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Bioreductive Activation of a Series of Analogues of 5-Aziridinyl-3-Hydroxymethyl-1-Methyl-2-[1H-indole-4,7-dione] prop-β-en-α-ol (EO9) by Human DT-Diaphorase

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ABSTRACT. The enzyme DT-diaphorase (NAD(P)H:quinone acceptor oxidoreductase, EC 1.6.99.2.; DTD) is believed to be a good target for enzyme-directed bioreductive drug development because elevated levels of enzyme activity have been described in several human tumour types and it plays a key role in the bioreductive activation of several quinone-based anticancer drugs. As part of an ongoing program to develop new bioreductive drugs, the ability of a series of indoloquinone compounds to serve as substrates for and to be bioreductively activated by purified recombinant human DTD was investigated. Of the seven compounds evaluated, EO9, EO68 and EO4 were substrates for human DTD, but only EO4 was reduced to a DNA cross-linking species, and this DNA damage was both concentration dependent and inhibited by dicoumarol. A broad spectrum of chemosensitivity was observed in the H460 non–small cell lung cancer cell line, with the most potent compounds being EO4 (IC_{50} = 23.9 nM), EO9 (IC_{50} = 34.5 nM) and EO68 (IC_{50} = 37.8 nM). Relatively minor structural changes resulted in major changes in both substrate specificity and cytotoxic potency. Comparative chemosensitivity studies demonstrated that EO4, EO9 and EO68 are preferentially toxic towards DTD-rich H460 cells compared with DTD-deficient H596 cells (ratio of IC_{50} values for H596 cells to H460 cells were 113.8, 92.2 and 103.9 respectively). In conclusion, this study has identified two new compounds that are substrates for human DTD, one of which (EO4) is reduced to a DNA cross-linking species. Further studies in a broad panel of cell lines and human tumour xenografts are warranted for EO4 and EO68 based upon the result of this study. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;11:1711–1718, 1996.

KEY WORDS. DT-diaphorase; EO9; indoloquinones; bioreductive drugs; cytotoxicity; DNA interstrand cross links

The enzyme DTD* is a cytosolic flavoprotein that catalyses the two-electron reduction of different compounds including quinones [1]. Its physiological function is believed to be the detoxification of quinones [2, 3], although in certain cases the hydroquinone generated may undergo further reactions leading to DNA-damaging species [4]. The ability of DTD to activate quinone-based compounds in conjunction with the fact that elevated levels of DTD activity have been documented in several human tumour types, particularly NSCLC [5–7], has led to considerable interest in this enzyme as a target for anticancer drug design [8]. The indoloquinone compound EO9 has been shown to be a good substrate for DTD and is bioreductively activated to a DNA-damaging species following reduction by DTD [9]. In addition, several groups have reported that the response of cell lines to EO9 *in vitro* can be predicted based upon the DTD enzyme activity in each cell line [10–13]. Therefore, EO9 meets many of the criteria specified in the concept of enzyme-directed bioreductive drug development initially proposed by Workman and Walton [14], the ultimate objective of which is to target drugs that are activated by specific reductase enzymes to patients whose tumours are rich in this enzyme. The novel mechanism of activation of EO9 and its activity against solid tumours *in vitro* and *in vivo* and its lack of myelosuppression in animal studies led to the selection of EO9 for clinical trial [15].

EO9 is currently undergoing phase I/II clinical evaluation under the auspices of the European Organisation for the Research and Treatment of Cancer (EORTC). Despite reports of partial remissions in two patients with adenocarcinomas of unknown origin and in one patient with bile duct cancer [16], little activity has been reported in phase II trials [17]. The reasons for the lack of activity of EO9 in the clinic are not known, although there are some concerns about the design of the clinical trials. In particular, DTD activity in patients' tumours were not measured routinely, and many of these patients may have had tumours with

* *Abbreviations:* DTD, DT-diaphorase or NAD(P)H:quinone acceptor oxidoreductase (EC 1.6.99.2); NSCLC, non–small cell lung cancer; IC_{50}, drug concentration required to reduce cell survival by 50% relative to controls; DCPIP, 2,6-dichlorophenol-indophenol; IPTG, isopropyl β-D-thiogalactopyranoside; EO9, 5-aziridinyl-3-hydroxymethyl-1-methyl-2-[1H-indole-4,7-dione]prop-β-en-α-ol; SR, selectivity ratio.

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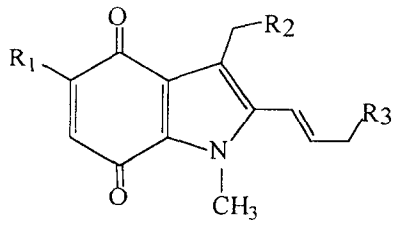
inherently low DTD activity. In addition, it is a widely held view that bioreductive drugs must be evaluated as part of a combination therapy with either radiation or chemotherapy because damage to the hypoxic fraction alone is not likely to be detected as a clinical response following treatment with bioreductive drugs [18]. An alternative explanation may be that pharmacological problems result in poor delivery of EO9 to tumours. Several pieces of evidence support this concept, particularly the fact that EO9 has a very short half-life in patients (7.8 ± 5.6 min) and in experimental models (1.9 min), and no EO9 could be detected in either the tissues or tumours of mice bearing the KHT tumours [16, 19]. In addition, DLD-1 human carcinoma cells exposed to EO9 as multicellular spheroids were significantly less responsive than were the same cells treated as monolayers [20]. Similar results were obtained by using multilayered postconfluent HT-29, SW620 and A2780 cells, which were more resistant to EO9 than were subconfluent cells [21]. Although several factors may account for these observations, drug penetration barriers may exist for EO9, resulting in suboptimal drug exposure conditions. Intratumoural injections of EO9 into DTD-rich and -deficient xenografts resulted in significant activity against tumours that are high in DTD compared with those with low DTD activity, suggesting that, if EO9 can be delivered to tumours, then preferential toxicity towards DTD-rich tumours may be achieved [22].

Despite the disappointing clinical results with EO9, DTD remains a good target for drug design because it is an enzyme that is able to activate certain quinone-based compounds and high levels of activity have been found in several human solid-tumour types. Methods of improving the delivery of EO9 to tumours [23] or the development of analogues of EO9, which retain the key characteristics of EO9 (i.e. bioreductive activation by DTD) but have more favourable pharmacological properties, are two ways of developing new therapies based upon DTD as a target. Thus, the principal objective of this study is to evaluate a series of seven novel analogues of EO9 to identify those compounds that are both good substrates for purified recombinant human DTD and activated to DNA cross-linking species following reduction by DTD. Preliminary evaluation of compounds in DTD-rich and -deficient NSCLC cell lines are also reported to determine structure activity relationships and to provide evidence of selectivity toxicity towards DTD-rich cells.

MATERIALS AND METHODS

Drugs

EO compounds were initially synthesised by Oostveen and Speckamp [24] and were obtained from the Screening and Pharmacology Group of the EORTC. Seven compounds were evaluated in this study, and their chemical structures are presented in Fig. 1. All compounds were dissolved in DMSO and stored at −20°C in small aliquots. Repeated freeze thawing was avoided and no loss of cytotoxic activity



Compound	R_1	R_2	R_3
EO1	$-OCH_3$	$OCOCH_3$	$OCOCH_3$
EO2	$-OCH_3$	OCO_2CH_3	OCO_2CH_3
EO4		$OCOCH_3$	$OCOCH_3$
EO9		OH	OH
EO15	$-NHC_6H_5$	OH	OH
EO68		NHC_6H_5	OH
EO72		OCO_2CH_3	OH

FIG. 1. Structures of EO compounds.

(as determined by the chemosensitivity protocols described below), was detected over a 6-month period.

Expression and Purification of Human DTD

Escherichia coli (JM 105) transformed with the expression plasmid pKK233-2 containing the full-length cDNA sequence for human DTD (derived from the H460 human NSCLC) was a gift from Dr. D. Ross (University of Colorado) [25]. Human DTD was expressed and purified based upon previously published protocols [26, 27], details of which are described below. A single colony of transformed JM 105 cells was inoculated into 5 ml of LB medium containing ampicillin (50 $\mu g\ mL^{-1}$) and incubated overnight at 37°C with constant agitation (200 cycles/min). Overnight cultures (2 mL) were transferred to conical flasks containing 800 mL of LB (plus ampicillin) and incubated at 37°C until the OD_{600} reached 0.6, whereupon IPTG was added to a final concentration of 2 mM. Following a 17-hr incubation at 37°C (200 cycles/min), cells were harvested by centrifugation and resuspended in 20 mL of buffer A (50 mM Tris, pH 7.5, 0.25 M sucrose). Cells were sonicated (Semat 250, Semat Technical, St Albans, UK) at 40% duty cycle with output setting at 4 for three bursts of 20 s each. Cell debris was removed by centrifugation at 13,800*g* (Beckman Optima™ TL Ultracentrifuge) for 10 min, followed by the centrifugation of the supernatant at 105,000*g* for 90 min at 4°C. The supernatant was applied to a reactive blue 2 (Sepharose CL-6B) affinity column that had been equilibrated with 500 mL of buffer A prior to the

addition of the sample. The column was washed with buffer A until no protein could be detected in the eluate. The column was washed with 50 mL of buffer B (0.05 M Tris, pH 8.9, 0.25 M sucrose, 2 M KCl) and DTD was eluted from the column in buffer C (0.02 M Tris, pH 10.0, 1 mM NADH). The purity of the enzyme preparation was determined by SDS-PAGE stained with coomassie blue. Specific enzyme activities were determined as the dicoumarol sensitive reduction of DCPIP at 600 nm. Assay conditions were 200 μM NADH, 40 μM DCPIP, 20 μM dicoumarol (when required), 2 μL of purified enzyme in a final volume of 1 mL Tris-HCl buffer (25 mM, pH 7.4) containing BSA (0.7 mg mL^{-1}). Protein concentration was determined by using the Bradford assay [28].

Enzyme Assays and Kinetic Analysis

DTD activity was assayed by measuring cytochrome c reduction at 550 nm on a Beckman DU 650 spectrophotometer [9]. Enzyme assays contained cytochrome c (70 μM), NADH (2 mM), purified DTD (0.866–1.733 μg) and drug concentrations ranging from 0 to 100 μM in a final volume of 1 mL Tris-HCl (50 mM, pH 7.4) containing 0.14% BSA. Reactions were carried out at room temperature (24.2 ± 0.62°C) and started by the addition of NADH. Rates of reduction were calculated from the initial linear part of the reaction curve (30 sec) and results were expressed in terms of μmol cytochrome c reduced/min/mg protein by using a molar extinction coefficient of 21.1 mM cm^{-1} for cytochrome c. Enzyme kinetic studies were designed according to the criteria defined by Henderson [29] and apparent K_m and V_{max} values were determined from plots of s/v against s.

DNA Interstrand Cross-Linking Assay

DNA cross linking was determined by a modified version of the method described by Hartley *et al.* [30], details of which are described elsewhere [31]. Very briefly, pRSET B plasmids (InVitrogen) were isolated from *E. coli* (JM 109) by small-scale plasmid preparation techniques. Plasmids (1 μg) were linearised by digestion with Nco 1 and 3′ end labelled with [α^{32}P] dCTP (3000 Ci/mmol, Amersham Life Science, UK) and Klenow fragment of DNA polymerase I. DNA was ethanol precipitated, washed in 70% ethanol and resuspended in sterile distilled water. Approximately 10 ng of labelled DNA was incubated with each compound at 25°C for 1 hr in 10 mM potassium phosphate buffer (pH 7.0) containing 0.14% BSA, NADH (100 μM), EDTA (1 mM), and DTD (1 μg). Dicoumarol (20 μM) was used to study the effects of inhibiting DTD activity. Reactions were terminated by ethanol precipitation of DNA, and samples were dissolved in 20 μL of strand separation buffer (30% DMSO, 1 mM EDTA, 0.04% Bromophenol blue). Samples were heated to 90°C for 2 min (except nondenatured control samples) and immediately cooled in an ice water bath. DNA samples were loaded into a 1% agarose gel and electrophoresed for 14–16 hr at 40 V in TAE gel and running buffer. Gels were dried at 80°C and the DNA was visualised by autoradiography.

Cell Culture and In Vitro *Chemosensitivity Studies*

H460 and H596 NSCLC cell lines were obtained from ATCC and were routinely maintained as monolayer cultures in RPMI 1640 medium supplemented with 10% foetal calf serum, sodium pyruvate (1 mM), L-glutamine (2 mM) and penicillin/streptomycin (50 IU/mL/50 μg/mL) and buffered with HEPES (25 mM). Cells were plated into 96-well culture plates (1–2 × 10^3 cells per well) and incubated overnight at 37°C in an humidified atmosphere containing 5% CO_2. Drugs were added to each well (8 wells/drug exposure) to give the desired range of final drug concentrations (final DMSO concentration was 0.1% in all cases). Following a 1-hr incubation at 37°C, the cells were washed twice with Hank's balanced salt solution and 200 μL of RPMI 1640 growth medium added to each well. Following another 6-day incubation at 37°C, cell survival was assessed by using the MTT assay, details of which are described elsewhere [32]. DTD activity was determined by measuring the dicoumarol sensitive reduction of DCPIP by using NADH as the electron donor [33].

RESULTS

Purification of DTD

Recombinant human DTD migrated as a single band on SDS-PAGE with a molecular weight of approximately 31 kDa (Fig. 2). The specific activity of the purified enzyme

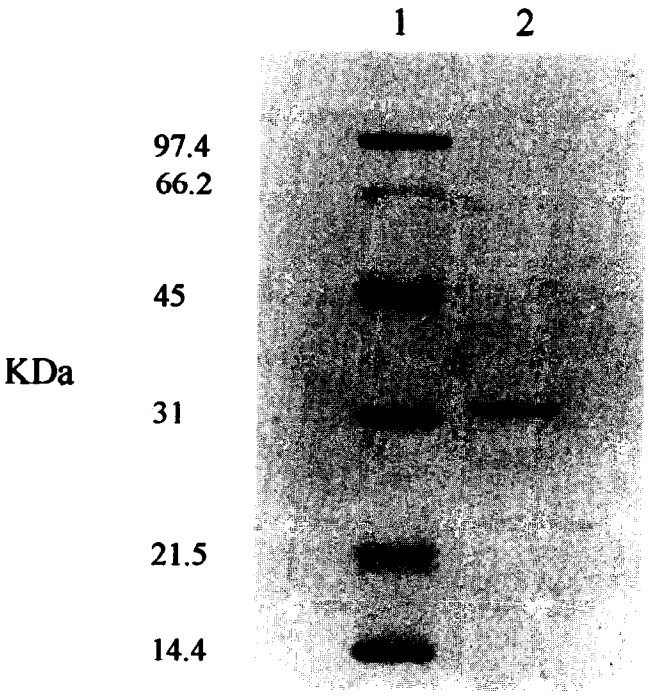


FIG. 2. SDS-PAGE analysis of purified human DTD. Lane 1: Molecular weight markers (low range markers, Biorad, UK). Lane 2: Purified human DTD. Gel was stained with Coomassie Blue.

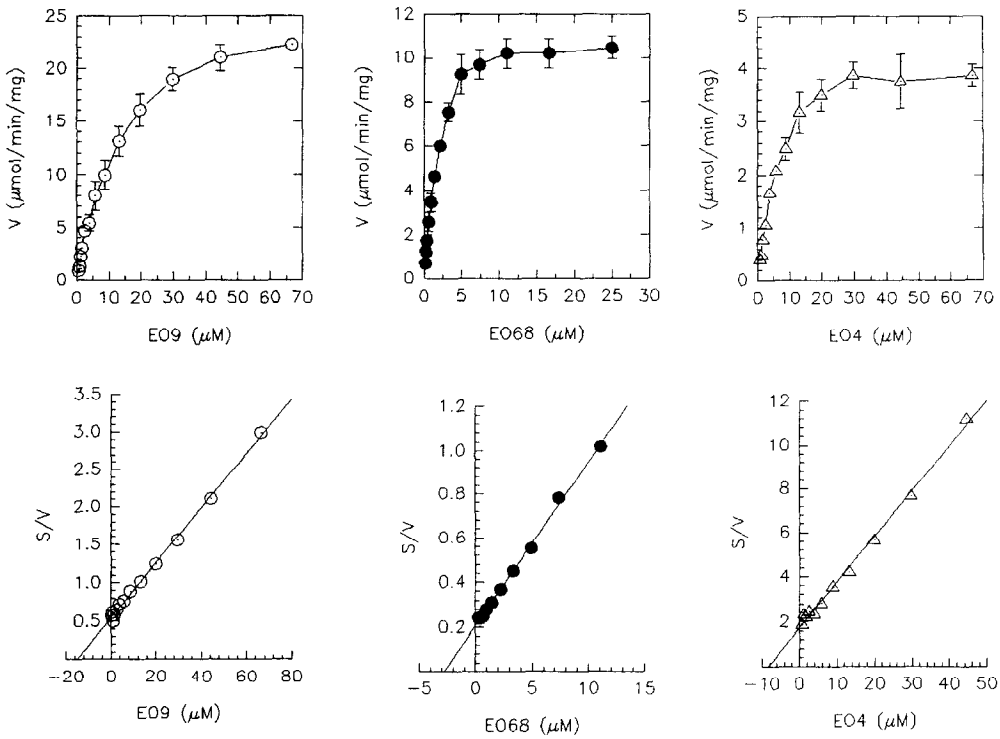


FIG. 3. Plots of v vs. s (top) and Hanes plots (bottom) of the metabolism of EO9 (○), EO68 (●) and EO4 (△) by purified human DTD. Each point represents the mean ± SD for three independent experiments.

was 139 μmol DCPIP reduced/min/mg protein and all activity was completely inhibited by dicoumarol.

Enzyme Kinetics

Of the compounds tested, only EO4, EO9 and EO68 are substrates for human DTD (Fig. 3). The most efficient substrate for DTD was EO9, followed by EO68 and EO4 (V_{max} = 28.07 ± 0.99, 12.74 ± 2.41 and 4.73 ± 0.72 μmol/min/mg, respectively; Table 1). Minor alterations in structure resulted in significant changes in substrate specificity. In particular, replacing the aziridine ring of EO9 with a phenylamino group (EO15) resulted in the complete loss of specificity (Fig. 1 and Table 1). Similar results were obtained with EO4 and EO1, where replacing the aziridine ring of EO4 with a methoxy group also resulted in a loss of substrate specificity (Fig. 1 and Table 1). In contrast, replacing the hydroxyl group at R_2 on EO9 with a phenylamino group (EO68) had a minor effect on substrate specificity. Replacing the hydroxyl group (R_2) of EO9 with a methyl carbonate group at R_2 (EO72) resulted in loss of substrate specificity (Fig. 1 and Table 1).

DNA Cross-Linking Studies

No DNA cross linking was observed with any of the compounds in the absence of DTD (Fig. 4A). Following the addition of DTD, only EO4 was bioreductively activated to a DNA cross-linking species (Fig. 4B). The cross linking of DNA following the reduction of EO4 by DTD was concentration dependent (Fig. 4C) and was completely inhibited by dicoumarol (Fig. 4D).

In Vitro *Chemosensitivity*

Against the DTD-rich H460 cell line (1,139 ± 120 nmol DCPIP reduced/min/mg protein), a broad spectrum of chemosensitivity exists ranging from inactive (EO15) to highly active (EO4) following a 1-hr drug exposure (Fig. 5 and Table 1). The most potent compounds (EO4, EO9 and EO68) are substrates for DTD. Minor changes in the structure of the EO compounds result in major changes in cytotoxic potency. These changes are in line with the struc-

TABLE 1. Relationship between substrate specificity and *in vitro* chemosensitivity for a panel of EO compounds

Compound	Apparent V_{max} (μmol/min/mg)	Apparent K_m (μM)	H460 IC_{50} Values (nM)
EO9	28.07 ± 0.99	14.99 ± 1.62	34.5 ± 3.7
EO68	12.74 ± 2.41	2.48 ± 0.18	37.8 ± 5.9
EO4	4.73 ± 0.72	8.27 ± 1.48	23.9 ± 3.0
EO1	0	0	498.7 ± 82.1
EO2	0	0	77.8 ± 7.7
EO15	0	0	>2,000
EO72	0	0	520.1 ± 81.4

All values represent the means ± SD of at least three independent experiments.

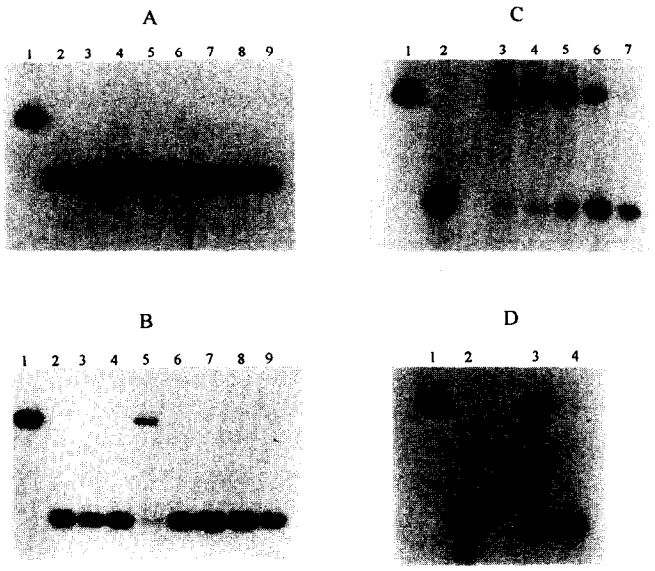


FIG. 4. Cross linking of PRSET B plasmid DNA by EO compounds in the absence of human DTD (A) and in the presence of DTD (B). Lanes 1 and 2 in both panels represent control nondenatured and denatured DNA, respectively. Lanes 3–9 represent compounds EO1, EO2, EO4, EO9, EO15, EO68 and EO72, respectively (drug concentrations were 100 μM in all cases). Concentration-dependent cross linking of DNA by EO4 (C). Lanes 1 and 2 represent control nondenatured and denatured DNA, respectively. Lanes 3–7 represent cross linking by EO4 at 200, 100, 50, 25 and 12.5 μM, respectively. Dicoumarol inhibition of EO4 DNA cross linking (D). Lanes 1 and 2 represent control nondenatured and denatured DNA, respectively. Lanes 3 and 4 represent cross linking by EO4 (200 μM) in the absence and presence of dicoumarol (20 μM), respectively.

ture activity relationships for enzyme kinetics described above where loss of substrate specificity for DTD generally results in a loss or significant reduction in cytotoxic potency (Table 1). The only exception to this is EO2, which is relatively potent against H460 cells (IC_{50} = 77.9 ± 7.7 nM; Table 1), despite the fact that it is not an efficient substrate for DTD.

The response of DTD-rich H460 cells and DTD-deficient H596 cells (DTD activity = 0 nmol/min/mg) to selected EO compounds is presented in Table 2 and Fig. 6. EO4, EO9 and EO68 are selectively toxic to the DTD-rich H460 cell line as compared with the DTD-deficient H596 cell line (Table 2). SRs were defined as the ratio of IC_{50} values for H596 cells divided by the IC_{50} value for H460 cells and SR values of 113.8, 92.2 and 103.9 were obtained for EO4, EO9 and EO68, respectively (Table 2).

DISCUSSION

The identification of compounds that are both substrates for specific reductase enzymes and activated following reduction by these enzymes forms the cornerstone of the concept of "enzyme-directed bioreductive drug development" as proposed by Workman and Walton [14]. In this study, EO9, EO68 and EO4 proved to be substrates for purified human DTD at pH 7.4 and under aerobic conditions with V_{max} values of 28.07, 12.74 and 4.73 μmol/min/mg, respectively (Fig. 3 and Table 1). Of all the compounds tested, only EO4 was reduced by DTD to a DNA cross-linking species under these conditions (Fig. 4). EO4 is the most potent of the compounds tested against the DTD-rich H460 cell line, which may reflect the fact that DNA interstrand cross links are more toxic to cells than are single-strand breaks [34, 35]. In terms of their selectivity towards DTD-rich cells, this study demonstrates that EO4, EO9 and EO68 are selectively toxic towards H460 cells compared with DTD-deficient H596 cells, with SRs (SR = IC_{50} H596 divided by IC_{50} values for H460 cells) of 113, 92.2 and 103.9, respectively. The SRs presented in this study compare favourably with other published studies using the same pair of cell lines treated with Mitomycin C (SR = 11), MeDZQ (SR = 17), PDZQ (SR = 1.8), Streptonigrin (SR = 86) and EO9 (SR = 62), all of which are substrates for human DTD [36]. These results provide preliminary evidence for selectivity towards DTD-rich cells, and further studies are required to expand the panel of cell lines evaluated. Other factors such as activation by one-electron reductases or differences in DNA repair capacity may determine the final outcome of chemotherapy *in vitro* and these areas need to be addressed. Because quinones are substrates for other enzymes, most notably cytochrome b5 reductase [37], cytochrome P450 reductase [38] and xanthine dehy-

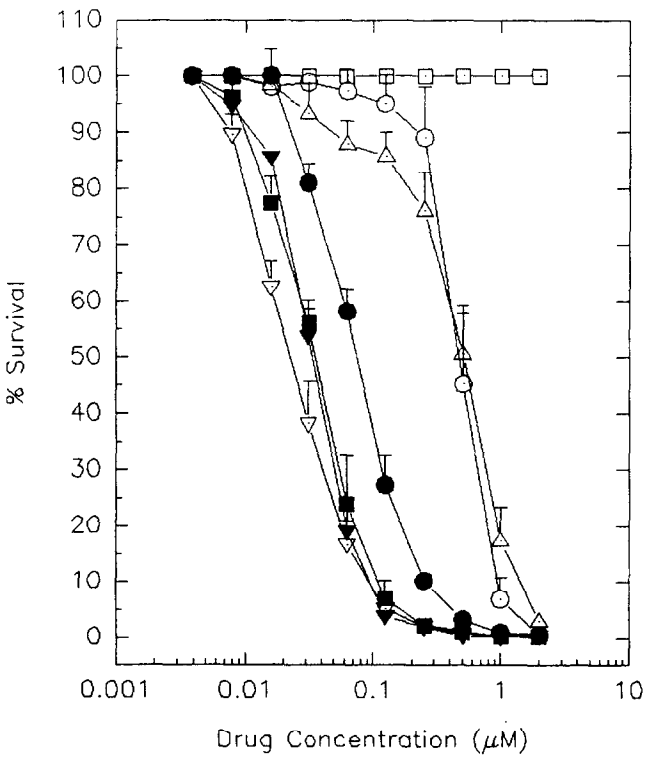


FIG. 5. Response of H460 cells following a 1-hr exposure to EO1 (○), EO2 (●), EO4 (▽), EO9 (▼), EO15 (□), EO68 (■) and EO72 (△). Each point represents the mean ± SD for three independent experiments.

TABLE 2. Response of H460 and H596 cells to EO4, EO9 and EO68

Cell lines	DTD activity (nmol/min/mg)	IC_{50} Values (nM) EO4	EO9	EO68
H596	0	2,720 ± 960	3,180 ± 410	3,930 ± 450
H460	1,139 ± 120	23.9 ± 3.0	34.5 ± 3.7	37.8 ± 3.7
SR		113.8	92.2	103.9

SR is defined as the ratio of IC_{50} values for H596 cells to IC_{50} values for H460 cells. All values represent the means ± SD for at least three independent experiments.

drogenase [39], the role of these enzymes in the activation of EO4 and EO68 will be addressed in future studies. The fact that EO2 is active against H460 cells despite the fact that it is not a substrate for DTD suggests that other enzymes may be involved in the mechanism of action of these compounds. Nevertheless, the results of this study suggest that EO4 and, to a lesser extent, EO68 meet the criteria for enzyme-directed bioreductive drug development. If these compounds are selectively toxic towards DTD-rich cells *in vitro* and in experimental *in vivo* models, they could be good candidates for clinical development.

Structure-activity studies demonstrate that relatively minor changes in structure can have a major effect on both substrate specificity and chemosensitivity. The most marked example of this is in the case of EO9 and EO15, where replacing the aziridine ring of EO9 with a phenylamino group leads to a loss of substrate specificity (Table 1) and complete loss of cytotoxicity (Fig. 5 and Table 1). Similar results were obtained for EO1 and EO4, where replacing the aziridine ring of EO4 with a methoxy group

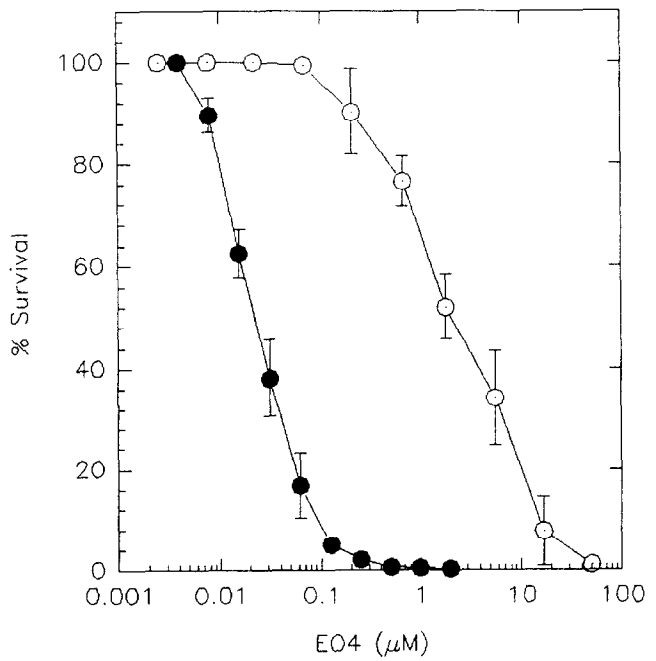


FIG. 6. Response of H460 (●) and H596 (○) cells to EO4. Each point represents the mean ± SD for three independent experiments.

results in complete loss of substrate specificity and a significant reduction in cytotoxic potency (Fig. 5 and Table 1). Thus, the aziridine ring structure appears essential for both substrate specificity and cytotoxicity, and these findings are in broad agreement with those of Bailey *et al.* [40]. However, the fact that EO7, which has a methoxy group rather than the aziridine ring of EO9, is a good substrate for DTD suggests that the aziridine ring structure is not an absolute requirement for reduction by DTD [40]. In the case of EO72, replacing the hydroxyl group at the R_2 position of EO9 with a methyl carbonate group (i.e. EO72) also results in the loss of substrate specificity and a reduction in cytotoxicity (Fig. 5 and Table 1). Replacing the hydroxyl group of EO9 with a phenylamino group as in the case of EO68 has little effect on substrate specificity and has no effect on cytotoxicity (Fig. 5 and Table 1). Although the reasons for these structure activity relationships are not fully understood, the major changes in substrate specificity and cytotoxicity are probably due to either the two-electron reduction potential of compounds or steric effects at the active site of DTD, and it will be interesting to study these effects once full details of the crystal structure of DTD are published.

In this study, no interstrand DNA cross links were produced following the reduction of EO9 by DTD, which is in marked contrast to previously published studies [41]. The reasons for the discrepancy are not clear, although two possible explanations exist. First, there are differences in the species of DTD used in each study, and this may be significant because rat DTD reduces quinone-based drugs (including EO9) with greater efficiency than does the human DTD [42, 43]. Species-dependent differences in the rate of EO9 metabolism are unlikely to account for differences in the type of DNA damage induced because the products of EO9 metabolism by DTD (i.e. the hydroquinone) are likely to be the same in both cases. The second scenario is based upon the fact that differences exist between the two studies in terms of the oxygenation status under which EO9 is reduced by DTD. The metabolism of EO9 is influenced by oxygen levels, and the toxic products generated following reduction by DTD may be different in oxic and hypoxic conditions. Recent studies have demonstrated that the hydroquinone form of EO9 is unstable in air, resulting in the formation of the parent compound and hydrogen peroxide [44, 45]. In air, EO9 is reduced to a

species capable of inducing DNA single-strand breaks in plasmid DNA, and this fact was attributed to alkylation of the DNA because DNA damage was not influenced by superoxide dismutase [9]. As catalase or metal chelators were not used in this study, an alternative explanation is that the strand breaks were produced as a consequence of the formation of hydrogen peroxide [45]. Under hypoxic conditions, the hydroquinone is stable, although it is not clear whether this species is toxic to cells [46]. In cell cultures, DTD protects cells from the toxic effects of EO9 under hypoxic conditions and the hydroquinone may be either less toxic than the semiquinone [46] or readily detoxified via conjugation with glucoronides, sulphates or glutathione [45]. The fact that interstrand DNA cross links have been reported following the reduction of EO9 by DTD under hypoxic conditions would favour the argument that the hydroquinone is detoxified in cells rather than the fact that it is an unreactive species. Both single-strand breaks and DNA interstrand cross links have been observed in Walker 256 mammary carcinoma cells treated with EO9 by alkaline elution techniques [47], although the role of one-electron reductases in generating these effects cannot be eliminated, particularly because dicoumarol had little effect on toxicity [47] and EO9 can be reduced to a DNA interstrand cross-linking species by xanthine oxidase [41].

In conclusion, this study has identified two novel analogues of EO9 that are substrates for human DTD, one of which (EO4) is activated to a DNA cross-linking species following enzymatic reduction under aerobic conditions and at physiological pH values. Both compounds are similar to EO9 in that selective toxicity towards DTD-rich H460 cells compared with DTD-deficient H596 cells is obtained with both EO4 and EO68. In view of the pharmacokinetic and pharmacological problems associated with EO9, a major requirement for the successful development of analogues will be to demonstrate that analogues of EO9 have improved pharmacological properties (i.e. longer plasma half-life and improved tissue penetration) relative to EO9. Both EO4 and, to a lesser extent, EO68 meet the criteria set out in the concept of enzyme-directed bioreductive drug development, and further studies in a panel of cell lines with a broad range of DTD activity and in DTD-rich and -deficient human tumour xenografts are warranted based on the results presented in this study.

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