Biochemical Pharmacology, Vol. 59, pp. 831–837, 2000.
© 2000 Elsevier Science Inc. All rights reserved.

ELSEVIER

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00391-3

Pharmacological Properties of a New Aziridinylbenzoquinone, RH1 (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone), in Mice

Paul M. Loadman, Roger M. Phillips, Lissa E. Lim and Mike C. Bibby*
CLINICAL ONCOLOGY UNIT, UNIVERSITY OF BRADFORD, BRADFORD BD7 1DP U.K.

ABSTRACT. RH1(2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone) has shown preferential activity against human tumour cell lines which express high levels of DTD (EC 1.6.99.2; NAD(P)H:quinone oxidoreductase, NQO1, DT-diaphorase) and is a candidate for clinical trials. EO9 (3-hydroxy-5-aziridinyl-1-methyl-2-[1*H* indole-4,7-dione]prop-β-en-α-ol) is a known substrate for DTD but clinical trials were disappointing, as a result of rapid plasma clearance and reversible dose-limiting kidney toxicity. It is an obvious concern that RH1 does not exhibit the same limitations. We therefore describe the antitumour activity and pharmacology of RH1 in mice and compare its pharmacological characteristics to those of EO9. Significant antitumour activity ($P = 0.01$) was seen for RH1 (0.5 mg/kg, i.p.) against the high DTD-expressing H460 human lung carcinoma. Pharmacokinetic analysis of RH1 in mice showed a $t_{1/2}$ of 23 min with an area under the curve of 43.0 ng hr mL^{-1} resulting in a calculated clearance of 5.1 mL min^{-1}, 10-fold slower than EO9. RH1 was also more stable than EO9 in murine blood, where the breakdown was thought to be DTD-related. NADH-dependent microsomal metabolism of RH1 and EO9 in both liver and kidney was slow (<100 pmol/min/g tissue), reflecting the low microsomal DTD expression (<35 nmol/mg/min). Liver cytosol metabolism was rapid for both compounds (>4500 pmol/min/g tissue), although DTD levels were low (21.4 ± 0.6 nmol/mg/min). DTD activity in the kidney cytosol was high (125 ± 8.2 nmol/mg/min) and EO9 was rapidly metabolised (4396 ± 1678 pmol/min/g), but the metabolic rate for RH1 was 7-fold slower (608 ± 86 pmol/min/g), even though RH1 was shown to be an excellent substrate for DTD (V_{max} = 800 μmol/min/mg and a K_m of 11.8 μM). The two DTD substrates RH1 and EO9 are clearly metabolised differently, suggesting that RH1 may have different pharmacological properties to those of EO9 in the clinic. BIOCHEM PHARMACOL **59**;7: 831–837, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. DT-diaphorase; EO9; RH1; metabolism; pharmacokinetics; bioreductive

In the search for more selective anti-tumour agents, there is considerable interest in the field of bioreductive drug development. Bioreductive drugs are designed to take advantage of some of the unique features of solid tumours, in particular reduced oxygen tension and the overexpression of certain reductase enzymes [1]. DTD† (EC 1.6.99.2) is one of several reductase enzymes that are capable of reducing quinone-type compounds to DNA-damaging species and is able to use NADPH or NADH as an electron donor [2]. Other reductases include the one-electron reductases NADPH-cytochrome P450 reductase and NADH cytochrome b_5 reductase. However, interest has focused on the two-electron reductase DTD, as it has been shown to be overexpressed in certain clinical tumours, in particular NSCLC (non-small cell lung carcinoma) [3, 4]. DTD has therefore been identified as a target for drug development and its current status has recently been reviewed [5].

Several quinone-containing anticancer agents can be reduced by DTD, including the aziridinylbenzoquinones AZQ (2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone; diaziquone) [6] and MeDZQ, with the antitumour antibiotic mitomycin C being probably the most well known and clinically most successful of the compounds whose activity can be influenced by DTD [7]. RH1 (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone) (Fig. 1), has recently been synthesised as a water-soluble analogue of MeDZQ and has been shown to have preferential activity against human tumour cell lines which express high levels of the two-electron reductase DTD [8]. It is currently a candidate for clinical trial in both the U.K. and the U.S.A., and as an extension of these studies we describe here the first antitumour data for RH1 against the high DTD-expressing human lung carcinoma, H460.

EO9 (3-hydroxy-5-aziridinyl-1-methyl-2-[1*H* indole-4,7-dione]prop-β-en-α-ol) is a synthetic indoloquinone related

* Corresponding author: Dr. Paul M. Loadman, Clinical Oncology Unit, University of Bradford, Bradford BD7 1DP, U.K. Tel. +44 (01274) 233226; FAX +44 (01274) 233234; E-mail: P.M.Loadman@Bradford.ac.uk

† *Abbreviations:* DTD, (EC 1.6.99.2; NAD(P)H:quinone oxidoreductase, NQO1); DMXAA, 5,6-dimethylxanthenone-4-acetic acid; AUC, area under the plasma concentration versus time curve; MeDZQ, (2,5-diaziridinyl-3,6-dimethyl-1,4-benzoquinone) and DCPIP, 2,6-dichlorophenolindophenol.

Received 8 June 1999; accepted 21 September 1999.

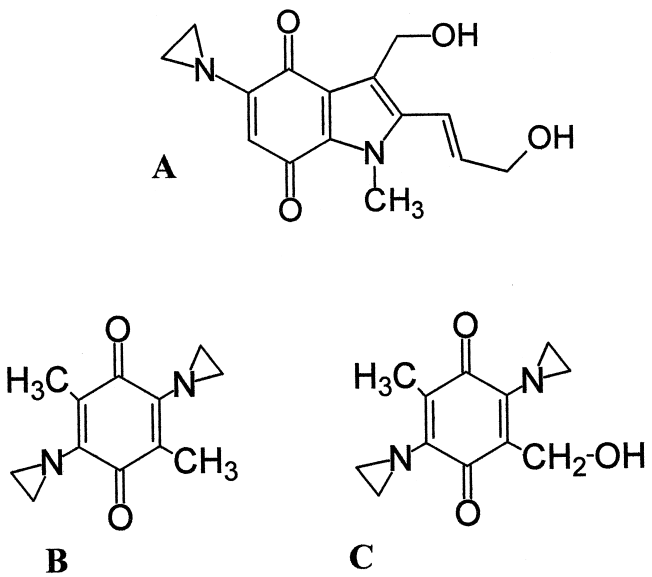


FIG. 1. Chemical structures of (A) EO9, (B) MeDZQ, and (C) RH1.

in structure to mitomycin C and a particularly good substrate for DTD [9]. Preclinical antitumour activity was previously demonstrated for EO9 against several solid tumour types [10]. This preclinical activity allowed EO9 to go to clinical trial. DTD-rich tumours are sensitive to substrates such as EO9 under aerobic conditions in *in vitro* experimental tumour systems and a good correlation exists between enzyme expression and sensitivity [11]. However, in the hypoxic fraction the influence of other one-electron reductases would assume more importance, and so tumours with low DTD expression may also be sensitive to EO9 [11]. Unfortunately, the results of an EORTC clinical trial of EO9 were disappointing, with no responses seen in the phase II trial [12, 13]. Several explanations have been given for this lack of activity. Two of the key problems identified were the rapid plasma clearance in conjunction with poor penetration through avascular tissue, resulting in poor drug delivery of EO9 to the tumour [14, 15], and the dose-limiting but reversible kidney toxicity in the form of proteinuria [12]. The rapid clearance of the drug with a $t_{1/2}$ of 10 min in patients [14] was predictable from preclinical data, as the plasma half-life in rodents was 3 min or less [16, 17]. With the benefit of hindsight, the kidney toxicity may also have been expected due to the presence of DTD in normal kidney tissue [18]. It is an obvious concern that RH1 and subsequent DTD substrates not suffer the same limitations and that lessons are learned from the failure of EO9. Recent studies addressing the pharmacological problems associated with EO9 have shown that the rapid clearance of EO9 in mice is in part due to the rapid metabolism of EO9 in red blood cells [19]. Murine blood has high DTD levels relative to human blood in which EO9 is stable. It follows that other tissues, including the kidneys, which express high levels of DTD are also likely to influence the systemic clearance of EO9 or other related DTD substrates. It is therefore important to understand the factors that influence the pharmacokinetics of RH1, as a short pharmacokinetic half-life or rapid clearance from the systemic circulation is unlikely to allow sufficient time for adequate tumour penetration of the drug into the bioreductive environment in which the drug was designed to act [15].

We therefore propose to study the antitumour activity and pharmacokinetics of RH1 in the mouse and investigate the influence of DTD on the *in vitro* metabolism of RH1 and EO9 in blood, liver, and kidney. These are the first studies to describe the pharmacological properties of RH1 and should give an indication as to whether similar pharmacokinetic problems to those which contributed to failure of EO9 will be encountered with RH1.

MATERIALS AND METHODS

Antitumour Activity

All animal experiments were carried out under a project licence approved by the Home Office, London, U.K., and UK CCCR guidelines [20] were followed throughout. The H460 is a high diaphorase-expressing lung carcinoma [21] and was grown subcutaneously as a xenograft in NCR-Nu mice of approximately 25 g (supplied by the NCI). H460 tumours were transplanted as fragments by trocar. Groups of 5–10 tumour-bearing mice were treated with RH1 at the previously established single i.v. maximum tolerated dose (MTD) of 0.5 mg kg^{-1}. Treatment commenced when tumours could be reliably measured by calipers, and therapeutic effects were assessed by twice weekly caliper measurements of the tumour. Tumour volumes were determined by the formula $a^2 \times b/2$, where a is the smaller and b the larger diameter of the tumour. Graphs were plotted of relative tumour volume against time and antitumour activities assessed by Mann–Whitney analysis of time taken for tumour to double in volume.

Pharmacokinetic Studies

CHEMICALS. All solvents used were of HPLC grade and were purchased from Fisher Scientific. All chemicals were of analytical grade and purchased from Sigma. Triple distilled water was used throughout. RH1 was synthesised by Dr. Robert Hargreaves of the Paterson Institute for Cancer Research, Manchester, U.K. Stock solutions of RH1 and EO9 were prepared in DMSO at a concentration of 5 mM and stored at −20°. DMXAA was a gift from the Cancer Research Campaign, U.K.

CHROMATOGRAPHIC ANALYSIS OF RH1. The HPLC system consisted of a System Gold Beckman 126 Programmable Solvent Module (Beckman Instruments U.K. Ltd.), a Beckman 507 Autosampler, which was cooled to 4° by the Grant Cooling Unit LTD6 (Grant Instruments), and a Beckman 168 Photo-Diode Array Detector. Data were processed using System Gold software (Beckman). Chro-

matographic separation of RH1 was achieved using a RP-18 end-capped LiChrospher column (5 μm, 250 × 4 mm) (Phenomenex), a mobile phase consisting of ammonium acetate (0.0482 M) and methanol (70%:30%) with the pH adjusted to 7.0, and a flow rate of 1.0 mL min^{-1}. Detection was at the λmax for RH1 (328 nm).

CHROMATOGRAPHIC ANALYSIS OF EO9. The analysis of EO9 used the same conditions as for RH1, but with a mobile phase consisting of methanol:water:phosphate buffer (0.5 M, pH 7.0) (43%:56%:1%) and detection at 270 nm [22].

EXTRACTION FROM BIOLOGICAL SAMPLES. Protein was precipitated in biological samples by the addition of acetonitrile:methanol (75%:25%) with a solvent:sample ratio of 3:1. Typically, 100 μL of sample was mixed with 300 μL solvent and centrifuged at 7000 *g* for 5 min. The supernatant (40 μL) was then injected into the HPLC system. Control (drug-free) samples were used both for calibration purposes and to calculate extraction efficiencies. Control samples were spiked with EO9 or RH1 at the appropriate concentration and extracted as described above. Saline spiked with the appropriate concentration of drug was used as 100% control. Recovery of EO9 or RH1 from biological samples was calculated as a percentage of the saline controls. Calibration curves for both RH1 and EO9 were generated over the range of 0–20 μM.

PHARMACOKINETIC SAMPLING. At various time points after administration of RH1 (0.5 mg kg^{-1}, i.v.) to NMRI mice of approximately 25 g weight (B & K Universal), mice were anaesthetised and blood samples taken via cardiac puncture. The blood was kept at 4° until centrifugation (1000 *g* × 5 min at 4°) and the plasma separated and stored at −20°. RH1 was extracted from plasma as described. The pharmacokinetic parameters were estimated by standard non-compartmental methods. The terminal elimination rate (K_{el}) was calculated using linear regression analysis of the terminal log-linear portion of the curve. The AUC was calculated using the trapezoidal rule. V_D, the volume of distribution at t = 0, was calculated from the equation Dose/C(0), where C(0) is the theoretical concentration at t = 0. Clearance was determined as Dose/AUC. The calculations have been described in detail elsewhere [23].

In Vitro *Metabolism Studies*

STABILITY OF RH1 IN BUFFERS. RH1 was diluted to a final concentration of 20 μM. The diluents used were saline, PBS, and a range of phosphate buffers at pH 3.0, pH 5.0, pH 7.0, and pH 8.0 (0.1 M). Samples were taken at timed intervals between 0–3 hr and analysed by HPLC. Peak area values for both drugs were then converted to percentages, with the value at t = 0 representing 100%. Graphs of percentage remaining versus time were plotted and log-linear regression used to calculate $T_{1/2}$ values for each curve.

STABILITY IN WHOLE BLOOD AND PLASMA. NMRI mice were used for blood and metabolism studies so that a direct comparison with the studies for EO9 could be made. Mice were anaesthetised with diethyl ether and whole blood taken by cardiac puncture into heparinized tubes kept on ice. The plasma was obtained following centrifugation of the blood at 4° (7000 *g* for 5 min). Human blood was obtained from healthy volunteers and processed as described for mice. Blood or plasma samples were incubated at 37° and spiked with either RH1 or EO9 to give a final concentration of 20 μM. Samples were taken at timed intervals and the drug extracted with solvent as described previously.

LIVER AND KIDNEY METABOLISM STUDIES. Livers and kidneys were excised from male non-tumour-bearing NMRI mice and placed on ice. They were weighed, diluted 1 in 4 with PBS, and homogenised with an Ultra-Turrax homogeniser (Janke and Kundel). The homogenates were centrifuged at 9360 *g* for 20 min using a Beckman Optima TL Ultracentrifuge with a Beckman TLA 100.4 rotor. The supernatant was removed and further centrifuged at 104,000 *g* for 60 min. The microsomal fraction (pellet) was washed and resuspended in PBS. All procedures were carried out at 4°. Microsomal and cytosolic preparations were then incubated at 37° in a Grant QBT4 heating block (Grant Instruments). NADH was added to give a concentration of 200 μM, and finally samples were spiked with RH1 or EO9 to give a final concentration of 20 μM. At timed intervals, aliquots were taken and extracted as described. Protein concentrations were measured by the Bradford assay [24] using BSA as a calibration protein. Rates of decay for the two compounds were expressed both in pmol/min/mg protein and as pmol/min/g tissue. Metabolism experiments with kidney cytosolic fractions were repeated with the inclusion of 100 μM DMXAA, a known inhibitor of DTD [25], and the rates calculated as described above. The concentration of 100 μM was chosen as it reflected the therapeutically achievable plasma levels in mice [26].

DT-diaphorase Assay and Substrate Specificity

The DTD enzyme activity was determined spectrophotometrically as described previously by measuring the dicoumarol-selective reduction of DCPIP [21]. DTD activity is calculated as dicoumarol-inhibitable DCPIP reduction. The reaction was initiated by the introduction of 10 μL of microsomal (0.6 mg mL^{-1}) or cytosolic (0.3 mg mL^{-1}) preparation. Results were expressed as nmol/min/mg protein. Kinetic studies, using purified human DTD [21] and designed to measure the efficiency of RH1 as a substrate for DTD, were identical to those used previously for EO9 [21]. The reduction of cytochrome *c* was monitored at 550 nm

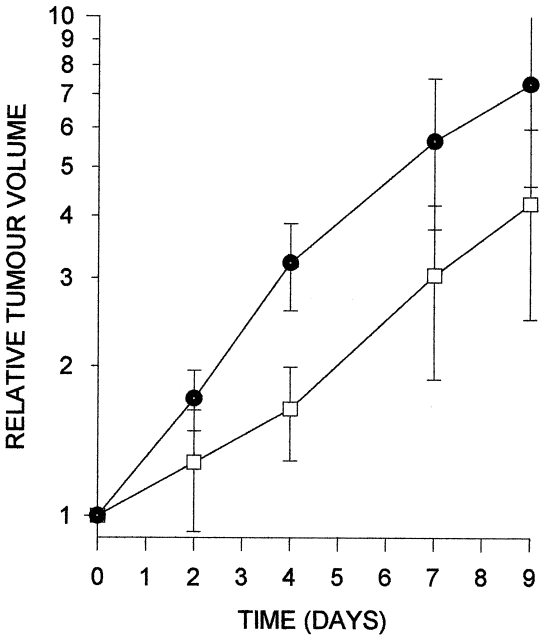


FIG. 2. Antitumour activity of RH1 against H460 human tumour xenograft following i.p. administration of RH1 (0.5 mg/kg) to NMRI mice showing untreated (●) and RH1-treated (□) mice (±1 SD). The data shown are the mean values (±1 SD) of eight mice per point.

using RH1 at concentrations ranging from 0.125 to 50 μM, and apparent K_m and V_{max} values calculated as described by Henderson [27] by plotting s/v against s.

RESULTS

Antitumour Activity

Figure 2 shows the *in vivo* antitumour activity of RH1 against the high DTD-expressing H460 tumour. There was a significant ($P = 0.01$) growth delay of 2.5 days for the tumour doubling time following i.p. administration of RH1 at a dose of 0.5 mg/kg.

Pharmacokinetic Studies

CHROMATOGRAPHY. Chromatographic separation was good using the system described with no interfering substances apparent in the control (drug-free) samples tested, which included blood, plasma, liver, and kidney extracts. Calibration curves were linear over the range tested and extraction efficiencies were >80% with sample variation <10%.

PHARMACOKINETICS. The pharmacokinetic data for RH1 are shown graphically in Fig. 3. The theoretical concentration at t = 0, C(0) was 405 ng mL^{-1}, giving a volume of distribution (V_D) of 30.8 mL. The curve of RH1 plasma concentration versus time is clearly biphasic, with an initial $t_{1/2}$ of 2.9 min and a terminal $t_{1/2}$ of 23 min. These data lead to a calculated AUC of 43.0 ng hr mL^{-1} and a clearance value of 5.14 mL min^{-1}.

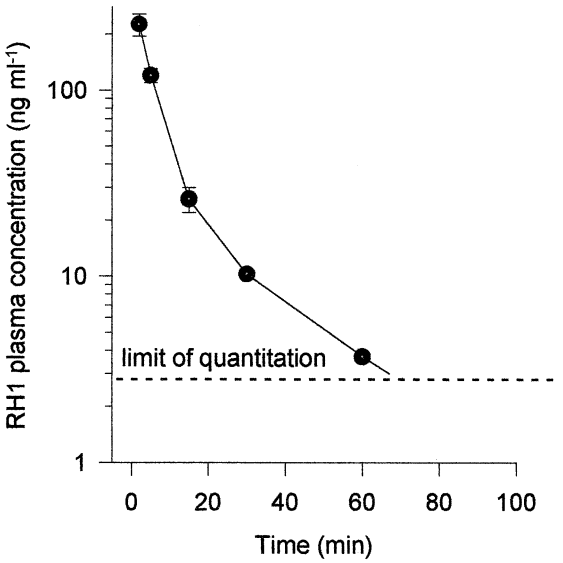


FIG. 3. Plasma concentrations of RH1 following i.v. administration of RH1 (0.5 mg/kg). The data shown are the mean values (±1 SD) of three mice per time point.

Stability of RH1

STABILITY IN BUFFERS. As expected, RH1 was unstable in acid pH with $t_{1/2}$s for parent compound of <0.1, 0.42, 2.43, and >13 hr for pH 3, 5, 7, and 8, respectively. Breakdown products detected by HPLC are likely to be the half ring-opened RH1 and fully ring-opened RH1.

STABILITY IN BLOOD. In blood, details of the disappearance of both EO9 and RH1 are shown in Table 1. EO9 was quite unstable in murine blood with a half-life of 0.26 hr, but RH1 was unexpectedly more stable with a half-life three times that of EO9 at 0.96 hr. Both compounds were much more stable in the human blood with half-lives of 1.16 and 1.31 hr, respectively. Disappearance of either compound in plasma was <5% over the 3-hr duration of the experiments, hence a calculated half-life of >10 hr.

In Vitro *Metabolism*

Microsomal pellets were resuspended in buffer to give a protein concentration of 6 mg mL^{-1}. Using only NADH as a cofactor, metabolism in both the kidney and liver microsomes was slow, with some metabolic rates being too slow to determine over a period of 3 hr (Table 2). However,

TABLE 1. Stability of RH1 and EO9 at a concentration of 20 μM in whole blood and plasma

	Blood		Plasma	
	Mouse	Human	Mouse	Human
RH1	0.96 ± 0.21	1.16 ± 0.40	>10	>10
EO9	0.26 ± 0.01	1.31 ± 0.38	>10	>10

The data show $t_{1/2}$ (hr) ± 1 SD (N = 3).

TABLE 2. Summary of metabolic rates for both EO9 and RH1 incubated in kidney and liver cytosolic and microsomal fractions

	Kidney		Liver	
Compound	Cytosol	Microsomes	Cytosol	Microsomes
EO9 + (DMXAA)	48.3 ± 18.4 (10.3 ± 7.9)	9.92 ± 4.5 (8.11 ± 6.6)	54.2 ± 3.9	13.3 ± 4.6
RH1 + (DMXAA)	5.69 ± 0.93 (6.15 ± 0.41)	8.48 ± 1.73 (2.17 ± 1.55)	41.7 ± 19.1	6.6 ± 0.02
EO9 + (DMXAA)	4396 ± 1678 (936 ± 72)	34.2 ± 15.3 (28.0 ± 22)	5540 ± 363	79.3 ± 27
RH1 + (DMXAA)	416 ± 63 (450 ± 28)	29.4 ± 6.0 (7.52 ± 5.4)	4904 ± 2231	39.6 ± 0.14

Rates are expressed as pmol/min/mg protein (top) and pmol/min/g tissue (bottom). Data shown are the means ± 1 SD (N = 3). Data in brackets indicates the inclusion of DMXAA as in inhibitor of DTD.

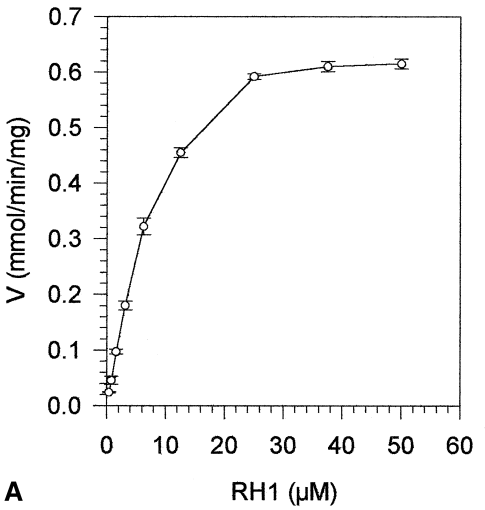
both compounds were rapidly metabolised in the liver cytosol (Table 2), in fact so rapidly that the cytosolic preparations needed diluting 20-fold in total before the metabolic rate could be accurately calculated over an adequate period of time. Although rates were similar when calculated per mg protein, both compounds disappeared nearly 100 times more quickly in the cytosol of the liver than in the microsomes when the weight of tissue was taken into account and results were expressed on a per gram tissue basis. There was no significant difference in metabolic rates between either compound in the cytosolic fractions of the liver. The microsomal fractions of the kidney gave similar results to those of the liver, with metabolism very slow. Surprisingly though, EO9 and RH1 consistently had different metabolic rates when incubated in kidney cytosolic preparations, in the presence of NADH (Table 2), with the rate of disappearance of EO9 being 6- to 7-fold greater than that of RH1. The addition of DMXAA to the kidney cytosolic preparation inhibited EO9 metabolism by up to 75% (Table 2), indicating a clear involvement of DTD in the kidney metabolism of EO9.

DT-diaphorase Assay and Kinetic Analysis

Levels of DTD activity in each of the tissue fractions used in this study are shown in Table 3. By far the highest levels of DTD are found in the kidney cytosol (125 ± 8.2 nmol/mg/min), with much lower levels in both the liver cytosol (21.4 ± 0.6 nmol/mg/min) and kidney and liver microsomal fractions (12.8 ± 5.6 and 33.7 ± 2.5 nmol/mg/min, respectively). Details of the kinetic studies are shown in Fig. 4. RH1 is shown to be an excellent substrate for DTD, with a V_{max} of 800 μmol/min/mg and a K_m of 11.8 μM compared to the previously published value for EO9 of 20.1 μmol/min/mg and 15.0 μM, respectively [21].

TABLE 3. Table of specific DT-diaphorase activity (nmol/min/mg) in murine whole blood, cytosolic, and microsomal preparations

Biological matrix	Specific activity (±1 SD) (nmol/min/mg)
Blood	14.8 ± 6.0
Liver cytosol	21.4 ± 0.6
Liver microsomes	33.7 ± 2.5
Kidney cytosol	125.7 ± 8.2
Kidney microsomes	12.8 ± 5.6



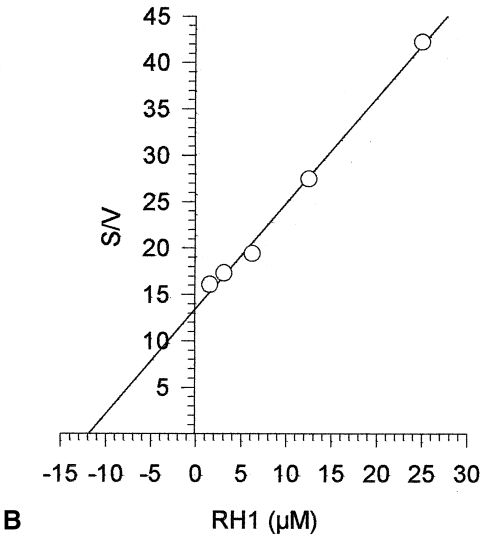


FIG. 4. Plot of v versus s (A) and Hanes plot (B) of the metabolism of RH1 by purified human DTD. Each point represents the mean (±1 SD) of three independent experiments. v = initial velocity (μmol/min/mg) and s = substrate concentration (μM).

DISCUSSION

RH1 is a novel aziridinylbenzoquinone that was synthesised as a substrate for DTD and is shortly to enter clinical trial. It is unusually potent and we describe here the first report of its significant antitumour activity against the high DTD-expressing H460 human lung carcinoma. This study has also investigated several of the factors that are likely to influence the pharmacokinetics of RH1 and compared these to the other DTD substrate EO9. It has been acknowledged for some time that the pharmacokinetic properties of EO9 may have contributed to the disappointing outcome of the clinical trials. The rate at which anticancer agents generally are cleared from the systemic circulation will influence their ability to penetrate a solid tumour mass [15] and hence their antitumour properties.

At first sight, RH1 would appear to present similar pharmacokinetic problems to those of EO9, i.e. rapid disappearance from the systemic circulation. With an AUC of 0.043 μg hr mL^{-1}, calculated clearance values show that clearance of RH1 (5.1 mL min^{-1}) was in fact 10-fold slower than that reported for EO9 (49.7 mL min^{-1}) [17], with initial volumes of distribution (V_D) also being quite different (RH1 = 30.8 mL, EO9 = 80.2 mL). Although the initial disappearance of RH1 was almost as rapid (2.9 min) as that of EO9 (1.8 min), the pharmacokinetic curve for RH1 was clearly biphasic as opposed to monophasic for EO9, which means that RH1 had a longer terminal $t_{1/2}$ of 23 min. These values are similar to those reported by Khan *et al.* [28], although the AUC was slightly higher and thus the clearance less rapid in our study. This is likely due to differences in route of administration: Khan *et al.* administered RH1 via the i.p. route, suggesting the RH1 may not have been completely absorbed.

Systemic metabolism in murine blood is likely to influence the pharmacokinetics of EO9 in mice, though probably not in humans, as EO9 is relatively stable in human blood. Surprisingly, the metabolism of EO9 and RH1 is not comparable in murine blood, with RH1 being relatively stable. This was unexpected, as the blood metabolism was considered to be DTD-related [19]. Further metabolism studies in liver and kidney cytosolic preparations clearly show the large influence these organs have on the clearance of EO9 from the mouse. NADH-related metabolism in kidney and liver cytosols is remarkably rapid for EO9, and this of course is the most likely explanation for the very short plasma half-lives of the compound. Even though kidney DTD levels in humans are not as high as in the mouse [18], it is likely that kidney metabolism of EO9 in humans is extensive, as proteinuria is the dose-limiting toxicity. The liver metabolism seen here is likely influenced by several other NADH-dependent one-electron reductases such as NADH cytochrome b_5 reductase, as liver cytosolic levels of DTD were found to be low. Indeed, the metabolism of RH1 in the liver cytosol could be largely inhibited (by 50%) using *p*-hydroxymercuribenzoate (pHMB), a known inhibitor of NADH:cytochrome b_5 reductase, an enzyme present in rat liver cytosol [29] (data not shown). However, the metabolism of RH1 was quite different from that of EO9, being extremely rapid in the liver cytosol but consistently 7-fold lower in the DTD-rich kidney. This is difficult to explain, as RH1 is an exceptionally good substrate for DTD. It may well explain, though, why RH1 has a slower clearance than EO9 in mice, and this 7-fold decrease in kidney metabolism may contribute to the 10-fold decrease in clearance of RH1. It also suggests that RH1 may be less kidney-toxic than EO9, which is an important clinical consideration.

Inhibition studies using dicoumarol, one of the most commonly used DTD inhibitors, were unsuccessful, as dicoumarol has only limited solubility. The use of DMXAA, however, inhibited the metabolism of EO9 in the kidney by up to 75%, suggesting the involvement of DTD in the kidney metabolism of this compound. The pH stability studies demonstrate the acid-labile nature of RH1 as well as highlighting the hydrolysis products that are likely to be the half ring-opened and fully ring-opened products of RH1. Further *in vitro* studies with purified human DTD and HPLC (data not shown) confirm the studies of Winski *et al.* [8], which showed that RH1 is in fact rapidly reduced by DTD to the hydroquinone. It is important to note that none of the breakdown products were detected during the metabolism studies.

This study has described the anti-tumour activity, pharmacokinetics, and NADH-dependent metabolism of the new benzoquinone and highlighted several pharmacological and metabolic differences between RH1 and EO9. The decreased kidney metabolism of RH1 is thought to contribute to the decreased systemic clearance of RH1 relative to EO9. The metabolic differences described here between the two compounds are likely to have both pharmacokinetic and toxicological consequences in the clinic and may have implications for other potential substrates of DTD.

This work was supported by War on Cancer, Bradford, U.K. We would also like to acknowledge the helpful contribution of Dr. John Butler, Paterson Institute for Cancer Research, Manchester, U.K., to this study. Dr. Butler also provided the HPLC conditions used. EO9 was a gift from the Screening and Pharmacology Group of the EORTC.

References

1. Brown JM and Giaccia AJ, The unique physiology of solid tumors: Opportunities (and problems) for cancer therapy. *Cancer Res* **58:** 1408–1416, 1998.
2. Ernster L, DT-diaphorase; A historical review. *Chem Scripta* **27A:** 1–13, 1987.
3. Schlager JJ and Powis G, Cytosolic NAD(P)H:(quinone-acceptor)oxidoreductase in human normal and tumor tissue: Effects of cigarette smoking and alcohol. *Int J Cancer* **45:** 403–409, 1990.
4. Malkinson AM, Siegel D, Forrest GL, Gazdar AF, Oie HK, Chan DC, Bunn PA, Mabry M, Dykes DJ, Harrison SD and Ross D, Elevated DT diaphorase activity and messenger Rna content in human non-small cell lung carcinoma: Relation-

ship to the response of lung tumor xenografts to mitomycin C1. *Cancer Res* **52:** 4752–4757, 1992.
5. Phillips RM, Human DT-diaphorase as a candidate for enzyme-directed bioreductive drug development. *Drugs of the Future* **21:** 1247–1256, 1996.
6. Fisher GR, Donis J and Gutierrez PL, Reductive metabolism of diaziquone (AZQ) in the S9 fraction of MCF-7 cells. II. Enhancement of the alkylating activity of AZQ by NAD(P)H: quinone-acceptor oxidoreductase (DT-diaphorase). *Biochem Pharmacol* **44:** 1625–1635, 1992.
7. Cummings J, Spanswick VJ, Tomasz M and Smyth JF, Enzymology of mitomycin C metabolic activation in tumour tissue. *Biochem Pharmacol* **56:** 405–414, 1998.
8. Winski SL, Hargreaves RH, Butler J and 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* **4:** 3083–3088, 1998.
9. Walton MI, Smith PJ and Workman P, The role of NAD(P)H:quinone reductase (EC 1.6.99.2, DT-diaphorase) in the reductive bioactivation of the novel indoloquinone antitumour agent EO9. *Cancer Commun* **3:** 199–206, 1991.
10. Hendriks HR, Pizao PE, Berger DP, Kooistra KL, Bibby MC, Boven E, Dreef-van der Meulen HC, Henrar RE, Fiebig HH, Double JA, Hornstra HW, Pinedo HM, Workman P and Schwartsmann G, EO9: A novel bioreductive alkylating indoloquinone with preferential solid tumour activity and lack of bone marrow toxicity in preclinical models. *Eur J Cancer* **29A:** 897–906, 1993.
11. Robertson N, Haigh A, Adams GE and Stratford IJ, Factors affecting sensitivity to EO9 in rodent and human tumour cells *in vitro*: DT-diaphorase activity and hypoxia. *Eur J Cancer* **30A:** 1013–1019, 1994.
12. Pavlidis N, Hanauske AR, Gamucci T, Smyth J, Lehnert M, te Velde A, Lan J and Verweij J, A randomized phase II study with two schedules of the novel indoloquinone EO9 in non-small-cell lung cancer: A study of the EORTC early clinical studies group (ECSG). *Ann Oncol* **7:** 529–531, 1996.
13. Dirix LY, Tonnesen F, Cassidy J, Epelbaum R, Huinink WW, Pavlidis N, Sorio R, Gamucci T, Wolff I, te Velde A, Lan J and Verweij J, EO9 phase II study in advanced breast, gastric, pancreatic and colorectal carcinoma by the EORTC early clinical studies group. *Eur J Cancer* **32A:** 2019–2022, 1996.
14. Verweij J, Aamdal S, Schellens J, Koier I and Lund B, Clinical studies with EO9, a new indoloquinone bioreductive alkylating cytotoxic agent. *Oncol Res* **6:** 519–523, 1994.
15. Phillips RM, Loadman PM and Cronin BC, Evaluation of a novel *in vitro* assay for assessing drug penetration into avascular regions of tumours. *Br J Cancer* **77:** 2112–2119, 1998.
16. Workman P, Binger M and Kooistra KL, Pharmacokinetics, distribution and metabolism of the novel bioreductive alkylating indoloquinone EO9 in rodents. *Int J Radiat Oncol Biol Phys* **22:** 713–716, 1992.
17. Bibby MC, Sleigh NR, Loadman PM and Double JA, Potentiation of EO9 anti-tumour activity by hydralazine. *Eur J Cancer* **29A:** 1033–1035, 1993.
18. Segura-Aguilar J, Cremades A, Llombart-Bosch A, Monsalve E, Ernster L and Romero F, Activity and immunohistochemistry of DT-diaphorase in hamster and human kidney tumours. *Carcinogenesis* **15:** 1631–1636, 1994.
19. Phillips RM, Loadman PM and Jarret CM, Species-dependent differences in the metabolism of idoloquinones by whole blood. *Br J Cancer* **75:** (Suppl 1): 20, 1997.
20. Workman P, Twentyman P, Balkwill F, Balmain A, Chaplin D, Double J, Embleton J, Newell D, Raymond R, Stables J, Stephens T and Wallace J, United Kingdom Co-Ordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia (Second Edition). *Br J Cancer* **77:** 1–10, 1998.
21. Phillips RM, Bioreductive activation of a series of analogues of 5-aziridinyl-3-hydroxymethyl-1-methyl-2-[1*H*-indole-4,7-dione] prop-β-en-α-ol (EO9) by human DT-diaphorase. *Biochem Pharmacol* **52:** 1711–1718, 1996.
22. Phillips RM, Hulbert PB, Bibby MC, Sleigh NR and Double JA, *In vitro* activity of the novel indoloquinone EO9 and the influence of pH on cytotoxicity. *Br J Cancer* **65:** 359–364, 1992.
23. Bibby MC, Loadman PM, al-Ghabban AF and Double JA, Influence of hydralazine on the pharmacokinetics of tauromustine (TCNU) in mice. *Br J Cancer* **65:** 347–350, 1992.
24. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein–dye binding. *Anal Biochem* **72:** 248–254, 1976.
25. Phillips RM, Inhibition of DT-diaphorase (NAD(P)H:quinone oxidoreductase, EC 1.6.99.2) by 5,6-dimethylxanthenone-4-acetic acid (DMXAA) and flavone-8-acetic acid (FAA): Implications for bioreductive drug development. *Biochem Pharmacol* **58:** 303–310, 1999.
26. McKeage MJ, Kestell P, Denny WA and Baguley BC, Plasma pharamcokinetics of the antitumour agents 5,6-dimethylxanthenone-4-acetic acid, xanthenone-4-acetic acid and flavone-8-acetic acid in mice. *Cancer Chemother Pharmacol* **28:** 409–413, 1991.
27. Henderson PF, Statistical analysis of enzyme kinetic data. In: *Enzyme Assays: A Practical Approach*. (Eds. Eisenthal R and Danson MJ), pp. 277–316. IRL Press, Oxford University Press, Oxford, 1993.
28. Khan P, Abbas S, Hargreaves RH, Caffrey R, Megram V and McGown A, Development and validation of a sensitive solid-phase extraction and high-performance liquid chromatographic assay for the novel bio-reductive anti-tumour agent RH1 in human and mouse plasma. *J Chromatogr B Biomed Sci App* **729:** 287–295, 1999.
29. Barham HM, Inglis R, Chinje EC and Stratford IJ, Development and validation of a spectrophotometric assay for measuring the activity of NADH: cytochrome b_5 reductase in human tumour cells. *Br J Cancer* **74:** 1188–1193, 1996.