Development of a New Isogenic Cell-Xenograft System for Evaluation of NAD(P)H:Quinone Oxidoreductase-Directed Antitumor Quinones: Evaluation of the Activity of RH1

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ABSTRACT

Purpose: The purpose of our study was to develop and validate an isogenic cell line pair that differs only in the expression of NAD(P)H:quinone oxidoreductase (NQO1) that can be used to examine the in vitro and in vivo role of NQO1 in the bioactivation of the antitumor quinone RH1 (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone), a compound currently in Phase I clinical trials.

Experimental Design: MDA-MB-468 (MDA468) human breast adenocarcinoma cells, homozygous for a polymorphism in NQO1 (NQO1*2/*2) and with low levels of NQO1 activity, were stably transfected with human NQO1 to generate a clone (NQ16) expressing very high NQO1 activity. We examined levels of other reductases and looked at biochemical systems that might influence response to antitumor quinones to validate that the isogenic cell line pair differed only in the expression of NQO1. The 3-(4,5-dimethylthiazol-2,5-diphenyl)tetrazolium (MTT) assay was used to determine the differential toxicity of various quinones, including the most recent NQO1-directed antitumor quinone, RH1, between the two cell lines. Human tumor xenografts were established from both MDA468 and NQ16 cells, and the antitumor activity of RH1 was evaluated.

Results: Levels of cytochrome P450 reductase, cytochrome b5 reductase, soluble thiols, and superoxide dismutase in the NQ16 line were unchanged from the parental line. The functional significance of wild-type NQO1 expression was confirmed by measurement of the differential toxicity of compounds activated or deactivated by NQO1 in the two cell lines. The toxicity of the NQO1-directed antitumor quinones RH1 and streptonigrin were markedly greater and the toxicity of menadione, which is detoxified by NQO1, was ameliorated in the NQ16 line. High levels of NQO1 expression were observed throughout xenograft tumors established from the NQ16 cell line. RH1 treatment was effective at statistically reducing tumor volume in NQ16 xenografts at all of the doses tested [0.1, 0.2, 0.4 mg/kg every day for 5 days], whereas only the highest dose of RH1 resulted in a significant reduction in tumor volume in MDA468 xenografts.

Conclusions: The MDA468/NQ16 isogenic cell line pair is a useful model system for evaluating the role of NQO1 in the bioactivation of antitumor quinones in both cell lines and xenografts. In addition, our data demonstrate that the novel antitumor quinone RH1, is effectively activated by NQO1 both in vitro and in vivo.

INTRODUCTION

Studies assessing the role of NAD(P)H:quinone oxidoreductase (NQO1) in the bioactivation of antitumor quinones have primarily been carried out in tumor cell lines expressing low and high NQO1 activity (1–4) or in tumor cell line panels in which drug sensitivity was correlated to NQO1 expression retrospectively (5, 6). Results with different cell lines are confounded by genotypic differences such as differential activities of other reductases and different response mechanisms to stress. Bioreductive activation is a complex process and teasing out the contribution of a single reductase requires controlling as many variables as possible. The NQO1*2/*2 polymorphism (7, 8) is a point mutation (C609T) in the human NQO1 gene that results in a protein reported to have no NQO1 activity because of rapid proteasomal degradation of the NQO1*2 protein (9). Cell lines homozygous for this polymorphism have been used as a molecular tool to develop isogenic cell lines that differ only in their expression of wild-type NQO1. Isogenic cell lines are useful for assessing the role of NQO1 in the bioactivation of antitumor quinones.

We have previously described one isogenic cell line pair, the human BE colon adenocarcinoma cell line (genotyped NQO1*2/*2) and its wild-type NQO1 expressing isogenic clone BE NQ-7, with respect to the levels of NQO1 and other reductases and differences in susceptibility to NQO1 substrates (10). The BE/BE NQ7 isogenic pair has only been used for in vitro studies and was found to be unsuitable for in vivo studies because of an extremely slow growth rate (data not shown). Sharp et al. (11) also generated an isogenic pair of cell lines differing only in NQO1 activity using the BE colon cancer cell
line as a parental cell line but using a different expression plasmid. Sharp et al. (11) used these lines to generate xenograft tumors but found a substantial decrease in tumor NQO1 activity (from 1400 in cells to 6.2 nmol cytochrome c reduced/min/mg protein in tumor homogenates) concomitant with tumor growth. Other investigators have observed the same phenomenon; NQO1 expression decreased in cells when grown as solid tumor xenografts (12).

We developed another isogenic cell line pair using the MDA-MB-468 (MDA468) human ductal breast carcinoma cell line. We report on the characterization of this cell line pair in terms of levels of NQO1 and other reductases, cellular levels of nonprotein thiols and of superoxide dismutase to verify that the isogenic pair of cell lines had similar abilities to respond to alkylating and oxidative stress. The characterization of the cell line pair was completed by assessing the functional significance of wild-type NQO1 expression by comparing the toxicity of compounds that are either activated or deactivated by NQO1 in both cell lines.

We performed initial in vivo testing of the MDA468 transfected cell line (NQ16) to evaluate tumorigenicity, wild-type NQO1 protein expression and histological similarities to the human tumor from which the cell line was derived. Xenograft tumors originating from the MDA468 parental line and its isogenic line NQ16, differing only in the expression of NQO1, were used to examine the role of NQO1 in the in vivo bioactivation of the antitumor quinone RH1 (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone), a compound currently in Phase I clinical trials.

MATERIALS AND METHODS

Materials. Cytochrome c, 2,6-dichlorophenol-indophenol (DCPIP), dicoumarol, 5,5’-dithio-bis(2-nitrobenzoid acid) (Ellman’s reagent), glutathione, menadione, 3-(4,5-dimethylthiazol-2,5-diphenyl)tetrazolium (MTT), NADH, streptonigrin (SN), trichloroacetic acid and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). RH1 was synthesized as described previously (10) with stock solutions made up in DMSO. Precast Cambrex PAGEr GOLD 12% Tris Buffer A 

(25 mM Tris-HCl (pH 7.4), 250 mM sucrose containing 5 μM flavin mononucleotide) and sonicated for determination of protein concentration as described previously (16) or were lysed in 5% trichloroacetic acid, vortexed immediately, and centrifuged to pellet cellular protein. The acidified supernatant was distributed into two tubes on ice. Cells were centrifuged, rinsed in PBS, and recentrifuged. The pellets were either brought up in Buffer A [25 mM Tris-HCl (pH 7.4), 250 mM sucrose containing 5 μM flavin mononucleotide] and sonicated for determination of protein concentration as described previously (16) or were lysed in 5% trichloroacetic acid, vortexed immediately, and centrifuged to pellet cellular protein. The acidified supernatant was mixed with 0.4 M Tris-HCl (pH 8.9), and 5,5’-dithio-bis(2-nitrobenzoid acid) was added to a final concentration of 100 μM. Absorbance at 412 nm was determined, and results were expressed as nanomoles of acid-soluble thiols per mg protein calculated on the basis of a μM glutathione calibration curve.

Growth Inhibition Assays. Toxicity was determined by growth inhibition (MTT) assays as described previously (20).

Alkaline Comet Assay. DNA damage was evaluated by the single-cell gel electrophoresis method, commonly known as the alkaline comet assay, as described previously (20), including modifications by Ward et al. (21) to detect DNA cross-linking. A minimum of 200 comets on each slide were visually scored as belonging to one of four classes according to the degree of DNA damage observed, in a similar fashion to the method of other investigators (22–24). Slides were scored blinded. Comet classes are as follows: class 0, intact, well-defined nucleus, no DNA damage; class 1, defined nucleus with light tail formation, <25% DNA in the tail; class 2, defined nucleus but weak fluorescence with 25 and 75% DNA in the tail; class 3, nucleus no longer well defined and tail consists of more than 75% of the DNA.

The visual classification system was verified independently using Euclid Comet Analysis software (St. Louis, MO) from images captured with a Nikon Coolpix 990 digital camera.
mounted to the microscope. The total number of comets in classes 0 and 1 (less than 25% DNA in the tail) and in classes 2 and 3 (greater than 25% DNA in the tail) were summed and expressed as a percentage of the total comets counted for each slide.

Establishment of Xenograft Tumors. All of the experiments involving animals were approved by the University of Colorado Health Sciences Center Animal Care and Use Committee and were carried out according to approved protocols. Female athymic nude mice (NCr nu/nu; National Cancer Institute, Fredrick, MD) were received at 5–6 weeks of age and were allowed to acclimate for 2 weeks in sterile microisolator cages with constant temperature and humidity. Mice had free access to food and water. After inoculation with tumor cells, mice were monitored daily and were weighed twice weekly; digital caliper (Mitutoyo, Japan) measurements began when tumors were visible.

In Vivo Model Development. Cells in log phase growth were harvested on the day of use and were injected s.c. at a concentration of 1 × 10^6 (left shoulder), 2 × 10^6 (right shoulder and left flank), and 4 × 10^6 cells (right flank) with 0.1 ml of a 75:25 unsupplemented medium/Matrigel suspension to define the concentration of cells necessary to initiate tumor growth. NQO1 activity in tumor homogenates was measured as described above. Tissue sections (4 μm) were cut from formalin-fixed, paraffin-embedded blocks and were stained as described below (see “Immunohistochemistry”).

RH1 Antitumor Activity. Cells in log phase growth were harvested on the day of injection and an equal number of animals were inoculated s.c. bilaterally in the flanks with 0.1 ml of a 75:25 unsupplemented medium/Matrigel suspension containing 4 × 10^7 cells/ml MDA468 or NQ16 cells. When tumors had grown to ~200 mm³ (4 weeks after cell implantation), animals bearing bilateral tumors were randomized into a control and three drug-treatment groups of seven to eight animals per cell line. RH1 (stock solution in DMSO) was diluted into sterile saline just before i.p. injection (0.1 mg/kg, 0.2 mg/kg, or 0.4 mg/kg) daily for five consecutive days (every day for 5 days). The doses of RH1 and schedule of treatment used in this study were based on the maximum tolerated dose (0.45 mg/kg/day i.p.; every day for 5 days) as determined by the National Cancer Institute in their xenograft studies. Control animals received an equal volume of DMSO in sterile saline.

Tumor volume was calculated by the formula \( V = \frac{L \times W^2}{2} \) (25), where \( L \) is the longer measurement of the tumor and \( W \) is the smaller tumor measurement. The increase in tumor volume from the start of treatment \( V_0 \) until the value at any give time \( V_t \) was calculated for each tumor and was expressed as the relative tumor volume \( \frac{V_t}{V_0} \) on the day of measurement. The mean of these values was used to calculate the ratio between treated (T) and control (C) tumors \((T/C \times 100\%)\) as an indication of drug efficacy. Growth inhibition is expressed as \( 100\% - (T/C \times 100\%) \). Optimal values are the lowest percentage \( T/C \) or the highest growth inhibition reached on a specific day after treatment ended (26). Tumor doubling time \( t_{50} \) was calculated by the formula \( t_{50} = \frac{\log 2}{\log \left( \frac{V_t}{V_0} \right)} \), where \( V_0 \) is the tumor volume at the start of treatment, \( V_t \) is the tumor volume at time \( t \) and \( t \) is the time (in days) of the second measurement. Negative values indicate tumor regression (27). Mice were euthanized ~3 months after tumor implantation, and tumors were removed for the determination of NQO1 activity and were paraffin-embedded for histological evaluation.

NQO1 activity was determined after excised tumors were weighed, cut in half, diluted 1:5 in Buffer A, and homogenized on ice for 2 min with a Kontes glass-glass homogenizer. The homogenates were probe sonicated on ice for 30 s, then were centrifuged at 13,000 × g for 10 min. at 4°C. The supernatant was removed and stored at −80°C until activity was measured.

Immunohistochemistry. Xenograft tumor tissue sections (4 μm) were cut from formalin-fixed, paraffin-embedded blocks. H&E staining was performed according to standard histological procedure. Immunodetection of NQO1 was performed as described previously (13) except for the addition of the mouse-on-mouse immunodetection kit according to the manufacturers instructions (Vector, Burlingame, CA).

Statistical Analysis. Statistical analysis was performed using NCSS (Kaysville, UT) software. The two-sample \( t \) test was used to evaluate differences between treatment and control, and \( P < 0.05 \) were considered significant. Data are presented as mean ± SE.

RESULTS

Characterization of the MDA468 Isogenic Cell Line Pair. A wide range of NQO1 activity was detected in the transfected clones. Activity was continuously measured in transfected clones to ensure maintenance of stable NQO1 expression. The NQ16 clone expressed the highest NQO1 activity level measured and has maintained very high NQO1 activity levels in culture. NQO1 activity levels of parental and transfected NQ16 cells are shown in Table 1. Immunoblot analysis with 5 μg of cell cytosol of the MDA468 parent and the transfected NQ16 cell line demonstrated the presence of NQO1 protein in the transfected NQ16 cells (Fig. 1A). PCR-RFLP confirmed the presence of the NQO1*I1 genotype as well as the genomic NQO1*I2 allele in the NQ16 cell line (Fig. 1B). NQ16 cells demonstrate both of the DNA sequences, whereas the parental MDA468 cell line has only the NQO1*I2 allele. Fluorescence in situ hybridization (FISH) assays indicated the plasmid

Table 1 Average reductase activity

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>MDA468</th>
<th>NQ16</th>
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<tbody>
<tr>
<td>NQO1 (nmol DCPIP/min/mg protein)</td>
<td>9.5 ± 2.2</td>
<td>4797.4 ± 137.5</td>
</tr>
<tr>
<td>Cytochrome b5 reductase</td>
<td>20.4 ± 2.2</td>
<td>20.2 ± 1.9</td>
</tr>
<tr>
<td>Cytochrome P450 reductase</td>
<td>7.0 ± 0.8</td>
<td>6.9 ± 0.6</td>
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* NQO1, NAD(P)H:quinone oxidoreductase; DCPIP, 2,6-dichloro-phenol-indophenol.
 containing the wild-type NQO1 cDNA had integrated into chromosome 3 of the NQ16 transfected cells (data not shown). Multiple copies of the gene insert were not detected by fluorescence in situ hybridization. To confirm the fluorescence in situ hybridization results, real-time PCR (Laragen Inc., Los Angeles, CA) determined that two copies of the wild-type NQO1 gene were present in the NQ16 cells.

To ensure that transfection of the MDA468 cell line with wild-type NQO1 did not alter biochemical systems that might influence response to antitumor quinones, the enzymatic activity of other reductive enzymes and levels of nonprotein cellular thiols were monitored in the parental MDA468 and transfected NQ16 cells. No statistical difference (P < 0.05) was observed in the activities of cytochrome P450 reductase or cytochrome b5 reductase between the parental MDA468 cells and the NQ16 cells transfected with wild-type NQO1 (Table 1). No significant differences (P < 0.05) in total nonprotein cellular thiols, used as an indication of available glutathione, were observed between MDA468 (5.0 ± 0.7 nmol acid-soluble thiols/mg protein) and NQ16 cells (4.7 ± 0.7 nmol acid-soluble thiols/mg protein). In addition, no apparent differences in levels of cytosolic superoxide dismutase (Cu/Zn superoxide dismutase) protein levels were found by immunoblot using β-actin as a loading control (data not shown).

To ensure that this isogenic cell line pair responded similarly to compounds that were not substrates for NQO1, the toxicity of cisplatin was evaluated in these cell lines. Cisplatin can form inter- and intrastrand cross-links in DNA (28) independent of NQO1 status. No statistical difference was observed in the cytotoxicity of cisplatin to the MDA468 parental cells and the NQ16 NQO1-transfected cells as measured by the MTT assay. The IC50 ± SE for the MDA468 parental line was calculated as 0.57 ± 0.05 µM and 0.54 ± 0.06 µM for the NQ16 cell line. Cisplatin also displayed equivalent DNA cross-linking in both MDA468 and NQ16 cell lines. After treatment with cisplatin (50 µg/ml) and subsequent irradiation, ~46% of all of the comets counted, in either cell line, retained more than 75% of the DNA in the head of the comet (Fig. 2). This is a 2-to-5-fold increase over the amount of DNA that remained in the head of control cells (after irradiation). Retardation in the amount of DNA migration into the tail of the comet after irradiation is an indication of cross-linked DNA (29).

As a validation of the functional significance of the presence of wild-type NQO1, the toxicity of several compounds known to be substrates of NQO1 were tested in the MDA468/NQ16 isogenic pair. SN, an aminoquinone antitumor antibiotic, is a highly efficient substrate for NQO1 (30, 31) with cytotoxicity correlated to NQO1 activity (11, 20, 32). SN was significantly (P < 0.05) more toxic to NQ16 cells than to the parental MDA468 cells (Table 2). Menadione (2-methyl-1,4-naphthoquinone), a vitamin K3 analog (33) can arylate and undergo one electron reduction resulting in the formation of superoxide anion and hydrogen peroxide as a result of redox cycling. NQO1 plays a protective role in the metabolism of menadione because it prevents the oxidative stress caused by this compound through two-electron reduction to the hydroquinone. Consistent with the role of NQO1 in the detoxification of menadione, it was signif-

Fig. 2 Percentage of comets formed in each category (black bars, <25% DNA in the tail; white bars, >25% DNA in the tail) after treatment with cisplatin compared with controls. Both cisplatin-treated and control cells were irradiated with 10 Gy before processing for the comet analysis. Irradiation induces a given amount of strand breaks in both treated and control cells. Broken DNA can migrate into the tail of the comet. Cross-linked DNA will not migrate into the tail and is identified by an increase in the percentage of total comets counted with less than 25% DNA in the tail compared with controls. Two hundred comets were counted per experiment; two separate experiments were conducted.
significantly less toxic \((P < 0.05; \text{higher IC}_{50})\) to NQ16 cells that express wild-type NQO1 than to MDA468 cells (Table 2). Finally, RH1, the novel antitumor agent developed specifically to be bioactivated by NQO1, was significantly more toxic to NQ16 cells than to the parental MDA468 cells (Table 2). The IC\(_{50}\) of RH1 was about 10-fold lower in NQ16 cells that express high levels of wild-type NQO1 protein compared with the IC\(_{50}\) for the parental MDA468 cells.

**Development of an in Vivo Model.** The NQ16 stably transfected cell line was found to be tumorigenic in NCr nu/nu mice, exhibiting a doubling time of 7.6 days. NQO1 activity levels \((2958.4 \pm 92 \text{ nmol DCPIP reduced/min/mg protein})\) were \(\sim 77\%\) of those measured in the particular cell culture used for implantation \((3856.8 \pm 325 \text{ nmol DCPIP reduced/min/mg protein})\). Tumors derived from NQ16 cells (Fig. 3A) demonstrated a similar appearance to human breast adenocarcinoma (Fig. 3B) with tumor cells disposed in cords embedded in connective tissue and solid tumor cell nests. In addition, immunostaining of tumor tissue for NQO1 protein (Fig. 3C) demonstrated relatively even expression of NQO1 in tumor cells.

**RH1 Antitumor Activity Studies.** Approximately 7 weeks after implantation of MDA468 or NQ16 cells, the majority of tumors of both cell lines had grown to 200 mm\(^3\) and the calculated pretreatment tumor doubling times were similar, 12.9 days for MDA468 tumors and 11.8 days for NQ16 tumors.

RH1 treatment \((0.4 \text{ mg/kg and } 0.2 \text{ mg/kg})\) of mice bearing NQ16 tumors (Fig. 4) resulted in a significant reduction \((P < 0.05)\) in tumor volume between treated groups and controls as early as 5 days after the treatment period ended. Low-dose RH1 \((0.1 \text{ mg/kg})\) also resulted in a significant reduction in tumor volume between treated mice and controls, although additional time was required \((3 \text{ days})\) for the difference to become observable. Complete regression of a single tumor was observed for each of two mice that received high-dose RH1. RH1 exhibited antitumor activity in a dose-dependent manner against NQ16 tumors growing in athymic mice.

High-dose RH1 treatment \((0.4 \text{ mg/kg})\) was required to significantly \((P < 0.05)\) reduce tumor volume compared with controls in mice bearing MDA468 tumors starting 8 days after the treatment period ended (Fig. 5). RH1 treatment \((0.2 \text{ mg/kg})\) produced a significant difference \((P < 0.05)\) in tumor volume between treated and control animals at only two individual time points \((19 \text{ days and } 33 \text{ days after RH1 treatment was complete})\), but not at any intervening period. Low-dose RH1 treatment did not significantly reduce the tumor volume in treated mice compared with control mice at any time.

The efficacy of RH1 treatment is depicted graphically (Fig. 5).
Isogenic Cell System for Evaluation of RH1

Fig. 4 NQ16 tumor growth depicting measured tumor volume (cm$^3$) over time. Each plot point represents the mean of all animals in the treatment group (seven to eight). Error bars were omitted for better visual clarity. Circles, high-dose RH1 (0.4 mg/kg) treatment group; triangles, medium-dose RH1 (0.2 mg/kg) treatment group; diamonds, low-dose RH1 (0.1 mg/kg) treatment group. □, control animals that received an equivalent volume of DMSO in saline. *, the time point when tumor volume in treatment groups was statistically different from controls as determined by the Student’s $t$ test with equal variance.

Fig. 5 MDA468 tumor growth depicting measured tumor volume (cm$^3$) over time. Each plot point represents the mean of all animals in the treatment group (seven to eight). Error bars were omitted for better visual clarity. Circles, high-dose RH1 (0.4 mg/kg) treatment group; triangles, medium-dose RH1 (0.2 mg/kg) treatment group; diamonds low-dose RH1 (0.1 mg/kg) treatment group. □, control animals that received an equivalent volume of DMSO in saline. *, the time point when tumor volume in treatment groups was statistically different from controls as determined by the Student’s $t$ test with equal variance.

6, A–C) as the percentage treatment:control (T:C) ratio. Fig. 6A is a comparison of the efficacy of high-dose RH1 (0.4 mg/kg) in NQ16 tumors and MDA468 tumors. At this dose, the optimal percentage T:C for NQ16 tumors was 10.6% (at day 26 after the end of RH1 treatment) and 24% for MDA468 tumors (day 22). At the medium dose of RH1 (0.2 mg/kg; Fig. 6B), an optimal percentage T:C was 41% (day 15) for NQ16 tumors and 52% (day 33) for MDA468 tumors. In NQ16 tumors, the lowest RH1 dose (Fig. 6C) resulted in an optimal percentage T:C of 52% (day 26), 20% below the optimal percentage T:C of 74% (at day 26) for MDA468 tumors.

Toxicity Evaluation. Weight loss was used as a surrogate measure for systemic drug toxicity (25, 34). Before initiation of treatment with RH1, weights remained steady in both MDA468 and NQ16 tumor-bearing animals. There were no significant differences in body weight between treated and control MDA468 tumor-bearing animals at any concentration of RH1. A significant decrease in body weight from control animals was observed in all RH1-treated mice bearing NQ16 xenograft tumors 5 weeks after treatment was initiated (1 week before death). Animals lost up to 14% body weight over the period after the initiation of treatment until death, although no toxicity-related deaths occurred (death within 2 weeks of the start of treatment). No signs of overt drug toxicity were seen in any animals.

NQO1 Activity in Tumor Tissue. The activity of NQO1 in tumor homogenates is presented in Table 3. Although the NQO1 activity in NQ16 tumors was less than that measured in NQ16 cells in vitro, this cell line displays such high activities that a decrease should have little effect on the levels of enzyme available to activate RH1. No significant differences in NQ1 activity of tumor homogenates between treatments within each cell line were seen.

DISCUSSION

NQO1 has been used as a target for the enzyme-directed approach to the design of antitumor agents because NQO1 is expressed at high levels throughout many human solid tumors (35–38). Various models have been used to study the role of NQO1 in the cytotoxicity of NQO1-directed antitumor agents. Because of the complex nature of quinone bioactivation, an excellent model for in vitro and in vivo studies is one of an isogenic pair of human cell lines differing only in the expression of wild-type human NQO1. Several such lines have been generated in our laboratory, and the MDA468 human breast adenocarcinoma and its isogenic line, NQ16, were characterized for this study to ensure conformity between them aside from expression of NQO1. The parental cell line is homozygous for the NQO1*2 polymorphism that results in the production of a mutated form of the NQO1 protein (NQO1*2 protein) which undergoes rapid proteasomal degradation (9). The activity of cytochrome P450 reductase and cytochrome b$_2$ reductase were similar between the MDA468/NQ16 isogenic pair as were levels of superoxide dismutase and glutathione. These results indicate that the isogenic pair should metabolize quinones and antitumor agents similarly via one electron reductases. Detoxification of reactive electrophiles and superoxide mediated via soluble thiols and superoxide dismutase, respectively, would also be expected to be similar.

Cisplatin was used as part of the validation of the MDA468 isogenic pair of cell lines because its activation and toxicity are independent of NQO1. MDA468 cells and NQ16 cells exhibited similar cytotoxicity and degree of cross-linking of DNA after exposure to cisplatin. In contrast, the isogenic cell line pair exhibited marked differences in toxicity when exposed to NQO1 substrates. Reduction of SN by NQO1 results in an unstable hydroquinone that can react with molecular oxygen to produce reactive oxygen species and regenerate the parent quinone. Hydroxyl radicals are produced in the presence of Cu$^{2+}$ and Fe$^{2+}$ in a Fenton-type reaction that results in single- and double-strand DNA breaks, the ultimate source of cellular damage (39). SN was found to be substantially more toxic to NQ16 cells expressing NQO1 than to MDA468 cells. These results correspond to data obtained by others in which SN was shown to
have the strongest correlation between NQO1 activity levels and toxicity in 31,000 compounds tested (40). Furthermore, SN induced more DNA damage in NQ16 cells than in MDA468 cells as determined by the comet assay (data not shown). Over 99% of the comets formed after SN treatment of NQ16 cells had more than 75% of the DNA in the tail, whereas only about 40% of the comets formed after MDA468 cells were treated with SN had more than 75% of the DNA in the tail. Two-electron reduction of menadione by NQO1 prevents redox cycling that occurs when menadione undergoes one-electron reduction. In keeping with the protective role of NQO1 in the detoxification of menadione, the transfected NQ16 cells were less sensitive to menadione than were the parental MDA468 cells. Menadione may be a better substrate for one-electron reductases than for NQO1 because the presence of NQO1 in NQ16 cells did not provide a substantial decrease in toxicity compared with the MDA468 cells (1.6-fold). This small difference in toxicity to menadione was also seen by Gustafson et al. (41) using Chinese hamster ovary cells transfected with human wild-type NQO1 (1.4-fold difference between isogenic lines). The final compound used to validate the MDA468/NQ16 isogenic cell line model was RH1. RH1 is “activated” upon two-electron reduction of the quinone, and the two aziridinyl functional groups undergo protonation that results in aziridine ring opening. The resulting bifunctional alkylating species can covalently bind to DNA and form inter- and intranuclear cross-links (42). RH1 was more toxic to the NQ16 cell line than to the parental MDA468 cell line, consistent with what was observed in the BE colon carcinoma cells transfected with wild-type NQO1 (10).

We then evaluated the ability of the NQ16 stably transfected cell line to create a competent xenograft model (acceptable growth rate, NQO1 activity, uniform NQO1 expression, and histological characteristics of the xenograft tumor). Tumors that were initiated from NQ16 cells grew slowly but formed well-differentiated tumor architecture that mimicked human breast adenocarcinoma, a characteristic that is desirable for predictive screening of potential anticancer drugs (43). Although the NQ16 tumors had lower NQO1 activity levels than the cell line used to establish them the large differential in NQO1 activity between isogenic lines was retained. The reason for the down-regulation of NQO1 activity in cells grown as solid tumors xenografts is unclear. Tumors with central areas of necrotic cells could have contributed to the dilution of activity levels because of nonexpressing cells.

The in vivo evaluation of RH1 validated the in vitro studies that showed a difference in sensitivity to RH1 between NQ16 cells and the parental MDA468 cells. A statistically significant and dose-dependent inhibition of tumor growth, as assessed by tumor volume, was demonstrated in NQ16 human tumor xenografts treated with all doses of RH1, and a complete regression of two tumors was observed after treatment at high-dose RH1. In contrast, only at high doses was RH1 effective in arresting tumor growth and reducing tumor volume in MDA468.

### Table 3 NAD(P)H:quinone oxidoreductase (NQO1) activity in MDA468/NQ16 tumor homogenates

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NQO1 activity (nmol DCPIP&lt;sup&gt;a&lt;/sup&gt; reduced/min/mg of protein)</th>
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<tbody>
<tr>
<td></td>
<td>MDA468</td>
</tr>
<tr>
<td>Control</td>
<td>5.7 ± 1.2</td>
</tr>
<tr>
<td>Low-dose RH1</td>
<td>5.1 ± 1.2</td>
</tr>
<tr>
<td>Medium-dose RH1</td>
<td>11.6 ± 3.0</td>
</tr>
<tr>
<td>High-dose RH1</td>
<td>21.3 ± 11.4</td>
</tr>
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<sup>a</sup> DCPIP, 2,6-dichlorophenol-indophenol.
tumor xenografts. One possible explanation for the response of MDA468 tumor xenografts to high-dose RH1 may be the high levels of NQO1*2 protein in MDA468 cells. The MDA468 cells carry four copies of chromosome 16 or its derivates (data not shown) and have the highest amount of NQO1*2 protein of any of the NQO1*2/2 cell lines that we have tested. As a result, we were able to detect low levels of NQO1 catalytic activity in parental MDA468 cells (Table 1) and in MDA468 xenograft tumor homogenates (Table 3). These very low activity levels, presumably resulting from high protein levels, may be sufficient to bioactivate extremely efficient substrates such as RH1. Alternately, RH1 may be activated by other one- and two-electron reductases in tumor cells or by mouse NQO1. Phillips et al. (44) speculated NQO1 activity measurements in tumor xenograft tissue may not accurately reflect NQO1 activity of the tumor cell due to the contribution of mouse stromal tissue that express NQO1 protein. Mouse NQO1 is very similar to human NQO1 in terms of substrate specificity (45) and could readily bioactivate RH1.

It is also important to note that RH1 was not overly toxic to mice, using body weight as an indicator of toxicity. Although NQ16 tumor-bearing mice lost body weight, this occurred in the last week of the study. We speculate that weight loss could have resulted from a buildup of unmetabolized or toxic proteins released from tumor cells undergoing apoptosis or necrosis.

In summary, we have demonstrated that the MDA468/NQ16 pair of cell lines represents a useful model system for evaluating the activity of NQO1-directed antitumor quinones in both cell lines and xenografts. Our data also demonstrate increased activity of the antitumor quinone RH1 in both NQ16 cell lines and xenografts, validating the role of NQO1 in the bioactivation of RH1 in vivo. These data add to the evidence that NQO1 can be exploited as an activating enzyme for chemotherapeutic quinones.

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REFERENCES


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