>10</sub> determined in the previous study, a dose level of 9.0 mg/kg was chosen for toxicology studies. Body weights and bone marrow counts were comparable in test and control mice. A slight anaemia was observed during the first week after dosing. Thereafter, blood values of treated mice were comparable to those of control mice. There were grossly visible signs of irritation at the site of injection in the tail in most treated animals for several days after treatment. Gross examination at autopsy did not reveal any additional treatment-related changes. During microscopic evaluation, signs of inflammatory reaction in the tail at the injection site were confirmed.

*Multiple dose i.p. toxicity study in mice.* The dose of 1.5 mg/kg did not cause drug-related deaths in these experiments. Body weights of drug-treated animals were lower than controls from day 9 until day 28 with recovery after day 28. During the dose period a slight anaemia was observed, which disappeared during the recovery period. Though the total numbers of leucocytes in treated and control mice were comparable, a small increase in numbers of neutrophils and a small decrease in numbers of lymphocytes were observed in EO9 treated mice during the whole observation period. Minor changes in organ weight were observed in testes, spleen, liver and kidneys. However, the only abnormalities on gross and microscopic examination were congestion of blood vessels and haemorrhagic spots in the jejunum on days 9 and 28. No significant bone marrow effects were observed.

*Single dose i.v. study in rats.* A dose of one-tenth of the mouse equivalent LD<sub>10</sub> (2.7 mg/m<sup>2</sup>) was found to be safe in rats. Body weights of treated (0.45 mg/kg) and control rats were comparable. There were no changes in haematological parameters or organ weights. Gross examination at autopsy did not reveal any treatment-related changes. Upon microscopy, no bone marrow toxicity was found.

*Multiple dose i.v. study in rats.* Multiple dosing of EO9 at 2.7 mg/m<sup>2</sup> or 0.45 mg/kg did not induce treatment-related macroscopic or microscopic changes, except for inflammation at the site of injection in the tail. No myelotoxicity was observed. Therefore, the dose of 2.7 mg/m<sup>2</sup> was recommended as the starting dose in human trials.

Table 8 summarises the main findings of animal toxicology studies of EO9.

### DISCUSSION

EO9 is a novel synthetic bioreductive indoloquinone, structurally-related to MMC but exhibiting several important differences. In this study, we demonstrate that EO9 exerts significant antitumour effects in various preclinical tumour models. Notably, it possesses preferential *in vitro* activity against solid tumour cell lines with minimal effects against leukaemia models. Relatively low concentrations of EO9 proved to be effective against a broad spectrum of human solid tumour lines in the NCI disease-oriented panel. For example, EO9 showed especially good activity against colon, melanoma and central nervous system cell lines. In comparison to MMC, EO9 was a more potent antiproliferative agent in the disease-oriented human tumour cell line panel, showing a 2–3 log difference in GI<sub>50</sub> values and also a clearly distinct cytotoxicity profile. Similar results were observed previously in a panel of human (K562, HCLo, HCT-18, HRT-18, HT-29, DLD-1, MCF-7) and

murine (MAC15A, MAC16, MAC26, WEHI-3B) tumour lines using a 96 h continuous exposure [6].

EO9 exerted similar effects on MCF-7 and its multidrug resistant variant, MCF-7/ADR, which is resistant to MMC. Previously, Stratford *et al.* [27] have demonstrated that EO9 was equally effective against the CHO-K1 cell line and its MMC-resistant variant.

Reduction in pH decreases the chemical stability and increases the cytotoxicity of EO9 and MMC in a similar way [6, 28, 29]. However, the observed marked differences in cytotoxicity profile between EO9 and MMC *in vitro* support enzymological observations indicating that these quinone agents may differ in their mechanism of action. A particular line which is being pursued is the potentially more prominent role of the enzyme DT-diaphorase (DTD) in the bioactivation of EO9. Thus, at physiological pH, EO9 was shown to be an excellent substrate for reduction by rat and human DTD and activation of EO9 but not MMC by the purified Walker rat tumour enzyme was shown to produce species which generate single strandbreaks in co-incubated plasmid DNA [4, 30]. Moreover, DTD-rich Walker tumour cells develop both DNA single strandbreaks and DNA crosslinks after exposure to EO9 [5]. Furthermore, a direct correlation between DTD activity in the tumour and sensitivity to EO9 has been demonstrated in the MAC16 and 26 tumours *in vivo* and in a pair of human colon carcinoma cell lines *in vitro* [31, 32]. An additional correlation was seen within a larger *in vitro* panel [33]. In contrast, although MMC is bioactivated by DTD at low pH [34], this reaction is not seen at physiological pH [3, 4, 35]. Since other enzymes and additional factors are also likely to play a role in the sensitivity to EO9, it will be important to establish the overall significance of DTD. In particular, Workman and co-workers have recommended an "enzyme-directed" approach to the use of bioreductive agents based on the enzyme profile of the target human tumour [36, 37]. Thus patients who might show a particularly good response could be selected on the basis of an advantageous enzyme profile (e.g. a high DTD content).

*In vivo*, EO9 showed antitumour activity in two (MAC16 and MAC26) out of four refractory murine adenocarcinomas and in three out of five human tumour xenografts: gastric GXF97, ovarian MRI-H-207, and breast MAXF449. No activity was observed in the P388 murine leukaemia *in vivo*. Antitumour activity has also been shown in human small cell lung cancer xenograft OC-Tol [38]. These *in vivo* data confirmed the broad spectrum of activity and the preference for solid tumours observed in the *in vitro* experiments.

The directly comparative results of MMC in GXF97 and MRI-H-207 reported here, together with historical data of MMC in LXFL529, MAXF449, and RXF423 (not shown) which were similar to EO9, indicated that both compounds had comparable activity profiles in this small panel of human solid tumours.

Animal toxicological studies of EO9 identified gastrointestinal tract toxicity as dose-limiting. Interestingly, no significant bone marrow effects were observed in these studies. Considering that the clinical use of MMC is mostly limited by the occurrence of myelotoxicity, the lack of bone marrow toxicity of EO9 represents a highly favourable characteristic of this compound.

The observation of enhanced antitumour effects of EO9 under hypoxic conditions is also noteworthy. We show here that EO9 exhibits a 3-fold greater killing of hypoxic compared to oxic EMT6 mouse mammary tumour cells. Moreover, EO9 has also demonstrated preferential cytotoxicity against hypoxic V79 and KHT cells *in vitro* and again there was an improvement in the hypoxic cell specificity over MMC [27, 39]. Moreover,

Table 8. Summary of animal toxicology studies of EO9

Type of study	Dose and schedule	Toxicities
Studies in mice		
Lethality	Single dose i.v.	LD <sub>10</sub> = 9.2 mg/kg (7.4–10.5)* males 9.9 mg/kg (8.0–11.3)* females LD <sub>50</sub> = 13 mg/kg (11.6–15.4)* males 14 mg/kg (12.3–17.0)* females
Acute toxicity	Single dose i.v. 9 mg/kg	Transient anaemia No changes in total white blood cell counts and leucocyte subpopulations No bone marrow toxicity
Chronic toxicity	Multiple dose i.p. 1.5 mg/kg, ×5 for 4 weeks once a day	Transient anaemia Increased neutrophil and decreased lymphocyte numbers No bone marrow toxicity Reversible changes in gastrointestinal tract (local haemorrhage and necrotic spots, congested blood vessels in jejunum, lymphoid hyperplasia in Peyer's patches)
Safety studies in Wistar rats		
Acute toxicity	Single dose i.v. 0.45 mg/kg	No changes in red and white blood cell count No bone marrow toxicity No treatment related changes
Chronic toxicity	Multiple dose i.v. 0.45 mg/kg once a week ×4	No changes in red and white blood cell counts No bone marrow toxicity No treatment related changes

\*95% confidence limits.



substantial activity towards hypoxic cells was also noted in the KHT sarcoma *in vivo* [37]. In addition, preliminary studies have indicated that the cytotoxicity of EO9 in mice can be increased by co-administration of the vaso-active drug hydralazine (Bibby *et al.*, manuscript in preparation). Taking into account that hypoxia is considered to be one of the reasons for the failure of cytotoxic therapy in solid tumours in the clinic, hypoxic cell cytotoxicity might represent a favourable characteristic of this compound. Nevertheless, in contrast to such developmental bioreductive agents as the aziridinyl nitroimidazole RB6145 and the benzotriazine di-*N*-oxide SR4233 [39, 40] EO9 does retain considerable potency for killing aerobic tumour cells as well. This may again be a unique and attractive feature for EO9. Whereas the above-mentioned agents would be less likely to exhibit single agent activity, EO9 does have the capacity to do this, as shown here by the *in vivo* antitumour studies. Nevertheless EO9 might find optimal use in combination with radiation or other drugs. In the latter context, the lack of myelosuppression for EO9 may be a distinct advantage.

On the basis of its uniquely interesting and attractive preclinical antitumour activity and toxicology profile, EO9 has been selected for clinical evaluation within the framework of the EORTC. A phase I clinical trial of this compound given by the i.v. bolus every 3 weeks administration is well underway, and a second study using weekly administration has been activated.

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## Feature Articles

# An Unusual Case of Haematuria

Jim Cassidy and Stanley B. Kaye

A 40-YEAR-OLD MAN presented with a 3-month history of painless haematuria. He had been tetraplegic from the age of 21 following a road traffic accident, and had a neurogenic bladder with frequent episodes of urinary infection. He was otherwise well with no history of weight loss and no symptoms referable to the respiratory or cardiovascular systems. He was a life-long non-smoker, lived with his wife and an adopted daughter. Despite his disability he was in full-time employment as a bank clerk. He had no prior exposure to chemicals or asbestos, and at presentation was taking only muscle relaxants for lower limb spasms.

Examination on referral revealed that he was tetraplegic in keeping with his history of cervical trauma. He was otherwise well with no evidence of weight loss and no palpable lymphadenopathy. Examination of the chest and abdomen revealed no abnormality. At presentation he had an indwelling urinary catheter.

His full blood count was normal, but routine biochemistry revealed the following abnormalities; sodium 124 mmol/l [normal range (NR) 135–144] chloride 89 mmol/l (NR 97–108), creatinine 59  $\mu$ mol/l (NR 60–110), calcium 2.19 mmol/l (NR 2.20–2.65) with a normal serum albumin at 37 g/l. Liver transaminases, gamma glutaryltranspeptidase and bilirubin were all within normal limits. Chest X-ray was normal and urinary cytology was uninformative.

An intravenous urogram and cystogram was performed which revealed some clubbing of the renal calyces with loss of cortex but the appearances had not deteriorated in comparison with a prior examination some 5 years ago. The bladder had a typical neurogenic appearance with marked wall thickening but no space-occupying lesion could be demonstrated, even after direct introduction of contrast to the bladder via the urinary catheter.

He then proceeded to cystoscopy which revealed a white lesion occupying the dome of the bladder. Biopsies were taken which revealed infiltration of the bladder wall and muscle by a undifferentiated small cell carcinoma. Histologically this was indistinguishable from bronchial small cell carcinoma. Staining for gonadotrophins was negative, no other special stains were used. Tumours of this type are rare but can occur as primary bladder carcinoma and carry a poor prognosis [1]. In histological appearances they are indistinguishable from small cell bronchial carcinoma, and tend to behave in a similarly aggressive manner. Ectopic hormone production has been recorded [2, 3]. The pathological diagnosis prompted the performance of an isotope bone scan which was normal, and a computed tomography (CT) scan of abdomen and pelvis. The CT appearances of the lung and mediastinum were normal. The bladder was seen to be contracted with tumour infiltration through the wall; there was evidence of bilateral involvement of the lymphatics of the external and common iliac chains. The appearance of the kidneys was in keeping with chronic pyelonephritis. No other abnormality was seen.

Our current treatment policy in "good prognosis" bronchial small cell carcinoma is intensive combination chemotherapy followed by consolidation radiotherapy to the chest. Therefore, we decided to adopt a similar treatment for this patient. We anticipated that myelosuppression may be very troublesome in view of our experience with this regimen and because of his chronic bladder infections we were concerned that this might further compromise his tolerance of therapy, we therefore chose to use doses around 25% lower than in lung cancer patients. Bone marrow was not performed as this is not currently part of our staging policy for bronchial small cell carcinoma. He was commenced on ICE (ifosfamide 5 g/m<sup>2</sup>, mesna 8 g/m<sup>2</sup>, carboplatin 300 mg/m<sup>2</sup>, etoposide 150 mg days 1 and 2 intravenously and 300 mg orally on day 3, all given in a 4-week cycle), with continuous administration of a co-trimoxazole (two tablets twice daily) as prophylaxis of neutropenic septicemia. On day 12 he was readmitted with a fever over the preceding 24 h. On

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