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Relationship between NAD(P)H:quinone oxidoreductase 1 (NQO1) levels in a series of stably transfected cell lines and susceptibility to antitumor quinones[1]

Shannon L. Winski[a], Elizabeth Swann[b], Robert H.J. Hargreaves[c], Donna L. Dehn[a], John Butler[c,d], Christopher J. Moody[b], David Ross[a,*]

[a]*Department of Pharmaceutical Sciences, School of Pharmacy and Cancer Center, Campus Box C238, University of Colorado Health Sciences Center, Denver, CO, 80262, USA*
[b]*University of Exeter, Exeter, UK*
[c]*Christie Research Center, Manchester, UK*
[d]*Department of Biological Sciences, Salford University, Salford, UK*

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Abstract

To investigate the importance of NAD(P)H:quinone oxidoreductase 1 (or DT-diaphorase; NQO1) in the bioactivation of antitumor quinones, we established a series of stably transfected cell lines derived from BE human colon adenocarcinoma cells. BE cells have no NQO1 activity due to a genetic polymorphism. The new cell lines, BE-NQ, stably express wild-type NQO1. BE-NQ7 cells expressed the highest level of NQO1 and were more susceptible [determined by the thiazolyl blue (MTT) assay] to known antitumor quinones and newer clinical candidates. Inhibition of NQO1 by pretreatment with an irreversible inhibitor, ES936 [5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione], protected BE-NQ7 cells from toxicity induced by streptonigrin, ES921 [5-(aziridin-1-yl)-3-(hydroxymethyl)-1,2-dimethylindole-4,7-dione], and RH1 [2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone]. RH1 was evaluated further by clonogenic assay for cytotoxic response and was more cytotoxic to BE-NQ7 cells than to BE cells. Cytotoxicity was abrogated by inhibition of NQO1 with ES936 pretreatment. Using a comet assay to evaluate DNA cross-linking, BE-NQ7 cells demonstrated significantly higher DNA cross-links than did BE cells in response to RH1 treatment. DNA cross-linking in BE-NQ7 cells was observed at very low concentrations of RH1 (5 nM), confirming that NQO1 activates RH1 to a potent cross-linking species. Further studies using streptonigrin, ES921, and RH1 were undertaken to analyze the relationship between NQO1 activity and quinone toxicity. Toxicity of these compounds was measured in a panel of BE-NQ cells expressing a range of NQO1 activity (23–433 nmol/min/mg). Data obtained suggest a threshold for NQO1-induced toxicity above 23 nmol/min/mg and a sharp dose–response curve between the no effect level of NQO1 (23 nmol/min/mg) and the maximal effect level (>77 nmol/min/mg). These data provide evidence that NQO1 can bioactivate antitumor quinones in this system and suggest that a threshold level of NQO1 activity is required to initiate toxic events. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: NQO1; Antitumor quinones; Enzyme-directed drug development; Preclinical research; *In vitro* testing

1. Introduction

Antitumor quinones are a major class of therapeutic agents. MMC is the prototype antitumor quinone, which requires reductive activation [1]. It is used clinically with success in both single and combination therapies [2,3]. Other compounds in this class include streptonigrin and EO9 and newer quinones such as RH1,[1] which is currently under consideration for clinical trials. Intracellular bioactivation of antitumor quinones requires enzymatic reduction and can be accomplished by both one- and two-electron reductases. The result of bioactivation is the production of both mono- and bifunctional alkylating species that can

* Corresponding author. Tel.: +1-303-315-6077; fax: +1-303-315-0274.
E-mail address: David.Ross@uchsc.edu (D. Ross).

Abbreviations: MMC, mitomycin C; NQO1, NAD(P)H:quinone oxidoreductase 1 or DT-diaphorase; MeDZQ, 2,5-diaziridinyl-3,6-dimethyl-1,4-benzoquinone; DCPIP, 2,6-dichlorophenol-indophenol; MTT, thiazolyl blue; P450R, NADPH:cytochrome P450 reductase; b5R, NADH:cytochrome b_5 reductase; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; FBS, fetal bovine serum; and MEM, Eagle's minimum essential medium.

[1] In accordance with the policy of the University of Colorado Health Sciences Center, D.R., R.H.J.H., and J.B. declare a patent interest in RH1.

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cross-link DNA and generate reactive oxygen species [for review, see Refs. 1,4, and 5]. Enzymes that may be important in activating MMC through one-electron reduction are P450R (EC 1.6.2.4) [6,7], b5R (EC 1.6.2.2) [8], and xanthine oxidase (EC 1.2.3.2) [9]. Two-electron reduction can be accomplished by xanthine dehydrogenase (EC 1.1.1.204) [10] and NQO1 (DT-diaphorase, EC 1.6.99.2) [11]. Affinity constants of these enzymes for MMC are relatively similar, and bioactivation may predominantly depend on the levels of enzymes in the cell [12]. Although the specific activity for MMC is relatively low for these enzymes, many more efficient substrates have been developed in the hopes of exploiting these activation pathways.

Using NQO1 as the principal target to design chemotherapeutic agents is of significant interest due to the fact that it is expressed at high levels throughout many tumor tissues. NQO1 activity and gene expression were elevated above normal tissue levels in lung, colon, liver, and breast tumors [13–15]. NQO1 was elevated in NSCLC compared with its level in SCLC and uninvolved tissue [16]. A recent study demonstrated that expression of NQO1 increases with malignant progression in colon cancer [17]. In a study of enzyme levels in NSCLC, colon, and head and neck tumors, the level of NQO1 was 5- to 25-fold greater than that of P450R and b5R [18]. *In vitro* data also correlate NQO1 levels with sensitivity to MMC and EO9. One study compared enzyme levels of NQO1, P450R, and b5R with sensitivity to MMC and EO9 in a panel of 69 tumor cell lines [19]. NQO1 levels significantly correlated with drug sensitivity, and, in general, NQO1 was expressed at higher levels relative to the other enzymes measured. In addition to enzyme levels, overall bioactivation of antitumor quinones is affected by a number of factors including pH, oxygenation, and tumor microenvironment.

Due to the complex nature of reductive bioactivation, it is necessary to study these issues in carefully designed, human-derived models. To examine the role of NQO1 in a controlled manner, we established a series of cell lines that stably express wild-type human NQO1 protein. These cells were derived from BE human colon adenocarcinoma cells, which do not demonstrate significant NQO1 activity due to a polymorphism in the NQO1 gene [20,21]. The new cells, BE-NQ, stably express wild-type human NQO1 protein and are more susceptible to MMC, MeDZQ, and RH1 [22]. Levels of P450R and b5R are relatively low in the parent cell line and are unchanged by transfection [22]. In the present study, we extended these results to include antitumor quinones that are activated by NQO1, and we also tested new potential candidates. Included are studies of the indolequinones ES921 and ES923 that were identified as potential antitumor quinones in previous studies [23,24]. (Definitions of the ES compounds are found in the next section.) We also present data utilizing a specific inhibitor of NQO1, ES936 [25]. This work has allowed characterization of the contribution of NQO1 in the bioactivation of antitumor quinones and more specifically, to define the relationship between NQO1 enzyme levels and cell killing in human tumor cells.

2. Materials and methods

2.1. Materials

Cell culture medium was obtained from Life Technologies, and FBS was from Gemini BioProducts, Inc. Other cell culture materials were purchased from Fisher Scientific. Mouse anti-human NQO1 primary antibody was derived from hybridoma clones A180 and B771, which are maintained by University of Colorado core facilities, and peroxidase-conjugated goat anti-mouse antibody was obtained from Jackson ImmunoResearch Laboratories. Precast 12% acrylamide gels were purchased from Bio-Rad. RH1 [2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone] was synthesized as described previously [22]. ES1352 [9-(hydroxymethyl)-7-(2-methylaziridin-1-yl)-1,2-dihydro-3*H*-pyrrolo[1,2-*a*]indole-5,8-dione], ES936 [5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione], ES921 [5-(aziridin-1-yl)-3-(hydroxymethyl)-1,2-dimethylindole-4,7-dione], and ES923 [3-(hydroxymethyl)-1,2-dimethyl-5-(2-methylaziridiny1-yl)indole-4,7-dione] were synthesized according to published procedures [23]. AZQ [diaziquone, 2,5-bis(carboethoxyamino)-3,6-diaziridinyl-1,4-benzoquinone] was obtained from the National Cancer Institute, and EO9 [3-hydroxy-5-aziridinyl-1-methyl-2-(1*H*-indole-4,7-indione)-prop-β-en-α-ol] was a gift from Dr. John Butler. Immobilon-P western blotting membrane, MTT, DCPIP, streptonigrin, and all other chemicals were purchased from the Sigma Chemical Co.

2.2. Cell culture

Stable transfection of wild-type human NQO1 cDNA into BE cells to generate BE-NQ cells is described elsewhere [22]. BE human colon adenocarcinoma cells were obtained from Dr. Neil Gibson (University of Southern California) and were mycoplasma-negative. BE and BE-NQ cells were maintained at 37° in a humidified atmosphere with 5% CO_2. The cells were grown as monolayers in MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The presence of the selection agent, geneticin (G418), was not required to maintain the stable expression of NQO1 in the BE-NQ cell lines.

2.3. Characterization of stably transfected BE cells (BE-NQ)

Stable expression of NQO1 was measured as the dicoumarol-inhibitable fraction of DCPIP reduction in the cell cytosol as described [26] with modifications [27] and was normalized to total cytosolic protein [28]. The presence of NQO1 was confirmed by immunoblot analysis as described

[29]. Protein bands identified immunologically were quantitated by imaging densitometry (Fluor-S Multiimager; Bio-Rad).

2.4. Toxicity assays

Quinone toxicity was determined by growth inhibition (MTT) and clonogenic survival assays. For the MTT assay, cells were seeded at 1000 cells/well in a 96-well plate and 16 hr later were treated with test compounds in serum-free MEM. Drug-containing medium was removed after 2 hr and replaced with serum-supplemented medium. Cell viability was measured by MTT after 96 hr as described [30]. IC_{50} values were defined as the drug concentration that resulted in a 50% reduction in cell number compared with untreated controls. These values were derived from semilog plots of percentage control versus drug concentration. For comparison, the toxicity of RH1 was determined by a colony-forming assay, using a similar dosing regimen. Cells were seeded depending on expected cell kill (100–5000 cells/100 mm dish) and then allowed to attach for 5 hr. RH1 was dissolved in DMSO for dosing; the final DMSO concentration was 0.1%. This concentration did not affect the growth or the viability of the cells tested. Cells were exposed to RH1 in serum-free MEM for 2 hr, and then the medium was removed and replaced with serum-supplemented MEM. Colonies were allowed to form for 10 days at which time colonies in the vehicle-treated control plates were greater than 50 cells in size. Colonies were fixed for 2 min in 4% acetic acid/8% methanol, stained for 5 min in 0.2% crystal violet in PBS containing 10% formalin, and then rinsed with distilled water. Colonies were counted blind and in random order.

2.5. Determination of DNA cross-linking by the comet assay

DNA cross-linking in response to RH1 treatment was evaluated by single cell alkaline gel electrophoresis (comet assay). The assay is a standard alkaline comet assay with modifications to allow for detection of DNA interstrand cross-links as described [31]. Detection of cross-links is based upon introducing a fixed amount of single-strand breaks in the DNA and then visualizing inhibition of DNA movement through an electrophoretic field caused by interstrand cross-linking. To do this, the cells were treated with RH1 for 2 hr and suspended in ice-cold serum-supplemented medium. The cells were then irradiated with 30 Gy (cobalt source) after which the samples were processed for normal alkaline comets. A suspension of treated cells was embedded in 1% low melting point agarose and transferred to slides coated with poly-L-lysine and agarose. Cells were lysed for 1 hr in ice-cold lysis buffer (2.5 M NaCl, 10 mM Tris–HCl, 100 mM EDTA, 1% Triton X-100, 1% DMSO). After removal of the lysis buffer and rinsing in distilled deionized water (ddH_2O), the slides were submerged in alkali unwinding buffer (50 mM NaOH, 1 mM EDTA, 1% DMSO) for 30 min and electrophoresed (0.6 V/cm, 45 mA, 25 min) in the same buffer. The slides were then removed and neutralized in 500 mM Tris–HCl (pH 8.0), followed by rinsing in PBS. The slides were dried overnight, stained with 2.5 μg/mL of propidium iodide for 30 min, and rinsed with ddH_2O to remove excess stain. The slides were examined on a Nikon Eclipse TE300 (Nikon Inc.) equipped with epifluorescence capabilities and a CCD camera (Princeton Instruments). Comet images (minimum of 100) were captured and quantitated with NIH Image version 1.62. DNA cross-linking is defined by the following equation: % DNA cross-linked = 100 − (100A/B), where A is the % DNA in the tail of drug-treated samples + 30 Gy, and B is the % DNA in the tail of vehicle-treated samples + 30 Gy.

2.6. Inhibition of NQO1 using ES936 in cytotoxicity assays

The cytotoxicity of streptonigrin, RH1, and ES921 was measured by the MTT assay in BE and BE-NQ7 cells after pretreatment with ES936. ES936 is a potent inhibitor of purified recombinant human NQO1 [23] and does not affect the activity of purified one-electron reductases, P450R and b5R [25]. Cells were pretreated for 1 hr with 25 nM ES936 in MEM supplemented with 10% FBS. At this concentration, there was no observed toxicity to BE or BE-NQ7 cells as determined by MTT growth-inhibition curves. The presence of serum did not affect the ability of ES936 to inhibit NQO1 in cell-free solutions or in intact cells (data not shown). ES936 pretreatment of BE-NQ7 cells under these conditions resulted in 98% inhibition of NQO1 activity. After the 1-hr pretreatment period, the medium containing the inhibitor was removed, and cells were treated with the drug of interest in serum-free medium, as described above. NQO1 activity was repressed throughout the drug treatment phase.

3. Results

3.1. Expression of wild-type NQO1 in BE cells

Transfected cells were screened for the wild-type DNA sequence and NQO1 activity [22]. Five cell lines were identified that exhibited stable expression of NQO1 in the absence of selection medium. Activity was measured throughout the toxicity experiments and remained stable for these cell lines at the levels indicated (Table 1). The relative level of NQO1 expression was measured by densitometric analysis of protein bands from immunoblot analysis (Fig. 1). Comparison of activity and protein expression demonstrated a linear correlation ($R^2 = 0.987$), indicating that the expressed protein was functional. The BE-NQ7 cell line exhibited the highest activity and was chosen to compare the toxicity of antitumor quinones. Activity of NQO1 in these cells was independent of cell confluency (data not shown). Levels of the one-electron reductases, P450R and

Table 1
NQO1 activity in BE cell lines transfected with human NQO1 cDNA

Cell line	NQO1 activity[a]
BE	2.0 ± 0.7
BE-NQ2	23 ± 4*
BE-NQ3	77 ± 2*
BE-NQ4	86 ± 2*
BE-NQ5	210 ± 20*
BE-NQ7	433 ± 38*

[a] Activity is expressed as nmol DCPIP reduced/min/mg of total cytosolic protein after inhibition with dicoumarol. Values represent means ± SEM of a minimum of three independent experiments. NQO1 activity was stable in the transfected cell lines, and expression was maintained in the absence of G418 selection medium.

* Activity higher than parent BE cells $P < 0.05$ (ANOVA, Dunnett's *t*-test for multiple comparisons to a single control).

b5R, and the two-electron reductase, xanthine dehydrogenase, were not significantly different between the transfected and parental cell lines [22].

3.2. Toxicity and selectivity of antitumor quinones in BE and BE-NQ7 cells

To screen a number of compounds, toxicity was tested in BE-NQ7 cells and compared with toxicity observed in the BE parental cell line. Growth inhibition was determined 96 hr post drug treatment, using the MTT assay. Data are expressed as IC_{50} values (Table 2), defined as the drug concentration required to inhibit cell growth by 50% of vehicle-treated controls. For comparison, compounds are listed in order of substrate specificity for purified, recombinant human NQO1 according to published values [32,33] (Table 2). With the exception of ES936, all compounds tested were significantly more toxic (lower IC_{50} value) to BE-NQ7 cells than to the parent BE cells. The IC_{50} value of ES936, an inhibitor of NQO1 [23,25], in BE-NQ7 cells was not significantly lower than that found in the parent cell line. To express the relative selectivity of the compounds for BE-NQ7 cells, IC_{50} values for BE and BE-NQ7 were utilized to calculate a selectivity ratio (SR) (Table 2). Streptonigrin, ES921, ES923, and the previously characterized RH1 [22] were the most selective to BE-NQ7 cells (SR = 43, 23, 17, and 17, respectively). EO9, which was highly selective in other models [19], and ES1352, which was selective in NSCLC lines [23], were only 2- to 4-fold more selective to the BE-NQ7 cell line. Short-term MTT assay may be more reflective of inhibition of growth rather than cell killing, which can be measured more accurately by long-term clonogenic survival assays [34]. To augment growth inhibition studies, long-term clonogenic survival data were generated for RH1 and MMC (Table 3). The concentrations of RH1 and MMC required for a 90% cell kill (C10) were significantly lower for BE-NQ7 cells than for the parent cells, demonstrating selective cytotoxicity. The ability of RH1 to cross-link DNA was determined by the comet assay. Treatment of BE-NQ7 cells with RH1 resulted in significantly more DNA cross-links than occurred in BE cells at equimolar concentrations (Table 4). RH1 did not cause significant single-strand breakage according to this assay (data not shown), implicating DNA cross-linking as the major toxic effect from this compound. In the BE-NQ7 cells, significant cross-linking was observed at concentrations as low as 5 nM after only 2 hr of treatment, while cross-linking was not observed in the NQO1-null BE cells below 50 nM RH1.

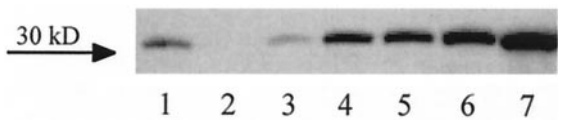

Fig. 1. Expression of NQO1 in stably transfected BE-NQ cells. NQO1 protein in cell cytosols prepared from G418-resistant cell lines was confirmed by immunoblot analysis. Lane 1, purified recombinant NQO1 standard (5 ng); lane 2, BE (parent cell line) cell cytosol (1 μg); lanes 3–7, BE-NQ2, 3, 4, 5, and 7 cell cytosols (1 μg).

3.3. Effect of NQO1 inhibition on the toxicity of streptonigrin, RH1, and ES921

The effect of NQO1 inhibition on the toxicity of streptonigrin, RH1, and ES921 was assessed in the BE and BE-NQ7 cell lines. ES936 was chosen as a specific inhibitor [23,25] over a more commonly used inhibitor, dicoumarol. In comparison to dicoumarol, ES936 was a more effective inhibitor of NQO1 in intact cells (1000-fold), and ES936 did not inhibit P450R or b5R [25]. Dicoumarol can inhibit other enzymes including b5R [35] and P450R [36] and may stimulate xanthine dehydrogenase [37]. Pretreatment of the BE-NQ7 cells with ES936 resulted in protection from toxicity induced by streptonigrin, RH1, and ES921 (Fig. 2). At the concentration chosen for the pretreatment protocol, ES936 did not induce toxicity in BE or BE-NQ7 cells and repressed NQO1 activity by 98% for the drug treatment period (data not shown). In colony-forming assays, ES936 pretreatment abrogated the cytotoxicity induced by RH1 in BE-NQ7 cells. These data provide clear evidence that NQO1 bioactivates these antitumor quinones in this system.

3.4. Relationship between NQO1 level and toxicity

Using IC_{50} values generated from MTT assays, streptonigrin, RH1, and ES921 demonstrated very high selectivity and were evaluated further for toxic response in a panel of BE-NQ cell lines expressing a range of NQO1 activities. Toxicity as growth inhibition was measured by the MTT assay, and IC_{50} values were calculated to express toxicity (Fig. 2). The IC_{50} value for the BE-NQ2 cell line, which expressed low NQO1 activity (23 nmol/min/mg), was not significantly different from that of the BE parent cell line for streptonigrin, RH1, or ES921. This implied that the level of expression in these cells was insufficient to activate these compounds and that a threshold

Table 2
Summary of IC_{50} values and selectivity ratios for BE and BE-NQ7 cells

Compound	Rate of compound metabolism by NQO1[a] [μmol/min/mg]	IC_{50} [μM]		SR[b]
		BE	BE-NQ7	
ES936	ND	0.13 ± 0.01	0.16 ± 0.03	
ES1352	2.22 ± 0.41	88 ± 31	41 ± 7*	2
ES923	2.01 ± 0.43	22 ± 20	2.0 ± 1.1*	17
ES921	3.35 ± 0.65	13 ± 3	0.56 ± 0.05**	23
AZQ	5.2 ± 0.3	29 ± 5.7	11 ± 2*	3
EO9	7.7 ± 2.0	1.00 ± 0.2	0.23 ± 0.03***	4
Streptonigrin	51 ± 4	3.5 ± 0.7	0.081 ± 0.006***	43

Growth inhibition of a group of known and potential antitumor quinones was tested by MTT assay in BE and BE-NQ7 cells. The level of drug required to achieve 50% growth inhibition (IC_{50}) after 96 hr of treatment is expressed as mean concentration ± SD of a minimum of three determinations. The selectivity ratio (SR) was calculated as (IC_{50} BE)/(IC_{50} BE-NQ7).

[a] The rate of metabolism by human NQO1 is included for comparison and noted as μmol NADH oxidized/min/mg of purified recombinant human NQO1 as previously reported [32,33]. ND, not detectable.

[b] MMC and RH1 were 3- and 17-fold more selective, respectively, to BE-NQ7 cells, as reported previously [22].

*–*** Significantly lower than BE value: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (Student's *t*-test).

level above 23 nmol/min/mg for NQO1 was required to observe selective toxicity in this system. In the four cell lines with greater NQO1 activities (77–433 nmol/min/mg), an interesting pattern of response was observed. Toxicity in these cell lines treated with streptonigrin, RH1, and ES921 was significantly increased compared with BE cells but significant differences among the four lines were not observed (ANOVA, Scheffe F test for multiple comparisons, $P < 0.05$). These data suggest that a maximal toxic response was reached for these compounds and that the dose–response curve for NQO1 level and toxicity was steep (between 23 and 77 nmol/min/mg).

4. Discussion

It is generally believed that both one- and two-electron reduction can activate MMC and other antitumor quinones. Bioactivation is dependent upon the levels of these enzymes in the cell, and competition of the various reductases for quinone substrate may occur. This illustrates the importance of determining bioactivation in a system where the enzyme of interest is varied in an independent fashion. To this end, we have established a series of cell lines derived from BE human colon adenocarcinoma cells, which have no NQO1 activity due to a polymorphism in the NQO1 gene [20,21]. BE cells were stably transfected with wild-type human NQO1 cDNA and express functional NQO1 protein. Previously, the BE-NQ7 cell line was found to be more susceptible than the parent cell line to MMC, MeDZQ, and RH1 [22]. In the present study, these experiments were extended to demonstrate that the transfected cells were also susceptible to other known antitumor quinones (streptonigrin, EO9, and AZQ). Newer compounds that were selective for BE-NQ7 cells included the indolequinones, ES921 and ES923, and a mitosene, ES1352. Importantly, there was no difference in cytotoxicity between BE and BE-NQ7 cells for ES936, a recently characterized inhibitor of NQO1. In the present study, we have confirmed the importance of NQO1

Table 3
Colony-forming assay for RH1 in BE and BE-NQ7 cells

Cell line	ES936 pretreatment	C10 [nM]	
		RH1	MMC
BE	−	26.9 ± 2.5	2280 ± 320
	+	30.3 ± 2.4	
BE-NQ7	−	3.4 ± 0.8*	757 ± 100*
	+	20.9 ± 2.3**	

The cytotoxicity of RH1 was tested by the colony-forming assay in BE and BE-NQ7 cells. Cytotoxicity is expressed as C10, which is defined as the concentration at which 10% of the cells remain as viable colonies compared with vehicle-treated controls. Values represent means ± SD of three determinations. Significance was determined by ANOVA, Scheffe F-test for multiple comparisons.

* Significantly different ($P < 0.05$) from BE (−) control.

** Significantly different ($P < 0.05$) from untreataed (−) BE-NQ7 cells.

Table 4
DNA cross-linking in colon carcinoma cells treated with RH1

RH1 [nM]	% Cross-links	
	BE	BE-NQ7
0	ND[a]	ND
5	ND	24.3 ± 7.0*,**
10	ND	29.6 ± 4.2*,**
50	26.1 ± 6.4*	54.2 ± 3.0*,**
100	34.9 ± 6.2*	72.3 ± 5.1*,**

DNA cross-linking was determined by the comet assay after a 2-hr drug exposure. Cross-linking is defined as the percent of DNA in the "tail" relative to vehicle-treated controls. Values represent mean percent cross-links ± SD of three determinations. Significance was determined by ANOVA, Scheffe F-test for multiple comparisons.

[a] ND, not detected.

* Significantly different ($P < 0.05$) from vehicle-treated control.

** Significantly different ($P < 0.05$) from BE cells at an equimolar dose.

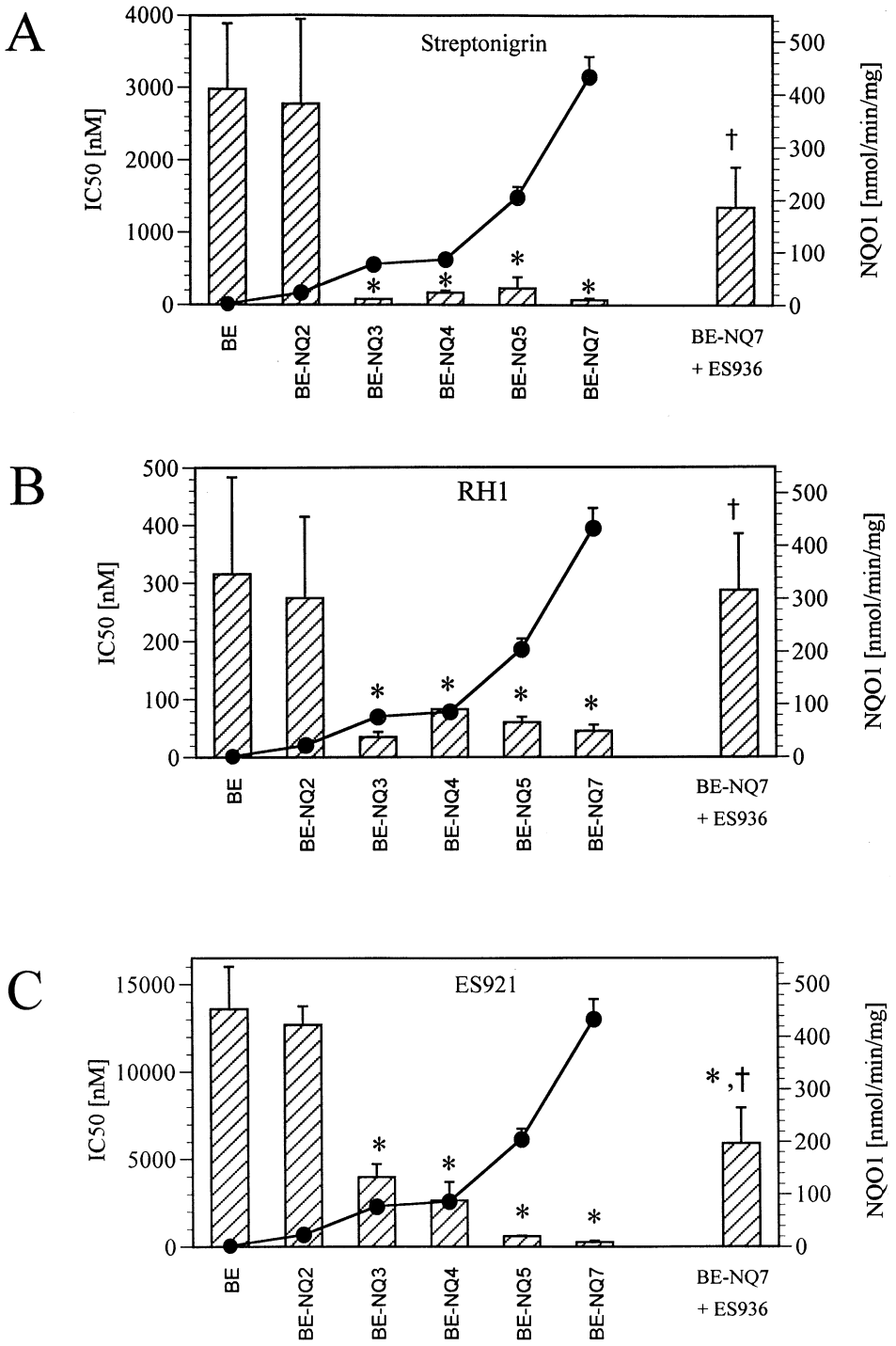

Fig. 2. Comparison of growth inhibition induced by streptonigrin (A), RH1 (B), and ES921 (C) and NQO1 activity levels in a series of BE-NQ cell lines. Cell viability was measured in BE-NQ cells 96 hr post-treatment with streptonigrin, RH1, or ES921. Growth inhibition is expressed as IC_{50} (the drug concentration resulting in 50% growth inhibition). The IC_{50} values (hatched bars) were calculated from semilog plots of percent viability versus drug concentration and are presented as the means ± SD of a minimum of three independent experiments. NQO1 activity levels (λ) represent the means ± SEM of activity measurements collected throughout the length of the experiment. Key: (*) statistically different ($P < 0.05$) from the BE parent cell line, and (†) BE-NQ7 cells statistically different ($P < 0.05$) from ES936-pretreated BE-NQ7 (ANOVA, Scheffe F-test for multiple comparisons).

in bioreductive activation by utilizing ES936, an irreversible inhibitor of NQO1. BE-NQ7 cells were protected from toxicity induced by streptonigrin, RH1, and ES921 after preincubation with ES936 to inhibit NQO1. Demonstration of significant DNA cross-linking at very low drug concentrations and exposure time implicates reductive activation by NQO1 as a key toxic event in this system. Taken together, these data point to the direct involvement of NQO1

in the activation of antitumor quinones in the transfected BE cell system.

There have been a number of attempts to identify key characteristics of antitumor quinones that are required for metabolic activation, but defining a single variable that ultimately leads to a potential clinical agent has remained elusive. Chemical reduction potential and *in vitro* substrate specificity do not correlate for naphthoquinones [38], aziridinylbenzoquinones [39], or indolequinones [23,24]. The presence of an aziridine group is consistently desirable in eliciting a toxic response [23,24,40,41]. Comparison of the SR for ES1352 and ES923 in the present study indicated that the fused ring structure of ES1352 was not advantageous for NQO1 activation in BE-NQ7 cells. This is in agreement with results in a similar study comparing ES923 and a fused ring system analog of ES1352 [24]. Differences in metabolic potential and ultimate toxicity are more likely influenced by the toxic metabolite that is generated and other microenvironmental factors such as pH and oxygen tension.

Interesting patterns of response were observed in comparing the toxicity of streptonigrin, RH1, and ES921 with the level of NQO1 in the BE-NQ cell panel. In five transfected cell lines that expressed different levels of NQO1, the toxicity of streptonigrin, ES921, and RH1 demonstrated that a threshold level of NQO1 activity above 23 nmol/min/mg was required to observe significant toxicity in this system. Streptonigrin and RH1 were maximally toxic to BE-NQ cells expressing 77 nmol/min/mg and higher, suggesting saturation of the toxic effect. Similar trends are reported in other transfection models [29,42]. In a series of Chinese hamster ovarian cell lines that were stably transfected with human NQO1 cDNA, streptonigrin and MeDZQ were not toxic to cells expressing 19 nmol/min/mg and were maximally toxic to cells expressing between 256 and 3527 nmol/min/mg [29]. Mikami and co-workers [42] transfected human NQO1 cDNA into St-4, a human stomach carcinoma cell line, which is deficient in NQO1 activity, to generate a panel of five cell lines expressing NQO1 activity between 144 and 1420 nmol/min/mg. Examining the susceptibility of these cells to MMC demonstrated a similar saturation of toxicity at NQO1 levels greater than 144 nmol/min/mg [42]. This behavior extends to panels of tissue-matched cell lines that constitutively express a range of NQO1 activities. Studies correlating susceptibility to MMC and MeDZQ with NQO1 activity demonstrate similar saturation results for stomach, colon, and lung cancer cell lines [33,42]. The cause of this saturation may be attributed to cellular uptake or metabolism but may also be related to a specific toxic lesion. In support of this, Mikami and co-workers [42] discovered that maximal toxicity in St-4 cells transfected with NQO1 was accompanied by saturation of MMC–DNA adducts.

It remains a possibility that NQO1 influences other pathways in cells such as SAPK/JNK and NF-κB-related systems [43]. In addition, aziridinylbenzoquinones are known to induce p21 [44,45], which could also play an important role in the cytotoxic responses to the antitumor quinones observed in this work. Another interesting observation is the fact that quinone compounds increase expression of the pro-apoptotic protein Bad [46]. Antitumor quinones such as mitomycin C are known to preferentially induce apoptosis in human tumor cells containing elevated NQO1 levels [47], suggesting additional molecular mechanisms that may modulate the activity of compounds such as RH1.

In summary, our results provide evidence that NQO1 is responsible for bioactivation of antitumor quinones in human tumor cells stably transfected with wild-type human NQO1. In addition, our results suggest that a threshold level of NQO1 is required to observe significant cytotoxicity and that above this threshold a maximally toxic response occurs. Future studies on the nature of damage caused by NQO1-directed antitumor agents will be necessary to fully understand the complex relationship between NQO1 levels and ultimate toxicity.

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