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Factors influencing the induction of DT-diaphorase activity by 1,2-dithiole-3-thione in human tumor cell lines

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Abstract

NAD(P)H:(quinone acceptor)oxidoreductase (DT-diaphorase) is a two-electron reducing enzyme that activates bioreductive antitumor agents and is induced by a wide variety of compounds including 1,2-dithiole-3-thione (D3T). We investigated factors influencing DT-diaphorase induction in fourteen human tumor cell lines. Four cell lines had basal DT-diaphorase activity that was increased by D3T treatment (group A), six cell lines had basal DT-diaphorase activity but the activity was not increased by D3T (group B), and four cell lines had low enzyme activity without, or with, D3T (group C). Two cell lines in group A and two cell lines in group B had a C to T polymorphism at base 609 in the NQO_1, DT-diaphorase gene, in one allele, while all four cell lines in group C were homozygous mutants. The base 609 mutant NQO_1 gene produces a protein with little enzyme activity. In group A, D3T increased NQO_1 mRNA and wild-type protein, and also increased mutant protein in the two heterozygous cell lines. In group B, the inducer slightly increased NQO_1 mRNA, did not increase the wild-type protein, but did increase the mutant protein in the two heterozygous cell lines. In group C, D3T increased NQO_1 mRNA as well as its mutant enzyme product. Transfection of the mutant NQO_1 gene into cells with two wild-type alleles did not alter DT-diaphorase activity. The results suggest that the lack of induction of DT-diaphorase activity is transcriptional in nature, that basal and induced expression of DT-diaphorase are regulated independently, and that mutant NQO_1 does not act as a dominant-negative to suppress DT-diaphorase activity. Published by Elsevier Science Inc.

Keywords: DT-diaphorase; Induction; Human tumor; NQO_1; Dominant-negative

1. Introduction

DT-diaphorase (EC 1.6.99.2) is a flavoprotein that catalyzes obligatory two-electron reduction of quinones, quinone imines, and nitrogen oxides, and requires NADH or NADPH as an electron donor for enzymatic activity [1]. DT-diaphorase is an activator of some anticancer drugs and is also an important detoxifying enzyme [1–3]. Several different human DT-diaphorase genes have been identified [4,5], but NQO_1 appears to be the most important for drug activation [1]. NQO_1 is 20 kb in length, has six exons and five introns, and produces three RNA transcripts (2.7, 1.7, and 1.2 kb); however, the 1.7-kb transcript is usually in low abundance [6]. DT-diaphorase consists of two identical protein subunits of approximately 30 kDa [1]. Enzyme levels vary in different human tissues [7], but the enzyme is highly inducible in normal and tumor cells and tissues [8–11].

Expression of DT-diaphorase is thought to be transcriptionally controlled [1]. NQO_1 has a xenobiotic response element, an ARE, and an AP2 binding site [4]. The ARE contains an AP1 binding site, two AP1-like sites, and a GCA box, and appears to be important for basal expression and induction of DT-diaphorase [6]. Inducers of DT-diaphorase may produce a redox signal that, through Ref-1, increases binding of the Fos-Jun complex to the AP1 site, producing increased NQO_1 transcription [12]. An NF-κ-B response element may also be involved in enzyme induction [13].

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Abbreviations: DT-diaphorase, NAD(P)H:(quinone acceptor)oxidoreductase; ARE, antioxidant response element; MMC, mitomycin C; EO9, 3-hydroxymethyl-5-aziridinyl-1-methyl-2-(1*H*-indole-4,7-dione)prop-β-en-α-ol; D3T, 1,2-dithiole-3-thione; SSC, 0.15 M sodium chloride, 0.015 M sodium citrate buffer solution; and PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism

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A C to T point mutation at base 609 of the NQO_1 gene, resulting in a proline to serine change at amino acid 187 of the protein, has been identified in human tumor and normal cells [14–16]. Cells with one mutated allele expressed varied levels of DT-diaphorase activity, but cells with this mutation in both alleles had no detectable enzyme activity [15,16]. Incidence of the mutation in the general population has been reported to be approximately 50%, with 10% being homozygous for the mutation; however, the incidence varies in different ethnic groups [15–18]. The mutant NQO_1 protein has not been detected previously in human cells, but has been expressed in bacteria [15,19] and COS (monkey kidney) cells [20] and has $<$15% of the activity of the wild-type protein.

Several other molecular variations have been observed in the NQO_1 gene. We found a T to C substitution at base 456, which results in an arginine to tryptophan change at amino acid 139 [21]. This mutant protein has approximately 40% of the activity of wild-type protein in reducing the antitumor agent, MMC, and is expressed at lower levels [22]. In addition, an alternatively spliced NQO_1 mRNA lacking exon 4 has been found in human tumor cells and tissues [21,23,24]. The protein representing the exon 4 deletion was not detected, but expression in *Escherichia coli* produced a 26 kDa protein with no enzymatic activity [22].

DT-diaphorase is a Phase II detoxifying enzyme that may be important in protecting cells from reactive metabolites and carcinogens [1,3]. It is induced by a wide variety of compounds including dithiolethiones, isothiocyanates, and Michael reaction acceptors, many of which can inhibit carcinogenesis by acting as blocking agents [25,26]. Thus, inducers of DT-diaphorase are under active investigation as cancer chemopreventive agents [27,28].

DT-diaphorase is also an important activating enzyme for bioreductive antitumor agents such as MMC [2,29] and EO9 [30]. These agents require intracellular activation by enzymes like NADPH:cytochrome P450 reductase (EC 1.6.2.4) [31] or DT-diaphorase [2]. Generally, cell lines [2,29] or tumor specimens [32] with higher levels of DT-diaphorase are more sensitive to MMC.

We have investigated the use of DT-diaphorase inducers to selectively increase enzyme activity in tumor cells, as a way of enhancing the effectiveness of bioreductive antitumor agents. We showed that the dithiolethione; D3T, can selectively increase DT-diaphorase activity in human [10,33] and murine [11] tumors, and that this enhanced the antitumor activity of MMC and EO9. However, we found that DT-diaphorase activity was not induced in ten of thirty-eight human tumor cell lines examined [10]. Since lack of induction of DT-diaphorase activity may hinder the use of enzyme inducers to improve chemotherapy or for cancer chemoprevention, we investigated why DT-diaphorase activity was not induced in some human tumor cell lines.

2. Materials and methods

2.1. Materials

All media for cell culture, as well as sense and antisense primers, were obtained from Gibco BRL. *Hin*fI was obtained from Promega; Taq polymerase and dNTP were from Amersham Pharmacia Biotech. Reagents for the DT-diaphorase assay were from the Sigma Chemical Co. Protein was quantitated using the Bio-Rad DC kit; acrylamide:bis (29:1) was also from Bio-Rad. The transfection reagents, LT1 and Fugene 6, were obtained from Panvera and Roche (Laval), respectively. Base 609 mutant NQO_1 protein was a gift from Dr. David Ross, School of Pharmacy, University of Colorado Health Sciences Center.

2.2. Cells

Conditions for cell culture were as described previously [10]. MCF-7 cells were obtained from the American Type Culture Collection and were designated MCF-7(ATCC). These cells were grown in the same medium as MCF-7 cells previously obtained from Dr. A. H. Greenberg, Manitoba Institute of Cell Biology [10], designated MCF-7(AHG). All other cell lines were obtained and cultured as previously described [10].

2.3. Induction of DT-diaphorase activity

Cells were incubated without, or with, 100 μM D3T for 48 hr. DT-diaphorase activity was measured as described previously, using menadione as the electron acceptor [10]. Activity was expressed as nanomoles per minute per milligram of protein.

2.4. PCR-RFLP and DNA sequencing

Genomic DNA was extracted from 5×10^6 cells, using the Genomic Prep DNA Isolation Kit (Amersham Pharmacia Biotech) and following the manufacturer's instructions. PCR-RFLP was performed using the sense and antisense primers described by Traver *et al.* [15] using 1 μL of the genomic DNA solution and a mixture containing 50 μM dNTP, 2.5 mM $MgCl_2$, 0.375 μM of each primer, and 2 Units of Taq polymerase. Thermal cycling conditions were 1 cycle of 94° for 1 min, 35 cycles of 94° for 30 sec, 64° for 30 sec, and 72° for 30 sec, and a final cycle of 72° for 5 min. Thermal cycling was performed in a Perkin-Elmer GeneAmp PCR system 9600. The PCR products were digested with 16 Units of *Hin*fI, and the fragments were isolated on a 2.5% agarose gel containing 0.05 μg/mL of ethidium bromide.

PCR products that had not been treated with the restriction enzyme were purified (Qiagen QIAquick PCR Purification Kit, Qiagen Inc.) and sequenced to confirm the C to T point mutation at base 609 using an ABI Prism 310

Genetic Analyzer (Perkin-Elmer). The PCR product was purified following the manufacturer's protocol and resuspended in 30 μL water. Generally, 2 μL of the purified PCR product was sequenced in a 5-μL volume containing 0.33 μL Big Dye, 1.67 μL sequencing buffer (Perkin-Elmer), and 15 pmol antisense primer.

2.5. Northern blot analysis

Cells were incubated without, or with, 100 μM D3T for 24 hr. Total RNA was extracted from cells, using TRIzol (Gibco BRL), and 20 μg of total RNA per lane was subjected to denaturing electrophoresis in 1% agarose-formaldehyde gels. Gels were transferred overnight in 10X SSC to nitrocellulose membranes (Bio-Rad). A 1.3-kb cDNA probe to NQO_1 was labelled with [α-^{32}P] dCTP using the Ready To Go Labeling Kit (Amersham Pharmacia Biotech) and hybridized following the method of Church and Gilbert [34]. The hybridization signal was detected by PhosphorImager screens and quantitated using the PhosphorImager mode of the ImageQuant program (Molecular Dynamics). β-Actin was used as a loading control.

2.6. Western blot analysis

Cells were incubated without, or with, 100 μM D3T for 48 hr. Protein was extracted from cells in lysis buffer consisting of 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, and protease inhibitors (Complete Mini, Boehringer Mannheim). Protein ranging from 10–50 μg per lane was loaded on a 0.75-mm thick 12.5% SDS-polyacrylamide gel with a 4% stacking gel (Hoefer Protein Electrophoresis Applications Guide) and run at 9.5 mA overnight. To enable separation of the wild-type and mutant bands for quantitation, a 16-cm separating gel was used and run until a 28-kDa marker of the Benchmark Prestained Protein Ladder (Gibco BRL) was 1 cm from the bottom of the gel. The samples were transferred to a nitrocellulose membrane (Bio-Rad) at 0.8 mA/cm^2 of membrane for 90 min using a continuous buffer system in a semi-dry blotting apparatus (LKB Bromma Nova Blot, Amersham Pharmacia Biotech). The membrane was blocked overnight with 4% BSA in Tris-buffered saline/0.25% Tween-20. Then the membrane was washed and incubated with a polyclonal NQO_1 antibody [21] for 2 hr, followed by incubation with a donkey anti-rabbit secondary antibody (Amersham Pharmacia Biotech) for 45 min. Secondary antibody was detected using the ECL detection kit (Amersham Pharmacia Biotech) with typical exposure times to x-ray film being 5–90 sec. The x-ray films were scanned and quantitated using the densitometer mode of the ImageQuant program.

2.7. Plasmids

pRc/CMV is a 5.5-kb mammalian reporter vector containing ampicillin and neomycin selectable markers. The 1.4-kb wild-type human or mutant NQO_1 cDNA was inserted into pRc/CMV at the *Hin*d-III cloning site to give the pRc/CMV/NQO_1 WT and pRc/CMV/NQO_1 609 plasmids, respectively. The mutant human NQO_1 cDNA was designed to contain the C to T point mutation at base 609. DNA sequencing of the pRc/CMV/NQO_1 WT and pRc/CMV/NQO_1 609 plasmids was performed to verify the mutation. pCMVβ is a 7.2-kb mammalian reporter vector containing an ampicillin selectable marker designed to express the *E. coli* β-galactosidase gene with eukaryotic translation initiation signals.

2.8. Transfections into human tumor cells

HCT116 human colon cancer cells were grown in 60-mm plates. Cells were plated at 5×10^5 cells and incubated overnight. A sterile coverslip was included in each plate to measure transfection efficiency. One microgram pCMVβ was co-transfected with 4 μg pRc/CMV, pRc/CMV/NQO_1 609, or pRc/CMV/NQO_1 WT using 15 μL of LT-1 following the transfection protocol as outlined in the product manual from Panvera. Following 6 hr of exposure to the plasmid, cells were incubated for an additional 42 hr to allow for plasmid protein expression.

PC-3 human prostate cancer cells were grown in 60-mm plates. Cells were plated at 5×10^5 cells and incubated overnight. A sterile coverslip was included in each plate to measure transfection efficiency; 0.8 μg pCMVβ was co-transfected with 3.2 μg pRc/CMV, pRc/CMV/NQO_1 609, or pRc/CMV/NQO_1 WT using 9 μL of Fugene 6 following the transfection protocol as outlined in the product manual from Roche. The cells were exposured to the plasmids for 48 hr.

The transfection efficiency of the pRc/CMV plasmids was determined from β-galactosidase expression in co-transfection experiments, measured using X-gal (Promega) staining solution, and was corrected for the amount of plasmid DNA used in the transfection. Non-transfected control cells and cells transfected with pRc/CMV, pRc/CMV/NQO_1 609, or pRc/CMV/NQO_1 WT were assessed for NQO_1 mutant and wild-type protein expression by western blot analysis and for DT-diaphorase activity.

2.9. Statistical analysis

DT-diaphorase activities in control and D3T-treated cells were analyzed by a two-tailed *t*-test comparing the significance of the difference of the mean activities. Levels of NQO_1 wild-type protein and DT-diaphorase activities in control and transfected HCT116 or PC-3 cells were analyzed by two-tailed *t*-tests comparing the significance of the difference of the mean NQO_1 wild-type protein or DT-diaphorase activities in control and transfected cells.

Table 1
Induction of DT-diaphorase activity in human tumor cell lines by D3T

Cell line	Tumor type	DT-diaphorase activity (nmol min^{-1} mg $protein^{-1}$)		Ratio of D3T treated/control	*P*
		Control	D3T treated		
Group A: Cell lines induced by D3T					
HL-60	Leukemia	4.0 ± 0.5	31.6 ± 3.6	7.9	< 0.001
T47D	Breast	27.8 ± 1.2	97.6 ± 4.5	3.5	< 0.001
NCI-H661	Lung	118.9 ± 13.0	295.3 ± 27.4	2.5	< 0.001
MCF-7(ATCC)	Breast	198.2 ± 22.4	363.6 ± 42.0	1.8	< 0.01
Group B: Cell lines not induced by D3T					
Detroit 562	Head and neck	173.3 ± 28.0	199.4 ± 39.4	1.1	NS[a]
NCI-H520	Lung	204.7 ± 28.0	239.5 ± 32.3	1.2	NS
FaDu	Head and neck	363.2 ± 47.3	463.3 ± 36.1	1.3	NS
DU145	Prostate	586.0 ± 46.5	577.3 ± 36.9	1.0	NS
MCF-7(AHG)	Breast	939.1 ± 88.3	981.2 ± 96.5	1.0	NS
HepG2	Liver	1168.8 ± 125.0	1281.9 ± 91.6	1.1	NS
Group C: Cell lines with no consistently detectable DT-diaphorase activity					
MDA-MB-231	Breast	ND[b]	1.7 ± 0.5		
NCI-H596	Lung	ND	3.6 ± 0.5		
Colo320DM	Colon	2.2 ± 2.2	ND		
MDA-MB-468	Breast	4.4 ± 2.4	ND		

Cells were incubated without, or with, 100 μM D3T for 48 hr, and enzyme activity was measured as described in the text. Values are the means ± SEM of 3–15 determinations. Statistical significance was determined using a two-tailed *t*-test comparing the significance of the difference of the mean DT-diaphorase activity in cells incubated without, or with, D3T.

[a] NS, not significant, $P \geq 0.05$.

[b] ND, not detectable (<1 nmol min^{-1} mg $protein^{-1}$).

3. Results

3.1. Induction of DT-diaphorase activity in human tumor cell lines by D3T

The ability of D3T to induce DT-diaphorase activity was measured in fourteen human tumor cell lines (Table 1). The tumor cell lines represented seven different tumor types including lung, head and neck, breast, colon, leukemia, prostate, and liver tumors. The control level of DT-diaphorase activity in these cells ranged from not detectable to 1168.8 ± 125.0 nmol min^{-1} mg $protein^{-1}$. Cells were incubated without, or with, 100 μM D3T for 48 hr, and enzyme activity was measured. Cells were incubated for 48 hr because we [10,11] and others [13,35] have shown that induction of DT-diaphorase activity reaches a maximum at approximately 48 hr in a number of cell lines. In addition, we have shown previously [10] that 100 μM D3T produces the maximum induction of DT-diaphorase activity in HL-60 cells without toxicity to these cells. The fourteen cell lines were classified into three groups based on their control level of DT-diaphorase activity and the ability of D3T to increase enzyme activity. Group A consisted of four cell lines [HL-60, T47D, NCI-H661, and MCF-7(ATCC)] that had a measurable control level of DT-diaphorase activity and significantly increased enzyme activity following D3T treatment. Group B consisted of six cell lines [Detroit 562, NCI-H520, FaDu, DU145, MCF-7(AHG), and HepG2] that had a measurable control level of DT-diaphorase activity but no increase in enzyme activity following D3T treatment. Group C consisted of four cell lines [MDA-MB-231, NCI-H596, Colo320DM and MDA-MB-468] that had DT-diaphorase activity that was not consistently detectable either before or after treatment with D3T.

3.2. Detection of NQO_1 mutation in human tumor cell lines

The presence of a C to T substitution at base 609 of the NQO_1 gene in the human tumor cell lines was detected by PCR-RFLP analysis [15]. The mutation at base 609 was detected in eight of the cell lines (Fig. 1), and the C to T substitution was confirmed in these cells by DNA sequencing. Four of these cell lines were homozygous for the mutation, while the remaining four were heterozygous. Of the cell lines in group A, two were homozygous wild-type [T47D and NCI-H661], while two had one mutant allele [HL-60 and MCF-7(ATCC)]. Four of the cell lines in group B were homozygous wild-type [Detroit 562, FaDu, DU145 and HepG2] and two were heterozygous for the mutation [NCI-H520 and MCF-7(AHG)]. All four cell lines in group C were homozygous for the mutation at base 609.

3.3. Northern blot analysis of induction of NQO_1 mRNA

The effect of D3T on induction of NQO_1 mRNA was determined. Cells were incubated without, or with, 100 μM D3T for 24 hr, and NQO_1 mRNA was measured by northern

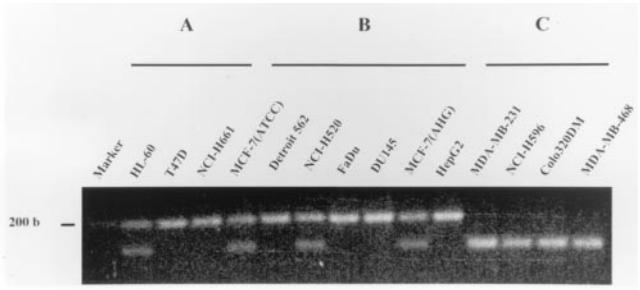

Fig. 1. Identification of the C to T substitution at base 609 of the NQO_1 gene in human tumor cell lines by PCR-RFLP analysis. Genomic DNA was extracted from 5×10^6 cells using the Genomic Prep DNA Isolation Kit. PCR-RFLP was performed using the sense and antisense primers described by Traver *et al*. [15], as described in the text. The PCR products were digested with *Hin*fI, and the fragments were isolated on a 2.5% agarose gel containing 0.05 μg/mL of ethidium bromide. Group A: cell lines induced by D3T; Group B: cell lines not induced by D3T; Group C: cell lines with no consistently detectable DT-diaphorase activity.

blot analysis (Fig. 2). Cells were incubated for 24 hr because we found that in NCI-H661, FaDu, and HepG2 cells NQO_1 mRNA reached a maximum at 24 hr and either remained the same or decreased slightly at 48 hr (data not shown). All the cell lines showed two mRNA transcripts at 1.2 and 2.7 kb. The 1.2 kb band was quantitated for cells incubated without, or with, D3T for each cell line (Table 2). All four cell lines in group A showed increased enzyme mRNA following incubation with D3T that ranged from 2.8- to 12.2-fold. The six cell lines in group B showed small increases in enzyme mRNA that ranged from 1.2- to 2.6-fold. All four cell lines in group C showed increased NQO_1 mRNA following incubation with D3T, with the increases ranging from 3.0- to 10.4-fold.

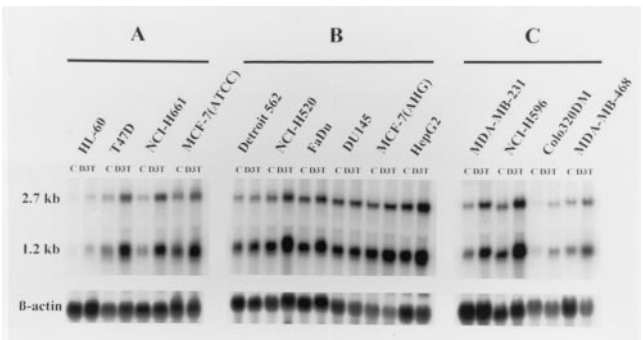

Fig. 2. Effect of D3T on NQO_1 mRNA in human tumor cell lines. Cells were incubated without, or with, 100 μM D3T for 24 hr. RNA was extracted using TRIzol and subjected to denaturing electrophoresis on 1% agarose-formaldehyde gels. RNA was transferred to nitrocellulose membranes and probed with a ^{32}P-labelled 1.3-kb cDNA of NQO_1. The RNA was probed with a ^{32}P-labelled probe for β-actin as a loading control. Group A: cell lines induced by D3T; Group B: cell lines not induced by D3T; Group C: cell lines with no consistently detectable DT-diaphorase activity. C, control; D3T, D3T-treated.

Table 2
Relative induction of NQO_1 mRNA by D3T in human tumor cell lines

Cell line	Tumor type	Ratio of D3T treated/control (1.2-kb band)
Group A: Cell lines induced by D3T		
HL-60	Leukemia	12.2
T47D	Breast	3.3
NCI-H661	Lung	7.0
MCF-7(ATCC)	Breast	2.8
Group B: Cell lines not induced by D3T		
Detroit 562	Head and neck	2.2
NCI-H520	Lung	1.6
FaDu	Head and neck	1.6
DU145	Prostate	1.2
MCF-7(AHG)	Breast	2.4
HepG2	Liver	2.6
Group C: Cell lines with no consistently detectable DT-diaphorase activity		
MDA-MB-231	Breast	4.8
NCI-H596	Lung	3.0
Colo320DM	Colon	10.4
MDA-MB-468	Breast	6.9

Cells were incubated without, or with, 100 μM D3T for 24 hr. RNA was extracted using TRIzol and subjected to denaturing electrophoresis on 1% agarose-formaldehyde gels. RNA was transferred to nitrocellulose membranes and probed with a ^{32}P-labelled 1.3-kb cDNA of NQO_1. The 1.2-kb band was quantitated using a PhosphoImager and was corrected for differences in loading by using β-actin as a loading control. Results represent the averages of duplicate gels from 2–4 separate experiments.

3.4. Western blot analysis of induction of NQO_1 protein

The effect of D3T on induction of NQO_1 protein was determined by western blot analysis. Cells were incubated without, or with, 100 μM D3T for 48 hr, and NQO_1 protein was detected by western blot analysis (Fig. 3). Two protein bands were detected, a 30-kDa band identical to purified wild-type NQO_1 protein and a 29.5-kDa band identical to purified mutant protein. The two protein bands were quantitated by densitometry for cells incubated without, or with, D3T for each cell line (Table 3). All four cell lines in group A showed increased wild-type NQO_1 protein after treatment with D3T. The two cell lines in this group that were heterozygous for the mutation also showed increased mutant protein after incubation with D3T. All six cell lines in group B showed no increase in wild-type NQO_1 protein. Both cell lines in this group that were heterozygous for the mutation showed an increase in the level of mutant protein. All four cell lines in group C showed large increases in mutant NQO_1 protein following treatment with D3T. In general, the increases in mutant protein levels were greater than the increases in wild-type protein levels following D3T treatment. However, the level of mutant protein in cells that were heterozygous for the mutation was still lower than the level of wild-type protein after treatment with D3T (see Fig. 3).

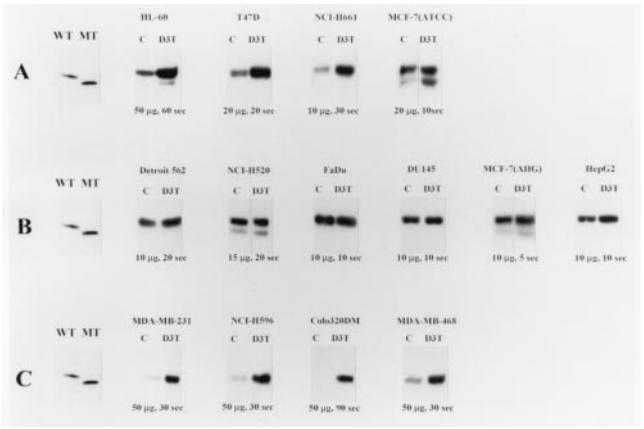

Fig. 3. Effect of D3T on NQO_1 protein in human tumor cell lines. Cells were incubated without, or with, 100 μM D3T for 48 hr. Protein was extracted and run on a 16-cm, 12.5% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membranes and probed with NQO_1 antibody and then with a secondary antibody. The secondary antibody was detected using an ECL detection kit. Group A: cell lines induced by D3T; Group B: cell lines not induced by D3T; Group C: cell lines with no consistently detectable DT-diaphorase activity. C, control; D3T, D3T-treated; WT, wild-type NQO_1 protein; MT, mutant NQO_1 protein.

3.5. Transfection of the NQO_1 gene into human tumor cells

HCT-116 (human colon cancer) cells were transfected with pRc/CMV, pRc/CMV/NQO_1 609, or pRc/CMV/NQO_1 WT plasmids. The transfection efficiency ranged from 61.6 to 69.5% (Table 4). Expression of wild-type NQO_1 protein was not changed significantly in the pRc/CMV or pRc/CMV/NQO_1 609 transfected cells compared with non-transfected cells (Fig. 4). In contrast, cells transfected with pRc/CMV/NQO_1 WT showed a 6.9-fold increase in expression of this protein. Cells transfected with pRc/CMV/NQO_1 609 also showed expression of mutant protein and the ratio of expression of mutant/wild-type protein, corrected for transfection efficiency, was 5.8 $\pm$ 1.5 (Table 4). There was no significant difference in DT-diaphorase activity between non-transfected cells and cells transfected with pRc/CMV or pRc/CMV/NQO_1 609; however, enzyme activity in cells transfected with pRc/CMV/NQO_1 WT was increased significantly (Table 4).

PC-3 (human prostate cancer) cells were transfected with pRc/CMV, pRc/CMV/NQO_1 609, or pRc/CMV/NQO_1 WT plasmids. The transfection efficiency ranged from 58.2 to 61.4% (Table 5). Expression of wild-type NQO_1 protein was not changed significantly in the pRc/CMV or pRc/CMV/NQO_1 609 transfected cells compared with non-transfected cells (Fig. 5). In contrast, cells transfected with pRc/CMV/NQO_1 WT showed an 8.4-fold increase in expression of this protein. Cells transfected with pRc/CMV/NQO_1 609 also showed expression of mutant protein and the ratio of expression of mutant/wild-type protein, corrected for transfection efficiency, was 2.7 $\pm$ 0.3 (Table 5). There was no significant difference in DT-diaphorase activity between non-transfected cells and cells transfected with pRc/CMV or pRc/CMV/NQO_1 609; however, enzyme activity in cells transfected with pRc/CMV/NQO_1 WT was increased significantly (Table 5).

Table 3
Relative induction of NQO_1 protein by D3T in human tumor cell lines

Cell line	Tumor type	Ratio of D3T treated control: Wild-type protein (30-kDa Band)	Ratio of D3T treated control: Mutant protein (29.5-kDa Band)
Group A: Cell lines induced by D3T			
HL-60	Leukemia	6.9	NC[a]
T47D	Breast	4.4	
NCI-H661	Lung	5.5	
MCF-7(ATCC)	Breast	2.4	3.4
Group B: Cell lines not induced by D3T			
Detroit 562	Head and neck	1.3	
NCI-H520	Lung	1.1	1.7
FaDu	Head and neck	1.3	
DU145	Prostate	0.9	
MCF-7(AHG)	Breast	1.2	2.7
HepG2	Liver	1.1	
Group C: Cell lines with no consistently detectable DT-diaphorase activity			
MDA-MB-231	Breast		9.9
NCI-H596	Lung		8.9
Colo320DM	Colon		NC
MDA-MB-468	Breast		6.5

Cells were incubated without, or with, 100 μM D3T for 48 hr. Protein was extracted and run on a 16-cm, 12.5% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membranes and probed with NQO_1 antibody and then with a secondary antibody. The secondary antibody was detected using an ECL detection kit, and x-ray films were scanned and quantitated using the densitometer mode of the ImageQuant program (Molecular Dynamics). Results represent the averages of duplicate gels from two separate experiments.

[a] NC, ratio could not be calculated due to the absence of a band in the untreated cells.

4. Discussion

DT-diaphorase is a Phase II enzyme that plays an important role in detoxifying chemically reactive metabolites and carcinogens, and may be important in an early cellular defense against tumorigenesis [1,3]. It is induced by a wide variety of chemical compounds including dithiolethiones, isothiocyanates, Michael reaction acceptors, and heavy metals [25,26], many of which can inhibit carcinogenesis by acting as blocking agents [26]. DT-diaphorase has also been shown to be an important activating enzyme for bioreductive antitumor agents like MMC [2,29]. We have been investigating the use of inducers of DT-diaphorase to selectively increase the level of DT-diaphorase activity in tumor cells as a way of enhancing the effectiveness of bioreductive

Table 4
Transfection of mutant and wild-type NQO_1 cDNA into HCT116 human colon tumor cells

Transfection	Transfection efficiency (%)	Transfected/Control wild-type protein	Mutant/ Wild-type protein[a]	DT-diaphorase activity (nmol min^{-1} mg $protein^{-1}$)
Control (no plasmid)				135.5 ± 14.4
pRc/CMV	69.5 ± 6.4	0.9 ± 0.1		132.9 ± 12.2
pRc/CMV/NQO_1 609	68.1 ± 5.9	1.4 ± 0.2	5.8 ± 1.5	142.6 ± 15.5
pRc/CMV/NQO_1 WT	61.6 ± 1.9	6.9 ± 1.4*		193.4 ± 13.8*

HCT116 (human colon cancer) cells were co-transfected with pRc/CMV, pRc/CMV/NQO_1 609 or pRc/CMV/NQO_1 WT and pCMVβ plasmids. Transfection efficiency was determined as described in the text, and ratios of wild-type and mutant NQO_1 protein expression were obtained from western blots. Results represent the means ± SEM of 3–5 determinations. Statistical significance was determined using two-tailed *t*-tests to compare the significance of the differences of the mean NQO_1 wild-type protein or the DT-diaphorase activities in control and transfected cells.

[a] Corrected for transfection efficiency.

* $P < 0.05$.

antitumor agents. We showed that the dithiolethione, D3T, can selectively increase DT-diaphorase activity in human [10,33,36] and murine [11] tumors, and that this can enhance the antitumor activity of MMC and EO9. However, DT-diaphorase was not induced in approximately 25% of the human tumor cell lines investigated [10]. In the present study, we examined mechanisms that might be responsible for the lack of induction of DT-diaphorase in the human tumor cell lines.

We investigated induction of DT-diaphorase by D3T in fourteen human tumor cell lines representing various tissue types. Four of these cell lines had basal enzyme expression and DT-diaphorase activity was increased significantly after incubation with D3T (group A). Six of the cell lines had basal expression of DT-diaphorase but showed no significant increase in enzyme activity after D3T treatment (group B). Four of the cell lines had very low enzyme activity before, or after, D3T treatment (group C). There was no obvious relationship between tumor type and induction of DT-diaphorase activity in these cell lines.

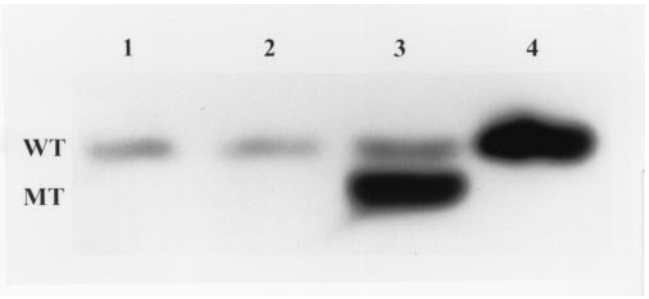

Fig. 4. Expression of NQO_1 wild-type and mutant protein in transfected HCT116 human colon cancer cells. Cells were co-transfected with pCMVβ and pRc/CMV, pRc/CMV/NQO_1 609, or pRc/CMV/NQO_1 WT using the LT-1 transfection reagent. Protein was extracted, and 10 μg was run on a 16-cm, 12.5% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membranes and probed with NQO_1 antibody and then with a secondary antibody. The secondary antibody was detected using an ECL detection kit. Lane 1, non-transfected cells; lane 2, cells transfected with pRc/CMV; lane 3, cells transfected with pRc/CMV/NQO_1 609; and lane 4, cells transfected with pRc/CMV/NQO_1 WT. WT, wild-type NQO_1 protein; MT, mutant NQO_1 protein.

PCR-RFLP analysis [15] detected a C to T substitution at base 609 of the NQO_1, DT-diaphorase, gene in eight of the cell lines (Fig. 1). The C to T substitution was confirmed in these cells by DNA sequencing. Two of the cell lines in group A [HL-60 and MCF-7(ATCC)], and two of the cell lines in group B [NCI-H520 and MCF-7(AHG)], had one mutant allele. All four of the cell lines in group C were homozygous for the mutation at base 609.

In the four tumor cell lines in group A [HL-60, T47D, NCI-H661 and MCF-7(ATCC)], treatment with the inducer resulted in an increase in NQO_1 mRNA and normal protein. In the two cell lines in this group that were heterozygous for the C to T mutation at base 609, there was also an increase in the level of mutant protein, although the amount of this protein was still less than the amount of normal protein in the cells.

All six tumor cell lines in group B had intermediate to high control levels of DT-diaphorase activity, demonstrating that they are capable of expressing active wild-type protein. Therefore, the lack of enzyme induction in these cells must be due to a failure in the induction process. The DU145 and FaDu cells had very small increases in NQO_1 mRNA of 1.2- and 1.6-fold, respectively, and no increases in wild-type protein. The NCI-H520 and MCF-7(AHG) cells had increases in NQO_1 mRNA of 1.6- and 2.4-fold, respectively. While the wild-type protein in these cells was not increased, they did have increases in mutant protein of 1.7- and 2.7-fold, respectively. Thus, the increased mRNA seen in these cells may have been due to a selective increase in transcription of the mutant, but not the wild-type, NQO_1 allele. In contrast, the Detroit 562 and HepG2 cells had larger increases in NQO_1 mRNA of 2.2- and 2.6-fold, respectively, but there was no increase in wild-type NQO_1 protein in these cells. Overall, these results suggest that the lack of enzyme induction in the cell lines in group B is due either to a transcriptional or pre-transcriptional defect. However, we cannot rule out the possibility that in some of the cell lines in this group, particularly the Detroit 562 and HepG2 cells, the lack of enzyme induction is due to a defect at the post-transcriptional level.

Table 5
Transfection of mutant and wild-type NQO_1 cDNA into PC-3 human prostate cancer cells

Transfection	Transfection efficiency (%)	Transfected/Control wild-type protein	Mutant/ Wild-type protein[a]	DT-diaphorase activity (nmol min^{-1} mg $protein^{-1}$)
Control (no plasmid)				86.2 ± 10.7
pRc/CMV	58.2 ± 8.9	1.0 ± 0.2		91.7 ± 11.2
pRc/CMV/NQO_1 609	58.6 ± 9.9	1.8 ± 0.8	2.7 ± 0.3	89.7 ± 8.8
pRc/CMV/NQO_1 WT	61.4 ± 13.4	8.4 ± 1.8*		126.3 ± 17.0*

PC-3 (human prostate cancer) cells were co-transfected with pRc/CMV, pRc/CMV/NQO_1 609, or pRc/CMV/NQO_1 WT and pCMVβ plasmids. Transfection efficiency was determined as described in the text, and ratios of wild-type and mutant NQO_1 protein expression were obtained from western blots. Results represent the means ± SEM of 3–5 determinations. Statistical significance was determined using two-tailed *t*-tests to compare the significance of the differences of the mean NQO_1 wild-type protein or the DT-diaphorase activities in control and transfected cells.

[a] Corrected for transfection efficiency.

* $P < 0.05$.

The four cell lines in group C were all homozygous for the mutation at base 609. These findings confirm an earlier report for the NCI-H596 cells [15] and provide the first demonstration that the MDA-MB-231, Colo320DM and MDA-MB-468 cells are homozygous mutants. The mutant protein is present in all the cells after D3T treatment, but the lack of enzyme activity in the cells likely reflects the reported low activity of the mutant protein [15,19,20]. While the level of NQO_1 induction in these cells lines in group C was relatively high, the level of mutant protein in these cells following induction was still lower than the level of wild-type protein in the cell lines in groups A and B. This is consistent with the suggestion that the mutant protein may be less stable than the wild-type protein [15,20].

DT-diaphorase activity is dependent upon formation of a dimer of the NQO_1 protein [1]. Thus, cells that are heterozygous for the NQO_1 mutation may form a heterodimer. If this heterodimer has low DT-diaphorase activity, the mutant NQO_1 protein might act as a dominant-negative to decrease enzyme activity. Since our studies suggested that mutant NQO_1 protein may be more easily induced than wild-type protein, this might make it more difficult to induce DT-diaphorase activity in cells that are heterozygous for the mutation. To test whether the mutant NQO_1 protein acts as a dominant-negative, we transfected a plasmid containing a mutant NQO_1 cDNA into HCT116 human colon cancer cells and PC-3 human prostate cancer cells, which have two wild-type NQO_1 alleles. The pRc/CMV/NQO_1 609 transfected cells showed unchanged expression of the wild-type NQO_1 protein, but also expressed the mutant protein. The ratio of mutant/wild-type NQO_1 protein was 5.8 ± 1.5 in the HCT116 cells and 2.7 ± 0.3 in PC-3 cells that were actually transfected with the pRc/CMV/NQO_1 609 plasmid, high enough that all the DT-diaphorase activity should have been inhibited in these cells if the mutant protein acted as a dominant-negative. Since the transfection efficiency in this study was 68.1 ± 5.9% in the HCT116 cells and 58.6 ± 9.9% in the PC-3 cells, decreases of approximately 70 and 60% in the DT-diaphorase activity of the two transfected cells, respectively, would have been expected. However, enzyme activity in the pRc/CMV/NQO_1 609 transfected cells was not significantly different from control cells or cells transfected with the pRc/CMV plasmid. Thus, mutant NQO_1 protein does not appear to act as a dominant-negative to decrease DT-diaphorase activity.

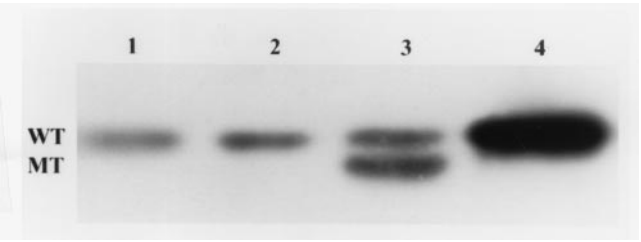

Fig. 5. Expression of NQO_1 wild-type and mutant protein in transfected PC-3 human prostate cancer cells. Cells were co-transfected with pCMVβ and pRc/CMV, pRc/CMV/NQO_1 609, or pRc/CMV/NQO_1 WT using the Fugene 6 transfection reagent. Protein was extracted, and 10 μg was run on a 16-cm 12.5% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membranes and probed with NQO_1 antibody and then with a secondary antibody. The secondary antibody was detected using an ECL detection kit. Lane 1, non-transfected cells; lane 2, cells transfected with pRc/CMV; lane 3, cells transfected with pRc/CMV/NQO_1 609; and lane 4, cells transfected with pRc/CMV/NQO_1 WT. WT, wild-type NQO_1 protein; MT, mutant NQO_1 protein.

We also transfected the plasmid containing the wild-type NQO_1 cDNA into HCT116 and PC-3 cells. The pRc/CMV/NQO_1 WT transfected HCT116 and PC-3 cells showed 6.9- and 8.4-fold increased expression of the wild-type NQO_1 protein compared with the control cells, respectively. DT-diaphorase activity in these cells was also increased significantly by 1.4- and 1.5-fold, respectively. It is not clear why the relative increase in DT-diaphorase activity was smaller than the relative increase in wild-type protein in these cells; however, increases in protein levels do not always correlate directly with increases in enzyme activity as was observed with some of the cell lines in group A, particularly the NCI-H661 cells.

These results provide evidence that induction of DT-diaphorase in human tumor cells is likely regulated at, or

prior to, transcription. The lack of DT-diaphorase induction in some cells may be due to the absence of, or mutations in, transcriptional elements that control enzyme induction in the 5′ flanking region of the NQO_1 gene. Alternatively, there may be defects that prevent induction of necessary transcription factors, or changes in the cellular target for the inducing agent. However, since all of the cell lines in group B that were not induced by D3T had intermediate to high basal levels of DT-diaphorase expression, these results suggest that basal and induced expression of DT-diaphorase may be regulated independently. The mutation at base 609 results in cells with little or no DT-diaphorase activity when both alleles of the gene carry the mutation. If only one allele carries the mutation, the cells have basal expression of DT-diaphorase, and some cell lines show enzyme induction while others do not. In addition, it does not appear that the mutant NQO_1 gene acts as a dominant-negative to decrease DT-diaphorase activity in cells that express both wild-type and mutant protein.

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