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The reductive activation of the antitumor drug RH1 to its semiquinone free radical by NADPH cytochrome P450 reductase and by HCT116 human colon cancer cells

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Abstract

RH1 (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone), which is currently in clinical trials, is a diaziridinyl benzoquinone bioreductive anticancer drug that was designed to be activated by the obligate two-electron reductive enzyme NAD(P)H quinone oxidoreductase 1 (NQO1). In this electron paramagnetic resonance (EPR) study we showed that RH1 was reductively activated by the one-electron reductive enzyme NADPH cytochrome P450 reductase and by a suspension of HCT116 human colon cancer cells to yield a semiquinone free radical. As shown by EPR spin trapping experiments RH1 was reductively activated by cytochrome P450 reductase and underwent redox cycling to produce damaging hydroxyl radicals in reactions that were both H_2O_2- and iron-dependent. Thus, reductive activation by cytochrome P450 reductase or other reductases to produce a semiquinone that can redox cycle to produce damaging hydroxyl radicals and/or DNA-reactive alkylating species may contribute to the potent cell growth inhibitory effects of RH1. These results also suggest that selection of patients for treatment with RH1 based on their expression levels of NQO1 may be problematic.

Keywords: *RH1, cytochrome P450 reductase, EPR, quinone, semiquinone radical, hydroxyl radical*

The bioreductive drug, 2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone (RH1, Figure 1) has shown good antitumor activity *in vitro* [1,2] and *in vivo* [3]. Bioreductive anticancer drugs must be reductively activated within the tumor to produce their active cytotoxic form. Preferential activation may occur in solid hypoxic tumors that contain over-expressed reductases [4]. Water-soluble RH1, which is a tetra-substituted diaziridinyl benzoquinone, is currently in phase I clinical trials for the treatment of advanced solid tumors in the UK [5,6]. RH1 was specifically designed to be reductively activated in solid tumors by NAD(P)H quinone oxidoreductase 1 (NQO1, DT-diaphorase) (EC 1.6.99.2) [2,7]. Patient NQO1 enzyme expression levels are being determined in this ongoing clinical trial to correlate with drug efficacy [6] with the view to selecting patients for treatment with RH1 based on their NQO1 expression levels. RH1 is, in fact, a good substrate for NQO1 and two-electron reduction by NQO1 can activate the aziridine groups to alkylate DNA and produce interstrand cross links [2,8,9]. Studies with isogenic cell lines expressing differing levels of NQO1 have shown that cells with higher levels of NQO1 are more sensitive to RH1, but only within a narrow window of NQO1 levels [8,9]. However, it has also been shown that RH1 causes NADPH oxidation by NADPH cytochrome P450 reductase (cytochrome P450 reductase) (EC 1.6.2.4) [10], suggesting that it is a substrate for this enzyme. Thus, redox cycling of RH1

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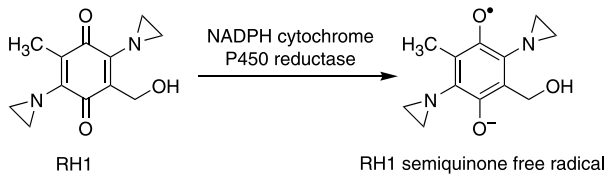

Figure 1. Structure of the anticancer drug RH1 and its cytochrome P450 reductase reductively activated free radical semiquinone form.

through one-electron reduction to its semiquinone free radical and reoxidation back to RH1 resulting in formation of reactive oxygen species may also contribute to its cytotoxicity.

A recent study has also shown that the potency of RH1 in the NCI 60-tumor cell panel is independent of NQO1 levels and that the NQO1inhibitor dicumarol has no impact on the sensitivity profiles of RH1 response [11]. Together these results suggest that alternative reductive pathways for activation of RH1 may be operative [11]. We also recently showed that NQO1 induction did not enhance the growth inhibitory effects of RH1 in HCT116 cells *in vitro* or in HL60 cells *in vivo* [12]. This may have been due to a narrow window of NQO1 activity [8], or alternatively, due to activation by cytochrome P450 reductase or other reductases. We also recently showed by electron paramagnetic resonance spectroscopy (EPR) that a series of benzoquinone mustard compounds and the anticancer indolequinone EO9 are reduced by cytochrome P450 reductase to their semiquinone free radical forms [13]. This EPR study was carried out to see if cytochrome P450 reductase and HCT116 colon cancer cells were able to reductively activate RH1 to its semiquinone free radical form in order to determine if reductive activation mechanisms other than through NQO1 were possible. While several diaziridinyl benzoquinone analogs that were the subject of earlier drug development have been shown to be reductively activated to their semiquinone by cytochrome P450 reductase [14–16], no similar studies have been carried out on RH1 which is currently in clinical trials. For these reasons it is important to characterize the reaction of RH1 with cytochrome P450 reductase.

Materials and methods

Materials and cell culture

The human recombinant cytochrome P450 reductase was from Gentest (Woburn, MA, USA). RH1 was a generous gift from Dr David Ross (University of Colorado Health Sciences Center, Denver, CO, USA). Deferasirox (ICL670) was a gift from Novartis Pharma (Basel, Switzerland). Unless indicated, other chemicals were from Sigma (St Louis, MO, USA). The HCT116 human colon carcinoma cells were obtained from ATCC (Manassas, VA, USA) and were cultured as described [12]. The HCT116 cells were trypsinized, pelleted and resuspended in 0.9% NaCl and loaded into the Teflon tubing for the EPR experiments.

EPR experiments

A freshly prepared 15 μl aliquot of the drug in the reaction system indicated 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 and inserted into a quartz EPR tube open at both ends, and placed in the EPR cavity as described [13,17]. The EPR spectra were recorded with a Bruker (Milton, Canada) EMX EPR spectrometer. Prepurified grade thermostated (37°C) argon or air (400 l/h) 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 9.25 GHz, modulation amplitude 0.5 or 2.0 G as indicated, time constant 0.02 s, 1024 data points/scan and a 50 or 100 G scan range as indicated. RH1 was dissolved in dimethyl sulfoxide (DMSO) and was present in the final reaction mixture at the concentrations indicated. The 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (100 mM) spin trapping experiments were carried out under oxic conditions in the presence of the secondary spin trap DMSO (10%). In the cell experiments the final concentration of DMSO was 1%. In order to correct for a sloping baseline for the EPR experiments with HCT116 cells the EPR spectrum of the control containing only 0.9% NaCl was subtracted from the other spectra.

Results

EPR measurement of cytochrome P450 reductase-induced RH1 semiquinone free radical formation

As shown by the EPR spectrum in Figure 2A, RH1 in the cytochrome P450 reductase system produced a multi-line semiquinone free radical EPR signal at $g \sim 2.0$ under hypoxic conditions. The complex EPR spectrum seen is typical of a semiquinone radical with several hyperfine splittings, which is likely due to spin coupling to the aziridinyl nitrogen atoms and the hydrogen atoms. It was also observed (Figure 2B) that the RH1-induced EPR signal that was produced under hypoxic conditions was rapidly and completely eliminated upon the reintroduction of air. In the absence of cytochrome P450 reductase, but with NADPH and RH1 in the system, no detectable EPR signal was seen (Figure 2C) which indicates that NADPH alone does not detectably reduce RH1.

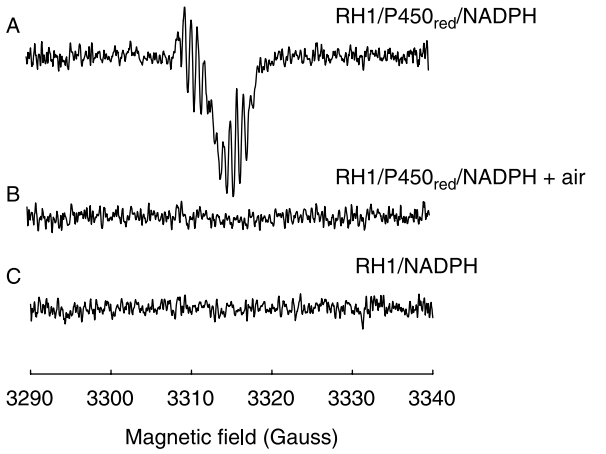

Figure 2. RH1 is reductively activated to its semiquinone free radical by cytochrome P450 reductase. (A) Multiline EPR spectrum of RH1 semiquinone radical produced by the cytochrome P450 reductase/NADPH reaction system under hypoxia. (B) EPR spectrum observed after the addition of air to the sample above. (C) EPR spectrum observed in the system containing RH1 and NADPH only. The complete system contained 15 μg/ml cytochrome P450 reductase, 2 mM NADPH, 1 mM RH1, 10% DMSO and Tris buffer (pH 7). The instrumental settings were: modulation amplitude 0.5 G, 37°C, and a 50 G scan range. A total of 10 spectra were accumulated over 7 min and averaged. $P450_{red}$/NADH indicates NADPH and cytochrome P450 reductase were in the reaction mixture.

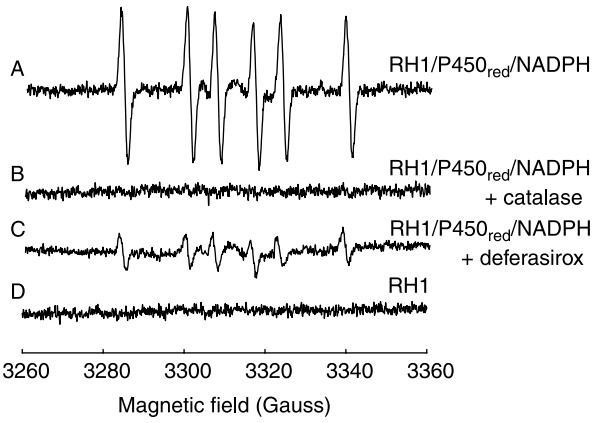

Figure 3. RH1 redox cycles in the cytochrome P450 reductase system to produce hydroxyl radicals. (A) Six-line 1:1:1:1:1:1 EPR spectrum of the hydroxyl radical-derived methyl radical adduct of DMPO produced by RH1 in the cytochrome P450 reductase system. (B) EPR spectrum of complete system but with 100 μg/ml of catalase. (C) EPR spectrum of complete system but with 100 μM of the ferric ion chelator deferasirox. (D) RH1spectrum in the absence of cytochrome P450 reductase. The complete system contained 15 μg/ml cytochrome P450 reductase, 2 mM NADPH, 1 mM RH1, 10% DMSO at pH 7. The instrumental parameters were as in Figure 2 but with a modulation amplitude of 2 G and a sweep width of 100 G. $P450_{red}$ indicates the presence of NADPH cytochrome P450 reductase in the reaction system.

EPR spin trapping experiments with RH1 in the cytochrome P450 reductase system

It is well known that the superoxide radical anion ($O_2^{\cdot -}$) reacts with DMPO to form DMPO–$O_2^{\cdot -}$, which then decays rapidly to DMPO–$OH^{\cdot}$ [18]. Thus, in order to distinguish hydroxyl radical production from $O_2^{\cdot -}$ production, spin trapping experiments were carried out on RH1 in the cytochrome P450 reductase system employing 10% (v/v) DMSO as a secondary radical trap. As shown in Figure 3A a six-line spectrum with a 1:1:1:1:1:1 ratio of peak heights was seen, which is characteristic of a carbon-centered radical expected for the methyl radical adduct of DMPO (DMPO–$CH_3^{\cdot}$) [19]. Thus, RH1 can redox cycle to produce damaging hydroxyl radicals. The hyperfine splitting constants observed were: $A_N = 16.1$ G, $A_H = 22.9$ G, which is also characteristic of DMPO–$CH_3^{\cdot}$ [19]. The hydroxyl radicals produced in this system reacted rapidly with DMSO and produced a methyl radical that then reacted with DMPO to produce DMPO–$CH_3^{\cdot}$. As seen in Figure 3B, in the presence of catalase to scavenge H_2O_2, the six-line hydroxyl radical-derived spectrum was reduced to below detectable levels which indicates that hydroxyl radical production reaction was H_2O_2-mediated. The addition of 100 μM deferasirox to the reaction system, which is a strong Fe^{3+} chelator (overall formation constant of $10^{38.6}\,M^{-2}$) [20], reduced the peak-to-peak amplitude of the six-line spectrum by approximately 80% (Figure 3C), indicating that hydroxyl radical formation was, at least in part, iron-dependent. We previously showed that the $Fe^{3+}(deferasirox)_2$ complex was effectively unable to be reductively activated by cytochrome P450 reductase [21]. The fact that the Fe^{3+} chelator deferasirox was not 100% effective in reducing hydroxyl radical formation might have been due to some small amount of redox cycling by the $Fe^{3+}(deferasirox)_2$ complex, or possibly through metal-independent formation of hydroxyl radicals by reaction of hydrogen peroxide with the quinone as has been suggested [22].

EPR measurement of RH1 semiquinone radical formation in HCT116 human colon cancer cells

In order to increase EPR sensitivity to detect low levels of RH1 semiquinone formation in the HCT116 cell suspension, the EPR spectra in these experiments were recorded at a modulation amplitude of 2 G. Similar to the result shown in Figure 2A, the addition of RH1 to the cytochrome P450 reductase system produced a semiquinone EPR signal (Figure 4A). However, the fine structure in the spectrum of Figure 4A was largely unresolved due to the much higher modulation amplitude used to record the signal. As shown in Figure 4B the addition of RH1 to the HCT116 cell suspension under hypoxic conditions produced a small, but detectable, RH1 semiquinone free radical EPR signal. The semiquinone spectrum in the cell suspension can be identified by comparison to the similar peak-to-peak line width observed in the spectrum shown in Figure 4A, and also by the peak

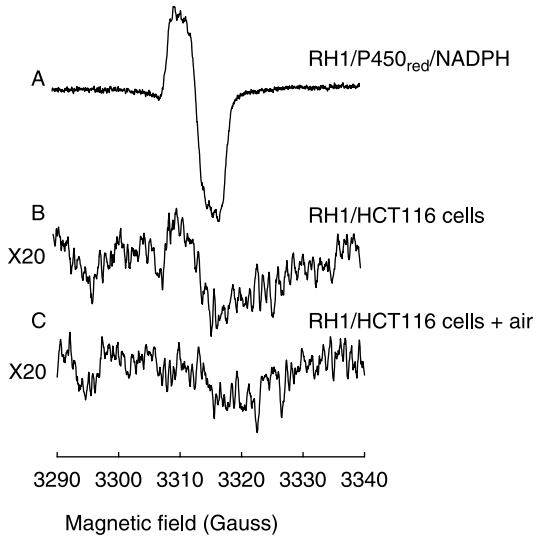

Figure 4. Hypoxic HCT116 colon cancer cells reduce RH1 to its semiquinone free radical. (A) EPR spectrum of RH1 semiquinone radical produced by the cytochrome P450 reductase system as described in the caption to Figure 2, but recorded at a modulation amplitude of 2 G to increase sensitivity. (B) EPR spectrum of the RH1 semiquinone radical produced by a suspension of HCT116 cells (2.5×10^8/ml in 0.9% NaCl) in 1 mM RH1. (C) EPR spectrum observed after the addition of air to the HCT116 cell suspension above. The instrumental parameters were as in Figure 2 but with a modulation amplitude of 2 G and a sweep width of 50 G. The vertical scale in (B) and (C) is 20-fold less than in (A).

position. The addition of air to the system reduced the peak-to-peak amplitude of the EPR signal by approximately 75% (Figure 4C). This result also indicates that the signal was an RH1 semiquinone. The small signal remaining in the presence of air, and shifted up field by approximately 2 G, was probably due to free organic and inorganic paramagnetic species in the cell. Using similar methods we previously demonstrated the formation of a doxorubicin semiquinone EPR signal in cardiac myocytes [17] and in Chinese hamster ovary cells [23].

Discussion

The EPR results of Figure 2A showed that RH1 was reductively activated to its semiquinone by cytochrome P450 reductase, which indicated that RH1 was a good substrate for this enzyme. Because the RH1 semiquinone was shown to accumulate, these results also suggest that the RH1 semiquinone was a poorer substrate for cytochrome P450 reductase than was RH1 itself. While our results show that cytochrome P450 reductase reductively activated RH1 to its semiquinone (Figure 2), it is unknown whether this was the specific reductase enzyme that was responsible for semiquinone formation observed in HCT116 cells (Figure 4B).

Semiquinones react very quickly with oxygen [5] to produce $O_2^{\cdot}$, which in turn dismutates to produce H_2O_2, which can then go on to produce hydroxyl radicals by the iron-dependent Fenton reaction [24]. The EPR spin trapping results of Figure 3 showed that the cytochrome P450 reductase/NADPH system produced hydroxyl radicals in a reaction that was both H_2O_2- and iron-dependent. The small quantities of iron required for this reaction probably came from adventitious iron in the reaction mixture. Generation of hydroxyl radicals by RH1 that is redox cycling may provide a way by which RH1 can, in part, contribute to its potent cell growth inhibitory effects. It has been postulated that formation of reactive oxygen species that damage cells through redox cycling of quinones likely only occurs at relatively high concentrations and that the predominate cell damaging event that occurs at the much lower cell growth inhibitory concentrations of RH1 is likely due to alkylation of DNA subsequent to its reductive activation [5]. The reactive DNA alkylating species produced upon either one- or two-electron reduction is likely the protonated aziridine of RH1 [5]. Even if formation of reactive oxygen species is not responsible for cell growth inhibition, enzymatic reduction of RH1, whether one- or two-electron, to enable alkylation of DNA is still required [5]. The fact that RH1-treated HCT116 cells produced the RH1 semiquinone (Figure 4B) indicates that one-electron reductive enzymes in these cells were able to reductively activate RH1. Thus, these results provide a possible mechanism by which RH1 may damage cells and inhibit their growth.

In conclusion, these EPR studies showed that cytochrome P450 reductase reduced the antitumor drug RH1 to a semiquinone free radical, and that following this reduction RH1 underwent redox cycling under oxic conditions to produce potentially damaging hydroxyl radicals. In addition, we have shown that human colon cancer HCT116 cells were also to reduce RH1 to its semiquinone form. One-electron reductive activation of RH1 by cytochrome P450 reductase to produce reactive oxygen species and/or to a reactive DNA alkylating form provide alternate mechanisms other than through NQO1 by which RH1 may exert its potent cell growth inhibitory effects. If reaction of cytochrome P450 with RH1 is a significant alternative pathway, as the results of this study suggest, then selection of patients for treatment with RH1 based on their expression levels of NQO1 [6] may be problematic.

Acknowledgements

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