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Preclinical Evaluation of the Pharmacodynamic Properties of 2,5-Diaziridinyl-3-Hydroxymethyl-6-Methyl-1,4-Benzoquinone

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Abstract

Purpose: The purpose of our study was to investigate the cellular accumulation, DNA cross-linking ability, and cellular toxicity of RH1 (2,5-diaziridinyl-3-[hydroxymethyl[-6-methyl-1,4-benzoquinone), a novel DNA alkylating agent currently in clinical trials. In addition, the *in vivo* efficacy of RH1 formulated in different vehicles was also compared.

Experimental Design: RH1 is activated by the two-electron reducing enzyme NQO1 [NAD(P)H:quinone oxidoreductase] forming a potent cytotoxic agent that cross-links DNA. We have used whole blood, cell lines, and primary explanted tumor cultures to measure both the cellular accumulation, DNA cross-linking, and cytotoxicity of RH1. Furthermore, the pharmacokinetic and pharmacodynamic characteristics of RH1 formulated in different vehicles were measured *in vivo* using the validated comet-X assay in mice bearing human tumor xenografts.

Results: Accumulation of RH1 was shown to be both time and concentration dependent, reaching a maximum after 2 hours and correlated well with DNA cross-linking measurements. DNA cross-linking *in vitro* could be detected at low (1-10 nmol/L) concentrations after as little as 2 hours exposure. In primary tumor cultures, RH1 induces much higher levels of DNA cross-links at lower doses than either mitomycin C or cisplatin. *In vivo* efficacy testing using polyvinyl pyrrolidone, saline, or cyclodextrin as vehicles showed DNA cross-links readily detectable in all tissues examined and was enhanced when given in cyclodextrin compared with polyvinyl pyrrolidone or saline.

Conclusions: RH1 represents a potent bioreductive anticancer drug, which may prove effective in the treatment of cancers, particularly those that overexpress NQO1. DNA cross-linking can be reliably measured in tissue using the validated comet-X assay.

Within the field of anticancer drug discovery, quinones have traditionally represented a major source of active compounds (1, 2). These agents are unique in cancer therapeutics as they require enzyme catalyzed drug metabolism (bioreduction) before conversion to intermediates that can either generate toxic-free radical species or bind to DNA and form covalent adducts (3). Two mechanisms have been proposed to explain their anticancer efficacy: bioactivation under hypoxic conditions normally only present in solid tumors (4, 5) and selectively due to overexpression of reductase enzymes in tumor relative to normal tissues (6, 7).

Many one and two electron reductases are capable of activating bioreductive drugs, including NADH cytochrome b5 reductase (8, 9), cytochrome *P*450 reductase (10, 11), and NQO1 (diphtheria toxin-diaphorase, EC 1.6.99.2; NAD(P)H:quinone oxidoreductase; 12 – 14). However, particular emphasis has been placed on NQO1 in enzyme directed drug development since its activity and gene expression have been shown to be elevated relative to uninvolved counterpart in a number of intrinsically drug resistant solid malignancies including lung, colon, and liver cancer (15 – 18). In addition, being a two-electron reductase quinone containing drugs metabolized by NQO1 are not dependent on the presence of hypoxic conditions in tumors for the full expression of their activity (19, 20). Nevertheless, significant NQO1 expression is present in number of normal tissues including the respiratory tract epithelium, kidney podocytes, and reproductive system (21 – 23), which may represent a potential mechanism of drug toxicity.

Whereas numerous *in vitro*-based studies have shown that NQO1 plays a key role in the mechanism of action of a number bioreductive drugs, such as clinically active mitomycin C (MMC; 14, 24 – 28), it is clear that there exits a far from simple relationship between *in vivo* drug activity and clinical efficacy and NQO1 expression (29 – 32) both under hypoxic and aerobic conditions (33, 34).

RH1 [2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone] is a water-soluble derivative that was identified from a large number of novel disubstituted aziridinylbenzoquinones due to its exceptionally high affinity for NQO1 as a

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substrate (35, 36). Upon reduction, it forms difficult to repair DNA interstrand cross-linked adducts with a unique sequence specificity of GCC (37). Unlike MMC, the products of RH1 quinone reduction are more stable and less likely to generate toxic reactive oxygen species (38). *In vitro*, a good correlation has been established between NQO1 levels and potency of RH1 in human non–small cell lung cancer and colon cancer cells (35, 36, 39). Thus, it is anticipated that RH1 will be selectively activated within tumors overexpressing NQO1, giving maximal antitumor activity with reduced toxicity in normal tissues.

RH1 is presently undergoing a Cancer Research UK sponsored phase I clinical trial at two different centres in the United Kingdom. Preclinical studies concerning efficacy, toxicology, and pharmacokinetics conducted before the commencement of this trial showed good activity in a number of xenograft models (40, 41), in particular non–small cell lung cancer and ovarian cancer and a significantly longer plasma half-life than related agents such as EO9 (42).[4] However, to date, there has been little reported data on the pharmacodynamic properties of RH1. In the present study, we have attempted to correlate drug intracellular accumulation to DNA cross-linking ability and NQO1 expression in a number of cell lines, primary tumor cultures, and volunteer blood using the comet-X assay. To aid development of a clinical formulation of the drug, the efficacy of RH1 delivered *in vivo* using different vehicles was investigated.

Materials and Methods

Chemicals. RH1 was synthesized as previously described (36). [^{3}H] RH1 was synthesized in the same manner with the tritium positioned on the $-CH_2OH$ group. The specific activity was 80 μCi/mmol. Cisplatin, MMC, and melphalan were obtained from Sigma (St. Louis, MO). MMC and RH1 were dissolved in PBS before use. Melphalan was dissolved in acidified ethanol and cisplatin was dissolved in 0.9% saline. All other reagents were of the highest purity commercially available.

Cell culture. The H460 non–small cell lung cancer cell line expressing high levels of NQO1 activity was used to determine primary pharmacodynamic variables. BE colon carcinoma cells [homozygous for a polymorphism in NQO1 (NQO1*2/*2)] and BE cells transfected with full-length NQO1 wild-type cDNA (BE-NQO1) were a kind gift from Professor David Ross (University of Colorado).

All cell lines were maintained as adherent cultures in RPMI 1640 supplemented with 10% FCS. Cells were maintained in log phase growth by weekly serial dilution and were screened for *Mycoplasma* contamination monthly. Primary ovarian tumor material was collected at the time of surgery with informed patient consent and full ethical approval. Tumor samples were cut into small pieces and explanted into primary culture as previously described (43). Blood was obtained from healthy volunteers with informed consent.

Growth inhibition studies. Both established cell lines and primary cultures were plated into 96-well plates (Falcon, Bedford, MA) at between 500 and 2,000 cells per well. Cells were allowed to attach for 24 hours before the addition of drugs. After 5 days, continuous exposure at 37°C, growth inhibition was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (44). Briefly to each well were added 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (3 mg/mL) and the cells incubated for 3 hours. Following incubation, all media and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were aspirated, the reduced formazan was solubilized in 200 μL of DMSO. The absorbance was then read at 540 nm. Growth inhibition curves were constructed and IC_{50} values determined. Substantial growth in control wells was observed for primary tumor cells.

NQO1 activity measurements. Cell and tissue pellets were immediately frozen at −80°C. Samples were allowed to thaw on ice and then lysed in Cytobuster (Merck, Darmstadt, Germany). NQO1 activity was measured as the rate of dicumarol-inhibitable 2,6-dichlorophenol-indophenol reduction (41). Activity was expressed as nmol 2,6-dichlorophenol-indophenol reduced per minute per mg protein.

Comet-X assay. The comet-X assay is a sensitive semiquantitative method of determining DNA interstrand cross-links. The assay requires the introduction of a fixed number of random strand breaks into the DNA of cells post-drug treatment. The effect of DNA cross-linking drugs is to retard the migration of this fragmented DNA in a dose-dependent manner. Cross-linking is expressed as a decrease in the fraction of DNA in the comet tail compared with irradiated only control cells (42, 43, 45). Cells were harvested by trypsinization and 20,000 cells/mL were resuspended in fresh medium in a 24-well plate. The cells were allowed to attach overnight before treatment. Following treatment with drugs at 37°C for between 2 and 24 hours, cells were processed using the comet-X assay. Briefly, cells were trypsinized, transferred to plastic Bijou tubes then immediately chilled on ice. Drug treated and control irradiated samples were then subjected to 20 Gy irradiation using a Cesium-137 source (0.4 Gy/min). All samples were maintained on ice to prevent repair. Control nonirradiated, nondrug-treated cells were maintained on ice in the same manner as treated samples.

Cell suspension (0.5 mL) was added to low–melting point agarose and the resultant mixture pipetted onto a precoated glass microscope slide and allowed to gel. The slides were then immersed in ice-cold lysing solution for 1 hour then washed thrice for 15 minutes in fresh double-distilled water. Slides were then immersed in alkali unwinding solution (50 mmol/L NaOH, 1 mmol/L EDTA buffered to pH 12.5) and left for 45 minutes to allow the DNA to unwind before being subjected to electrophoresis at 0.6 V/cm for 25 minutes. Each slide was then neutralized 0.4 mol/L Tris-HCl (pH 8.0), stained with Sybr Gold solution (1:10,000 dilution in water), and examined at 250× magnification under an epifluorescent microscope (Zeiss-Jenamed, Jena, Germany). Images were captured using Komet V5.1 software (Kinetic Imaging, Liverpool, United Kingdom). Fifty images from each of two duplicate slides were captured and analyzed. The % DNA present in tail of the comets was recorded and % DNA cross-linked calculated.

Accumulation of [^{3}H] RH1. Cells (10^6) were plated into 30-mm dishes and allowed to attach overnight. RH1 was added to a final concentration between 0.5 and 20 μmol/L and cells were incubated at 37°C for up to 8 hours. Periodically, cells were washed four times with ice-cold PBS and lysed in 1 mL of 1 mmol/L NH_4OH for 1 minute. Lysates were placed in scintillation vials. Each plate was then washed thrice with 1 mL of PBS and the washings also placed into the scintillation vial. Scintillant (Ecoscint) was added and the lysates counted using a Perkin-Elmer Counter. DPM per sample was calculated.

Whole blood was treated with 5 μmol/L RH1 as described above and cell fractions were separated using lymphoprep. Isolated peripheral blood lymphocytes (PBMC) and erythrocytes were then washed solubilized and tritium activity determined as for cultured cells.

The efficacy of RH1 in vivo. Before commencement of a phase I clinical trial, the effect of various vehicles on the efficacy of RH1 *in vivo* was determined. These studies used *nu/nu* mice bearing xenografts of the human breast carcinoma cell line NQ16 [homozygous for a polymorphism in NQO1 (NQO1*2/*2)] transfected with wild-type NQO1 (*1/*1) and expressing very high levels of enzyme (46). All animals had palpable tumors (200 mm^2) and the average NQO1 activity was 1,774 ± 191 nmol per minute per mg protein. Animals were treated at 0.56 mg/kg representing a 2/3 maximum tolerated dose (41) for 4 hours for DNA cross-linking studies. RH1 was formulated in saline, polyvinyl pyrrolidone (PVP), or cyclodextrin. Following treatment, the animals were sacrificed and tumor and normal tissues

[4] Development and Therapeutics Program, National Cancer Institute, unpublished data.

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were sampled for processing using the comet-X assay. Briefly, cells from blood, colon, lung, kidney, and tumor were excised into ice-cold HBSS containing 10% DMSO. Gentle compression of the tissue using the rounded end of a 1ml syringe plunger was sufficient to release enough cells for Comet analysis. Cells were diluted to $\sim 2 \times 10^5$/mL in ice-cold HBSS and then irradiated at 15 Gy to introduce a fixed number of strand breaks. DMSO was added to a final concentration of 10% and the samples were then frozen at −80°C before Comet-X analysis.

All animal experiments were carried in accordance with United Kingdom Coordinating Committee on Cancer Research guidelines (47) and as part of a project license issued by the UK Home Office (personal license no. 40/1720).

Results

Radiolabeled drug accumulation. The accumulations of [^{3}H] RH1 in cells and whole blood are shown in Fig. 1A-C. Accumulation in NQO1-rich H460 cells was gradual and equilibrium was reached within 2 hours (Fig. 1A) followed by a circa 30% decrease over the next 6 hours. This initial drug accumulation was subsequently shown to be dose dependent over a range of 0.5 to 20 μmol/L (Fig. 1B). Accumulation into the cellular compartment of whole blood is shown in Fig. 1C, where it can be seen that a much lower level of drug accumulates slowly over a 4-hour period in PBMCs. In contrast, erythrocytes seem to bind low levels of drug with no net accumulation over a 12-hour period.

DNA interstrand cross-linking. H460 cells (NQO1 activity; 5,341 ± 581 nmol per minute per mg protein) were treated at varying concentrations of RH1 for 2 hours (where intracellular accumulation of RH1 is maximum) and the results are shown in Fig. 2A. It can be seen that the maximum cross-linking occurs between 10 and 20 nmol/L with >85% of DNA migration being retarded. The rate of DNA cross-linking after a 20-nmol/L treatment is shown in Fig. 2B. Significant DNA cross-linking occurs within 30 minutes of treatment. With maximum cross-linking occurring between 4 and 8 hours.

Figure 2C shows the effect of varying doses of RH1 on the NQO1-deficient BE cell line. DNA cross-linking was relatively low and only 50% cross-linking was observed at 160 nmol/L. Restoration of NQO1 activity in BE cells by transfection (765 ± 146 nmol per minute per mg protein) resulted in a significant increase in DNA cross-linking and nearly 80% DNA cross-linking was observed for the same dose. Whereas intracellular accumulation in BE cells is similar to that observed in H460 cells which overexpress NQO1 (data not shown), H460 cells only required 5 nmol/L to produce circa 50% DNA cross-linking.

Experiments using PBMCs isolated from whole blood, added to RH1, showed a low level of DNA cross-linking, saturated above 20 nmol/L (Fig. 2C). The DNA cross-linking activity of three clinically used anticancer drugs (melphalan, MMC, and cisplatin) in primary cultures of cancer cells was compared with that of RH1 (Fig. 3). DNA cross-linking occurred at a significantly lower concentration (ca. 100×) with RH1 than with any of the other drugs used. The order of potency was RH1 > MMC > melphalan > cisplatin.

DNA cross-linking after a 2-hour exposure to drug followed by 24 hours in fresh medium is shown in Table 1. Repair (3-68%) of residual cross-links compared with initial treatment occurred with all drugs in all cultures. However, no statistically significant differences in repair were found between these tumor cultures.

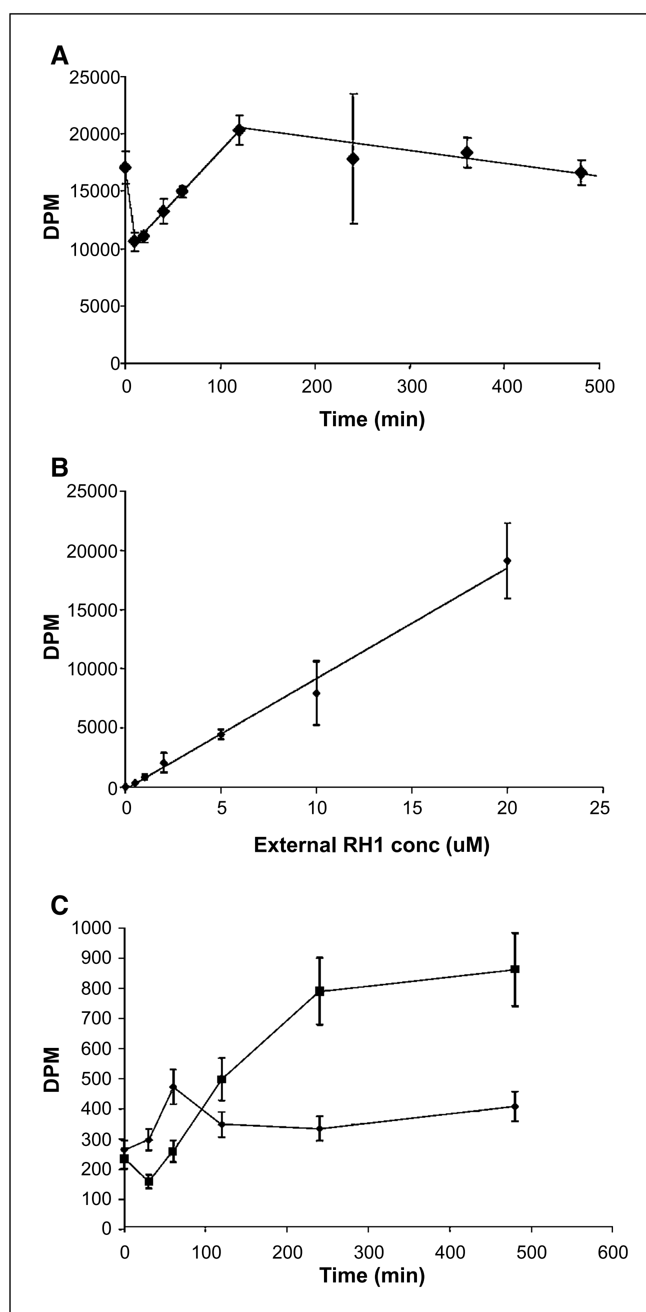

Fig. 1. *A*, kinetics of intracellular accumulation of [^{3}H] RH1 (20 μmol/L) in H460 cells. *B*, intracellular accumulation of RH1 in H460 cells treated with increasing concentrations of [^{3}H] RH1 for 2 hours at 37°C. *C*, intracellular accumulation of [^{3}H] RH1 in the cellular compartment of whole blood. Whole blood was spiked with 5 μmol/L [^{3}H] RH1 and erythrocytes (●) and PBMCs (■) were isolated at various times as described in Materials and Methods. *Point,* mean of three replicates; *bars,* ±SE.

Growth inhibition studies on primary tumors. Cytotoxicity assays comparing a panel of clinical anticancer drugs and RH1, showed that RH1 was >10 times more toxic than Paclitaxol and >50 times more toxic than protypical bioreductive drug MMC in these primary tumor cultures (Table 2). Measurable NQO1 activity (826 ± 53 nmol per minute per mg protein) was detected in only one of the four primary cultures used (i.e., CCU49).

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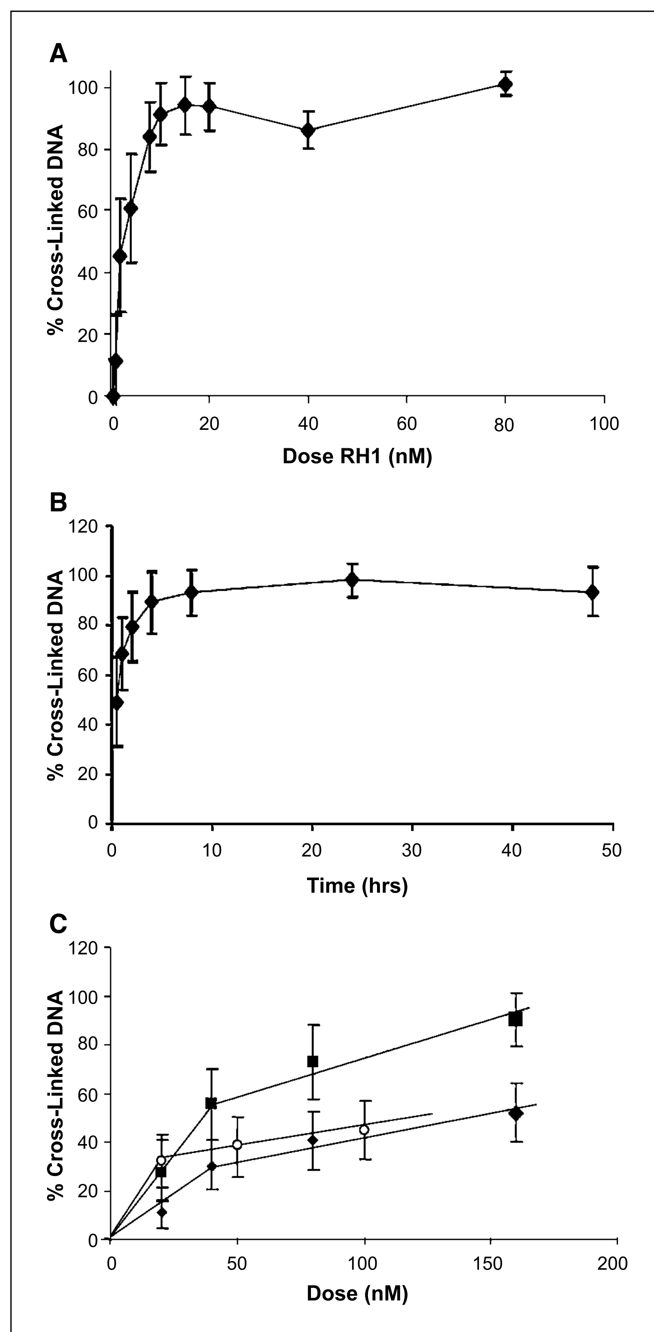

Fig. 2. *A*, dose-dependent DNA cross-linking in NQO1-expressing H460 cells. Cells were treated for 2 hours with a range of RH1 concentrations between 0.5 and 20 nmol/L. DNA cross-linking was measured using the comet-X assay. *B*, rate of formation of DNA interstrand cross-links in H460 cells treated at 20 nmol/L with RH1. *C*, rate of formation of DNA interstrand cross-links in NQO1-deficient BE cells (♦), BE cells transfected with NQO1 (■), or PBMCs (○). Point, mean of two slides on which a minimum of 50 comets was scored.

In vivo efficacy. The biodistribution of DNA cross-links is shown in Fig. 4. RH1 when delivered *in vivo* in cyclodextrin shows the greatest overall DNA cross-linking in all tissues compared with saline or PVP. DNA cross-linking was not detected in kidney when PVP was used or in colon when either saline or PVP was used. Of all the tissues showing DNA cross-linking, the kidney seemed to have the lowest level (8-15%). In contrast, both blood and tumor showed

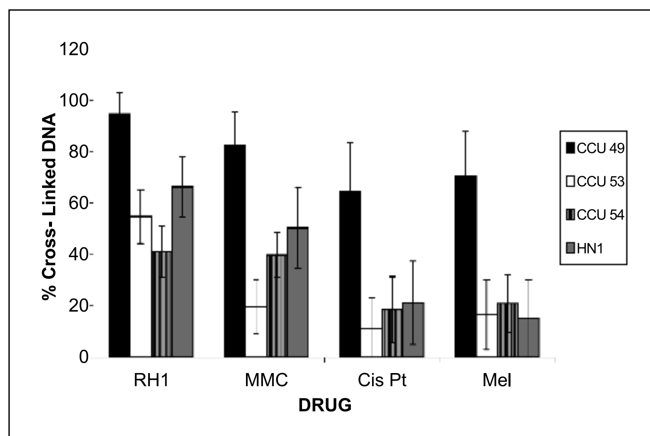

Fig. 3. DNA cross-linking in primary tumor cell cultures after a 2-hour exposure to 20 nmol/L RH1, 1 μmol/L MMC, 3 μmol/L cisplatin, or 3 μmol/L melphalan. CCU49, CCU53, and CCU59 were ovarian carcinomas and HN1 an adenoid cystic carcinoma of the head and neck. Point, mean of two slides on which a minimum of 50 comets was scored; *bars*, ±SE.

substantial cross-linking (20-50%). The level of DNA cross-linking in tumor tissue achieved when RH1 was delivered in cyclodextrin was significantly ($P < 0.01$) higher than obtained using saline.

Discussion

Drug accumulation. The novel bioreductive anticancer drug RH1 has now entered a phase I clinical trial. We describe here the first comprehensive report of its pharmacodynamic characteristics in human blood, cell lines, xenografts, and primary tumor cultures.

RH1 accumulates in cultured cells in a time- and dose-dependent manner. Intracellular accumulation reaches steady state after 2 hours of exposure corresponding to the maximum level of DNA interstrand cross-links measured by the comet-X assay. We have previously reported similar accumulation studies using radiolabeled AZQ and BZQ, two diaziridinyl benzoquinones structurally similar to RH1 (48). In these studies, BZQ is rapidly accumulated in cells, equilibrium being reached within 30 minutes. In contrast AZQ continued to accumulate slowly over several hours. The reason for this difference was attributed to the ease of reduction of AZQ by one-electron reductases to the

Table 1. Repair of DNA cross-links

	% DNA repair			
Cell line	**RH1**	**MMC**	**Melphalan**	**Cisplatin**
CCU 49	18.6	23.6	41.6	16.6
CCU 53	43.9	47.2	67.9	22.3
HN1	14	37.6	3.3	31.1

NOTE: The amount of cross-linked DNA repaired was calculated from [(1 − the fraction of DNA cross-linked at 24 hours) / (the fraction of DNA cross-linked at 2 hours)] × 100. Each point is a mean of two slides on which minimum of 50 comets were scored, ±SE.

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Table 2. Cytotoxicity of RH1 and five anticancer drugs measured in primary tumor cultures

	Drugs						
Cell Line	RH1	MMC	Cisplatin	Melphalan	Doxorubicin	Paxclitaxol	NQO1 activity
HN1	7.1	1,750	>5,000	60,000	80	6	ND
CCU53	7.5	>100	>2,000	>5,000	18	196	ND
CCU54	2.6	100	930	9,000	135	15	ND
CCU49	1	80	900	3,500	20	15	826 (53)

NOTE: Each value represents the mean IC_{50} from three individual curves. The coefficient of variation for each mean was <20% in each case. NQO1 activity as measured by the 2,6-dichlorophenol-indophenol assay is expressed as nmol/min/mg protein; value in parenthesis denotes SD.
Abbreviation: ND, not detected.

semiquinone, which could then be effluxed from the cell. Indeed it is possible that reducing such quinones to either the semiquinone or hydroquinone enables cells to effectively reduce the intracellular drug concentration by conjugation and/or efflux. BZQ is a poor substrate for either one or two electron reduction and is consequently rapidly equilibrated without significant efflux.

RH1 is accumulated with kinetics intermediate between BZQ and AZQ. RH1 is a poorer substrate for the one-electron reductases than AZQ and some studies suggest that its cytotoxicity is not increased in cells overexpressing such enzymes (46, 49). RH1 is however, efficiently reduced to its hydroquinone by NQO1 activity. RH1 accumulation using the NQO1-deficient cell line BE was identical to the cell lines described here (data not shown), suggesting such accumulation is not influenced by NQO1 activity and that the generation of the hydroquinone species does not facilitate enhanced efflux. General drug efflux is however occurring with RH1 because intracellular drug concentrations are reduced to 30% of their maximum over a period of several hours. The cause of this is unclear and may simply be due to degradation the drug in the culture medium, although RH1 is stable in buffer at temperatures below 40°C for over 26 hours (50). Alternately, there could be changes in membrane permeability occurring at high drug concentrations. However, it should be stressed that no morphologic changes were observed over this time period. RH1 accumulation in PBMCs was slow only reaching a maximum after 4 hours of exposure. The half-life of RH1 in human blood has been previously reported as 1.16 hours (42) in spiked samples. Although PBMCs contain little measurable NQO1 activity, human plasma does (38-48 nmol per minute per mg protein). Whereas this is lower than in murine plasma, it may still be sufficient to reduce RH1 to its hydroquinone, whereupon it would become much more easily glucuronidated and consequently less likely to accumulate within cells.

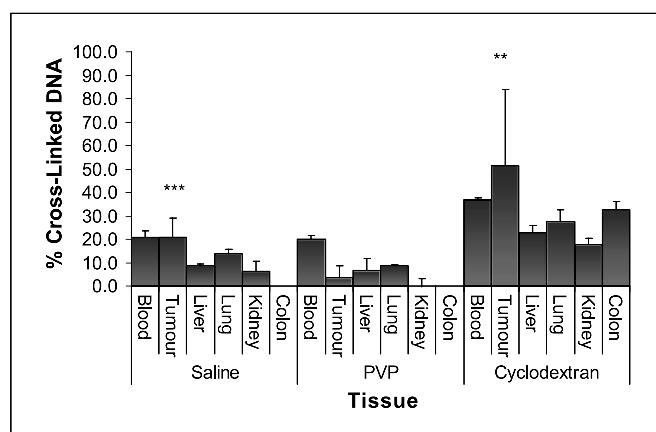

Fig. 4. Biodistribution of DNA cross-links in mouse tissue and human tumor xenografts 4 hours following a single i.p. injection of RH1 (0.56 mg/kg). *Column,* mean of two slides from each of two animals. A minimum of 50 cells per slide was measured. ***, $P < 0.01$, statistically significant compared with RH1 delivered in saline.

DNA interstrand cross-linking. The results from the comet-X assay show that production of DNA interstrand cross-links is both time and dose dependent. Cross-linking essentially mirrors intracellular drug accumulation and is maximal within 2 to 4 hours. The comet-X assay is sensitive enough to discriminate differences between responsive and drug-resistant cells (43) as well as PBMCs from patients before and after treatment with alkylating agents (51). In the present study, DNA interstrand cross-links were detected after as little as 1 nmol/L RH1 treatment. In addition, the low dose-dependent accumulation of DNA cross-links observed in NQO1-deficient BE cells can be enhanced by transfecting functional NQO1 back into these cells. Clearly, RH1 can be activated in the absence of NQO1 activity; however, the presence of even moderate levels of NQO1 (7-fold less than that seen in H460 cells) enhances activity greatly. These results are in good agreement with previous reports using these cell lines (31). PBMCs isolated from whole blood treated with RH1 show low levels of DNA cross-links consistent with the slow drug accumulation described above. Our previous studies on human and mouse PBMCs have shown NQO1 activity to be very low and much lower than the minimum required for enhanced sensitivity to RH1 (31).[5]

Many reports suggest bioreductive drugs can be activated by both one- and two-electron reducing enzymes. The principle one electron reducing enzyme involved in the bioreduction of drugs is NADH:cytochrome *P*450 reductase. Whereas the P450 reductases are widely distributed in the NCI 60 tumor cell line panel, they do not seem overexpressed in any particular tumor type (27). One-electron reductases have a role in chemotherapy

[5] <20 nmol per minute per mg protein, unpublished data.

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because they are able to form reactive radicals under both aerobic (oxygen-derived species) and hypoxic (drug-derived species) conditions. RH1 can be reduced by other reductases such as P450 reductase, b5 reductase, and xanthine oxidase and subsequently react with nucleophiles such as DNA. RH1 will therefore be activated in cells and tissues lacking NQO1 activity and produce reactive species. Whereas this occurs at concentrations much higher than in cells overexpressing NQO1, the drug is still extremely potent at nanomolar concentrations. Drugs which have a reduction potential lower than that of oxygen (−155 mV) can, in fully oxygenated cells, undergo futile redox cycling, resulting in reduced cytotoxicity (12). RH1 has a reduction potential of −220 mV and could potentially redox cycle in air that may result in reduced cytotoxicity, particularly in cells expressing little or no NQO1 activity. A possibility supported by the fact that cell lines with very low NQO1 but overexpressing P450 reductase do not show enhanced sensitivity to RH1 (49). NQO1 has been reported to be up-regulated under hypoxic conditions (33, 34). Consequently, under hypoxia, RH1 is likely to be more stable following any one-electron reduction. Furthermore, in cells that also overexpress NQO1, it may exhibit enhanced cytotoxicity. Consequently, NQO1 is pivotal for a more rapid and extensive activation of RH1.

Whereas many studies clearly show RH1's excellent antitumor activity, no attempt has been made to assess the degree of DNA cross-linking in either tissues or tumor. Whole animal biodistribution studies measuring DNA damage following alkylating agent treatment have been conducted using the comet assay (52). We believe however that this is the first report of a biodistribution study of a bioreductive anticancer drug delivered in three different vehicles.

A clear increase in DNA cross-linking is observed when RH1 is delivered in cyclodextrin compared with saline, a vehicle commonly used in studies such as this (40, 41, 46). DNA cross-links were observed in all target tissues examined when using cyclodextrin, but by far, the highest level was observed in the tumor. In contrast, PVP seemed much less favorable in producing cross-links, especially to tumor tissue. Both blood and tumor gave the highest level of cross-links using either saline or cyclodextrin to deliver RH1. This data suggest that drug delivery and pharmokinetic determinants may play an equally important role to enzymology *in vivo*. Clearly, cross-linking is occurring in all tissues measured, but overall it is much less than that observed in the NQO1-rich tumor. Cross-linking levels are lowest when RH1 is delivered in PVP with a reduction in the differential between tumor and normal tissue. Cross-linking *in vivo* (20-40%) correlates well with that measured *in vitro* RH1 spiking experiments (30%). These results suggest that detection of DNA cross-links in PBMCs and biopsy material is feasible and may prove consistent with results from others using clinically derived material (51). Interestingly, levels of cross-links in kidney seem the lowest of all tissues examined using all three vehicles. This is consistent with the observation that RH1 is metabolized slowly in mouse kidney compared with the bioreductive drug EO9 (42). It is conceivable that this may predict low or no renal toxicity in man, despite high levels of NQO1 in kidney podocytes (22, 23). Clearly, the use of cyclodextrin as a vehicle for RH1 may enhance this drugs efficacy.

***Ex vivo* chemosensitivity.** The screening of primary tumor material in *ex vivo* assays to determine chemosensitivity has been explored and yielded variable results. Correlations between patient response/survival and performance in such assays has been observed in common cancers such as liver, ovary, and stomach (53–55). We have previously reported the use of *ex vivo* drug screening in human tumor explants using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and the comet-X assay (15). In this study, we have attempted to directly compare the novel anticancer drug RH1 with existing clinical drugs. RH1 is clearly highly toxic in the nanomolar range of concentrations, much more so than either doxorubicin or paclitaxol (10- to 20-fold more toxic). Moreover, RH1 is some 50 times more toxic than the pro typical bioreductive drug MMC. The cross-linking potency of RH1 is superior to both MMC and cisplatin, with RH1 producing cross-linking at nmol/L concentrations as apposed to μmol/L concentrations for the MMC or cisplatin.

The repair of drug induced DNA cross-links is important and can be followed using the Comet assay (51, 56). In this study, estimations of DNA cross-linking after 2 hours of treatment followed by a 24 drug-free recovery phase suggests that RH1 adducts are indeed repaired. The rate of repair seems similar to that of either cisplatin or MMC in ovarian tumors but less so in the head and neck tumor line. Both the cytotoxcity and cross-links induced by RH1 are much higher in the ovarian tumor culture CCU49. The NQO1 activity of this culture is substantial (826 nmol per minute per mg protein) but not detectable in the other cultures tested. This supports our suggestion that by comparison the efficacy of RH1 is much higher in tumors and cell lines that express high levels of NQO1.

In conclusion, RH1 is a novel bioreductive-alkylating agent activated by reduction to a potent DNA cross-linking agent. This cross-linking ability is enhanced in tumors that overexpress NQO1. It has excellent *in vivo* antitumor activity and drug efficacy is improved when the drug is delivered in cyclodextrin rather than saline or PVP. It is currently undergoing a phase I clinical trial at the Christie Hospital and Southampton General Hospital. Using the validated comet-X assay described here, DNA interstrand cross-linking in peripheral blood lymphocytes and tumor biopsy material will be measured as a primary pharmacodynamic end point.

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