The importance of DT-diaphorase and hypoxia in the cytotoxicity of RH1 in human breast and non-small cell lung cancer cell lines

J. Y. Kim[a,b], A. V. Patterson[c], I. J. Stratford[c] and J. H. Hendry[a]

The diaziridiny/benzoquinone RH1 is shortly to enter a phase I clinical trial. The drug was originally designed as a substrate for the enzyme DT-diaphorase (DTD) such that metabolic activation of the drug would lead to toxicity. To evaluate this, we have measured the toxicity of RH1 in a pair of non-small cell lung cancer (NSCLC) cell lines of widely differing levels of DTD and in MDA231 breast cancer cells which have been engineered to overexpress DTD. In addition, we have explored the importance of the putative one-electron reductase, P450 reductase, by assessing the toxicity of RH1 in MDA231 cells engineered to overexpress the enzyme. All drug exposures were carried out under hypoxic and aerobic conditions. Those cells with the highest levels of DTD, i.e. D7 versus MDA231 wt and H460 versus H596, are substantially more sensitive to RH1 than the cell lines expressing low DTD activity. Those cells with the lowest levels of DTD activity, i.e. MDA231 wt, R4 and H596, show much greater sensitivity to RH1 under hypoxic conditions compared to aerobic conditions. Finally, overexpression of P450 reductase, i.e. comparing MDA231 wt with R4, has little, if any, impact on the toxicity of RH1 under hypoxic or aerobic conditions. In summary, RH1 can be effective in killing cells containing high levels of DTD and may be useful in treating tumors expressing this enzyme. *Anti-Cancer Drugs* 15:71–77 © 2004 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2004, **15**:71–77

Keywords: bioreductive agent, DT-diaphorase, hypoxia, RH1

[a]Cancer Research UK Group of Experimental Radiation Oncology, Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Manchester, UK, [b]National Cancer Center, Korea and and [c]Department of Pharmacy and Pharmaceutical Sciences, University of Manchester, UK.

Correspondence to I. J. Stratford, School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Oxford Road, Manchester M13 9PL, UK. Tel: +44 161 275 2487; fax: +44 161 275 8342; e-mail: ian.stratford@man.ac.uk

Received 27 July 2003 **Revised form accepted** 17 September 2003

Introduction

The enzyme DT-diaphorase (DTD, NQO1 [NAD(P)H quinone oxido-reductase]) is of major importance for activating quinone-based alkylating agents and markedly elevated levels of this enzyme have been reported in a range of human tumors. The observation that survival of 42 non-small cell lung cancer (NSCLC) patients who were treated with the mitomycin C (MMC)/ifosfamide regimen was better than that of the rest of the patients [1], coupled with the fact that human NSCLC tissues overexpress the enzyme NQO1, accelerated the search for the development of more specific NQO1 enzyme-directed bioreductive agents. However, although the development of more specific prodrugs metabolized by NQO1 has been pursued for some time, MMC is still the only drug which has been routinely used in a clinical setting. Despite its active clinical use as a bioreductive agent, the toxicity of MMC often prevents its wider use in the clinic and the mechanism of its bioactivation is not fully understood. DTD is known to be the major enzyme responsible for the aerobic toxicity of MMC and a positive correlation of the level of DTD activity with the clinical tumor response to MMC has been reported by some authors [2,3]. MMC also shows greater toxicity towards hypoxic relative to aerobic tumor cells. Generally, this hypoxic cytotoxicity is moderate and the mechanism of MMC cytotoxicity under hypoxic conditions still remains to be fully elucidated [4,5].

For a new generation of bioreductive drugs to be clinically useful, they should be more selective to tumor tissues, either by the enhanced distribution of metabolizing enzymes toward the tumor tissues or by higher hypoxic selectivity. RH1 has been developed as a DTD-directed bioreductive agent and is on the verge of entering a clinical phase I study. RH1 is more water soluble compared to its parent compound methyl diazyridinine quinone (MeDZQ) and its cytotoxicity more specifically depends on the presence of DTD than previously developed indoloquinone bioreductive agents [6,7]. We have previously studied the cytotoxicity of RH1 and its combined effect with γ-irradiation on three isogenic breast cancer cell lines [8], which showed that RH1 had increased cytotoxicity in cells overexpressing DTD. In the present study, the cytotoxicity of RH1 was examined under aerobic and hypoxic conditions using a pair of human NSCLC cell lines with contrasting DTD activity [naturally DTD-high and DTD-null cancer cells (H460/H596)]. We also studied three isogenic breast cancer cell lines, MDA 231 wild-type cells which have no DTD

0959-4973 © 2004 Lippincott Williams & Wilkins DOI: 10.1097/01.cad.0000109531.35346.1b

activity by virtue of gene mutation, and two stably transfected lines D7 and R4 expressing high levels of DTD and P450 reductase, respectively. The benefit of this investigation is that we are more aware of the fact that a different genetic background of the cells may affect the selectivity of RH1 based on DTD. In addition to this, knowledge of the cytotoxicity of RH1 in different tumor cell types would help to select the patient group which would be most suitable for the clinical use of this new drug. To pursue these objectives, we used two endpoints in measuring cytotoxicity, i.e. growth inhibition and clonogenic survival, to compare our results to other reports which used one or other of the assays. Second, the cytotoxicity of RH1 under hypoxic conditions was investigated in parallel to that under oxic conditions. Since the reduction pathway and the role of different reducing enzymes are not fully understood under different microenvironmental conditions for this agent, we also investigated the possible role of the one-electron reduction enzyme, using a P450 reductase-transfected MDA231 cell line.

Materials and methods

Cells and cultures

The NCI H460 and 596 cell lines were derived from large cell and squamous cell carcinomas of the lung, respectively. Cells were obtained from the NCI. The MDA MB231 cell line was established from an advanced human breast cancer. It has a homozygous mutation for a 609 C → T in the NQO1 gene, giving a Pro → Ser187 amino acid sequence change, resulting in the loss of enzymatic activity [9]. It is also known to express low levels of P450 reductase [10]. Cells were maintained at 37°C in RPMI 1640 medium, supplemented by 1% glutamine and 10% fetal calf serum. The MDA231 D7 and R4 cells were derived by transfection of the human NQO1 gene and P450 reductase gene, respectively, into the parent cell line [11]. Puromycin (3 μg/ml) was added to the medium to maintain the clonal characteristics of the cells [9,12,13].

Enzyme measurement

Cell pellets were collected every 10 passages from all three cell lines and were examined for enzyme activity.

Preparation. of cell lysates

Cells ($1–2 \times 10^7$) in exponential growth phase in T175 flasks were washed twice with phosphate-buffered saline (PBS) and harvested using EDTA/trypsin. Following centrifugation at 13 000 r.p.m., 50–100 μl of protease inhibitor was added to the cell pellets according to the pellet size. Cell pellets were sonicated for 10 s twice and allowed to stand for 10 min in ice before being repelleted by centrifugation at 13 000 r.p.m. for 10 min. All samples were kept in ice during the procedure. The protein concentration of the cell lysates was determined by the Bio-Rad protein estimation method, using bovine serum albumin (BSA) as the standard.

DTD activity

The activity of DTD was determined spectrophotometrically as the dicoumarol-inhibitable proportion of the NADH-dependent reduction of dichloro indophenol (DCPIP) [14]. The method uses DCPIP as an electron acceptor, which loses color upon reduction. Dicoumarol, a diaphorase-specific inhibitor, was used to confirm the specificity of the reaction with DCPIP and to exclude the possible role of other reductases. The amount of DCPIP remaining in the samples with and without dicoumarol was determined using the corresponding standard curves. Briefly, 100 μl of extract was placed in an acid-cleaned quartz cuvette containing 2.7 ml of buffer (25 mM Tris–HCl, pH 7.4, 700 μg/ml BSA), 100 μl of NADH (6 mM) and 100 μl of DCPIP (1.2 mM). The cuvette was rapidly shaken and the absorbance at 600 nm was read over 100 s using a sample with no enzyme as a reference. The assay was then repeated with a fresh sample containing 10 μl of the inhibitor dicoumarol (10 mM in DMSO). The dicoumarol sensitive activities (the rate of OD change without inhibitor – rate of OD change with inhibitor) were used as a measure of DTD activity. The final activities were calibrated against protein concentration and expressed as nM/min/mg protein.

NADPH:cytochrome P450 reductase activity

NADPH:cytochrome P450 reductase is a microsomal flavoprotein enzyme and its activity was determined spectrophotometrically as the NADPH-dependent reduction of cytochrome *c* which was used as an artificial electron acceptor. In this assay, oxidized (ferric) cytochrome *c* is converted to reduced (ferrous) cytochrome *c* which, unlike the oxidized form, has a characteristic absorption maximum at 550 nm. Each sample comprised 400 μl of cytochrome *c* (final concentration 50 μM) and 100 μl of 10 mM potassium cyanide (final concentration 1 mM) and 10–300 μg lysate protein (10–100 μl volume) made up to 0.98 ml with 100 mM phosphate buffer, pH 7.6. The content was gently mixed and the sample was put into the 37°C compartment of the spectrophotometer. The enzyme reaction was started by adding 20 μl of 10 mM NADPH (final concentration 200 μM) to the test cuvette and the increase in absorbance with time was recorded at 550 nm for 3 min against a blank without NADPH. Initial rates of reaction were based on an extinction coefficient of 21 mM^{-1} cm^{-1} calculated [15] and expressed as nmol cytochrome *c* reduced/min/mg of cell lysate protein.

Clonogenic cytotoxicity

Cells (5×10^4 to 10^5) were seeded in T25 or T75 flasks and grown to day 3–4 to obtain cells in exponential growth phase. The number of the cells by the time of drug treatment was kept constant around 2×10^5.

Table 1 The DTD and P450 reductase enzyme activity of the cells

Cells	Transfected with	Original activity of the enzymes	
		DTD[a] (nmol/min/mg protein)	P450 reductase (nmol cytochrome *c*/min/mg)
MDA231 wt	–	19	5.5
D7	DTD	5570	21
R4	P450 reductase	21	337
H460		580	17.6[b]
H596		0	7.3[b]

[a]Apparent dicoumarol-inhibited activity.
[b]Saunders *et al.* [22]

For drug treatment, monolayer cell cultures were rinsed twice with sterile PBS and replaced with medium containing 0–30 nM of RH1. Different concentrations of RH1 were prepared immediately before each experiment by dilution of 1 mM RH1 frozen stock solution into the medium without puromycin. The cytotoxic effect of RH1 was determined by exposing cells to drug for 3 h under oxic and hypoxic conditions at 37°C. For hypoxic experiments, the cells were grown in the dishes with low oxygen solubility (Permanox) until the time of the experiments and the dishes were moved into the hypoxic incubator as soon as the drug was administered. After 3 h of drug exposure, cells were rinsed with EDTA and trypsinized for a standard clonogenic cell survival assay. Plating numbers were selected between 2×10^2 to 1×10^5 per dish depending on the plating efficiency of the cells and the drug doses, so that the optimal colony numbers of 10–100 could be obtained at the incubation. For every dose of drug, at least three different dilutions of cell solutions were seeded with three replicate dishes for each dilution. Individual experiments were repeated 2–4 times. After plating, dishes were incubated at 37°C for 14 days, and colonies were stained and fixed with 1% crystal violet. Colonies with over 50 cells were counted.

MTT growth inhibition assay

To optimize the conditions for the assay, preliminary experiments were performed for each cell line, testing various cell numbers between 1200 and 3000 per well and incubating the plate for 5–8 days. The number of seeded cells and the incubation period after drug treatment was carefully selected for the individual cell line to make certain that they underwent at least five or six cell doublings and the optical density for each untreated control well was in the range of 1.5–2 when measured at the end of the incubation. Briefly, cells in exponential growth phase were washed twice in sterile PBS and harvested by trypsinization. After counting, cells were seeded into 96-well plates except for the first column, which contained medium only. After allowing the cells to attach for 4 h, the RH1 stock solution was diluted into eight different concentrations and 100 µl of drug solution was added to each well. All hypoxic exposures were conducted under conditions where residual oxygen in the gas phase was removed by passage over palladium catalyst in the *In Vivo* 400 hypoxic incubator. All plastics and media were pre-incubated in hypoxia for at least 24 h to remove any residual oxygen, prior to use. After 3 h of incubation under aerobic and hypoxic conditions, the drug was removed and the wells were rinsed twice with PBS. An aliquot of 200 µl of new medium was added and the plates were incubated in a 37°C 95% O_2/5% CO_2 incubator for 5–7 days depending on the cell lines. MTT (50 µl, 3 mg/ml) was added at the end of incubation and then aspirated after 3 h. The formazan crystals were solubilized in 200 µl DMSO and the plates were read at 540 nm by a spectrophotometer with subtraction at 690 nm.

Results

Enzyme activity of the cells

DTD and P450 reductase activity was determined for all cell lines, and values are given in Table 1. For the transfected cell lines, cell pellets were collected every 10 passages and were examined to test the stability of the transfection. The activity of DTD remained within the range of 6000–13 000 nmol/min/mg protein for D7 cells. For the R4 cell line, the activity of the P450 reductase varied in the range of 60–100 nmol cytochrome *c*/min/mg. Thus, although there was some change in the activity of each enzyme during passage, this was not systematic and in each case enzyme activity was substantially greater than the corresponding parental cells. The other tumor cells showed no change in levels of DTD and P450 reductase activity during passage.

Drug-sensitivity studies

We first established that the experimental conditions we used were sufficient to induce hypoxia. To test this we used the agent tirapazamine and assessed the toxicity of the drug by MTT assay following hypoxic or aerobic exposure. Values of clonogenic survival of MDA231 wt, R4 and D7 cells are given in Table 2, and clearly indicate that, at the drug concentration used, exposure to tirapazamine under the conditions we have used to generate hypoxia gives substantially greater cell killing. We infer from this and from previous experience in our laboratory that the experimental conditions are sufficient for us to regard them as inducing hypoxia.

Table 2 Tirapazamine cytotoxicity under oxic and hypoxic conditions

Cells	Tirapazamine concentration (μM)	Cell survival		SR (oxic/hypoxic)
		Oxic	Hypoxic	
MDA231				
R4	5	0.30	0.0022	140
WT	30	0.71	0.0021	340
D7	30	0.56	0.0010	560

A single tirapazamine dose was used for each experiment and the doses were chosen according to the suggested IC_{50} dose for breast and lung cancer cell lines [20–22].

Table 3 The cytotoxicity of RH1 measured by IC_{50} growth inhibition

Cell line	IC_{50} (nM) ± SD (MTT)[a]		HCR
	Oxic	Hypoxic	
MDA231 wt	10.7 ± 0.17	1.7 ± 0.17	5.8 ± 0.79
D7	0.5 ± 0.03	0.3 ± 0.03	1.7 ± 0.3
R4	8.9 ± 1.31	1.1 ± 0.39	10.2 ± 1.78
H460	1.2 ± 0.08	0.7 ± 0.11	3.6 ± 0.83
H596	42.9 ± 16.1	4.5 ± 0.30	9.9 ± 0.88

For the work with RH1 we used the MTT assay to derive values of IC_{50}, the concentration required to reduce proliferation by 50%, in each of the cell lines under aerobic and hypoxic conditions. These values of IC_{50} are given in Table 3 and representative growth inhibition curves are given in Figure 1. The results show three major findings.

(i) As predicted, those cells with highest levels of DTD, i.e. D7 versus MDA231 wt and H460 versus H596, are substantially more sensitive to RH1 than the cell lines expressing low activity of DTD.
(ii) Those cells with the lowest levels of DTD activity, i.e. MDA231 wt, R4 and H596, show much greater sensitivity to RH1 under hypoxic conditions compared to aerobic conditions.
(iii) Overexpression of P450 reductase, i.e. comparing MDA231 wt with R4, has little, if any, impact on the toxicity of RH1 under hypoxic or aerobic conditions.

These observations were confirmed in the MDA231 cell lines by carrying out RH1 toxicity experiments under hypoxic and aerobic conditions, and assessing drug sensitivity by clonogenic assay. Representative survival data is given in Figure 2 and from these curves we derived values of IC_{80}, the concentration required to kill 80% of the cells. These values are given in Table 4 and show that for RH1 the clonogenic survival data completely support the conclusion drawn from the results obtained following the MTT assay.

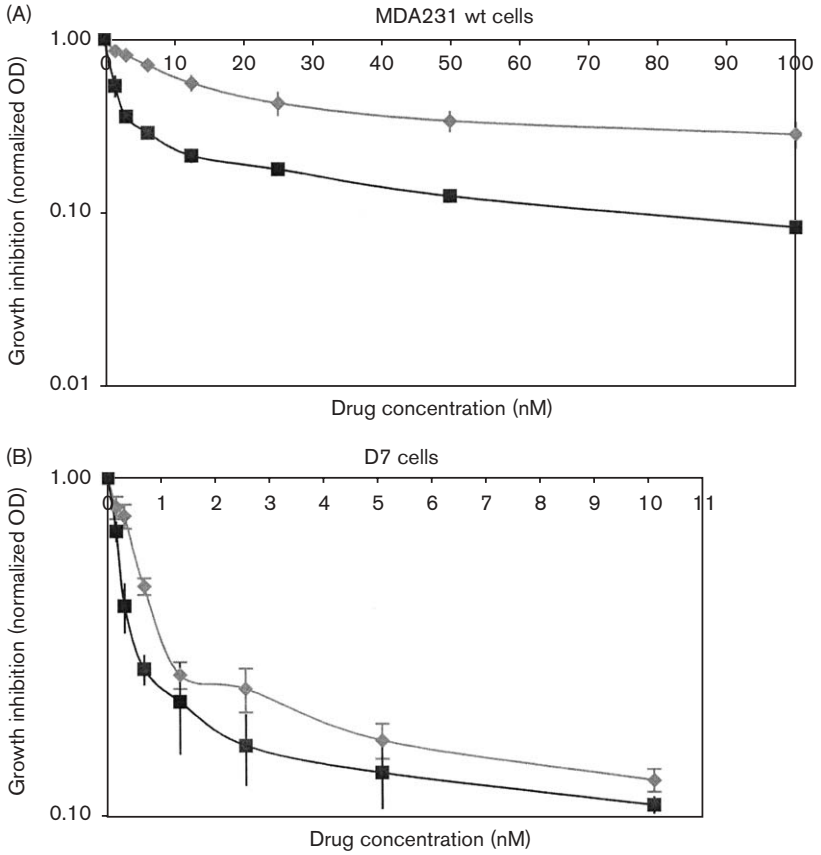
Discussion

The selective toxicity of RH1 based on DTD expression has been studied previously on different sets of cancer cells, HT29 and BE colon cancer cells, MDA231 human breast cancer cells and their DTD-transfected counterparts, and a pair of NSCLC cells lines with differing DTD activity [6–8].

Human NSCLC cells have been of particular interest because of past studies showing that DTD is overexpressed in NSCLC tissues, but not in normal lung [16,17]. In our previous study [8], we found less difference in cytotoxicity between DTD-high and DTD-null human breast cancer cell lines than was previously reported for human NSCLC cells [6,7]. Because the findings from the two studies were based on the different assays measuring cytotoxicity (growth inhibition versus clonogenic assays), we performed a series of experiments to determine the cytotoxicity of RH1 on H460/H596 cell pairs and our isogenic breast cancer cells at the same time, using both clonogenic and MTT growth inhibition assay. Using the MTT assay, a 2-fold higher aerobic toxicity for MDA231 D7 was observed compared to H460 cells and this was recapitulated using the clonogenic assay (data not shown). IC_{50} values are in accordance with the data reported by Winski *et al.* [6,7]. The higher cytotoxicity of the MDA231 D7 cell line is well explained by the higher DTD activity in these transfected cells, which is approximately 10-fold higher than that of naturally DTD-overexpressing H460 cells.

The ratio of IC_{50} for the DTD-rich and DTD-null cells which we define as the selectivity ratio (SR) was 20- and 40-fold for the breast isogenic cell pair and for the H460/H596 NSCLC cell pair, respectively. However, the ratio was much less when measured by clonogenic survival assay for the MDA231 cell pair. A similar trend was observed when a pair of colon cancer cells was used,

Fig. 1

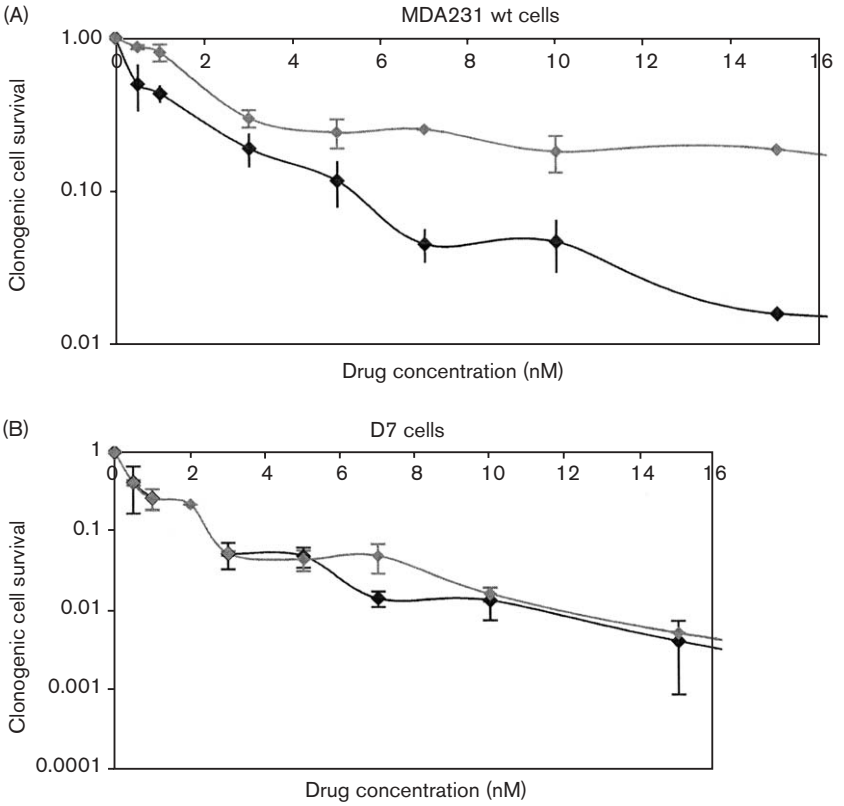


Toxicity of RH1 under aerobic (diamonds) or hypoxic (squares) conditions measured by the MTT proliferation assay. Data points are derived from at least three independent experiments. (A) MDA231 wt cells; (B) D7 cells.

where the SR for HT29 versus BE was shown to be 17 using a growth inhibition assay and 7 using a clonogenic assay [7].

The second objective was to examine the drug's cytotoxicity under hypoxic conditions as opposed to oxic conditions. Bioreductive drugs are generally expected to show more cytotoxicity under hypoxic conditions because the reduction pathway often occurs by a separate one-electron transfer process, which can be reversed by the presence of oxygen, leading to less toxic or easy-to-repair metabolites. Generally for quinone-based bioreductive drugs, hypoxic enhancement of cytotoxicity is not expected to be high since the reduction process is dominated by DTD regardless of the oxygen status and the reduced products are stable in the presence of oxygen. MMC, which is a prototype of the quinone-based bioreductive drug, has a hypoxic cytotoxicity ratio (HCR) of around 3. However, it would be inappropriate to directly compare the HCR of MMC to that of RH1 since the metabolism of MMC is not as strongly dependent on DTD activity as for RH1. Although there are several studies showing the correlation with DTD and P450R activity and the drug's cytotoxicity or tumor response [2], the bioactivation processes for MMC is not fully understood. Our result of RH1 cytotoxicity toward DTD-rich and poor cells is very similar to that reported for another DTD-directed bioreductive agent, EO9. *In vitro* tests of EO9 had shown the high HCR for cells with no DTD activity in a range of 100–300 and 1.0–1.7 for cells with high DTD levels [18,19]. Of note is that the P450 reductase-overexpressing cells show identical values of IC_{50} as the wild-type cells in air and hypoxia. This contrasts with EO9 [20], the nitroimidazole RSU1069 [10] and tirapazamine [21,22]. The toxicity of MMC and porfiromycin under both aerobic and hypoxic conditions was also increased by overexpressing P450 reductase, although the increases in toxicity were smaller than with the other bioreductive drugs [10,23]. Thus, RH1 is unusual in that it does not depend on the one-electron reductive enzyme P450 reductase for toxicity. An explanation for this would be that P450 reductase-mediated metabolism can occur, but the one-electron reduced product is *not* toxic and/or is sufficiently stable

Fig. 2



Toxicity of RH1 under aerobic (light diamonds) or hypoxic (dark diamonds) conditions measured by the clonogenic assay. Data points are derived from at least three independent experiments. (A) MDA231 wt cells; (B) D7 cells.

Table 4 Values of IC_{80} (values were derived by eye from survival curves generated from three independent experiments for each cell line)

Cell line	IC_{80} (nM)		HCR
	Air	Hypoxic	
MDA231 wt	9.3	2.8	3.4
D7	2.5	2.5	1.0
R4	7.8	2.5	3.1

that, in air, back oxidation to the parent compound occurs. Under hypoxic conditions two sequential one-electron reductions occur to convert the quinone to the semi-quinone to the hydroquinone and it is the latter moiety (which in air would be formed directly by DTD) that is the toxic species, this sequential reduction not being dependent on the level of P450 reductase over the range examined.

Although we found an enhanced hypoxic cytotoxicity to RH1, this result should be taken with caution because the hypoxic cytotoxicity of quinone-based bioreductive agents varies in response to the subtle changes in oxygen concentration of the tumor microenvironment. For example, the 3-fold enhanced hypoxic cytotoxicity of MMC was only achieved in extreme hypoxia where cells were radioresistant [4]. In that study, the hypoxic cytotoxicity of MMC was already reduced at 0.02% oxygen (around 0.15 mmHg). It was already pointed out that much of the variation observed between laboratories could be due to the different oxygen levels used in the different experiments [24]. We used a very low oxygen level (below 0.01%) in this study. Further investigation is needed to investigate the cytotoxicity of RH1 in various levels of hypoxia to find out whether the enhanced cytotoxicity of RH1 under hypoxic conditions is exploitable or not.

From the above observations, it is likely that there is difference between cell lines in terms of drug cytotoxicity and this should be taken into consideration in selecting a patient cohort who will benefit from the use of this agent. The variously reported SR values are valuable, but the more important point than SR *per se* obtained from DTD-rich and poor tumor cells would be how this new agent acts on normal cells in a different way compared to DTD-rich or DTD-poor hypoxic tumor cells. At the same time, studies on the pattern of cellular damage and mechanism

of repair in DTD-rich or DTD-poor hypoxic tumors are necessary to predict the appropriate clinical application and the toxicity of this new agent.

References

1 Gurney H, de Campos ES, Dodwell D, Kamthan A, Thatcher N. Ifosfamide and mitomycin in combination for the treatment of patients with progressive advanced non-small cell lung cancer. *Eur J Cancer* 1991; **27**:565–568.

2 Gan Y, Mo Y, Kalns JE, Lu J, Danenberg K, Danenberg P, Wientjes MG, *et al.* Expression of DT-diaphorase and cytochrome P450 reductase correlates with mitomycin C activity and human bladder tumors. *Clin Cancer Res* 2001; **7**:1313–1319.

3 Wang X, Doherty G, Leith M, Curphey T, Begleiter A. Enhanced cytotoxicity of mitomycin C in human tumour cells with inducers of DT-diaphorase. *Br J Cancer* 1999; **80**:1223–1230.

4 Marshall RS, Rauth AM. Oxygen and exposure kinetics as factors influencing the cytotoxicity of porfiromycin, a mitomycin C analogue, in Chinese hamster ovary cells. *Cancer Res* 1988; **48**:5655–5659.

5 Rauth AM, Mohindra JK, Tannock IF. Activity of mitomycin C for aerobic and hypoxic cells *in vitro* and *in vivo*. *Cancer Res* 1983; **43**:4154–4158.

6 Winski SL, Hargreaves RH, Butler J, Ross D. A new screening system for NAD(P)H:quinone oxidoreductase (NQO1)-directed antitumor quinones: identification of a new aziridinylbenzoquinone, RH1, as a NQO1-directed antitumor agent. *Clin Cancer Res* 1998; **4**:3083–3088.

7 Winski SS, Swann E, Hargreaves RH, Dehn DL, Butler J, Moody CJ, *et al.* Relationship between NAD(P)H:quinone oxidoreductase 1 (NQO1) levels in a series of stably transfected cell lines and susceptibility to antitumor quinones. *Biochem Pharmacol* 2001; **61**:1509–1516.

8 Kim JY, West C, Valentine H, Ward T, Patterson A, Stratford I, *et al.* Evaluation of the bioreductive agent RH1 as a potential radiosensitiser. *Radiother Oncol* 2003; in press.

9 Wu K, Eng E, Knox R, Chen S. Demonstration of the activation of prodrug CB1954 using human DT-diaphorase mutant Q104Y-transfected MDA-MB-231 cells and mouse xenograft model. *Arch Biochem Biophys* 2001; **385**:203–208.

10 Patterson A, Saunders M, Chinje E, Talbot D, Harris AL, Stratford IJ. Overexpression of human NADPH:cytochrome *c* (P450) reductase confers enhanced sensitivity to both tirapazamine (SR4233) and RSU 1069. *Br J Cancer* 1997; **76**:1338–1347.

11 Cowen RL, Patterson AV, Telfer BA, Airley R, Hobbs S, Phillips RM, *et al.* Viral delivery of P450 reductase recapitulates the ability of constitutive overexpression of reductive enzymes to potentiate the activity of Mitomycin C in human breast cancer xenografts. *Mol Cancer Therap* 2003; in press.

12 Hobbs S, Jitrapakdee S, Wallace J. Development of a bicistronic vector driven by the human polypeptide chain elongation factor 1 alpha promoter for creation of stable mammalian cell lines that express very high levels of recombinant proteins. *Biochem Biophys Res Commun* 1998; **252**: 368–372.

13 Hoff M, Christoffels V, Labruyere W, Moorman A, Lamers W. Electrotransfection with 'intracellular' buffer. *Methods Mol Biol* 1994; **48**:185–197.

14 Ernster L. DT-diaphorase. *Methods Enzymol* 1967; **10**:309–317.

15 Masters BSS, Williams CH, Kamin H. The preparation and properties of microsomal TPNH-cytochrome *c* reductase from pig liver. *Methods Enzymol* 1967; **10**:565–573.

16 Malkinson AM, Siegel D, Forrest GL, Gazdar GF, Oie HK, Chan DC, *et al.* Elevated DT-diaphorase activity and messenger RNA content in human non-small cell lung carcinoma: relationship to the response of lung tumor xenografts to Mitomycin-D. *Cancer Res* 1992; **52**: 4752–4757.

17 Smitskamp-Wilms E, Giaccone G, Pinedo H, Laan B, Peters G. DT-diaphorase activity in normal and neoplastic human tissues: an indicator for sensitivity to bioreductive agents? *Br J Cancer* 1995; **72**:917–921.

18 Adams GE, Stratford IJ. Bioreductive drugs for cancer therapy: the search for tumor specificity. *Int J Radiat Oncol Biol Phys* 1994; **29**: 231–238.

19 Robertson N, Haigh A, Adams G, Stratford I. Factors affecting sensitivity to EO9 in rodent and human tumour cells *in vitro*: DT-diaphorase activity and hypoxia. *Eur J Cancer* 1994; **30A**:1013–1019.

20 Saunders M, Patterson A, Chinje E, Harris A, Stratford I. NADPH:cytochrome *c* (P450) reductase activates tirapazamine (SR4233) to restore hypoxic and oxic cytotoxicity in an aerobic resistant derivative of the A549 lung cancer cell line. *Br J Cancer* 2000; **82**:561–656.

21 Patterson A, Barham H, Chinje EC, Adams G, Harris AL, Stratford IJ. Importance of P450 reductase activity in determining sensitivity of breast tumour cells to the bioreductive drug, tirapazamine (SR3233). *Br J Cancer* 1995; **72**:1144–1150.

22 Saunders MP, Patterson AV, Chinje EC, Harris AL, Stratford IJ. P450 reductase activates tirapazamine to restore oxic and hypoxic cytotoxicity in a resistant derivative of the A549 lung cancer cell line. *Br J Cancer* 1999; **82**:651–656.

23 Belcourt MF, Hodnick WF, Rockwell S, Sartorelli AC. Differential toxicity of mitomycin C and porfiromycin to aerobic and hypoxic Chinese hamster ovary cells overexpressing human NADPH:cytochrome *c* (P450) reductase. *Proc Natl Acad Sci USA* 1996; **93**:456–460.

24 Brown J, Siim B. Hypoxia-specific cytotoxins in cancer therapy. *Semin Radiat Oncol* 1996; **6**:22–36.