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Phase I pharmacokinetic and pharmacodynamic study of the bioreductive drug RH1

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Background: This trial describes a first-in-man evaluation of RH1, a novel bioreductive drug activated by DT-diaphorase (DTD), an enzyme overexpressed in many tumours.

Patients and methods: A dose-escalation phase I trial of RH1 was carried out. The primary objective was to establish the maximum tolerated dose (MTD) of RH1. Secondary objectives were assessment of toxicity, pharmacokinetic determination of RH1 and pharmacodynamic assessment of drug effect through measurement of DNA cross linking in peripheral blood mononuclear cells (PBMCs) and tumour, DTD activity in tumour and NAD(P)H:quinone oxidoreductase 1 (NQO1) polymorphism status.

Results: Eighteen patients of World Health Organization performance status of zero to one with advanced refractory solid malignancies were enrolled. MTD was 1430 μg/m^2/day with reversible bone marrow suppression being dose limiting. Plasma pharmacokinetic analysis showed RH1 is rapidly cleared from blood ($t_{1/2}$ = 12.3 min), with AUC increasing proportionately with dose. The comet-X assay demonstrated dose-related increases in DNA cross linking in PBMCs. DNA cross linking was demonstrated in tumours, even with low levels of DTD. Only one patient was homozygous for NQO1 polymorphism precluding any conclusion of its effect.

Conclusions: RH1 was well tolerated with predictable and manageable toxicity. The MTD of 1430 μg/m^2/day is the dose recommended for phase II trials. The biomarkers of DNA cross linking, DTD activity and NQO1 status have been validated and clinically developed.

Key words: chemotherapy, DT-diaphorase, pharmacodynamic, pharmacokinetic, phase I, RH1

introduction

Quinones traditionally represented a source of active compounds in cancer medicine, which require bioreduction to intermediates that either generate toxic free radical species or bind to DNA to form covalent adducts. The obligatory two-electron reductase, DT-diaphorase [DTD; (NAD(P)H: quinone oxidoreductase 1: NQO1)] has been the focus of much attention and can activate a number of xenobiotics, including mitomycin C, nitrobenzamides, orthonaphthoquinones and aziridinyl benzoquinones [1–5]. DTD is overexpressed in many cancers compared with the surrounding normal tissue, and high level expression is frequently seen in non-small-cell lung cancer (NSCLC), colorectal, liver and breast carcinomas [6]. DTD is encoded by four genetic loci (NQO1-4) [7] and, in man, the majority of DTD is coded for by the *NQO1* gene [8]. A single-point nucleotide polymorphism (SNP), known as NQO1*2, results in a lack of detectable DTD activity [9] and there is ethnic variation in the occurrence of this SNP, with 4.4% of Caucasians and 20.3% of Asians having the homozygous genotype [10]. Heterozygotes have intermediate activity compared with wild-type individuals [11].

RH1 (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone), a novel water-soluble diaziridinylbenzoquinone, which is cytotoxic at low nanomolar concentrations [12, 13], was selected from a panel of diaziridinylbenzoquinones as an excellent substrate for DTD [14]. Upon reduction, RH1 forms DNA interstrand cross-linked adducts with a unique sequence specificity for GCC [15]. Unlike mitomycin C, RH1 quinone reduction products are more stable, less likely to generate toxic reactive oxygen species and less likely to be influenced by hypoxia than mitomycin C [16]. In keeping with RH1 being an excellent substrate for DTD, it is significantly

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more cytotoxic *in vitro* in cells that overexpress DTD, naturally or through transfection with NQO1, than in cells with low DTD [13, 17–20]. Preclinical *in vivo* xenograft testing demonstrated significant activity in NSCLC, breast, colorectal and ovarian xenograft models [12, 19]. RH1 was selected for clinical development by Cancer Research UK (CR-UK) and National Cancer Institute (NCI), and detailed toxicology was carried out by the NCI in collaboration with CR-UK. The main toxic effects were myelosuppression and local injection site inflammation. A starting dose level of 40 μg/m^2/day for 5 days was suggested for human trials based on this being one-tenth of the maximum tolerated dose (MTD) in the most sensitive species, the dog.

This CR-UK sponsored, two-centre, dose-escalation study is the first clinical evaluation of RH1. The primary objective was to assess safety and tolerability of RH1 in patients with advanced solid tumours and to determine the MTD. Secondary objectives were to determine the pharmacokinetics (PK) of RH1 and evaluate mechanistic pharmacodynamic endpoints, including DNA cross linking in peripheral blood mononuclear cells (PBMCs) and tumour cells, to determine DTD activity in tumour biopsies and to evaluate the impact of NQO1 polymorphism.

patients and methods

eligibility criteria

Patients were eligible if they had histologically proven cancer, refractory to conventional treatment, or for which no conventional therapy existed. Patients were ≥18 years with a life expectancy of at least 3 months and a World Health Organization performance status of zero or one. Patients needed to have haemoglobin ≥9.0 g/dl, absolute neutrophil count (ANC) ≥1.5×10^9/l, platelets ≥100×10^9/l, 24 h creatinine clearance (CrCl) ≥50 ml/min, serum bilirubin ≤1.5 × upper limit of normal (ULN) and alanine transferase and asparate transferase no more than 2.5 × ULN (unless due to tumour in which case up to 5 × ULN was permissible). The main exclusion criteria were other anticancer therapy within 4 weeks (6 weeks for nitrosureas and mitomycin C); persistent toxic manifestations of previous treatment, apart from alopecia; current malignancies at other sites, excluding adequately treated cone-biopsied *in situ* carcinoma of the cervix uteri and basal or squamous cell carcinoma of the skin; pregnancy or lactation; uncontrolled concomitant non-malignant disease and known positivity for hepatitis B, C or HIV. Patients were required to take contraceptive precautions while on the trial and for 6 months afterwards. All patients provided written informed consent. Approval was obtained from an independent ethics committee according to national and local requirements. The study was carried out in accordance with International Conference on Harmonisation-Good Clinical Practice guidelines and the Declaration of Helsinki.

treatment and assessments

Prestudy screening was carried out within 7 days of trial entry. RH1 was formulated in 20% cyclodextrin and administered as a 10 min infusion but was extended to 30 min for patients 13–18 to reduce venous pain. Cycles were administered every 3 weeks and the starting dose was 40 μg/m^2/day on days 1–5. The starting dose for the study equated to 1/10th of the MTD in the most sensitive species (dog) determined by preclinical toxicology. Dose escalation was based on a modified Fibonacci series (40, 80, 135, 200, 265, 350, 460, 610, 810, 1080, 1430 and 1905 μg/m^2/day). Cohorts of single patients were recruited until drug-related toxicity of grade 2 or higher was observed and thereafter dose levels were expanded to three patients. If one of three patients developed dose-limiting toxicity (DLT), the cohort was expanded to a maximum of six patients. Dose escalation continued until >30% of patients in a cohort experienced DLT. Treatment continued to six cycles or until there was evidence of disease progression or if DLT was reached. Patients who showed clinical benefit or response from treatment but developed DLT could continue in study at a lower dose level. The following parameters were needed for retreatment: ANC ≥1.5×10^9/l, platelets ≥100×10^9/l and CrCl >50 ml/min and a 2-week treatment delay was allowed. Routine prophylactic antiemetics were not given, but patients could receive standard antiemetics as needed.

DLT was defined as any of the following treatment-related events: grade 4 neutropenia lasting ≥5 days; febrile neutropenia (grade 3 or higher) with infection; grade 4 thrombocytopaenia lasting ≥5 days or associated with active bleeding or requiring platelet transfusion; grade 3 or 4 non-haematologic toxicity (excluding grade 3 nausea and grade 3 or 4 vomiting or diarrhoea in patients who had not received optimal antiemetics and/or antidiarrhoeal therapies) and treatment-related death. The MTD was defined as the dose level of RH1 below that which ≥30% of patients at that dose level experienced DLT.

safety and tolerability

Adverse events were graded according to the NCI–Common Toxicity Criteria (NCI–CTC) version 2.0 and patients were assessed daily during drug treatment and at least weekly at other times. During grade 4 myelosuppression patients were seen daily until recovery to grade 3 or lower. Upon completion of treatment, patients were followed up for at least 4 weeks or until drug-related toxic effects returned to baseline or until the patient started another antitumour treatment.

pharmacokinetic analysis

Heparinised blood samples were collected pretreatment and at 0, 2, 5, 10, 15, 20, 40, 60, 120, 240 and 480 min and 24-h postinfusion during day 1 and day 5 dosing in the first cycle. Samples were centrifuged within 30 min at 1000 *g* for 10 min and plasma stored at −80°C until analysis. RH1 concentrations were determined using a sensitive validated liquidated chromatography–mass spectrometry-mass spectrometry (LC-MS-MS) method following solid-phase extraction. Briefly, patient plasma samples and a freshly prepared plasma standard curve were spiked with a fixed concentration of an internal standard, 2,5-diaziridinyl-1,4-benzoquinone (DZQ). Analytes were extracted using solid-phase extraction cartridges (100 mg Chromabond EASY; Macherey-Nagel, Düren, Germany) Eluted samples were assayed using an HPLC-MS-MS method (Waters Alliance HT2790 separations module coupled to a Waters-Micromass Quattro-LC mass spectrometer, HPLC column Nucleosil 100-5 C18 Nautilus; Macherey-Nagel). Detection was by multiple reaction monitoring with transitions of 253.1–217.2 for RH1 and 191.05–114.22 for DZQ. The assay was linear over a concentration range of 0–500 ng/ml, with a coefficient of variation (CV) for both accuracy and precision of <15% across the range. PK model building and parameter estimation was carried out using non-linear mixed effects modelling (NONMEM version V—first-order conditional estimation method). PK parameters of RH1 were determined from fitting of 1-, 2- or 3-compartment kinetic models to the population concentration time profiles. A proportional error model was used for the residual error and the final model was determined by reduction in the minimum objective function and graphical diagnostics. Patient covariates (age, weight, etc.) were evaluated during the model building process, with the contribution of a covariate determined by reduction in the minimum objective function and graphical diagnostics.

pharmacodynamic assessments

Blood samples were obtained predose and then at 5, 10, 20 and 40 min, 1, 2, 4, 8 and 24 h post-dosing on days 1 and 5 and in patients treated

at ≥810 μg/m^2/day samples were also obtained on days 8, 15 and 22. The comet-X assay previously validated for clinical use [21] was used to assess DNA cross linking in PBMCs and tumour cells. Fresh tumour biopsies, obtained by excision, were obtained pretreatment and at day 5 in cycle 1 and were immediately placed in Hanks balanced salt solution, minced and run in the comet-X assay. DTD levels in tumour biopsies were determined with Western blotting of snap-frozen tumour biopsies and by immunohistochemistry (IHC) of formalin-fixed specimens. Absolute DTD activity was measured using the 2,6-dichlorophenolindophenol (DCPIP) assay [22]. Patients were genotyped to assess their NQO1 polymorphism status [9].

tumour response evaluation

Antitumour activity was evaluated according to the RECIST criteria [23]. Baseline computed tomography imaging was carried out within 4 weeks of study treatment and after every two cycles.

results

patients and treatment

Eighteen patients with advanced solid tumours were treated at dose levels of 40–1905 μg/m^2/day (Table 1). Fourteen patients were male and four female; the mean age was 57.5 years and the most common tumour type ($n = 8$) was colorectal cancer. Seventeen of the 18 had previous chemotherapy, 2 had received immunotherapy and 1 had taken hormonal therapy. The mean number of previous systemic regimens was 2.7. The dosing cohorts are shown in Table 2. Reasons for discontinuation of RH1 included disease progression ($n = 14$), adverse events ($n = 2$) and completion of the study protocol ($n = 2$). The median number of cycles was 2 (range 1–6).

Table 1. Patient characteristics ($n = 18$)

Sex, number	
Male	14
Female	4
Age, years	
Mean	57.5
Range	30–72
World Health Organization performance status	
0	6
1	12
Tumour type	
Colorectal	8
Gastric	3
Non-small-cell lung cancer	2
Melanoma	2
Merkel cell carcinoma	1
Pancreatic	1
Renal	1
Prior treatment (patients may have received more than one type of prior therapy)	
Surgery	15
Radiotherapy	5
Chemotherapy	17
Immunotherapy	2
Hormonal therapy	1
Mean number of prior systemic regimens (range)	2.7 (0–5)
Median number of prior systemic regimens (range)	3 (0–5)

adverse events

All patients were evaluable for toxicity and RH1 was well tolerated up to and including doses of 1430 μg/m^2/day. DLTs occurred in two of two patients treated at 1905 μg/m^2/day; patient 12 developed grade 3 anaemia and thrombocytopaenia, with grade 2 haemoptysis, while patient 13 had grade 3 anaemia, fatal pneumonia and neutropenic sepsis. Treatment-related adverse events are summarised in Table 3. The most frequently occurring treatment-related adverse events were fatigue (39%), nausea (39%), anaemia (39%), leucopenia (33%), neutropenia (33%), lymphopenia (33%), thrombocytopaenia (33%), vomiting (22%) and venous pain (22%). Nausea and vomiting was generally mild and controlled with standard antiemetics. Venous pain during infusion was seen at 1905 μg/m^2/day and the infusion time was increased to 30 min for subsequent patients, which reduced this problem (patients 13–18). No renal toxicity was seen, apart from transient grade 1 increase in serum creatinine in one patient and serial monitoring of urinary *N*-acetyl-beta-D-glucosaminidase (NAG) levels did not reveal acute or cumulative tubular toxicity. There were no significant hepatic, cardiac or neurologic adverse events and no clinical alopecia. Dose-related myelosuppression represented the main drug-related toxicity and DLT. Cohort 11 was expanded to six patients and this dose level was well tolerated confirming the MTD as 1430 μg/m^2/day.

pharmacokinetics

Plasma RH1 levels were determined by LC-MS-MS method over a concentration range of 0–500 ng/ml. The intraassay

Table 2. Dose level

Cohort	Dose level (μg/m^2/day)	Number of patients	Number of cycles completed	Number of DLT events
1	40	1	2	0
2	80	1	2	0
3	135	1	2	0
4	200	1	2	0
5	265	1	6	0
6	350	1	4	0
7	460	1	2	0
8	610	1	2	0
9	810	1	2	0
10	1080	1	4	0
11	1430	6	1 ($n = 1$)	0
			2 ($n = 3$)	
			4 ($n = 1$)	
			6 ($n = 1$)	
12	1905	2	3 ($n = 2$)	2[a]

[a]One grade 3 anaemia and grade 3 thrombocytopaenia and 1 grade 5 neutropenic sepsis.
DLT, dose-limiting toxicity.

Table 3. Adverse events related to RH1 ($n = 18$) (worst grade per patient NCI–CTC version 2.0)

Adverse event	Dose level ($\mu g/m^2$/day)	n	Maximum CTC grade				Total
			1	2	3	4	n (% total)
Fatigue	40–1080	10	0	0	0	0	0
	1430	6	0	6	0	0	6
	1905	2	1	0	0	0	1
	Total	18	1	6	0	0	7 (39)
Nausea	40–1080	10	0	0	0	0	0
	1430	6	2	3	0	0	5
	1905	2	2	0	0	0	2
	Total	18	4	3	0	0	7 (39)
Vomiting	40–1080	10	0	0	0	0	0
	1430	6	2	1	0	0	3
	1905	2	1	0	0	0	1
	Total	18	3	1	0	0	4 (22)
Phlebitis	40–1080	10	0	0	0	0	0
	1430	6	0	2	0	0	2
	1905	2	0	2	0	0	2
	Total	18	0	4	0	0	4 (22)
Haemoglobin	40–1080	10	0	0	0	0	0
	1430	6	1	1	2	0	4
	1905	2	1	2	0	0	3
	Total	18	2	3	2	0	7 (39)
WBC	40–1080	10	1	0	0	0	1
	1430	6	1	1	2	0	4
	1905	2	0	1	1	0	2
	Total	18	1	2	3	0	6 (33)
Neutrophils	40–1080	10	1	0	0	0	1
	1430	6	1	1	1	0	3
	1905	2	1	0	1	0	2
	Total	18	3	1	2	0	6 (33)
Platelets	40–1080	10	0	0	0	0	0
	1430	6	3	0	0	0	3
	1905	2	2	0	1	0	3
	Total	18	5	0	1	0	6 (33)
Lymphocytes	40–1080	10	0	0	0	0	0
	1430	6	0	2	3	0	5
	1905	2	0	0	1	0	1
	Total	18	0	2	4	0	6 (33)

NCI, National Cancer Institute; CTC, Common Toxicity Criteria; WBC, white blood cell.

coefficient of variation (CV) at 0.5 μg/ml was 1.7% with a lower limit quantification of 5 ng/ml. Data for 15 patients were evaluable for NONMEM population PK analysis. A 3-compartment model gave the best fit of the clinical data (Tables 4 and 5). None of the examined clinical covariates significantly improved the objective function or fit of the data. There was good agreement between observed and population predicted individual predicted plasma concentrations, with a uniform spread of weighted residuals ~zero (Figure 1).

pharmacodynamics

All 18 patients were evaluable for pharmacodynamic analysis by comet-X assay and genotyping. Four patients underwent paired tumour biopsies for functional assessment of tumour DTD activity (patients 10, 15, 16 and 18).

Table 4. Description of the PK models and results of model building and selection

Model	N	Model description	Objective function	Δ Objective function
PK(1)	2	One-compartment	893.3	–
PK(2)	4	Two-compartment	765.9	−127.4
PK(3)	6	**Three-compartment**	748.3	−17.6
PK(4)	6	Three-compartment with influence of WT on CL	747.2	−1.1
PK(5)	6	Three-compartment with influence of WT on V	751.2	+2.9
PK(6)	6	Three-compartment with influence of WT on CL and V	748.8	+0.5
PK(7)	6	Three-compartment with influence of HT on CL	748.2	−0.1
PK(8)	6	Three-compartment with influence of BSA on CL	747.7	−0.6
PK(8)	6	Three-compartment with influence of age on CL	749.1	+0.8
PK(9)	7	Three-compartment with CrCL acting on a renal clearance component	747.1	−1.2

The best fit model is shown in bold. N is the number of parameters. PK, pharmacokinetics; WT, weight; HT, height; CL, clearance; BSA, body surface area; CrCL, creatinine clearance.

Table 5. Final parameter estimates of RH-1 according to the best fit model, PK(3)

Parameter	Mean	SE[a]	BSV[b] (SE)
CL (l/min)	0.54	0.276	0.946 (0.438)
V1 (l)	19.7	0.279	0.733 (0.661)
$CL_{ic,1}$ (l/min)	1.82	0.261	0.790 (0.511)
V2 (l)	332	0.147	0.308 (0.997)
$CL_{ic,2}$ (l/min)	1.30	0.148	0.004 (0.805)
V3 (l)	11.5	0.348	0.417 (0.466)
Proportional error	0.279	0.188	–

[a]Expressed as a CV of the mean estimate.
[b]SE expressed as a CV of the BSV term.
SE, standard error; BSV, between-subject variability; CV, coefficient of variation.

DNA cross linking in PBMCs. At 40–200 $\mu g/m^2$/day little DNA cross linking was detectable on day 1 and only low level cross linking was seen by day 5. At dose levels >200 $\mu g/m^2$/day, DNA cross linking (>10%) was detectable by the end of day 1 and throughout day 5. On day 1, the highest levels of cross linking occurred 4–8 h post dose and DNA cross linking at 4–8 hours post dose on days 1 and 5 as a function of dose level is shown in Figure 2A. Cross linking in PBMCs was greatest at doses of 610–1080 $\mu g/m^2$/day with 25% of the DNA being cross-linked by day 5. At the two highest dose levels, the measured degree of DNA cross linking was lower than at 610–1080 $\mu g/m^2$/day. It is probable that in these patients the dose of RH1 depleted the most damaged population of PBMCs, leaving more moderately damaged cells with relatively lower levels of cross linking. In keeping with this, grade ≥2 lymphopenia was seen in 6 of 6

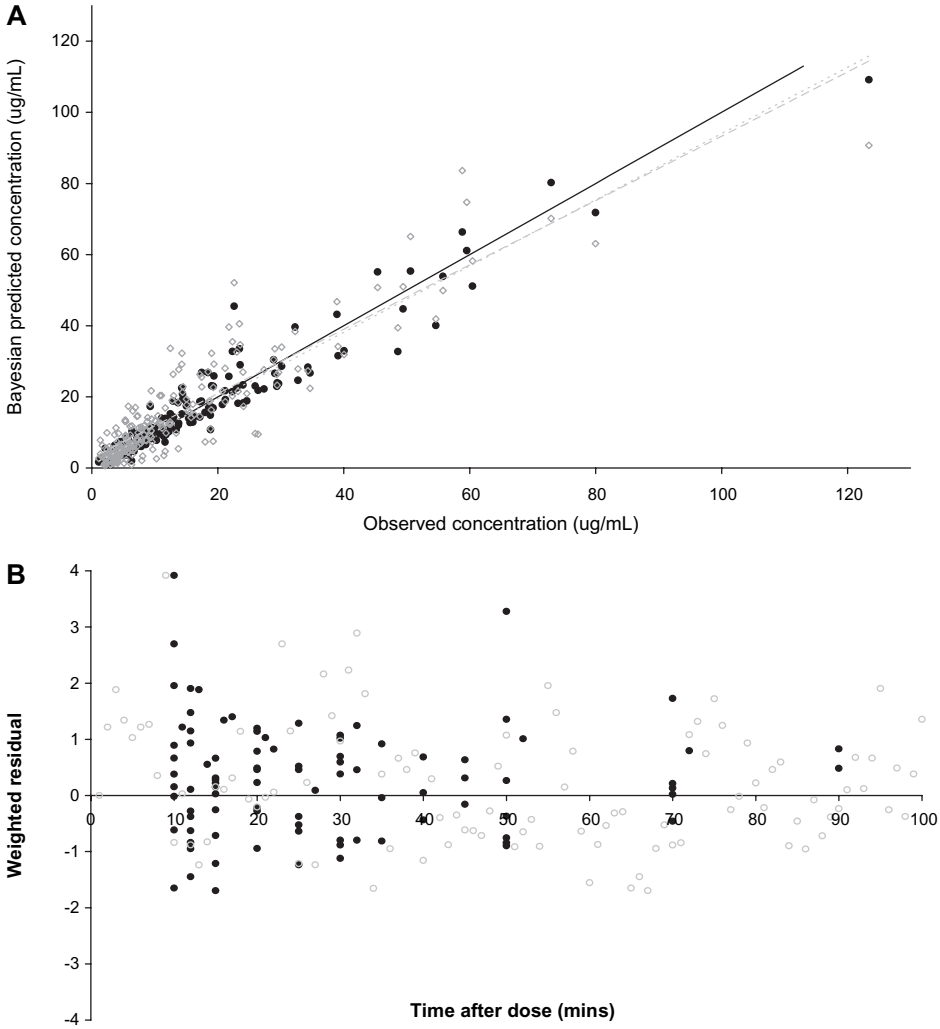

Figure 1. (A) Population-derived individual-predicted (solid circles) and population-predicted (grey circles) plasma RH1 concentrations versus observed data according to the best fit three-compartment pharmacokinetics model. The diagonal line represents a perfect match of the values. The grey dotted and grey dashed lines are regressions of individual predicted ($R^2 = 0.9335$) and population predicted ($R^2 = 0.8153$) values, respectively. (B) Weighted residuals versus time after end of infusion on day 1 (solid circles) and day 5 (grey circles).

patients at 1430–1905 μg/m^2/day but was seen in 0 of 10 patients at lower dose levels. DNA cross linking during the first cycle for the six patients treated at the MTD dose of 1430 μg/m^2/day is shown in Figure 2B. Following a rise in cross linking during dosing, there is a subsequent modest fall with cross linking persisting to day 22. As discussed above, the observed fall seen in day 8, 15 and 22 sampling at the MTD dose may be secondary to clearance of the most severely damaged cells since at lower doses of 460–810 μg/m^2/day persistence of DNA cross linking was seen.

tumour biopsy results. Paired biopsies (pre- and posttreatment) were collected in four patients and two were evaluable for DNA cross linking, DTD IHC and DCPIP analysis. Patient 10 (1080 μg/m^2/day) showed moderate IHC staining for DTD in tumour cells, with a DTD activity of 73.8 nM/min/mg ±7.2% and 48% DNA cross linking in the tumour biopsy compared with 35% cross linking in PBMCs at this time point. In contrast, patient 15 (1430 μg/m^2/day) had higher DTD activity of 121 nM/min/mg ±2.6% with strong IHC staining in tumour, but tumour cross linking was only 6.8% compared with 37% in PBMCs at the same timepoint.

NQO1 polymorphism status. Of the 18 patients genotyped, 11 (61.1%) were found to be homozygous for the wild-type allele while 6 were heterozygous (33.3%). One patient (patient 18) was found to be homozygous representing 5.5% of this patient population (Figure 3). The frequency of these genotypes was consistent with that expected in the Caucasian population studied—4.4% in the published literature [10]. There was no relationship between NQO1 genotype and RH1 PK.

tumour response evaluation

All 18 patients were evaluable for response. Seven (39%) patients had stable disease and 11 (61%) patients had progressive disease. No complete or partial responses were documented in this refractory patient population.

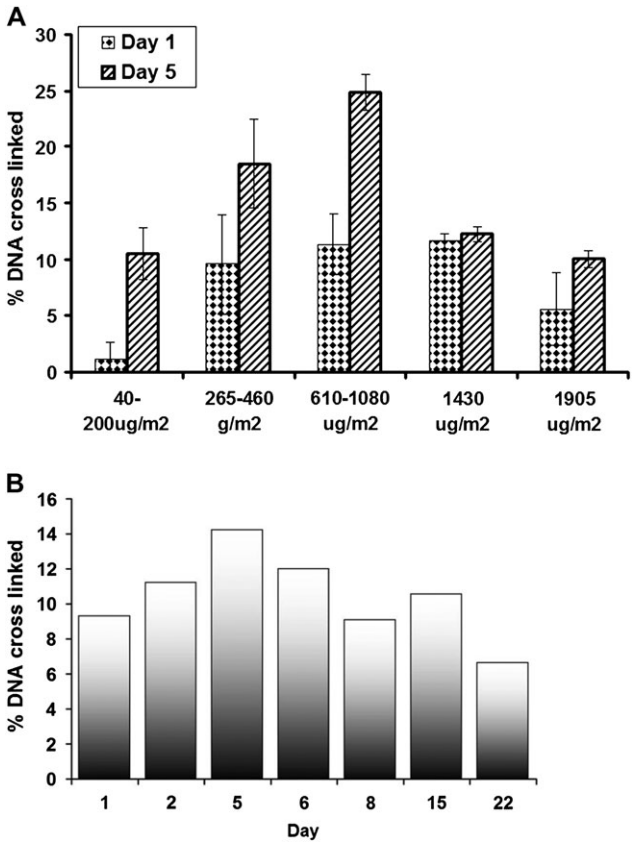

Figure 2. (A) Comet-X assay measurements of percentage of DNA cross-linked in peripheral blood mononuclear cells observed 4–8 h following RH1 treatment on day 1 and 5. The percentage of DNA cross-linked is shown versus increasing doses of RH1. Error bars represent standard deviation. (B) Extent and duration of percentage of DNA cross-linked following administration of RH1 at the MTD (1430 μg/m^2/day). These data are for the six patients treated at the maximum tolerated dose.

discussion

In this first-in-man phase I study, the MTD of RH1 administered i.v. over 30 min on days 1–5 every 3 weeks was 1430 μg/m^2/day, and this is the dose recommended for phase II study. Treatment with RH1 was generally well tolerated and the emergence of dose-limiting myelosuppression was consistent with the preclinical toxicology data. A formulation of RH1 in 20% cyclodextrin was selected to reduce the local injection site toxicity seen in preclinical toxicology studies with RH1 when administered in water. Venous pain responded to an increase in infusion duration and a 30-min infusion is recommended for future use. Although preclinical studies did not identify kidney as a major organ for RH1 toxicity, there was concern about the potential for this toxicity because high levels of DTD protein occur in human kidney [24], and the clinical development of the related drug, E09, was severely limited by its renal toxicity [25]. This study incorporated three serial measures of renal function: serum creatinine, 24-h urinary creatinine clearance and urinary NAGs. Renal toxicity was not encountered.

The preclinical toxicology data carried out in rodents and dog yielded a starting dose that proved conservative for humans and so 12 dose levels were required to reach DLT. Using single patient cohorts with a dose-doubling approach until grade 2 toxicity reduced the number of patients required and the entire study was completed with 18 patients. RH1 clinical development involved collaboration between CR-UK and the NCI, and preclinical toxicology carried out by the NCI routinely includes a non-rodent species while CR-UK previously published data to support a rodent-only toxicology approach as an adequate basis on which to proceed to most trials in man [26]. In retrospect, rodent-only toxicology for RH1 would have provided a less conservative starting dose and reduced the number of dose-escalation steps.

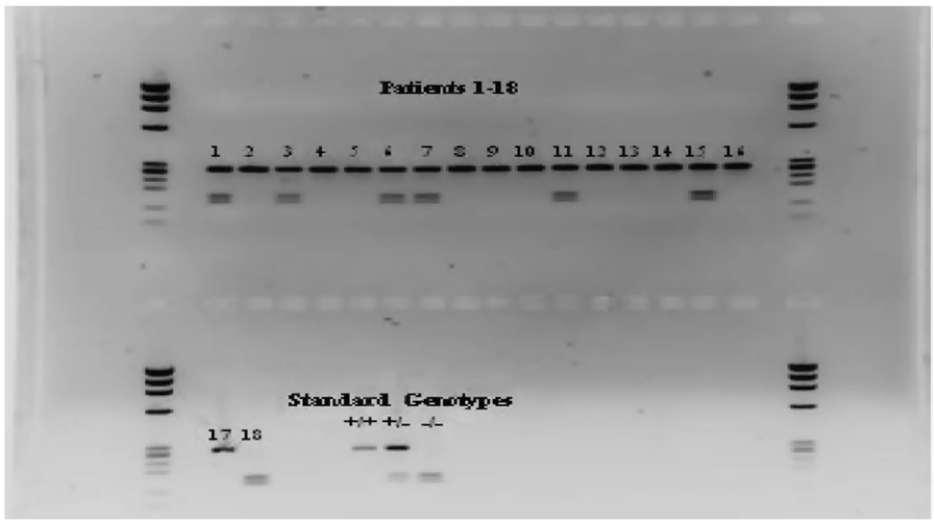

Figure 3. Restriction fragment length polymorphism assay for the NQO1 polymorphism. The presence of an upper single band denotes a wild-type NQO1 genotype while an upper single and a double lower band denotes a heterozygous genotype. Individuals who are homozygous for the NQO1 single-point nucleotide polymorphism have only a lower double band.

In preclinical studies with short infusions of RH1 in 20% cyclodextrin, the plasma distribution and elimination half-life of RH1 in rat and dog were brief (~2 and 20 min, respectively). The patient plasma concentration–time data were best fit by a three-compartment model and the plasma half-life of RH1 was similarly short in man, with a mean elimination half-life of 12.3 min. Loadman et al. [17] compared the clearance of RH1 in mice with that of E09 and reported that the clearance of RH1 was 10 times slower than the clearance of E09. Human results are consistent with this preclinical observation as the clearance of RH1 (0.54 l/min) is ~10 times lower than the clearance of E09 in human subjects (5.08 l/min) [27]. None of the demographic or patient variables studied had significant influence on the model fit to the clinical data and future larger trials of RH1 with larger patient numbers are required to identify possible covariate effects.

The pharmacodynamic results confirm that RH1 is a bifunctional alkylating agent capable of cross-linking DNA in man. The comet-X analysis of both PBMCs and tumour cells represents a novel pharmacodynamic approach to the study of DNA cross linking agents [21]. The comet-X assay was relatively robust in its validation and as dose escalation proceeded, a dose-related trend emerged with higher doses of RH1 being associated with more pronounced DNA cross linking in PBMCs. However, at the highest dose levels, there was evidence of a decline in the measured levels of DNA cross linking in assayed PBMCs. This apparent decline may be because the most severely damaged PBMCs are cleared from the blood leading to an altered population of assayed PBMCs. Such a conclusion is supported by the observations of reduced PBMC numbers at dose levels ≥1080 μg/m^2/day. In preclinical studies using spiked whole blood, the uptake of RH1 was maximal after 4 hours exposure and cross linking was maximal at 4–8 h following treatment [21]. The clinical data show a similar time course to the *ex vivo* experimental data.

As a 'proof of principle', the comet-X analysis in the tumour biopsies confirms that RH1 reaches its biological target and produces DNA cross linking. Although the number of patients studied is small, the paired tumour biopsy data show that DNA cross linking can occur in the presence of relatively low levels of DTD activity and conversely, that higher tumour DTD activity can be associated with relatively lower tumour DNA cross linking. It is possible that maximal enzymatic RH1 activation requires a relatively low level of DTD activity making additional DTD activity above a notional threshold superfluous. It is also possible that RH1 activation *in vivo* is occurring at non-tumour sites or that RH1 can be activated by non-DTD mechanisms. There is emerging work that although RH1 is a good substrate for DTD that it also has some activation independent of DTD expression. For example, although RH1 has greater activity in cells expressing DTD [12, 19, 28], the *in vitro* cytotoxicity profiles for RH1 are not markedly affected by the NQO1 inhibitor dicoumarol [29] and others have identified that semiquinone free radical formation from RH1 (at least *in vitro*) can be caused by several reductases, such as NQO2 [20, 30–32]. Our biopsy data suggest that taken in isolation tumour DTD levels do not represent a suitable criterion for future patient selection in studies of RH1.

Bioreductive drugs are theoretically well suited to improving selectivity through an enzyme-directed approach to tumour targeting and the *in vitro* and *in vivo* features of RH1 fulfil some but not all the required features, namely that the agent is a good substrate for DTD and this enzyme is highly overexpressed in some tumours. However, RH1 like other bioreductive agents studied to date is not uniquely specific for a single reductive enzyme and there remains an incomplete knowledge of the structural features required for tight enzyme selectivity. Further work to define these features is required for the future optimisation of bioreductive approaches.

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disclosure

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references

1. Ross D, Beall HD, Siegel D et al. Enzymology of bioreductive drug activation. Br J Cancer 1996; 74: S1–S8.
2. Walton MI, Smith PJ, Workman P. The role of NAD(P)H:quinone oxidoreductase (EC 1.6.99.2, DT-diaphorase) in the reductive bioactivation of the novel indoloquinone antitumor agent E09. Cancer Commun 1991; 3: 199–206.
3. Boland MP, Knox RJ, Roberts JJ. The differences in kinetics of rat and human DT diaphorase result in a differential sensitivity of derived cell lines to CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide). Biochem Pharmacol 1991; 41: 867–875.
4. Pardee AB, Li YZ, Li CJ. Cancer therapy with β-lapachone. Curr Cancer Drug Targets 2002; 2: 227–242.
5. Danson S, Ward T, Butler J et al. DT-Diaphorase: a target for novel anticancer drugs. Cancer Treat Rev 2004; 30: 437–449.
6. Workman P. Enzyme-directed bioreductive drug development revisited: a commentary on recent progress and future prospects with emphasis on quinone anticancer agents and quinone metabolising enzymes, particularly DT-diaphorase. Oncol Res 1994; 6: 461–475.
7. Edwards YH, Potter J, Hopkinson DA. Human FAD-dependent NAD(P)H diaphorase. Biochem J 1980; 187: 429–436.
8. Jaiswal AK, McBride OW, Adesnik M et al. Human dioxin-inducible cytosolic NAD(P)H:menadione oxidoreductase. cDNA sequence and localization of gene to chromosome 16. J Biol Chem 1988; 263: 13572–13578.
9. Traver RD, Horikoshi T, Danenberg KD et al. NAD(P)H:quinone oxidoreductase gene expression in human colon carcinoma cells: characterisation of a mutation which modulates DT-diaphorase activity and mitomycin sensitivity. Cancer Res 1992; 52: 797–802.
10. Kelsey KT, Ross D, Traver RD et al. Ethnic variation in the prevalence of a common NAD(P)H quinone oxidoreductase polymorphism and its implications for anticancer chemotherapy. Br J Cancer 1997; 76: 852–854.
11. Misra V, Grondin A, Klamut HJ et al. Assessment of the relationship between genotypic status of a DT-diaphorase point mutation and enzymatic activity. Br J Cancer 2000; 83: 998–1002.
12. Dehn DL, Winski SL, Ross D. Development of a new isogenic cell-xenograft system for evaluation of NAD(P)H:quinone oxidoreductase-directed antitumor quinones: evaluation of the activity of RH1. Clin Cancer Res 2004; 10: 3147–3155.
13. Ward TH, Danson S, McGown AT et al. Pre-Clinical evaluation of the Pharmaceutical properties of RH-1(2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone). Clin Cancer Res 2005; 11: 2695–2701.

14. Winski SL, Hargreaves RH, Butler J et al. A new screening system for NAD(P)H:quinone oxidoreductase (NQO1)-directed antitumor quinines: identification of a new aziridinylbenzoquinone, RH1, as a NQO1-directed antitumour agent. Clin Cancer Res 1998; 4: 3083–3088.
15. Berardini MD, Souhami RL, Lee CS et al. Two structurally related diaziridinylbenzoquinones preferentially crosslink DNA at different sites upon reduction with DT-diaphorase. Biochemistry 1993; 32: 3306–3312.
16. Xing C, Skibo EB. Sigmatropic reactions of the aziridinyl semiquinone species. Why aziridinyl benzoquinones are metabolically more stable than aziridinyl indoloquinones. Biochemistry 2000; 39: 10770–10780.
17. Loadman PM, Phillips RM, Lim LE et al. Pharmacological properties of a new aziridinylbenzoquinone, RH1 (2,5-diaziridinyl-3-(hydroxyl)-6-methyl-1,4-benzoquinone), in mice. Biochem Pharmacol 2000; 59: 831–837.
18. Winski SL, Swann E, Hargreaves RH et al. Relationship between NAD(P)H:quinone oxidoreductase 1 (NQO1) levels in a series of stably transfected cell lines and susceptibility to antitumor quinines. Biochem Pharmacol 2001; 61: 1509–1516.
19. Cummings J, Ritchie A, Butler J et al. Activity profile of the novel aziridinylbenzoquinones MeDZQ and RH1 in human tumour xenografts. Anticancer Res 2003; 23: 3979–3983.
20. Kim J-Y, Patterson AV, Stratford IJ et al. The importance of DT-diaphorase and hypoxia in the cytotoxicity of RH1 in human breast and non-small cell lung cancer cell lines. Anticancer Drugs 2004; 15: 71–77.
21. Danson S, Ranson M, Denneny O et al. Validation of the comet-X assay as a pharmacodynamic assay for measuring DNA cross-linking produced by the novel anticancer agent RH1 during a phase I clinical trial. Cancer Chemother Pharmacol 2007; 60: 851–861.
22. Chen HH, Ma JX, Forrest GL et al. Expression of rat liver NAD(P)H:quinone-acceptor oxidoreductase in Escherichia coli and mutagenesis in vitro at Arg-177. Biochem J 1992; 284: 855–860.
23. Therasse P, Arbuck SG, Eisenhauer EA et al. New guidelines to evaluate the response to treatment in solid tumours. J Natl Cancer Inst 2000; 92: 205–216.
24. Zappa F, Ward T, Pedrinis E et al. NAD(P)H: quinone oxidoreductase 1 expression in kidney podocytes. J Histochem Cytochem 2003; 51: 297–302.
25. Schellens JH, Planting AS, van Acker BA et al. Phase I and pharmacologic study of the novel indoloquinone bioreductive alkylating cytotoxic drug E09. J Natl Cancer Inst 1994; 86: 906–912.
26. Newell DR, Burtles SS, Fox BW et al. Evaluation of rodent-only toxicology for early clinical trials with novel cancer therapeutics. Br J Cancer 1999; 81: 760–768.
27. Schellens JH, Dombernowsky P, Cassidy J et al. Population pharmacokinetics and dynamics in phase II studies of the novel bioreductive alkylating cytotoxic indoloquinone EO9. Anticancer Drugs 2001; 12: 583–590.
28. Dehn DL, Inayat-Hussain SH, Ross D. RH1 induces cellular damage in an NAD(P)H:quinone oxidoreductase 1-dependent manner: relationship between DNA cross-linking, cell cycle perturbations, and apoptosis. Pharmacol Exp Ther 2005; 313: 771–779.
29. Tudor G, Alley M, Nelson CM et al. Cytotoxicity of RH1: nAD(P)H:quinone acceptor oxidoreductase (NQO1)-independent oxidative stress and apoptosis induction. Anticancer Drugs 2005; 16: 381–391.
30. Hasinoff BB, Begleiter A. The reductive activation of the antitumor drug RH1 to its semiquinone free radical by NADPH cytochrome P450 reductase and by HCT116 human colon cancer cells. Free Radic Res 2006; 40: 974–978.
31. Begleiter A, Leith MK, Patel D et al. Role of NADPH cytochrome P450 reductase in activation of RH1. Cancer Chemother Pharmacol 2007; 60: 713–723.
32. Yan C, Kepa JK, Siegel D et al. Dissecting the role of multiple reductases in bioactivation and cytotoxicity of the antitumor agent 2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone (RH1). Mol Pharmacol 2008; 74: 1657–1665.