Establishment of an Isogenic Human Colon Tumor Model for NQO1 Gene Expression: Application to Investigate the Role of DT-Diaphorase in Bioreductive Drug Activation In Vitro and In Vivo

SWEE Y. SHARP, LLOYD R. KELLAND, MELANIE R. VALENTI, LISA A. BRUNTON, STEVE HOBBS, and PAUL WORKMAN

CRC Centre for Cancer Therapeutics, The Institute of Cancer Research, Sutton, Surrey, United Kingdom

Received April 8, 2000; accepted July 6, 2000 This paper is available online at http://www.molpharm.org

ABSTRACT

Many tumors overexpress the NQO1 gene, which encodes DT-diaphorase (NADPH:quinone oxidoreductase; EC 1.6.99.2). This obligate two-electron reductase deactivates toxins and activates bioreductive anticancer drugs. We describe the establishment of an isogenic human tumor cell model for DT-diaphorase expression. An expression vector was used in which the human elongation factor 1a promoter produces a bicistronic message containing the genes for human NQO1 and puromycin resistance. This was transfected into the human colon BE tumor line, which has a disabling point mutation in NQO1. Two clones, BE2 and BE5, were selected that were shown by immunoblotting and enzyme activity to stably express high levels of DT-diaphorase. Drug response was determined using 96-h exposures compared with the BE vector control. Functional validation of the isogenic model was provided by the much greater sensitivity of the NQO1-transfected cells to the known DT-diaphorase substrates and bioreductive agents streptonigrin (113- to 132-fold) and indoloquinone EO9 (17- to 25-fold) and the inhibition of this potentiation by the DT-diaphorase inhibitor dicoumarol. A lower degree of potentiation was seen with the clinically used agent mitomycin C (6- to 7-fold) and the EO9 analogs, EO7 and EO2, that are poorer substrates for DT-diaphorase (5- to 8-fold and 2- to 3-fold potentiation, respectively), and there was no potentiation or protection with menadione and tirapazamine. Exposure time-dependent potentiation was seen with the diaziquone analogs methyl-diaziquone and RH1 [2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone], the latter being an agent in preclinical development. In contrast to the in vitro potentiation, there was no difference in the response to mitomycin C when BE2 and BE vector control were treated as tumor xenografts in vivo. This isogenic model should be valuable for mechanistic studies and bioreductive drug development.

NADPH:quinone oxidoreductase (EC 1.6.99.2, DT-diaphorase) is an obligate two-electron reducing enzyme of interest because it deactivates toxins and carcinogens and activates bioreductive antitumor agents (e.g., see Riley and Workman, 1992a; Ross et al., 1993, 1996; Workman, 1994; Rauth et al., 1997; Stratford and Workman, 1998). Expression of DT-diaphorase is higher in some human tumor cell lines and cancer tissues (notably colon, lung, breast, and liver) versus the normal equivalents (Schlager and Powis, 1990; Belinsky and Jaiswal, 1993; Smitskamp-Wilms et al., 1995; Fitzsimmons et al., 1996; Marin et al., 1997). Bioreductive antitumor agents activated by DT-diaphorase include mitomycin C (Siegel et al., 1990b; Cummings et al., 1998), diaziquone (AZQ) (Siegel et al., 1990a), the indoloquinone EO9 (Walton et al., 1991, 1992b; Plumb et al., 1994) and streptonigrin (Beall et al., 1996; see also Boyer, 1997; Stratford and Workman, 1998, for recent reviews).

The most extensively studied form of DT-diaphorase is encoded by the NQO1 gene (Robertson et al., 1986; Jaiswal, 1991). The NQO1 gene promoter contains the antioxidant response element, xenobiotic response element, and activator protein 2 elements, which all have the potential to regulate gene expression and may contribute to higher activity in tumors (Jaiswal, 1994). A commonly used approach to discover agents activated by DT-diaphorase is to determine the sensitivity of tumor cell panels differing in DT-diaphorase expression (Robertson et al., 1992; Collard et al., 1995; Fitzsimmons et al., 1996) or to compare specific, nonisogenic, paired cell lines (Siegel et al., 1990a,b; Plumb and Workman, 1994; Beall et al., 1996,
1998). Many low DT-diaphorase tumor lines, such as BE, have a homozygous disabling point mutation in NQO1 (Traver et al., 1992). Although useful, such nonisogenic paired cell line models suffer from the disadvantage that differences in the expression of a range of genes other than DT-diaphorase could also affect drug sensitivity.

To provide a better model for determining the role of NQO1 in activating bioreductive agents, we established cell lines that are isogenic apart from their level of NQO1 gene expression. To do this, we constructed a mammalian bicistronic expression vector containing the wild-type NQO1 gene in which expression is driven by the powerful human elongation factor 1α promoter. After stable transfection into the NQO1 mutant BE human colon tumor cell line, clones expressing DT-diaphorase were selected for further evaluation. Sensitivity to various bioreductive agents, including established DT-diaphorase activated drugs, was determined in NQO1 transfected versus vector control BE lines. After functional validation, we used the isogenic model to answer two questions: (1) what is the role of DT-diaphorase in the bioactivation of various developmental bioreductive agents in vitro? and (2) what is the relative contribution of DT-diaphorase to the antitumor efficacy of mitomycin C in solid tumors in vivo? As this work was nearing completion, a similar model was reported by Winski et al. (1998) and the results are compared with that and other models.

Materials and Methods

Drugs and Chemicals. Streptonigrin (Sigma Chemicals, Poole, UK), the indoloquinones EO9, EO2, and EO7 (Dr. Hans Hendriks, NDDO, Amsterdam, the Netherlands) and diaziquones (AZQ, MeDZQ, and RH1; Dr. J. Butler and Dr. R. Hargreaves, University of Salford, UK) were made up at 2 mM (except RH1 at 10 mM) in dimethyl sulfoxide. Tirapazamine (Professor Ian Stratford, University of Manchester, UK) at 10 mM was dissolved in dimethyl sulfoxide. Cisplatin (at 1 mM stock in 0.9% saline) was obtained from the Johnson Matthey Technology Center (Reading, Berkshire, UK). Menadione (Sigma) was dissolved at 1 mM in sterile water. The chemical structures of these drugs are shown in Fig. 1. All other chemicals were purchased from Sigma, unless otherwise stated.

Cell Culture. The BE and HT29 human colon tumor cell lines grew as monolayers in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum (Life Technologies, Paisley, Scotland, UK), 2 mM L-glutamine, 0.5 μg/ml hydrocortisone, and minimal essential medium nonessential amino acids in a 6% CO2/94% air atmosphere. Cells were free of Mycoplasma species.

Construction of DT-Diaphorase Expression Plasmids. The bicistronic expression vector pEFIRES-P (Hobbs et al., 1998) was used to express the human NQO1 gene (Jaiswal, 1991) in BE cells. This plasmid uses the human elongation factor 1α promoter to produce a bicistronic message containing the gene of interest followed by the internal ribosome entry site sequence from the encephalomyocarditis virus, which directs internal translation initiation of the gene for puromycin resistance, thus predisposing all puromycin resistant clones toward expression of the test gene. Full details of the construction and use of this plasmid (F373) are published elsewhere (Hobbs et al., 1998). A plasmid containing the cDNA for human NQO1 DT-diaphorase was obtained from Dr. S. Chen via Professor R. Knox (Chen et al., 1995). The coding region was amplified from this vector by polymerase chain reaction (PCR) with the proof-reading polymerase Pfu (Stratagene, La Jolla, CA); unique NcoI sites were incorporated in the primers HDIAFOR1:5'-ACCAGAGCCATG-GTCGGCAG and HDIABACK1:5'-TTTGATACCATGGTAGAAGG (NcoI sites underlined) to enable subcloning. The PCR product was first blunt-cloned into the plasmid pBluescript II SK(+) at the EcoRV site. A separate modified form of pBluescript II SK(+) was prepared in which the section of the multiple cloning site between the EcoRV and HincII sites (both blunt cutters) was replaced with the sequence CCTCGAGTCACCATGAT (C, G, A, T). This introduced an extra XhoI site (underlined) followed by a Kozak sequence for good translation initiation in eukaryotic cells (Kozak, 1984) and a unique NcoI site. The NQO1 PCR insert was then excised as a NcoI fragment and cloned into this modified Bluescript at the NcoI site.

This plasmid uses the human elongation factor 1α promoter to drive expression of the test gene preceded by an internal ribosome entry site (IRES) sequence from the encephalomyocarditis virus, which directs internal translation initiation of the gene for puromycin resistance, thus predisposing all puromycin resistant clones toward expression of the test gene. Full details of the construction and use of this plasmid (F373) are published elsewhere (Hobbs et al., 1998). A plasmid containing the cDNA for human NQO1 DT-diaphorase was obtained from Dr. S. Chen via Professor R. Knox (Chen et al., 1995). The coding region was amplified from this vector by polymerase chain reaction (PCR) with the proof-reading polymerase Pfu (Stratagene, La Jolla, CA), unique NcoI sites were incorporated in the primers HDIAFOR1:5'-ACCAGAGCCATG-GTCGGCAG and HDIABACK1:5'-TTTGATACCATGGTAGAAGG (NcoI sites underlined) to enable subcloning. The PCR product was first blunt-cloned into the plasmid pBluescript II SK(+) at the EcoRV site. A separate modified form of pBluescript II SK(+) was prepared in which the section of the multiple cloning site between the EcoRV and HincII sites (both blunt cutters) was replaced with the sequence CCTCGAGTCACCATGAT (C, G, A, T). This introduced an extra XhoI site (underlined) followed by a Kozak sequence for good translation initiation in eukaryotic cells (Kozak, 1984) and a unique NcoI site.

The NQO1 PCR insert was then excised as a NcoI fragment and cloned into this modified Bluescript at the NcoI site. A recombinant vector was then selected in which the open reading frame (ORF) followed the XhoI site and Kozak sequence in the sense orientation. The ORF bearing the Kozak sequence and NcoI start codon was excised from this plasmid as an XhoI fragment using the downstream XhoI site remaining in the Bluescript multiple cloning site and cloned into the XhoI site of the vector pCR-Script Cam. Recombinants in which the insert was present in the forward and reverse orientations were first cut with Asp718 and then treated with Klenow DNA polymerase and dNTPs to flush the ends. The Klenow was heat-sacrificed, and the DNA was cleaned and subjected to a second digestion with SalI to isolate the ORF. The vector pEFIRES-P was prepared by cutting

Fig. 1. Chemical structures of the drugs used.
with XhoI followed by Klenow fill-in and a separate digest with Xhol. In this form, it accepted the blunt/SalI inserts bearing the NQO1 (DT-diaphorase) ORF, producing the final plasmids P388 (antisense) and F397 (sense) (Fig. 2). Insert identities were confirmed by diagnostic PCR, restriction digests, and dideoxy sequencing using standard methods (Sambrook et al., 1989).

Transfection of the NQO1 Gene into BE Cells. BE cells were seeded into six-well plates at $3 \times 10^5$/well and allowed to attach and enter exponential growth for 2 days. Five micrograms of vector DNA in Lipofectamine (Life Technologies) was added to cells for 6 h under serum-free conditions. The cells were then washed with PBS and growth medium was added. Two days later, cells were trypsinized and divided into 24-well plates in the presence of 0.3 to 0.5 $\mu$g/ml puromycin (concentrations predetermined to kill all nontransfected cells). Two transfected lines, BE-F397 clone 2 (BE2) and BE-F397 clone 5 (BE5), were selected for detailed study and comparison with the BE empty vector (F373) control.

Western Blotting. The Western blot analysis of DT-diaphorase was performed according to Sharp et al. (1994), using the rat DT-diaphorase polyclonal antibody (from Professor R. Knox; Fitzgerald Pharmaceuticals, Sunnyvale, CA).

Enzyme Assay for DT-Diaphorase and Other Reductases. DT-diaphorase activity was measured by a spectrophotometric assay in which the rate of reduction of cytochrome $c$ was monitored at 550 nm (Walton et al., 1991). Briefly, cells ($2 \times 10^5$) were harvested and lysed at 4°C for 1 h and then centrifuged. The supernatants were used for protein determination (bicinichonic acid assay; Pierce Chemical, Rockford, IL) and Western blot analysis. The human colon cell line HT29 previously shown to overexpress DT-diaphorase (Traver et al., 1995) was used as the positive control. Densitometry analysis was carried out using the ImageQuant program (Molecular Dynamics, Sunnyvale, CA).

Results

DT-Diaphorase Protein Expression. Figure 3 shows the expression of DT-diaphorase in HT29 (positive control), BE vector control, and the two DT-diaphorase transfected lines, BE-F397 clone 2 (BE2) and BE-F397 clone 5 (BE5). DT-diaphorase was readily detected in all cell lines except for the BE vector control. The BE2 line expressed approximately 10 times the activity of the positive control. The BE5 line expressed approximately 20 times the activity of the positive control.
1.5-fold higher DT-diaphorase protein than the BE5 line, as measured by densitometry.

**DT-Diaphorase Activity.** Table 1 shows the activity of DT-diaphorase in all cell lines. There was no detectable activity in the BE vector control. DT-diaphorase transfected cell lines (BE2 and BE5) exhibited at least a 650- to 700-fold increase in activity over BE vector control and a 1.4- to 1.5-fold higher activity than HT29, which was reported previously to possess constitutively high enzyme activity (Traver et al., 1992; Riley et al., 1993; Plumb and Workman, 1994; Fitzsimmons et al., 1996). There were no differences in the activity of two other reductive enzymes that may contribute to drug sensitivity, cytochrome P450 reductase, and cytochrome b₅ reductase, between the BE vector control and BE2 cell lines (Table 1).

**Sensitivity to Bioreductive Agents.** For functional validation, we determined sensitivity to streptonigrin and indoloquione EO9, which are strongly activated by DT-diaphorase (Walton et al., 1991, 1992b; Beall et al., 1996). With both agents, the sensitivities of the HT29 line and of each of the NQO1 transfected clones was much greater than that of BE vector control, as measured by growth inhibition after 96-h exposure (Fig. 4, A and B). The degree of potentiation (sensitization ratio, IC₅₀ in BE vector control/IC₅₀ in either BE2 or HT29) was 132-fold for streptonigrin and 16.7-fold for EO9 in the BE2 compared with BE vector control cells, and 198-fold and 16.7-fold for streptonigrin and EO9, respectively, in HT29 cells compared with BE vector control. Thus, transfection of wild-type BE cells with NQO1 increased sensitivity to streptonigrin and EO9 to equal that of the intrinsically sensitive and constitutively DT-diaphorase rich HT29 line. In contrast, there was no difference in sensitivity between the lines with the directly DNA damaging drug, cisplatin; IC₅₀ ± SD values were 10.7 ± 2.2 μM for BE vector control cells, 8.3 ± 2.5 μM for BE2 cells, 8.5 ± 4.0 μM for BE5 cells, and 8.0 ± 3.2 μM for HT29. No sensitization was observed in either the BE isogenic pair or the HT29/BE vector control pair for the benzotriazine-di-N-oxide bioreductive agent, tirapazamine (Table 2). This agent is activated by one-electron reductases rather than DT-diaphorase (Walton and Workman, 1990; Riley and Workman, 1992b; Walton et al., 1992a; Fitzsimmons et al., 1994; Patterson et al., 1997, 1998; Evans et al., 1998). Interestingly, with the commonly used DT-diaphorase substrate and oxidative stress-inducing agent menadione, there was no difference in sensitivity between BE2 and BE vector control cells (Table 2).

Although subject to some criticism (e.g., Ross et al., 1993; Workman, 1994) modulation of drug sensitivity by the inhibitor dicoumarol (Ernster, 1987) is often used to indicate the involvement of DT-diaphorase. Figure 5A shows that there was no difference in sensitivity to either streptonigrin or EO9 in the absence or presence of 100 μM dicoumarol (maximum nontoxic concentration for a 2-h exposure) in the BE vector control line. However, sensitivity of BE2 cells to streptonigrin and EO9 were decreased by 103-fold and 65-fold, respectively (Fig. 5B). This supports the view that the potent growth inhibitory effects of streptonigrin and EO9 in the NQO1-transfected BE2 cells are mediated by DT-diaphorase.

The sensitivities of the cell lines to other known or putative DT-diaphorase substrates and bioreductive agents were also determined after 96-h exposure to the drugs (Table 2). Compared with streptonigrin and EO9, there was a lower but significant sensitization to mitomycin C (mean of 6.4-fold in the BE DT-diaphorase clones) as a result of NQO1 transfection. Interestingly, a greater sensitization (22-fold) was ap-

---

**TABLE 1**

Activity of DT-diaphorase and other reductive enzymes in HT29, BE vector control, BE2 and BE5 cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Enzyme Activity Reduction of Cytochrome c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DT-diaraphorase</td>
</tr>
<tr>
<td>HT29</td>
<td>940 ± 200</td>
</tr>
<tr>
<td>BE vector control</td>
<td>2.0</td>
</tr>
<tr>
<td>BE2</td>
<td>1400 ± 500</td>
</tr>
<tr>
<td>BE5</td>
<td>1300 ± 200</td>
</tr>
</tbody>
</table>

Values are mean ± SD of triplicate experiments.

ND, not determined.

* Lower limit of detection.

---

![Fig. 4. Dose response curves for streptonigrin (A) and EO9 (B) in BE vector control (▲), BE2 (▼), BE5 (■), and HT29 (●). C, sensitization ratio, IC₅₀ in BE vector control versus HT29, BE2, or BE5, for streptonigrin (■) or EO9 (●). Results are representative experiment of three determinations](image)
parent when comparing the HT29 naturally high DT-diaphorase colon line with the BE vector control, suggesting the involvement of additional genes in HT29. For two EO9 analogs, EO2 and EO7 (Bailey et al., 1992; Phillips, 1996), sensitization was considerably lower than for EO9: a mean of 2.6-fold for EO2 and 6.8-fold for EO7 obtained in the BE DT-diaphorase clones. When comparing HT29 cells with the BE vector control cells, a similar sensitization (3.3- and 3.6-fold) was observed for both EO2 and EO7. These results are consistent with the behavior of EO2 and EO7 as poorer substrates for DT-diaphorase compared with EO9 (Bailey et al., 1992; Phillips, 1996).

There was no change in sensitivity in BE2 and BE5 versus BE vector control lines to either of the simple 1,4-benzoquinone-based bioreductive agents, AZQ and MeDZQ, after 96-h continuous drug exposure. In contrast, there was greater sensitivity in HT29 cells (3.7-fold for AZQ and 10-fold for MeDZQ) versus the BE vector control. For RH1, a close analog of MeDZQ in preclinical development, a 4-fold increase in sensitivity was observed for the transfected BE2 cells versus BE vector control. However, this was much less

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>96-h IC50</th>
<th>Streptonigrin EO9</th>
<th>Mitomycin C EO2</th>
<th>EO7</th>
<th>AZQ</th>
<th>MeDZQ</th>
<th>RH1</th>
<th>Tirapazamine</th>
<th>Menadione</th>
</tr>
</thead>
<tbody>
<tr>
<td>BE vector control</td>
<td>7.9 ± 1.9</td>
<td>0.5 ± 0.9</td>
<td>22 ± 0.3</td>
<td>1.0 ± 0.08</td>
<td>25.3 ± 10.1</td>
<td>1.2 ± 0.9</td>
<td>0.2 ± 0.02</td>
<td>0.007 (0.06)</td>
<td>ND</td>
</tr>
<tr>
<td>BE2</td>
<td>0.06 ± 0.02</td>
<td>0.02 (112.9)</td>
<td>0.02</td>
<td>0.008 (25.0)</td>
<td>0.02</td>
<td>0.1 (5.5)</td>
<td>0.02</td>
<td>0.022 (20.0)</td>
<td>0.0005 (0.37)</td>
</tr>
<tr>
<td>BE5</td>
<td>0.07 ± 0.04</td>
<td>0.02 (112.9)</td>
<td>0.02</td>
<td>0.008 (25.0)</td>
<td>0.02</td>
<td>0.1 (5.5)</td>
<td>0.02</td>
<td>0.022 (20.0)</td>
<td>0.0005 (0.37)</td>
</tr>
<tr>
<td>HT29</td>
<td>0.04 ± 0.01</td>
<td>0.01 (197.5)</td>
<td>0.03</td>
<td>0.006 (3.3)</td>
<td>0.03</td>
<td>0.02 (22.0)</td>
<td>0.03</td>
<td>0.05 (3.3)</td>
<td>0.005 (3.6)</td>
</tr>
</tbody>
</table>

Table 3
Optimum treated/control (%) values for HT29, BE vector control, and BE2 xenografts

<table>
<thead>
<tr>
<th>Xenograft</th>
<th>Day</th>
<th>Treated/Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>10*</td>
<td>69.9</td>
</tr>
<tr>
<td>BE vector control</td>
<td>24</td>
<td>39.2</td>
</tr>
<tr>
<td>BE2</td>
<td>25</td>
<td>40.4</td>
</tr>
</tbody>
</table>

* Experiment with HT29 xenograft was terminated on day 10 because the tumor became ulcerated.

Fig. 5. The effect of the absence (■) and presence (○) of dicoumarol (100 μM) on the sensitivity of BE vector control (A) and BE2 (B) cells to a 2-h exposure to streptonigrin or EO9. SD was less than the symbol size where not indicated.
that 40-fold greater sensitivity seen with HT29, suggesting (as with mitomycin C) the involvement of additional genes. Because of the relatively small effects seen with MeDZQ and RH1 using a 96-h treatment and in view of their behavior as good DT-diaphorase substrates (Beall et al., 1995), we examined 2-, 6-, and 24-h exposures in BE2 and HT29 cells. Differences in sensitivity compared with the BE vector control were generally greater using the shorter drug exposures (Fig. 6). For MeDZQ the maximum differentials were 13.3-fold for BE2 (at 24 h) and 26-fold for HT29 (at 2 h). With RH1, the maximum differentials were 25-fold for BE2 and 100-fold for HT29 (both seen at 24 h). The relationship between sensitivity and exposure time was not simple, but differentials were generally greater for the intrinsically high DT-diaphorase HT29 line than for the transfected BE2 line, compared with BE vector control.

**In Vivo Antitumor Efficacy of Mitomycin C.** The presence of DT-diaphorase in the HT29 and BE2 cells grown as a solid tumor xenograft in nude mice was confirmed by Western blotting (Fig. 7). The activities of DT-diaphorase in these xenografts were $18.3 \pm 2.3$ and $6.1 \pm 0.4$ nmol/min/mg of protein for HT29 and BE2, respectively, with no detectable activity in the BE vector control. Figure 8 shows that responses to mitomycin C (2 mg/kg, i.p. injected, days 0, 4, and 8) were similar in BE vector control, BE2, and HT29 (Table 3). In particular, the optimum treated/control (%) values were similar at 39% for BE vector control on day 24 and 40% for BE2 on day 25 and slightly higher at 70% on day 10 for HT29. No responses were seen in similar studies with streptonigrin (not shown) but the quinone ansamycin Hsp 90 inhibitor 17-allylamino-17-demethoxy geldanamycin showed greater activity in HT29 and BE2 than in BE vector control xenografts (Kelland et al., 1999).

**Discussion**

The enzyme-directed strategy for bioreductive anticancer drug development aims to improve therapeutic selectivity by exploiting increased expression of reductases in tumor versus normal tissue (Workman 1994; Ross et al., 1996; Stratford and Workman, 1998). The enzyme that has received the most attention is DT-diaphorase, encoded by the gene NQO1, which shows high level expression in many tumor cell lines and human cancers (Schlager and Powis, 1990; Riley and Workman, 1992; Robertson et al., 1992; Belinsky and Jaiswal, 1993; Smitskamp-Wilms et al., 1995; Fitzsimmons et al., 1996; Marin et al., 1997).

Here, we transfected the wild type human NQO1 gene into a human colon tumor cell line BE, which has null DT-diaphorase activity due to a homozygous disabling point mutation in NQO1 (Traver et al., 1992). This polymorphism in position 609 of NQO1 (proline to serine change in amino acid 197) is present in 5 to 20% of patients depending on ethnic background (Kelsey et al., 1997). Two stable, high-expressing DT-diaphorase clones were selected (termed BE2 and BE5) and expression confirmed by Western blotting and enzyme activity. The activity of the BE2 and BE5 clones was 1300 to 1400 nmol/min/mg compared with $2$ nmol/min/mg for the BE vector control, an increase of at least 650-fold. DT-diaphorase protein levels and activities were about 50% higher in BE2 and BE5 than in the constitutively active HT29 human colon tumor cell line, for which values are similar to previous reports (e.g., Plumb and Workman, 1994; Beall et al., 1996; Fitzsimmons et al., 1996). The activity of two other representative quinone reductase enzymes that may metabolize these antitumor quinones, cytochrome P450 reductase and cytochrome $b_5$ reductase, were similar. Xanthine oxidase/xanthine dehydrogenase was not determined but other studies reported no detectable activity in HT29 and BE cells (Beall et al., 1996; Winski et al., 1998).

In addition to catalytic activity, further functional validation of our transfected BE models for NQO1/DT-diaphorase was provided by the marked sensitization observed in the transfected BE2 cells to the known DT-diaphorase substrates.
and bioreductive agents streptonigrin (Beall et al., 1995, 1996) and indoloquinone EO9 (Robertson et al., 1992; Walton et al., 1992b; Beall et al., 1995, Collard et al., 1995; Fitzsimmons et al., 1996; Bailey et al., 1997). Potentiation arising from NQO1 transfection was particularly impressive with streptonigrin. This is consistent with observations that when data on 31,000 compounds tested against the NCI 60 human tumor cell line panel were analyzed for a relationship between sensitivity and DT-diaphorase expression, streptonigrin showed the strongest correlation (Paull et al., 1994). Also adding to the functional validation of our isogenic BE model, the DT-diaphorase inhibitor dicoumarol (Ernster, 1987) protected BE2 cells, but not vector controls, from streptonigrin and EO9 cytotoxicity. In contrast, BE2, BE5, and the vector control showed similar sensitivities to the directly DNA damaging drug cisplatin, as noted previously for BE and HT29 cells (Plumb and Workman, 1994).

The role of DT-diaphorase in bioactivating mitomycin C, the prototype bioreductive alkylating agent, is controversial (e.g., Workman et al., 1989; Workman, 1994; Cummings et al., 1998), and involves one-electron (cytochrome P450 reductase, xanthine oxidase, cytochrome b5 reductase, or xanthine dehydrogenase; e.g., see Hoban et al., 1990; Gustafson and Pritsos, 1992; Hodnick and Sartorelli 1993; Patterson et al., 1997; Cummings et al., 1998) as well as DT-diaphorase (e.g., see Siegel et al., 1990b; Ross et al., 1993, 1996). Most cellular data show a positive correlation between DT-diaphorase expression and mitomycin C cytotoxicity (Siegel et al., 1990b; Robertson et al., 1992; Ross et al., 1993, 1996; Plumb and Workman, 1994). This relationship was seen in the NCI 60 human tumor cell line panel for mitomycin C and also the indoloquinone EO9 (Fitzsimmons et al., 1996). Furthermore, previous studies have showed HT29 colon cells to be 6- to 18-fold more sensitive to mitomycin C than BE cells (Siegel et al., 1990b; Plumb and Workman, 1994, respectively) and the high DT-diaphorase H460 lung cells to be 11-fold more sensitive than DT-diaphorase negative H596 (Beall et al., 1995). However, across 15 human lung breast and colon tumor lines, no correlation existed between DT-diaphorase levels and sensitivity (Robertson et al., 1992). Our results for mitomycin C using the HT29 versus BE comparison agree with the previous reports, with HT29 cells being 22-fold more sensitive. Moreover, our results using the BE2 and BE5 transfected lines, where a mean of 6.4-fold potentiation was observed relative to BE vector control cells, are supportive of a significant contributory role for DT-diaphorase in mediating the in vitro cellular response to mitomycin C. On the other hand, the greater sensitivity of HT29 compared with BE2 and BE5 implicates additional genes governing mitomycin C sensitivity.

After clinical trials with mitomycin C and EO9 (Boyer, 1997), the aromatic N-oxide, tirapazamine is the latest bioreductive drug, in this case targeted specifically at hypoxic tumor cells, to enter the clinic; it is currently undergoing phase III evaluation in combination with chemotherapy (Von Pawel and Von Roemeling, 1998). We found no difference in sensitivity to tirapazamine between the high- and low-DT-diaphorase BE clones, or between control BE and HT29 cells. These results are consistent with previous nonisogenic cell comparisons and enzymology studies (Walton and Workman, 1990; Riley and Workman, 1992a,b; Walton et al., 1992a; Fitzsimmons et al., 1994; Patterson et al., 1994, 1998; Plumb and Workman, 1994; Evans et al., 1998). Although DT-diaphorase catalyzes a reduction that would detoxify tirapazamine (Walton and Workman, 1990; Riley and Workman, 1992b), this clearly does not play a role in cellular sensitivity, where activation by one-electron reductases predominates (Fitzsimmons et al., 1994; Patterson et al., 1997; Evans et al., 1998).

Our results showing a lack of effect of NQO1 transfection on menadione toxicity are somewhat surprising, because menadione was the standard agent used in classical experi-

![Fig. 8. Tumor growth curves for animals bearing BE vector control (A), BE2 (B), or HT29 (C) xenografts to mitomycin C (2 mg/kg i.p. days 0, 4, and 8) or control untreated.](image-url)
ments to show that DT-diaphorase protects isolated hepatocytes against quinone toxicity (Thor et al., 1982). In fact, those experiments relied solely on dicoumarol modulation, and protection was relatively modest at around 1.5-fold when analyzed in terms of dose modification. Transfection of human DT-diaphorase into Chinese hamster ovary cells led to a slight decrease (1.4-fold) in their sensitivity to menadione (Gustafson et al., 1996). One reason for the lack of effect of NQO1 transfection in our isogenic model is that further enzymes may be required to assist any protection afforded by DT-diaphorase; e.g., Phase 2 conjugating enzymes may stabilize the menadione hydroquinone for subsequent excretion in vivo. Other possible reasons for the differences seen are that the tumor cells used here may be insensitive to oxidative stress and the resultant mitochondrial injury or that cell death/apoptosis was measured in hepatocytes, whereas we used a cell growth endpoint.

The indoloquinone analogs, EO2 and EO7, showed much less potentiation in BE2 and BE5 cells compared with EO9, consistent with their relative abilities to act as DT-diaphorase substrates (Bailey et al., 1992; Phillips, 1996). In agreement, the H460 (high DT-diaphorase) and H596 (low DT-diaphorase) lung cell lines showed no difference in sensitivity to EO2, which was not a substrate for DT-diaphorase, whereas there was a 92-fold difference in sensitivity to EO9 (Phillips, 1996). We found a 2- to 3-fold increase in EO2 sensitivity in the NQO1-transfected BE lines and a 3-fold increase in HT29, both compared with BE vector controls.

Interestingly, in the case of the aziridyl benzoquinones AZQ (diaziquone) and MeDZQ, the BE2 and BE5 NQO1-transfected lines showed similar sensitivity to BE vector control after 96-h drug exposure. In contrast, HT29 cells were 3.7- and 10-fold more sensitive to AZQ and MeDZQ, respectively, than BE vector controls. Previous studies in BE versus HT29 colon cancer cells using AZQ reported minimal sensitization with ratios of 2 and 0.6 (Siegel et al., 1990a; Plumb and Workman 1994). With MeDZQ, sensitization ratios of 5 to 6 and 32 were reported for the HT29 to BE comparison (Gibson et al., 1992; Winski et al., 1998). High sensitization ratios (17 and 29) were reported for MeDZQ in the H596 versus H460 lung cancer pair (Beall et al., 1995; Winski et al., 1998, respectively). The higher sensitization ratios observed for MeDZQ versus AZQ correlate with MeDZQ acting as a better substrate for human DT-diaphorase than AZQ (Gibson et al., 1992; Beall et al., 1995). An in vivo study using three nonisogenic nonsmall cell lung xenografts suggested that the antitumor activity of MeDZQ depended on tumor levels of both DT-diaphorase and cytochrome P450 reductase (Cummings et al., 1996). Our results with the BE2 and BE5 human NQO1 transfected cells indicate that, at least in our isogenic human colon tumor model, DT-diaphorase per se does not influence AZQ or MeDZQ cytotoxicity after 96-h drug exposure. This suggests that the greater sensitivity of HT29 compared with BE cells is caused by factors other than NQO1.

The effects of transfection of NQO1 into various cell types show differences with respect to sensitivity to bioreductive drugs. Hodnick et al. (1995) stably transfected cDNA encoding rat DT-diaphorase into Chinese hamster ovary cells and observed an increase in sensitivity to mitomycin C in the DT-diaphorase transfected line. However, the stable expression of human DT-diaphorase in Chinese hamster ovary cells resulted in no change in sensitivity to mitomycin C, although the potency of streptonigrin was enhanced a very modest 1.7-fold, with EO9 increased 2.9-fold and MeDZQ 4-fold (Gustafson et al., 1996). Human NQO1 has also been stably transfected into a DT-diaphorase negative human gastric cancer cell line, St-4 (Mikami et al., 1996), resulting in 5- to 10-fold higher sensitivity to mitomycin C, in agreement with the 5.5- and 7.3-fold potentiation observed in our transfected BE clones. Wiemels et al. (1999) observed a 2-fold increase in sensitivity to streptonigrin after transfection of human HL60 leukemia cells with NQO1. During completion of our present work, Winski et al. (1998) also reported transfection of human NQO1 into the BE colon line. The latter isogenic model was created using a monocistronic vector and CMV promoter. Our use of a bicistronic vector allows the NQO1 and selectable marker genes to be driven off the same powerful elongation factor 1α promoter, leading to even higher levels of stable DT-diaphorase expression. Compared with vector control, the transfected BE-NQ7 line of Winski et al. (1998) was 3-fold more sensitive to mitomycin C, 7-fold more sensitive to MeDZQ, and 17-fold more sensitive to a novel water-soluble closely related analog of MeDZQ, RH1. RH1 has recently shown activity against human cell lines expressing high DT-diaphorase levels and has pharmacological properties different from those of EO9 (Loadman et al., 2000). Winski et al. (1998) used a 2-h drug exposure and their results for MeDZQ and RH1 contrast with the lack of potentiation we observed using 96-h drug exposure. We routinely use 96-h exposure followed by SRB readout as a convenient high throughput cell-screening assay. A similar protocol with 48-h exposure is employed in the NCI 60 human tumor cell panel. Validation of this methodology for use with bioreductive agents under nonhypoxic conditions was provided by the correlation in NCI panel between DT-diaphorase expression and sensitivity to streptonigrin, mitomycin C, and EO9 (Paull et al., 1994; Fitzsimmons et al., 1996). This correlation held true for the same agents using 96-h exposure in our BE isogenic model. However, disparity between our own results with MeDZQ and RH1 and those of Winski et al. (1998) led us to investigate the effects of varying exposure time. We found that length of exposure to MeDZQ or RH1 markedly influenced sensitization by high DT-diaphorase expression. For MeDZQ, the fold increase in sensitivity (BE2 versus BE vector control) was 5.1 with 2-h exposure (similar to Winski et al., 1998) and over 10-fold for 6- and 24-h exposures. For RH1, potentiation was similar using 2-h (2.3-fold), 6-h (1.4-fold), or 96-h exposure (4.0-fold), but 25-fold using 24-h drug exposure. The reasons for these differences are unclear. They may relate to the relative contributions of DT-diaphorase versus other reductases for different exposure times. It is difficult to predict which, if any, in vitro exposure time will predict for behavior in vivo. Pharmacokinetic properties will probably have a significant impact. In vivo studies will be required to shed light on this issue.

To our knowledge, there are no published data on bioreductive agents using isogenic models in the intact animal. In fact, there are few studies extending the in vitro cell line findings into in vivo models. Xenografts derived from non–small-cell lung cancer cell lines with high DT-diaphorase were more responsive to mitomycin C than tumors derived from small-cell lung lines containing lower DT-diaphorase (Malkinson et al., 1992). In contrast, a negative correlation
was observed between response of four human xenografts to mitomycin C and DT-diaphorase activity (Nishiyama et al., 1993). We compared the sensitivity of the positive control HT29 line, the NQO1-transfected BE2 line, and the BE vector control when grown as solid tumor xenografts in nude mice. DT-diaphorase expression and activity were retained in the BE2 cell line when grown in this way, whereas the BE vector control line was negative. The DT-diaphorase activities present in the HT29 and BE2 line, however, were lower than those observed for the same cells in vitro, possibly because of the presence of stromal tissue or microenvironmental factors. We found no difference in mitomycin C sensitivity between low- and high-DT-diaphorase isogenic BE xenografts. We conclude that for this clinically used agent, a DT-diaphorase-mediated differential in vitro sensitivity of around 7-fold for the BE2 line, obtained using 96-h exposures under conventional ‘aerobic’ conditions, does not translate into a significant difference in response in the context of a solid tumor xenograft in vivo. Tumor sensitivity to bioreductive agents in vivo is likely to be affected by several bioreductive enzymes, and also by microenvironmental factors, particularly hypoxia (Plumb et al., 1994; Plumb and Workman, 1994; Workman, 1994; Cummings et al., 1998; Stratford and Workman, 1998). We intend to investigate the role of these factors using our isogenic BE model. Subject to drug availability, we will also expand these studies to other bioreductive agents, including those with greater differentials in the isogenic BE pair in vitro. Unfortunately, the BE, BE2, and HT29 lines were not sensitive to streptonigrin as xenografts. However, we have shown with the quinone ansamycin 90-kDa heat-shock protein inhibitor 17-allylamino-17-demethoxy geldanamycin that greater activity is seen in HT29 and BE2 than in vector control xenografts, which is consistent with the in vitro data (Kelland et al., 1999).

We believe that our isogenic model should be valuable for mechanistic work and drug screening. Our isogenic model is similar to that recently described by Winski et al. (1998), but there are differences in construction, validation, and applications between the two models. A potential advantage is our use of the bicistronic expression vector, which allows the NQO1 gene and the selectable marker (puromycin resistance) to be driven in a single transcript off the same powerful promoter (human elongation factor 1α), leading to the selection of NQO1-transfected BE clones. Using our model, we confirm the involvement of DT-diaphorase in the cellular sensitivity to mitomycin C, indoloquinone EO9, MeDZQ, RH1, and streptonigrin. In addition, we report interesting effects of the time of bioreductive drug exposure on the potentiation of cytotoxicity in our NQO1-transfected line, and these have implications for the use of such isogenic models as an investigative tool and as a screen in drug development. The extension of our isogenic model to investigate the role of DT-diaphorase expression in vivo should prove useful. In addition to further understanding the role of NQO1 in the mechanism of action of bioreductive drugs, there are also potential applications in identifying toxins, carcinogens, and chemopreventive agents that act via DT-diaphorase. The lack of potentiation of mitomycin C sensitivity in NQO1-transfected xenografts strengthens the need to identify novel agents that act selectively on DT-diaphorase-rich tumors in vivo.

References
Fitzsimmons SA, Lewis AD, Riley RJ and Workman P (1994) Reduction of 3-amino-1,2,4-benzotriazine-1,4-di-N-oxide (tirapazamine; WIN 53075; SR 4233) to a DNA-damaging species: A direct role for NADPH-cytochrome P450 oxidoreductase. Carcinogenesis 15:1003–1011.
Loh SY, Mistry P, Kelland LR, Abel G and Harrap KR (1992) Reduced drug accumulation as a major mechanism of acquired resistance to cisplatin in a human
ovarian carcinoma cell line: Circumvention studies using novel platinum (II) and (IV) ammine/amine complexes. Br J Cancer 66:1109–1115.


Send reprint requests to: Prof. Paul Workman, CRC Centre for Cancer Therapeutics, The Institute of Cancer Research, Block E, 15 Cotswold Rd., Sutton, Surrey, SM2 5NG, UK. E-mail: paulw@icra.