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ORIGINAL ARTICLE

Role of NADPH cytochrome P450 reductase in activation of RH1

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Abstract
Purpose RH1 is a new bioreductive agent that is an excellent substrate for the two-electron reducing enzyme, NAD(P)H quinone oxidoreductase 1 (NQO1). RH1 may be an effective NQO1-directed antitumor agent for treatment of cancer cells having elevated NQO1 activity. As some studies have indicated that RH1 may also be a substrate for the one-electron reducing enzyme, NADPH cytochrome P450 reductase (P450 Red), P450 Red may contribute to the activation of RH1 where NQO1 activities are low and P450 Red activities are high. The mean P450 Red activity in the human tumor cell line panel used by NCI for evaluation of new anticancer agents is 14.8 nmol min^{-1} mg $prot^{-1}$, while the mean NQO1 activity in these cell lines is 199.5 nmol min^{-1} mg $prot^{-1}$. Thus, we investigated whether P450 Red could play a role in activating RH1.
Methods Reduction of RH1 by purified human P450 Red was investigated using electron paramagnetic resonance and spectroscopic assays. The ability of RH1 to produce DNA damage following reduction by P450 Red was studied using gel assays. To determine the role of P450 Red in activation of RH1 in cells, cell growth inhibition studies with inhibitors of P450 Red and NQO1 were carried out in T47D human breast cancer cells and T47D cells transfected with the human P450 Red gene (T47D-P450) that have P450 Red activities of 11.5 and 311.8 nmol min^{-1} mg $prot^{-1}$, respectively.
Results Reduction studies using purified P450 Red and NQO1 confirmed that RH1 can be reduced by both enzymes, but redox cycling was slower following reduction by NQO1. RH1 produced DNA strand breaks and crosslinks in isolated DNA after reduction by either P450 Red or NQO1. DPIC, an inhibitor of P450 Red, had no effect on cell growth inhibition by RH1 in T47D cells, and had only a small effect on cell growth inhibition by RH1 in the presence of the NQO1 inhibitor, dicoumarol, in T47D-P450 cells.
Conclusions These results demonstrated that P450 Red does not contribute to the activation of RH1 in cells with normal P450 Red activity and plays only a minor role in activating this agent in cells with high levels of this enzyme. These studies confirmed that P450 Red could activate RH1 and provided the first direct evidence that RH1 could produce both DNA strand breaks and DNA crosslinks after reduction by P450 Red. However, the results strongly suggest that P450 Red does not play a significant role in

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activating RH1 in cells with normal P450 Red activity.

Keywords RH1 · NADPH cytochrome P450 reductase · Reduction · Activation

Abbreviations

RH1	2,5-Diaziridinyl-3-(hydroxymethyl) -6-methyl-1,4-benzoquinone
P450 Red	NADPH cytochrome P450 reductase
NQO1	NAD(P)H quinone oxidoreductase 1
T47D-P450	T47D cells transfected with the human NADPH cytochrome P450 reductase gene
EPR	Electron paramagnetic resonance
DPIC	Diphenyliodonium chloride
IC_{50}	Concentration of drug that reduced the relative absorbance fraction to 0.5

Introduction

The bioreductive drug, 2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone (RH1) has demonstrated significant antitumor activity in vitro [31, 37] and in vivo [7, 16]. Bioreductive agents are a class of anticancer drugs that require activation by reductive enzymes like the one-electron reducing enzyme, NADPH cytochrome P450 reductase (P450 Red) (EC 1.6.2.4) [23, 28] or the two-electron reducing enzyme NAD(P)H quinone oxidoreductase 1 (NQO1; DT-diaphorase) (EC 1.6.99.2) [27, 29]. The relative contribution of these enzymes to the reductive activation of bioreductive agents is dependent on the drug, enzyme, oxygen level and pH [2, 13, 18, 27, 32]. RH1 is a very efficient substrate for NQO1 [37] and reduction by NQO1 results in activation of the aziridine groups and subsequent DNA alkylation with interstrand cross-links formed preferentially at 5′ GCC 3′ sequences [4, 7]. Because RH1 is a very good substrate for NQO1, this agent was considered ideal for use in an enzyme-directed tumor targeting strategy to treat tumors, like non-small cell lung cancer, that have high NQO1 activity [17]. RH1 is currently in a clinical trial [7].

Although RH1 is an excellent substrate for NQO1 and this enzyme plays an important role in activating this agent [37, 38], Cenas [6, 20] showed that RH1 could be a substrate for both NQO1 and P450 Red (Fig. 1). However, Kim [15] found no increase in RH1 activity in MDA-MB231 cells transfected with P450 Red compared with parent cells. MDA-MB231 cells have no NQO1 activity due to an inactivating NQO1 mutation. Tudor [36] studied the toxicity of RH1 in the NCI's 60 tumor cell line panel and found that although the sensitivity of RH1 was marked in cell lines expressing NQO1, there was no correlation between RH1 cytotoxicity and NQO1 activity. They also found a high sensitivity to RH1 in leukemia and lymphoma cell lines with low or absent NQO1 expression. While studies suggest that NQO1 is the most important activating enzyme for RH1, there is evidence that P450 Red can activate this bioreductive antitumor agent. Thus, it is possible that P450 Red may contribute to the activation of RH1 in circumstances where NQO1 activities are low and P450 Red activities are high. P450 Red activities in the human tumor cell line panel used by NCI for evaluation of new anticancer agents range from approximately 2.5–63 nmol min^{-1} mg $prot^{-1}$ with a mean of 14.8 nmol min^{-1} mg $prot^{-1}$, while NQO1 activities in these cell lines range from approximately 0–5,000 nmol min^{-1} mg $prot^{-1}$ with a mean of 199.5 nmol min^{-1}mg $prot^{-1}$ [9]. Furthermore, DNA damage produced by RH1 following reduction by P450 Red has not been studied in detail. In this study we investigated the reduction of RH1 by P450 Red and its role in the DNA damaging activities and cell growth inhibition of RH1.

Materials and methods

Materials

RH1 was obtained from Professor David Ross (University of Colorado Health Sciences Center, Denver, CO, USA). Streptonigin was obtained from Sigma (St. Louis, MO, USA) and tirapazamine was prepared as described previously [30]. T47D human breast cancer cells and T47D cells transfected with the human P450 Red gene, (T47D-P450) were obtained from Professor Ian Stratford (University of Manchester, Manchester, UK). Both cell lines were grown in 10% FBS in Hams F12:DMEM 1:1 media and the T47D-P450 cells were grown in the presence of puromycin. Purified human P450 Red was purchased from BD Biosciences (San Jose, CA, USA), pBR322 plasmid DNA was from New England Biolabs (Pickering, ON, Canada) and human NQO1 was provided by Dr. Sushu Pan (University of Pittsburgh, Pittsburgh, PA, USA).

Electron paramagnetic resonance (EPR) studies

A freshly prepared 15:l aliquot of the drug in the reaction mixture (10 mM RH1, 2 mM NADPH,

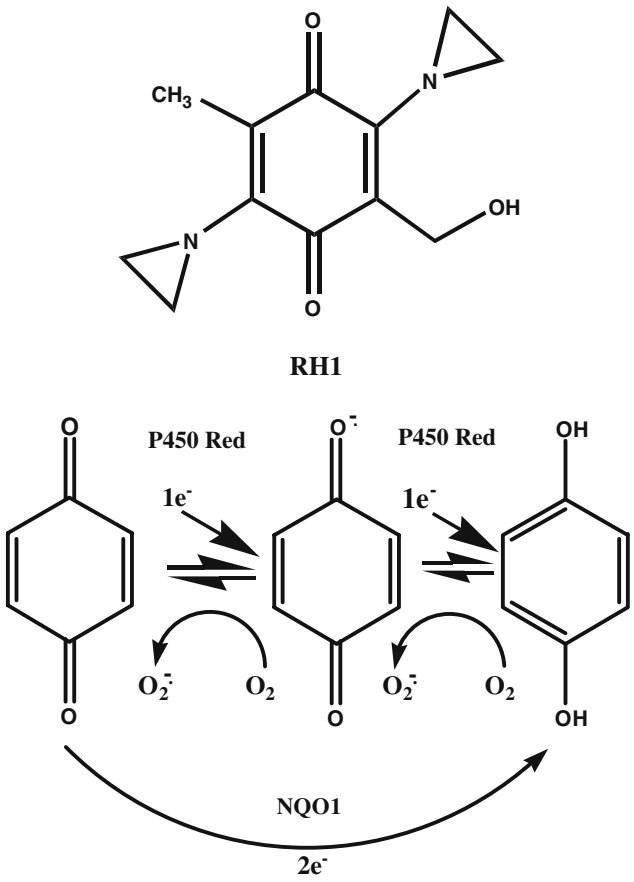

Fig. 1 Structure and reduction pathways for RH1

16 μg prot ml^{-1} P450 Red in 50 mM Tris buffer at pH 7.4 containing 10% DMSO for P450 Red; or 1 mM RH1, 97:M FAD, 0.4 mM NADH, 0.0005% Tween 20, 1.1 g prot ml^{-1} NQO1 in 0.5 mM Tris buffer at pH 7.4 containing 1% DMSO for NQO1) was injected into an 8 cm length of gas-permeable Teflon tubing (Zeus Industrial Products, Raritan, NJ, USA) which was then folded at both ends, inserted into a quartz EPR tube open at both ends and placed in the EPR cavity as previously described [1, 12]. The EPR spectra were recorded with a Bruker (Milton, Canada) EMX EPR spectrometer. Pre-purified grade thermostated (37°C) argon (400 l h^{-1}) or air as indicated was flowed continuously over the sample while the spectra were recorded. Recording of the first-derivative EPR spectra was started approximately 2 min after the sample was prepared. A total of 10 spectra (42 s/scan) were recorded over 7 min and their signals were averaged. For recording of the spectra the instrument settings were: microwave power 20 mW, modulation frequency 100 kHz, microwave frequency 9.24 GHz, modulation amplitude 2.0 G, time constant 0.02 s, gain 20,000, 1,024 data points/scan, magnetic field centered at 3315 G, and a 50 G scan range.

Reduction studies

Buffers (0.05 M phosphate pH 7.8 for P450 Red or 0.05 M Tris pH 7.4 for NQO1) were pre-incubated in rubber septum-sealed Starna cuvettes (Starna Cells, Atascadero, CA, USA) with 100% nitrogen or air bubbled into the buffer via 2′′ 20 gauge needles for 2 h. RH1 (50 μM final concentration), NADPH (200 μM final concentration) and enzyme (1.2 μg prot ml^{-1} final concentration for P450 Red or 0.22 μg prot ml^{-1} final concentration for NQO1) were added via a gas-tight syringe and the cuvettes were transferred to the spectrophotometer. Reductions were monitored using the scanning kinetics module in a Varian Cary 1 (Mississauga, ON, Canada) spectrophotometer and kinetics continuums at 370 nm were calculated [20].

DNA strand breaks

Reaction mixtures in buffers (0.05 M phosphate pH 7.8 for P450 Red or 0.05 M Tris pH 7.4 for NQO1) were RH1 (50 μM), NADPH (4 mM), pBR322 plasmid DNA and enzyme (4.8 μg prot ml^{-1} for P450 Red or 0.88 μg prot ml^{-1} for NQO1). Following a 2 h incubation in air, the reactions were terminated by addition of 1 mM diphenyliodonium chloride (DPIC) (to inhibit P450 Red) or 10 μM dicoumarol (to inhibit NQO1). DNA strand breaks were determined using a modification of a gel-based assay we have used previously [10]. Briefly, following isolation of the DNA by centrifugation, the DNA pellets were dissolved in 15 μl loading dye (30% sucrose in 10 mM Tris–HCl (pH 8)-1 mM EDTA) and were then run on a 1% agarose gel with no ethidium bromide for 45 min at 75 V. DNA strand breaks are presented as the amount of relaxed DNA as a percent of the total DNA. All values were corrected for a background level of strand breaks in the plasmid DNA of approximately 15%. Control experiments containing RH1 but no P450 Red or NQO1 produced no DNA stand breaks above the background.

Similar experiments were carried out under hypoxic conditions in a hypoxia chamber (Forma Scientific Anaerobic System, Model 1025, Fisher Scientific, Winnipeg, Canada) maintained under 95% nitrogen:5% carbon dioxide. Reaction buffers were incubated in the hypoxia chamber for 5 days prior to the initiation of the reaction. All other reagents were incubated in the hypoxia chamber for 0.5 h prior to initiation of the reaction. Following 2 h incubation in the hypoxia chamber, the reactions were terminated by addition of 1 mM DPIC (to inhibit P450 Red) or 10 μM dicoumarol (to inhibit NQO1). DNA strand break formation was analyzed as described above.

DNA crosslinking

Reaction mixtures in cell free buffers (0.05 M phosphate pH 7.8 for P450 Red or 0.05 M Tris pH 7.4 for NQO1) were RH1 (100 μM), NADPH (400 μM), linearized ^{32}P end-labeled pBR322 DNA and enzyme (1.2 μg prot ml^{-1} for P450 Red or 0.22 μg prot ml^{-1} for NQO1). Buffers were gassed with 100% nitrogen for 1 h prior to addition of the other reaction mixture components. Following 45 min incubation under hypoxic conditions, the reactions were terminated by addition of 1 mM DPIC (to inhibit P450 Red) or 10 μM dicoumarol (to inhibit NQO1). DNA crosslinks were determined using a modification of a gel-based assay we have used previously [10]. Briefly, following isolation of the DNA by centrifugation, the DNA pellets were dissolved in 20 μl strand separation loading dye (35% DMSO, 1 mM Na_2EDTA pH 7, 10 mM Tris pH 7, 0.05% bromophenol blue, 0.05% xylene cyanol). DNA strand separation consisted of incubation of the DNA in a 70°C water bath for 3 min, followed by immediate cooling in an ice-water bath for 30 min. The DNA was then run on a 1% agarose gel with no ethidium bromide for 45 min at 75 V. DNA crosslinks are presented as the amount of double stranded DNA as a percent of total DNA. Control experiments containing RH1 but no P450 Red or NQO1 produced no DNA crosslinks.

Enzymatic assays

P450 Red activities in cells were determined in cell sonicates using a standard method [33] with the addition of potassium cyanide [25]. P450 Red activity is reported as nmol cytochrome c reduced per minute per milligram of protein. NQO1 activities in cells were determined in cell sonicates as previously described [3]. NQO1 activity is reported as nmol 2,6-dichlorophenolindophenol reduced per minute per milligram of protein.

Cell growth inhibition

Cells under aerobic conditions were treated with buffer, 0.1 mM DPIC alone, 0.2 mM dicoumarol alone or 0.1 mM DPIC and 0.2 mM dicoumarol for 1 h and then with varying concentrations of RH1, streptonigrin or tirapazamine for an additional 1 h in the presence of the inhibitors. Cell growth inhibition was determined by MTT assay [14] and is presented as the relative absorbance calculated from the ratio of the absorbance from drug treated cells to absorbance from untreated cells. IC_{50} values were defined as the concentration of drug that reduced the relative absorbance to 0.5 as determined from the linear regression lines of RH1 dose versus the logarithm of the relative absorbance curves. The cell growth inhibition activities were compared statistically using a t test comparing the significance of the difference of the slopes of the dose versus the logarithm of the relative absorbance curves.

Results

EPR studies

Formation of RH1 semiquinone free radical following reduction by P450 Red or NQO1 was monitored by EPR. Reduction of RH1 by P450 Red under hypoxic conditions resulted in a semiquinone free radical EPR signal at $g \sim 2.0$ due to the RH1 semiquinone (Fig. 2a),

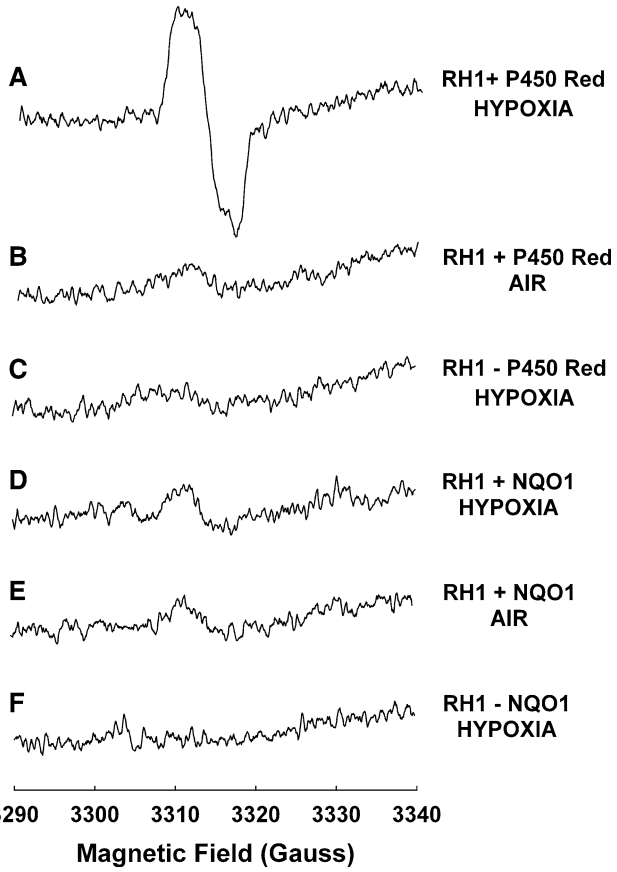

Fig. 2 EPR spectra following reduction of RH1 by P450 Red or NQO1. A freshly prepared aliquot of RH1 in the reaction mixture containing P450 Red or NQO1 was injected into an 8 cm length of gas-permeable Teflon which was then folded at both ends, inserted into a quartz EPR tube open at both ends and placed in the EPR cavity. The EPR spectra were recorded with a Bruker EMX EPR spectrometer. Argon or air as indicated was flowed continuously over the sample while the spectra were recorded. Recording of the first-derivative EPR spectra was started approximately 2 min after the sample was prepared. A total of 10 spectra (42 s/scan) were recorded over 7 min and their signals were averaged

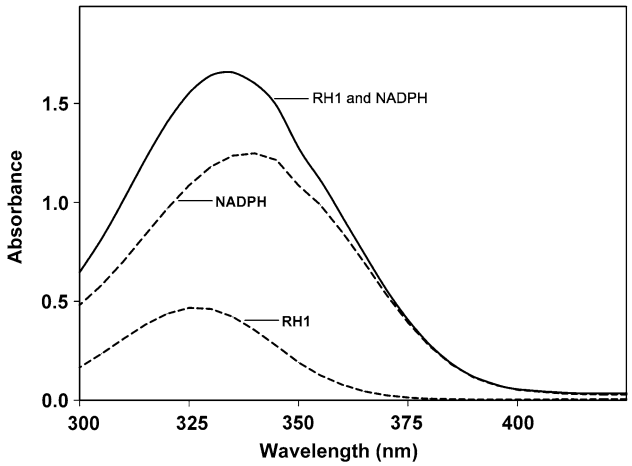

Fig. 3 UV/Visible spectra of RH1 and NADPH. Spectra of RH1 (50 μM), NADPH (200 μM) or a mixture of RH1 (50 μM) and NADPH (200 μM) in buffer were obtained using a Varian Cary 1 spectrophotometer

which decreased to a very weak signal when air was introduced into the reaction tube (Fig. 2b). Reduction of RH1 by NQO1 under hypoxic conditions produced a small EPR signal characteristic of the RH1 semiquinone free radical (Fig. 2d). As shown in Fig. 2e this weak semiquinone signal was decreased only slightly after the introduction of air. In control experiments, no significant EPR semiquinone signals were seen under hypoxia with RH1 in the absence of P450 Red or NQO1 (Fig. 2c, f).

Reduction of RH1 by P450 Red and NQO1

Reduction of RH1 by P450 Red or NQO1 under hypoxic and aerobic conditions was monitored by spectroscopic scans at various times. RH1 has an absorbance maximum at 326 nm while NADPH, which was used as the electron donor for P450 Red and NQO1, has an absorbance maximum at 340 nm. In all experiments 4 equivalents of NADPH were used for each equivalent of RH1. The starting reaction mixture showed a single peak with a maximum at 331 nm resulting from absorbance by both RH1 and NADPH. Reduction was monitored by following the loss of absorbance at 370 nm, where approximately 90% of the absorbance is due to NADPH (Fig. 3). Loss of NADPH was determined from the loss of absorbance after correction for RH1 and background absorbance.

For reduction by P450 Red under hypoxic conditions (Fig. 4a), there was a loss of approximately one equivalent of NADPH in the first 4 min with little further loss of NADPH after that time. In contrast, under aerobic conditions there was a loss of >90% of the NADPH by 20 min.

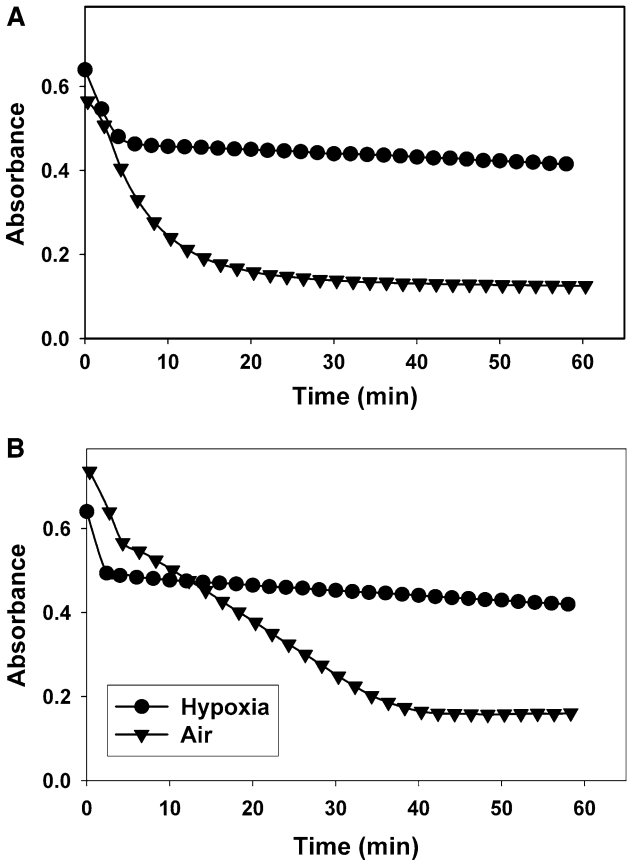

Fig. 4 Reduction of RH1 by P450 Red and NQO1. **a** For reduction by P450 Red, 0.05 M phosphate buffer pH 7.8 was pre-incubated in rubber septum-sealed Starna cuvettes with 100% nitrogen or air bubbled into the buffer via 2′′ 20 gauge needles for 2 h. RH1 (50 μM), NADPH (200 μM) and P450 Red (1.2 μg prot ml^{-1}) were added via a gas-tight syringe and the cuvettes were transferred to the spectrophotometer. **b** For reduction by NQO1 0.05 M Tris buffer pH 7.4 was pre-incubated in rubber septum-sealed Starna cuvettes with 100% nitrogen or air bubbled into the buffer via 2′′ 20 gauge needles for 2 h. RH1 (50 μM), NADPH (200 μM) and NQO1 (0.22 μg prot ml^{-1}) were added via a gas-tight syringe and the cuvettes were transferred to the spectrophotometer. Reductions were monitored using the scanning kinetics module in a Varian Cary 1 spectrophotometer and kinetics continuums at 370 nm were calculated

For reduction by NQO1 (Fig. 4b) under hypoxic conditions there was a loss of approximately one equivalent of NADPH in the first 2 min with little further loss of NADPH after that time. In contrast, under aerobic conditions there was a loss of approximately one equivalent of NADPH in the first 2 min, and this was followed by a slower loss of NADPH to <10% of the original level over an additional 40 min.

DNA strand break and DNA crosslink formation

To measure the formation of DNA strand breaks by RH1 following reduction by P450 Red or NQO1, RH1,

NADPH and enzyme were incubated in air or under hypoxia in the presence of pBR322 plasmid DNA for 2 h. DNA strand breaks were measured by a gel assay [10]. Reduction of RH1 by P450 Red in air resulted in 52.8 ± 9.2% of the DNA having strand breaks, and reduction by NQO1 in air resulted in 68.1 ± 5.9% of the DNA having strand breaks (Table 1). RH1 did not produce any DNA strand breaks in the absence of P450 Red or NQO1. Under hypoxia, reduction of RH1 by P450 Red resulted in a significantly lower level of DNA strand breaks compared with reduction by P450 Red in air, 25.3 ± 3.6% ($P < 0.008$), and reduction by NQO1 under hypoxia also resulted in a significantly lower level of DNA strand breaks compared with reduction by NQO1 in air, 26.4 ± 4.9% ($P < 0.001$) (Table 1).

To measure the formation of DNA crosslinks by RH1 following reduction by P450 Red or NQO1 RH1, NADPH and enzyme were incubated under hypoxia in the presence of linearized pBR322 plasmid DNA for 45 min. DNA crosslinks were measured by a gel assay [10]. Reduction of RH1 by P450 Red resulted in 22.9 ± 8.3% of the DNA having crosslinks, and reduction by NQO1 resulted in 30.2 ± 6.5% of the DNA having crosslinks (Table 1). RH1 did not produce any DNA crosslinks in the absence of P450 Red or NQO1.

Table 1 DNA strand breaks and crosslinks produced by RH1 after reduction by P450 Red or NQO1

	P450 Red	NQO1
Air		
% DNA strand breaks (*N*)	52.8 ± 9.2 (9)	68.1 ± 5.9 (7)
Hypoxia		
% DNA strand breaks (*N*)	25.3 ± 3.6 (11)[a]	26.4 ± 4.9 (10)[b]
Hypoxia		
% DNA crosslinks (*N*)	22.9 ± 8.4 (8)	30.2 ± 6.5 (12)

P450 Red or NQO1, RH1, NADPH and enzyme were incubated in air or under hypoxia in the presence of pBR322 plasmid DNA for 2 h for measurement of DNA strand breaks. P450 Red or NQO1, RH1, NADPH and enzyme were incubated under hypoxia in the presence of linearized pBR322 plasmid DNA for 45 min for measurement of DNA crosslinks. DNA strand breaks and crosslinks were measured by gel assays [10]. The values represent the mean ± SE

N, number of determinations

[a] Significantly different from % DNA strand breaks after reduction by P450 Red in air ($P < 0.008$)

[b] Significantly different from % DNA strand breaks after reduction by NQO1 in air ($P < 0.001$)

Effect of inhibitors on P450 Red and NQO1 activities in T47D and T47D-P450 cells

To determine the effect of DPIC on P450 Red and NQO1 activity, T47D, human breast cancer cells, or T47D-P450 cells (T47D cells stably transfected with the human P450 Red gene) were incubated without, or with, 0.1 mM DPIC for 1 or 2 h and P450 Red and NQO1 activities were measured. The level of P450 Red activity was 11.5 ± 1.5 nmol min^{-1} mg $prot^{-1}$ in T47D cells and 311.8 ± 8.7 nmol min^{-1} mg $prot^{-1}$ in T47D-P450 cells (Fig. 5). Incubation with DPIC for 1 h significantly decreased the levels of P450 Red activity to 0.7 ± 0.2 nmol min^{-1} mg $prot^{-1}$ in T47D cells and 19.8 ± 3.1 nmol min^{-1} mg $prot^{-1}$ in T47D-P450 cells, and these P450 Red activities were similar when the cells were incubated with DPIC for 2 h (Fig. 5). NQO1 activities in T47D and T47D-P450 cells were 80.1 ± 29.4 and 66.8 ± 13.0 nmol min^{-1} mg $prot^{-1}$, respectively. DPIC did not significantly inhibit NQO1 activity in either cell line.

To determine the effect of dicoumarol on P450 Red and NQO1 activity, T47D, human breast cancer cells

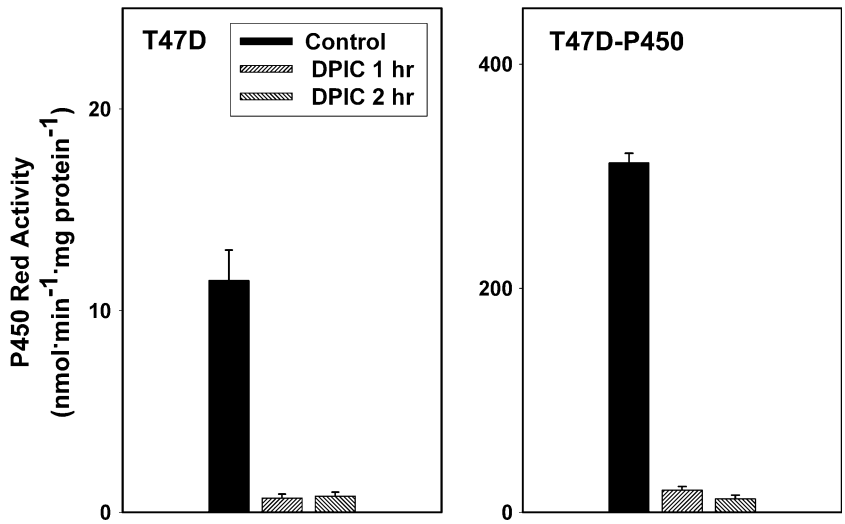

Fig. 5 Inhibition of P450 Red activity by DPIC in T47D and T47D-P450 cells. T47D or T47D-P450 cells were incubated without DPIC, or with 0.1 mM DPIC for 1 or 2 h. P450 Red activity was measured [25, 33] and is reported as nmol cytochrome c reduced per min per milligram of protein. The *bars* represent the mean ± SE of three or four determinations

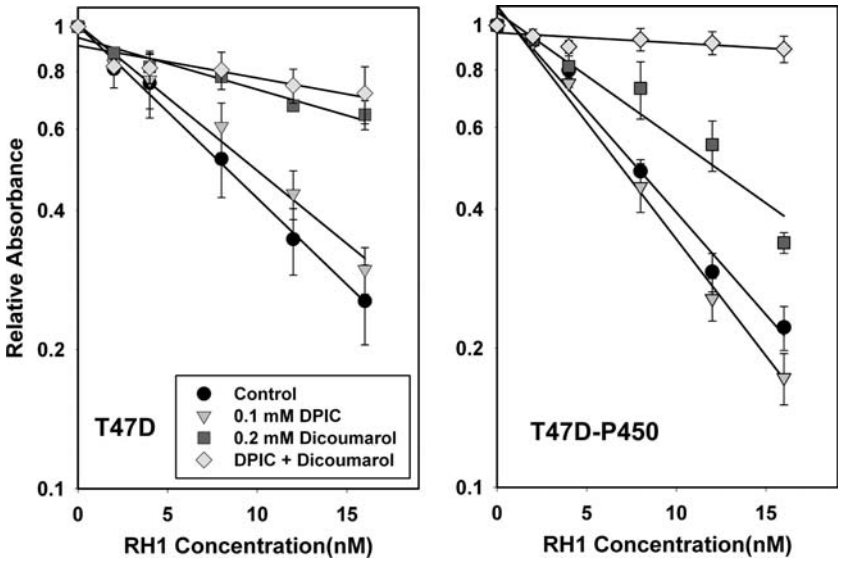

Fig. 6 Inhibition of cell growth inhibitory activity of RH1 in T47D and T47D-P450 cells. T47D or T47D-P450 cells were incubated with buffer, 0.1 mM DPIC alone, 0.2 mM dicoumarol alone or 0.1 mM DPIC and 0.2 mM dicoumarol for 1 h and then with varying concentrations of RH1 for an additional 1 h in the presence of the inhibitors. Cell growth inhibition was determined by MTT assay [14] and is presented as relative absorbance. *Points* mean ± SE of four or five determinations; *lines* linear regression lines

or T47D-P450 were incubated without, or with, 0.2 mM dicoumarol for 1 h and then with 1 mM or 100 μM tirapazamine, respectively, or 30 or 15 nM streptonigrin, respectively, for an additional 1 h in the presence of the inhibitor. Cell growth inhibition was determined by MTT assay. The relative absorbance with 1 mM tirapazamine in T47D cells was 0.73 ± 0.05 in the absence of dicoumarol and 0.65 ± 0.02 in the presence of dicoumarol and these values were not significantly different. The relative absorbance with 100 μM tirapazamine in T47D-P450 cells was 0.69 ± 0.06 in the absence of dicoumarol and 0.49 ± 0.09 in the presence of dicoumarol and these values were not significantly different. In contrast, the relative absorbance with 30 nM streptonigrin in T47D cells was 0.44 ± 0.047 in the absence of dicoumarol and 0.96 ± 0.03 in the presence of dicoumarol and these values were significantly different ($P < 0.001$). The relative absorbance with 15 nM streptonigrin in T47D-P450 cells was 0.25 ± 0.06 in the absence of dicoumarol and 0.63 ± 0.03 in the presence of dicoumarol and these values were significantly different ($P < 0.001$). These results represent the mean ± SE of 3–6 determinations.

Cell growth inhibition by RH1

T47D or T47D-P450 cells under aerobic conditions were treated with buffer, 0.1 mM DPIC alone, 0.2 mM dicoumarol alone or 0.1 mM DPIC and 0.2 mM dicoumarol for 1 h and then with varying concentrations of RH1 for an additional hour in the presence of the inhibitors. Cell growth inhibition was determined by MTT assay [14]. RH1 was highly growth inhibitory in T47D and T47D-P450 cells (Fig. 6) with IC_{50} values of 8.3 ± 1.7 and 7.7 ± 0.6 nM, respectively (Table 2), that were not statistically different. Incubation of the cells with DPIC alone did not significantly effect RH1 cell growth inhibition in either cell line. Incubation of the cells with dicoumarol alone significantly decreased the growth inhibition activity of RH1 in both cell lines ($P < 0.05$), but the effect was greater in the T47D cells. The IC_{50} values in the presence of dicoumarol were 25.4 ± 2.7 and 11.9 ± 1.1 nM in T47D and T47D-P450 cell, respectively. Incubation of T47D cells with DPIC and dicoumarol did not decrease the growth inhibition of RH1 to a greater extent than dicoumarol alone. However, incubation of T47D-P450 cells with DPIC and dicoumarol produced a significantly greater decrease of RH1 cell growth inhibition than dicoumarol alone with the IC_{50} increasing to 57.3 ± 7.5 nM ($P < 0.05$).

Discussion

RH1 is a bioreductive alkylating agent that is a highly efficient substrate for the reductive enzyme, NQO1 [37]. Reduction of the benzoquinone activates the aziridines resulting in alkylation and crosslinking of DNA, which are believed to be responsible for the cytotoxic effects of this agent [4, 7]. Because RH1 is such a good substrate for NQO1, this agent was considered ideal for use in an enzyme-directed tumor targeting strategy to treat tumors with high levels of this enzyme [17, 27] and is currently being tested in the clinic [7]. However, several recent studies have suggested that RH1 may also be a substrate for the reductive enzyme, P450 Red [6, 20], and have raised some doubt about the specificity

Table 2 Cell growth inhibition by RH1 in T47D and T47D-P450 cells

	T47D IC_{50} (nM)	T47D-P450 IC_{50} (nM)
Control	8.3 ± 1.7	7.7 ± 0.6
0.1 mM DPIC alone	9.7 ± 1.2	6.8 ± 0.5
0.2 mM Dicoumarol alone	25.4 ± 2.7[a]	11.9 ± 1.1[a]
0.1 mM DPIC + 0.2 mM Dicoumarol	30.8 ± 9.1[a]	57.3 ± 7.5[a,b]

T47D or T47D-P450 cells were incubated with buffer, 0.1 mM DPIC alone, 0.2 mM dicoumarol alone or 0.1 mM DPIC and 0.2 mM dicoumarol for 1 h and then with varying concentrations of RH1 for an additional 1 h in the presence of the inhibitors. Cell growth inhibition was determined by MTT assay [14] and is presented as IC_{50} values (concentration of drug that resulted in 50% reduction in the relative absorbance) as determined from the linear regression lines of the RH1 concentration versus relative absorbance curve. Values represent the mean ± SE of four or five determinations. The cell growth inhibitions were compared statistically using a *t* test comparing the significance of the difference of the slopes of the concentration versus relative absorbance curves

[a] Significantly different from Control ($P < 0.05$)

[b] Significantly different from Dicoumarol alone ($P < 0.05$)

of RH1 activation by NQO1. Therefore, we examined the ability of P450 Red to reduce RH1 and the contribution of this enzyme to the activation of RH1 in human cancer cells.

To determine if RH1 is a substrate for P450 Red we used EPR studies to measure formation of an RH1 semiquinone free radical following reduction by this enzyme. Since P450 Red is a one-electron reducing enzyme, transfer of a single electron to RH1 should result in the formation of a semiquinone free radical (Fig. 1). This has been demonstrated previously for other quinones [26]. Reduction of RH1 by P450 Red under hypoxia produced an EPR signal within 10 min, due to the RH1 semiquinone free radical (Fig. 2a). When air was introduced into the reaction tube, this signal rapidly decreased to about 10% of its hypoxic level likely as a result of re-oxidation of the semiquinone (Fig. 2b). The formation of equilibrium concentrations of semiquinones under aerobic conditions have been reported [39]. This may be due to the quinone undergoing a hydrolytic reduction to produce the hydroquinone (or its corresponding anion) or that hydroquinone is a contaminant [39]. The quinone and the hydroquinone may then undergo an equilibrium disproportionation reaction ($Q + QH_2 \equiv 2Q^{\bullet-} + 2H^+$) to yield low steady state levels of the semiquinone [22, 35]. In the case of RH1 in the presence of P450 Red there may also have been some formation of hydroquinone by further reduction of the semiquinone by P450 Red and subsequent disproportionation.

NQO1 is an obligate two-electron reducing enzyme that reduces quinones directly to hydroquinones (Fig. 1). Thus, reduction of RH1 by NQO1 should result in direct formation of a hydroquinone, without intermediate formation of a semiquinone. When RH1 was reduced with NQO1 under hypoxia, a small EPR signal characteristic of the RH1 semiquinone free radical was observed (Fig. 2d), and this semiquinone signal was decreased only slightly after the introduction of air (Fig. 2e). The semiquinone was likely produced by reduction of RH1 to the hydroquinone by NQO1 and a subsequent disproportionation reaction. In control experiments in the absence of P450 Red or NQO1, no significant EPR signals were observed.

In order to confirm that RH1 was reduced by P450 Red and to examine the rate of reduction of the quinone and re-oxidation of the semiquinone and hydroquinone, we studied reduction of this agent by P450 Red and NQO1 using a spectroscopic assay to follow loss of the electron donor, NADPH. NADPH can serve as an electron donor for both P450 Red and NQO1. Although there was an overlap of the absorbance spectra of RH1 and NADPH, for these studies we measured the consumption of NADPH by following the loss of absorbance at 370 nm, where approximately 90% of the absorbance is due to NADPH [20].

For all the reduction studies a fourfold molar excess of NADPH was used so that the initial reduction of RH1 and subsequent redox cycling under aerobic conditions could be followed. Reduction of RH1 by P450 Red under hypoxia consumed approximately one equivalent of NADPH in the first 4 min with little further loss of NADPH after this time (Fig. 3), suggesting that the RH1 was fully reduced to the hydroquinone within 4 min. Reduction by NQO1 resulted in loss of approximately one equivalent of NADPH in the first 2 min with little further loss of electron donor (Fig. 3). This result suggests that RH1 is reduced to the hydroquinone by both enzymes. Under aerobic conditions, there was a loss of >90% of the NADPH by 20 min with P450 Red, while >90% of the NADPH was gone by 40 min with NQO1. These results suggests that redox cycling of RH1 is rapid following reduction by P450 Red, probably because the initially formed semiquinone is rapidly re-oxidized to the quinone in the presence of oxygen. This finding is consistent with the EPR studies, which showed a rapid decrease of the semiquinone free radical signal when air was introduced (Fig. 2a, b). In contrast, redox cycling of RH1 was slower following reduction by NQO1 probably because re-oxidation of the initially formed hydroquinone is slower than re-oxidation of the semiquinone [20]. This may explain the small EPR semiquinone signal following reduction of RH1 by NQO1 and introduction of air. If re-oxidation of the RH1

hydroquinone to the semiquinone is slow compared to re-oxidation of the semiquinone to the quinone, there would be little accumulation of the intermediate semiquinone after reduction by NQO1.

Reaction of semiquinones or hydroquinones with oxygen results in the formation of superoxide radical anions, which can dismutate to produce H_2O_2 and subsequently hydroxyl radical by the iron-dependent Fenton reaction [11]. These reactive oxygen species produce a variety of damage to cells, including DNA strand breaks, that can lead to cell death [5, 11, 19, 21]. Because our studies demonstrated that redox cycling occurred following reduction of RH1 by P450 Red or NQO1 under aerobic conditions, we examined whether RH1 produced DNA strand breaks after reduction by these enzymes using a cell free assay. RH1 produced significant levels of DNA strand breaks after reduction by either P450 Red or NQO1 under aerobic conditions (Table 1). The production of significant levels of DNA strand breaks following reduction by NQO1 was somewhat unexpected since redox cycling was slower following reduction of RH1 by NQO1 compared with P450 Red. However, in these studies there may have been more redox cycling because a large excess of NADPH was used and the DNA was incubated with RH1 for 2 h. Thus, DNA strand breaks can be produced following reduction by NQO1 or P450 Red, and this damage may contribute to the cytotoxic activity of RH1 in cells.

In a control experiment we carried out the DNA strand break experiments in a hypoxia chamber under 95% nitrogen:5% carbon dioxide. In the absence of oxygen the reduced products of RH1 would not be expected to undergo re-oxidation and we would not expect to see the formation of DNA strand breaks. As expected, the level of DNA strand breaks produced after reduction of RH1 by either P450 Red or NQO1 under hypoxia was significantly reduced. This provides evidence that the DNA strand breaks produced by RH1 in air resulted from the formation of reactive oxygen species. The low level of DNA strand breaks that we observed under hypoxia may have been due to the introduction of small amounts of oxygen when the reagents for the assay were brought into the hypoxia chamber 0.5 h prior to the start of the reductions. Alternatively, some DNA strand breaks may have resulted from the alkylation of DNA bases by RH1.

Although it had been shown previously that reduction of RH1 by NQO1 results in activation of the aziridine groups and formation of DNA crosslinks [4, 7], there has been no previous data showing that reduction by P450 Red would produce DNA crosslinks. We found that RH1 did produce significant DNA crosslinking following reduction by P450 Red (Table 1). This result, along with the finding that RH1 can produce DNA strand breaks after reduction by P450 Red, suggests that P450 Red could contribute to the activation of RH1 and to the cytotoxic activity of this agent.

To determine if P450 Red does contribute to RH1 activation in cells, we investigated the effects of DPIC, an inhibitor of P450 Red, and dicoumarol, an inhibitor of NQO1, on the cell growth inhibition activity of RH1 in T47D human breast carcinoma cells and T47D-P450 cells. P450 Red activity was approximately 30-fold higher in T47D-P450 cells compared with the parental T47D cells and approximately five-fold higher than the highest activity normally found in tumor cells. P450 Red activities in the human tumor cell line panel used by NCI for evaluation of new anticancer agents ranged from approximately 2.5–63 nmol min^{-1} mg $prot^{-1}$ with a mean of 14.8 nmol min^{-1} mg $prot^{-1}$ [9]. Incubation of the cells with 0.1 mM DPIC for 1 or 2 h decreased P450 Red activity by 94% in both cell lines (Fig. 5). NQO1 activities in the T47D and T47D-P450 cells were similar and were relatively low compared with cell lines in the NCI human tumor cell line panel that had NQO1 activities ranging from approximately 0–5,000 nmol min^{-1} mg $prot^{-1}$ with a mean of 199.5 nmol min^{-1} mg $prot^{-1}$ [9]. DPIC did not significantly inhibit the activity of NQO1 in either cell line.

Dicoumarol acts as an excellent inhibitor of NQO1 at low concentrations, but may also inhibit P450 Red activity at higher concentrations. However, because dicoumarol acts as a competitive inhibitor of both enzymes, it is not possible to directly measure the extent of enzyme inhibition produced by this inhibitor in cells. Thus, these effects have been measured indirectly by determining the effect of dicoumarol on the cytotoxicity of drugs that are specifically activated by NQO1 or P450 Red. Previous studies have shown that 0.1 or 0.2 mM dicoumarol was sufficient to fully inhibit the cell growth inhibition of streptonigrin, a bioreductive agent whose cytotoxicity correlates with cellular NQO1 activity, in cell lines with NQO1 activities five-fold to 22-fold higher than those in T47D and T47D-P450 cells [31, 34]. We showed in this study that 0.2 mM dicoumarol completely inhibited the cell growth inhibition of streptonigrin in T47D cells and most of the cell growth inhibition of this agent in T47D-P450 cells. This suggests that the concentration of dicoumarol used in these studies was sufficient to inhibit all of the NQO1 in the T47D and T47D-P450 cells. The small amount of cell growth inhibition by streptonigrin remaining in the T47D-P450 cells in the presence of dicoumarol may reflect a weak ability of P450 Red to activate streptonigrin at high enzyme

levels. In contrast, dicoumarol did not inhibit the growth inhibition activity of tirapazamine in T47D or T47D-P450 Red cells. Tirapazamine is an anticancer agent whose cell growth inhibition under aerobic conditions correlated with 450 Red activity [24]. These results suggest that the concentration of dicoumarol used in these studies was sufficient to inhibit NQO1 activity in the T47 D and T47D-P450 cells, but that this concentration of dicoumarol did not inhibit P450 Red in these cells.

RH1 was highly effective in inhibiting cell growth in both T47D and T47D-P450 cells with IC_{50} values of 8.3 ± 1.7 and 7.7 ± 0.6 nM, respectively. Incubation of the cells with DPIC alone did not significantly effect RH1 cell growth inhibition in either T47D or T47D-P450 cells (Fig. 6; Table 2). This suggests that P450 Red is not a major contributor to the activation of RH1 in either cell line. In contrast, incubation of the cells with dicoumarol significantly inhibited the cell growth inhibition of RH1 in both cell lines (Fig. 6; Table 2), but this effect was greater in the T47D cells where dicoumarol inhibited nearly all of the RH1 activity. This finding confirms previous studies showing that NQO1 was the major contributor to the activation of RH1 in a variety of human tumor cell lines [8, 37, 38]. However, incubation with dicoumarol did not completely inhibit RH1 cell growth inhibition in the T47D-P450 cells suggesting that another enzyme may be contributing to activation of this agent in these cells. Incubation of T47D cells with DPIC and dicoumarol did not significantly affect RH1 cell growth inhibition to a greater extent than dicoumarol alone (Fig. 6; Table 2). In contrast, incubation of T47D-P450 cells with both inhibitors produced significantly greater inhibition of cell growth by RH1 than dicoumarol alone (Fig. 6; Table 2), suggesting that in the absence of NQO1 activity, very high levels of P450 Red activity may be able to activate some of the RH1.

These studies confirmed that RH1 was a substrate for P450 Red and demonstrated that this enzyme could activate RH1. The rate of redox cycling after reduction of RH1 by P450 Red was faster than redox cycling following reduction by NQO1. This probably occurred because redox cycling following P450 Red reduction involves re-oxidation of the RH1 semiquinone, while redox cycling following NQO1 reduction involves a slower initial re-oxidation of the RH1 hydroquinone to the semiquinone followed by re-oxidation of the semiquinone. This study also provided the first direct evidence that RH1 could produce both DNA strand breaks and DNA crosslinks after reduction by P450 Red. Despite these findings, P450 Red did not appear to contribute significantly to the activation of RH1 in T47D cells. This is likely due to the more efficient reduction of RH1 to the hydroquinone by NQO1. Although P450 Red did appear to play a minor role in activating RH1 in the T47D-P450 cells, this study supports previous findings suggesting that this enzyme does not play a significant role in activation of RH1 in cancer cells with normal levels of P450 Red activity. Furthermore it suggests that P450 Red is not responsible for activation of RH1 in normal hematopoetic cells which generally have low NQO1 activity but normal levels of P450 Red activity.

The lack of correlation between NQO1 activity and sensitivity to RH1 reported previously [36] may be due to the fact that only very low levels of NQO1 activity are required to activate the low concentrations of RH1 used to treat cells. Thus, most tumor cell lines have more than sufficient NQO1 to activate all of the RH1 used in treatment and would be expected to show similar sensitivities to this drug. Alternatively, unidentified enzymes, in addition to NQO1, may also contribute to activation of RH1 in cells.

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