
Jenny Wu¹, Peter H Clingen¹, Victoria J Spanswick¹, Maria Mellinas-Gomez¹, Tim Meyer¹, Igor Puzanov², Duncan Jodrell³, Daniel Hochhauser¹ and John A Hartley¹,*

¹CR-UK Drug-DNA Interactions Research Group, UCL Cancer Institute, Paul O’Gorman Building, Huntley Street, London WC1E 6BT, UK
²Vanderbilt-Ingram Cancer Centre, Vanderbilt University Medical Centre, Nashville, TN, USA
³Department of Oncology, University of Cambridge, Addenbrooke’s Hospital, Cambridge CB2 0QQ, UK

*To whom correspondence should be addressed:
Professor John A Hartley
UCL Cancer Institute
Paul O’Gorman Building
University College London
72 Huntley Street
London
WC1E 6BT
Tel: +44 (0) 20 7679 6055
Fax: +44 (0) 20 7679 6925
Email: john.hartley@ucl.ac.uk
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**Statement of Translational Relevance**

γ-H2AX foci are induced following DNA interstrand cross-linking drug treatment. This paper documents the pre-clinical evaluation of this assay and pharmacodynamic results from two Phase I studies in solid tumors evaluating different dosing schedules of the novel cross-linking agent SJG-136 (SG2000). This first use of the immunochemical measurement of γ-H2AX to detect DNA damage response to a DNA cross-linking agent in lymphocytes and solid tumor in a clinical trial setting suggests that it may have a role in cancer drug development and treatment optimization in the future.
ABSTRACT

Purpose: To evaluate γ-H2AX foci as a pharmacodynamic marker for DNA damage induced by DNA interstrand cross-linking drugs.

Experimental Design: γ-H2AX foci formation was validated pre-clinically in comparison to the comet assay, and evaluated pharmacodynamically in two Phase I studies of different dosing schedules of the novel cross-linking agent SJG-136 (SG2000).

Results: The measurement of γ-H2AX foci in human fibroblasts and lymphocytes in vitro was >10-fold more sensitive than comet assay measurement of cross-linking, with peak γ-H2AX response 24 hours after the peak of cross-linking. In lymphocytes from a Phase I study (3-weekly schedule), γ-H2AX foci were detectable 1 hour following the end of administration and in all patients maximum response was observed at 24 hours. Significant levels of foci were still evident at days 8 and 15 consistent with the known persistence of the DNA damage produced by this agent. In two tumor biopsy samples, foci were detected 4 hours post-infusion with levels higher than in lymphocytes. Extensive foci formation was also observed prior to the third dose in cycle 1 in lymphocytes from a second Phase I study (daily x 3 schedule). These foci also persisted with a significant level evident prior to the second cycle (day 21). An increased γ-H2AX response was observed during the second cycle consistent with a cumulative pharmacodynamic effect. No clear relationship between foci formation and administered drug dose was observed.

Conclusion: This is the first use of γ-H2AX as a pharmacodynamic response to a DNA cross-linking agent in a clinical trial setting.
INTRODUCTION

The histone protein H2AX is phosphorylated in response to ionising radiation resulting in γ-H2AX (1). Discrete nuclear foci form rapidly at sites of DNA double strand breaks which can be visualised by immunohistochemistry (2). An essential role of γ-H2AX is in the recruitment and accumulation of DNA repair and cell cycle checkpoint proteins to sites of DNA double strand break damage (1,3), including sites of replication fork collapse (4). Because of the sensitivity and utility for detection of DNA double strand breaks, γ-H2AX has emerged as a potentially useful biomarker with clinical implications (5,6). Persistence of these foci is considered to be an indicator of cell sensitivity after radiotherapy or treatment with radiomimetic drugs (7-9). We, and others, have also shown that the DNA damage produced by DNA interstrand cross-linking agents, including mechlorethamine, cisplatin, mitomycin C and psoralen plus UV, can also induce γ-H2AX foci (9-13), raising the possibility that the measurement of γ-H2AX foci could be used as a sensitive pharmacodynamic marker of DNA damage by cross-linking agents in the clinic (6).

SJG-136 (SG2000, NSC 694501) is a rationally designed pyrrolobenzodiazepine dimer which interacts sequence selectively in the minor groove of DNA (14). It spans six base pairs with a preference for 5’-purine-GATC-pyrimidine sequences, binding covalently to the N2-positions of guanine on opposite strands of DNA to form highly cytotoxic DNA interstrand cross-links spanning four base pairs (14-16). Based on the potent antitumor activity of SJG-136 in vitro and against several human tumors in vivo (16,17) it has progressed to Phase I clinical trials in USA (through NCI) and in UK (through CR-UK) (18-20).
Detailed pharmacokinetic and pharmacodynamic studies were included as part of the initial Phase I studies of SJG-136. The primary pharmacodynamic assay was a modification of the single cell gel electrophoresis (comet) assay to measure the formation of DNA interstrand cross-links in peripheral blood lymphocytes, and tumour tissue where available (21). This method has previously been validated and used successfully in several early phase trials involving DNA interstrand cross-linking agents (22-25). In addition, γ-H2AX foci formation was evaluated as a novel, and potentially more sensitive, pharmacodynamic endpoint. This paper documents the pre-clinical evaluation of this assay in comparison to the comet assay, validation in vitro and in vivo, and pharmacodynamic results from two Phase I studies in solid tumors evaluating different dosing schedules of SJG-136. This first use of the immunochemical measurement of γ-H2AX to detect DNA damage response to a DNA cross-linking agent in lymphocytes and solid tumor in a clinical trial setting suggests that it may have a significant role in cancer drug development and treatment optimization in the future.

Materials and Methods

Drug

SJG-136 was synthesized as described previously (14). For in vitro experiments 1 mM stock solutions were prepared in methanol and stored at -20°C. All subsequent drug dilutions were freshly prepared in serum free medium prior to drug treatment. All other chemicals were from Sigma Chemical Co., Poole, UK unless otherwise stated.

Cell Lines and Peripheral Blood Lymphocytes

The normal human fibroblast AGO1522B (AGO) cell line was kindly provided by Dr. Kevin Prise (Gray Cancer Institute) and maintained in alpha Minimum Essential
Medium containing 2mM L-glutamine, 20% foetal calf serum (FCS), 1% non-essential amino acids, 100U/ml penicillin and 100 U/ml streptomycin. Cells were grown in a humidified atmosphere at 37°C and 5% carbon dioxide (CO₂) and maintained in exponential growth. The cells were kept at low passage, returning to original frozen stocks every 3 to 6 months, and tested regularly for *Mycoplasma*.

Normal peripheral blood lymphocytes (PBLs) were isolated using the Vacutainer® CPT™ system (Becton Dickinson, Oxford, UK). Samples were centrifuged at 1500g for 20 minutes at room temperature. The fluffy mononuclear layer at the interface of the two layers was removed using a Pasteur pipette and transferred to a 15 ml tube. 10 ml cold RPMI 1640 tissue culture media was then added and the tube gently inverted and centrifuged immediately at 200g for 5 minutes at 4°C. The supernatant was then discarded and the cell pellet resuspended in RPMI 1640 containing 10% foetal calf serum and 2mM L-glutamine.

**Patient Samples**

PBL samples were taken for pharmacodynamic analysis from two Phase I clinical trials of SJG-136 in patients with advanced cancer performed in the UK and USA. In the CRUK trial whole blood samples (8ml) were collected predose, 1h, 4h, 24h, day 8 and day 15 after the end of infusion for cycle 1 and pre-, 1h, 4h, day 8 and day 15 after the end of infusion for cycle 2. PBLs were then isolated using the Vacutainer® CPT™ system described above with final re-suspension in 2ml RPMI 1640 containing 20% foetal calf serum and 10% dimethylsulphoxide and stored at -80°C until analysis. Samples were analysed for DNA interstrand cross-linking using the Single Cell Gel Electrophoresis (Comet) assay (21) and the same samples (Cycle 1 only) were then used for the quantification of γ-H2AX foci induction as the research endpoint.
Tumour biopsies were also obtained from two patients prior to treatment and between 2-4 h post-infusion. A single cell suspension was prepared from the tumour within 30 minutes of collection. The tumour was placed in a Petri dish with a small amount of cold RPMI 1640 medium and using two scalpel blades the tumour was chopped using a cross cutting action until a suspension of cells was formed. The cell suspension was then transferred to a 15ml tube and centrifuged at 200g for 5 min at 4°C. The supernatant was then discarded and the cells re-suspended in 2ml RPMI 1640 containing 20% foetal calf serum and 10% dimethylsulphoxide and stored at -80°C until analysis.

In the NCI trial PBLs were isolated using the Vacutainer® CPT™ system described above and samples taken from the patients prior to the first and third doses of SJG-136 during Cycles 1 and 2. As in the CRUK trial the formation of DNA interstrand cross-links were measured using a modification of the Single Cell Gel Electrophoresis (Comet) assay was measured as a pharmacodynamic endpoint and the same samples were then used for the quantification of γ-H2AX foci induction as a research endpoint.

All the patient samples were independently coded prior to analysis, processed and the number of γ-H2AX foci scored blind.

**In vitro Drug Treatment**

For the single cell gel electrophoresis (Comet) assay, AGO cells and normal PBLs were treated with SJG-136 for 1 hour at 37°C and 5% CO₂ in a humidified atmosphere. For AGO cells, drug treatments were carried out in a 6 well plate and following drug removal cells were post-incubated in medium supplemented with serum. For normal PBLs, the drug was removed by centrifugation at 200g for 5
minutes. The supernatant was removed and cells were re-suspended in drug-free full media. Cells were then incubated at 37°C and 5% CO₂ in a humidified atmosphere for the required pos-incubation time.

For γ-H2AX foci induction, AGO cells and PBLs to be treated ex vivo were incubated with SJG-136 for 1 hour at 37°C and 5% CO₂ in a humidified atmosphere. For AGO cells, drug treatments were carried out in 8 well LAB-TEK® II chamber slides™ (Nalge Nunc International, Hereford, UK) and the medium was replaced with drug-free medium following treatment with SJG-136. For PBLs, the drug was removed by centrifugation at 200g for 5 minutes, the supernatant removed and cells resuspended in drug-free full media. Cells were then incubated at 37°C and 5% CO₂ in a humidified atmosphere as required.

**Determination of DNA interstrand cross-link formation**

The details of the Single Cell Gel Electrophoresis (Comet) assay used to measure DNA interstrand cross-linking and repair are described in detail elsewhere (21). All procedures were carried out on ice and in subdued lighting. All chemicals were obtained from Sigma Chemical Co. (Poole, U.K.) unless otherwise stated. Immediately before analysis cells were diluted to give a final concentration of 2.5 x10⁴ cells/mL and irradiated (15Gy) in order to deliver a fixed number of random DNA strand breaks. After embedding cells in 1% agarose on a pre-coated microscope slide, the cells were lysed for 1 hour in lysis buffer (100mM disodium EDTA, 2.5M NaCl, 10mM Tris-HCl pH 10.5) containing 1% Triton X-100 added immediately before analysis, and then washed every 15 minutes in distilled water for 1 hour. Slides were then incubated in alkali buffer (50mM NaOH, 1mM disodium EDTA, pH12.5) for 45 minutes followed by electrophoresis in the same buffer for 25 minutes
at 18 V (0.6V/cm), 250mA. The slides were finally rinsed in neutralising buffer (0.5M Tris-HCl, pH 7.5) then saline.

After drying the slides were stained with propidium iodide (2.5µg/mL) for 30 minutes then rinsed in distilled water. Images were visualised using a NIKON inverted microscope with high-pressure mercury light source, 510-560nm excitation filter and 590nm barrier filter at x20 magnification. Images were captured using an on-line CCD camera and analysed using Komet Analysis software 4.02 (Andor Technology, U.K.). For each duplicate slide 25 cells were analysed. The tail moment for each image was calculated as the product of the percentage DNA in the comet tail and the distance between the means of the head and tail distributions (26). DNA interstrand cross-linking was expressed as percentage decrease in tail moment compared to irradiated controls calculated by the formula:

\[
\% \text{ decrease in tail moment} = \left[ 1 - \frac{(T_{Di} - T_{Cu})}{(T_{Ci} - T_{Cu})} \right] \times 100
\]

Where \( T_{Di} \) = tail moment of drug-treated irradiated sample; \( T_{Cu} \) = tail moment of untreated, unirradiated control; \( T_{Ci} \) = tail moment of untreated, irradiated control

\[\gamma\text{-H2AX immunofluorescence}\]

For AGO cells, 2 x 10^4 cells per well were seeded in an 8 well LAB-TEK® II chamber slides™ (Nalge Nunc International, Hereford, UK) and incubated overnight at 37°C. Cells were treated with SJG-136 for 1 hour after which the drug was removed and cells incubated at 37°C in drug-free medium. Cells were fixed with ice cold methanol:acetone (50:50) for 8 minutes at 4°C. Cells were washed 3 times with cold phosphate buffered saline (PBS) then permeabilized with 0.5% Triton X-100 in PBS
for 5 minutes at room temperature. Cells were then blocked overnight at 4°C with blocking buffer (0.1% Triton X-100, 0.2% skimmed dry milk in PBS). Blocked cells were incubated with anti-phospho-histone H2A.X (Ser139) monoclonal antibody (Millipore, U.K) for 1 hour at a 1:10,000 dilution in blocking buffer at room temperature. After washing 3 times with washing buffer (0.1% Triton X-100 in PBS), cells were then incubated for 1 hour at room temperature with Alexa Fluor® 488 goat anti-mouse secondary antibody (InVitrogen, UK) in blocking buffer. Cells were then washed with PBS.

For ex vivo treatment of PBLs, cells were treated with 10 nM SJG-136 for 1 hour after which the drug was removed by centrifugation at 200g, cells re-suspended and incubated at 37°C in drug-free medium. For both ex vivo treated PBLs and patient samples (PBL and tumour), 5 x10^4 cells were adhered to Vision BioSystems™ Plus slides by cytopinning at 650rpm for 5 minutes at room temperature. Slides were then dried at room temperature. Cells were fixed with ice cold methanol:acetone (50:50) for 15 minutes at 4°C. Cells were washed 3 times with cold PBS then permeablized for 15 minutes at room temperature. Cells were then blocked in blocking buffer (0.1% Triton X-100, 0.2% skimmed dry milk in PBS) overnight at 4°C. Blocked cells were incubated overnight at 4°C with anti-phospho-histone H2A.X (Ser139) monoclonal antibody (Millipore, U.K) at a 1:1000 dilution in blocking buffer. After washing 3 times with washing buffer (0.1% Triton X-100 in PBS), cells were then incubated for 4 hours at room temperature with Alexa Fluor® 488 goat anti-mouse secondary antibody (InVitrogen, UK) in blocking buffer. Cells were then washed with PBS.

For all cell lines, normal lymphocytes and patient samples, cells were counterstained with 2μg/mL propidium iodide for 2 minutes. Slides were then rinsed in distilled water for 30 minutes, mounted with Vectashield® (Vector Laboratories, Peterborough, UK)
and the edges sealed with clear nail polish. Images were visualised using Perkin Elmer Ultraview ERS Suite v 3.0.0 and confocal microscopy consisting of Zeiss Axiovert 200 inverted fluorescence microscope (x40 oil objective) equipped with 14 bit ECCD camera and argon and krypton gas excitation lasers at 488nm and 568nm. Z-stack acquisition using optimal slice distancing was performed on each microscope image. Images were then compressed to produce a maximum intensity projection (MIP) image to allow maximum foci detection. Foci were counted in 50 cells per time point and results are expressed as mean number of foci per cell from three independent experiments. All clinical samples were read blind to avoid any potential bias.

**Statistical analysis**

Statistical analysis was performed using Prism 5.02 (GraphPad Software Inc.). For samples taken from the Cancer Research UK Phase I trial, all post-treatment samples were compared with pre-treatment samples by using repeated one-way anova analysis. For the NCI Phase I trial the samples were compared by using one-tailed unpaired t test. The significance level was expressed as ns = non-significant, *p<0.05, **p<0.005, ***p<0.001.

**Results**

**γ-H2AX foci formation following exposure to SJG-136 in vitro and in vivo**

We have previously shown that exposure of cells to conventional clinical cross-linking agents and the novel minor groove sequence selective cross-linking agent SJG-136 can induce γ-H2AX foci (10,27). Figure 1A shows a dose response of γ-H2AX foci formation in individual human fibroblast AGO cells 24 hours following a 1 hour exposure to SJG-136. Foci are observed at doses as low as 10pM reflecting the known efficiency of DNA interaction of this agent (14-16). Figure 1B shows the dose
response in AGO cells of $\gamma$-H2AX foci formation above background 24 hours after 1 hour SJG-136 exposure (red) compared to the measurement of DNA interstrand cross-linking using the single cell gel electrophoresis (comet) assay (blue) immediately after 1 hour drug exposure. The data clearly show that the $\gamma$-H2AX response is at least 10-fold more sensitive than direct measurement of DNA interstrand cross-linking using the comet assay.

The reason for the difference in timing at which the assays are performed is shown in figure 1C. DNA interstrand cross-links form rapidly following exposure to SJG-136 and have reached a peak in AGO cells at the end of the 1 hour treatment. The non-distorting cross-links produced by this agent persist and are still detectable at high levels at 120 hours in these cells. In marked contrast, the $\gamma$-H2AX response is delayed and reaches a peak at 24 hours following the 1 hour treatment. The response then declines to around 30% of the peak level by 120 hours (Figure 1C). Importantly, a similar $\gamma$-H2AX response is observed in human lymphocytes treated ex vivo with SJG-136, although in this case the peak level at 24 hours is approximately 60% of that observed in AGO cells at the same dose of drug (Figure 1C).

The ability of the assay to detect $\gamma$-H2AX at pharmacologically relevant doses was tested in vivo. Data from a representative xenograft experiment are shown in Supplementary Figure 1. Doses of SJG-136 of 0.15 and 0.3mg/kg iv were chosen to give dose-dependent tumor growth delay and measurable levels of cross-linking in tumor, peaking at 2 hours, as measured by the comet assay. Levels of $\gamma$-H2AX foci increased with dose in both lymphocytes and tumor and were considerably higher at 24 hours than at the peak of crosslinking (2 hours) consistent with in vitro data. Both background and drug-induced levels of foci were higher in tumor than in lymphocytes.
Prior to use on clinical samples, validation experiments to determine the variability of the assay were performed in human lymphocytes treated ex vivo with SJG-136 for 1 hour followed by post-incubation for 24 hours. Inter- and intra-assay data showing the high reproducibility of the assay are shown in Supplementary Figure 2A and B. In addition, during analysis of all clinical samples, aliquots of internal standard samples of treated lymphocytes stored at -80°C were run. Data from the same internal standards from 10 runs individual clinical sample analyses are shown in Supplementary Figure 2C.

**γ-H2AX foci formation in the CRUK Phase I clinical trial of SJG-136**

The Cancer Research UK study was designed as a two centre Phase I dose escalation study of SJG-136 given as a 10 minute intravenous infusion every 21 days in patients with solid tumours (18). Seventeen patients were entered into the study and sixteen patients were treated. Doses administered ranged from 15-240 μg/m². The schema for the dosing schedule and timing of sample for pharmacodynamic analysis are shown in Figure 2 (top). The γ-H2AX foci data in lymphocytes from the individual patients at the different dose levels are shown in Figure 3A with the pooled data in Figure 3B. All samples were read blind to avoid any potential bias. The background level of γ-H2AX foci in the pre-dose samples ranged from 0-10 foci/cell. Foci are detectable in all lymphocyte samples as early as 1 hour after the end of drug infusion (Figure 3A) although the overall level did not reach significance over the pre-dose level (Figure 3B). A significant level of foci formation was, however, observed at 4 hours (p<0.005) which then reached a peak at 24 hours. Interestingly, there is no clear dose response in γ-H2AX foci formation. Statistically significant levels of foci are still evident in the day 8 (p<0.005) and day 15 (p<0.05) samples.
In two patients with melanoma (both treated with 45 μg/m²) it was possible to obtain both pre-dose and post-treatment (4 hour) tumor biopsy samples. The pre-dose biopsy samples had a greater background level of γ-H2AX foci than in lymphocytes. This level, however, increased by >4-fold in patient 12 and >3-fold in patient 13 following treatment (Figure 3C).

**γ-H2AX foci formation in the NCI Vanderbilt Phase I clinical trial of SJG-136**

In the NCI trial SJG-136 was administered via 20 minute intravenous infusion using a daily × 3 dosing schedule every three weeks with steroid premedication (dexamethasone, 8 mg/day, Day -1 through Day 3) and early diuretic treatment with spironolactone (19). The starting dose of SJG-136 was 20 μg/m²/day which was escalated to 25, 30 and 35 μg/m²/day. The schema for the dosing schedule and timing of samples for pharmacodynamic assessment is shown in Figure 2 (lower). The γ-H2AX foci data in lymphocytes from the individual patients at the different dose levels are shown in Figure 4A. The background level of γ-H2AX foci in the cycle 1 pre-dose samples was below 10 in all samples with the exception of one patient who had a level above 20. In the cycle 1 day 3 samples (just prior to the administration of the third daily dose) γ-H2AX foci were observed in all samples ranging from 4-34 foci/cell. This level of foci was highly significant (p<0.001) compared to the pre-cycle 1 sample (Figure 4B), although no clear dose response was observed. 18 days later, just prior to the first administration of the second cycle (pre C2, Figure 4) the level of foci had decreased in all samples but the mean level was still significantly (p<0.005) above that in the pre cycle 1 samples (Figure 4B). Cycle 2 day 3 (C2D3) gave the highest level of γ-H2AX foci which was significantly higher than the pre-cycle 1 samples (p<0.001), but also significantly higher than the C1D3 samples (p<0.05, Figure 4B) indicating a cumulative DNA damage response with cycle.
Discussion

The current study was undertaken to evaluate the potential of γ-H2AX foci formation as a clinical pharmacodynamic marker of DNA damage produced by DNA cross-linking agents and its repair. We have previously shown in vitro that γ-H2AX can act as a highly sensitive and general marker of DNA damage induced by nitrogen mustard and platinum drugs and that it showed promise for predicting cellular chemosensitivity to interstrand cross-linking agents (10). γ-H2AX is likely marking sites of double strand breaks generated after lesion processing by structure specific endonucleases. A decline in γ-H2AX after the peak of formation suggests the resolution of the intermediate double strand breaks by downstream pathways e.g. homologous recombination repair, translesion DNA synthesis etc.

The novel DNA minor groove cross-linking agent was chosen for the current study since it has previously been shown to be a highly efficient cross-linking agent (16) and the two Phase I studies of this agent, using different dosing schedules where appropriately timed samples were already being taken for other pharmacodynamic analyses, made it ideal for the clinical evaluation of γ-H2AX response. In the pre-clinical studies the measurement of γ-H2AX foci was found to be at least ten fold more sensitive than the comet assay at detecting DNA damage induced by SJG-136. The modified comet assay directly measures DNA interstrand cross-links, the critical cytotoxic lesion produced by bifunctional alkylating drugs and SJG-136. Although capable of producing other damage to DNA including intrastrand cross-links (28), SJG-136 rapidly and efficiently produces interstrand cross-links in cells which persist (16, 29). This is in contrast to more conventional cross-linking drugs such as the nitrogen mustard and platinum drugs where the helix distorting DNA interstrand cross-links account for less than 5% of damage to the DNA and are repaired more readily (16, 30). In contrast to the comet assay, the γ-H2AX assay does not directly
measure the drug-induced lesion but is indicating a damage response and acts as a more general surrogate marker for the detection of the DNA damage produced by interstrand cross-linking agents. The γ-H2AX response following cross-linking agent exposure does not require cells to be actively dividing since it is observed in lymphocytes.

Interestingly, the γ-H2AX response following SJG-136 is markedly different to that produced by conventional cross-linking drugs. We have previously shown that the peak of γ-H2AX foci formation is approximately 1 hour following the peak of interstrand cross-linking (measured using the comet assay) for nitrogen mustard and platinum drugs (10). In the case of SJG-136, however, the current study shows that the γ-H2AX response does not peak until 24 hours after the peak of the cross-linking. This may reflect the non-distorting nature of the cross-links produced by this agent (14,16) and which may evade early detection. In addition to the increased sensitivity of the γ-H2AX assay another potential advantage is that while the comet assay is restricted to detecting the induction and initial incision/unhooking of DNA interstrand cross-links, it cannot detect defects in the downstream processing of unhooked cross-links, or the repair of cross-link-associated DNA double strand breaks by homologous recombination (31). We have previously shown that γ-H2AX foci persist in cells defective in both the initial unhooking of interstrand cross-links (ERCC1 defective) and homologous recombination repair (XRCC3 defective) (10).

In the samples from the CRUK study γ-H2AX foci were detectable at 1 hour following the end of treatment and in all patients the maximum response was observed at 24 hours, showing the same time course as was observed in the lymphocytes treated ex vivo. Significant levels of foci were still evident at day 8 and day 15 consistent with the known persistence of the DNA damage produced by this agent. In the two biopsy
samples that were available DNA damage was clearly detected 4 hours post-infusion with foci levels much higher than in the lymphocyte samples taken at the same time point. This clearly indicates that a significant level of drug had reached the tumor. It is interesting to note that in these two patients the level of foci in the tumor at 4 hours was greater than the level in lymphocytes from the same patients at 24 hours, the peak time of $\gamma$-H2AX response.

Extensive foci formation was also observed just prior to the third dose in cycle 1 in the lymphocytes from the NCI study. These foci were also shown to persist and a significant level was evident just prior to the second cycle (day 21). Consistent with this was the fact that low levels of cross-linking were observed just prior to cycle 2 in some patients measured using the comet assay (19). An increased $\gamma$-H2AX response was observed during the second cycle consistent with a cumulative pharmacodynamic effect. It is interesting to note that in neither clinical study was there a clear relationship between lymphocyte foci formation and administered drug dose (Supplementary Figure 3A,B) despite a clear dose response observed in vitro. In both clinical studies linear drug pharmacokinetics was observed across the dose ranges tested. In the NCI study the low levels of cross-linking observed in samples taken during cycle 1 correlated better with the systemic exposure to SJG-136 (as assessed by AUC or Cmax) than with the administered dose levels (19). The absence of measurable cross-linking in the CRUK study and relatively low levels detected in the NCI study suggest that the levels produced were near the limits of detection of this assay for this drug. In the NCI study there was no clear relationship between foci formation and crosslinking (Supplementary Figure 3C). The loss of $\gamma$-H2AX observed in lymphocytes may not necessarily represent repair but loss of damaged cells which may partly explain the lack of dose response observed. It is interesting to note, however that no significant myelosuppression was observed on
the NCI study (19). The γ-H2AX assay, however, provided conclusive evidence of drug-DNA interaction by SJG-136 in lymphocytes from all patients, and in tumor in the two patients where it was possible to evaluate.

One patient unexpectedly had a very high background of γ-H2AX foci in lymphocytes. In addition, the level of background in the tumor biopsy samples was found to be higher than in lymphocytes, as observed *in vivo* (Supplementary Figure 1). Most circulating lymphocytes are quiescent and DNA synthesis is limited. In tumor, the fraction of cells in S phase may be expected to have a higher γ-H2AX response than cells in other phases. Background levels of γ-H2AX have been shown to vary widely between cells in different tissues, culture or different cell lines and it has been found that elevated levels of γ-H2AX are present in a number of human cancer cell model systems (32,33) suggesting that an increased level of DNA damage is a general characteristic of cancer development. In addition, the extent of γ-H2AX response to cross-links can also vary between different cells (as observed in this study for AGO cells and lymphocytes) and between different interstrand cross-linking agents (10,12,13).

The current study clearly demonstrates that measurement of γ-H2AX foci can act as a surrogate clinical marker of DNA damage induced by DNA interstrand cross-linking agents such as the novel sequence selective minor groove cross-linking agent SJG-136. The current clinical studies utilised primarily lymphocytes where the time course and persistence of γ-H2AX foci formation could be studied with good sensitivity. The data in biopsy material was limited in the current study due to the design of the clinical protocols, but importantly showed that the assay was also applicable to target tumor tissue. Encouragingly, in the CRUK study higher levels of foci were observed in tumor than in time-matched lymphocytes. Future studies should examine the
relationship between cross-linking drug-induced $\gamma$-H2AX foci formation and persistence in relation to clinical antitumor response. Indeed, $\gamma$-H2AX foci is being measured directly in tumor cells in two further studies of SJG-136: a Phase II study in ovarian cancer and a Phase I/II study in haematological malignancies, and the results of these analyses will be presented in due course. A recent study has shown the potential of monitoring drug-induced $\gamma$-H2AX as a pharmacodynamic biomarker in individual circulating tumor cells (34), two studies have used flow cytometry measurement of H2AX phosphorylation following drug treatment in leukaemic cells (35,36) and an immunofluorescence assay for $\gamma$-H2AX has been validated as a pharmacodynamics marker of topoisomerase I inhibitor activity (37). The current study is the first to use the immunochemical measurement of $\gamma$-H2AX to detect DNA damage response to a DNA cross-linking agent in lymphocytes and solid tumor in a clinical trial setting. The central position of $\gamma$-H2AX in DNA damage detection and repair and its ease of detection suggest that it may have a significant role in cancer drug development and treatment optimization in the future (6).

Disclosure of potential conflicts of interest

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Figure Legends

**Figure 1.** A. Dose response of γ-H2AX foci (green) in human fibroblast AGO cells 24 hours following a 1 hour exposure to SJG-136. Red staining of nuclei is by propidium iodide. B. Comparison of interstrand cross-link formation measured by the comet assay (immediately after treatment) and DNA damage response measured as γ-H2AX foci formation (24 hours after treatment) in AGO cells following a 1 hour exposure to SJG-136. Data are the mean ± s.d. from three independent experiments with 50 cells counted per dose. C. Persistence of interstrand cross-links (blue) and formation and disappearance of γ-H2AX foci (red) in AGO cells (solid line) and human lymphocytes (dotted line) following a 1 hour exposure to 100nM SJG-136. Data are the mean ± s.d. from three independent experiments with 50 cells counted per time point.

**Figure 2.** Schema of the dosing schedule for SJG-136 and the timing of samples for γ-H2AX foci analysis in the two Phase I clinical trials.

**Figure 3.** Results of the γ-H2AX foci analysis from the CRUK Phase I study of SJG-136. A. Mean number of foci per cell for the 16 patients presented by dose of SJG-136 administered. B. Pooled data from all patients showing statistical significance (repeated one-way anova) of the number of foci per cell compared to the pre-dose sample. The level of significance was: ns = non-significant, * p<0.05, ** p<0.005. C. Number of foci per cell in pre-dose and post-treatment (4 hour) tumor biopsy samples from patients 12 and 13 showing statistical significance post-treatment (* p<0.05, *** p<0.001).
Figure 4. Results of the γ-H2AX foci analysis from the NCI Vanderbilt Phase I study of SJG-136. A. Mean number of foci per cell for the patients presented by dose of SJG-136 administered at pre-Cycle 1 (Pre C1), cycle 1 day 3 (C1D3), pre-Cycle 2 (Pre C2 and Cycle 2 day 3 (C2D3). B. Pooled data from all patients showing statistical significance (one-tailed unpaired t test) of the number of foci per cell of C1D1, Pre-C2 and C2D3 to the Pre-C1 sample, and also C2D3 compared to C1D3. The significance level was: * p<0.05, ** p<0.005, *** p<0.001.
Figure 1

A

Control 0.01nM 0.1nM

1nM 10nM

B

% decrease in tail moment
mean no of foci/cell

0.01 0.1 1 10 100 1000
SJM-136 (nM)

C

% decrease in tail moment
mean no of foci/cell

0 24 48 72 96 120
Post-incubation time (hrs)
Figure 2

Cancer Research UK Phase I - Three Weekly Schedule

NCI Phase I - Daily x 3 Schedule
Figure 3

A

No of foci / cell

Predose 1hr 4hr 24hr Day 8 Day 15

15µg/m²
30µg/m²
45µg/m²
60µg/m²
120µg/m²
240µg/m²

B

No of foci / cell

Predose 1hr 4hr 24hr Day 8 Day 15

ns
**
**

C

Patient 12
No of foci / cell

Predose 4hr

***

Patient 13
No of foci / cell

Predose 4hr

*

Figure 4
Clinical Cancer Research

γ-H2AX foci formation as a pharmacodynamic marker of DNA damage produced by DNA cross-linking agents: results from two Phase I clinical trials of SJG-136 (SG2000)


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