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CHEMOSENSITIVITY TO THE INDOLOQUINONE EO9 IS CORRELATED WITH DT-DIAPHORASE ACTIVITY AND ITS GENE EXPRESSION

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Abstract—EO9, a new bioreductive indoloquinone alkylating agent, requires activation by a two-electron reduction, which can be catalysed by the NAD(P)H:quinone oxidoreductase DT-diaphorase (DTD) (EC 1.6.99.2). Seven human and four murine tumor cell lines from different histological origins were evaluated for their DTD enzyme activity (evaluated using dichlorophenolindophenol and EO9 as substrates), DTD gene expression and chemosensitivity to EO9. In general the cell lines could be divided into two groups: leukemic cells which were relatively resistant to EO9 ($IC_{50} \geq 0.5\ \mu M$) and had no measurable DTD activity, and solid tumor cells, which were more sensitive to the drug ($IC_{50} < 0.06$ nM) and contained a high DTD activity (>90 nmol/min/mg). The expression of the DTD gene was measured by semiquantitative PCR in the human cell lines and an excellent correlation between gene expression and enzyme activity was observed ($r^2 = 0.94$). A higher DTD gene expression also correlated with higher chemosensitivity to EO9. Protection of chemosensitivity to EO9 by dicoumarol, a strong and specific inhibitor of DTD activity, was dependent on duration of exposure and concentration of dicoumarol. Inhibition was best observed by short exposure to dicoumarol and EO9 together, demonstrating that bioactivation of EO9 by DTD is essential. In conclusion, DTD activity and expression appear to predict sensitivity to EO9 in a variety of cell lines. Evaluation of activity or expression in patients' tumor samples might predict the response to EO9.

Key words: DT-diaphorase; EO9; semiquantitative PCR; cell lines; *in vitro* chemosensitivity testing; SRB; enzyme kinetics

EO9 (3-hydroxymethyl-5-aziridinyl-1-methyl-2-(1H-indole-4,7-dione)-propenol) is the lead compound of a series of novel bioreductive alkylating indoloquinones, synthesized as analogs of MMC† [1] and has shown potent cytotoxic activity *in vitro* [2–4]. Apart from being a better hypoxic cell cytotoxin than MMC, EO9 can function as an effective radiation sensitizer *in vivo* when administered post-irradiation [5–7]. In the *in vitro* disease oriented screening panel of the US National Cancer Institute (NCI) EO9 clearly showed greater cytotoxicity in solid tumor than in leukemic cell lines [4]. EO9 recently entered phase I and II clinical trials under the auspices of the EORTC-NDDO (New Drug Development Office) [8]. Although structurally related to MMC, a drug with activity against a variety of human cancers [9], EO9 showed a different spectrum of activity. Since both drugs require metabolic activation, this may be due to the involvement of different enzymes in the reductive bioactivation pathway.

Walton *et al.* [10] showed that EO9 is a substrate for human and rodent DTD enzymes (EC 1.6.99.2, also known as NQO, QAO or NMOR [11]), whereas MMC can be activated by NADPH:cytochrome P450 oxidoreductase, xanthine oxidase, cytochrome b5 reductase or NADPH:cytochrome *c* reductase [12–14]. MMC can also be reduced by DTD at physiological pH [15, 16], but has been shown to be a less favorable substrate (high K_m, low V_{max}) than EO9 [10]. At pH 7.8 the enzyme is inhibited by the reactive product of this reaction, due to crosslinking of the two subunits of DTD [16].

DTD is an obligate two-electron donating flavoenzyme, found in the cytosolic fraction of many tissues and especially in the liver [11, 17]. DTD catalyses the two-electron transfer between the reduced pyridine nucleotides NADH and NADPH and several substrates, such as quinones. The function of the isoenzymes (NQO1, NQO2, NQO4) found in several tissues of rat, mouse and man is still unclear [18, 19]. DTD probably has a function in the biosynthesis of vitamin K since anticoagulants, such as dicoumarol and warfarin, are strong inhibitors of this enzyme, resulting in a decreasing amount of vitamin K dependent clotting factors [11, 17].

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† Abbreviations: DTD, DT-diaphorase; MMC, mitomycin C; NQO, NAD(P)H:quinone oxidoreductase; NMOR, NAD(P)H:menadione oxidoreductase; NCI, National Cancer Institute; AZQ, diaziquone; DCPIP, dichlorophenolindophenol; DMEM, Dulbecco's modified Eagles medium; SRB, sulforhodamine B; MTT, 3-[4,5-dimethylthiazol-2,5-diphenyl] tetrazolium; PCR, polymerase chain reaction; dNTP, deoxynucleoside triphosphate; AMV-RT, avian myeloblastosis virus reverse transcriptase; NSCLC, non small cell lung cancer; and SCLC, small cell lung cancer.

Notably, Edwards *et al.* showed that some of the diaphorase isoenzymes were much less inhibited by dicoumarol than diaphorase 4, which is DT-diaphorase [18]. Another proposed function of DTD is a role in cellular protection against xenobiotics and radicals, by circumventing the toxic redox cycling [20, 21].

On the other hand, DTD is able to activate quinones, such as EO9 and AZQ, to DNA damaging metabolites [10, 22–25]. Bioreductive metabolism can generate more than one reactive center (reduction to hydroquinone, presence of leaving groups and aziridine rings with DNA alkylating properties). In this way intra- and interstrand crosslinks can be formed. Since in some tumors a higher DTD activity has been shown than in the surrounding normal tissue [25–27], the use of prodrugs such as EO9 could be advantageous. The use of inactive prodrugs that need activation by reductive enzymes, may be extremely interesting in determining the selectivity of the drug for solid tumors with hypoxic regions [2, 25].

The first indication of a relationship between the presence of DTD and sensitivity to EO9 was made by Workman *et al.* [28] in a pair of murine adenocarcinomas. This was further evaluated by Walton *et al.* [29] and this correlation was confirmed and extended in a panel of 15 human tumor cell lines (lung, breast) *in vitro* by Robertson *et al.* [30]. In this study we determined in a panel of 11 different cell lines the chemosensitivity to EO9 and the activity and gene expression of DTD, and we found a strong correlation between these three parameters.

MATERIALS AND METHODS

Chemicals. EO9 was synthesized as part of a program on synthesis of bioreductive indoloquinones supported by the Dutch Cancer Society [1]. It was obtained from the EORTC New Drug Development Office (Amsterdam, The Netherlands) and was stored as a 2 mM stock solution in 96% ethanol at −20°. DCPIP (2,6-dichlorophenolindophenol) and gentamicin were obtained from Merck (Darmstadt, Germany). Dicoumarol (bis-hydroxycoumarin) was from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and stored in 50 mM NaOH at −20°. NADH was from Boehringer Mannheim (Mannheim, Germany), AMV Reverse Transcriptase (AMV-RT), RNasin and the deoxynucleoside triphosphates (dNTPs) were purchased from Promega (Leiden, The Netherlands). AmpliTaq DNA polymerase was obtained from Perkin Elmer (Gouda, The Netherlands). All other chemicals were of standard analytical quality and commercially available.

Cell lines and culture conditions. The human colon tumor lines HT29 and SW620, the human prostate cancer cell line PC3 and the human leukemia CCRF-CEM were obtained from ATCC (Rockville, MD, U.S.A.); the human ovarian cancer cell line A2780 was provided by Dr R.F. Ozols (NCI, Bethesda, MD, U.S.A.); the human squamous-cell carcinoma of the larynx UM-SCC-11B was obtained from Dr T. Carey (Ann Arbor, MI, U.S.A.); the human breast cancer cell line MCF-7 was obtained from Dr K.H. Cowan (NCI, Bethesda, MD, U.S.A.) and the murine leukemia P388 was a gift from Dr P. Lelieveld (TNO, Rijswijk, The Netherlands). The murine fibroblast, NIH3T3 and its TRK and RAS transformed variants were described recently [31].

Cells were grown, free of mycoplasma infection, as monolayers (except the leukemic cell lines, growing as cell suspensions in RPMI medium) in HEPES buffered DMEM supplemented with 5% heat inactivated fetal calf serum (Gibco, BRL, Paisley, U.K.) and 1 mM L-glutamine in 80 cm^2 flasks in a 37°, 5% CO_2, 95% humidified air incubator and were subcultured twice a week.

Chemosensitivity assays. Chemosensitivity assays were performed in 96 well microtiter plates using the SRB assay for the adherent cell lines [32] and a modified MTT assay for leukemic cells [33]. The number of plated cells provided exponential growth for the duration of the assay. At 24 hr after plating, cells were exposed for 72 hr to serial dilutions of EO9 (10^{-5}–10^{-10} M in DMEM medium supplemented with gentamicin 50 μg/mL) in triplicate. The IC_{50} was defined as the concentration of EO9 inducing 50% growth inhibition [31]. Experiments were repeated at least three times.

The influence of dicoumarol on EO9 cytotoxicity was assessed in four schemes: (a) 24 hr exposure to a concentration range of EO9 (10^{-5}–10^{-10} M) followed by a washing step and addition of dicoumarol (1,10 μM) for 48 hr; (b) 24 hr dicoumarol (1,10 μM) followed by a washing step and addition of EO9 in the concentration range mentioned above for 48 hr; (c) dicoumarol (1,10 μM) together with EO9 (10^{-5}–10^{-10} M) for 72 hr and (d) 3 hr exposure to dicoumarol (50 μM) together with EO9 in the same concentration range, followed by a washing step and culture in drug-free medium for 69 hr. At the end of all assays the SRB test was performed. IC_{50} of EO9 in the presence of dicoumarol was compared to the IC_{50} of EO9 alone for each incubation time (3, 24, 48, 72 hr).

DTD enzyme activity. Cells were trypsinized, washed, counted and suspended in 25 mM Tris buffer (pH 7.4) at 0.5×10^6 cells/mL. Cells were lysed by sonication (five times 5 sec on ice) and cell extracts were centrifuged for 20 min at 14,000 rpm at 4°. A sample was taken for protein measurement [34], subsequently BSA was added directly to the supernatant to a final concentration of 1% to act as a DTD-activator [35]. Samples were assayed immediately after isolation, according to Ernster [36]. DTD activity was measured in cytosolic cell extracts at 25° in 25 mM Tris buffer (pH 7.4) containing 0.2 mM NADH, 40 μM 2,6-dichlorophenol-indophenol (DCPIP), using 0.5–40 μg cellular protein per assay. The total volume of the reaction mixture was 3 mL and the reaction was initiated by addition of DCPIP and the cell extract. The reduction of DCPIP was followed spectrophotometrically at a wavelength of 600 nm. Enzyme activity was calculated as the dicoumarol (50 μM) inhibitable fraction, using a molar extinction coefficient (ε) for DCPIP of $21 \times 10^3\ M^{-1}cm^{-1}$. To determine the K_m values of DTD for several cell lines the assay was performed as described above, with various DCPIP concentrations, ranging from 5 to 160 μM.

Table 1. DTD activity, gene expression and chemosensitivity to EO9 (IC_{50}) in 11 cancer cell lines

Cell line*	Origin	EO9 IC_{50}† (μM) ± SD	DTD activity† nmol/min/mg ± SD	(%)‡	DTD relative gene expression $10^3 \times$ DTD/β actin ± SD	(%)‡
A2780	Ovarian	0.006 ± 0.002	220 ± 57	(32)	26 ± 4	(29)
MCF-7	Breast	0.006 ± 0.003	290 ± 31	(42)	29 ± 1	(31)
SW620	Colon	0.014 ± 0.01	402 ± 142	(59)	65 ± 3	(75)
3T3-TRK	Fibroblast	0.02 ± 0.01	267 ± 58	(39)	‖	
PC3	Prostate	0.029 ± 0.01	182 ± 56	(27)	13 ± 4	(15)
NIH3T3	Fibroblast	0.03 ± 0.01	91 ± 58	(13)	‖	
UM-SCC-11B	Larynx	0.03 ± 0.01	100 ± 10	(15)	1.3 ± 0.5	(2)
HT29	Colon	0.033 ± 0.01	682 ± 168	(100)	88 ± 7	(100)
3T3-RAS	Fibroblast	0.06 ± 0.02	139 ± 50	(20)	‖	
P388	Leukemia	0.5 ± 0.2	ND		‖	
CCRF-CEM	Leukemia	1.8 ± 1.3	ND		0.3 ± 0.1	(0.3)

* Arranged in the order of increasing IC_{50}.
† IC_{50} and activities are means ± SD of three to 10 experiments.
‡ Relative to values in HT29.
§ Ratio $\times 10^{-3}$ and are ±SD of three to four experiments.
‖ The expression could not be measured in murine cell lines because the primer set used for DTD gene did not recognize the murine cDNA sequence.
ND, below detection limit.

DTD activity was also measured using EO9 as substrate in 3 mL Tris buffer containing 26 μM EO9, 77 μM cytochrome *c* as final electron acceptor (550 nm: $\varepsilon = 18.5 \times 10^3\ M^{-1}cm^{-1}$) and 0.2 mM NADH [37]. When calculating the enzyme activity one should consider the fact that two equivalents of cytochrome *c* are reduced by 1 equivalent NADH.

Semiquantitative PCR. DTD gene expression was measured using the PCR assay as described by others [38, 39]. The expression in these cell lines was high enough to be detected by ethidium bromide staining of the agarose gel after the PCR amplification, therefore no additional steps were performed to increase the sensitivity of the method. Total RNA was isolated from 5×10^6–2×10^7 cells according to the guanidine isothiocyanate lysis method and CsCl gradient centrifugation [40]. Ten micrograms of RNA were reverse transcribed for 45 min at 37°, in a reaction mixture (total 50 μL) containing 50 mM Tris, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM of each deoxynucleoside triphosphate (dNTP), 10 mM dithiothreitol, 20 units RNasin, 15 μg random hexamer primers and 20 units of AMV-RT. The synthesis of cDNA was stopped by heating the samples for 2 min at 95°. cDNA was diluted in water (10–10^6 times) and stored at −20°. The linear amplification range was established for each gene in every cDNA sample. Varying amounts of cDNA were amplified in a reaction mixture of 50 μL, containing 0.2 mM of each dNTP, 10 mM Tris–HCl (pH 8.3), 44 mM KCl, 2 mM $MgCl_2$, 1 unit of AmpliTaq DNA polymerase and 25 pmol of each primer. Primers of DTD (DTD-54 and DTD-56) were kindly provided by P. Danenberg, Los Angeles, U.S.A. [38]. Primers for β-actin were synthesized with a DNA synthesizer (Pharmacia/LKB gene assembler plus, Pharmacia, Uppsala, Sweden) by the methoxy-phosphoramitide method, according to sequences BA-67 and BA-68 (without the T7 promotor sequences) as described by Horikoshi *et al.* [38]. Thirty-five amplification cycles were run following the pattern of 1 min at 93.5°; 1 min at 60° and 1 min at 72°, in a PCR Thermocycler 60-2 (Biomed, Tecnolab, Alkmaar, The Netherlands). Subsequently, 10 μL of the reaction mixture was loaded on a 1.5% agarose gel and run for 2 hr at 100 V. The gel was stained with ethidium bromide and photographed. The intensity of the bands was measured by densitometry using a Cybertech CS1 Image Processor (Cybertech, Berlin, Germany). The amount of DTD amplified product was compared to that of the internal standard β-actin within the linear amplification range using the following calculation:

$$\text{Ratio of PCR products} = \frac{\text{intensity DTD}}{\text{volume of DTD}} \times \frac{\text{volume of } \beta\text{-actin}}{\text{intensity } \beta\text{-actin}},$$

where intensity = optical density of scanned bands on gel.

RESULTS

DTD enzyme activity and EO9 chemosensitivity

For each cell line linearity for time and protein was assessed, and a narrow linearity range was observed (0.5–10 μg protein/assay) in all of them. The DTD reaction was linear for at least 3 min except for cell lines with a high specific activity, such as HT29. In those cases, the initial rate was determined by extrapolating the tangent of the first min. The enzyme activity was inhibited by more than 95% by addition of 50 μM dicoumarol.

Table 1 summarizes the activity of DTD and the chemosensitivity to EO9 for the tested cell lines. The DTD activity varied between not detectable and 682 nmol DCPIP/min/mg protein and was highest in HT29 cells. In the relative resistant

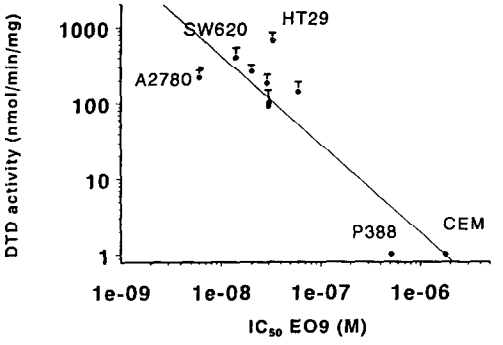

Fig. 1. Double logarithmic plot of DT-diaphorase activity vs IC_{50} of EO9 in 11 cell lines. The enzyme activity of the leukemic cell lines is set to 1 nmol/min/mg, which is the limit of detection of the assay. Values ± SD were derived from at least three experiments.

leukemic cell lines, no DTD activity could be detected, even when cytosolic protein up to 40 μg per assay was used. For three cell lines (A2780, SW620, HT29), the enzyme activities were previously compared in cells isolated from exponential growing cultures and confluent cultures and no significant difference was observed in cells collected in different phases of growth [41]. The chemosensitivity of the various cell lines varied 300-fold, with IC_{50} values ranging from 6 nM to 1.8 μM EO9.

There was a good correlation between the logarithm of the DTD activity and the sensitivity to EO9 ($r^2 = 0.80$) (Fig. 1). The cell lines in this panel can be divided into two groups, the sensitive cell lines with a high enzyme activity and less sensitive cell lines with no detectable DTD activity. The first group contains all solid tumor cell lines, while only leukemic cell lines are in the second group. The inactivity of EO9 on leukemic cell lines is in agreement with the data of the NCI screening and with the observation that EO9 is not myelotoxic [4, 42].

When looking at the enzyme activity data, slight misalignments between cytotoxicity and enzyme activity were found, such as with HT29, which would be expected to be the most sensitive cell line, based on DTD activity. It is, however, possible that measurement of the maximal enzyme activity was not completely precise since only one substrate concentration (40 μM DCPIP), derived from literature was used [36, 43]. In order to establish whether different enzyme properties would explain this phenomenon, we determined the enzyme kinetics of DTD in three cell lines, SW620, HT29 and A2780. The K_m values were very similar for HT29, A2780 and SW620 (33 ± 6, 34 ± 5.5, 43 ± 9 μM DCPIP, respectively; means ± SEM of 3–4 separate experiments). Strikingly, a pronounced substrate inhibition

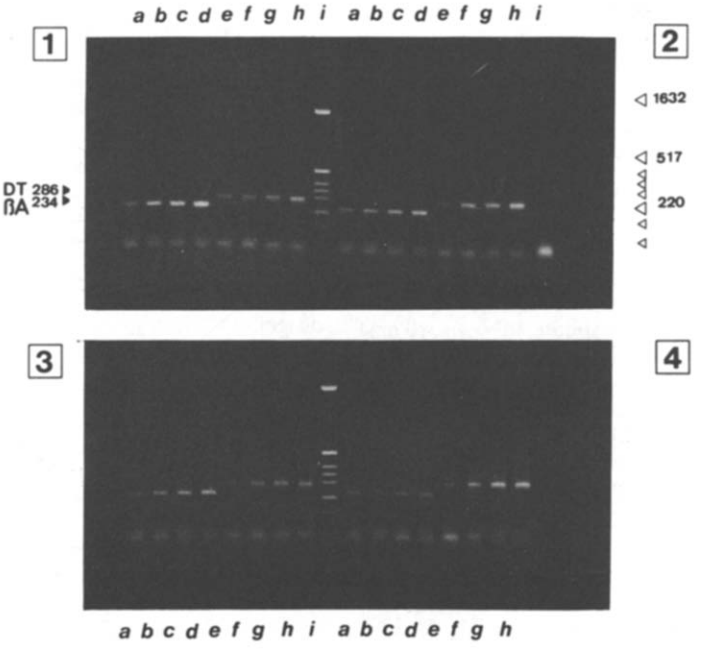

Fig. 2. Amplified PCR products run on an ethidium bromide stained agarose gel. The lower bands (lanes a–d) are β actin amplification products, the higher bands (lanes e–h) are DTD amplification products. Panel 1: lanes a–h, 0.01; 0.03; 0.05; 0.1; 0.1; 0.3; 0.5; 1 nL of SW620 cDNA; lane i, marker, pBR322 DNA digested with Hinf1. Panel 2: lanes a–h, 0.01; 0.03; 0.05; 0.1; 1; 5; 10; 20 nL of PC3 cDNA; lane i, negative control (no cDNA). Panel 3: lanes a–h, 0.01; 0.03; 0.05; 0.1; 0.1; 0.3; 0.5; 1 nL of HT29 cDNA; lane i, marker, pBR322 DNA digested with Hinf1. Panel 4: lanes a–h, 0.006; 0.01; 0.03; 0.05; 0.6; 1; 3; 5 nL of A2780 cDNA.

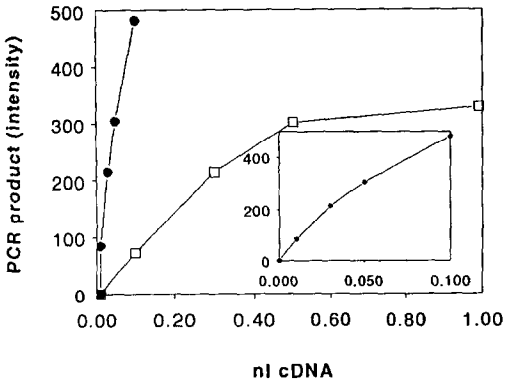

Fig. 3. Linear relationship between volume of cDNA amplified and the amount of PCR product, measured as intensity of the bands on gel. Various amounts of HT29 cDNA amplified with the β actin primer set (●), various amounts of HT29 cDNA amplified with the DTD primer set (□). *Inset*, expansion of the β actin graph.

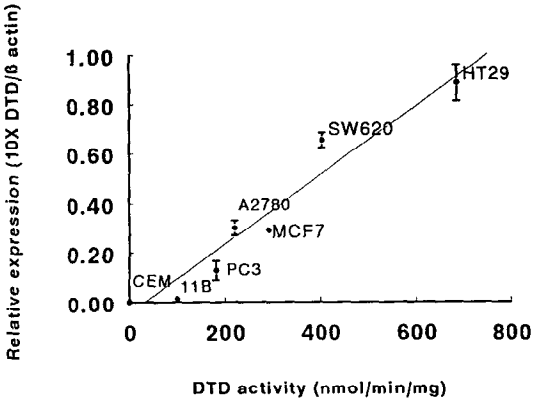

Fig. 4. Correlation between DTD expression (10 × DTD/β actin ratio) and enzymatic activity in seven human cancer cell lines.

was observed in all the cell lines at concentrations above 80 μM, but for A2780 even occurring at 40 μM DCPIP, a concentration often used for DTD activity measurements [11, 44].

Substrate specificity

Although DTD accepts many substrates (mostly quinones) the rate at which the reduction takes place may differ among substrates and enzyme extracts. We therefore compared the activities of the enzyme extracts of some of the cell lines with EO9 as a substrate. The activities in the tested cell lines were (in nmol EO9 reduced/min/mg protein) 28 for HT29, 15 for SW620, 7 for A2780, and 18 for MCF7, which is 20–40 times lower than the reduction of DCPIP. Of importance is that almost the same ranking order is observed as with DCPIP as substrate, so there was no different pattern of substrate specificity within these cell lines.

Although more appropriate for this study, EO9 was not used as the standard substrate in the DTD activity measurement due to less favorable enzyme kinetics of the human enzyme, as described by Walton [10, 45].

EO9 reduction could not completely be inhibited by 100 μM dicoumarol, as was also reported by Walton [9]. A combination of the following factors can be held responsible for that. Firstly, as pointed out by Preusch [46], EO9 is a less efficient substrate for the enzyme than e.g. DCPIP, which leads to the necessity for higher concentrations of inhibitor. Secondly it is possible that more reducing enzymes are present that act on EO9, such as the 1-electron reductase NADPH : cytochrome P450 reductase, that are not inhibited by dicoumarol. Although this is important for the activation of EO9 this assay gives less information about the DTD activity *per se*.

DTD gene expression

For each cell line multiple dilutions of the cDNA samples were used as a template in order to establish a linear range (Figs 2 and 3), as only in this range can the relative expression be calculated. For β-actin this range was between 0.005 and 0.05 nL of the cDNA (equivalent to the original cDNA, derived as described in material and methods). For DTD, amounts from 0.1–0.5 nL cDNA (HT29) up to 100–500 nL cDNA (CEM) were amplified in order to detect DTD expression in a linear range. Table 1 and Fig. 4 show the excellent agreement between DTD activity and gene expression ($r^2 = 0.94$). As expected, the expression of the DTD gene was also related to the chemosensitivity to EO9 ($r^2 = 0.6$) (not shown). The murine cell lines were not included since the murine and human DTD genes are not homologous in the amplified region.

Modulation of sensitivity to EO9 by dicoumarol

In order to determine how EO9 is activated intracellularly by DTD, we used the DTD inhibitor dicoumarol to modulate the chemosensitivity to EO9. Experiments were performed in the three cell lines used for the enzyme kinetics. Dicoumarol at the concentrations utilized (1, 10 μM for 72 hr or 50 μM for 3 hr) exhibited no growth inhibition by itself, however at higher concentrations and with continuous exposure, dicoumarol was more growth inhibitory on A2780 cells than on the colon cell lines (result not shown). Addition of dicoumarol after EO9 had no effect at all (scheme a). Dicoumarol had no significant effect when HT29 cells were exposed continuously to 1 μM of this DTD inhibitor together with EO9. Addition of this low concentration of dicoumarol together with EO9 or 24 hr prior to EO9 had a slight (1.3–3 times) but significant inhibiting effect on cytotoxicity of EO9 on SW620 and A2780 cells (Table 2, schemes b, c). However, when added for a short time at higher dicoumarol concentrations, for all the tested cell lines a larger and comparable (7–15 times) effect was found (scheme d).

DISCUSSION

In this paper we demonstrate a correlation between

Table 2. Effect of dicoumarol

Cell line	Ratio (EO9 + dic) IC_{50}/EO9 IC_{50}		
	Scheme a*	Schemes b, c*	Scheme d*
A2780	1 ± 0.1	2 ± 0.5	10, 27
SW620	1 ± 0.1	2.5 ± 0.5	9, 15
HT29	1 ± 0.2	1 ± 0.3	10, 14

Values ± SE derived from three experiments unless otherwise indicated.
* Schemes as described in Materials and Methods.

DT-diaphorase activity, DTD gene expression and chemosensitivity to the bioreductive indoloquinone EO9 in 11 cell lines of different histological origin. There was a striking difference between leukemic cell lines, which were resistant to EO9 and contained no detectable DTD activity, and solid tumor cell lines with a high DTD activity and which were more sensitive to EO9.

Our data are supportive of previously demonstrated correlations between EO9 sensitivity and DTD activity [28–30], but are contrary to results of Collard and Double, who described three cell lines with the same sensitivity, but with a broad variation in DTD activity [47]. Apart from adding different types of cell lines, this study gives an extra dimension to the subject of enzyme directed drugs, by measuring the gene expression by PCR. Semiquantitative PCR appears to be a convenient method to determine the amount of DTD mRNA, even in samples in which DTD enzyme activity was not measurable (CCRF-CEM). The excellent correlation between enzyme activity and DTD expression in colon cell lines, as was shown by Traver *et al.* [39], is extended by the present data using a set of cell lines from different histological origin, showing that the original observation of a linear relation can be generalized.

The cell lines in our study can be divided into two groups: sensitive cell lines, containing high DTD activity (>90 nmol/min/mg) and relatively resistant cell lines with no measurable enzyme activity. Especially when combining our data with the results of Robertson [30], who used NSCLC, SCLC and breast cancer cell lines, the gap between the two clusters is filled and the causal link between enzymes activity and chemosensitivity gets even more evident. Unfortunately, it is not possible to calculate a correlation coefficient from the combined data from literature, because of the use of different substrates (menadione, DCPIP).

Although EO9 is found inactive against some leukemias and bone marrow [4, 42], it is not allowed to assume that all leukemic cells have a low DTD and low sensitivity, since Phillips [14] reported one leukemic cell line, K562, with a low DTD activity, which was as sensitive as HT29 ($IC_{50} = 5 \times 10^{-8}$ M).

When comparing the results for the murine NIH3T3 cell line and its transformed variants, the TRK transformed line has three times more DTD than the parent and is more sensitive, although not to the same extent. The H-RAS transformed variant has 1.5 times more DTD but is rather less sensitive to EO9. So the transformed lines do differ from the parent, although both to a different extent. Insertion of the v-Ha-RAS oncogene in human lung cancer cell lines was only enhancing the DTD activity, when the transfection resulted in altered growth patterns [44]. Apparently, transfection with H-RAS in our transformed NIH3T3 variant was not sufficient to affect the chemosensitivity.

When studying DTD activity in relation to some other bioreductive agents, no correlation with chemosensitivity to MMC or porfiromycin was found [30]. In addition to the correlation between DTD activity and sensitivity to EO9 or MMC, several authors attempted to correlate the DTD expression with sensitivity to MMC [39, 44]. Malkinson *et al.* [44] found a correlation between the amount of DTD mRNA expression (by northern blotting) and enzyme activity in several lung cancer cell lines and a non-linear relation with the effect of MMC on xenografts of the same cell lines. For a MMC resistant subset of the colon lines tested by Traver *et al.*, the expression of DTD was found to be in agreement with the sensitivity to MMC [39]. Although the extent of the role of DTD in the activation of MMC is still unclear [48, 49], our data support a major role for DTD in the activation of EO9.

We investigated further the small discrepancies found for some of the cell lines in the correlation curve between enzyme activity and sensitivity. The K_m values, determined in order to detect abnormalities in enzyme kinetics, did not differ significantly, leading to the conclusion that the affinity of these enzymes for DCPIP could not be the cause for the differences in sensitivity. The substrate inhibition was already observed at 40 μM DCPIP in A2780 cells and demonstrates that the V_{max} value is relatively higher in A2780 cells compared to other cell lines, than would be predicted based on the enzyme measurement reported in Table 1. This kind of substrate inhibition was also found by Walton *et al.*, when using menadione/cytochrome *c* as substrate but not with EO9 [10, 45]

These questions about the enzyme kinetics would not be encountered when evaluating the gene expression. However, the expression of DTD in, e.g. HT29 cells, is also not proportional to EO9 sensitivity. It is possible that differences in isoenzyme content are present. Since it is known that the genes of NQO1 and NQO2 (the isoforms of DTD) are

substantially different, they could not have been detected with the PCR primer set we used.

Other variables that may play a role in determining the sensitivity to EO9 are for example the intrinsic sensitivity to alkylating agents (repair of DNA damage), or the presence of other enzymes such as NADPH: P450 reductase, which are associated with the performance of the drug under hypoxia (1-electron reductases) [7]. These parameters could have influenced the shape of the curve in Fig. 1.

When using long exposures to a low concentration of dicoumarol in the HT29 cell line, dicoumarol could not reverse the effect of EO9, whereas a small reversal was achieved in two other cell lines (Table 2). However, dicoumarol had a definite effect on the three tested cell lines when using a shorter treatment with a higher concentration, in line with the theory of Preusch, stating that higher inhibitor concentrations are necessary when the second substrate (quinone) is less efficiently reduced [46]. The larger effect of a short exposure time may also be linked to the short half-life of EO9 itself [50]. The inhibiting effect of dicoumarol should be taken in account when patients use EO9 in combination with oral anticoagulants, some known to be inhibitors of DTD [17, 36].

PCR methods usually have a major advantage over enzyme activity assays because of their greater sensitivity. However, in the case of DTD the activity assay is very sensitive too, as one needs less than 10^5 cells, a number which is higher than that required for RNA isolation, but achievable by tumor biopsy. In general in the prediction of sensitivity to a drug that needs activation by a target enzyme, the evaluation of enzyme activity should be preferred to the gene expression, as mutations can give rise to inactive enzymes without significantly altering expression levels [39]. Since we observed an excellent agreement between activity and expression for DTD both approaches can be used, although the simpler enzyme assay appears to be informative enough.

Preclinical studies suggested a high activity of EO9 in solid tumors; this is probably due to the fact that EO9 can be activated by DTD in both euoxic and hypoxic regions [4, 7]. Identification of those tumor types with high levels of DTD will possibly identify tumor types with high sensitivity to EO9 *in vivo*. Several studies have evaluated EO9 or MMC sensitivity mostly in murine tumor models [3, 28, 29, 44]. Sensitive tumors appeared to have a higher DTD content than resistant tumors. This together with the present data warrant the investigation of DTD activity in tumor samples from patients, and eventually correlate it with sensitivity to bioreductive agents such as EO9.

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