Pergamon

European Journal of Cancer Vol. 30A, No. 7, pp. 1013–1019, 1994

0959-8049/94 $7.00+0.00

0959-8049(93)E0062-9

Factors Affecting Sensitivity to EO9 in Rodent and Human Tumour Cells *In vitro*: DT-Diaphorase Activity and Hypoxia

N. Robertson, A. Haigh, G.E. Adams and I.J. Stratford

Twenty-three human tumour cell lines (lung, breast, and colon) and eight rodent cell lines were evaluated for their sensitivity to the quinone-based anticancer drug EO9 [3-hydroxymethyl-5-aziridinyl-1-methyl-2-(1H indole-4,7-dione)prop-β-en-α-ol]. Sensitivity was compared with the intracellular levels of DT-diaphorase, and cell lines showing highest enzyme activity tended to be the most sensitive to EO9. The role of DT-diaphorase in determining drug sensitivity was confirmed by using the enzyme inhibitor dicoumarol, which protects cells containing high levels of DT-diaphorase from the cytotoxic action of EO9. Hypoxia increased the cytotoxicity of cells containing low but not high levels of DT-diaphorase, implying that both 1- and 2-electron reductive activation processes can be important for expression of EO9 toxicity. It is concluded that EO9 is a potentially useful agent in the enzyme directed approach to the use of bioreductive drugs in cancer therapy.

Key words: EO9, bioreductive drugs, DT-diaphorase, hypoxia
Eur J Cancer, Vol. 30A, No. 7, pp. 1013–1019, 1994

INTRODUCTION

EO9 (3-hydroxymethyl-5-aziridinyl-1-methyl-2[H-indole-4,7-dione]-prop-β-en-α-ol; NSC382459) has recently entered phase I clinical trials in Europe under the auspices of the EORTC (European Organisation for Research and Treatment of Cancer). This agent was originally synthesised as an analogue of mitomycin C [1], but extensive screening against tumour models *in vitro* and *in vivo* showed the two drugs to have substantially different patterns of antitumour activity [2, 3]. Mitomycin C can be reductively activated by the obligate two-electron-donating flavoenzyme DT-diaphorase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC.1.6.99.2][4, 5]. However, in comparison, EO9 is a far more efficient substrate than mitomycin C for both human and rat DT-diaphorase which may explain, at least in part, the differences in cellular sensitivity to the two drugs [6]. Metabolism of EO9 by purified DT-diaphorase from rat Walker tumour cells results in substantial damage to plasmid DNA [6]. It is therefore possible that cellular expression of DT-diaphorase will be a major factor influencing response of cells to EO9. In a preliminary study by Robertson and colleagues [7] of 15 human breast, lung and colonic cell lines having a 500-fold range in DT-diaphorase activity, it was shown that high DT-diaphorase levels were clearly associated with drug sensitivity. A consequence of this is the potential application of EO9 to the 'enzyme-directed' approach to bioreductive drug development, first put forward by Workman and Walton [8]. Thus, tumours suitable for treatment with EO9 could be selected on the basis of high levels of DT-diaphorase. Some evidence supporting this approach has been obtained with a pair of mouse colon tumours *in vivo* [9]. In this work, the tumour showing a 16-fold higher level of DT-diaphorase activity was more responsive to EO9 than the tumour expressing lower levels of enzyme.

Adams and colleagues [10] carried out a study *in vivo* of the effects of EO9 in the murine KHT sarcoma in combination with X-irradiation. EO9 alone had no effect in this tumour and this is consistent with the low levels of DT-diaphorase measured in cells from KHT tumours (97 nmoles cytochrome c reduced/min/mg protein; Robertson, unpublished results). However, when given after irradiation, EO9 substantially increased the killing of KHT tumour cells. The dose of X-rays used in these experiments was 10 Gy, which is sufficient to sterilise the majority of aerobic tumour cells [11], leaving the residual radiation resistant hypoxic cell population. It is these hypoxic cells that EO9 is able to kill when given post-irradiation. This hypoxia-mediated bioreductive drug activity *in vivo* is similar to that observed for agents such as the alkylating nitroimidazole, RB 6145 [12] and the benzotriazene-di-*N*-oxide, tirapazamine (SR 4233) [13, 14]. The ability of EO9 to kill hypoxic KHT tumour cells was subsequently confirmed *in vitro* [15].

The purpose of this work is to extend initial observations of the dependence on DT-diaphorase for sensitivity of human tumour cells to EO9, and determine the effect of hypoxia on drug sensitivity in these cells. Further, a panel of rodent cells with deficiencies in DNA repair and/or levels of cytochrome P450 reductase or DT-diaphorase, have been used to gain further insights into the mechanisms of action of this indoloquinone bioreductive drug.

MATERIALS AND METHODS

Cells and culture

Table 1 lists the human tumour cells used in this work. The lung lines are representative of the major histological types seen

Correspondence to I.J. Stratford.
The authors are at the MRC Radiobiology Unit, Chilton, Didcot, Oxfordshire OX11 0RD, U.K.
Revised 13 Dec. 1993; accepted 21 Dec. 1993.

Table 1. DT-diaphorase activity and sensitivity to EO9 in human cells in vitro

Cell line	Origin	IC_{50}(nM) EO9	DT-diaphorase activity
HBL 100	Breast	1270	<1†
MCF7		89	560
MDA 231		285	8
MDA 468*		1720	63
SkBr3		22	1750
T47D*		5110	39
ZR75*		580	2110
H249*	SCLC	3710	2.5
N417*		210	116
H526*		165	7.5
H841*		51	440
L47		15	415
L51		112	14
L88		42	1920
L279		160	3.5
A549*	NSCLC	7	4980
H226*		79	1000
H322*		15	4510
H358*		19	3270
H460*		4	5020
H522*		32	550
H647*		9	4970
HT29	Colon	157	2750

*Results quoted are an update of those reported previously [7]. †Below the limit of detection, which in this system is ~ 1 nmole cytochrome C reduced/min/mg protein.

clinically. The non-small cell lung cancer (NSCLC) cell lines include three adenocarcinomas, two bronchio-alveolar, one large cell and one squamous carcinoma cell line. The breast cancer cell lines are derived from adenocarcinomas. The panel also includes the breast epithelial cell line HBL100 which is immortalised but non-tumorigenic.

Rodent cell lines and mutants derived from them are listed in Table 2. IRS-1 cells were derived from V79 cells by mutation with EMS [16], and are characterised by sensitivity to radiation [16] and mitomycin C [16, 17]. CHO-MMC® cells were produced by sequential exposure of CHO-K1 cells to increasing concentrations of mitomycin C [18]. These cells are characterised by mitomycin C resistance [17, 18], which is thought to be due to impaired ability to activate the drug because of low levels of cytochrome P450 reductase [18]. AA8 is another wild type CHO cell line, and UV-41 an excision repair deficient mutant derived from it that exhibits hypersensitivity to mitomycin C [19]. S9-32 was isolated by selection of UV-41 cells in 2 ng/ml mitomycin C and S162 was isolated from a repair proficient transfectant of UV-41 by selection in 200 ng/ml mitomycin C [20]. S9-32 is excision repair deficient while S162 is repair proficient; both cell lines show 4 to 10 times lower levels of activity of DT-diaphorase compared to AA8 and UV-41, respectively [21].

Table 2. Values of IC_{90} for pairs of rodent cell lines exposed to EO9 for 3 h under aerobic or hypoxic conditions

Cells		IC_{90} (μM) Air	IC_{90} (μM) N_2	Differential toxicity
V79		0.18	0.015	
IRS-1		0.12	0.0025	12
	Ratio	1.5	6	48
CHO-AA8		0.5	0.03	
CHO-UV41		0.022	0.001	17
	Ratio	23	30	22
CHO-K1		0.67	0.03	
CHO-MMC®		8.3	0.17	22
	Ratio	0.08	0.18	49

All cell lines were maintained in exponential growth phase in RPM1 1640 medium supplemented with 10% fetal calf serum, penicillin and streptomycin.

Drug sensitivity

MTT assay. This proliferation assay is based on the ability of viable cells to convert a soluble tetrazolium salt, MTT (3-4,5-dimethylthiazole-2,5-diphenyl tetrazolium bromide) into purple formazan crystals [22]. The optical density of the dissolved crystals is proportional to the number of viable cells, although the absorbance varies with cell line as the assay depends on the activity of mitochondrial dehydrogenases [23]. The conditions for carrying out the assay in human tumour cells have been described previously [7, 23]. Initial experiments to determine cellular sensitivity in air involved plating 10^3–10^4 cells (depending on cell line) into each well of a 96-well plate prior to drug exposure. Cells were than exposed to the drug continuously for 4 days at 37°C. Alternatively, 3-h drug exposures were used, in which 2×10^4–10^5 cells were plated into each well of a 24-well plate (glass or plastic wells, as appropriate) 2 h prior to drug exposure. Cells were then exposed to drug for 3 h at 37° in air or hypoxia, or in the presence of dicoumarol. The drug was then removed, 0.5 ml of fresh medium was added and the cells allowed to grow for 3 days. Subsequently, MTT was added and cells incubated for a further 4 h. After this time, culture medium and unconverted MTT were removed, the formazan crystals dissolved in DMSO and optical density measured on a multiwell spectrophotometer (Titertek Twin reader, ELISA, Flow-Labs, High Wycombe, U.K.) using a 550 nm filter with blanking at 700 nm. Values of IC_{50}, the concentration of EO9 required to reduce optical density to 50% of the control, was used as a measure of cellular sensitivity to a given treatment.

Clonogenic assay. For the Chinese hamster cells, this was carried out by seeding 200 cells from an exponentially growing culture into 6 cm glass or plastic plates, and allowing them to attach for 2 h. Subsequently, cells were treated with various concentrations of EO9 for 3 h at 37°C under either hypoxic (glass plates) or aerobic conditions. Drug was then removed, replaced with fresh medium and surviving cells scored as colonies 7–10 days later. Values of IC_{90}, the concentration of EO9 required to reduce survival by 90% compared to untreated controls, were derived from at least three independent experiments, and are used as the measure of cellular sensitivity.

Enzyme activity. For measurement of enzyme activity, cells were harvested, pooled and centrifuged. Pellets were washed twice in ice cold PBS and finally resuspended in a hypotonic buffer (10 mM HEPES/KOH pH 7.9, 1.5 mM MgCl, 10 mM KCl, 0.5 mM DTT). The suspensions were homogenised and centrifuged at 4000 rpm for 10 min (4°C). Supernatants were flash frozen and stored in liquid nitrogen. DT-diaphorase activity was measured at 37°C using menadione as the substrate

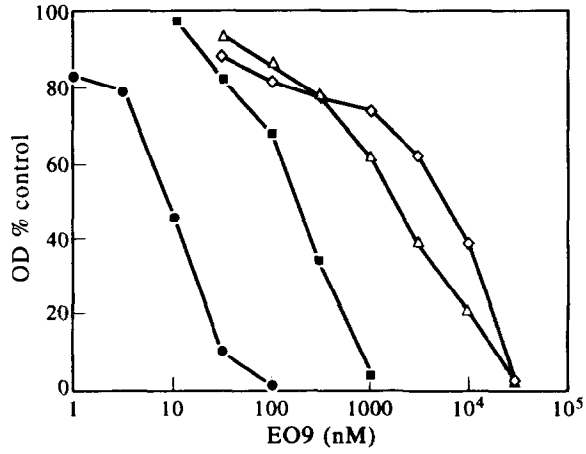

Figure 1. The sensitivity of human tumour cells to EO9 following a 4-day exposure in air as determined by the MTT assay. ●, A549; ■, HT29; △, MDA 468; ◇, T47D.

and cytochrome C as the terminal electron acceptor. Activity was calculated as that fraction which was inhibited by dicoumarol. Values quoted are nmoles cytochrome C reduced per min per mg protein.

RESULTS

Human tumour cells

Initial experiments were carried out to determine cellular sensitivity to EO9 following a 4-day exposure in air. Typical survival curves derived from individual experiments are given in Figure 1, and illustrate the wide range of sensitivity (1000-fold) that exists within this panel of cell lines. From such curves, values of IC_{50} can be obtained. Geometric mean values of IC_{50} were calculated from three to six replicate experiments, and these are listed for each cell line in Table 1.

Measurements of the level of activity of DT-diaphorase were made, and results derived from between three and 27 individual determinations for each cell line are recorded in Table 1. The enzyme activity in this panel of cell lines covers a 5000-fold range. The NSCLC show a uniformly high level of DT-diaphorase. In contrast, enzyme activity is more variable and generally lower in the breast and small cell lung cancer (SCLC) cell lines.

The dependence of cellular sensitivity to EO9 on DT-diaphorase activity is illustrated in Figure 2, which updates and extends

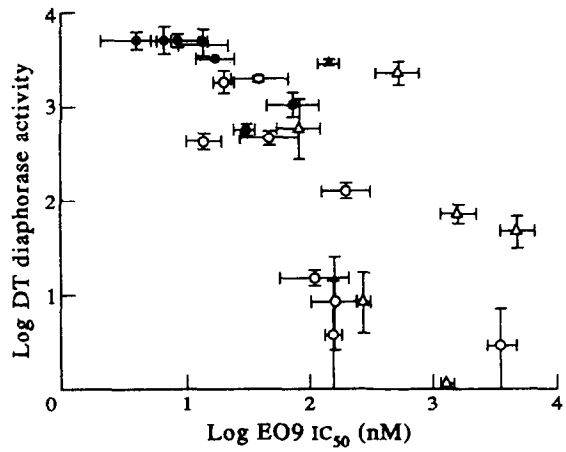

Figure 2. A comparison of EO9 sensitivity (4-day exposure in air) and DT-diaphorase activity in human cell lines *in vitro*. ●, NSCLC; ○, SCLC; △, breast; ▲, colon. Bars indicate standard errors of the mean.

our original observations [7]. This data shows a clear trend for high DT-diaphorase activity to be accompanied by sensitivity to EO9.

The importance of DT-diaphorase in the activation and toxicity of EO9 in the human tumour cells has been further evaluated by carrying out short-term, 3-h aerobic exposures to EO9 in the presence of 0.2 mM dicoumarol, a potent inhibitor of DT-diaphorase activity. A set of survival curves are shown in Figure 3 for a cell line that expresses high levels of DT-diaphorase, H322, and a cell line with low activity, T47D. In the H322 cells that are sensitive to EO9, dicoumarol provides substantial protection against the action of the bioreductive drug. The change in IC_{50} is about 40-fold. In contrast, the low expressing cell line, that is resistant to EO9, is not protected by dicoumarol. In fact, there appears to be a small increase in sensitivity. Values of IC_{50} were determined from at least three replicate paired experiments and these values are given in Table 3 for a range of cell lines. These results further confirm the importance of DT-diaphorase in determining the activity of EO9 in air, since those cell lines with high enzyme activity are protected by dicoumarol, whereas no effect is observed in the two lines tested that have low activity of DT-diaphorase.

Previously published data have indicated that EO9 is more potent when given to cells under anaerobic conditions [15, 24]. Therefore, the human lines were used to assess the influence of DT-diaphorase on the expression of EO9 toxicity in hypoxic cells. Figure 4 shows a set of survival curves for H322 and T47D cells that have units of DT-diaphorase activity of 4510 and

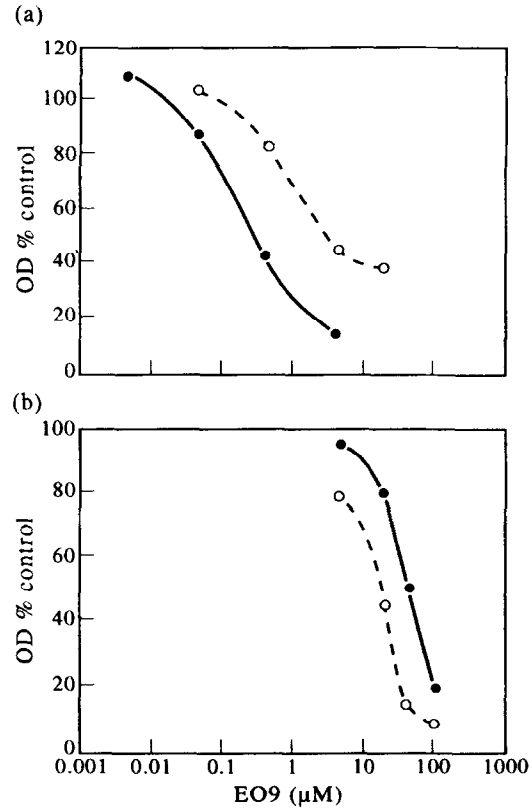

Figure 3. Survival curves showing the effect of 0.2 mM dicoumarol on the activity of EO9 in cell lines having either (a), high (H322) or (b), low (T47D) levels of DT-diaphorase activity. ●, EO9 alone; ○, EO9+dicoumarol. Cells were exposed to drug(s) for 3 h in air, washed and allowed to grow for a further 4 days before use of the MTT assay.

Table 3. The effect of 3-h exposure to EO9 on the sensitivity of human tumour cells under aerobic or hypoxic conditions

Cell line	IC_{50} (μM) Air	N_2	Differential toxicity
A549	0.1	0.06	1.7
H-647	0.3	0.2	1.5
H-322	0.2	0.2	1.0
HT29	0.2	0.2	1.0
MDA 468	15.1	0.05	302
T47D	38.6	0.3	129

Table 4. Effect of dicoumarol on the sensitivity of human tumour cells exposed to EO9 for 3 h in air

Cell line	IC_{50} (μM) EO9	EO9+DIC	Protection factor
A549	0.1	0.7	7.0
H-647	0.3	7.8	26.0
H-322	0.3	11.3	38.7
HT29	0.2	7.0	35.0
MDA 468	19.7	10.0	0.5
T47D	46.7	22.5	0.5

39, respectively. In the high expressing cell line, the gaseous conditions have no influence on EO9 sensitivity. In contrast, in T47D cells, the sensitivity to EO9 is increased over 100-fold when exposure is in hypoxia. Values of IC_{50} were determined from at least three replicate paired experiments and these values are given in Table 4 for a range of cell lines. These results provide additional data showing that hypoxia has little effect in those cell lines that have high activities of DT-diaphorase and are already sensitive to EO9.

However, the two cell lines examined that have low DT-diaphorase activity show substantial increases in sensitivity when exposure is in hypoxia. This implies that one-electron reduction processes, that are potentially reversible by oxygen, can be important in the activation of EO9, but this is only revealed when exposure to drug is carried out under anaerobic conditions in cells with low DT-diaphorase activity.

Rodent cell lines

The role of DT-diaphorase in the activation and toxicity of EO9 has been further examined by using cell lines derived from CHO-AA8 that show lower enzyme activity [21]. Cell kill curves derived by clonogenic cell survival are given in Figure 5. The value of IC_{90} for the repair proficient AA8 wild type line is 0.5 μM. This is increased to 7 μM for the S162 cells that are also repair proficient [20, 21], but deficient in DT-diaphorase relative to the AA8 cells. The IC_{90} for the repair deficient UV-41 cells is 22 nM. This 20-fold increase in sensitivity compared to the AA8 cells suggests DNA as a target for the action of EO9, and implies that cross-link formation may be important for the expression of EO9 toxicity. The S932 cells are repair deficient and also have low levels of DT-diaphorase. The IC_{90} for these cells is 0.25 μM, which is 10-fold higher than the UV41 cells from which they are derived. Those results further support the contention that DT-diaphorase is important in determining the toxicity of EO9 in air.

Pairs of Chinese hamster cell lines have been evaluated for their sensitivity to EO9 in air or in hypoxia. All these cell lines are low in DT-diaphorase compared to the majority of the human cells referred to above ([18, 21]; Robertson unpublished), so it might be expected that exposure under hypoxic conditions would result in greater cell kill by EO9 than is seen in air. This is illustrated by the examples of representative paired survival

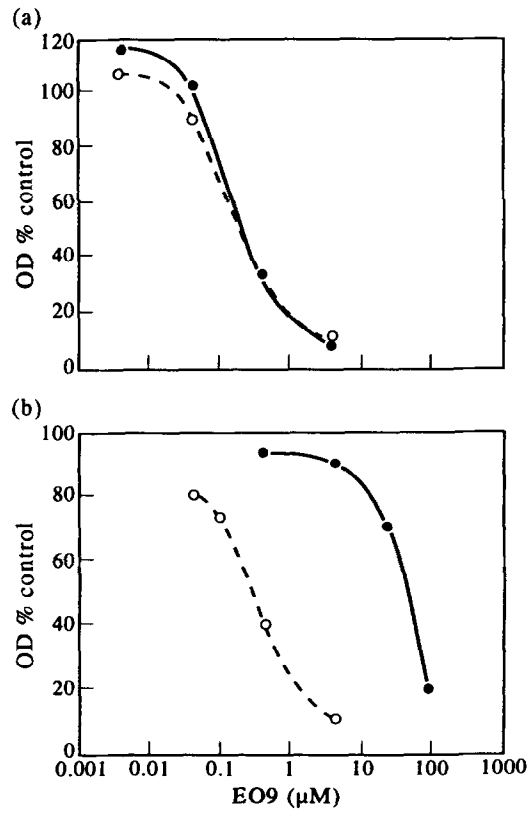

Figure 4. Survival curves showing the influence of exposure conditions: ●, air or ○, hypoxia: on cellular sensitivity to EO9. (a), H322 cells (high activity of DT-diaphorase); (b), T47D cells (low activity of DT-diaphorase). Cells were exposed to EO9 for 3 h washed and allowed to grow for a further 4 days before use of the MTT assay.

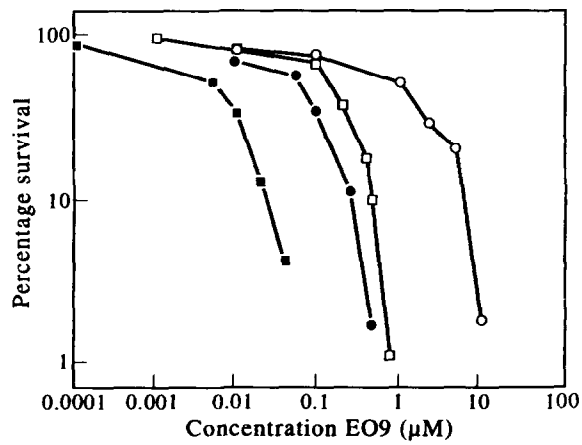

Figure 5. Representative survival curves for CHO cells exposed to EO9 for 3 h in air. □, AA8 wild type; ○, S162 DT diaphorase deficient; ■, UV-41 repair deficient; ●,S9/32 repair deficient and DT-diaphorase deficient.

curves for V79 and IRS-1 cells that are given in Figure 6. Values of IC_{90}, derived from survival curves drawn by eye through pooled data from up to six separate experiments for each pair of cells, are given in Table 2.

The AA8/UV41 pair show a similar increase in sensitivity to EO9 in hypoxia, whereas for V79/IRS-1 pair the effect of EO9 in hypoxia is greatest in the IRS-1 cells, i.e. a 4-fold increase in differential (Figure 6). The increased effectiveness in hypoxia in the IRS-1 cells, which mis repair DNA damage [25], suggests there may be a difference in the damage caused under aerobic versus hypoxic conditions. For example, one- and two-electron reductions may yield different species (or species in different proportions), and as a consequence in nitrogen the IRS-1 cells may have more difficulty in processing the lesions so formed.

The CHO-MMC® cells are resistant to mitomycin C [17, 18], and this is attributed to lowered expression of cytochrome P450 reductase in these cells relative to CHO-K1. Cross resistance is also seen for EO9 in air. Both cell lines show a substantial increase in activity in hypoxia with a slightly greater effect (larger differential) being shown in the MMC® cells.

DISCUSSION

The indolequinone EO9 is effectively metabolised by DT-diaphorase [6], a finding that led to the proposal by Riley and Workman [5] that tumours expressing high levels of this reductase would be good targets for treatment with EO9. This proposal has been tested by us [7] and extended in the present work, by measuring the activity of DT-diaphorase in whole cell lysates from 23 human cell lines, and making a comparison with the sensitivity of these cells to EO9. Enzyme activity varies over a 5000-fold range, and EO9 sensitivities, expressed as values of IC_{50}, vary by over 1 000. Cells derived from NSCLC all show high levels of DT-diaphorase, and this is reflected by these lines having the greatest sensitivity in EO9. In contrast, cells from breast or SCLC show much more variable levels of enzyme activity (1 000-fold range) and, on the whole, are more resistant to EO9 than the NSCLC cells. Higher levels of DT-diaphorase in NSCLC verses SCLC have been reported previously [7, 26] and the data reported here are consistent with these earlier observations. Further, these results suggest that, in the clinical evaluation of EO9, NSCLC should be considered as a tumour type more likely to be responsive to treatment with EO9.

The importance of DT-diaphorase for determining the activity of EO9 is further illustrated by the effect of the enzyme inhibitor dicoumarol (Figure 3, Table 3). It should be noted that the use of dicoumarol to specifically probe for the importance of DT-diaphorase in bioreductive drug activation in whole cells could be compromised by other biochemical properties of dicoumarol [27, 28]. Nevertheless, the data showing the dicoumarol mediated protection against EO9 toxicity in high DT-diaphorase expressing cell lines, together with results showing resistance to EO9 in pairs of CHO cell lines with lowered DT-diaphorase activity, are consistent with a dominant role for two-electron reduction, catalysed by DT-diaphorase, in the bioactivation of EO9 in air.

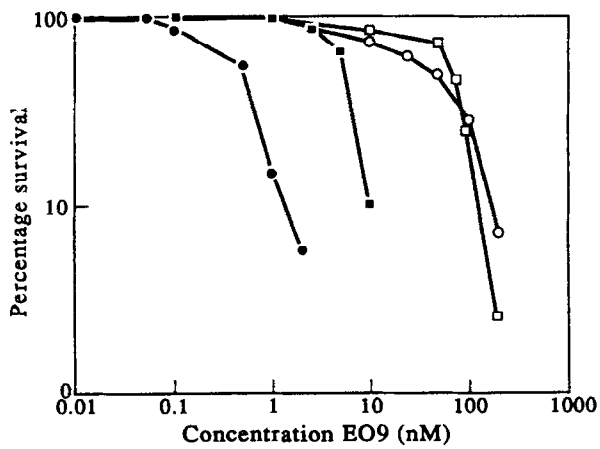

Figure 6. Representative survival curves for V79 and IRS cells exposed to EO9 for 3 h in air or nitrogen. □, ■, V79 cells; ○, ●, IRS-1 cells. Open symbols, air; closed symbols, N_2.

Two-electron reduction of EO9 by DT-diaphorase yields the corresponding hydroquinone. Reductive activation of quinones, to give potentially toxic species, can also occur by initial one-electron reduction to give a semiquinone. This can be carried out by enzymes such as cytochrome P450 reductase in a process that may be reversible by oxygen [29]. The subsequent level of the ultimate damaging species will then be governed by any disproportionation reaction between the semi- and hydroquinones [29, 30]. The potential contribution of one-electron reductive activation to subsequent toxicity is revealed in experiments carried out under anaerobic conditions. The work in this paper, supported by data reported elsewhere [15, 31, 32], shows that in both human tumour cells and rodent cells with relatively low levels of DT-diaphorase, exposure to EO9 under hypoxic conditions substantially increases toxicity. Whereas in cells with high DT-diaphorase activity, treatment in hypoxia does not alter toxicity. This implies that both one- and two-electron reductive processes can be operative. In cells expressing high levels of DT-diaphorase, metabolism of EO9 by this enzyme dominates in both air and hypoxia. In cells with low DT-diaphorase activity, metabolism by enzymes, such as P450 reductase, assume more importance, such that under conditions where oxygen is not present to reverse the one-electron reduction process (i.e. in hypoxia), toxicity is shown to increase. In support of this, reduction of EO9 by purified cytochrome P450 has now been demonstrated [33].

Some mechanistic interpretation of the function of EO9 has been made by examining the effects of structural analogues in a cell line with extremely high levels of DT-diaphorase [34]. Replacement of the aziridine group in the drug with methyl aziridine results in a 50-fold reduction in toxicity which is accompanied by a substantial decrease in metabolism by DT-diaphorase. In contrast, replacement of the aziridine with methoxy causes only a slight reduction in metabolism by DT-diaphorase, but toxicity is decreased 100-fold. Thus, the aziridine group in EO9 contributes to cell killing by modulating activation by DT-diaphorase, and also through an enzyme-independent effect, probably involving DNA alkylation. The EO9 molecule has three potential sites for alkylation, the aziridine at C7 and the two leaving groups at C10 and C1 (numbering is analogous to that used for MMC, which cross-links via C10 and C1).

The repair deficient cell lines IRS-1 and UV-41 are substantially more sensitive (~50-fold) to treatment with MMC in air compared to their parental lines ([17], Haigh unpublished data). The UV-41 cells show cross-sensitivity to EO9, but this is not observed with the IRS-1 cells, which have a similar sensitivity to their V79 parent line. This strongly suggests that the nature of the damage caused by MMC and EO9 in air is different, and this probably has its basis in the alkylating properties of the C7 aziridine in EO9.

Further inspection of the V79/IRS-1 results (Table 3, Figure 6) indicates that the difference in sensitivity between the cells, which is 1.5 in air, is increased to 6 in hypoxia. This may be due to different lesions being produced from EO9 in hypoxia compared to air and to which the IRS-1 cells are sensitive. Similar experiments have been carried out by Hughes and colleagues [35] with MMC in air and nitrogen, using other repair deficient rodent cell lines, and they concluded that MMC also formed different lesions in hypoxia compared with air.

Human cells with high DT-diaphorase activity exposed to EO9 for 3 h in air have IC_{50} values of around 0.2 μM, whereas human cells low in DT-diaphorase have values of IC_{50} 100-fold greater (Tables 3 and 4). The value of IC_{90} for the rodent V79 cells given the same exposure was also 0.2 μM, but these cells are low in DT-diaphorase (53±2.5 units of activity). Further, the sensitivity of the CHO-K1 cells is the same order as the V79 cells, and yet the CHO-K1 cells have undetectable DT-diaphorase ([18], Robertson unpublished). The most likely explanation for this is differences in substrate specificity for the DT-diaphorase derived from rodent versus human cells. This is currently being evaluated by using whole cell lysates from the different cell types and measuring rates of reduction of a variety of compounds, including some related to EO9, that are thought to be substrates for DT-diaphorase.

In conclusion, those cells highest in DT-diaphorase tend to be the most sensitive to EO9. For cells that are resistant in air, because of their low DT-diaphorase levels, this resistance can be overcome in hypoxia. Further, in rodent tumour cells, which have low levels of DT-diaphorase, substantial activity in EO9 in hypoxia relative to aerobic cells can be demonstrated both *in vitro* [15] and *in vivo* [10]. Thus, compounds such as EO9 may not only be useful for targeting aerobic tumour cells rich in DT-diaphorase, but also in targeting radio- and chemoresistant hypoxic cells in solid tumours.

1. Oostveen EA, Speckamp WN. Mitomycin analogs I. Indoloquinones as (potential) bisalkylating agents. *Tetrahedron* 1987, **43**, 255–262.
2. Hendriks HR, Pizao PE, Berger DP, *et al.* EO9: a novel bioreductive alkylating indoloquinone with preferential solid tumour activity and lack of bone marrow toxicity in preclinical models. *Eur J Cancer* 1993, **29A**, 897–906.
3. Phillips RM, Hulbert PB, Bibby MC, Sleigh NR, Double JA. *In vitro* activity of the novel indoloquinone EO9 and the influence of pH on cytotoxicity. *Br J Cancer* 1992, **65**, 359–364.
4. Siegel D, Gibson NW, Preusch PC, Ross D. Metabolism of mitomycin C by DT-diaphorase: role in mitomycin C-induced DNA damage and cytotoxicity in human colon carcinoma cell. *Cancer Res* 1990, **50**, 7483–7489.
5. Riley RJ, Workman P. DT-diaphorase and cancer chemotherapy. *Biochem Pharmacol* 1992, **43**, 1657–1669.
6. Walton MI, Smith PJ, Workman P. The role of NAD(P)H: quinone reductase (EC1.6.99.2,DT-diaphorase) in the reductive bioactivation of the novel indoloquinone antitumour agent EO9. *Cancer Commun* 1991, **3**, 199–206.
7. Robertson N, Stratford IJ, Houlbrook S, Carmichael J, Adams GE. The sensitivity of human tumour cells to quinone bioreductive drugs: what role for DT-diaphorase? *Biochem Pharamacol* 1992, **44**, 409–412.
8. Workman P, Walton MI. Enzyme-directed bioreductive drug development. In Adams GE, Breccia A, Fielden EM, Wardman P, eds. *Selective Activation of Drugs by Redox Processes*. New York, Plenum Press, 1990, 173–191.
9. Walton MI, Bibby MC, Double JA, Plumb JA, Workman P. DT-diaphorase activity correlates with sensitivity to the indoloquinone EO9 in mouse and human colon carcinomas. *Eur J Cancer* 1992, **28A**, 1597–1600.
10. Adams GE, Stratford IJ, Edwards HS, Bremner JCM, Cole S. Bioreductive drugs as post-irradiation sensitizers: comparison of dual function agents with SR 4233 and the mitomycin C analogue EO9. *Int J Rad Oncol Biol Phys* 1992, **22**, 717–720.
11. Stratford IJ, Adams GE, Godden J, Howells N. Induction of tumour hypoxia post-irradiation: a methd for increasing the sensitizing efficiency of misonidazole and RSU 1069 *in vivo*. *Int J Radiat Biol* 1989, **55**, 411–422.
12. Cole S, Stratford IJ, Adams GE, Fielden EM, Jenkins TC. Dual-function 2-nitroimidazoles as hypoxic cell radiosensitizers and bioreductive cytotoxins: *in vivo* evaluation in KHT murine sarcomas. *Rad Res* 1990, **124**, S38–S43.
13. Zeman EM, Brown JM, Lemmon MJ, Hirst VK, Lee W. SR4233: a new bioreductive agent with high selective toxicity for hypoxic mammalian cells. *Int J Radiat Oncol Biol Phys* 1986, **12**, 1239.
14. Stratford IJ. Bioreductive drugs in cancer therapy. *BJR* 1992 (Suppl. 24), 128–135.
15. Adams GE, Bremner J, Edwards HS, *et al.* Bioreductive drugs—dual function agents and related compounds. In Dewey WC *et al.*, eds. *Radiation Research. A Twentieth-Century Perspective*, Vol.II. Academic Press, 1992, 802–806.
16. Jones NJ, Cox R, Thacker J. Isolation and cross-sensitivity of X-ray-sensitive mutants of V79-4 hamster cells. *Mutation Res* 1987, **183**, 279.
17. Keohane A, Godden J, Stratford IJ, Adams GE. The effects of three bioreductive drugs (mitomycin C, RSU-1069 and SR4233) on cell lines selected for their sensitivity to mitomycin C or ionising radiation. *Br J Cancer* 1990, **61**, 722–726.
18. Hoban PR, Walton MI, Robson CN, *et al.* Decreased NADPH: cytochrome P-450 reductase activity and impaired drug activation in a mammalian cell line resistant to mitomycin C under aerobic but not hypoxic conditions. *Cancer Res* 1990, **50**, 4692–4697.
19. Thompson LH, Brookman KW, Dillehay LE, Mooney CL, Carrano AV. Hypersensitivity to mutation and sister-chromatid-exchange induction in CHO cell mutants defective in incising DNA containing UV lesions. *Somatic Cell Genet* 1982, **8**, 759–773.
20. Dulhanty AM, Li M, Whitmore GF. Isolation of Chinese hamster ovary cell mutants deficient in excision repair and mitomycin C bioactivation. *Cancer Res* 1989, **49**, 117–122.
21. Dulhanty AM, Whitmore GF. Chinese hamster ovary cell lines resistant to mitomycin C under aerobic but not hypoxic conditions are deficient in DT diaphorase. *Cancer Res* 1991, **51**, 1860–1865.
22. Mossmann T. Rapid colourimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J Immunol Meth* 1983, **65**, 55–61.
23. Carmichael J, De Graff WF, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semi-automated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 1987, **47**, 936–941.
24. Stratford IJ, Adams GE, Bremner JCM, *et al.* The assessment of bioreductive drug toxicity *in vitro* and in experimental tumours *in vivo*. In Adams GE, Breccia A, Fielden EM, Wardman P, eds. *Selective Activation of Drugs by Redox Processes*. New York, Plenum Press 1990, 203–212.
25. Collins AR. Mutant rodent cell lines sensitive to ultraviolet light, ionizing radiation and cross-linking agents: a comprehensive survey of genetic and biochemical characteristics. *Mutation Res DNA Repair* 1993, **293**, 99–118.
26. Malkinson AM, Siegel D, Forrest GL, *et al.* Elevated DT-diaphorase activity and messenger RNA content in human non-small cell lung carcinoma: relationship to the response of lung tumour xenografts to mitomycin C. *Cancer Res* 1992, **52**, 4752–4757.
27. Workman P, Walton MI, Powis G, Schlager JJ. DT-diaphorase questionable role in mitomycin C resistance, but a target for novel bioreductive drugs? *Br J Cancer* 1989, **60**, 800–802.
28. Preusch P, Siegel D, Gibson NW, Ross D. A note on the inhibition of DT-diaphorase by dicoumarol. *Free Radical Biol Med* 1991, **11**, 77–80.
29. Wardman P. Bioreductive activation of quinones: redox properties and thiol reactivity. *Free Radical Res Comms* 1990, **8**, 219–229.
30. Hoey BM, Butler J, Swallow AJ. Reductive activation of mitomycin C. *Biochemistry* 1988, **27**, 2608–2614.
31. Plumb JA, Workman P. Unusually marked hypoxic sensitisation to indoloquinone EO9 and mitomycin C in a human colon tumour cell lines that lacks DT-diaphorase activity. *Int J Cancer* 1994 (in press).
32. Robertson N, Haigh A, Stratford IJ. Quinone bioreductive drugs: interplay between hypoxia and reductive enzymology for activation and toxicity. *Br J Cancer* 1993, **67**, (Suppl. 20) Abstr 1.2.

33. Bailey SM, Lewis AD, Patterson LH, Fisher GR, Workman P. Free radical generation following reduction of EO9: involvement in cytotoxicity. *Br J Cancer* 1993, **67**(Suppl. 20), abstract 1.4.
34. Bailey SM, Suggett N, Walton MI, Workman P. Structure-activity relationships for DT-diaphorase reduction of hypoxic cell directed agents: indoloquinones and diaziridinyl benzoquinones. *Int J Rad Oncol Biol Phys* 1992, 22, 649–653.
35. Hughes CS, Irvin CG, Rockwell S. Effect of deficiencies in DNA repair on the toxicity of mitomycin C and porfiromycin to CHO cells under aerobic and hypoxic conditions. *Cancer Commun* 1991, 3, 29–35.

Acknowledgements—We are grateful to Dr Hans Hendricks from the EORTC NDDO in Amsterdam for the supply of EO9, Dr Peter Twentyman for supply of the small cell lung cancer cell lines designated L- and Drs Ann Dulhanty and Gordon Whitmore for the cells derived from CHO-AA8. The support of Dr S. Houlbrook and Professor J. Carmichael is gratefully acknowledged. Thanks are also due to David Papworth (MRC Radiobiology Unit) for carrying out the statistical analyses, and Richard Knox and Frank Friedlos for use of facilities at the Institute of Cancer Research where measurements of DT-diaphorase were made. This work was funded in part by the U.S. NCI grant no. P01-CA-55165.

Pergamon

European Journal of Cancer Vol. 30A, No. 7, pp. 1019–1022, 1994

0959–8049/94 $7.00+0.00

0959-8049(94)E0031-X

Increases in Leucocyte and Platelet Counts Induced by the Alkyl Phospholipid Hexadecylphosphocholine

L.C. Pronk, A.S.Th. Planting, R. Oosterom, T.E. Drogendijk, G. Stoter and J. Verweij

Hexadecylphosphocholine (HePC) is a new alkyl phospholipid that has been shown to have antitumour activity *in vitro* and *in vivo*. *In vivo* studies have shown absence of bone marrow toxicity at therapeutic doses. In addition, at the highest dosage group in rats, an increase in white blood cell counts (WBC) was observed. To study the presence of a similar phenomenon in man, frequent measurements of haematological parameters were performed in a series of phase II studies. 70 patients were treated with daily doses of 100–200 mg of the oral formulation of HePC. WBC and platelet counts were performed weekly. In a subgroup of 23 patients serum levels of haemopoietic growth factors were measured before and during treatment. A significant increase in WBC and platelet counts was seen in 74 and 73% of the patients, respectively. In 4 patients, bone marrow showed normal cellularity, and in 1 patient, bone marrow culture showed normal numbers and sizes of colony forming units. No abnormal levels or trends over time of cytokines were observed. We conclude that oral HePC induces an increase in WBC and platelet counts in the majority of those treated.

Key words: cytokines, hexadecylphosphocholine, leukocytosis, thrombocytosis
Eur J Cancer, Vol. 30A, No. 7, pp. 1019–1022, 1994

INTRODUCTION

ETHER LIPIDS and related drugs have recently entered the stage of clinical testing. Hexadecylphosphocholine (HePC) is a new alkyl phospholipid with antitumour activity in human leukaemic cell lines, and in methylnitrosurea (MNU)-induced and dimethylbenzanthracene (DMBA)-induced breast carcinoma models in the rat [1–6].

In vivo studies in mice and rats showed absence of bone marrow toxicity at therapeutic doses [1, 2, 5, 6]. In addition, at the highest dosage in rats, a significant increase in the total white blood cell (WBC) counts was noted, mainly related to an increase in the absolute number of granulocytes [1, 2, 4, 5]. Bone marrow examinations did not show augmentation of granulocyte precursor cells [2]. To study the presence of a similar effect in man, we prospectively analysed WBC and platelet counts in 70 patients included in phase II studies, in which the oral formulation of HePC was administered. In a subgroup of patients, serum haemopoietic growth factor levels were measured before and during treatment with HePC.

Correspondence to J. Verweij.
L.C. Pronk, A.S.Th. Planting, G. Stoter and J. Verweij are at the Dept. of Medical Oncology; R. Oosterom is at the Dept. of Clinical Chemistry; and T.E. Drogendijk is at the Dept. of Medical Statistics, Rotterdam Cancer Institute, Dr. Daniel den Hoed Kliniek, P.O. Box 5201, 3008 AE Rotterdam, The Netherlands.
Revised 11 Nov. 1993; accepted 29 Nov. 1993.

PATIENTS AND METHODS

Patients

Phase II studies were performed with oral HePC in non-small cell lung cancer, colorectal cancer, squamous cell head and neck cancer and soft tissue sarcomas.

Criteria for inclusion were histological proof of malignancy, performance status WHO $\leq$ 2, oral informed consent, age $\geq$ 18