Cancer Chemother Pharmacol (2007) 60:851–861
DOI 10.1007/s00280-007-0432-9

ORIGINAL ARTICLE

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

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Received: 21 September 2006 / Accepted: 22 January 2007 / Published online: 2 March 2007

Abstract
Purpose RH1 is a novel anticancer agent with potent DNA-cross linking activity. RH1 has the potential to be activated within tumors over expressing NQO1, giving maximal antitumour activity with reduced toxicity in normal tissues. RH1 has recently completed a Cancer Research UK sponsored phase I clinical trial at two different centers in the United Kingdom. The comet-X assay was a secondary endpoint in this trial and assay validation was necessary. We describe here this validation process. Whilst it is impossible to cover all variations/conditions of a pharmacodynamic assay, we have strived to evaluate and demonstrate that this assay conforms to the three R's of validation, that is robustness, reliability and reproducibility.
Methods K562 and peripheral blood mononuclear cells were treated with either radiation alone, or with a combination of radiation and drug. These samples were then embedded in low melting point agarose and subjected to a modified version of the alkaline single cell gel electrophoresis (Comet) assay, described here as the comet-X assay. Variations in the preparation, electrophoresis, storage and scoring of these samples was investigated. In addition radiation and drug dose response curves were constructed. Finally stability of QC standards was investigated over a 30-month period.
Results We have demonstrated a linear radiation-dose response in cells up to 20 Gy and drug induced DNA cross-linking up to 50 nM. From the radiation dose response curves we were able to show that the relative inaccuracy measured against a global mean value was less than 25% and the relative (within day) imprecision was less than 30% over all doses. Between day runs produced an intra assay imprecision of 21.2%. Variables involved in the electrophoresis process showed the voltage across all slides in the tank ranged from 3.1 to −2.0 (mV) whilst the current ranged from 0.8–5.5 mA. QC standards were prepared from PBMCs of healthy donors and frozen at −80°C. The stability of these frozen QC standards was measured over a 30-month period. No significant deterioration in any of the control, irradiated or drug treated samples was observed.
Conclusions The comet-X assay has been shown to be a robust, reliable and reproducible assay. It is ideally suited for the evaluation of the pharmacodynamic effects of DNA cross-linking agents undergoing early clinical trials. Furthermore, this assay may provide valuable data, in conjunction with pharmacokinetics, when measuring toxicity and efficacy as part of the RH1 phase I clinical trial.

Keywords Comet-X assay · SCGE · RH1 · DNA cross-linking · Validation

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Introduction

The single cell gel electrophoresis (SCGE) or comet assay was first described by Ostling and Johanson [1].

This assay can be used for direct visualisation and quantification of DNA damage to individual cells in a population. With this assay rapid generation of data from low numbers of almost any cell type can be achieved independent of cell proliferation and the reader is directed to an extensive review of applications by Fairburn et al. [2]. More recent citations include a number of studies such as assessment of DNA damage secondary to ionising radiation [3, 4], cytotoxic agents [3, 5–9], genotoxins [10–12] and DNA repair activity [10, 13]. This basic alkaline assay was modified by Ward et al. [14] to measure DNA cross-linking and is referred to as the comet-X assay. We and others have shown that this assay is a sensitive semi quantitative method of determining DNA inter-strand cross-links [14–16]. Central to the assay is the introduction of a fixed number of random strand breaks into the DNA of cells post-drug treatment. DNA cross-linking drugs effectively retard the migration of this fragmented DNA in a dose-dependent manner. We have previously used the comet-X assay extensively as a pharmacodynamic end point in the pre clinical study of the in vivo efficacy of the novel DNA-cross linking diaziridinylbenzoquinone RH1 (2,5-diaziridinyl-3-[hydroxymethyl[-6-methyl-1,4- benzoquinone) [14, 16].

RH1 is a novel anticancer agent with potent DNA-cross linking activity. It is an excellent substrate for and can be activated by the two electron reductase enzyme DT-diaphorase (DTD, EC 1.6.99.2; NAD(P)H:quinone oxidoreductase). RH1 has the potential to be activated within tumors over expressing NQO1, giving maximal antitumor activity with reduced toxicity in normal tissues. Preclinical studies showed good activity in a number of xenograft models [17–21]. Activation of RH1 is not confined exclusively to NQO1 expression [20, 22]. Whilst one electron reduction of RH1 can occur this does not enhance cytotoxicity under hypoxic conditions [20]. It is clear however that in those cells and tissues that over express NQO1, enhanced cytotoxicity is observed. Consequently, NQO1 is pivotal for a more rapid and extensive activation of RH1.

In order to establish its tolerability in human subjects with advanced solid tumours RH1 has recently entered a Cancer Research UK sponsored phase I clinical trial at two different centers in the United Kingdom.

The potential use of the comet assay as a biomarker of DNA breaks and the use of comet X as a biomarker of DNA cross linking has been recognised for many years. However, there are multiple points at which variability can be introduced, consequently the comet assay in general is more successful in the hands of some groups than others. This has prompted the publication of guidelines [23, 24] although such guidelines may not be universally applicable.

An integral component of the RH1 clinical trial is that pharmacodynamic (PD) assays are performed on patient-derived samples (plasma, peripheral blood mononuclear cells and tumour biopsies) in order to provide evidence of drug target interaction. In this instance the comet-X assay is used as a DNA cross-linking assay to monitor drug effects on peripheral blood mononuclear cells and tumour.

All assays when used in clinical trials are now subjected to more stringent regulatory requirements within the EU [25]. Cancer Research UK policy requires that the degree of validation is dependent on the order of priority designated to the pharmacodynamic assay. The comet-X assay is a secondary end-point in this trial and assay validation was necessary. We describe here such a validation process for the assay. Whilst it is impossible to cover all variations/conditions of a pharmacodynamic assay, we have strived to evaluate and demonstrate that the assay conforms to the three R's of validation, that is, it is reliable, reproducible and robust. To achieve this we have concentrated on three main areas. Firstly the slide electrophoresis, slide stability, storage and analysis, secondly sample transport and processing radiation/DNA cross-linking dosimitry and finally the generation and stability of internal quality control (QC) samples.

Materials and methods

RH1

RH1 was synthesised as previously described [17] and was dissolved in DMSO and diluted as necessary prior to use.

Cell culture

The human erythroleukaemic blast K562 cell line was obtained originally from the European Collection of Animal Cell Cultures (ECACC, UK). Cells were grown in RPMI 1640 medium supplemented with 1% L-glutamine and 10% heat inactivated foetal calf serum (Invitrogen) at 37°C in a humidified atmosphere with 5% carbon dioxide. They were maintained in log phase growth by weekly serial dilution. Cells were routinely screened for mycoplasma contamination by PCR and remained negative throughout the experiments described.

K562 cells were used for most validation experiments. Briefly cells were diluted to 2×10^4 cells/ml in RPMI medium in a total volume of 0.5 ml.

Peripheral blood mononuclear cell (PBMCs) preparation

PBMCs were harvested from five healthy volunteers with informed consent. From each, 6 ml of blood was collected into tubes containing EDTA. The blood was layered over 5 ml of lymphoprep (Invitrogen) and spun at 800*g* for 20 min. The buffy layer was removed and washed in 15 ml of ice cold Hanks Balanced Salt Solution (HBSS) (Sigma). Cells were finally re-suspended in ice cold HBSS, counted and diluted to a final concentration of 4×10^4 cells/ml prior to treatment. A sixth sample was prepared by pooling cells from each of the five volunteers.

Both K562 cells and PBMCs were transferred to bijou tubes (Sterilin, Staffordshire, UK) and placed on ice. Drug treated and control irradiated samples were then subjected to irradiation using a Cesium-137 source (0.4 Gy/min). All samples were maintained on ice to prevent repair. All experiments consisted a minimum of both control non-irradiated and irradiated cells.

External QC samples

Pooled PBMCs were isolated and prepared as described above then treated with either 10 or 25 nM RH1 for 2 h. Cells were then washed in ice cold HBSS and re-suspended at 4×10^4 cells/ml in plastic Bijou tubes and irradiated at 20 Gy. Samples were either analysed immediately or dimethyl sulfoxide (DMSO, Sigma) was added to a final concentration of 10% and samples were stored at −80°C for various lengths of time.

Slide preparation and electrophoresis equipment

Super frost slides (76 × 26 mm, BDH) were wiped with a 0.1% solution of poly-L-lysine (Sigma) and allowed to air dry. Once dry, 1% molten normal agarose (BMA, Rockland, US; dissolved in ddH_2O) was spread evenly over each slide and allowed to dry overnight. Pre coating the slides in way prevented the top agarose layer containing cells from slipping off during electrophoresis. Purpose made electrophoresis tanks with lids were constructed from black plastic with a single stretched platinum electrode at each end. The dimensions of the tank (W × L × D) were such that 1 l of electrophoresis buffer was needed to completely cover the slides which themselves were accommodated on purpose built trays. These trays were use to hold and transfer the slides during lysis and washing steps accomplished in tanks of similar dimensions to that of the electrophoresis tanks.

Two electrophoresis tanks were used throughout the study.

Comet-X assay

To 0.5 ml of cell suspension in bijou tubes 1 ml of molten (50°C) 1% low melting point (LMP) agarose (USB, Cleveland, US; dissolved in PBS) was added. This suspension was gently mixed then placed onto a microscope slide prepared as described above. A slide length borosilicate coverslip (50 × 22 mm, BDH) was placed onto each slide to spread the sample evenly and the slide was then transferred to an ice tray. When the agarose had cooled and set, the coverslip was removed; each slide was placed on a tray. Up to 24 slides were placed on each tray, using blank slides if necessary to fill in the gaps. The trays were then placed into a washing tank. Cells were lysed for 1 h in ice-cold comet lysis buffer (CLB, 2.5 M NaCl (Sigma), 10 mM Tris (BDH), 100 mM EDTA, pH 10.5–11 (BDH), 10 ml of 10% DMSO and 10 ml Triton X-100 (Sigma). CLB was made up and stored at 4°C prior to the experiment without DMSO and Triton, which were then added just before use. Following lysis the slides were washed thrice with ddH_2O for 15 min each time and then covered with 1 l of alkali unwinding buffer (50 mM NaOH (BDH) and 1 mM EDTA (BDH), pH 12.5). The slides were left in the unwinding buffer for 30 min and then electrophoresed at 21 V (0.6 v/cm) for 25 min. The slides were washed with a neutralising solution of 500 mM pH 8.0 Tris–HCl (BDH), rinsed with PBS and left to air-dry overnight. The slides were then stored in lightproof boxes prior to analysis. Prior to scoring, the slides were rehydrated and stained with the a 1/10,000 dilution of the fluorochrome SYBR GOLD (Molecular Probes, Eugene, US). The slides were viewed using fluorescence microscopy (Zeiss-Jenamed, Oberkochen, Germany) at 250× magnification. Fifty cells, 25 each on duplicate slides were captured and the data quantified using a computer package (Komet Version 6, Kinetic Imaging). The percentage DNA present in each comet was recorded and the % DNA cross-linked calculated [14]. Representative images of comets scored during these experiments are shown in Fig. 1.

Statistical methods

The basic parameter used in analysing the comets was % tail DNA. This figure was used to calculate % DNA

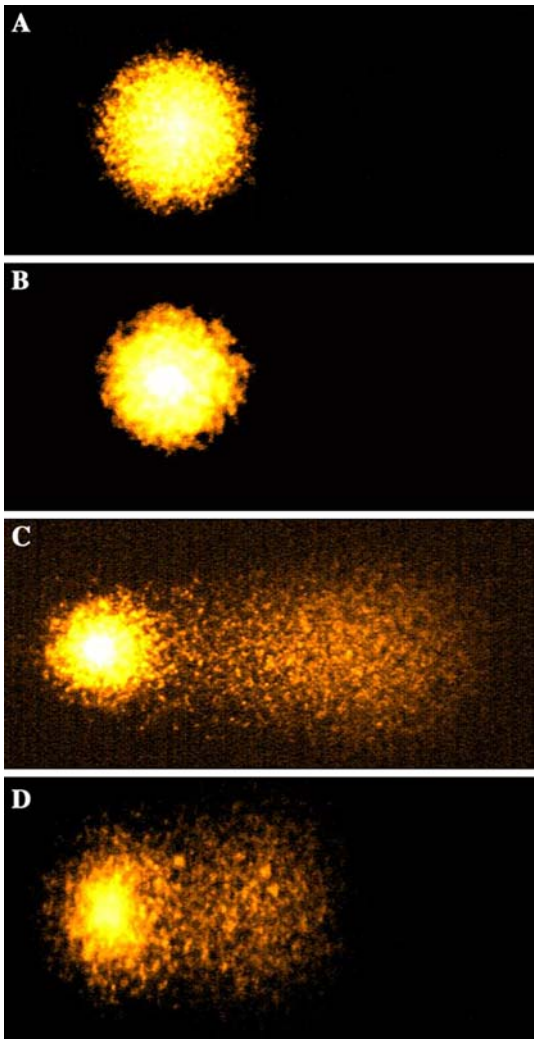

Fig. 1 Typical comets obtained from control and treated PBMCs using the comet-X assay. **a** Control nondrug nonirradiated comet. **b** No radiation drug only (10 nM) demonstrating little or no DNA damage from RH1 alone. **c** No drug, irradiation (20 Gy) alone, showing a substantial comet tail of fragmented DNA. **d** Irradiation following drug treatment (10 nM, *RH1*). Note the distinct retardation of DNA migration resulting in a shortened comet tail

cross-linked [14]. Statistical analysis was performed using two tailed *t* tests.

Results

Electrophoresis

Six slides, without cells, were placed at regular intervals within the electrophoresis tank. The slides were just covered with alkali unwinding buffer and electrophoresis performed for 25 min. During electrophoresis, using a modified probe, both the current and the voltage were measured in-gel on three different areas of each slide.

Initiation of both current and voltage was rapid and equalised within 5 min. Graphical representation of variations in both the current and the voltage within the gel during electrophoresis are shown in Fig. 2. The voltage across all slides ranged from 3.1 to 2.0, (mean 2.5 ± 0.3 SD) whilst the current ranged from 0.8 to 5.5 mA (mean 3.1 ± 1.4 SD).

K562 cells (unirradiated and irradiated) were embedded in agarose on slides. One slide of each was placed in the left upper quadrant, left lower quadrant, right upper quadrant, right lower quadrant and in the centre of the electrophoresis tank. The samples were then processed as described previously. Each experiment was repeated twice in each of the two tanks. Comparison of the means between slides in any of the four quadrants and the centre of the tanks are shown in Table 1. In irradiated samples some statistical difference ($P < 0.05$) between slide positions in experiments performed on different days was observed. When experiments were run on the same day no such differences were observed Furthermore, these differences in

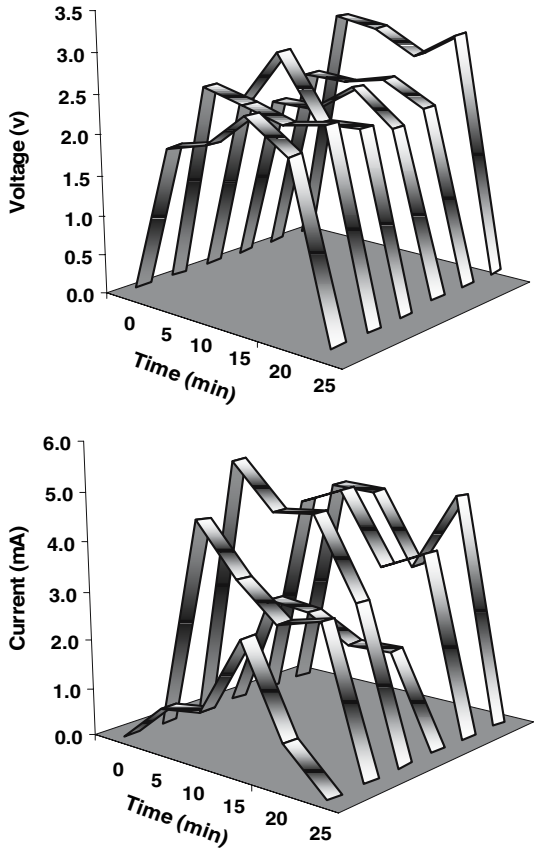

Fig. 2 The extent and duration of both current and voltage passing through each of six individual cell free agarose gels placed on slides as described in Materials and methods. The different positions in the electrophoresis tank include those listed in Table 1. (Each profile is the mean of three readings per time point; error bars are omitted for clarity)

Table 1 % Tail in the tail of control or irradiated PBMCs at various positions in the electrophoresis tank

Position	Control	Irradiated
Upper right quadrant	5.1 (5.1)	78.8 (4.7)
Upper left quadrant	4.0 (4.3)	77.1 (5.9)
Centre	5.8 (5.4)	78.2 (6.5)
Lower right quadrant	4.8 (4.4)	79.5 (7.1)
Lower left quadrant	5.4 (4.9)	81.3 (6.2)

Each point represents the mean and SD of 25 comets scored on each of two slides from each of two tanks on two independent assay runs

comet tails on slides in different positions were not reproducible for any given position in any tank between different experiments. No statistical differences in control slides were observed in any of the experiments performed.

Stability

K562 cells (10 unirradiated controls and 10 irradiated samples) were embedded in LMP agarose on slides. Half the slides were placed in CLB immediately and the others were kept on ice for 2 h and then placed in CLB. Then the samples were processed as described previously. There were no statistically significant differences between samples, which were processed immediately, and those, which were placed on ice for up to 2 h ($P > 0.05$). The CLB buffer can be made up without detergent or DMSO and stored at 4°C prior to use. It was necessary therefore to determine the effect of storage time on this buffer. A single large batch of CLB buffer was produced and stored at 4°C. Freshly prepared control and irradiated K562 cells were used to produce test slides, which were subsequently lysed and processed in this buffer. Samples of CLB were used fresh, 1, 4 and 8 weeks old and the results are shown in Table 2. Comparisons of means between control samples processed in either fresh CLB or stored CLB buffer just showed statistical significance ($P = 0.04$) after 8 weeks storage but not at 4 weeks. Irradiated samples however showed no such differences ($P = 0.2$).

Table 2 The stability of comet lysis buffer (CLB) determined by processing control or by irradiated K562 cells though the comet assay following lysis in fresh buffer or buffer stored without DMSO or Triton X-100 for up to 8 weeks

	Fresh buffer	Four weeks 4°C	Eight weeks 4°C
Control	4.62 (0.16)	5.05* (1.2)	6.0** (0.81)
Irradiated	72.9 (4.3)	76.7 (0.06)	73.3 (3.4)

Each point represents the mean and SD deviation of 25 comets scored on each of two slides

* $P = 0.057$, ** $P = 0.032$

Scoring

Slides from these stability experiments were allowed to air dry, scored immediately and then left in lightproof cardboard boxes for 1 month. After this time, the slides were rehydrated and scored again using the same software. Four slides from each of the two experimental runs were scored and analysed separately by two analysts. There was no statistical difference between fresh and stored sets of slides ($P = 0.075$). Likewise there was no statistically significant difference between the scores derived from either analyst ($P > 0.05$).

Radiation calibration curves for K562 cells

The comet-X assay requires the introduction of a fixed number of strand breaks the degree of retardation of which acts as a surrogate measurement of DNA cross linking. The introduction of a fixed number of breaks must be reproducible and accurate. The within day and between day variation of such a step needs to be determined. K562 cells were subjected to 5, 10, 15 and 20 Gy of radiation at 0.4 Gy/min using Cesium-137 source. Radiation calibration curves were constructed and are shown in Fig. 3. Two complete sets of experiments were performed on each of two different days. Both the inter- and intra-experimental variation was measured.

The measured response to ionising radiation was found to be linear up to 20 Gy. There were no significant differences between any of the radiation calibration curves ($P > 0.05$). Inter assay imprecision was found to be 31.8% whilst the Intra assay imprecision was 21.2%. The r^2 range was 0.98 ± 0.02. The highest mean control (no radiation) value was 18.05 ± 9.5 SD. The lowest level of quantification (LLOQ) was therefore set at 1 SD above this value and was shown to be 5 Gy.

Radiation calibration curves for PBMCs

Biological response to ionising radiation can be cell type dependant so it is essential to demonstrate, for each cell type used in this assay, a linear DNA damage response curve to ionising radiation. Blood from five healthy volunteers was collected with informed consent and processed to yield PBMCs as described above. A sixth pooled sample consisting of equal numbers of cells from all five donors was also created. The cells were irradiated at doses 0, 5,10,15 and 20 Gy.

The radiation calibration curves for each individual sample and for the pooled sample are shown in Fig. 4. All curves were observed to be linear. There was no statistical difference ($P > 0.05$) between any of the

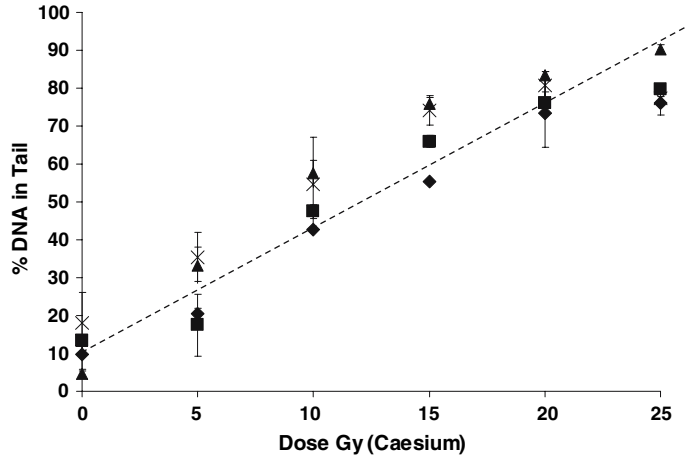

Fig. 3 Radiation calibration curves for K562 cells. Percentage of DNA in the tail of the comet was measured following increasing doses of irradiation. Each point represents the mean and SD of 25 comets scored on each of two slides from each of two tanks on two independent assay runs. *Symbols* represent individual experiments consisting of 25 comets scored from each of five slides

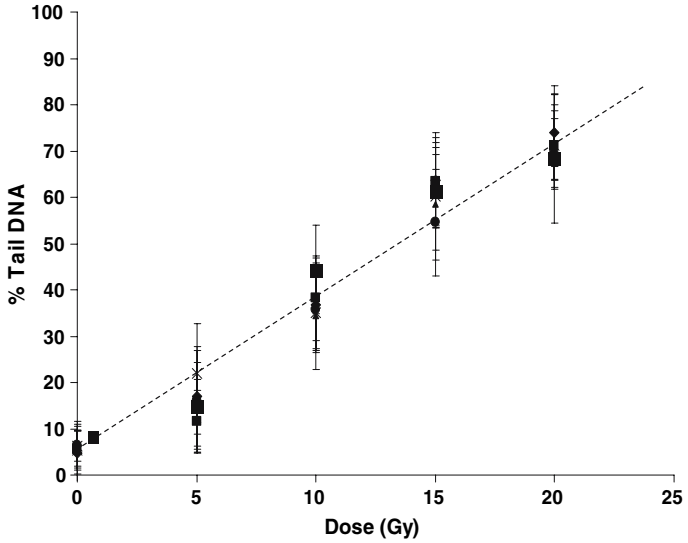

Fig. 4 Dose response curve representing mean percentage of DNA cross-linked in PBMCs after 2 h treatment with either 10 or 25 nM RH1. This experiment the introduction of a fixed number of strand breaks was accomplished using two radiation doses (15 or 20 Gy). The experiment was run using pooled samples from five healthy volunteers. Each point represents the mean and SD of 25 comets scored on each of two slides from each of two tanks on two independent assay runs. Symbols [(*filled triangle*) (*filled small square*)* (*filled big square*)] represent individual PBMC samples whilst the pooled PBMC sample is represented by (*filled circle*)

individual response curves and the pooled sample response curve. The r^2 range was 0.97 ± 0.017 SD. The LLOQ was between 2.5 and 3 Gy measured at 1 SD above the highest control (no radiation) dose. The inter assay variation using the mean of all radiation doses was 19.2 ± 4.2 SD.

Stability and transport of blood peripheral mononuclear cells

Free radical damage of DNA can occur on transport and storage of cells. Due to inherent delays of blood sampling in a clinical setting the stability of PBMCs with or without the addition of the free radical scavenger/cryo-preservation agent DMSO was investigated.

Peripheral blood was collected and processed as described. The resultant blood was counted then suspended in HBSS with or without the addition of DMSO (final concentration 10%). The effect of storage at room temperature or at 4°C and immediate or delayed (24 h) processing in the comet-X assay was investigated. The percentage of DNA in the comet tail and qualitative description of the cells is shown in Table 3. All samples processed either at room temperature or without DMSO present showed crenulation of the cells with >20% DNA present in the tail. Conversely samples run fresh or stored overnight at 4°C in

Table 3 Short-term stability of samples

	Immediate processing		Overnight at room temperature		Overnight at 4°C	
DMSO	+	−	+	−	+	−
Mean tail DNA	7.8 (5.0)	20.2 (12.5)	22.0 (10.2)	20.1 (9.5)	6.2 (4.8)	20.3 (10.3)
Description of cells	Spherical	Crenulated	Crenulated	Crenulated	Spherical	Crenulated

Percentage of DNA found in the tail of the comet when PBMCs were treated with or without DMSO and then processed immediately or left overnight at either room temperature or at 4°C

the presence of DMSO showed less than 20% DNA in the tail and in the spherical morphology. Statistical comparison of samples with and without DMSO processed either fresh or stored overnight at 4°C showed a significant statistical difference ($P < 0.05$).

Minimum and maximum limits of detection of DNA cross-linking by RH1

The purpose of these experiments was to determine both the minimum and maximum amount of detectable DNA cross-linking produced by the drug RH1. In addition, the optimum irradiation dose to detect minimum and maximum cross-linking was determined. Pooled PBMCs from the previous experiment were treated at 0, 5, 10, 25, 50 and 100 nM RH1 for 2 h at 37°C. The samples were then chilled on ice and irradiated at either 15 or 20 Gy and subsequently processed with an unirradiated control.

The maximum and minimum range of DNA cross-linking for each concentration of RH1 for both radiation doses was determined and the results shown in Fig. 5. A linear dose response curve was obtained up to 50 nM RH1 resulting in 90% or more of the DNA cross-linked. Further treatment above 50 nM did not significantly increase the level of DNA cross-linking observed. Pooled PBMCs (five donors) treated with RH1 concentrations (10 and 25 nM) designed to produce low (circa 20%) and high (circa 40%) levels of DNA cross-linking were used to create quality control (QC) standards. These QC standards were intended to be used alongside clinical samples each time the assay is run. A radiation dose of 20 Gy was selected as standard for this assay for this trial.

Accuracy and precision

Accuracy is the closeness of agreement between the value that is accepted as a reference value and the measured value. There are no quantitative reference standards available for the comet assay and therefore it is not possible to derive truly meaningful accuracy values for this assay. We were able to derive relative accuracy values by comparing the results derived from the pooled PBMC sample slides compared to those obtained from each of the individual sample slides. In total five individual samples were compared to the mean value derived from the pooled PBMC sample. A relative inaccuracy value (the % CV of these measurements) of less than 30% was achieved at all radiation doses used (Table 4).

Precision is the closeness of agreement (degree of scatter) between a series of measurements obtained from a multiple sampling of the same sample. Relative

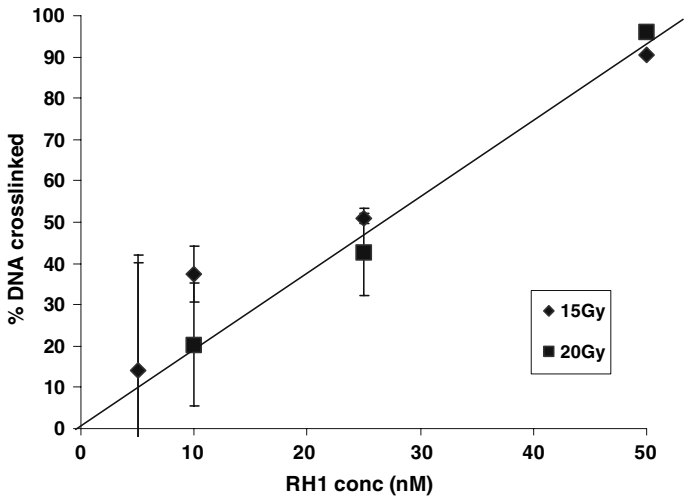

Fig. 5 Extent of DNA interstrand cross-linking in PBMCs following RH1 treatment. Assays were run using either 15 Gy (*filled diamond*) or 20 Gy (*filled square*) to introduce a fixed number of simple DNA strand breaks against which drug induced retardation of migration could be measured

Table 4 The mean percentages of accuracy and precision at each point along the PBL calibration curve

Dose (Gy)					
	0	5	10	15	20
Imprecision (%)	24.6	29.7	13.3	11.5	8.4
Inaccuracy (%)	17.63	22.5	10.3	2.3	2.8

Each measurement represents the individual sample means when compared to the pooled sample mean

precision could be determined by comparing the mean values derived from eight slides (two slides per sample in each of two tanks on each of two separate days) derived from each of five individuals. A relative imprecision (the % CV of these measurements) of less than 25% was achieved at all radiation doses used (Table 4).

Reference standard preparation

Each comet-X assay performed in the trial will have four QC standard included. Since there was no authenticated analytical reference standard, these primary QC standards were prepared at the start of validation. Briefly PBMCs from healthy volunteers were collected, pooled and then treated for 2 h at two concentrations of RH1 (low 10 and high 25 nM) and irradiated at 20 Gy to create the positive standards. Non-drug treated PBMCs were designated as positive control for cross-linking (no tail) as well as for absolute controls (no background DNA breaks) and irradiated PBMCs as both radiation standards and negative cross-linking (maximum tail length). All QC standards were frozen in 1 ml aliquots at −80°C in the presence of 10% DMSO. The stability of these frozen QC standards was determined before the trial started (fresh to 6 months storage) and subsequently as the clinical trial progressed (12–30 months). The results are shown in Table 5. Control untreated un-irradiated samples all had less than 10% DNA present in the comet tail (mean 3.8 ± 1.7). The irradiation alone controls showed between 45 and 77% DNA in the tail (mean of 61.3 ± 12.3 SD). The low dose RH1 controls (10 nM) showed between 7 and 18% DNA cross-linked (mean 11.3 ± 3.9 SD) whilst the high dose RH1 controls (25 nM) showed between 23 and 55% DNA cross-linked (mean 39 ± 10 SD).

Discussion

In early clinical trials proof of principle or of mechanism of action is often sought by evaluation of molecular pharmacodynamic (PD) endpoints [26]. Thus, the incorporation of robust PD endpoints into the drug development process is often the key to successful development of new agents [27]. Clinical and translational cancer research continues to be subjected to increasing regulatory requirements. In 2001 the European Parliament issued a directive on clinical trials in order to harmonise regulations throughout the European community, with the express aim of protecting the rights and safety of the individual [25], and this directive became law in the United Kingdom from the 1st of May 2004 (Statutory Instrument 1031, HMSO). According to these regulations trials will have to be conducted to Good Clinical Practise (GCP, as broadly defined by the ICH and recently published by the EU); while patient sample analysis will have to be performed to a quality system (as yet poorly defined) analogous to Good Laboratory Practise regulations as defined by the FDA (21 CFR part 58).

For Cancer Research-UK sponsored trials PD assays analysing critical biomarkers and molecular endpoints will be deemed acceptable (Cancer Research-UK Policy Document, 2001) if these laboratory-based methods can be demonstrated to pass stringent criteria for bioassay method validation according to internationally accepted performance criteria [28, 29]. These regulations require that such assays demonstrate an acceptable level of reproducibility (precision and accuracy), can be performed utilising quality control samples and that the stability of the samples and reagents is fully characterised at sample storage and analysis temperatures.

Table 5 Long-term stability of quality control (QC) standards froze at −80°C for up to 30 months

Time	Control irradiated	10 nM (RH1)	25 nM (RH1)
Immediate	45.0	8.5	37.1
24 h	61.2	7.4	31.9
7 days	54.0	12.3	34.4
1 month	48.7	14.6	55.4
3 months	49.8	14.6	31.4
6 months	56.0	7.0	23.4
1 year	74.8	13.5	49.8
12 months	77.0	10.2	38.2
18 months	70.9	18.1	50.3
22 months	75.4	6.5	37.6
30 months	45.0	8.5	37.1
Mean	61.3	11.3	39.0
SD	12.3	3.9	10.0

Pooled PBMC samples treated with low and high doses of RH1 along with control and irradiation alone samples were analysed using the standard Comet-X assay. Each value represents the mean and SD of 25 comets analysed from each of two slides from each of two electrophoresis tanks. The units of measurement for the irradiated controls are % DNA in the comet tail whilst that of the RH1 treated samples is % DNA cross-linked

RH1 (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone) is a water soluble bioreductive agent which upon reduction, forms difficult to repair DNA interstrand cross-linked adducts [17]. This novel agent is currently in phase I clinical trial and the comet-X assay is being used as a PD assay to detect DNA interstrand cross-links in peripheral blood mononuclear cells and tumour tissue. Before this assay could be used it was necessary to validate it in a laboratory setting. In this paper, we have described such a process of validation and confirmed that the comet-X assay is a robust, reproducible, and reliable assay capable of generating informative semi-quantitative results.

Our initial experiments using K562 cells focused on aspects of the lysis and subsequent electrophoresis of slides, including preparation, position in the tank, slide storage and scoring. We have shown that samples even after irradiation to produce high levels of DNA damage may be left embedded in LMP agarose on slides on an ice tray for up to 2 h prior to immersion in comet lysis buffer (CLB). This buffer itself may be stored for up to 8 weeks without significantly affecting the assay results. It has previously been suggested that inter-experiment variability exists due to differences in electrophoresis of individual samples and that such variation would be reduced by ensuring that slides are randomly distributed in the tank [23]. Our results suggest that the slide position does not cause any significant differences in the results obtained and that the samples can be placed in any position in the tank. Whilst some slides do produce statistically different results from the majority of the slides within the electrophoresis box, this is a random occurrence and more likely due to the gel preparation of individual slides than the actual position in the box. The measurements of both current and voltage within gels described in Fig. 2 are quite revealing. Little variation in voltage is observed between individual gels as expected as this is simply a measure of the electric potential passing through each gel. However, the measured current is much more variable between gels; consistent with the fact that current is a product of the resistance of the medium through which the voltage is passed. Consequently, individual gels should be cast so as to minimize potential difference. For this reason we choose to use a single layer of LMP gel to cover the entire slide. Such casting methods ensure an even distribution and thickness of agarose across the entire slide. Using this approach cells are much more widely distributed from each other ensuring ease of measurement. It has been suggested previously [23] that slides can be dried and scored some time after staining. Dried slides enable operators to score slides some time after electrophoresis. More importantly in the context of a clinical trial, the slides themselves will constitute raw data and as such must be archived. In such cases the ability to dry slides and store them would be an advantage. In this study, we can confirm that slides can be simply dehydrated and stored in lightproof cardboard boxes for up to at least one month or more prior to scoring without deterioration. Whilst this study confined itself to short term storage, slides within our laboratory have been stored without deterioration for several years. Additionally, we found no significant differences in inter-operator scoring such that several trained individuals are able to score slides from different experiments and derive similar results. We believe that the reason we achieve such low inter-operator variation is twofold. Firstly each operator is trained using a standard operating procedure, and secondly the parameter we choose to generate our primary data is % tail DNA. This measurement is felt to be most useful because it is the most simple and direct measurement possible with a variety of analysis packages as well as when scoring by eye. We believe along with others [12, 30] that this parameter is associated with less inter-electrophoresis or inter-experimenter variation than other contrived units of measurement such as tail moment [31].

Tissue collection and transport from the clinic to the laboratory should be accomplished as rapidly as possible to minimise damage associated with storage. Blood is the most readily available tissue for serial sampling and analysis. Blood is collected in EDTA tubes to minimise nuclease activity and it is preferable to isolate whole white cell populations [24]. In this study, we have demonstrated that once isolated, PBMCs samples can be stored at 4°C overnight provided they have 10% DMSO added to the storage media.

Since the radiation step is critical in this assay, a linear radiation-dose response had to be confirmed and the optimum dose of radiation selected for use in the assay.

It is well documented that variation in tissue samples from different individuals can be marked [5, 10]. For this reason we evaluated the radiation dosimitry using both normal and cancer cells. The inter assay variation was 19.2 for PBMCs from healthy volunteers, compared to 6.02 for K562 leukaemia cells reflecting possibly the clonogenic nature of cultured cells compared to the variation in DNA integrity found in lymphocytes from healthy volunteers [5, 10]. With both cell types a linear dose response curve was generated up to 20 Gy. The lower limit of detection was found to be 5–6 Gy. Higher sensitivity may well be possible using a higher pH alkali-unwinding buffer such as the commonly used pH 13 buffers. However, this would certainly increase the level

of basal DNA damage detected. In the case of phase I cancer patients who have different tumour types, stages of disease and have received a selection of treatments such basal damage could be quite substantial [6, 8]. Since only simple frank DNA breaks are required for this assay in order to measure the degree of retardation of DNA induced by cross-linking agents, there is little to be gained by increasing the sensitivity at the lower end of the radiation dosimitry. Twenty gray induces sufficient DNA damage against which cross-linking can be measured therefore in the trial, we have elected to use an irradiation dose of 20 Gy to introduce a fixed number of DNA strand breaks.

Similarly it was important to demonstrate a linear DNA cross-linking dose response following RH1 treatment. We have previously shown that RH1 induced DNA cross-links are detectable at nanometer doses using the Comet-X assay [21]. Here, we were able to demonstrate a linear dose response up to concentrations of 50 nM. This is within the pharmacokinetic range of plasma concentrations we expect to determine during the trial [21].

In our validation, intra-assay and inter-assay variations were not significantly different from which has been demonstrated previously [10, 12]. However, the use of internal standards is recommended as it controls for inter-experiment variation and so allows comparison of data between different experiments [12]. The use of such standards in a clinical trial that may last over two years calls in to question issues of stability and reproducibility. For long-term stability, it has previously been noted that storage of comet slides in CLB and cryo-preservation of blood and tissue in liquid nitrogen can lead to increases in DNA breakage [11]. Others have demonstrated the stability of patient samples after storage at −70/80°C for several months [6, 10]. In this study, we have been able to show that control, drug treated and irradiated QC samples can be cryo-preserved and stored at −80°C prior to analysis for up to 30 months without significant deterioration (Table 5). We believe that these rigorously conducted stability studies have been conducted for a much longer period than any reported so far. Whilst the CV for both the irradiated and high dose RH1 treated samples are low (<25%) and that of the low RH1 treated QC samples is quite high at 34%. This may reflect a combination of pharmacokinetic (drug concentration) and drug induced DNA damage contributing to the radiation induced strand breaks. Indeed this high variability is seen at the lower end of the RH1 dose response curve shown in Fig. 5 At higher doses of cross-linking agents the DNA would be more stable and such effects masked.

This study has considered many variables that have been addressed by a number of groups in the past [23, 24]. We have sought to look at the impact of all these variables in a detailed, statistically sound manner. Our validation has confined itself to variables considered to be most likely to influence reproducibility and accuracy in this assay. It does not however claim to have exhaustively covered all parameters. The basic alkaline comet assay being used by others in clinical trials [32] has shown a significant correlation between the comet assay data and those patients developing an elevated skin reaction to radiotherapy following breast-conserving surgery for breast cancer. Braybrooke et al. [6] showed that, in patients with melanoma treated with dacarbazine and tamoxifen, PBL strand breaks occurred with a peak at 48 h following chemotherapy and there was incomplete repair at 24 h. They also showed that a correlation between the comet assay data obtained and toxicity seen in patients as drug-induced emesis correlated with the percentage of DNA in the tail of the PBLs. The comet-X assay described here has previously been used to demonstrate DNA cross-linking in primary tumour material derived from patients [14] and has been shown to be robust and reproducible both in vitro and in vivo pre-clinical studies [21]. It is now validated for use in a phase I trial with the novel antitumour agent, RH1. Analysis of the data generated from the clinical trial will inform on how useful is the comet-X assay in determining both toxicity and alkylating activity of drugs in patients with cancer. It will be interesting to see how well this assay is able to delineate effects on lymphocytes compared to tumour cells. This correlative data will be collated and will be published on completion of the trial. We believe that a validated comet-X assay will be invaluable as a PD assay in future clinical trials. Furthermore, since it is the radiation step, which distinguishes this assay from the standard alkaline comet assay, then the validation of such a standard assay for clinical trial use should be more straightforward.

Acknowledgments This work was supported and funded by Cancer Research UK.

References

1. Ostling O, Johanson KJ (1984) Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun 123:291–298
2. Fairbairn DW, Olive PL, O'Neill KL (1995) The comet assay: a comprehensive review. Mutat Res 339:37–59
3. Olive PL, Banath JP (1993) Detection of DNA double-strand breaks through the cell cycle after exposure to X-rays, bleomycin, etoposide and 125IdUrd. Int J Radiat Biol 64:349–358

4. Moneef MA, Sherwood BT, Bowman KJ, Kockelbergh RC, Symonds RP, Steward WP, Mellon JK, Jones GD (2003) Measurements using the alkaline comet assay predict bladder cancer cell radiosensitivity. Br J Cancer 89:2271–2276
5. Woods JA, Young AJ, Gilmore IT, Morris ABilton RF (1997) Measurement of menadione-mediated DNA damage in human lymphocytes using the comet assay. Free Radic Res 26:113–124
6. Braybrooke JP, Houlbrook S, Crawley JE, Propper DJ, O'Byrne KJ, Stratford IJ, Harris AL, Shuker DE, Talbot DC (2000) Evaluation of the alkaline comet assay and urinary 3-methyladenine excretion for monitoring DNA damage in melanoma patients treated with dacarbazine and tamoxifen. Cancer Chemother Pharmacol 45:111–119
7. Tice RR, Strauss GH, Peters WP (1992) High-dose combination alkylating agents with autologous bone-marrow support in patients with breast cancer: preliminary assessment of DNA damage in individual peripheral blood lymphocytes using the single cell gel electrophoresis assay. Mutat Res 271:101–113
8. Vaghef H, Nygren P, Edling C, Bergh J, Hellman B (1997) Alkaline single-cell gel electrophoresis and human biomonitoring for genotoxicity: a pilot study on breast cancer patients undergoing chemotherapy including cyclophosphamide. Mutat Res 395:127–138
9. Hartley JM, Spanswick VJ, Gander M, Giacomini G, Whelan J, Souhami RL, Hartley JA (1999) Measurement of DNA cross-linking in patients on ifosfamide therapy using the single cell gel electrophoresis (comet) assay. Clin Cancer Res 5:507–512
10. Collins A, Dusinska M, Franklin M, Somorovska M, Petrovska H, Duthie S, Fillion L, Panayiotidis M, Raslova K, Vaughan N (1997) Comet assay in human biomonitoring studies: reliability, validation, and applications. Environ Mol Mutagen 30:139–146
11. Belpaeme K, Cooreman K, Kirsch-Volders M (1998) Development and validation of the in vivo alkaline comet assay for detecting genomic damage in marine flatfish. Mutat Res 415:167–184
12. De Boeck M, Touil N, De Visscher G, Vande PA, Kirsch-Volders M (2000) Validation and implementation of an internal standard in comet assay analysis. Mutat Res 469:181–197
13. Suggitt M, Fearnley J, Swaine DJ, Volpato M, Phillips RM, Bibby MC, Loadman PM, Anderson D (2003) Comet assay and flow cytometry analysis of DNA repair in normal and cancer cells treated with known mutagens with different mechanisms of action. Teratog Carcinog Mutagen Suppl 2:13–29
14. Ward TH, Butler J, Shahbakhti H, Richards JT (1997) Comet assay studies on the activation of two diaziridinylbenzoquinones in K562 cells. Biochem Pharmacol 53:1115–1121
15. Friedmann B, Caplin M, Hartley JA, Hochhauser D (2004) Modulation of DNA repair in vitro after treatment with chemotherapeutic agents by the epidermal growth factor receptor inhibitor gefitinib (ZD1839). Clin Cancer Res 10:6476–6486
16. Dehn DL, Winski SL, Ross D (2004) 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 10:3147–3155
17. Winski SL, Hargreaves RH, Butler J, Ross D (1998) 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
18. Loadman PM, Phillips RM, Lim LE, Bibby MC (2000) Pharmacological properties of a new aziridinylbenzoquinone, RH1 (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone), in mice. Biochem Pharmacol 59:831–837
19. Cummings J, Ritchie A, Butler J, Ward TH, Langdon S (2003) Activity profile of the novel aziridinylbenzoquinones MeDZQ and RH1 in human tumour xenografts. Anticancer Res 23:3979–3983
20. Kim JY, Patterson AV, Stratford IJ, Hendry JH (2004) 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 15:71–77
21. Ward TH, Danson S, McGown AT, Ranson M, Coe NA, Jayson GC, Cummings J, Hargreaves RH, Butler J (2005) Preclinical evaluation of the pharmacodynamic properties of 2,5-diaziridinyl-3-hydroxymethyl-6-methyl-1,4-benzoquinone. Clin Cancer Res 11:2695–2701
22. Tudor G, Alley M, Nelson CM, Huang R, Covell DG, Gutierrez P, Sausville EA (2005) Cytotoxicity of RH1: NAD(P)H:quinone acceptor oxidoreductase (NQO1)-independent oxidative stress and apoptosis induction. Anticancer Drugs 16:381–391
23. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35:206–221
24. Moller P (2006) The alkaline comet assay: towards validation in biomonitoring of DNA damaging exposures. Basic Clin Pharmacol Toxicol 98:336–345
25. Rosengren NFaB (2001) Directive 2001/20/EC of the European Parliment and of the Council of 4 April 2001 on the approximation of the laws, regulations and administrative provisions of the Member States relating to the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use. Official J Eur Communities L121:34–44
26. Rothenberg ML, Carbone DP, Johnson DH (2003) Improving the evaluation of new cancer treatments: challenges and opportunities. Nat Rev Cancer 3:303–309
27. Woude GF, Kelloff GJ, Ruddon RW, Koo HM, Sigman CC, Barrett JC, Day RW, Dicker AP, Kerbel RS, Parkinson DR, Slichenmyer WJ (2004) Reanalysis of cancer drugs: old drugs, new tricks. Clin Cancer Res 10:3897–3907
28. Shah VP, Midha KK, Findlay JW, Hill HM, Hulse JD, McGilveray IJ, McKay G, Miller KJ, Patnaik RN, Powell ML, Tonelli A, Viswanathan CT, Yacobi A (2000) Bioanalytical method validation–a revisit with a decade of progress. Pharm Res 17:1551–1557
29. Miller KJ, Bowsher RR, Celniker A, Gibbons J, Gupta S, Lee JW, Swanson SJ, Smith WC, Weiner RS (2001) Workshop on bioanalytical methods validation for macromolecules: summary report. Pharm Res 18:1373–1383
30. Collins AR, Dusinska M, Gedik CM, Stetina R (1996) Oxidative damage to DNA: do we have a reliable biomarker? Environ Health Perspect 104(suppl 3):465–469
31. Olive PL, Banath JP, Durand RE (1990) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. Radiat Res 122:86–94
32. Oppitz U, Schulte S, Stopper H, Baier K, Muller M, Wulf J, Schakowski R, Flentje M (2002) In vitro radiosensitivity measured in lymphocytes and fibroblasts by colony formation and comet assay: comparison with clinical acute reactions to radiotherapy in breast cancer patients. Int J Radiat Biol 78:611–616