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Development of a lyophilised RH1 formulation: a novel DT diaphorase activated alkylating agent

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Abstract

RH1 is a novel aziridinylbenzoquinone alkylating agent, which is activated in tumour cells by DT diaphorase. In common with previous aziridinylbenzoquinones, RH1 exhibits limited aqueous stability and solubility. The aim of this study was to examine the pharmaceutical properties of RH1 with a view to preparing a suitable formulation for clinical trial. Stability in a neutral phosphate-buffered solution was poor with a degradation half-life of 50 h at 55°C, indicating that lyophilisation was preferable. The reaction kinetics indicated a similarity with previous studies for base-catalysed degradation of aziridinylbenzoquinones. Intrinsic aqueous solubility at 0.5 mg mL^{-1} may be increased in solvent systems or by the use of polymers such as polyvinylpyrrolidone (PVP) or complexing agents like hydroxypropyl-β-cyclodextrin (HPBCD). In the latter case this increased solubility by an order of magnitude to around 5 mg mL^{-1}. Four potential formulations based on lyophilisation of RH1 (1 mg mL^{-1}) from buffered solution (pH 7, 0.01 M NaH_2PO_4) containing either 50 mg mL^{-1} mannitol, 40 mg mL^{-1} dextran, 20 mg mL^{-1} PVP or 50 mg mL^{-1} HPBCD were prepared and examined for stability characteristics. All formulations exhibited a temperature-dependent degradation. The mannitol and dextran formulations had limited stability and degraded rapidly at all temperatures. The PVP and HPBCD formulations degraded at elevated temperatures but remained stable for up to twelve months at 4°C. Examination of the degradation kinetics in the latter systems demonstrated similarity to the solution degradation mechanism, while in the former alternative degradation pathways appeared to be occurring. The chemical stability of RH1 in lyophilised formulations is dependent upon the excipient employed and storage temperature. Either the PVP or HPBCD formulation would be suitable clinical trial formulations of RH1. The results indicate that the choice of lyophilisation excipient for aziridinylbenzoquinones cannot be based on previous literature studies of related agents.

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Introduction

The biology of solid tumours produces within their central core a unique environment with reduced oxygen potential and an over-expression of certain reductase enzymes (Brown & Giaccia 1998; Brown 1999). This feature has led to the development of pro-drugs, which are only activated in reduced oxygen potential or by reductase enzymes (bioreductive agents) and therefore have increased tumour selectivity. One enzyme implicated in this pathway is NAD(P)H : quinone oxireductase (DT diaphorase), which is capable of reducing quinone compounds to DNA-damaging species and is able to use NADPH or NADH as electron donors

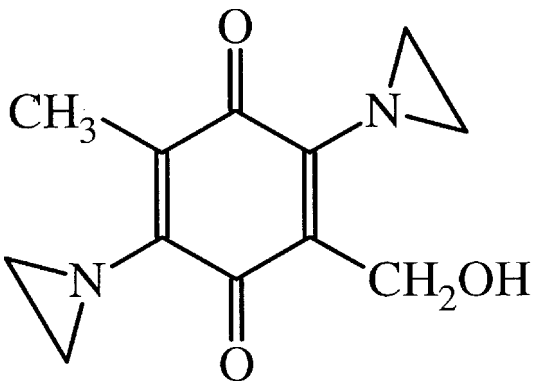

Figure 1 Chemical structure of RH1 (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone).

(Ernster 1987). A variety of anticancer drugs utilise this feature, for example the natural antitumour antibiotic mitomycin C (Chirrey et al 1995) or the synthetic aziridinylbenzoquinones such as AZQ (2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone) (Fisher et al 1992), BZQ (2,5-di-*N*-aziridinyl-3,6-di-(*N*-2-hydroxyethyl)benzoquinone) (Betteridge et al 1990) or EO9 (3-hydroxy-5-aziridinyl-1-methyl-2-(1*H* indole-4, 7-dione)prop-β-en-α-ol) (Hendriks et al 1993). These bioreductive agents are, however, not ideal. Mitomycin C, for example, is recognised to produce phlebitis at the site of injection (Bhardwaj et al 2000) and EO9, although active in model systems, was not active in a Phase II clinical trial (Dirix et al 1996). There is therefore a requirement for agents with improved activity. RH1 (Figure 1) (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone) has recently been synthesised as an analogue with increased water solubility and preferential activity against human tumour cell lines which express high levels of DT diaphorase (Winski et al 1998).

Several aziridinylbenzoquinones have been subjected to formulation studies and this particular molecular structure presents interesting formulation challenges. Aqueous solubility is usually limited (Jonkmandevries et al 1994) and pH-dependent (Vezin & Salole 1993) chemical instability via aziridinyl ring opening well recognised (Xing & Skibo 2000). No reported studies have attempted to enhance the solubility of these agents and formulations of AZQ employed dissolution in *N*,*N*-dimethylacetamide (Anon 1990). Acceptable stability has either required lyophilisation (Vezin & Salole 1993; Jonkmandevries et al 1994) or the provision of the material as a dry powder (Anon 1990). No single lyophilisation excipient has been employed; EO9 utilised lactose as a diluent (Jonkmandevries et al 1994) while BZQ required dextran (Vezin & Salole 1993).

To prepare RH1 for clinical trial, we have investigated potential solubilisation and stabilisation excipients for lyophilised formulations. Pre-clinical information indicates that the solubility of RH1 is low and a bulking agent will be required during lyophilisation to produce a reasonable cake. Mannitol has therefore been investigated as a classical lyophilisation excipient along with dextran, which was employed for BZQ. In addition polyvinylpyrrolidone (PVP) was investigated as a non-hydroxyl bearing excipient along with 2-hydroxypropyl-β-cyclodextrin (HPBCD) which should be able to enhance solubility, provide a suitable lyophilised cake and reduce toxicity at the site of administration.

Materials and Methods

Materials

RH1 was synthesised by High Force Research Limited using a synthetic method based upon that developed by R. H. J. Hargreaves of The Paterson Institute (Hargreaves et al 1999). Dextran was purchased from Sigma Aldrich, mannitol from Merck and HPLC-grade methanol from Rathburn Chemicals, Scotland. Polyvinylpyrrolidone (PVP, Plasdone, k value ca. 15) was provided by ISP Europe, Guildford, UK, and 2-hydroxypropyl-β-cyclodextrin (HPBCD) by Janssen, Belgium. All other chemicals were of analytical grade.

HPLC analysis

HPLC analysis was performed on a Spectraphysics AS3000 System using a UV3000 detector and P4000 pump. A Phenomenex C 8(2) 15×4.6 mm, 5 μm column with guard was utilised with a gradient elution program of water–methanol (70:30) for 6 min followed by a linear gradient to water–methanol (30:70) after 19 min. This was followed by a 10-min equilibration period at water–methanol (70:30). Detection was by UV at 230 nm and the samples were cooled to 4°C during the analysis.

Solution stability

RH1 was prepared at 0.5 mg mL^{-1} in 0.01 M NaH_2PO_4 and incubated at 4°C, 25°C, 40°C and 55°C in the dark, and at ambient temperature exposed to light. HPLC analysis for content was performed on days 0, 1, 2, 3, and 8.

Solubility studies

RH1 solubility was investigated in solutions of 10% and 60% v/v propylene glycol, 10 mg mL^{-1} and 30 mg mL^{-1} HPBCD, 20 mg mL^{-1} and 40 mg mL^{-1} polyvinylpyrrolidone (PVP), all buffered to pH 7 with 0.01 M NaH_2PO_4.

Differential scanning calorimetry (DSC)

DSC was performed using a Mettler-Toledo DSC821^e. Solutions of RH1 formulations were tested using a program of 20°C to −50°C at 5°C min^{-1}, holding for 5 min, and then from −50°C to 20°C at 5°C min^{-1}. The instrument was calibrated according to the manufacturer's instructions at regular intervals.

Lyophilised formulations

Four formulations of RH1 at a concentration of 1 mg mL^{-1} were prepared in either 20 mg mL^{-1} PVP, in 50 mg mL^{-1} HPBCD, in 40 mg mL^{-1} dextran or in 50 mg mL^{-1} mannitol. All solutions were adjusted to pH 7 in 0.01 M NaH_2PO_4. Type-1 10-mL clear glass vials were filled to 2 mL volume and all batches subjected to an identical freeze-drying program. Freezing: 20°C to −30°C over 3 h, holding at −30°C for 2 h; primary drying: −30°C to −5°C over 2 h, then −5°C to −10°C over 8 h; secondary drying: −10°C to 10°C over 2 h, then holding at 10°C for 6 h, total cycle time 23 h. All batches were prepared under cGMP in the Cancer Research Campaign Formulation Unit sterile suite facilities, University of Strathclyde, Glasgow.

Stability studies

Stability studies were conducted according to ICH guidelines with appropriate quantities of each test formulation stored at 4°C, 25°C and 40°C in the dark. Analysis was performed for RH1 content by HPLC, water content by Karl Fischer (Mettler-Toledo DL37-KF Coulometer), pH, reconstitution time and visual appearance were also examined.

Results and Discussion

RH1 instability in acidic or basic media was immediately apparent with rapid discolouration providing a clear visual indication of chemical breakdown, most notably in an acidic environment with a red to black colour change in the space of only a few hours. Thus development of a suitable formulation for intravenous administration of RH1 in Phase 1 clinical trial required examination of neutral-pH solutions. This is in contrast to BZQ where the pH of optimal stability was around 9 (Vezin & Salole 1993) but similar to AZQ where stability was greatest at pH 7 (Poochikian and Cradock 1981).

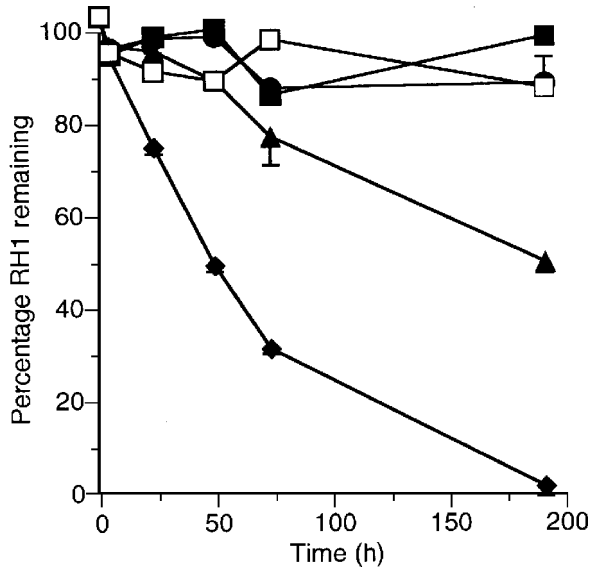

Figure 2 Percentage RH1 remaining in pH 7 phosphate buffer after storage at different temperatures. All solutions had an RH1 starting concentration of 0.5 mg mL^{-1} in 0.01 M NaH_2PO_4 at pH 7; RH1 concentration was measured by HPLC. ■, 4°C; ●, 25°C; ▲, 40°C; ◆, 55°C; □, ambient (uncontrolled temperature); all conditions in dark except ambient, which was exposed to normal laboratory lighting. Mean±range, n = 2.

Solutions of RH1 (0.5 mg mL^{-1}) at pH 7 in 0.01 M phosphate buffer were incubated at a range of temperatures and the percentage of RH1 remaining determined using HPLC (Figure 2). The 4°C solution showed little change over the time period studied (up to 8 days). The pseudo first-order reaction rate constants for the 25°C, 40°C and 55°C solutions were determined and an Arrhenius plot constructed. The linear regression line had a gradient of -4.544×10^3 h^{-1} ($R^2 = 0.9996$), extrapolation of which allowed calculation of the 4°C rate constant as 0.059×10^{-3} h^{-1}. Based on this calculated rate constant the time for an aqueous solution of RH1 stored at 4°C to reach 95% of the initial content was 36.3 days. It was therefore concluded that RH1 had insufficient stability in solution to provide a suitable shelf life for clinical trial use and a lyophilised formulation was therefore required.

The calculated activation energy of RH1 degradation in solution was 87 kJ mol^{-1} with a frequency factor of

Table 1 Solubility of RH1 in various systems.

System	Solubility (mg mL^{-1})
Water	0.5
Chloroform	$\geqslant$ 34
DMSO	$\geqslant$ 37
DMSO 25% v/v in buffer	$\leqslant$ 3.8
Propylene glycol 10% v/v in buffer	$\leqslant$ 3.3
Propylene glycol 60% v/v in buffer	$\leqslant$ 4.4
PVP 20 mg mL^{-1} in buffer	3.0
HPBCD 100 mg mL^{-1} in buffer	5.1
HPBCD 300 mg mL^{-1} in buffer	5.3

Buffer 0.01 M NaH_2PO_4 pH 7.

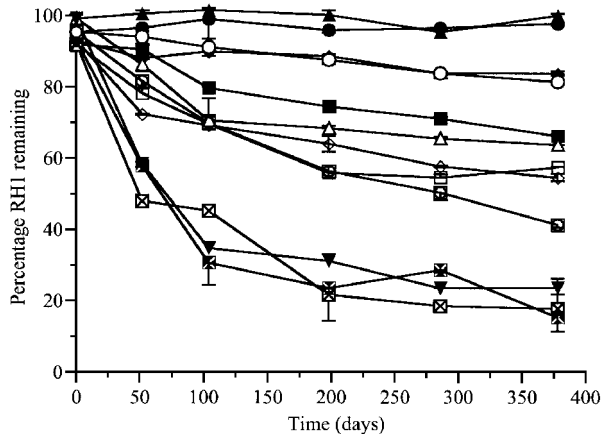

Figure 3 Percentage RH1 remaining in lyophilised formulations after storage at different temperatures. All formulations had an RH1 starting concentration of 1 mg mL^{-1} in 0.01 M Na H_2PO_4 at pH 7 and included either mannitol 50 mg mL^{-1}, dextran 40 mg mL^{-1}, PVP 20 mg mL^{-1} or HPBCD 50 mg mL^{-1}, lyophilised as 2 mL of solution in a 10-mL vial. RH1 content was determined by HPLC after reconstitution with water for injection. Mannitol: ■, 4°C; □, 25°C; ⊠, 40°C. Dextran: ◆, 4°C; ◇, 25°C; ▩, 40°C. PVP: ▲, 4°C; △, 25°C; ▼, 40°C. HPBCD: ●, 4°C; ○, 25°C; ◘, 40°C. Mean±range, n = 2.

4.1×10^8 s^{-1}. The activation energy is higher than values quoted for EO9 at pH 4 and 12 of 40.6 and 67 kJ mol^{-1}, respectively. However, these values were not obtained at the pH of maximal stability. The frequency factor is also higher but close to the EO9 value at pH 12 of 1.6×10^8 s^{-1} (Devries et al 1993). Previous research (Kusai et al 1982) with related aziridinylbenzoquinones has determined that high activation energies are associated with hydroxyl-mediated degradation. Although not conclusive this hints that RH1 degradation at pH 7 may be occurring via hydroxyl-mediated cleavage of the aziridine ring in a manner similar to other aziridinylbenzoquinones. Further research would be required to conclusively identify this pathway and associated degradation products.

Formulation development might be complicated by the low aqueous solubility of RH1, the reported maximum solubility being only 0.5 mg mL^{-1}. Solubility studies (Table 1) in a range of pharmaceutically acceptable buffered (pH 7) excipient or solvent systems identified two potential routes to increased solubility. PVP solutions increased RH1 solubility up to 3 mg mL^{-1} and HPBCD up to 5 mg mL^{-1}. This offered a maximum of a ten-fold increase in solubility and both systems were therefore pursued in the development of a lyophilised clinical trial formulation.

To identify freezing and melting behaviour of the PVP and HPBCD solutions prior to lyophilisation, DSC studies were performed. The PVP formulation exhibited an onset of freezing at −18.7°C and melted at −1.0°C when warmed; for HPBCD the values were −17.2°C and −1.3°C. This indicates that a conservative lyophilisation cycle would require freezing to at least −20°C followed by primary drying at a product temperature below −5°C. A pilot-scale lyophilisation confirmed the suitability of these temperatures for both formulations (data not shown). Four batches of RH1 were then produced – two test formulations using mannitol and dextran and two putative formulations using PVP and HPBCD. Lyophilisation was found to be complete within 23 h, based on a pressure-rise test during secondary drying.

Although subjected to identical lyophilisation conditions the starting water content of the batches varied considerably at 3.89 mg/vial for mannitol, 1.26 mg/vial for dextran, 0.60 mg/vial for PVP and 0.65 mg/vial for HPBCD. This difference reflects excipient performance during drying and the ratios of frozen, non-frozen and hydrogen-bound water present during lyophilisation.

During stability testing the measured pH of the reconstituted vials and the reconstitution time did not change. However, a temperature- and formulation-dependent colour change was noted from red to orange or dark orange, similar to the changes noted during solution stability testing. The water content also changed during storage, with mannitol formulations losing water while all other formulations slowly increasing in water content.

The chemical stability of RH1 lyophilised products is presented in Figure 3 and for all formulations there is a clear temperature-dependent degradation. After only 1 month the mannitol and dextran formulations exhibited degradation at all temperatures while PVP and

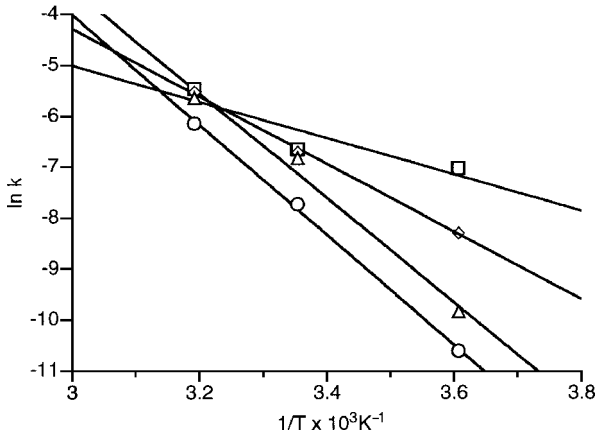

Figure 4 Arrhenius plot of lyophilised stability data. Data plotted is the pseudo first-order rate constants derived from Figure 2. Linear regression line plotted. Mannitol □ $R^2 = 0.8341$; Dextran ◇ $R^2 = 0.9978$; PVP △ $R^2 = 0.9863$; HPBCD ○ $R^2 = 0.9987$.

HPBCD formulations did not show significant change at low temperatures. This loss of RH1 was accompanied by an increase in the breakdown products detected by HPLC (data not shown). The extent of degradation did not appear to be related to water content as during stability testing formulations with different water contents produced similar degrees of RH1 degradation, indicating that the excipient is important in the degradation process. After extended storage periods only the PVP and HPBCD stored at 4°C remained viable formulations. For new products, an acceptable level of degradation is based on the proposed daily dose, the levels of impurity tested in toxicity studies and the toxicity and derivation of the degradation products (Anon 1999). The proposed RH1 clinical dose is approximately 30 mg, requiring the reporting of degradants above 0.1% content, the identification of degradants above 0.2% and the qualification of those above 0.5%. Although this study only measured RH1 content and not the degradation profile, the results infer that only the PVP and HPBCD formulations stored at low temperatures will meet the required criteria.

The pseudo first-order reaction rate constants for each formulation and temperature were determined and an Arrhenius plot constructed (Figure 4). The determined Arrhenius constants are presented in Table 2 and it is evident that there are differences between the mannitol, dextran and PVP or HPBCD formulations. The calculated activation energy for the mannitol degradation is lower than the value obtained in neutral phosphate buffer solution by a factor of three and the dextran figure is just under half the solution value. The values for PVP and HPBCD are almost identical to the solution value, and the PVP and HPBCD lines are parallel, differing only in the frequency factor. The HPBCD may provide a slightly enhanced stability due to entrapment of RH1 within the cyclodextrin toroid, although further work would be required to fully elucidate this. These results indicate that the degradation mechanisms occurring in the PVP and HPBCD lyophilised formulations are identical and probably similar to the solution-based mechanism, while in the mannitol and dextran formulations a potentially different reaction mechanism exists. In solution, acid-catalysed degradation (aziridinyl ring opening) of aziridinylbenzoquinones has a lower activation energy than base-catalysed degradation (aziridinyl ring cleavage to hydroxybenzoquinone) (Kusai et al 1982). The hydroxyl groups present on mannitol and dextran may therefore be acting as a Lewis acid and catalyse degradation. Previous aziridinylbenzoquinones have been lyophilised using similar excipients, EO9 for example utilised lactose as a bulking agent (Jonkmandevries et al 1994) while BZQ utilised dextran (Vezin & Salole 1993). In both cases at least twelve months of acceptable stability was obtained although both formulations were at a basic pH before lyophilisation.

Table 2 Calculated Arrhenius constants for lyophilised formulations.

Formulation	Activation energy (kJ mol^{-1})	Frequency factor (s^{-1})
Mannitol	29.7 ± 22.4	$3.43 \times 10^{-3} \pm 3.13 \times 10^{1}$
Dextran	55.3 ± 4.38	$7.24 \times 10^{1} \pm 6.02 \times 10^{1}$
PVP	84.2 ± 17.0	$8.27 \times 10^{6} \pm 8.26 \times 10^{6}$
HPBCD	90.7 ± 5.47	$2.62 \times 10^{7} \pm 2.34 \times 10^{7}$

Mean ± 33% confidence limit.

Conclusions

RH1, like previous aziridinylbenzoquinones, does not exhibit sufficient chemical stability in solution to warrant a liquid formulation. A suitable clinical trial formulation may be obtained after lyophilisation of a pH-7-buffered solution with either PVP or HPBCD as excipients. In the latter formulation RH1 solubility can be increased by an order of magnitude to 5 mg mL^{-1}. However, all lyophilised formulations exhibit a temperature-dependent stability and storage at reduced temperatures would be required. The chemical insta-

bility noted in lyophilised formulations employing mannitol or dextran as excipients is at variance to literature reports for previous aziridinylbenzoquinones. These differences suggest that the stability of aziridinylbenzoquinones in lyophilised formulations is complex and related to chemical structure, excipient choice and starting solution pH.

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