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SHORT COMMUNICATION

The Relative Importance of NADPH: Cytochrome *c* (P450) Reductase for Determining the Sensitivity of Human Tumour Cells to the Indolequinone EO9 and Related Analogues Lacking Functionality at the C-2 and C-3 Positions

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ABSTRACT. Analogues of EO9 (3-hydroxymethyl-5-aziridinyl-1-methyl-2[1*H*-indole-4-7-dione]prop-2-en-1-ol) which lack functionality at either the C-2 or C-3 position were synthesised. The aim was to establish the importance of each group towards toxicity and to give an indication as to whether substitution at either position altered activation and toxicity after metabolism by cellular NADPH: cytochrome *c* (P450) reductase (P450R). MDA231 breast cancer cells were transfected with the cDNA for human P450R and stable clones were isolated. These high P450R-expressing clones were used to determine the aerobic and hypoxic toxicity of EO9 and the two analogues that lacked functionality at either C-2 or C-3. The results showed that P450R was strongly implicated in the bioactivation of EO9 and its analogues under both of these conditions. This data also showed that the C-3 functionality was primarily implicated in hypoxic toxicity. BIOCHEM PHARMACOL **59**;8:993–996, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. cytochrome P450 reductase; indolequinone bioreductive drugs; EO9; transfected P450R

It is now well established that many solid tumours possess viable hypoxic cells that are resistant to radiation therapy and some forms of chemotherapy [1, 2]. Strategies designed to overcome this radioresistance include the use of bioreductive drugs, which exhibit selective toxicity towards hypoxic cells both *in vitro* and *in vivo* [3]. Of these agents, EO9 (**1**, NSC 382459; 3-hydroxymethyl-5-aziridinyl-1-methyl-2[1*H*-indole-4,7-dione]prop-2-en-1-ol) ([4]; see Fig. 1 for chemical structure) was developed as a bioreductive alkylating agent related to the naturally occurring antitumour antibiotic mitomycin C [5]. EO9, mitomycin C (MMC), and other related indolequinone derivatives are thought to undergo a similar mechanism of bioactivation utilising similar reductive enzymes [3, 6, 7]. The obligate 2-electron reductase, DTD¶ (NQ01, NADP(H) (quinone acceptor) oxidoreductase, EC 1.6.99.2), plays a dominant role in metabolising these indolequinones [8, 9]. EO9 itself is an efficient substrate for DTD, and this explains the very strong dependence on cellular levels of DTD for toxicity of EO9 [10, 11]. Analogues of EO9 have been synthesised, and two of them were shown to be even better substrates for DTD than EO9 ([12]; see Fig. 1 for chemical structures). The cytotoxicities of these analogues were evaluated *in vitro* in rodent and human cell lines [12, 13]. In comparison with EO9, 5-(aziridin-1-yl)-3-hydroxymethyl-1-methylindole-4,7-dione (**2**) was found to be more cytotoxic towards A549 cells under both aerobic and hypoxic conditions. This non-small cell lung cancer (NSCLC) cell line has high endogenous DTD activity. In the same study, 5-(aziridin-1-yl)-2-hydroxypropenyl-1-methylindole-4,7-dione (**3**) was shown to be less toxic towards this cell line, even though it was a better substrate for DTD than EO9. This data suggested that these quinones were perhaps not primarily activated by this two-electron reducing enzyme.

DTD-catalysed reduction is oxygen-independent but under hypoxia, additional one-electron reductases such as P450R (EC 1.6.2.4) will also contribute significantly to quinone activation and hypoxia-selective toxicity [14, 15]. In this study, we evaluated the role of P450R in the metabolism of these indolequinones. The MDA231 breast

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¶ *Abbreviations:* DTD, DT-diaphorase, NAD(P)H oxidoreductase; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; and P450R, NADPH: cytochrome *c* (P450) reductase.

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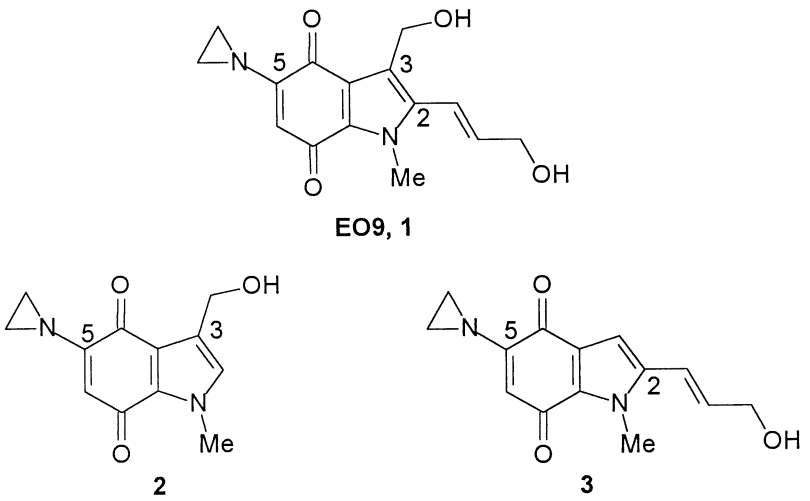

FIG. 1. EO9 and closely related analogues 2 and 3. Analogue 2 lacks a hydroxypropenyl group at C-2, whilst analogue 3 lacks a hydroxymethyl group at C-3.

cancer cells, which had been transfected with the cDNA for P450R, were exposed to EO9 and its two analogues. This cell line has low endogenous P450R and DTD activity. The aim was to determine the importance of each functional group at the C-2, C-3, and C-5 positions of the indole nucleus in terms of activation by P450R and resulting toxicity under both aerobic and hypoxic conditions.

MATERIALS AND METHODS

Cells and Enzyme Activity

A panel of four high P450R-expressing MDA231 clones were chosen, with a range of P450R activities from 25.32 to 189.56 nmol cytochrome *c* reduced/min/mg S-9 protein. When compared to the parental cell line, their activity was approximately 6 to 42 times greater (Table 1: fold increase in P450R indicated by the numerical designated to each clone). DTD, b_5 reductase, and total glutathione levels of the four clones were not significantly different when compared to the parental cell line. Thus, only the level of P450R activity differed significantly [16]. All cell lines were maintained in an exponential growth phase in RPMI-1640 medium supplemented with 2 mM glutamine and 10% foetal bovine serum (FBS). P450R-expressing clones were grown under identical conditions along with the selection agent, puromycin (2 μg/mL). Tissue culture media were prepared at Clare Hall (ICRF), whilst FBS and all other reagents of analytical grade were purchased from Sigma. EO9 was synthesised by a method described by Speckamp and Oostveen [4], whilst analogues 2 and 3 were synthesised by methods described by Naylor *et al.* [3] and Jaffar *et al.* [12, 13].

MTT Proliferation Assay

Drug–response curves were generated using the MTT proliferation assay, based on the ability of viable cells to convert a yellow tetrazolium salt (MTT) into mauve formazan crystals. The concentration of drug required to reduce the optical density to 50% of that of the untreated control wells was determined. This was termed IC_{50} and was recorded as the mean of at least three independent experiments (range 3 to 10), conducted on different days. The parental cell line and the P450R-expressing clones were exposed to each of the three drugs for 3 hr under aerobic or anoxic conditions. All media, plates, and other plastic material were placed into the anoxic cabinet for at least 24 hr prior to each anoxic experiment. After each 3-hr exposure, the drug was removed and fresh media instilled into each well. After 96 hr incubation at 37°, the MTT proliferation assay was performed. The IC_{50} results were expressed as the mean of between three and ten separate results (±SEM). The data was then analysed by methods described in Ref. 15 and the statistical significance was tested by the Student's *t*-test.

RESULTS AND DISCUSSION

P450R activity was shown to be strongly implicated in the activation of EO9 and analogues **2** and **3** under both aerobic and hypoxic conditions. The dependence of IC_{50} on P450R activity for EO9 and the two analogues is shown in Table 1 and Figure 2. Under both aerobic and hypoxic conditions, there was a highly significant relationship between P450R activity and toxicity for all of the drugs (Table 1). This is consistent with the findings of Patterson *et al.* [16], who showed a similar relationship with tirapazamine and RSU 1069 (hypoxic conditions only). Thus, these results suggest that under both aerobic and hypoxic conditions, P450R is implicated in the bioactivation and subsequent toxicities of these quinones.

Analogues **2** and **3** had similar activity under acute aerobic exposure, which may be accounted for by the

TABLE 1. IC_{50} values for parental line and clones after exposure to EO9 and analogues under aerobic and hypoxic conditions

	EO9			Analogue 2			Analogue 3		
Cell line	IC_{50} (air)¶	IC_{50} (N_2)** (μM)	HCR††	IC_{50} (air)¶ (μM)	IC_{50} (N_2)** (μM)	HCR††	IC_{50} (air)¶ (μM)	IC_{50} (N_2)** (μM)	HCR††
wt*	1.34 ± 0.92	0.338 ± 0.15	4.0	26.50 ± 3.00	3.54 ± 0.90	7.5	28.76 ± 9.06	8.16 ± 0.15	3.5
Rd 6†	1.05 ± 0.29	0.449 ± 0.29	2.3	15.07 ± 5.24	2.10 ± 0.55	7.2	10.53 ± 4.18	4.01 ± 0.29	2.6
Rd 16‡	1.25 ± 0.43	0.136 ± 0.08	9.2	9.88 ± 2.38	0.51 ± 0.41	19.4	8.40 ± 1.77	1.18 ± 0.08	7.1
Rd 22§	0.75 ± 0.35	0.079 ± 0.05	9.5	8.52 ± 2.59	0.28 ± 0.20	30.4	6.63 ± 1.11	1.24 ± 0.05	5.3
Rd 42‖	0.61 ± 0.06	0.036 ± 0.02	16.9	5.59 ± 1.12	0.18 ± 0.08	31.1	4.66 ± 1.64	0.46 ± 0.02	10.1

*wt is wild type.
†Rd 6 is transfected cell line with 6-fold increase in P450R over wt.
‡Rd 16 is transfected cell line with 16-fold increase in P450R over wt.
§Rd 22 is transfected cell line with 22-fold increase in P450R over wt.
‖Rd 42 is transfected cell line with 42-fold increase in P450R over wt.
¶IC_{50} (air) is exposure under aerobic conditions.
**$IC_{50}(N_2)$ is exposure under hypoxic conditions.
††HCR is the hypoxic cytotoxicity ratio.
Shown are IC_{50} values (micro M) for parental line and clones (6- to 42-fold increase in P450R activity) after exposure to EO9 and both analogues under aerobic and hypoxic conditions (mean ± SEM, N = 3 to 10).

presence of the C-5 aziridinyl ring, which even in the absence of reductive enzymes is probably prone to DNA alkylation and subsequent formation of *mono* drug–DNA adducts [13]. However, from the results in Table 1, EO9 is still at least a factor of 10 more potent than its analogues under aerobic conditions. This may be explained if EO9 is activated by enzymes other than DTD and P450R, which might have little effect on **2** and **3**. The fact that EO9 is more hydrophilic (due to the presence of both polar functionalities at the C-2 and C-3 positions) may also contribute to its higher potency.

These results showed that EO9 was still more potent than its closely related analogues, whilst compound **2** was more potent than compound **3** under hypoxic conditions. This suggested that neither the C-3 (hydroxymethyl) or C-2 (hydroxypropenyl) functionality alone contributes significantly to aerobic toxicity. However, under hypoxic conditions, the 3-hydroxymethyl functionality is more important than the 2-hydroxypropenyl function with respect to both enzyme activation and cytotoxicity. This is further indicated by the fact that analogue **3** has the greatest hypoxic cytotoxicity ratio (HCR) due to a decrease in aerobic toxicity rather than an increase in hypoxic cytotoxicity. This attribute may be beneficial in terms of this

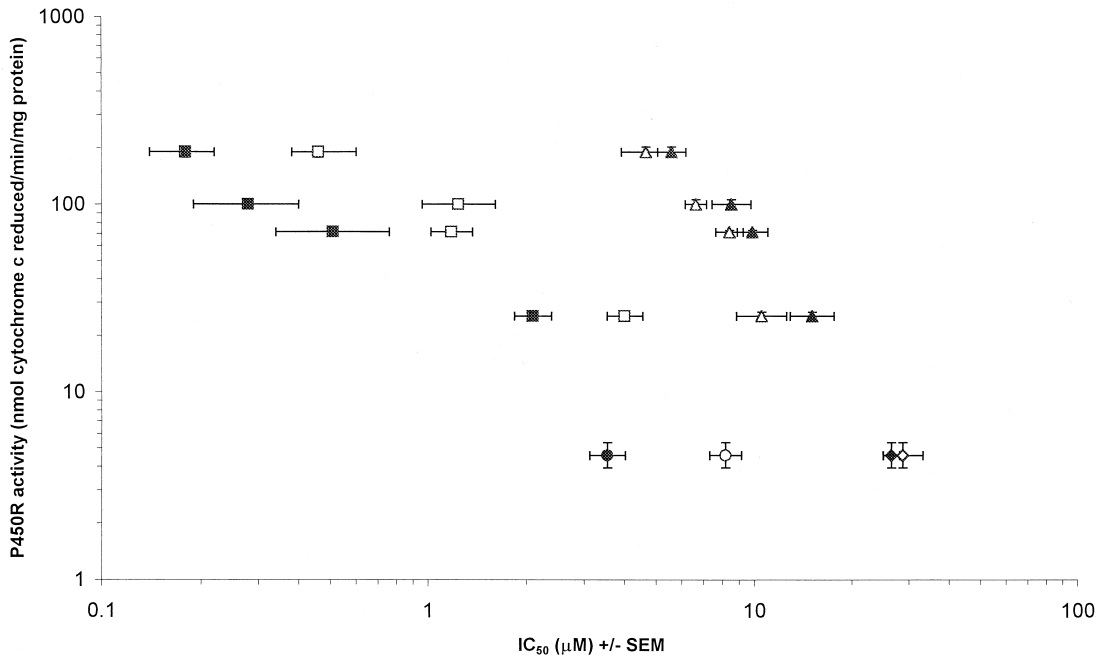

FIG. 2. Graph to show the relationship between P450R activity and IC_{50} for the clones after exposure to analogue 2 (▲ aerobic and ■ hypoxic conditions) and 3 (△ aerobic and □ hypoxic conditions). The parental line is also shown for analogue 2 (◆ aerobic and ● hypoxic) and analogues 3 (◇ aerobic and ○ hypoxic) for comparison (n = 3 to 10).

analogues ability to target hypoxic tumours with relative sparing of the adjacent (oxic) normal tissues.

Overall, these results confirmed that P450R is implicated in the bioactivation of these quinone bioreductive drugs under both aerobic and hypoxic conditions. These findings support the suggestion by Patterson *et al.* [16] that the introduction of P450R into tumours, possibly under the control of hypoxia-responsive elements, may lead to greater activation and effectiveness of bioreductive drugs. It may also be possible to design superior quinone bioreductive drugs by altering side group functionality.

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